THE ORIGINS AND PHENOTYPIC CONSEQUENCES OF REPETITIVE SEQUENCE VARIATION IN *DROSOPHILA MELANOGASTER*

A Dissertation

by

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ABSTRACT

Environmental stimuli can have a significant impact on gene expression patterns and this impact is not always confined to a single cell cycle, but can sometimes persist through multiple divisions or even transgenerationally. Such phenomena are often classified as epigenetic because genetic mutations are not thought to behave in such a directed, nonrandom manner. Studies concerning epigenetics often overlook unstable variation in repetitive heterochromatic sequences as a potential mode of transgenerational inheritance. Although they constitute a large fraction of most eukaryotic genomes, technological limitations have greatly hindered our understanding of the functional importance of such sequences.

Copy number variation in the unstable ribosomal RNA gene array (rDNA)—a specific class of repetitive sequence— modulates heterochromatin formation and influences the expression of a large fraction of the *Drosophila* genome. The primary aim of this study was to identify an environmental source of rDNA instability and to characterize the phenotypic consequences of the variation generated by that instability. Using genetic, cytological, and molecular assays, I discovered that increased dietary yeast concentration results in rDNA instability and copy number reduction in the soma and germline. Modulation of Insulin/TOR signaling produces similar results, indicating a role for known nutrient sensing signaling pathways in this process.

Previous studies suggest that rDNA deletions influence the regulation of a number of metabolically important genes. Supporting this, I found that variation in rDNA modulates the *Drosophila* starvation response and affects lipid metabolism. This effect is potentially mediated by differential rDNA transcription, suggesting a link between the cause of instability and its phenotypic outcome. Instability is not just thought to be a property of rDNA, but seems to occur via similar mechanisms in other repetitive sequences. I developed a novel polymerase chain reaction (PCR) technique to quantify simple pentameric sequence repeats and used it to discover previously uncharacterized natural and mutationally-induced variation on the heterochromatic *Y* chromosome. Taken together, these findings suggest a non-epigenetic mechanism through which the environment can influence gene expression patterns in a manner that is specific, heritable, and consequential.

ACKNOWLEDGEMENTS

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INTRODUCTION AND LITERATURE REVIEW

The Repetitive Genome

Nuclear genome size is highly variable amongst eukaryotic species and bears little relation to the perceived complexity of a given organism. This seemingly contradictory observation was originally termed “the C-value paradox”—C-value in this instance referring to the mass of an organisms haploid genome [1,2]. Over 50 years of gene discovery have made it abundantly clear that the coding potential of a genome does not correlate with it size [3,4]. Although the discovery of non-coding DNA has largely resolved the C-value paradox, the evolutionary significance and molecular function of such sequences remains controversial [5-8].

Throughout the 1960’s and 1970’s, researchers in the nascent field of comparative genomics used C_0T analysis to characterize genome complexity in a variety of organisms [9] [10]. In a typical C_0T experiment, genomic DNA is sheared into small fragments and then heated to disassociate the strands. As the solution is allowed to cool, the relative proportion of single-stranded DNA is monitored via spectrophotometry. Low complexity (i.e. repetitive) sequences anneal more quickly due to the relative abundance of complementary pairing partners, while complex (i.e. single copy) sequences take much longer to anneal. When percentage of single-stranded DNA is plotted as a function of

time, three broad sequence categories can be discerned: highly repetitive, middle repetitive, and low copy number [11]. The relative proportion of each of these categories across different taxa was found to be consistent with genome size and coding potential estimates. Specifically, organisms with larger genomes tend to have a greater proportion of repetitive sequences while organisms with smaller genomes have a greater proportion of single or low copy number sequences. The complex DNA identified by C_0T analysis largely corresponds to coding and regulatory sequences, while low complexity DNA corresponds to highly repetitive major and microsatellites as well as middle repetitive sequences such as transposable elements, telomeric repeats, or high copy number genes (i.e. Ribosomal RNA genes) [12-14]. For a variety of reasons—including their more obvious functional significance and relative ease of study—coding sequences and their associated regulatory elements have largely become the focus of molecular biology research.

Although repetitive sequences constitute a large proportion of many eukaryotic genomes, compared to their coding counterparts relatively little is known about them. Since they are largely not amenable to many standard molecular biology techniques (PCR and sequencing in particular), the precise copy-number and arrangement of most repetitive sequences is unknown [15,16]. Cytological analysis has revealed at least some generalities, as repetitive sequences tend to be clustered within centromeric or telomeric regions. These chromosomal regions were originally dubbed “heterochromatin” due to their

more intense staining pattern in comparison to “true” chromatin or “euchromatin” [17]. Contemporary sources define heterochromatin more broadly as the gene-poor, late-replicating, transcriptionally silent portion of the genome along with any of its associated proteins [18,19]. These proteins include a variety of silencing factors and chromatin remodelers that are thought to be responsible both for transcriptional inactivity as well as the cytologically compact appearance of heterochromatin. Euchromatin, in contrast, is home to most protein coding genes, is lighter staining, and appears cytologically “decondensed” [20].

Chromatin and Epigenetics

The fruit fly *Drosophila melanogaster* has been an excellent model system for understand the properties and function of heterochromatin and has been a useful genetic tool for identifying heterochromatic protein components. Much of this work was accomplished using a system known as position effect variegation (PEV) [21]. Generally speaking, PEV describes the phenomenon whereby a gene’s expression levels are influenced by its broader genomic context. The most widely known example of this in *Drosophila* is the *white-mottled 4* allele (Figure 1.1) [22]. *In(1)w*^{m} is the result of an X-ray induced inversion of the euchromatic arm of chromosome 1 (the *X* chromosome). The *white* gene (*w*), which is required for eye pigmentation, is normally located near the distal end of the *X* chromosome, but the *w*^{m4} inversion places it near centric heterochromatin [23]. Although the genomic context immediately surrounding the gene is unchanged, proximity to heterochromatin causes stochastic silencing

[24]. Phenotypically, this is readily visible as a variegated eye with red (active) and white (silenced) patches. That heterochromatic silencing could seemingly spread to non-heterochromatic sequences was of great interest because it suggested that silencing is not dependent upon a specific sequence. Furthermore, the observation that variegated patches are developmentally related suggested that once conferred, the silencing properties of heterochromatin were heritable and thus, potentially epigenetic [25].

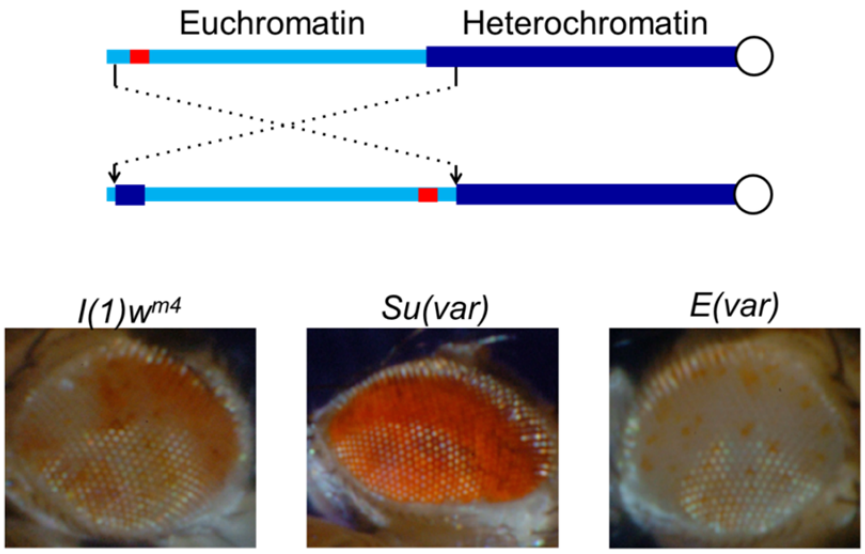

Figure 1.1 Position Effect Variegation (PEV) in Drosophila. The top illustration represents the *X* chromosome. Dark blue indicates heterochromatin while light blue indicates euchromatin. The white circle represents the centromere while the red box indicates the relative location of the *white* gene. The *I(1)w^{m4}* allele is a result of the inversion indicated by dashed arrows and is visible as a variegated eye (bottom left). Su(var) and E(var) mutations suppress (middle) or enhance (right) this effect. Figure adapted from [26].

To better understand the molecular underpinnings of heterochromatin, a number of research groups performed genetic screens for modifiers of PEV in *Drosophila* [27,28]. These screens uncovered many of the conserved protein components of heterochromatin. PEV modifiers are categorized as either suppressors, called Su(var)s, or enhancers of variegation, called E(var)s. Su(var) mutations result in the deposition of more eye pigment (Figure 1.1) and are found in genes encoding a variety of histone modifying and remodeling enzymes as well as components of RNA interference pathways [29]. E(var) mutations, which cause lighter eye pigmentation, are much less common and much less studied, but include some genes encoding transcriptional activators such as trithorax-like and GAGA factor [30].

Su(var) proteins exert their effect on gene silencing largely through the modification and positioning of histones—the basic structural component of most eukaryotic chromatin [31]. Throughout the cell cycle, DNA is packaged in nucleosomes which are composed of approximately 147 bp of DNA wrapped around a histone octamer containing two each of histones H2A, H2B, H3, and H4 [32]. Individual nucleosomes are typically spaced approximately 80 bp apart, and can be compacted by the addition of the linker histone H1 [33].

The position of nucleosomes along a segment of DNA influences the overall accessibility of the sequences therein [34]. Nucleosomal occupancy can prevent the binding of regulatory proteins to the sequences they govern and, thus, is an important regulator of transcription [35]. Perhaps as a consequence

of this, many active promotors and enhancers are maintained in an accessible state via the activity of chromatin remodeling proteins [36,37]. Furthermore, since the presence of nucleosomes impedes polymerase procession, many transcriptional elongation complexes include remodeling proteins [38-40]. In contrast, heterochromatin is often enriched with proteins required for the maintenance of nucleosome occupancy [41-43].

In addition to chromatin remodelers, heterochromatin formation and maintenance requires a variety of proteins involved in the addition or subtraction of histone post-translational modifications. Histones possess N-terminal tails which can be covalently modified in a number of ways [44]. Little is known about the structural role of these tails, so post-translational modifications are instead thought to function by regulating the types of proteins that can interact with a particular histone, which in turn modulate the transcriptional activity of the genomic region [45,46]. An example of this process, termed the histone code model, is heterochromatin protein 1a (HP1a). The chromodomain of HP1 allows it to bind to the tail of histone H3 when it is methylated at lysine nine (H3K9) [47,48]. Once bound, HP1 recruits a complex of silencing factors including more HP1a as well an H3K9 methyltransferase—SU(VAR)3-9—which helps maintain and propagate the modification [49,50]. Numerous genome-wide protein mapping studies (i.e. ChIP and DamID) have largely validated the histone code model insofar as particular combinations of histone modifications have been found to be associated with specific protein compliments and transcriptional

states [51,52]. For instance, one can predict, with a high degree of accuracy, which genes are active in a given cell by mapping the distribution of K9-acetylated H3 histones.

The discovery and characterization of chromatin-modifying proteins has been important not just for the shades of complexity that they add to our understanding of gene regulation, but for the potential role these proteins play in epigenetics—a concept that requires some elaboration. Historically, the term “epigenetic” referred to any non-genetic inheritance of some property or phenotype and was applied mostly to the process of multicellular development and differentiation [53,54]. Currently, most definitions include the added stipulation that the epigenetic information must be associated with a specific chromosomal location [55]. Thus, the inheritance of trans-acting cytoplasmic factors (transcription factors, repressors, small RNAs, etc.) would no longer be considered “epigenetic.”

Although the evolution of the term has led to considerable misunderstanding and misapplication, it is generally accepted that in order to be epigenetic, a phenomenon must be transmissible through one or more cell divisions, have a physical chromosomal location, and not be a consequence of a change in DNA [55,56]. The stable transmission of modified histones seemingly bears all the hallmarks of epigenetics and represents a conserved, chromosome-based mechanism through which gene expression patterns might be inherited. There are, of course, problems with this model. First, the actual

stability of chromatin state is unclear. Various studies using various methodologies have found that nucleosome turnover is greatest at active promoters and seems to correlate with the presence of transcriptional activators and active chromatin modifications while turnover of nucleosomes associated with repressors or silent chromatin modifications is reduced [57,58]. A 2010 study by Deal et al. supported the general relationship between transcriptional activity and nucleosome stability, finding that in *Drosophila* S2 cell culture, the mean nucleosome lifetime ranges from around one hour at transcriptional start sites to 1.5 hours at actively repressed loci [59]. Since S2 cells have a doubling time of approximately 15 hours, nucleosomes—even at repressed sites—would turn over multiple times during a cell cycle. Furthermore, as of yet there is no clear mechanism for the replication and transmission of modified histones during cell division—the question of whether or not histones or the protein complexes which modify them can even remain associated with DNA through the replication fork remains controversial and no semiconservative replication pathway has been identified [60-62].

In addition to questions of stability, it is unclear to what extent chromatin modifications predispose a locus to be either actively transcribed or silenced. Although many studies accept the presence of chromatin modifiers or modifications as evidence of epigenetic regulation—a problem reviewed here [56]—there is little evidence to suggest such factors actually drive transcriptional regulation. Instead, there is evidence to suggest that—much like transcription—

maintenance of chromatin state requires constant input (i.e. the presence of activators or repressors) [63-67]. Thus, while it is clear that many chromatin modifying enzymes are required for transcriptional regulation, their role in establishing any sort of transcriptional memory is suspect.

A much stronger case can be madefor DNA methylation being epigenetic [68]. In eukaryotes, cytosine methylation patterns are established and maintained by various DNA methyltransferases—DNMT3 is thought to mediate *de novo* methylation events while DNMT1 maintains these patterns through replication by interacting with hemi-methylated DNA [69]. While DNA methylation is certainly a chromosome-based phenomenon with a clear semiconservative replication mechanism, it is unclear to what extent it serves as any sort of transcriptional memory. For instance, DNA methylation is often associated with transcriptionally silent genes and many repressors actively recruit DNMTs. However, as is the case with histone methylation, in the event of activation—either by the introduction of an activator or knockdown of a repressor—DNA methylation is rapidly lost [70-72]. While there are several examples of phenomena that require DNA methylation, there is little evidence that general mRNA regulation is one [73-75]. Furthermore, unlike histone and their modifications, DNA methylation does not appear to be an especially conserved gene regulatory system, being largely absent in a number of model species including *Caenorhabditis elegans*, *Drosophila*, and yeast [76,77].

Alternatives to Epigenetics

There are other mechanisms available that can explain epigenetic phenomena—mechanisms which are, perhaps justifiably, overlooked because they cannot precisely be considered “epigenetic.” One such mechanism is the inheritance of non-coding, regulatory RNAs. Initially characterized in *C. elegans* [78], RNA interference (RNAi) has been found in a number of organisms including humans and models systems such as *Drosophila*, fission yeast, and mice. Most RNAi pathways work in the following fashion. Double-stranded RNA molecules—either exogenously or endogenously derived—are cleaved into small, approximately 20 bp fragments which are loaded onto one or more silencing complexes. The RNA-induced silencing complex (RISC) is directed to complementary mRNAs in the cytoplasm and either degrades them or blocks their translation depending on the degree of base pairing. Within the nucleus, small RNAs bind to the RNA-induced transcription silencing complex (RITS) which mediates the pre-transcriptional silencing of complementary genetic loci [79].

Small RNAs can be replicated via several known mechanisms which lead to the accumulation of a heritable pool within the cytoplasm [80,81]. This, along with the discovery of small transcription-activating RNAs derived from promoters and enhancers [82], suggests a clear mechanism for the inheritance of gene expression patterns. Furthermore, since pre-transcriptional silencing by the RITS complex is accompanied by the establishment of silencing chromatin

modifications, small RNAs may also underlie the inheritance of chromatin state. RNAi, as a mechanism, cannot strictly be considered “epigenetic” since it requires the transmission of cytoplasmic factors, and would likely only apply to mitotic or maternally-inherited phenomena. Paternal effects are, in general, much more difficult to account for. Cytoplasm is largely absent in sperm, so only the nucleus is inherited. Furthermore, during spermatogenesis histones are largely replaced by protamines, of which much less is known—though a system similar to the histone code has been proposed [83,84].

Another overlooked alternative to epigenetics might simply be genetics. Epigenetics studies rarely account for the possibility of induced genetic changes, which, to be fair, can often be difficult to identify. Although the genome is considered to be a relatively stable (apart from random mutation), a number of unstable elements have been identified—transposable elements (TEs) historically being the most well-known example [85]. TE mobility requires the expression of one or more products located within the element itself [86].* Upon mobilization TEs can disrupt gene function by either inserting directly into a gene or by inserting into some regulatory element. To avoid this, TEs are suppressed by a number of mechanisms including RNAi and heterochromatin formation, making them subject to the same regulatory pathways that are thought to underlie epigenetic phenomena [87]. A number of studies have found that TE

* In the case of DNA transposons, transposition requires the expression of a transposase enzyme which mediates the “cutting and pasting” of the element. Retrotransposons are transcribed as a whole and encode a reverse transcriptase which creates a DNA copy of the transcript which can integrate into the genome.

silencing can be disrupted by a variety of external factors and suggest that transposition may represent an important mechanism through which the environment might induce long-term changes in expression patterns [88-90].

Although much less understood, instability is also a characteristic of many of the other repetitive sequences found in heterochromatin. Like TEs, many of these sequences are associated with silent chromatin factors, and this association seems to be important for their stability [91]—a topic which will be discussed later in some detail. Unlike TEs, however, it is unclear what effect instability in such sequences might have on gene regulation [92]. While a number of length polymorphisms have been identified in human interspersed satellites—some of which are of disease relevance—these repeats, located throughout euchromatin, represent only a fraction of the repetitive genome [93,94]. Centric heterochromatin blocks lack unique flanking sequences making RFLP (restriction fragment length polymorphism) and PCR (polymerase chain reaction) analysis problematic, and their repetitive nature makes aligning contiguous sequences impossible. Although a number of studies have revealed a great deal of variation within various classes of heterochromatic repeats, the origins and phenotypic consequences of such variation are only just now being explored [95-98].

The *Y* Chromosome of *Drosophila melanogaster*

Addition and subtraction are both powerful tools for characterizing the functional components of any genetic system and heterochromatic sequences

are no exception. The difference in this case is one of scale. A point mutation or simple insertion/deletion in a highly repetitive sequence is unlikely to have an effect since many such sequences are non-coding and are present in hundreds or even millions of copies throughout the genome. For this reason, the *Drosophila Y* chromosome has been an indispensable tool for understanding the role of repetitive sequences in genome regulation. Necessary only for male fertility, the *Y* chromosome can be partially or completely removed or duplicated with no effect on sex determination or survival. *X/Y*, *X/0*, *X/Y/Y* males, and *X/X/Y* females can all be obtained, and apart from *X/0* males, all are fertile [99,100]. Furthermore, the *Y* chromosome is almost entirely repetitive containing only a handful protein coding genes [101,102]. The bulk of the chromosome is composed of blocks of tandemly repeated satellite sequences, while the remainder is made up of transposable elements, transposable element remnants, telomeric repeats, and rDNA [17,103].

In addition to being one of the strongest suppressors of PEV [104], recent studies have shown that uncharacterized variation on the *Y* chromosome can dominantly affect the expression of thousands of genes throughout the *Drosophila* genome [105]. Lemos and colleagues placed *Y* chromosomes obtained from geographically diverse populations (Massachusetts, Ohio, Zimbabwe, and Congo) into otherwise identical genetic backgrounds. They then performed transcriptional analysis via microarray to identify genes that were differentially regulated. Significant differences were found in up to a thousand

genes which mapped throughout the genome, demonstrating generality of *Y*-linked regulatory effects. Additional studies demonstrated that these chromosomes differentially affect variegation of the w^{m4} allele [106]. At the time, these results were attributed to “cryptic” variation on the *Y* chromosome—presumably unmapped repetitive sequence polymorphisms [92]. While until recently there was no way to investigate this hypothesis, subsequent studies performed in our lab have demonstrated that variation within the rDNA array is a significant contributor to *Y*-linked regulatory variation (YRV) [107].

Ribosomal RNA Genes (rDNA)

rDNA is a repetitive array of Ribosomal RNA (rRNA) genes found in most eukaryotes. Each cistron within the tandem array contains a promoter, located in the non-transcribed spacer sequence (*NTS*), and is individually transcribed by RNA polymerase I as a single pre-rRNA molecule that is processed into a number of rRNA subunits. The *Drosophila* rDNA cistron encodes a *35S* pre-rRNA which is processed into *18S*, *28S*, *5.8S* and *2S* molecules via the removal of several external (*ETS*) and internal transcribed spacer (*ITS*) sequences (Figure 1.2). Mature rRNA molecules combine with a number of ribosomal proteins as well as the RNA polymerase III transcribed *5S* rRNA to form a functional ribosome [108,109]. This entire process occurs within a subnuclear compartment called the nucleolus. Since rDNA is required for nucleolus formation, rDNA loci are sometimes referred to as nucleolus organizing regions

(NORs). In *Drosophila melanogaster*, there are two such arrays located on the *X* and *Y* chromosomes.

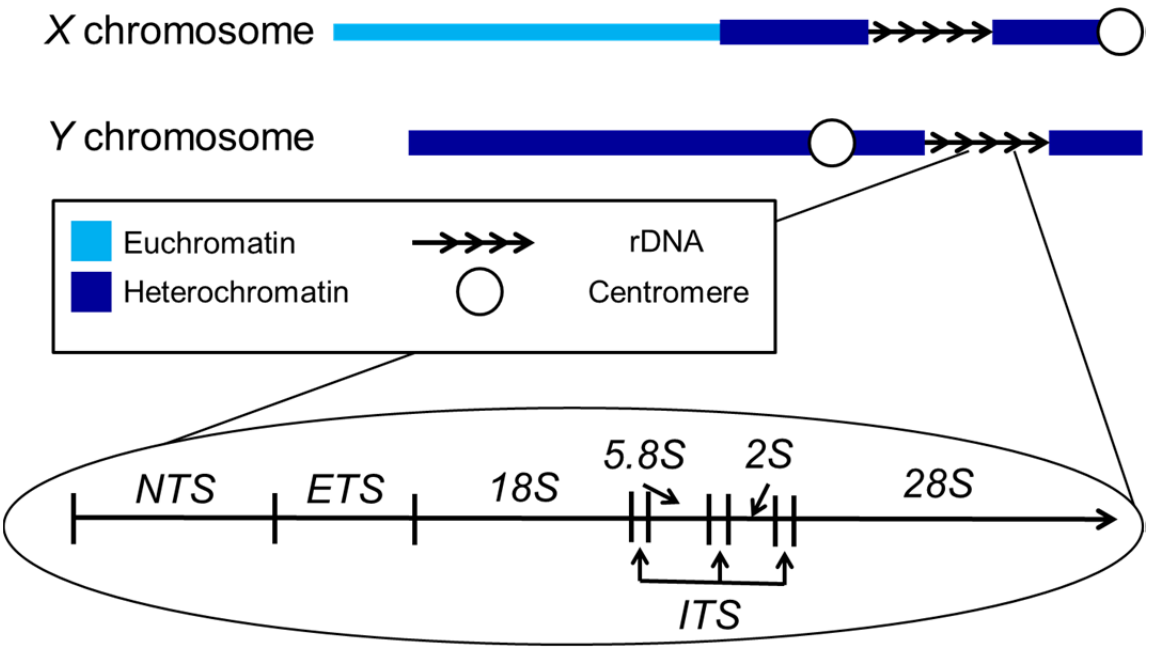

Figure 1.2 Location and sequence arrangement of *Drosophila* rDNA. rDNA cistrons are found within repetitive arrays on the *X* and *Y* chromosomes (top illustration). An individual cistron is illustrated below. *NTS* = nontranscribed spacer; *ETS* = external transcribed spacer; *ITS* = internal transcribed spacers. Adapted from [26] and [110].

rRNA transcription is estimated to account for approximately 50% of transcription at a given time and over 80% of steady state cellular RNA levels [111,112]. Despite this relative overrepresentation and the fact that it is frequently used as a loading control in transcription analyses, rRNA transcription is subject to regulation. Since an average wild-type array in *Drosophila* consists of approximately 150-250 copies of rDNA and only around 100 are thought to be

required, the redundant copies must be silenced [113]. Transcriptional repression is thought to be a relatively orchestrated process since select copies—or, in some cases, entire arrays—are preferentially inactivated [114].

Control of rDNA expression occurs at a number of levels. At the DNA level, rDNA transcription is regulated by one or more promotor/enhancer elements found within the *NTS* region. The number of these elements is variable between copies and positively correlates with RNA polymerase I recruitment and thus the transcriptional output of a given cistron [115-117]. Recruitment of RNA polymerase I is preceded by the binding of a pre-initiation complex composed of common and RNA polymerase I-specific components [118]. Given the importance of rRNA transcription in cellular growth and proliferation—both as an energy consumer and as a limiting factor of protein synthesis—many RNA polymerase I co-factors are under the regulation of numerous input pathways [119]. For example, the recruitment of transcription initiation factor IA (TIF-IA) requires phosphorylation by target of rapamycin (TOR), an important downstream target of many nutrient sensing pathways, including insulin/insulin-like signaling (IIS) [120,121]. As with other genes, chromatin is also an important regulator of rDNA. Transcription requires c-Myc [122,123]—a histone acetyltransferase-recruiting transcription factor—while repression requires the activity of methyltransferases and deacetylases such as SUVAR3-9 and SIR2 respectively [124]. The involvement of c-Myc and SIR2 is notable because it further links the regulation rRNA transcription to growth and proliferation. c-Myc

is a downstream component of EGF and similar growth, proliferation, and differentiation-signalling pathways, while SIR2 is a histone deacetylase whose activity is dependent upon NAD^+ and is therefore directly related to an organism's nutritional status.

The contribution of chromatin factors has suggested an epigenetic mechanism for some of the more peculiar aspects of rDNA regulation. All rDNA copies do not behave the same in regards to transcriptional activity. A single array will usually possess both highly active and completely silent copies and these can often be found directly adjacent to one another [125,126]. While this is presumably necessary to compensate for rDNA cistron redundancy, it does raise the question of why simply reducing the output of all cistrons isn't an option (i.e. by downregulating RNA polymerase I for instance). Furthermore, it remains largely unknown how silent and active copies are specified. In *Drosophila* and other arthropods, many silent cistrons are interrupted by one or more retrotransposons known as R1 and R2 elements [127,128]. Inserted cistrons are often silenced, but this is not always the case—some inserted cistrons are transcribed while some uninserted cistrons are silent [126]. On a much larger scale, separate rDNA arrays within the same nucleus can display dramatically different expression patterns—a phenomenon known as nucleolar dominance. In males, the *Y*-linked array is typically active while the *X*-linked array is silent—but not always. In females, both *X*-linked arrays are usually co-dominant, but again there are exceptions [129,130]. As is the case for individual cistrons, it is unclear

what specifies dominance/co-dominance—be it sequence variations, some epigenetic mark, or something else entirely.

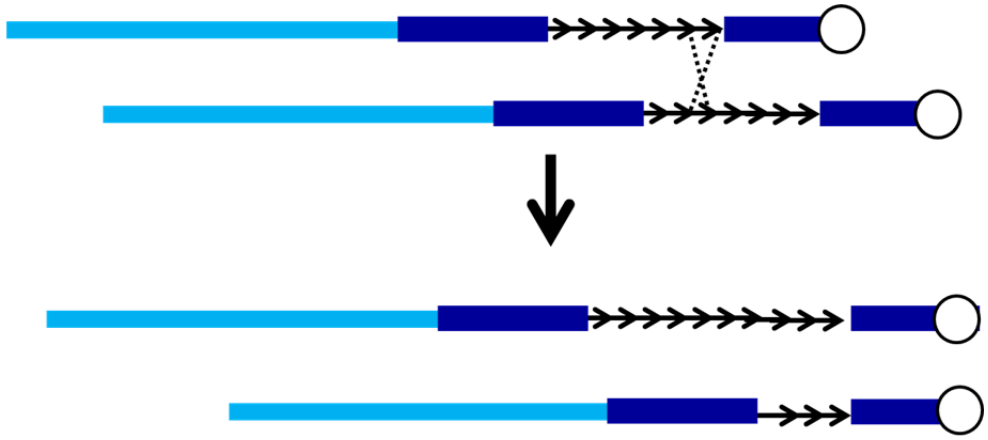

Figure 1.3 Interchromosomal rDNA recombination. When recombination (dashed line) occurs unequally between rDNA arrays (small arrows) located on separate chromosomes— homologous chromosomes or sister chromatids—the result is magnification of one array and partial deletion of the other.

rDNA Instability

Apart from energy concerns and translational capacity, regulation of rDNA is thought to be especially critical due to the relationship between rRNA transcription and rDNA stability. This proposed link is based on studies in *Drosophila* and *Saccharomyces cerevisiae* demonstrating that the removal of a variety of silencing factors (DCR-2, SUVAR3-9, HP1, PARP, CTCF, and SIR2) leads to an increase in rRNA transcription accompanied by an increase in DNA damage and hyper-recombination at the rDNA locus [110,131-134]. Typically, when damage such as a double strand break (DSB) occurs in a low-copy

number sequence (i.e. a single copy gene) the free ends are simply identified and rejoined in a process known as non-homologous end-joining. In the case of rDNA, or any repetitive sequence array, the large concentration of complementary sequence promotes strand invasion and repair via homologous recombination. When ***inter***chromosomal homologous recombination occurs between unlinked arrays, chromosomal fusions and/or unequal exchange can occur leading to either an increase or decrease in the size of a particular array (Figure 1.3). ***Intra***chromasomal recombination occurring within a single array results in the formation of extrachromosomal circles (eccDNA) (Figure 1.4) [135-137]. In closed mitotic systems, these circles are retained within the nucleus and accumulate over time. In the budding yeast, *S. cerevisiae*, extra-chromosomal rDNA circles are preferentially retained in the mother cell and are thought to contribute to cellular aging [138]. In open mitotic systems, such as *Drosophila* and mammals, non-segregating eccDNAs are lost from the nucleus upon the dissolution of the nuclear envelop and eventually degraded.

Although many regulatory factors have been identified, the precise mechanism underlying rDNA instability remains largely unknown. Apart from a few specific exceptions [139], instability is not thought to be an adaptive regulatory mechanism, rather it is assumed to be an unavoidable consequence of the arrangement and regulatory properties of rDNA. Current models can be divided into two basic categories, which I will refer to as the “transcription-dependent model,” and the “chromatin protection model.” Distinguishing these

models is difficult due to the inextricable link between transcription and chromatin dynamics. Since chromatin remodeling is required for transcription to occur, anything that induces hyper-transcription will necessarily induce the removal of silencing chromatin. Conversely, since chromatin modifications are required for proper transcriptional repression, removal of any silencing components will induce hyper-transcription. Analysis is further complicated by the fact that many rDNA regulatory components are required for survival and are involved in the transcriptional regulation of many other genes. Thus, mutations in factors regulating rDNA instability are often lethal and/or have highly complex phenotypic effects.

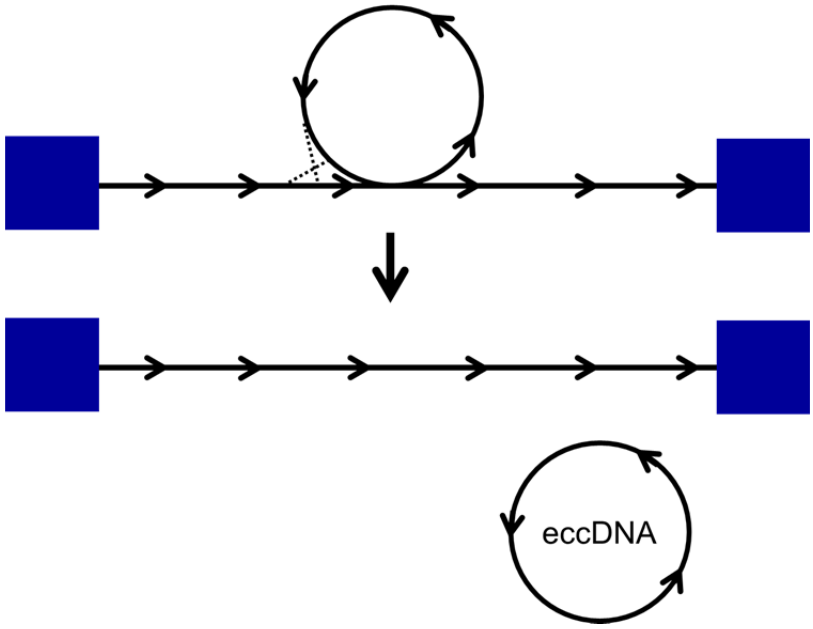

Figure 1.4 Intrachromosomal rDNA recombination. When recombination (dashed line) within an individual array (arrows) occur, the result is a deletion accompanied by the formation of an extrachromosomal circle (eccDNA)

The transcription-dependent model holds that some aspect of transcription induces DNA damage leading to hyper-recombination and instability. In yeast, damage is thought to result from a collision between transcription and replication machinery with both RNA polymerase I and Fob1—a replication fork blocking protein[140]. Much less is known about the mechanism of rDNA instability in *Drosophila*—a similar fork blocking mechanism is not known to exist and damage can be observed in post-mitotic cells suggesting that the phenomenon can occur independently of replication.

Another model for rDNA damage and instability is that silent chromatin confers a protective property to DNA, insulating it from a variety of possible damage sources (ionizing radiation, rogue nucleases, oxidizing agents, etc.). When silencing chromatin is removed, either by mutation or some normal process such as chromatin remodeling during transcription, this protective property is lost and damage rates increase. Thus, while the transcriptional machinery is not required to induce damage, highly transcribed regions would naturally experience a greater propensity for damaging events. Supporting this idea is a number of studies showing that repetitive sequences, in general, are susceptible to the same type of instability observed at the rDNA locus [131,136,141,142]. Using two-dimensional agarose gel electrophoresis, Cohen et al. found evidence for circular DNA fragments originating from tandemly repeated sequences including the histone cluster, the stellate locus, and centric heterochromatin. Similarly, Peng and Karpen found that mutations in a variety of

heterochromatin factors resulted in damage to the underlying repetitive DNA sequences—sequences which were thought to be largely transcriptionally silent [143].

rDNA Variation and the Consequences Thereof

While the mechanism underlying rDNA instability remains unresolved, the direct outcome of such instability is clear: rDNA arrays are highly variable – between laboratory stocks and natural populations, and even within individual organisms. Studies in *Drosophila* have found an up to six-fold difference in rDNA copy number in wild caught populations and anecdotal evidence from our lab and others suggests that many laboratory stocks possess significantly different array sizes [107,113,132,144]. Although the study of human rDNA is complicated by the fact that we possess five arrays on five different chromosomes, RFLP analysis has revealed a high degree of variation [145]. As would be expected, parents possessing unique rDNA variants produced children with RFLP patterns consistent with a blending of those variants. Perhaps unexpectedly, researchers found RFLP variants in children that were not present in their parents, suggesting the *de novo* appearance of rDNA polymorphisms in the germline or during development. This conclusion was further supported by the discovery of unique variants in differing tissue types.

Due in part to the heretofore “hidden” nature of repetitive sequence variation, the phenotypic consequences of such variation have been left largely unexplored. In *Drosophila*, rDNA was initially dubbed the *bobbed* (*bb*) locus due

to its association with a shortened bristle phenotype. This phenotype is visible when an rDNA-deficient *X* or *Y* chromosome is placed into a background completely lacking any other rDNA array. Although the small array is often enough for survival, flies possessing such arrays exhibit a range of bristle and cuticular phenotypes depending on the severity of the deletion with extreme deletions resulting in death during the larval stages (Figure 1.5). This phenotype is thought to stem from reduced translational capacity due to the decrease in rRNA production [146].

The *bobbed* phenotype has been used to analyze a variety of rDNA deletions, to screen for magnification events, and has been a generally helpful tool for understanding rDNA instability [144,147-149]. Its recessive quality, however, makes it less useful for understanding the phenotypic effects of the type of rDNA variation observed in nature. For instance, what effect might an rDNA deletion have in a wild-type genetic background where an extra array could compensate for the deleted copies? One theory, known as the “chromatin balance model,” makes a couple of predictions. This model suggests that there is a finite amount of heterochromatin factors in a cell and that if one were to introduce extraneous heterochromatic sequences then such factors would be diluted and heterochromatic silencing would be suppressed [26,150]. This effect is demonstrated by the strong suppression of PEV observed with the introduction of an extra *Y* chromosome ($w^{m4}/Y/Y$) [104]. Would the inverse be true? Would reducing a heterochromatic compartment, such as rDNA lead to

increased silencing capacity? Would a transcriptionally active locus such as rDNA even behave like heterochromatin that situation? These are the sort of questions that previous work from our lab sought to address.

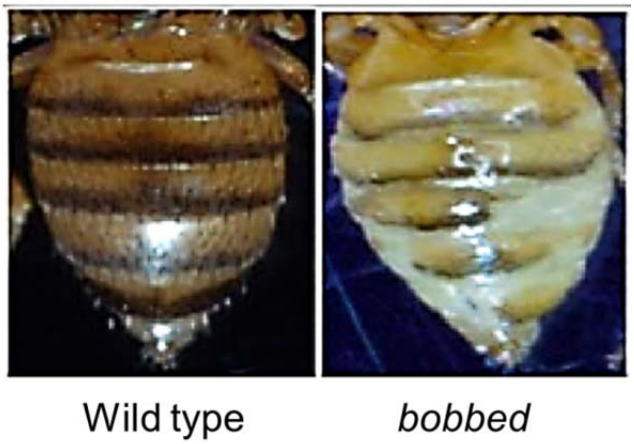

Figure 1.5 The *bobbed* phenotype. Severe rDNA deletions result in the *bobbed* phenotype which is characterized by the incomplete formation of the abdominal cuticle (right) and shortening of some bristles. Figure adapted from [26].

Paredes and Maggert created an isogeneic *Y*-rDNA deletion series via the expression of I-CreI, an endonuclease whose sole *Drosophila* target is found within the rDNA [151]. I-CreI expression was induced in males bearing a *Y* chromosome marked by the morphologically distinguishable *yellow* gene (called *Y, 10B*) and numerous isogenic lines were created from their progeny. *Y* chromosomes with rDNA deletions were identified by their ability to cause a bobbed or lethal phenotype when paired with the *C(1)DX* chromosome, a compound *X* chromosome completely lacking rDNA. *Y*-rDNA deletions were

quantified using Real Time PCR and the relative size of the deletion largely corresponded to the severity of the phenotype [152].

To test the effects of these *Y*-linked rDNA deletions on the strength of heterochromatin silencing, Paredes and Maggert paired them genetically with a variety of variegating alleles (w^{m4}, w^{m4h}, $Stubble^{v}$, and $light^{var}$*). Unexpectedly, they found that rather than being enhancers, *Y*-rDNA deletions were strong suppressors of heterochromatic silencing and that suppression again correlated with the severity of the deletion [132]. The relationship between rDNA copy number and PEV suppression has been further supported in experiments using naturally polymorphic chromosomes isolated from the wild, as well as experiments comparing differentially expressing cell patches from the same organism [107,132].

Although these results don't really clarify the chromatin balance model, they do suggest a dominant role for rDNA copy-number variation—natural or laboratory engineered—in the regulation of gene expression. Subsequent microarray studies have revealed that as many as 1500 genes—extrapolated to around 40% of the *Drosophila* coding genome—are sensitive to rDNA copy number [107]. Differentially expressed genes are located throughout the genome and exhibit no clear bias in regards to heterochromatin proximity. Furthermore,

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* *light* is a gene normally located within heterochromatin and requires heterochromatin proteins to be expressed. $light^{var}$ behaves in an opposite manner relative most variegating alleles in that mutations that decrease heterochromatin (*Su(var)*s) actually reduce its expression. *Y*-rDNA deletions were found to enhance the variegation of $light^{var}$ (i.e. reduce its expression), which is consistent with a role for rDNA in regulating heterochromatin stability.

no known heterochromatin protein genes were found to be differentially expressed making it difficult to formulate any clear hypotheses as to the mechanism underlying rDNA's effect upon PEV. Functional categories that *were* overrepresented include mitochondrial function and lipid metabolism.

Research Aims

Induced, phenotypically consequential variation within repetitive DNA sequences represents a largely unexplored avenue through which the environment might influence the long-term gene expression patterns of an organism or their offspring. Previous work in our lab identified variation within the rDNA in particular as a powerful regulator of genome-wide transcription levels [107,132]. While instability is a known property of rDNA arrays and a number of mutants have been shown to affect it [113,131,144], the extent to which the environment influences the process remained unknown. The first aim of the research presented here was to identify an environmental factor that could potentially underlie natural rDNA instability. Given that rDNA instability is related to rRNA transcription [140] which is in turn related to nutrient availability [119,153], I sought to test the effects of diet on heritable rDNA copy number—work which is summarized in Chapter II.

Apart from changes in gene transcription and PEV, little else is known about the phenotypic consequences of rDNA variation. Genes involved in lipid metabolism and mitochondrial function were found to be sensitive to rDNA copy number suggesting a link between rDNA variation and the regulation of cellular

metabolism [107,154]. The work discussed in Chapter III deals with the effects of *Y*-rDNA deletions on starvation resistance—a phenotype ultimately governed by both energy storage and consumption rates.

The large size and complex nature of the individual cistrons makes rDNA relatively easy to quantify and manipulate in comparison to the various satellite sequences (AACAC*n*, AAGAG*n*, etc.). Evidence suggests, however, that rDNA variation constitutes a fraction of *Y*-linked regulatory variation [107], indicating that satellite variation may also be important. The aim of the work detailed in Chapter IV was to develop a technique to more easily quantify satellite sequence variation and to use that technique to identify natural and mutationally-induced variants.

CHAPTER II

TRANSGENERATIONAL INHERITANCE OF DIET-INDUCED rDNA DELETIONS*

Introduction

It is clear that an organism's gene expression patterns are responsive to environmental input. Often, this influence is not limited to short-term regulatory changes, but can persist through multiple cell divisions and can, in some cases, be transmitted to offspring. Typically, such changes are identified as "epigenetic" and are thought to be mediated by a variety of chromatin modifications [155-162]. However, because genome stability—particularly of repetitive sequences—is modified by silencing involving repressive histone modifications, "epigenetic" perturbations may have both direct and long-term consequences: the former caused by disruption of silencing leading to chromatin changes, and the latter by creating transmissible changes to chromosomes that themselves may affect gene regulation. This consideration significantly adds to models of epigenetic inheritance which often overlook the ease with which histones and DNA methylation can be modified and the rapid rate at which they are turned over in non-dividing cells [59,163]. Recent [56,164-166] and previous [67] findings show epigenetic silencing is unstable even in non-dividing cells, making it a

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particularly difficult challenge to reconcile models of chromatin (e.g., histone) mediated epigenetic silencing with transgenerational (i.e., mitotic and/or meiotic) inheritance.

35S Ribosomal RNA gene (rDNA) transcription has been a powerful model for understanding the regulatory effects of chromatin modification because evidence suggests identical genes may adopt different stable activity states (expressed versus repressed) even when immediately juxtaposed [167-170]. Transcription from tandem repeated rDNA arrays accounts for approximately 50% of total transcription [111] and is regulated such that only a subset of the redundant copies are active in a given cell type, while the remainder are inactive and are accompanied by chromatin structure consistent with silencing [108,171]. Consequences of misregulation are severe, in part due to the tandem repeat of identical sequence. Mutations in silencing factors (e.g., gene products of the *dcr-2*, *Su(var)3-9*, *HP1/Su(var)205*, *PARP*, *CTCF*, and *sir2/sirt* loci) result in supernumerary mini- or micro-nucleoli and rDNA copy number reduction [110,131-134], possibly through increased frequency of intrachromosomal recombination resulting from the repair of transcription-induced damage [136,140]. The tendency for natural loss and the ability of some rDNA arrays to expand through unknown processes [135,137,149] contribute to striking variation in rDNA copy number in both wild and laboratory strains [14,109,113,172]. This variation, in turn, is a potent genetic modifier of a number of phenomena, including the regulation of ecologically- and metabolically-

relevant gene networks, the stability of genome structure and heterochromatin silencing, stress responses, and potentially metabolic function [26,132,133,154,173-180].

The relationship between rDNA transcriptional activity and rDNA array stability suggests a non-epigenetic mechanism through which the environment might induce heritable and consequential changes in the genome through long-term (i.e., permanent) modulation of genetic variation and epigenetic stability. Although the change may bear the hallmarks of epigenetic regulation (i.e., inducible, heritable, consequential), it may not technically be considered epigenetic because it involves chromosome changes [181]. Nonetheless, because rDNA copy number modulates the stability of epigenetic silencing [132,133,178], the origin of rDNA copy number variation is a significant concern to studies of “hidden” regulatory variation, heterochromatin, and epigenetics.

The aim of this study was to identify a natural source of rDNA copy number variation. Expanding on previous work suggesting that an increase in rDNA expression may lead to its loss, I hypothesized that rDNA copy number changes can be induced by modulating diet. In support of this hypothesis, I found that flies raised on high dietary yeast concentrations had increased supernumerary nucleoli as larvae, and somatic rDNA copy-number reductions as adults. Similar results were observed in flies expressing a hypermorphic insulin receptor allele, and were reproduced pharmacologically using human insulin *in vitro*, suggesting that diet-induced rDNA instability is mediated by

known nutrient signaling pathways. Drugs that inhibit expression of the rDNA mitigated instability and loss, suggesting the effects were a consequence of expression. Furthermore, adult males fed high-yeast diets produced offspring with fewer *Y*-linked rDNA copies demonstrating that the effect was transgenerationally heritable. These findings identify diet as a potential source for rDNA variation observed in natural populations and suggest a mechanism through which environmental conditions might result in induced "transgenerational" genome changes.

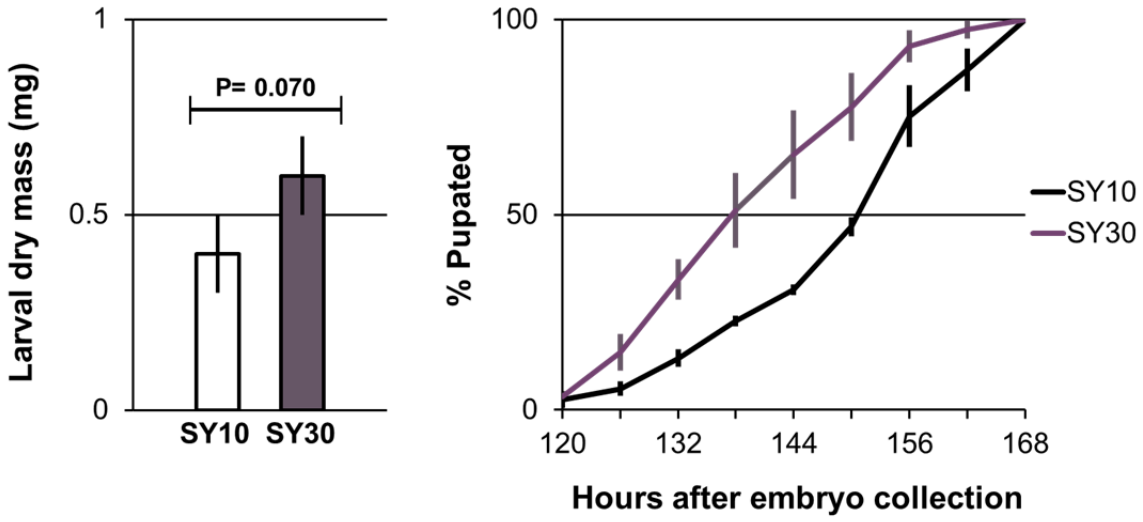


Figure 2.1 The effects of dietary yeast concentration on larval mass and development. The graph on the left shows the average dry weight of larvae raised on either SY10 or SY30.P-value calculated suing Student's t-test. Time to pupation is plotted on the right. Error bars represent standard deviation of three independent experiments.

Results

Previous work from yeast, filamentous fungi, *Drosophila*, plants, and experimental mammalian systems have suggested that rDNA expression increases in response to diet, while other work has shown that derepression of the rDNA results in instability and loss. Together, these imply that natural rDNA variation may be reflective of the nutritional history of a population. Because of the growing awareness of the importance of rDNA instability and copy number variation in phenotypic variation, stress response, and disease, I directly tested the hypothesis that rDNA copy number may be manipulated by affecting diet.

Dietary composition is an easily modified environmental variable in *Drosophila* laboratory studies and has been shown to influence a variety of complex phenotypes including rRNA transcription and ribosome biogenesis [124,153,182,183]; I therefore considered it as a potential source of “natural” rDNA instability and variability. In this study I used two experimental media based on those used in dietary restriction studies [184], consisting of a constant carbon source (5% sucrose) and altered concentrations of nutritional yeast (10%, and 30% w/v) These diets are referred to as SY10 and SY30. Larvae raised on SY30 were somewhat heavier than those raised on SY10 and developed to pupation approximately nine hours earlier (Figure 2.1). Apart from this observation, there were no obvious differences in terms of adult body mass or survival between flies reared on the different media. To obviate secondary effects on culture conditions based on crowding, and to assure similarity in

conditions as much as possible, I controlled density of eggs, larvae, and adults in vials by collecting eggs from petri dishes containing agar made from apple juice, suspending eggs in PBS, and pipetting identical volumes of eggs to SY10, SY30, or standard cornmeal medium.

I first confirmed that altered diet affected rRNA expression. I could discriminate accumulated mature rRNA products (*18S, 28S, 5.8S*) from actively-transcribed pre-rRNAs (*35S*) by detecting the quantity of cDNA derived from the pre-processed 5'-most sequence of the *35S* primary transcript containing the External Transcribed Spacer (*ETS*). The *ETS* is constitutively processed during maturation of the pre-rRNA *35S* transcript and quickly degraded, and is therefore used to measure de novo rDNA expression [133,178,185]. I compared male flies of genotype *yellow*^{1} *white*^{67c23}*/Dp(1;Y) y*$^{+}$*, P{w=RS5}10B* (henceforth *Y,10B*), upon which our lab has performed other studies of the rDNA [26,98,132,152,186]. I detected an approximately 50% increase in pre-rRNA levels in populations of second instar larvae raised on SY30 compared to Standard media—though there is considerable overlap. Results from a northern blot were consistent with these findings, confirming the suitability of these media for this study (Figure 2.2).

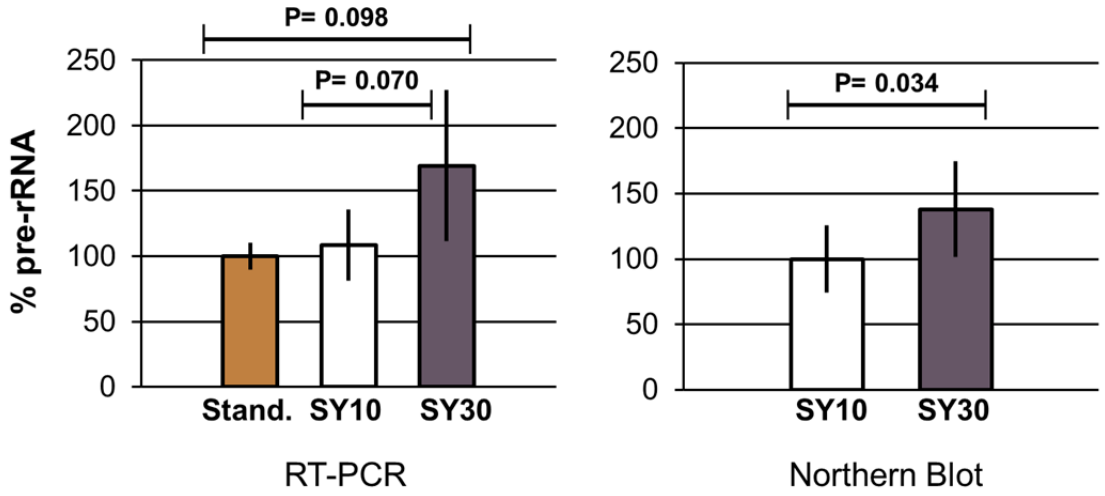


Figure 2.2 The effect of diet on rRNA expression levels. Real-Time PCR quantification of cDNAs derived from unprocessed (*ETS-18S* junction) pre-rRNA from larvae fed either SY10 or SY30 diets (Left). Values were normalized to the genomic DNA copies of $tRNA^{K\text{-}CTT}$ genes and proportions plotted relative to Standard-fed larvae (defined as 100%). Error bars report standard deviation of RNA quantities derived from 3-5 independent pools of larvae for each condition. Northern blot quantification is shown on the right. *35S* pre-rRNA values were normalized to mature rRNA levels and plotted relative to SY10. Error bars report standard deviation of three independent samples. All P-values were calculated using Student's t-test. Figure adapted from [187].

I next addressed whether an increase in dietary yeast concentration during development would result in rDNA loss. In interphase cells, the rDNA is the genetic location of the cytogenetic Nucleolus Organizing Region (NOR), and thus the foundation of the nucleolus. Even single rRNA genes are capable of forming tiny supernumerary nucleoli [188]. Consequently, nucleolar morphology is sensitive to the overall size, activity level, and integrity of the rDNA arrays. In *Drosophila*, rDNA damage is readily observed in larval salivary glands. Damage and repair are thought to lead to extrachromosomal circles, which coalesce mini- or micro-nucleoli in non-dividing or post-mitotic cells. Such "fragmentation" has

been observed in flies mutant for heterochromatin components [110,131-133], where it is thought to stem from aberrant intrachromosomal recombination resulting in the formation of extrachromosomal rDNA circles [91,136]. I performed immunofluorescence on larval salivary gland cells with an anti-fibrillarin antibody to detect the nucleolar dense fibrillar component which, in *Drosophila*, typically forms a single focus containing both *X*-linked and *Y*-linked rDNA arrays (Figure 2.3). I observed an elevated frequency of nucleolar fragmentation in SY30-fed larvae compared to SY10-fed larvae. Multiple nucleoli (defined as more than one discrete separate fibrillarin focus) were present in 40% ± 24% of the nuclei within single salivary glands dissected from SY30-fed larvae; in contrast multiple nucleoli were observed in 7% ± 6% of salivary gland nuclei from SY10-fed larvae.

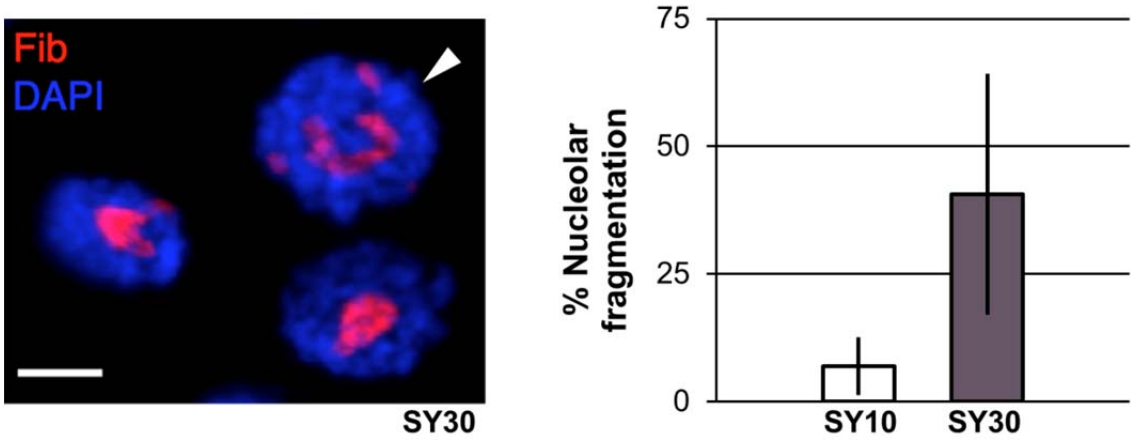


Figure 2.3 Diet affects nucleolar stability. To the left is a representative image of salivary nuclei taken from a third instar larvae raised on SY30. Nuclei are stained blue with DAPI and nucleoli are stained red with an anti-fibrillarin antibody. The white arrowhead indicates a fragmented nucleolus and the scale bar represents 20 μm. Nucleolar fragmentation in SY10 and SY30-fed larvae was quantified (right). Values represent the average percentage of fragmented nucleoli per 20X microscope field and error bars represent standard deviation between individual fields. 337 and 522 nuclei were scored in total for SY10 and SY30 respectively. Significantly more fragmentation was observed SY30-fed flies (P=0.023). Figure adapted from [187]

In dividing cells, acentric extrachromosomal rDNA circles are lost, effectively reducing rDNA copy number and shortening the rDNA array through development. To quantify rDNA loss stemming from diet-influenced extrachromosomal circle formation during development, I used RT-PCR to measure the rDNA abundance of flies raised as larvae on either SY10 or SY30. Relative copy number was quantified using genomic copy number of a tRNA gene as normalization resulting in a DNA-to-DNA proportion [152]. rDNA copy number differences were monitored by comparing the rDNA copy number in freshly eclosed F1 males to that of males taken from the “F0” parental stock (i.e.,

treated males compared to their fathers and uncles). Male adults were collected within hours of eclosion, thus any diet-influenced changes to rDNA copy number were the result of physiological effects initiated prior to metamorphosis. The rDNA copy number of flies raised on SY10 was indistinguishable from that of their sires, while those raised on SY30 exhibited an average copy number reduction of about 20% (Fig 2.4).

The quantified loss of rDNA due to SY30 is within the range of natural *Y*-linked rDNA variation [92,113,152], and within the experimental range used to demonstrate heterochromatin changes and gene regulatory variability by us and others [92,132] on the *Y*, *10B* chromosome specifically. Therefore, altered diet could in principle be responsible in entirety or in part for the natural rDNA copy number variance observed in natural populations isolated from the wild.

In yeast, it is thought that rDNA damage and loss is a consequence of a collision between the replication fork and transcriptional machinery at the rDNA array [140]. This model accounts for the transcription-dependent nature of rDNA recombination as well as the relationship between rDNA stability, cell division, calorie-restriction, and aging [134,182,189]. Much less is known about the regulation of rDNA stability in multicellular organisms and, therefore, the precise mechanism underlying my observations is unknown.

If instability is a consequence of changes in nutrient availability, then I reasoned that modulating known nutrient signaling pathways should produce similar results. In *Drosophila*, as in *C. elegans*, mouse, and human, the

insulin/insulin-like growth factor signaling (IIS) and TOR signaling networks mediate many cellular responses to nutrient availability, including ribosome biogenesis and rDNA expression [121,124,190]. I expressed a constitutively active form of the insulin receptor (InR.R418P) in larvae (using a *Ubiquitin* promoter to drive GAL4 expression in all cells) and observed the effect on nucleolar stability in salivary glands. I observed elevated levels of multiple nucleoli (approximately 41%) (Figure 2.5), indicating that activating the Insulin Receptor was sufficient to induce instability.

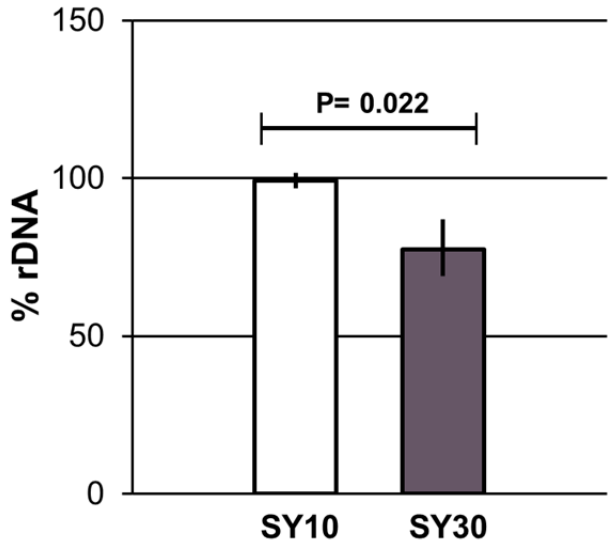


Figure 2.4 Quantification of somatic rDNA loss via RT-PCR. Real-time PCR analysis of *35S* rDNA copy number in adult males raised on SY10 or SY30 as larvae. rDNA levels are normalized against genomic copies of $tRNA^{K\text{-}CTT}$. Percentages are calculated relative to isogenic flies raised on standard food (defined as 100%). Error bars are standard deviation of three independent biological replicate populations. Figure adapted from [187]

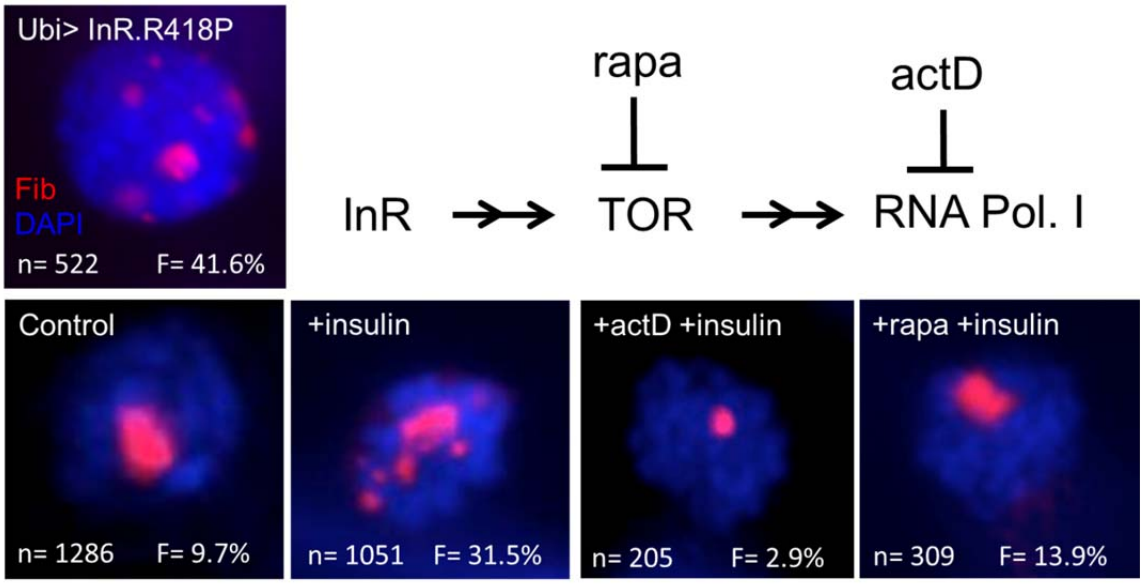


Figure 2.5 Perturbation of nutrient signaling affects nucleolar stability. The top left image shows a representative salivary gland nucleus from larvae expressing a hyperactive insulin receptor allele (*Ubi*>InR.R418P). Nucleolar fibrillarin is highlighted in red, "n" is the number of nuclei scored, and "F" is the frequency fragmented nucleoli observed. The bottom four images show nuclei from cultured salivary glands treated with various combinations of recombinant human insulin, actinomycin D (actD), and rapamycin (rapa). A diagram summarizes the relationship of these factors and RNA polymerase I activity. Figure adapted from [187].

In order to limit my view to acute cell-autonomous effects of nutrient signaling perturbation, I next opted to modulate the activity of these pathways pharmacologically in cultured larval salivary glands, which are a fully developed, post-mitotic tissue. In this way I could separate developmental defects (as a result of prolonged expression of a hypermorphic allele) from acute defects caused by altered cell physiology. I dissected larval salivary glands from flies expressing a fibrillarin-RFP fusion protein [191] whose expression was controlled by a heat shock responsive *hsp70* promoter. I did not induce

expression with heat shock because sufficient nucleolar RFP was detectable without heat shock. Salivary glands were cultured in *Drosophila* cell/tissue culture medium for 22 – 24 hours in the presence (or absence) of recombinant human insulin.

Treatment with insulin resulted in increased supernumerary nucleoli in live salivary glands (Fig 2.5). Exposure to either actinomycin D or rapamycin (two drugs which block RNA polymerase I activity, the former by inhibiting polymerase procession via intercalation and the latter by inhibiting TOR) for two hours prior to insulin addition abrogated the multiple nucleolar morphology. I confirmed that drug treatment reduced active rRNA expression by culturing eviscerated whole wild-type larvae in tissue culture medium for 24 hours in the presence of rapamycin or actinomycin D alone. RT-PCR quantification of cDNAs of pre-processed rRNA junctions (as in Fig 2.2) were reduced to 80% (+19.8%/-15.9%) and 69% (+13.1%/-11%) (N = 10 larvae for each condition), respectively, compared to control larvae cultured without any drug. The fact that nucleolar instability is enhanced by insulin and mitigated by rapamycin and actinomycin D suggests that the consequential effect on rDNA stability occurs downstream of the convergence of the activities of these pharmacological agents.

Wild-caught *Drosophila* strains exhibit a wide variance in rDNA copy number [14,113]; the source of this variance, however, is unknown. For this variability to be explained by environmentally induced instability during the life history of these chromosomes, germline rDNA would have to be susceptible to

environmental influence. To look for possible germline effects of diet, I used a genetic strategy to specifically measure copy number of *Y*-linked rDNA (Figure 2.6). I chose to focus my attention on the *Y*-linked array because it is preferentially active in males [130,178] and because of the ease with which the Y chromosome is manipulated genetically [152]. I genetically-isolated Y-linked rDNA arrays by crossing adult males to females bearing an rDNA-deficient compound *X* chromosome (*C(1)DX*). Female progeny of this cross were viable and carried the patroclinous *Y*-linked rDNA as their sole source of rRNA genes; thus, any differences between rDNA in daughters were due to permanent germline changes to the chromosomes occurring in the fathers.

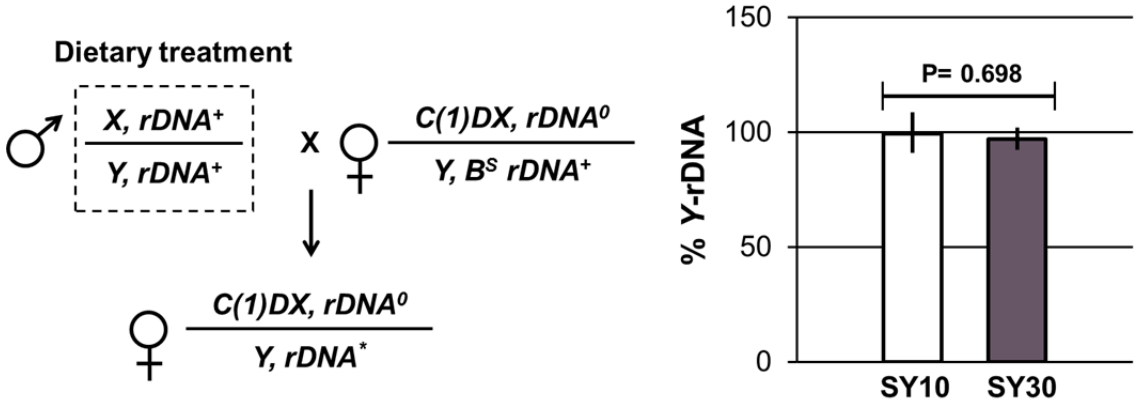


Figure 2.6 Larval diet does not induce germline rDNA copy number changes. *Y*-linked rDNA arrays from adults raised as larvae on Standard, SY10, and SY30 media were genetically isolated using the crossing scheme shown left. The progeny of this cross bear the array of interest (*Y, rDNA**) as their sole source of source of rDNA. Isolated arrays were quantified using RT-PCR (right). Percentages were calculated relative to males maintained on standard media and error bars represent SD of three biological replicates. Figure adapted from [187].

When male flies were raised as larvae on either SY10 or SY30 (Figure 2.6) and then moved to Standard food and outcrossed to *C(1)DX* virgin females, the progeny had no detectable difference in the rDNA copy number. Thus while the soma was undergoing diet-induced rDNA loss at this stage (Figure 2.4), the germline was not susceptible to diet induced loss of rDNA; this was not necessarily an unexpected result because the germline cells are thought to be relatively quiescent in larvae.

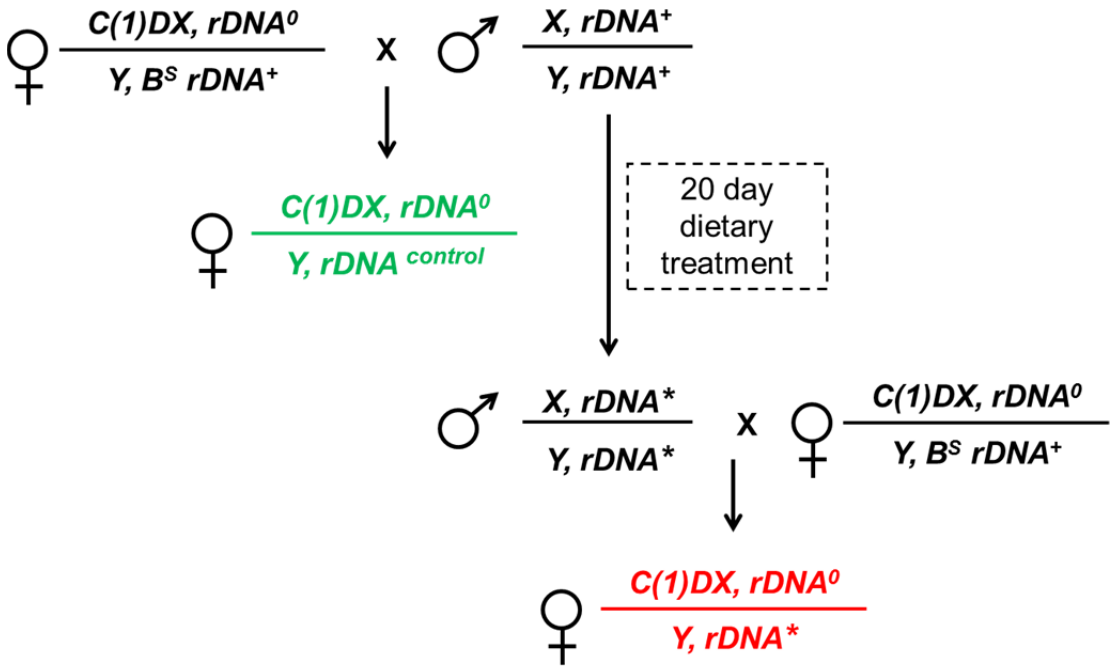
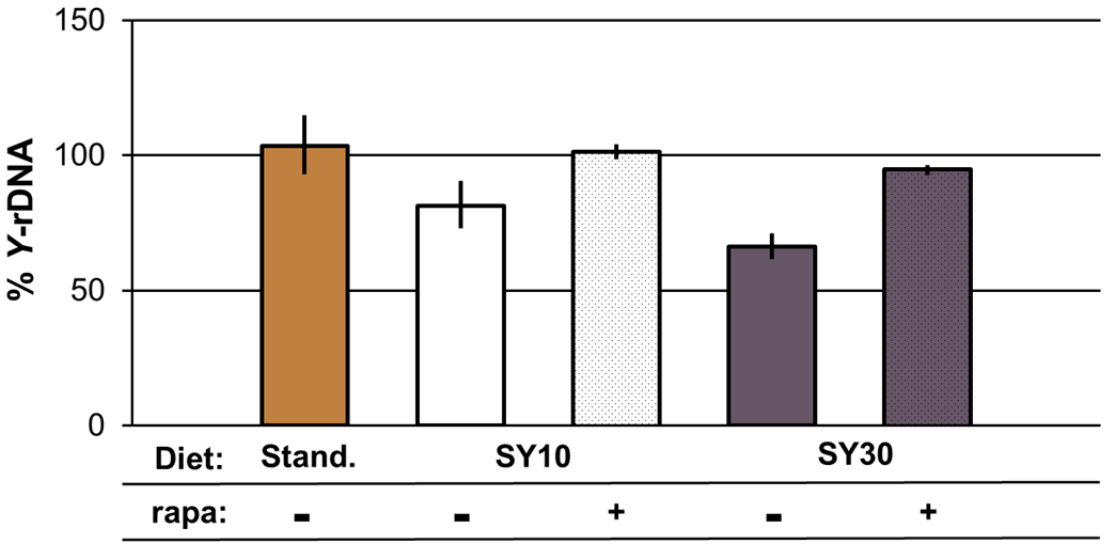


Figure 2.7 Crossing scheme used to isolate Y-linked rDNA arrays for RT-PCR. Newely eclosed males were crossed to *C(1)DX* virgins and their progeney (highlighed in green) were used as an control for subsequent comparisons. After this initial cross, the same males were placed on new media for a 20 day dietary treatment, after which they were crossed to fresh *C(1)DX* virgins in order to produce the progeny of interest (highlighted in red) . Figure adapted from [187].

Conversely, the adult germline was found to be sensitive to dietary conditions. Adult males (Figure 2.7), raised from eggs on Standard medium, were collected 1-4 days after eclosion and allowed to mate with *C(1)DX* virgin females on Standard food for one day; the female progeny of this cross served as the baseline “Control” for subsequent comparisons. The next day, the males were transferred to experimental conditions (Standard media, SY10, or SY30), with or without rapamycin, and were allowed to feed. Males were crossed with fresh *C(1)DX* virgins after 20 days. In this way, I was able to sample the germline of the same group of males before and after treatment.

Progeny of males aged on standard cornmeal molasses media had no detectable rDNA loss, supporting our lab’s anecdotal observation that rDNA arrays seem largely stable in stocks maintained under standard culturing conditions (Figure 2.8). In contrast, rDNA loss was observed in the progeny of males raised on both SY10 and SY30, loss being significantly greater in the latter. Loss was mitigated when 10 μM rapamycin was included in the SY10 and SY30 food. It has previously been confirmed that rapamycin concentrations up to 200 μM have no effect on *Drosophila* feeding rates [192] suggesting that the effects of rapamycin on germline rDNA loss are pharmacological in origin as opposed to behavioral. Spermatogenesis and germline stem cell proliferation in adults are regulated by both diet and nutrient sensing pathways [193,194]; it is likely that germline rDNA instability in response to increased dietary yeast

concentration is a result of the modulation of these pathways, along with any subsequent changes in rDNA regulation.



	vs. 100%	vs. Stand.	vs. rapa +	vs. SY30
Stand.	0.635	~	~	~
SY10	0.013	0.021	0.002	0.007
SY10 +	0.549	0.740	~	~
SY30	0.006	0.003	<0.001	~
SY30 +	0.040	0.225	~	~

P-values

Figure 2.8 Diet influences heritable rDNA copy number. RT-PCR was used to quantify rDNA copy number in the progeny of adult males raised on either standard (Stand.), SY10, or SY30 media with (+) or without (-) rapamycin (rapa). Percentages were calculated relative to control progeny collected prior to dietary treatment, which were defined as "100%." Error bars are standard deviation of at least three independent DNA samples. P-values were calculated using Student's t-test and are presented in the lower table. Column headings (blue) describe the hypothesis tested, while the various treatments are indicated in the row titles. P-values $\leq$ 0.05 are highlighted in yellow. Figure adapted from [187].

In order for a population to maintain a steady-state rDNA size, natural loss must be balanced by expansion. In *Drosophila*, rDNA magnification may serve this purpose, although magnification is not wide-spread and is only observed on some chromosomes under certain conditions [135,137,148,149]. To test for this sort of reversion of diet-induced rDNA loss, I established independent lines from SY30-fed males and kept them on Standard food as with any *Drosophila* strain. This allowed us to monitor transgenerational rDNA copy number for reversion or continued instability (Figure 2.9).

I tested pooled males from three such independent lines using RT-PCR and found that lost rDNA remained lost (Figure 2.10). This observation is consistent with published findings (as well as our anecdotal observations) that while some engineered rDNA deletions lines exhibited moderate (around 5%) expansion shortly after production [152], they have been otherwise stable, without selection, over many subsequent generations. Indeed, I tested one such line and found that in relation to the progenitor stock, magnification had not occurred after six years on Standard food, corresponding to no fewer than sixty generations. These observations suggest that, just like any other polymorphism, rDNA deletions (naturally-occurring or otherwise) persist over multiple generations and that magnification, in contrast, is relatively rare.

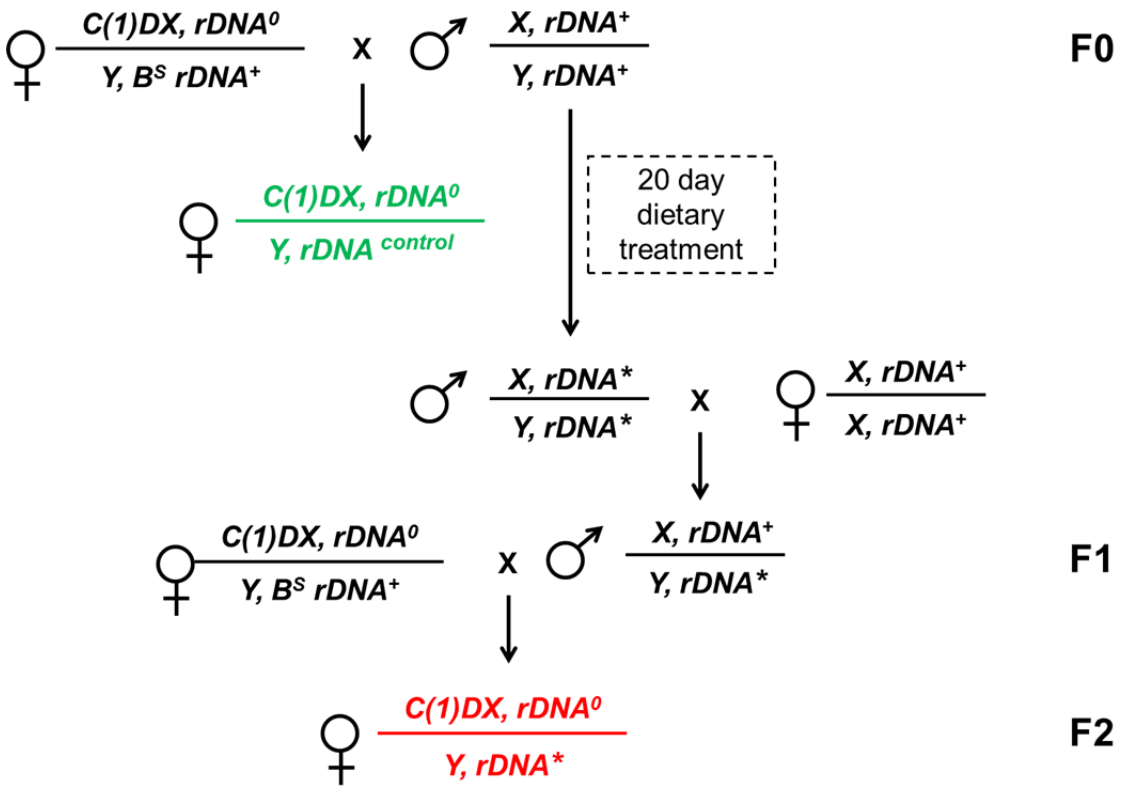


Figure 2.9 Crossing scheme used to isolate F2 progeny of treated males. As in Figure 2.7, control progeny (green) are collected prior to dietary treatment. SY30 treated males were then crossed to standard *X/X* females to produce F1 progeny which are maintained on standard media. F1 males were crossed to C(1)DX virgins to produce F2 progeny (red) for subsequent RT-PCR analysis. Figure adapted from [187].

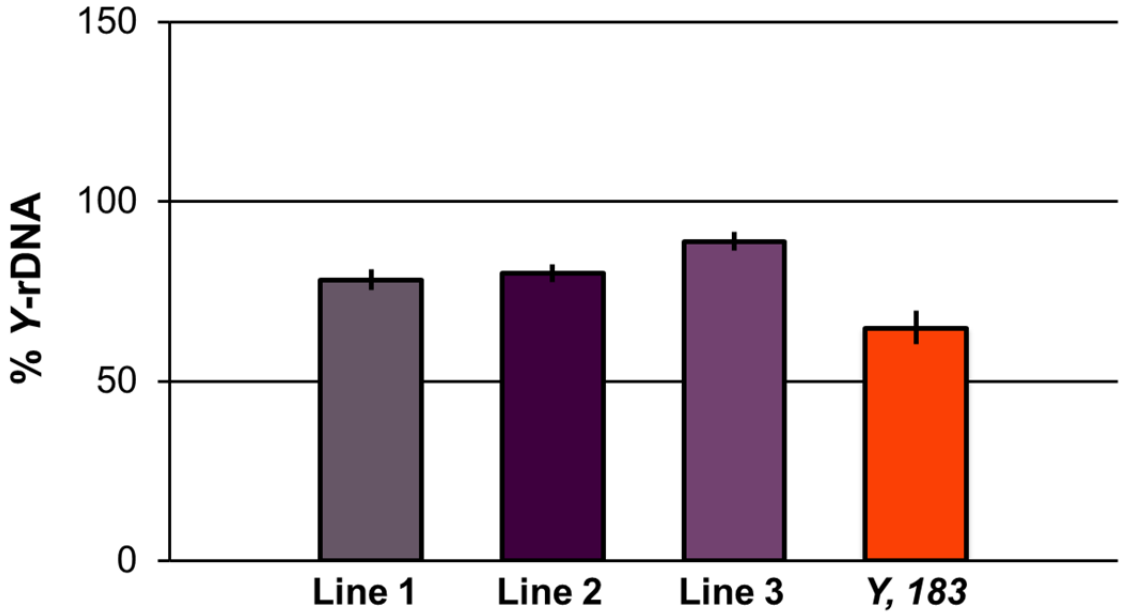


Figure 2.10 rDNA loss is transgenerationally stable. *Y*-linked rDNA copy number of three independent lines ("Lines" 1–3) established from SY30-fed males. *Y*-rDNA was isolated and quantified two generations after dietary treatment. Percentages calculated relative to *Y*-rDNA genetically isolated from F0 males (see Figure 2.9) prior to treatment. *Y, 183* is an engineered rDNA deletion line that has been maintained for over sixty generations on standard media. Error bars represent S.E.M. of 3-4 RT-PCR replicates for each pooled sample. Figure adapted from [187].

Discussion

In this work I established that ribosomal DNA (rDNA) copy number polymorphisms can be created by manipulating the diet of wild-type flies. By directly altering insulin-like signaling and phenocopying nucleolar instability in culture using recombinant insulin, I showed that normal IIS signaling can be a significant source of rDNA copy number variation in the soma. Diet-induced rDNA copy number changes occur in both the soma and germline. As a result, they are both permanent within an organism and are capable of being

transmitted to subsequent generations, hence may act as a record of the dietary history of an individual or for a population.

I believe that the diet- and IIS-induced rDNA instability I observe is a general, or at least common, feature of *Y*-linked rDNA because it has been measurable in males of many strains used in our work. For instance, I specifically tested two other *Y* chromosomes: a wild-type male from a laboratory *Canton-S* stock and a freshly wild-caught ("*Texas-B*") male. rDNA copy number of flies raised on SY10 or SY30 was compared and males bearing the *Canton-S Y* chromosome exhibited a 38% decrease in rDNA copy number, while the *Texas-B* chromosome exhibited an 8% decrease. Thus, while diet-induced loss appears to be a common feature of *Y*-linked rDNA genes, there are likely other genetic factors that influence the rate or bounds of loss. Additionally, the two presumably-unrelated transgenic lines (the *Y* from the UAS-InR strain as well as the *Y* from the Fibrillarin-RFP strain presented in Figure 2.5) both showed nucleolar instability under conditions with increased IIS signaling. Preliminary results suggested that the phenomenon of rDNA loss was less clear in females, who appeared to exhibit small amounts of loss that were not statistically robust. Because the biology of *X*-linked rDNA arrays differs from that of the *Y*-linked arrays [130,147,195], and the consequence of *X-X* exchange at the rDNA is very different from that of *X-Y* exchange, there was no reason to believe that the phenomenon is necessarily related and I have yet to pursue it further.

rDNA instability is observed in a number of eukaryotes and is associated with a variety of complex phenotypes including position effect variegation in *Drosophila* [132,178], replicative lifespan in yeast [189], plant size in flax [196], cancer progression in humans [197-199], and the aforementioned “hidden variation” of *Y*-linked Regulatory Variation. My findings provide a mechanism for the influence of diet on all of these processes. These findings are likely generally relevant to many organisms due to the conserved structure of ribosomal DNA arrays, the common copy number polymorphisms at that locus [14], and the common modes of rDNA regulation [200]. While I focused on diet, other processes that influence rRNA transcription (e.g. cell proliferation, DNA damage, determination and differentiation, stress, aging, temperature, etc. [180]) would presumably also affect rDNA stability via similar mechanisms, and thus, the rDNA may be a common mediator of induced and heritable effects.

CHAPTER III

Y-rDNA VARIATION MODULATES THE *DROSOPHILA* STARVATION RESPONSE

Introduction

Accounting for approximately 50% of transcription, ribosomal RNA (rRNA) is the most abundant product of the eukaryotic nucleus [111,112]. Being an essential component of the ribosome, rRNA is required for protein synthesis and thus ultimately a limiting factor for cell growth and division [201]. The regulation of rRNA transcription is a point of convergence for numerous signal transduction pathways including nutrient sensing (IIS, TOR, etc.) and proliferation, and is an important target of anti-cancer drugs [119,120,199].

The transcriptional output of most genes is a product of a number processes including RNA polymerase recruitment, initiation, and rate of elongation. rRNA transcription differs in that—in addition to the aforementioned factors [171]—the regulation of active versus in inactive copies is an important determinant of transcript levels [170]. In eukaryotes, most rRNAs (i.e. *18S, 28S, 5.8S*, etc.) are co-transcribed from individual cistrons which are present in multiple copies. These copies are typically arranged into one or more tandem arrays known as ribosomal DNA (rDNA) or nucleolar organizing regions (NORs) [108]. rDNA is often located within heterochromatic regions and many of the

silencing proteins found therein seem to be important for regulating the its transcriptional output [167].

Transcriptional regulation of the rDNA is not just important for maintaining appropriate steady-state rRNA levels, but is also linked to the stability of the array. Mutations in silencing factors, as well as natural conditions resulting in transcriptional upregulation, can lead to DNA damage, aberrant recombination, and genomic instability of the rDNA locus [110,131,133,136,140]. Perhaps as a consequence, the number of cistrons within an rDNA array is highly variable between populations, individuals, and even different cells within the same organism [109,113,132,172].

While rDNA instability is well-documented in a number of organisms and many of the factors regulating it have been identified [113,140,145,154], much less is known about the phenotypic consequences of the variation created by such instability. In *Drosophila,* severe rDNA deficiencies were first identified as the genetic source of the bobbed phenotype—a cuticular defect now known to result from diminished translational capacity [144,147-149]. *Drosophila* rDNA is located in redundant arrays the *X* and *Y* chromosomes, and typically only a fraction of one of these arrays (around 100 copies) is actually needed [113]. Pairing a deficient array with a chromosome completely lacking rDNA—*C(1)DX, rDNA*^{0}*,* for instance—results in either lethality or bobbed, depending upon the severity of the deficiency [146].

In contrast to bobbed studies, investigations into minor rDNA deletions—the type seen in the wild—are all relatively recent. Previous work from our lab resulted in the creation of an isogenic *Y*-linked rDNA deletion series [152]. Although these deletions were large enough to cause the bobbed phenotype when used as the sole source of rDNA, the effects of these chromosomes was tested in in a heterozygous (*X, rDNA*$^{+}$*/Y, rDNA*^{def}) background. *Y*-linked deletions were found to dominantly suppress heterochromatic silencing [132] and to influence the transcriptional regulation of thousands of genes across the genome [107]. Comparable results were seen with naturally variant *Y*-rDNA arrays, and analysis in humans has revealed a similar relationship between rDNA copy number and transcriptional variation [154,178].

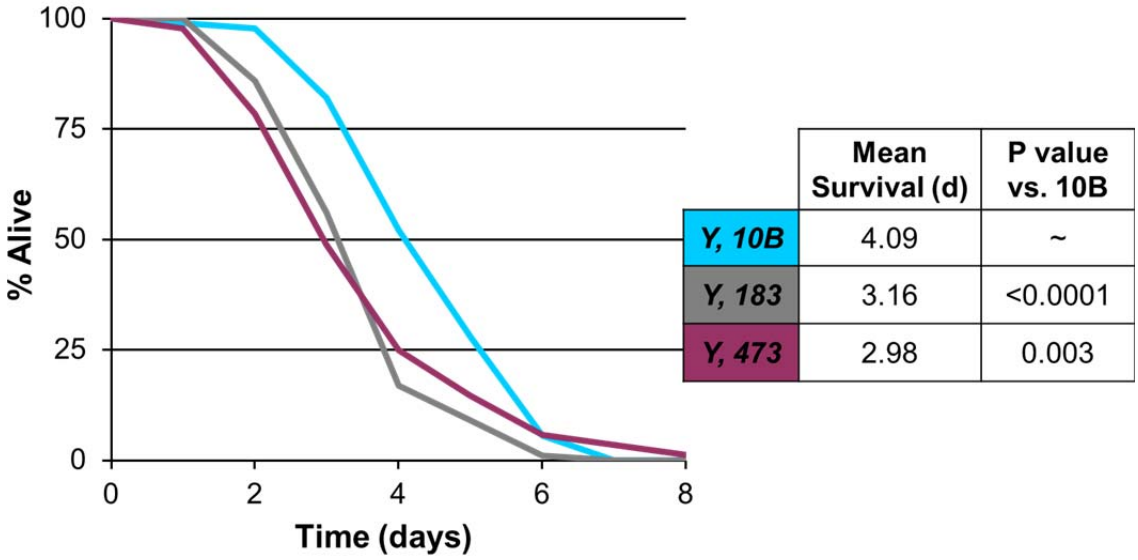
The extent to which these transcriptional differences translate into consequential phenotypes is unknown. In *Drosophila* and humans, numerous genes involved in lipid metabolism and mitochondrial function were found to be differentially transcribed [107,154], suggesting a potential role for rDNA variation in energy acquisition, storage, and/or consumption. The goal of this study was to explore this possibility further. I found that flies bearing *Y*-rDNA deletions were sensitive to starvation and that genotypic reversion rescued the phenotype. Furthermore, via a screen, I identified a *Y*-rDNA variant that was significantly more resistant to starvation than our “wild-type” strain. Taken together, these results clearly establish *Y*-rDNA as a polymorphic locus regulating starvation resistance. Attempts to reveal the mechanism underlying this phenomenon are

still underway, but have thus far revealed that starvation resistance in *Y*-rDNA variants correlates with the rate of triacylglyceride consumption (the major long-term energy storage molecule in most animals) as well as rRNA transcription levels. Potential mechanistic models and experimental approaches to test them are summarized in the discussion.

Results

Previous work from our lab has shown that *Y*-linked rDNA deletions dominantly modulate the expression of thousands of genes across the *Drosophila* genome, many of which are related to metabolic function [107]. Differential expression of lipid metabolism genes in particular suggested a role for *Y*-rDNA in regulation of lipid homeostasis. It was impossible to determine the nature of this regulation, however, since no clear patterns could be discerned in regards to expression levels—for instance, some lipases were overexpressed in Y-rDNA deletion lines while others were downregulated. A more direct way of interrogating organism-level defects in energy storage or acquisition is by measuring starvation resistance. In a typical experiment, flies are placed on media lacking food but containing abundant water. Time of death is recorded and used to generate survival curves. Mutants defective in either the storage or utilization of energy reserves—typically lipids or carbohydrates— have lower mean survival times while those that either store more energy or require less of it live longer [202,203].

To test the effects of rDNA copy number on starvation resistance (SR), I initially used three strains, first described in [152]. *Y, 10B* served as my control—representing an "average" *Y*-linked array—and was the progenitor of *Y, 183* and *Y, 473*, which represent "mild" and "severe" deletions respectively. Although these strains had previously been isogenized, prior to experimentation I backcrossed males from each stock twice to females of the *Y, 10B* stock in order to eliminate any potential background effects that might have arisen during stock maintenance. Three independent lines were established for each genotype. Adult males aged 1-4 days (post eclosion) were allowed to feed for five days on cornmeal molasses media before being transferred to starvation media composed of 1% agar and 1X phosphate-buffered saline. Survival curves are presented in Figure 3.1 along with statistics derived from these data. *Y, 10B* flies had a mean survival time of 4.09 days which is significantly longer than that of *Y, 183* (3.16 days) and *Y, 473* (2.98 days) flies, supporting a role for rDNA in metabolism, as was suggested by microarray data [107].



	Mean Survival (d)	P value vs. 10B
Y, 10B	4.09	~
Y, 183	3.16	<0.0001
Y, 473	2.98	0.003

Figure 3.1 *Y*-linked rDNA copy number influences starvation resistance. Males from each genotype (90 per genotype) were placed on starvation media (10 per vial) and monitored daily. Each data point represents data from nine replicate vials containing 10 flies each. Mean survival times were determined using the Kaplan-Meier method and P-values were calculated via log-rank test.

Although the *Y*-rDNA deletions were initially derived from a single progenitor chromosome (Y, 10B), and are thus theoretically isogenic apart from the rDNA, other repetitive sequences found on *Y* chromosome are known to be unstable [98,141]. To confirm that the SR effect is linked to the rDNA as opposed to instability-induced variation in some other Y-linked sequence, I next sought to rescue the phenotype by converting an rDNA deletion back to "wild-type" levels (i.e. genotypic reversion). rDNA deletions were originally created by expressing I-CreI—an rDNA specific endonuclease—in males and outcrossing their progeny to *C(1)DX* virgins. Female progeny of this cross possess the *Y*-linked array as their sole source of rDNA, and deletions could thus be identified

via the bobbed phenotype [152]. Deletion events are not the only outcome of this process; *Y*-rDNA can also acquire additional copies from the *X*-linked array resulting in magnification.

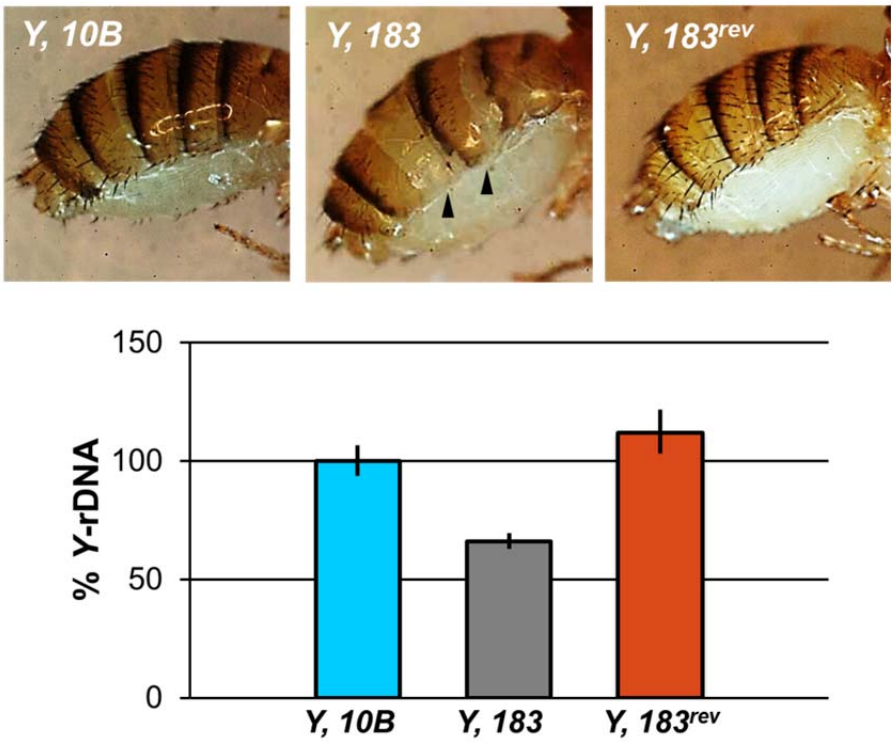


Figure 3.2 Genotypic reversion of the *Y, 183* deletion. When *Y, 183* is paired with *C(1)DX, rDNA*^{0}, a mild bobbed phenotype is observed (black arrowheads). *Y, 183*^{rev} reverts this phenotype (top right). rDNA was quantified using RT-PCR (bottom) to confirm magnification. Percentages were calculated relative to the *Y, 10B* chromosome and error bars represent S.E.M. of at least three replicates.

I reverted the *Y, 183* deletion by exposing it to I-CreI as before and screening for *C(1)DX*/*Y* flies that were no longer bobbed (Figure 3.2). One line was identified and found with *Y*-rDNA levels similar to *Y, 10B* indicating a

reversion of the *Y, 183* array to wild type levels. As expected, genotypic reversion largely rescued the SR phenotype (Figure 3.3) supporting my initial results by demonstrating that the SR phenotype most likely maps to the rDNA as opposed to some unknown *Y*-linked polymorphism.

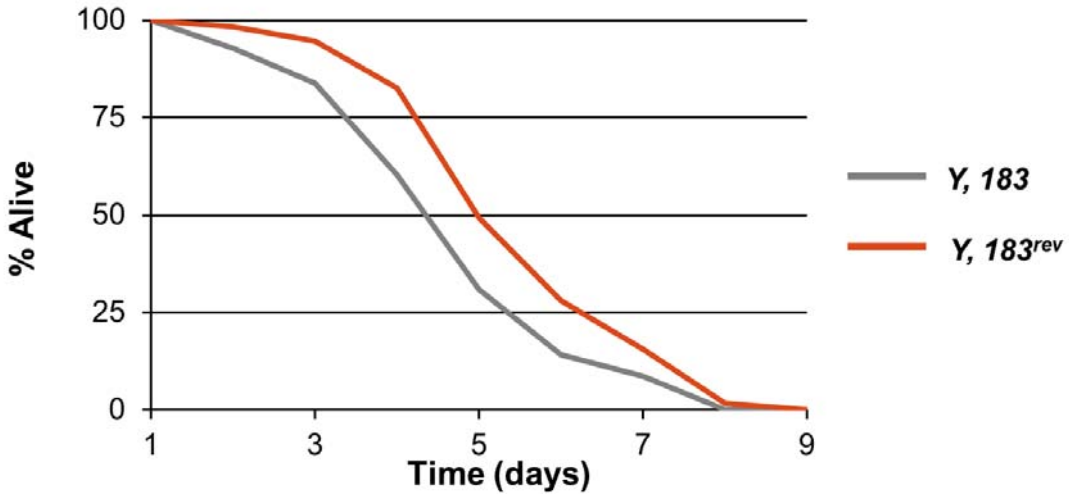


Figure 3.3 Reversion of Y, 183 rescues the SR phenotype. Survival curve analysis was performed for *Y, 183* and *Y, 183*^{rev} under starvation conditions (n=60 flies per genotype). *Y, 183*^{rev} had a mean survival time of 3.97 days compared to *Y, 183*'s 3.38 days (P=0.034, log-rank test). In this experiment, the *Y, 10B* control (not shown) had a mean survival of 3.75 days, which was not significantly different than that of *Y, 183*^{rev}.

The basic mechanisms regulating energy homeostasis are largely conserved in invertebrates and mammals [204]. In *Drosophila*, excess carbohydrates are stored either locally or in specialized organs known as fat bodies along with triacylglycerides (TAGs). Upon starvation, glycogen stores in the fat bodies are broken down and released into the hemolymph as trehalose. TAGs are released from the fat bodies into hemolymph where they are

hydrolyzed by lipases into free fatty acids which are further metabolized intracellularly.

Based on the prior transcriptional profiling results [107], I initially hypothesized that the SR phenotype observed in rDNA deletion lines was due to misregulation of lipid homeostasis—either a failure to accumulate enough TAG reserves prior to starvation or a defect in accessing those reserves. To address this hypothesis, I used thin layer chromatography (TLC) to separate lipids by degree of hydrophobicity and specifically quantify TAGs in fed and starved flies of all three genotypes (*Y, 10B, Y, 183* and *Y, 473*) (Figure 3.4). Under fully-fed conditions, I observed no significant difference in TAG levels between any of the genotypes. This finding was consistent with the fact that there was no measurable difference in body mass between any of the lines and suggested that the SR phenotype was not due to defects in either lipid acquisition or storage. Upon 24 hours starvation, a qualitative decrease was observed in TAG levels across genotypes with the rDNA deletion lines both having somewhat lower levels in comparison to *Y, 10B*. This trend was more pronounced after 48 hours starvation with *Y, 10B* flies having more TAGs than either *Y, 183* or *Y, 473*, indicating differing rates of consumption.[*]

[*] An important consideration for this experiment is that lipids were only extracted from living flies. Therefore, it is likely that *Y, 183* and *Y, 473* flies exhibiting the most severe TAG deficits would be excluded from analysis due to being dead. Thus, I suspect my analysis may significantly underestimate the TAG differences between lines.

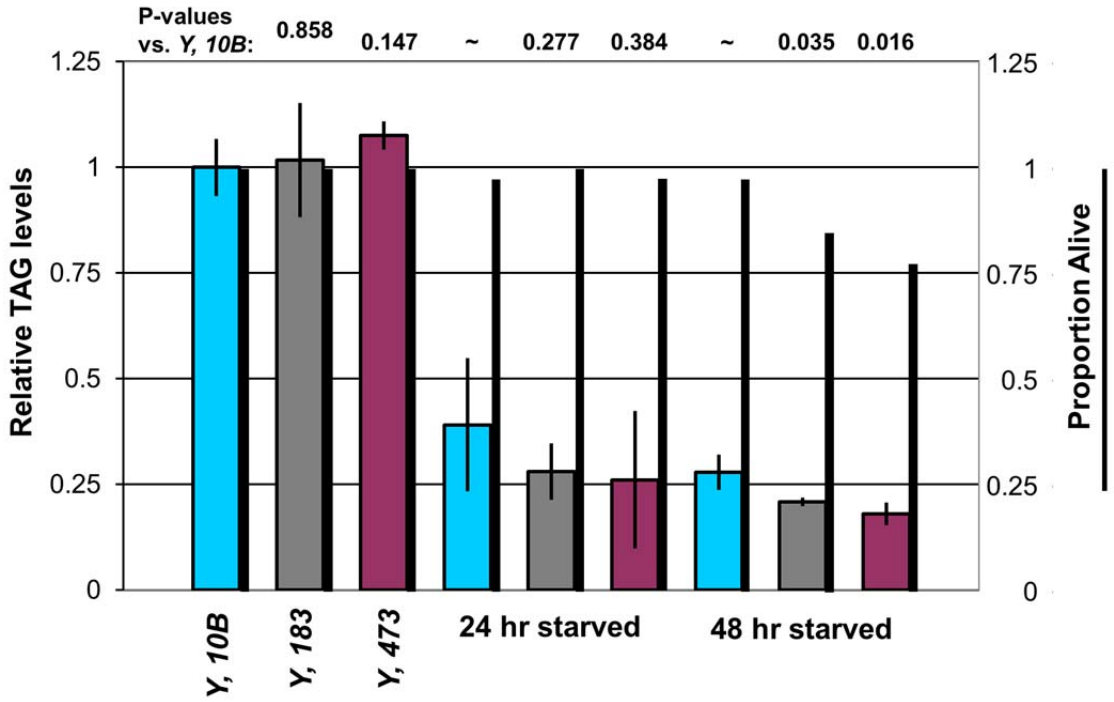


Figure 3.4 Quantification of triacylglycerides via TLC. Lipids were extracted from pools of five flies, separated using TLC and visualized with a general oxidizing stain. Developed plates were scanned and TAG bands were quantified using densitometry. TAG levels (blue, gray, and magenta bars) were calculated relative to the fed *Y, 10B* value and error bars represent standard deviation of four independent samples each containing 10 flies run on separate plates. P-values were calculated using Student's t-test and are presented above. TAG levels in the deletion lines were compared to the *Y, 10B* values in each respective treatment group (i.e. fed, 24h starved, and 48 hr starved) to determine significance. The proportions of flies alive at a given time point are represented as black bars plotted on the secondary left axis. These data are derived from the survival curves presented in Figure 3.1.

This finding suggested two possibilities concerning lipid metabolism and the SR phenotype. The first was that *Y*-rDNA deletion lines simply have greater energetic requirements, and thus catabolize their lipid reserves more quickly upon starvation. The second, more complicated possibility is that the deletions

induce specific regulatory changes which result in an accelerated breakdown of TAGs, but that the consumption of the intermediates produced by that breakdown is either normal or defective in some way. The former would indicate that the SR phenotype is unrelated to TAG metabolism, while the latter would suggest a defect in either fatty acid transport or beta-oxidation. To test this, I reanalyzed the TLC plates and quantified free fatty acid (FA) levels of flies starved 48 hours (Figure 3.5). FA levels were reduced in both deletion lines suggesting either normal or accelerated lipid catabolism rates—an increase would have been indicative of an accumulation of FAs and would have suggested a defect in either their transport or catabolism. This finding supports the hypothesis that *Y*-rDNA deletion lines have greater energetic requirements, and thus higher metabolic rates. Experiments are currently underway to measure O_2 consumption, which will be indicative of increased levels of aerobic respiration.

The fact that the SR phenotype could be rescued by reversion of the *Y, 183* deletion to wild-type suggested that the effect may be a function of rDNA copy number. This would be consistent with previous work from our lab showing that the dominant suppression of heterochromatic silencing (PEV) by *Y*-linked rDNA deletions was negatively correlated with the size of those arrays. To test this possibility and to provide a more robust point of comparison for future analyses, I next sought to use I-CreI to create a line that was even more starvation resistant than *Y, 10B*. I expressed I-CreI in males bearing the *Y, 10B*

chromosome and outcrossed them to *yw* females. I-CreI^{-} adult males were collected and placed on starvation media for eight days. Survivors were placed on cornmeal-molasses media and outcrossed to establish stocks (Figure 3.6).

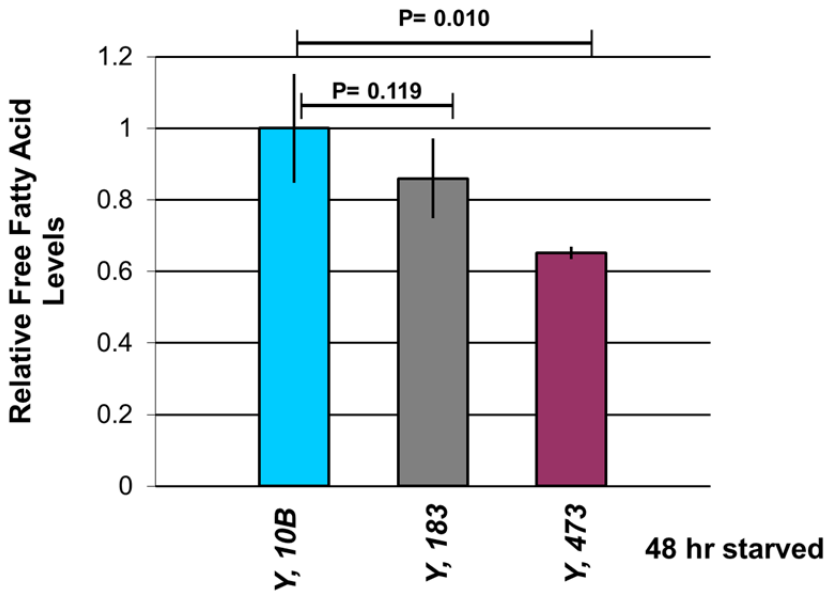


Figure 3.5 Free fatty acid levels in 48 hour starved flies. Lipids were extracted from pools of five flies, separated using TLC and visualized with a general oxidizing stain. Developed plates were scanned and free fatty acid bands were quantified using densitometry. All values were calculated relative to the fed *Y, 10B* value and error bars represent standard deviation of four independent samples run on separate plates. P-values were calculated using Student's t-test.

Approximately 1000 flies were screened in this manner, resulting in 14 viable stocks. All 14 stocks were reassayed and the longest living strain—named *Y, SSR* for Selected Starvation Resistant—was selected for further analysis. *Y, SSR* flies exhibit enhanced resistance to starvation compared to *Y, 10B* with a mean survival of 5.72 days, and TAG levels were significantly higher under

starvation conditions (Figure 3.7) supporting the conclusion that variation in lipid consumption rates underlies rDNA-linked variation in SR.

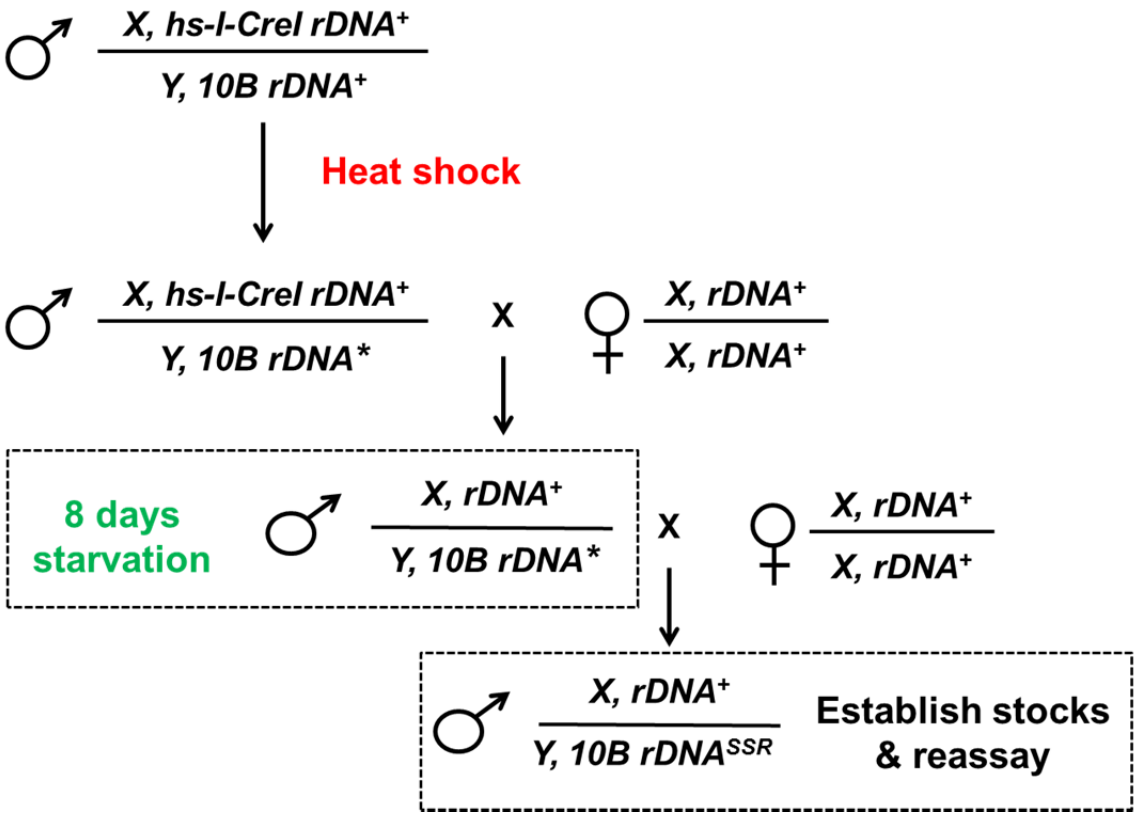


Figure 3.6 Crossing scheme for starvation resistance screen. *hs-I-CreI* is under control of the *hsp70* promoter and was induced in *Y, 10B* larvae via a 37ºC heat shock for 30 minutes. Adult males with rearranged rDNA (rDNA*) were crossed to wild type females. The adult male progeny of this cross were subjected to starvation for eight days to screen for resistance. Individual stocks were established from the surviving, fertile males and were retested.

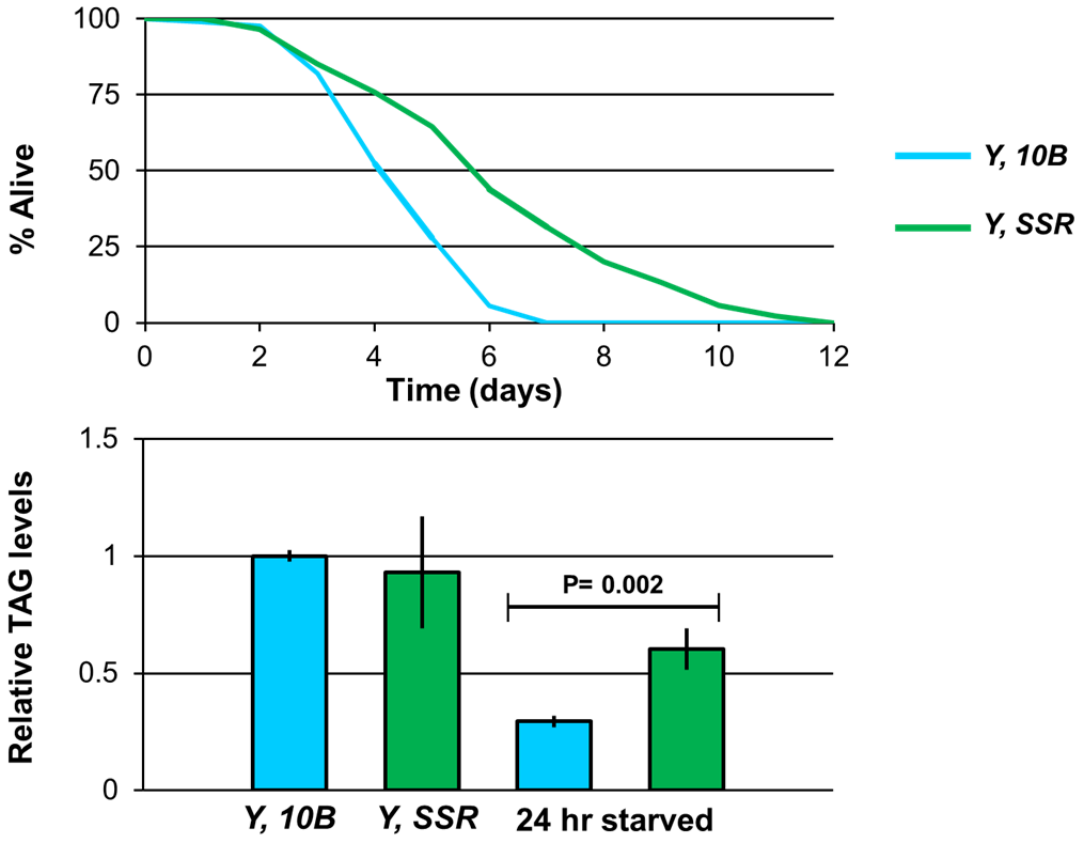


Figure 3.7 Starvation resistance is enhanced by *Y, SSR* while TAG consumption is reduced. *Y, 10B* and *Y, SSR* survival curve is plotted above (n=90 per genotype). *Y, SSR* lives significantly longer under starvation conditions (5.72 days; P<0.0001 log-rank test). After 24 hour starvation, greater TAG levels—as quantified via TLC—were observed in *Y, SSR* flies in comparison to *Y, 10B* (below). Error bars represent standard deviation of 3-4 samples and P-value were calculated using Student's t-test.

Previous results suggested a correlation between rDNA array size and starvation resistance so I therefore predicted that *Y, SSR* would possess a larger *Y*-rDNA array than *Y, 10B*. Using RT-PCR to quantify relative *Y*-rDNA copy number, I found that this was not the case. The *SSR* Y-linked array actually possessed around 12% fewer copies than *Y*, *10B*. This finding strongly suggests

that some different polymorphism underlies the observed variation in starvation resistance and TAG consumption rates (Figure 3.8). The bobbed phenotype illustrates the potential genotypic complexity associated with rDNA. Although typically the result of an rDNA deletion, large magnified arrays have been identified that induce the phenotype [152]. Similarly, the size of the *Y, SSR* rDNA array is comparable to that of several engineered deletion lines, yet it is not bobbed while those are. Other rDNA-linked polymorphisms such as promoter variants [115], retrotransposon insertions [127], and other unidentified polymorphisms may instead underlie these phenotypic effects.

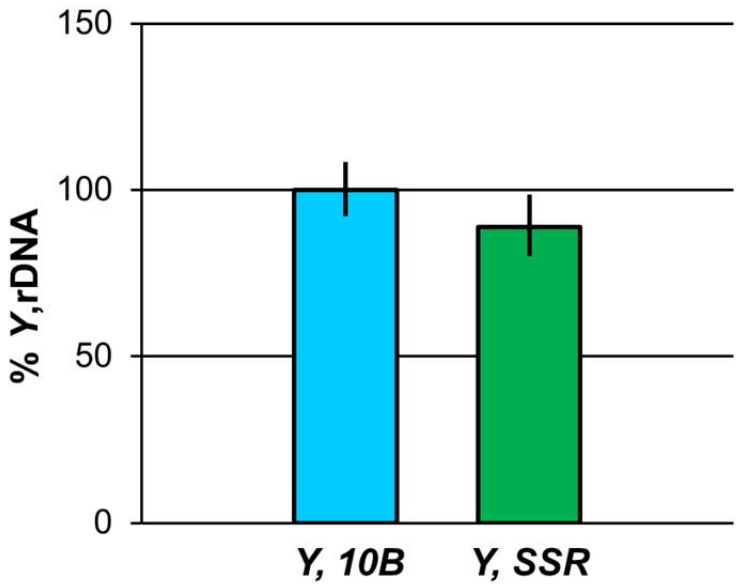


Figure 3.8 *Y, SSR* rDNA copy number. RT-PCR was used to measure *Y*-linked rDNA copy number in *C(1)DX/Y, SSR* flies. Percentage is calculated relative to *C(1)DX/Y, 10B* flies and error bars represent S.E.M. of 4-5 technical replicates derived from pooled samples of 20 flies.

Y, 183 and *Y, 473* flies consume TAGs at a greater rate than *Y, 10B* or *Y, SSR* flies, suggesting that differing energetic requirements may underlie the SR variation observed in these lines. *Y*-rDNA deletion strains have elevated mature rRNA levels and unstable nucleoli, both of which are indicative of rDNA hyper-transcription [132]. Could rRNA transcriptional variation be a proximal source of differing TAG consumption rates? Given the already high levels of rRNA transcription [111,112] as well as the integral role ribosomes play in cellular growth and metabolism, even a small increase could potentially have a substantial impact on an organism's energy consumption rate—a topic which further addressed in the discussion.

Steady state rRNA levels were originally measured in rDNA deletion lines using ethidium bromide stained RNA gels [132]. I used RT-PCR to quantify pre-rRNA levels [187] (Figure 3.9). Consistent with previous results, I found elevated pre-rRNA transcription in the deletion line *Y, 183* relative to *Y, 10B* (139% vs 100%; P=0.119), while *Y, SSR* was found to have significantly less transcription (49.5%, P=0.001). Taken together, these findings suggested a relationship between rRNA transcription and starvation resistance. If this is the case then I expected that treatment with actinomycin D—a drug which inhibits polymerase procession via intercalation [187]—would enhance starvation resistance. Newly eclosed adult males were fed standard culture media with or without 0.6 μM actinomycin D for five days prior to being placed on starvation media with or without the drug. Inclusion of actinomycin D significantly enhanced starvation

resistance of both the wild type (*Y, 10B*) and a Y-rDNA deletion strain (*Y, 473*), supporting my hypothesis that differential rRNA expression underlies the SR differences in variable Y-rDNA lines (Figure 3.10)

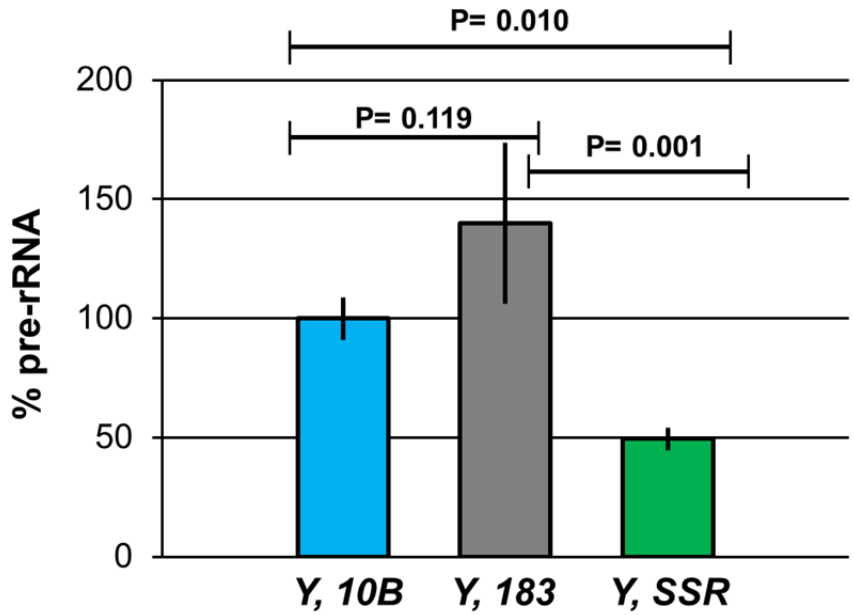


Figure 3.9 pre-rRNA expression levels negatively correlate with starvation resistance. RT-PCR quantification of cDNAs derived from unprocessed (*ETS-18S* junction) pre-rRNA from adult males of the indicated genotypes. Values were normalized to the genomic DNA copies of $tRNA^{K\text{-}CTT}$ genes and proportions plotted relative to *Y, 10B* (defined as 100%). Error bars report standard deviation of relative cDNA quantities derived from three independent pools of ten adults each. P-values calculated using Student's t-test.

Discussion

Outside of the laboratory environment, most animals face periods of food shortage ranging from limited access to specific resources to acute starvation. The physiological response to these conditions (i.e. starvation resistance) is thus

an important determinant of an organism's survival and overall fitness [203]. In *Drosophila*, SR is a quantitative trait governed by hundreds of different genetic loci [202]. Death, as a response to acute starvation, is thought to largely be a consequence of organ failure due to energy depletion. Therefore, genes regulating energy storage levels, utilization rates, or the cellular response to nutrient depletion are all potential modifiers of SR variation.

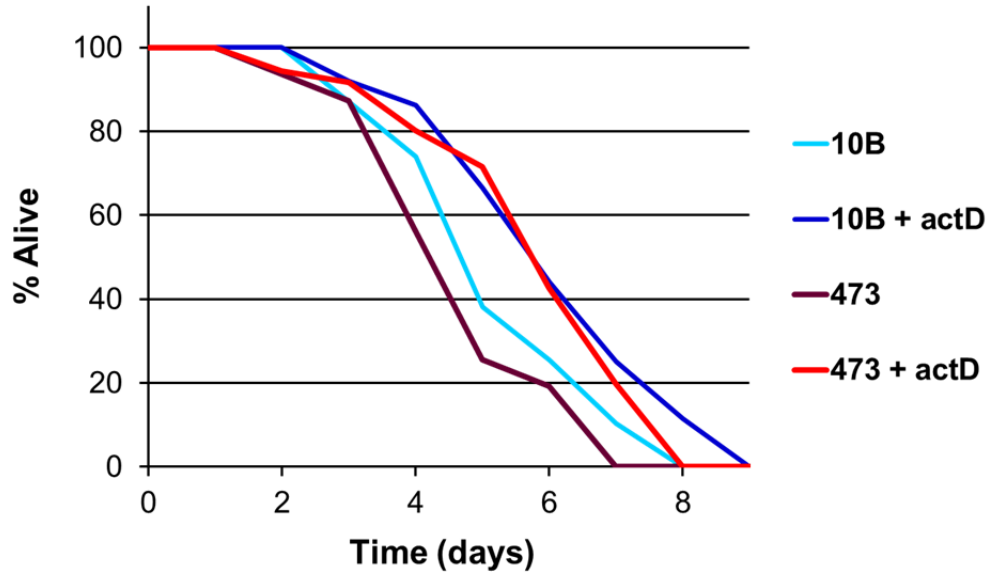


Figure 3.10 Actinomycin D treatment enhances starvation resistance. Survival curves were plotted for male *Y, 10B* and *Y, 473* flies (40 flies per condition) treated with (+actD) or without 0.6 μM actinomycin D. Flies werefed standard media, with or without the drug, five days prior to being transferred to starvation media. Treated *Y, 10B* flies lived significantly longer than untreated (5.94 vs. 4.64 days; P=0.001 log-rank test). Similar results were observed for *Y, 473* (5.7 vs. 4.23 days; P=0.002).

In most animals, lipids—triacylglycerides to be precise—are the primary mode of energy storage [204], and in *Drosophila*, the regulation of lipid

homeostasis is known to be a major component of SR [205]. Previous work from our lab identified “lipid metabolism” as an overrepresented class of genes differentially transcribed in *Y*-rDNA deletion strains [132]. In the work presented here, I demonstrate that these strains are also sensitive to starvation, suggesting some metabolic defect. Magnification of *Y, 183* rDNA to *Y, 10B* levels largely rescued the phenotype suggesting that the SR differences observed in this study may simply be a function of rDNA copy number. When I performed a selection screen for an exceptionally resistant strain, however, I found that this was likely an oversimplification. While the *Y, SSR* array is indeed larger than those of either *Y, 183* or *Y, 473*, it is actually somewhat smaller than that of *Y, 10B*.

While these findings collectively support my conclusion that *Y*-linked rDNA is a polymorphic locus regulating SR, they also suggests at number of interrelated possibilities concerning the genetics of this phenomenon. First, as previously mentioned, it is clear that copy number is not the only variable governing an arrays effect on SR. Low copy number may be the cause of the starvation sensitivity observed in the deletion strains, but enhanced resistance certainly doesn’t require magnification; noting, of course, that there is no reason the two (“resistance” versus “sensitivity”) are necessarily related. Second, it is likely—especially in the case of enhanced resistance—that rDNA polymorphisms other than copy number are in play. Promoter/enhancer variants found within the *NTS* are known to influence transcriptional output of a given cistron [115-117]. Thus, an array with “better” promoters (on average) may have

a higher level of basal rRNA transcription. R1 an R2 retrotransposable elements affect the activity of the arrays in which they are inserted as well as the functionality of the RNAs transcribed from the cistrons they interrupt [127]. For example, a large rDNA array composed primarily of R1/R2 inserted cistrons would be largely useless in that such copies are either silenced or their products are non-functional since inserted rRNAs are not packaged into ribosomes and are rapidly degraded [126]. These are just a few examples and there are likely other unknown polymorphisms within the rDNA which might influence SR. Next generation sequencing will likely be required to identify specific rDNA variants associated with either resistance or sensitivity to starvation.

As with PEV and transcriptional regulation, it remains unclear how *Y*-rDNA variation affects SR. I found similar levels of TAGs in all genotypes in fed conditions suggesting that defects in fat accumulation were not the root of SR variation. Utilization of fat stores does seem to be involved since upon starvation, they are depleted more rapidly in sensitive lines (*Y, 183* and *Y, 473*) compared to resistant lines (*Y, 10B* and *Y, SSR*). Free fatty acids are likewise consumed more rapidly in *Y, 183* and *Y, 473*, which is consistent with an elevation in metabolic rate in those lines. It is unclear if the differential transcription of lipid metabolism genes previously observed in fully fed flies bears a causal relationship to the SR phenotype or is itself a consequence of altered metabolic homeostasis. Tissue specific transcriptional profiling during both fed and starved conditions will be necessary to better understand the

transcriptional changes induced by variable *Y*-rDNA arrays. Monitoring metabolic rate—either by calorimetry or O_2 consumption/CO_2 production rates—will likewise be helpful in understanding the organism wide effects of rDNA variation.

A possible connection between rDNA sequence variation and metabolic rate is rRNA transcription. Assuming that biosynthesis of the various NTPs is energetically similar, a *Drosophila* rRNA molecule would require the equivalent of approximately 1,500 ATP molecules to produce. Beta-oxidation of fatty acids results in the net production of 14*n*-6 ATPs with *n* representing one-half the number of carbons in an even number chain [206]. Thus, the complete oxidation of an average fat body TAG (*n*=24) [207] would yield 990 ATP molecules. Using this rough calculation, I estimate that a 10% increase in rRNA levels would require the consumption of around 15% more TAGs. Nucleotide polymerization is not the only energetic requirement of rRNA transcription—chromatin remodeling and rRNA processing are both energetically costly phenomenon that would accompany increased rDNA expression. Consistent with this possibility, I found that pre-rRNA transcription levels were somewhat elevated in *Y, 183* flies in comparison to *Y, 10B* while levels in *Y, SSR* were significantly lower. If this relationship holds, then drugs or mutations inhibiting rRNA transcription should enhance starvation resistance while those causing hyper-transcription should result in greater sensitivity.

CHAPTER IV

SIMPLE SEQUENCE POLYMORPHISMS ON THE *DROSOSPHILA Y* CHROMOSOME*

Introduction

A significant portion of most eukaryotic genomes is composed of repetitive DNA elements [5]. It is estimated that as much as 1/3 of the genome of *Drosophila melanogaster* is composed of such sequences [16,208]. This fraction is largely confined to centric and telomeric regions where it forms constitutive heterochromatin, which is cytologically distinct in its appearance and genetically distinct in its properties. Constitutive heterochromatic sequences are largely of two types: middle repetitive sequences such as transposable elements, and highly repetitive major- and micro-satellite sequences [14,16,17,209-211]. Although highly-repetitive heterochromatic satellite sequences (e.g., AAGAG, AATAT, AAGAGAG) house a variety of biological phenomena including centromere function, chromosome cohesion and pairing, nuclear organization, control of recombination, species-compatibilities, replication rate, and gene regulatory variation [212-217], understanding their function mechanistically has lagged far behind sophisticated understanding of the function of euchromatic sequences. This is due in large part to the difficulty in handling these sequences

* Part of this chapter is reprinted with permission from "Simple quantitative PCR approach to reveal naturally occurring and mutation-induced repetitive sequence variation on the *Drosophila Y* chromosome" by JC Aldrich and KA Maggert, (2014). *PLoS ONE* 9(10). Copyright 2014 by Aldrich and Maggert.

with modern molecular biological approaches. Next-generation sequencing technology has increased the rate with which we have learned about the structure and variation of euchromatin, but the heterochromatic portion of the genome remains relatively ignored in its characterization [15,16], even very recently not rising to the level of notice in debate over the role of ‘‘junk’’ DNA [7,8].

The *Y* chromosome of *Drosophila* melanogaster is a useful tool for understanding the evolution of satellite sequences and their contribution to genome regulation [102,104]. The *Drosophila Y* chromosome is naturally variant, can be made supernumerary in males or females, is dispensable in males, has very few genes, is a component of numerous chromosome rearrangements, and its functional and sequence elements have been roughly mapped. Apart from genes necessary for male fertility and a small set of non-essential genes, the *Y* chromosome is almost entirely composed of repetitive DNA such as megabase-long blocks of satellite repeats –variously called alphoid repeats, alpha-heterochromatic repeats, satellite repeats, simple repeats, simple satellite repeats (SSRs), highly-repetitive DNAs, repetitious DNAs, etc. –as well as interspersed or clustered transposable elements, the repetitive Ribosomal RNA genes (rDNA), and other genetic elements [14,103,210]. *Y* chromosomes isolated from diverse populations affect a number of phenotypes including temperature sensitivity, sex ratio, heterochromatin formation, male fitness, innate immunity, and others [92,105,106,218,219] and may do so by

differentially influencing genome-wide transcription. Although some of these effects can be attributed to rDNA copy number polymorphisms [107,152], it is likely that the balance of unmapped variation lies within satellite sequence [96].

‘‘Complex’’ euchromatin contains ample sequence variation to analyze for function, while the sequence variation of satellites has fewer parameters in which it can vary. Blocks of satellite repeats can vary in their length (i.e., copy number), homogeneity (i.e., polymorphisms in the consensus repeat unit), punctuation (i.e., location, type, and copy number of transposable elements or transposable element remnants), orientation (e.g., AAGAG or CTCTT in relation to the centromere), juxtapositions (e.g., the types or arrangements of satellite repeats at junctions), or linkage (to specific chromosomal locations). There have been some attempts to explore these features, but it is difficult to apply standard molecular tools to understand the architecture of the heterochromatin. Currently, studies to address variation have chiefly measured linkage and copy number using fluorescence *in situ* hybridization or Southern blot analysis.

Acknowledging that no approach is perfect, and following on recent experiments [220,221] demonstrating the importance of rDNA copy number variation in heterochromatin formation and *Y*-linked Regulatory Variation, I wished to develop a similar method to quantify the copy number of satellite repeats that is (i) simple, (ii) robust, (iii) sensitive, (iv) quantifiable, (v) inexpensive, (vi) fast, and (vii) can be integrated with other approaches to provide an understanding of the arrangements of satellite DNAs.

‘‘Real-Time’’ or ‘‘Quantitative’’ Polymerase Chain Reaction (RT-PCR) has been successfully used to accurately quantify rDNA copy-number variation in numerous studies [130,152,178,186,222], and is theoretically directly applicable to any repetitive sequence element whose repeat unit is longer than the typical approximately 100 base pair product of RT-PCR. The absence of unique primer binding sites in blocks of short (e.g., pentameric or heptameric) satellites makes avoidance of primer-primer annealing the chief difficulty. An assay that circumvented this problem and allowed the amplification and quantification of simple telomeric repeats has been developed [223,224]. I thought this assay could in principle be adapted for heterochromatic satellites, which in many regards pose the same problems as telomeric DNA: short, homogenous, high copy number. In this study, I show the successful adaptation of this RT-PCR technique for the quantification of pentameric satellites. I validated precision using a dilution series and *Y* chromosome aneuploids, and found that geographically diverse *Y* chromosomes harbor previously uncharacterized satellite copy number polymorphisms. Furthermore, I applied the approach to discover that long-term exposure to a mutation affecting heterochromatin formation and genome stability, the *Su(var)205* locus which encodes the HP1a gene product, results in measurable changes in satellite copy number, suggesting that much like rDNA [113,131,144], satellite copy number stability is regulated by chromatin factors.

Results

Large blocks of simple pentameric repeats AACAC and AAGAC are constituents of the *Drosophila Y* chromosome [17,225], accounting for less than about 2% and about 20%, respectively, of the *Y*; the remaining balance largely resides in the pericentric heterochromatin of chromosome 2. In order to investigate copy number variation of these repeats, I adapted a Real-Time PCR (RT-PCR) assay, originally designed for quantifying telomeric repeat copy number by Cawthon [223,224], which would allow us to quantify their relative copy number. The reaction used primers with designed self-incompatibilities to disfavor primer-dimer formation and instead heavily favor template-dependent and product-dependent priming. The products of template-dependent synthesis created self-compatible products, which were preferentially amplified exponentially as is normal in PCR reactions.

Five design elements were incorporated into primer design. First, a ‘‘Forward’’ primer matching the repeat (e.g., AACAC) contained a base-pair change (therefore a mismatch with the repeat) every 5 nucleotides. Second, the ‘‘Reverse’’ primer (e.g., GTGTT) did so as well, but the mismatch was not the same as that on the ‘‘Forward’’ primer. Third, the primer set (Forward and Reverse) converged at a position in the repeat that was not complementary (i.e., they did not overlap at their 3’ ends). Fourth, the primers each contained five nucleotides at their 5’ends that were not homologous to the repeat. Fifth, the

primers had nucleotides at their 3’ ends such that the best primer-primer annealing configurations had minimally two 3’ mismatches [224].

This design balanced RT-PCR primers (i) effectively binding to and priming from the genomic satellite DNA repeat, (ii) exponentially amplifying from products of previous cycles of the ‘‘chain reaction’’ amplification, and (iii) avoiding primer-dimers forming between primers both directed at the same repetitious DNA sequence. Key to this end, introduced base pair mismatches (“First” and “Second” design elements above) were out of phase with each other and compromised the binding between primers and target genomic DNA, but more egregiously compromised binding with each other. This is clarified in Figure 4.1

Primer-dimers are a constant concern in primer design, and the repetitious nature of the target sequences makes avoidance difficult because there are multiple pairing arrangements that are a function of the repeat-length. I analyzed the number of possible base pairs forming given every degree of overlap between AACAC Forward and AACAC Reverse (Figure 4.1). The repeat unit length is clear as a local maximum every five nucleotides, flanked by two far-sub-optimal arrangements around each local maximum (i.e., offset by 4–5–6 nucleotides, 9–10–11, etc.). AACAC Forward and Reverse best pair with an offset of five nucleotides which creates eight internal mismatches, disrupts pairing of more than 3 consecutive bases, and leaves 3’ mismatches on both ends, which significantly inhibits polymerase elongation.

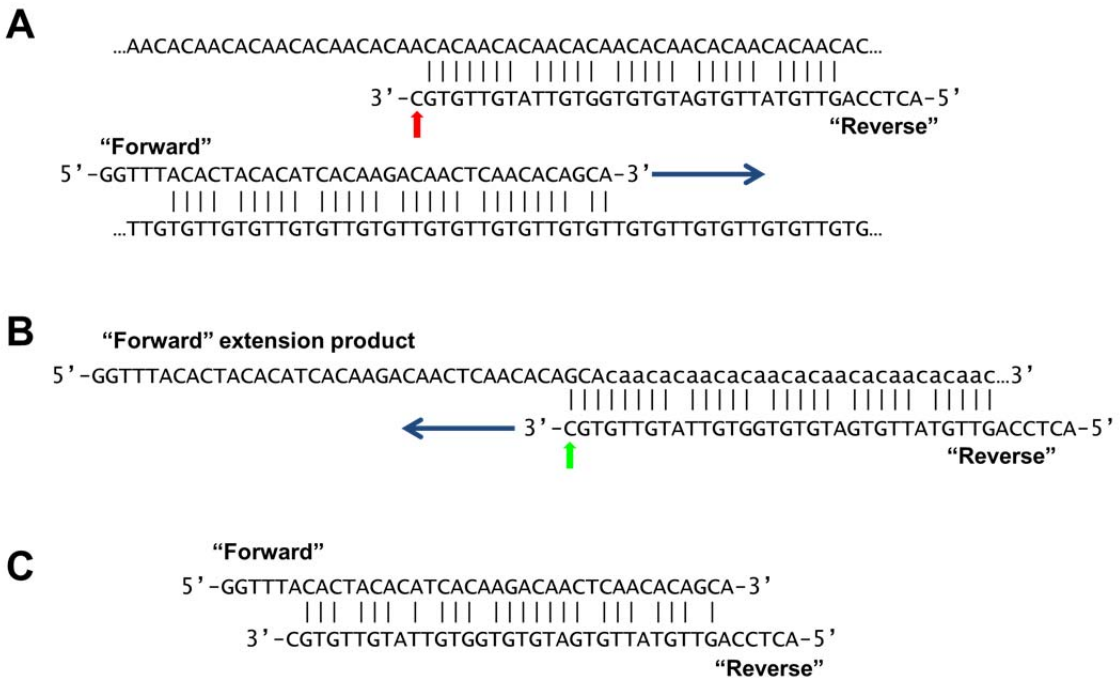


Figure 4.1 RT-PCR primer design features. (A) Representation of a homogenous block of AACAC repeat denatured and annealing to "Forward" and "Reverse" primers. Vertical lines indicate base pairs. During the first round of PCR, forward primer extension occurs (blue arrow) but reverse primer extension is inhibited by a 3' mismatch (red arrow). (B) During the second round of PCR, the reverse primer can anneal to the newly synthesized portion (lowercase) of the forward primer extension product—the mismatch is corrected in the forward sequence (green arrow). An intentional mismatch on the forward primer can pair with the 3' end of the reverse primer (green arrow) allowing extension to occur. (C) Extension is inhibited in the most optimal primer dimer pair due to 3' mismatches on both ends. Figure adapted from [98].

Additionally, this ''best'' match allows only ten nascent nucleotides (both 5-base pair 3'-overhangs) to be incorporated during a PCR extension, much less signal than would be included by even the worst priming of a previously amplified primer (40 nucleotides if the primers annealed at juxtaposed genomic repeats; 38 for the primer plus the 2 of non-overlap in the repeat unit,

…aacaCAacac…) or a valid genomic DNA-primed event. After the second successive cycle of priming and elongation, there are no longer any mismatches between primer and PCR-produced template, thus normal RT-PCR conditions are established.

To confirm the robustness of my assay, I performed RT-PCR reactions on isolated genomic DNA over an approximately 100-fold dilution range (1.23 ng– 100 ng per reaction) surrounding optimal conditions determined empirically in other studies [152,187]. Over an intermediate range (3.7 ng– 33.3 ng), I observed a very high correlation (R2= 0.99) between template concentration and quantification cycle (Cq, [226]) using primers directed at the copy number stable multicopy $tRNA^{K\text{-}CTT}$ gene [152], AACAC, or AAGAC (Figure 4.2), which matched mine and others' experience with amplification of the middle-repetitive *35S* ribosomal RNA gene [152] and others' experience with simple telomeric repeats [223]. In practice, to assure robustness, I routinely perform reactions using DNA concentrations falling within the middle of this range (about 4–10 ng/12 µl reaction). I recommend this concentration, however my results indicate that fluctuations in the DNA concentration due to variation in extraction or errors in preparation will have negligible influence over the result.

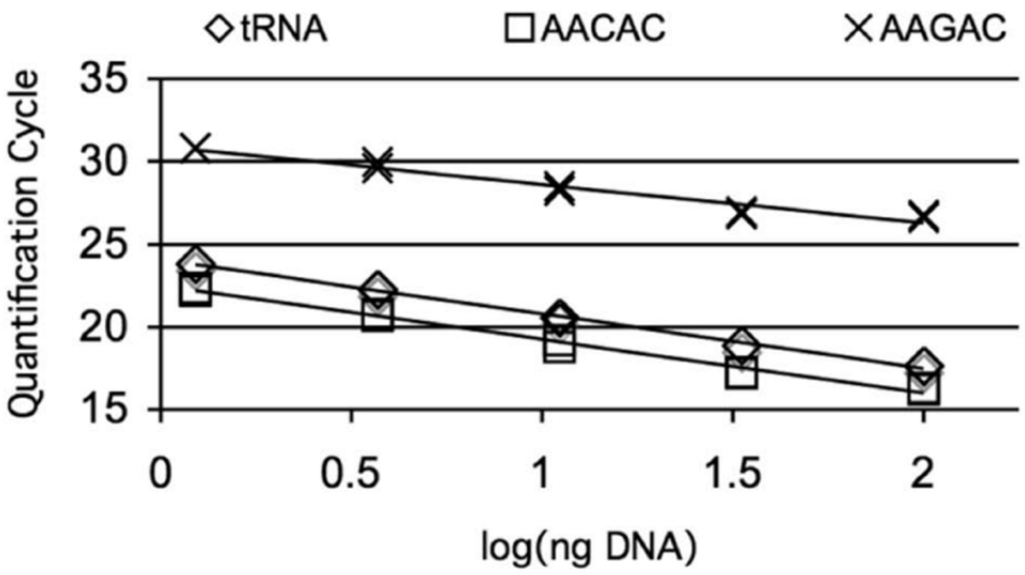


Figure 4.2 RT-PCR assay validation via dilution series. Quantification cycle (Cq) of duplicate qPCR reactions plotted as a function of template DNA per reaction. X axis represents log10 of an approximately 100-fold dilution series. Figure reprinted with permission from [98].

I analyzed the raw data from the RT-PCR reaction of each target for efficiency using the LinRegPCR software package [227]. This analysis ascertains closeness-to-doubling (efficiency) with each PCR cycle, thus a score of 2.0 is theoretically ideal. The efficiency values for $tRNA^{K\text{-}CTT}$, AACAC, AAGAC, and AAGAG, respectively, are 1.866 ±0.003, 1.876 ±0.004, 1.876 ±0.002, and 1.866 ±0.004 (each calculated from 12 reactions, errors are standard errors of the mean). Although these values are below theoretical maximal efficiency, they are all similar, thus any correction that would be applied to the data to account for subideal efficiencies would be applied equally to all values and are effectively canceled out when reporting relative values. These

efficiency values are within generally accepted guidelines (90%– 110%) despite the intentional mismatches in satellite-directed primer sets. Post-hoc melt-curve analysis confirmed that only single melting peaks were observed from these reactions, indicating single PCR products were amplified during RT-PCR (Figure 4.3), supporting the computational justification.

I next used the ΔΔCq method of analyzing RT-PCR results to quantify repeat copy-number of AACAC and AAGAC relative to that of the $tRNA^{K\text{-}CTT}$ gene [152]. Although these satellite repeats have been cytologically mapped, little information about their overall abundance in the genome is available. They are found on the *Y* chromosome, which can be removed or made supernumerary without defects in viability, allowing me to manipulate *Y* chromosome copy number to monitor the sensitivity of my assay. I collected infrequent (frequency = ~10^{-4}) spontaneous primary nondisjunctional exceptional progeny from a *yellow*^{1} *white*^{67c23}*/Y, 10B y*$^{+}$ stock [100], or created secondary nondisjunctional progeny (see Materials and Methods). *y*$^{+}$ females were crossed to euploid brothers and *y*^{1} *w*^{67c23}*/Y, 10B y*$^{+}$ */Y, 10B y*$^{+}$ progeny were identified by their duskier bodies, a consequence of the *Y*-terminal duplication of the *yellow*$^{+}$ gene translocation.

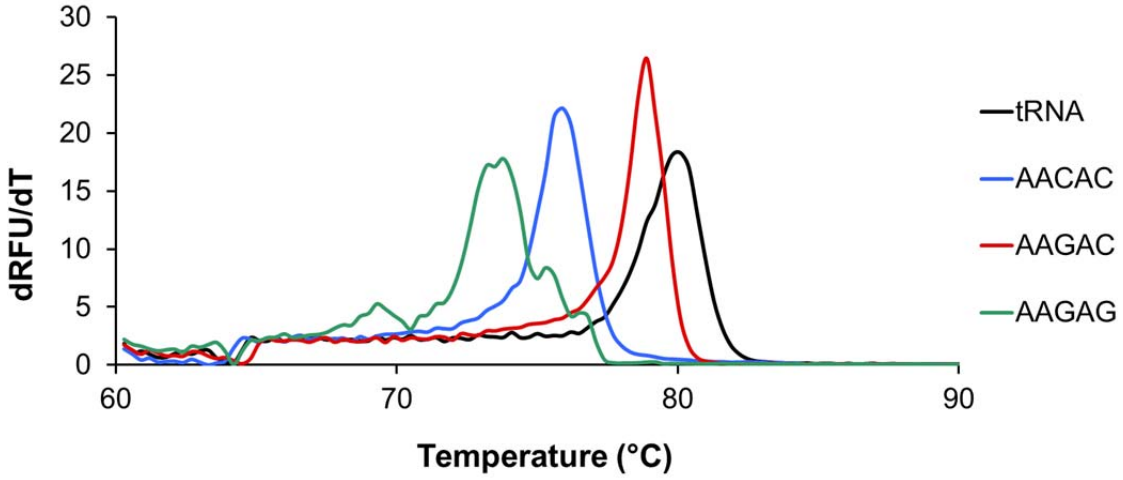


Figure 4.3 Melt curve analysis of RT-PCR products. First derivative with respect to temperature of Relative Fluorescence Units (RFU) is plotted over the indicated temperature range. Derivative was calculated by ΔY/ΔX for each temperature interval after maximal fluorescence was set at 100%. Single major peaks indicate monophasic melting, indicative of single RT-PCR products with relatively-homogenous melting profiles. Figure reprinted with permission from [98].

I determined copy number of pentameric AACAC and AAGAC in sibling X/Y and X/Y/Y males (Figure 4.4); data are shown as %AANAN (indicating either AACAC or AAGAC) with the values for $y^{1}\ w^{67c23}/Y,\ 10B\ y^{+}$ (my reference chromosomes) defined as 100%. AACAC and AAGAC are thus treated separately because one cannot support an a priori expectation that the AACAC and AAGAC primers sets should prime RT-PCR reactions with the same metrics (annealing temperature, elongation rate, fluorescence, efficiency, etc.). Similarly, determining absolute copy number (using known tRNA copy number as a multiplier) is not valid.

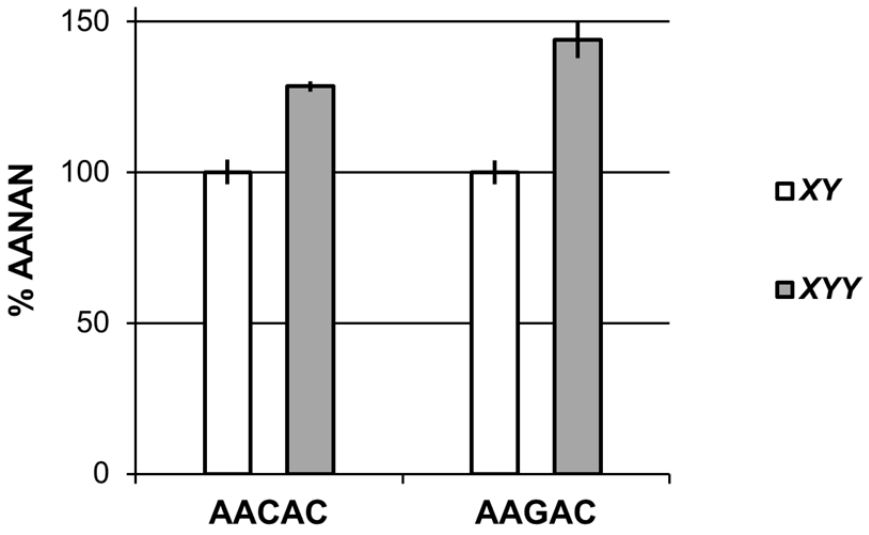


Figure 4.4 Quantification of satellite copy number in *X/Y* and *X/Y/Y* males. Percentages of each repeat type (%AANAN) in *X/X/Y* were calculated relative to X/Y (defined as 100%). Error bars represent standard error of the mean (S.E.M.) derived from triplicate reactions. Figure reprinted with permission from [98].

As expected, males with an extra *Y* chromosome possessed elevated AACAC and AAGAC repeats. By pooling siblings during DNA extraction, data on standard deviations between individuals was lost, hence the error bars report standard errors of the means (S.E.M). Based on these averages, I estimate that *Y*-linked blocks of AACAC and AAGAC contribute approximately 29% and 44% to the total amounts of those respective satellites to the euploid $y^1\ w^{67c23}$*/Y, 10B* y^+ genome.

It is of note that my estimate of *Y*-linked AAGAC levels differs from a previously published estimate of 69% [17]. While this discrepancy might simply reflect the differing sensitivities of RT-PCR and radiolabelled or fluorescence in

situ hybridization, it might also represent variation between different laboratory stocks. Repetitive sequence variation is of course not without precedence [96]. Examples include the expansion and contraction of ribosomal DNA in yeast and flies [113,140], as well as interspersed satellite copy-number polymorphisms in humans and plants [93,95]. Indeed, it is hypothesized that such variation may underlie the differential gene-regulatory effects of geographically divergent *Drosophila* Y chromosomes [92,105-107]

To address this possibility, I next asked if RT-PCR could be used to detect satellite copy number differences on three of the *Y* chromosomes used in studies of unidentified *Y*-linked regulatory variation, referred to as *Y, Ohio, Y, Congo*, and *Y, Zimbabwe*. I introduced each of these chromosomes into otherwise-isogenic backgrounds by multiple patrilineal backcross to strains bearing homozygous recessive mutations on the *X* and autosomes [102], effectively replacing all non-*Y* nuclear and cytoplasmic DNA (y^1 /*Y;* bw^1 *;* e^1 *;* ci^1 ey^1). In this way, I ensured that any observed satellite copy number differences were linked to the *Y* chromosome. Compared to *Y, Ohio* (our reference genotype for this experiment), AACAC levels were significantly higher (around 130%) on both *Y, Congo* and *Y, Zimbabwe* (P = 0.033 and 0.008, respectively, using Student's t-test), while *Y, Congo* possessed relatively fewer copies of both AAGAC (approximately 79%) and AAGAG (approximately 75%) (P = 0.038 and 0.037, respectively). No significant difference was observed in *Y, Zimbabwe*

AAGAC or AAGAG copy numbers compared to *Y, Ohio* (p = 0.098 and 0.862, respectively) (Figure 4.5).

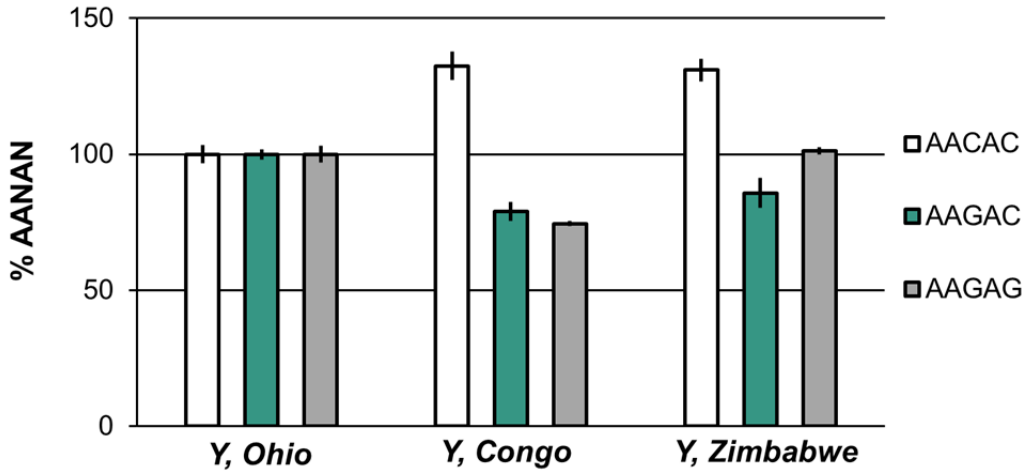


Figure 4.5 RT-PCR Quantification of *Y*-linked satellites in geographically divergent lines. DNA samples were obtained from males bearing *Y* chromosomes originally isolated from wild-caught flies. The genetic background of these males was otherwise isogenic. Percentages are relative to *Y, Ohio* (defined as 100%). Error bars represent S.E.M. of triplicate RT-PCR reactions. Figure reprinted with permission from [98].

To support these findings and compare my approach to alternative techniques, I used fluorescence in situ hybridization to detect AACAC sequences in larval neuroblast nuclei (Figure 4.6). Integration of data from ninety nuclei (thirty nuclei each from three separate brains dissected from sibling males) was largely consistent with my RT-PCR results: I confirmed significantly more AACAC in *Y, Congo* and Y, *Zimbabwe* compared to *Y, Ohio* (P = 0.036 and 0.008). The error bars in Figure 4.6 report standard deviation of integrated fluorescence from each nucleus and highlight the difficulty in quantification using

fluorescence hybridization, which is prone to vagaries in hybridization, photobleaching, and chromosome spread quality.

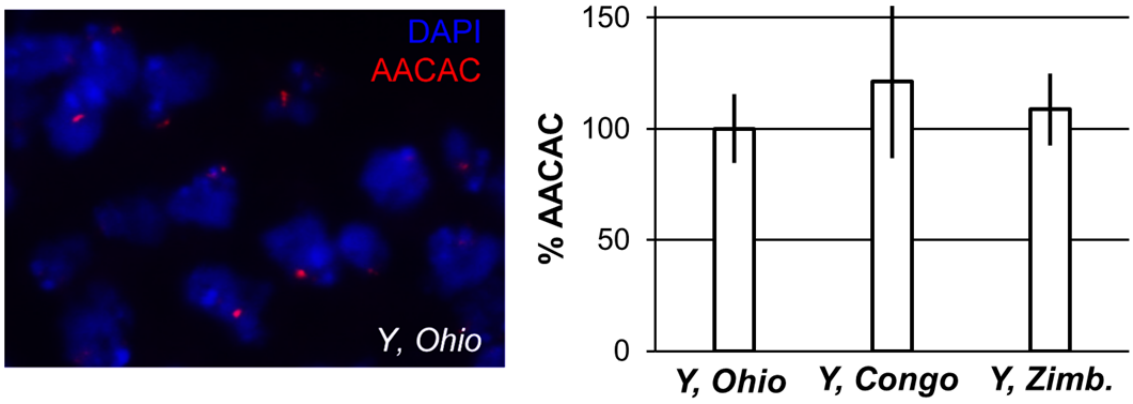


Figure 4.6 Quantification of AACAC in geographically divergent lines using fluorescent *in situ* hybridization. The left image was obtained using FISH to detect AACAC repeats (red) in squashed neuroblast cells derived from *Y, Ohio* larvae. DAPI stains DNA blue. Quantification of FISH signals is shown right. Percentages calculated relative to *Y, Ohio* (defined as 100%). Error bars represent standard deviation (S.D.) of nuclei from thirty neuroblasts from each of three separate preparations per genotype (N = 90). Tissue samples were obtained from males bearing *Y* chromosomes originally isolated from wild-caught flies; the genetic background of these males was otherwise isogenic. Figure reprinted with permission from [98].

Several models exist to explain repetitive sequence copy number variation of the type that is seen in wild-caught *Y* chromosomes. Polymerase slippage during replication is thought to be responsible for the changes in of simple sequence tracts while interchromosomal and intrachromosomal recombination events account for the gain or loss of larger portions of repetitive sequence [6,135,147]. Aberrant recombination in particular may be a common

mechanism linking copy number variation to the type of genomic instability observed at other repetitive arrays [136]. In *Drosophila*, rDNA stability is regulated by a variety of chromatin factors (e.g. Histone H3 Lysine-9 methyltransferase, HP1a, DCR-2, CTCF) [131,141,152]. Removal of these factors by mutation results in genomic instability, increased damage and repair defects in heterochromatin, and copy number changes [91,131,141].

To determine if mutations that alter heterochromatin-induced position effect variegation, rDNA expression, and rDNA stability also affect other satellite DNA copy numbers, I exposed our standard *Y* chromosome (*Y, 10B*) to a mutation hypothesized to destabilize heterochromatic repeats. The *Su(var)205* gene encodes heterochromatin protein 1a (HP1a), which is enriched at sites of heterochromatin and is required for heterochromatic silencing [41,228]. Notably, it is also required to maintain genomic stability in heterochromatin, and is involved in DNA repair of those sites [131,142]. Given these properties, I hypothesized that the *Su(var)205* mutation might act dominantly and induce satellite DNA copy number changes I placed *Y, 10B* into a *Su(var)205/+* mutant background and maintained it without selection for approximately 150 generations (approximately 6 years). In parallel I maintained a control *Y, 10B* in a control $y^1\ w^{67c23}$ background. After this, I moved the control *Y, 10B* and the six-year *Su(var)205* ‘‘tempered’’ counterpart ($Y,\ 10B^{t205}$) into the same isogenic background as above ($y^1\ ;\ bw^1\ ;\ e^1\ ;\ ci^1\ ey^1$) and quantified satellite copy number of AACAC, AAGAC, and AAGAG. I discovered that $Y,\ 10B^{t205}$ had 31%

more AACAC compared to *Y, 10B* (p = 0.007) and apparent but nonsignificant decreases in AAGAC and AAGAG (p = 0.300 and 0.168, respectively) (Figure 4.7).

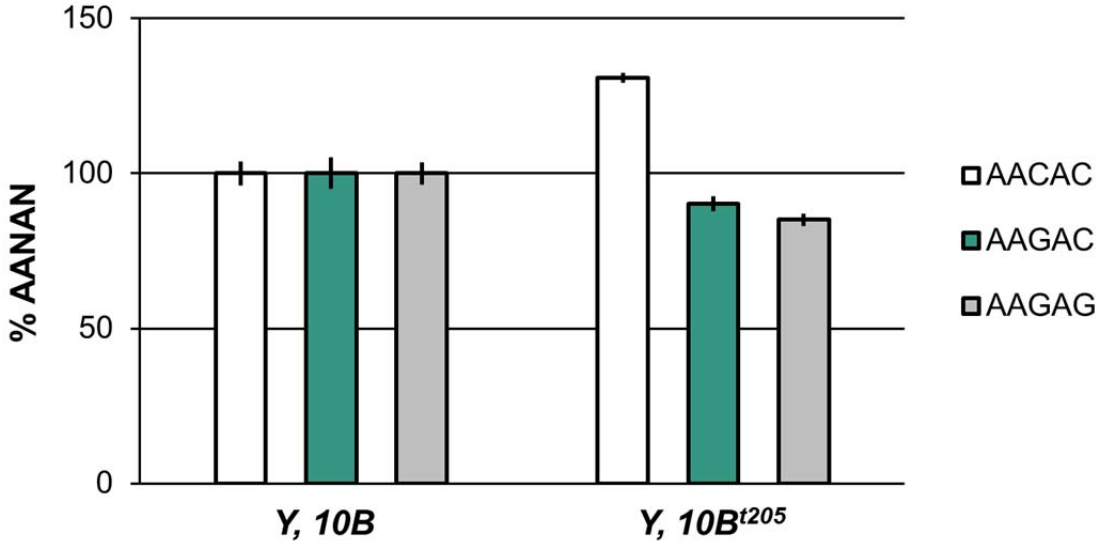


Figure 4.7 Long-term exposure to the *Su(var)205* mutation induces satellite copy number variation. Relative satellite copy number on *Y, 10B*^{t205} compared to *Y, 10B* (defined as 100%). The chromosomes are originally from a single progenitor, but the former was maintained for 6 years in a *Su(var)205*/*CyO* mutant background. Error bars represent S.E.M. of quadruplicate RT-PCR reactions. Figure reprinted with permission from [98].

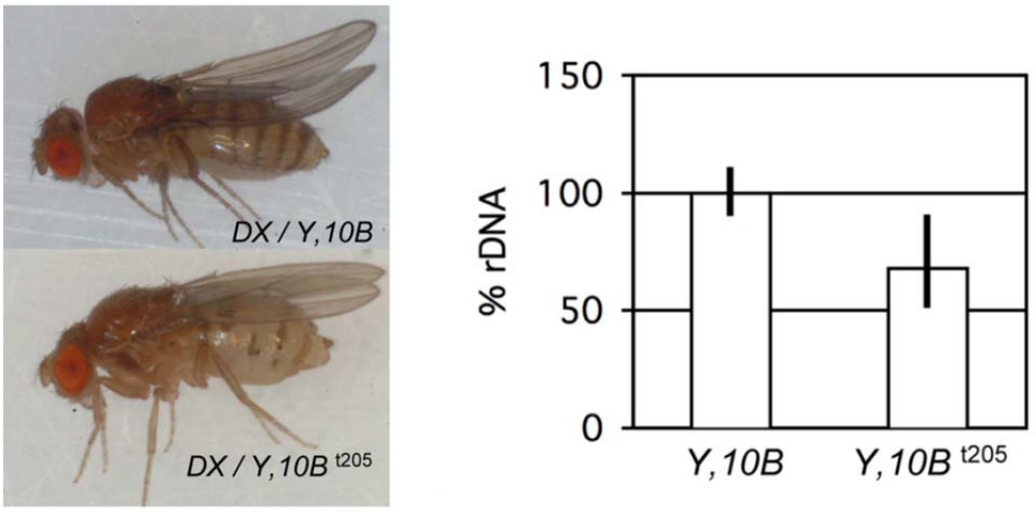


Figure 4.8 Long-term exposure to the *Su(var)205* mutation induces rDNA loss. Images of female flies of genotype *C(1)DX/Y, 10B* (top left) and *C(1)DX/Y, 10B*^{t205} (bottom left). The fly with *10B*^{t205} as sole source of rDNA exhibits a strong bobbed phenotype, indicating significant rDNA loss. RT-PCR determination of rDNA copy number in these flies is shown right. Error bars report S.E.M. of replicate RT-PCR reactions from pooled siblings. Figure reprinted with permission from [98].

Peng and Karpen have previously observed that mutations in *Su(var)205* destabilize the rDNA [131], and other studies have shown have shown that *Drosophila* strains with mutations in the methyltransferase responsible for creating the histone modification to which HP1a binds (*Su(var)3–9*) have few rDNA [130,152]. I therefore expected that in addition to destabilizing AACAC, and potentially AAGAC and AAGAG, rDNA copy number would be different between *Y, 10B* and *Y, 10B*^{t205}. Crossing these two chromosomes to females bearing a compound *X* chromosome devoid of rDNA (C(1)DX, rDNA^{0}) revealed that the latter expressed a bobbed phenotype of etched and herniated

abdominal dorsal cuticle, the manifestation of reduced translational capacity from reduced rDNA copy number (Figure 4.8). rDNA copy number quantification using RT-PCR confirmed a loss of rDNA in the *Y, 10B*^{t205} chromosome. Hence, exposure to *Su(var)205* mutation affects other repetitive DNAs of the *Y* chromosome.

Discussion

A number of methods currently exist for determining the copy number of satellite DNAs –the repetitive simple sequences that comprise nearly half of most eukaryotic genomes. These methods include quantification using fluorescence in situ hybridization [229], hybridization blots [17], and next-generation sequencing [230]. Each has benefits and drawbacks, therefore none are ideal, but all are useful depending on the specific investigation and limitations. The RT-PCR technique adapted for this study is simple in that it requires only routine DNA purification, two specially-designed satellite-specific primers, two ‘‘denominator’’ comparison primers, and is mathematically simple to calculate relative amplifications. With the growing awareness that repetitive satellite DNA in centric constitutive heterochromatin may be linked to ecological variation or disease proclivity, this technique fills a large and growing need. The approach I describe here is simple to perform, robust to fluctuations in DNA concentration or preparation, sensitive to small changes (I estimate 5% based on standard error) in satellite repeat copy number, very low-cost and rapid.

The total time from living organism to data is less than one day, making it rapid and useful for most purposes. The ability to perform analyses using as little as one nanogram of genomic DNA also allows independent assessment of satellite copy number in old samples, individuals, or dissected tissues, far below the useful detection limits of Southern blot analyses. The molecular nature allows satellite quantification even in cell types or organisms without established cytology. The rapidity, flexibility, and cost-effective nature of this assay makes it useful to a large number of investigators, even without resources for more expensive approaches (e.g., next-generation sequencing).

The design of primers should be broadly amenable to any satellite repeat. Although I only validated it here for pentameric repeat satellites, design of the mismatches are expected to be easier as the repeat length increases. Provided some foreknowledge of the repeat identities, use of this technique will allow investigators to begin to investigate questions about natural variation in copy number, or mutation- or treatment- induced changes to satellite copy number. To that end, between-satellite comparisons are not valid, nor are determinations of absolute copy number, using this technique. This is evident from the different Cq values in Figure 4.2, which I believe to be a function of the parameters of binding, priming, and elongation of different repeat sequences, or other factors that cannot be normalized across different primer or target sequences. However, between-organism comparisons of satellite copy numbers are valid, allowing

investigators to determine if mutations or treatments results in copy number variability.

I used both natural ecological variation and mutant analyses to validate my approach. Using RT-PCR, I noted heretofore undiscovered variation in satellite copy number in natural populations from wild-caught *Y* chromosomes from three different geographical sources. These polymorphisms, and others like them, may contribute to phenomena such as *Y*-linked Regulatory Variation or the ability of different chromosomes to variably suppress epigenetic heterochromatin-induced position effect variegation in trans [92,105,107,152].

I also discovered that a mutation in the *Su(var)205* gene, which encodes HP1a, results in satellite instability of a subset of repeat types. Previous cytological work showed that *Su(var)205* mutation, and a histone methyltransferase in the same chromatin modification pathway (*Su(var)3–9*) both act dominantly to cause nucleolar (rDNA) instability [130,131,141,152]. Moreover, the amount of damage (judged by repair foci in interphase cells) suggested that the damage was more widespread than just the rDNA [141]. Since it had not been mapped, it was undetermined if damage induced by Su(var)205 heterozygosity was limited to the soma or could affect germ cells, and thus be a source of satellite variability in natural populations.

I showed that a chromosome maintained long-term in a mutant of *Su(var)205* was induced to alter satellite copy number (Figure 4.7). This finding was striking because it shows that mutations thought to act ‘‘epigenetically’’ may

also act by altering chromosome structures at places that have not yet been investigated. HP1a appears to bind to all cytological heterochromatin, so the discovery that AACAC was increased in copy number while AAGAC and AAGAG were reduced was not predicted. Similar findings have been noted in a recent next-generation sequencing study of satellite sequence variation in *Drosophila* populations [97]. Wei and colleagues identified significant satellite variation in natural populations and found both positive and negative correlations between different sequences. This suggests at least some instability is nonrandom and that certain classes of sequences may be regulated independently of one another.

In regards to my findings, one possibility is that HP1a acts to stabilize some satellite sequences while destabilizing others. A number of studies establish a clear role for HP1a in the former [141,142], while the latter has no obvious mechanism but is nonetheless logically consistent with my observation. A second possibility is that HP1a acts to stabilize all satellite sequences but that gain and loss are both potential outcomes of instability. Similar to rDNA, instability may lead to loss through intrachromosomal recombination and either loss or gain through interchromosomal recombination. Additionally, increases in copy number could be accomplished by replication-coupled polymerase slippage, rolling-circle replication, re-replication, or some unknown event. A third possibility is that only some satellites are sensitive to HP1a reduction, but there is cross-talk between the different sequences. For instance, the *Su(var)205*

mutation may destabilize AAGAC and AAGAG while AACAC is initially unaffected. The loss of AAGAC and AAGAG sequence may in turn trigger some unknown mechanism leading to the stabilization or magnification of AACAC satellites independently of HP1a.

Our lab's anecdotal experience has been that stocks of some mutations – *Su(var)205* and *Su(var)3–9* among others – become stronger in their abilities to suppress variegation (their eponymous phenotype) after being established. While others have noted this, it has been informally accepted to be by selection as a consequence of the small populations and conditions of fly stock maintenance. My findings suggest that these mutations may also (or instead) induce copy number changes to unlinked satellite sequences, which themselves permanently alter phenotypes. The extent and consequence of such changes are unknown, but with RT-PCR copy number determination they may now be pursued.

CHAPTER V

MATERIALS AND METHODS

Fly Strains and Husbandry

All crosses were performed at 25°C and 80% humidity. Unless otherwise noted in the experiment, all stocks were maintained on standard cornmeal molasses medium. *C(1)DX,* y^1 f^1 bb^0 [146] was used to genetically isolate *Y* chromosomes for real-time PCR analysis and to visualize cuticular defects. y^1 w^{67c23}*/Dp(1;Y)* y^+ *, P{w = RS5}10B* (referred to in text as *Y, 10B*) was described previously [151], and was used for all feeding experiments in Chapter II and is the progenitor of rDNA deletion strains *Y, 183* (y^1 w^{67c23}*/Dp(1;Y)* y^+ *, P{w = RS5}10Bbb-183)*, *Y, 473* (y^1 w^{67c23}*/Dp(1;Y)* y^+ *, P{w = RS5}10BL-473)*[152], and *Y,* $10B^{t205}$ [98] as well as the strains created for this work *Y,* 183^{rev} and *Y, SSR* (Chapter III). I-CreI expression was achieved using the stock *P{*$v^{+t1.8}$*=hs-I-CreI.R}2A,* v^1[151].

Constitutively active insulin receptor expression (Figure 2.5) was performed using the following stocks: *w*; P{Ubi-GAL4}2/CyO, ,* y^1 w^{1118}*; P{UAS-InR. R418P}2,* [231], both of which were obtained from the Bloomington Drosophila Stock Center. w^{1118}*; P{*w^{+mC} *= UAS-mRFP-Fib}* was used for in vitro salivary gland culturing experiments to visualize nucleolar fibrillarin (Figure 2.5) [191], and was a gift from Dr. Patrick DiMario. Expression was under control of

the core *hsp70* promoter on the *pUAST* backbone, which provided ample expression to visualize without a GAL4 driver.

In Chapter IV, *X/Y/Y* males were generated by crossing spontaneously occurring $y^1\ w^{67c23}/y^1\ w^{67c23}/Y,\ 10B$ or $y^1\ w^{67c23}/y^1\ w^{67c23}/Y,\ B^S$ female primary nondisjunctants to $y^1\ w^{67c23}/Y,\ B^S$ *or* $y^1\ w^{67c23}/Y,\ 10B$ males, respectively. For the former, $y^1\ w^{67c23}/y^1\ w^{67c23}/Y,\ B^S$ virgins were crossed to $y^1\ w^{67c23}/Y,\ 10B$, then $y^1\ w^{67c23}/Y,\ 10B\ /Y,\ B^S$ male offspring backcrossed to $y^1\ w^{67c23}$ to create and maintain secondary nondisjunctional strains which produce large numbers of *X/Y/Y* males. *X/Y/Y* males were distinguished from their *X/Y* siblings by the severity of the Bar-stone or yellow$^+$ phenotypes. Geographically diverse *Y* chromosomes (*Y, Ohio, Y, Congo, and Y, Zimbabwe*) were obtained from Bernardo Lemos [105]. Chromosomes were placed in an isogenic background by crossing males to $y^1;\ bw^1\ ;\ e^4\ ;\ ey^R$ females and backcrossing to the maternal genotype until all four recessive markers were made homozygous. $Y,\ 10B^{t205}$ was generated by maintaining *Y, 10B in a* $y^1\ w^{67c23}$; $Su(var)205^{05}$*/ CyO* background for approximately six years.

Feeding Experiments

I used two experimental media for my feeding experiments in Chapter II. The first was based on SYA media used in dietary restriction studies [184] and contained 5% sucrose, 10% hydrolyzed yeast, 5% cornmeal (w/v), and 1% agar (w/v). These ingredients were boiled in deionized water and mixed until fully dissolved. Media was allowed to cool to 55°C before the addition of propionic

acid and tegosept to 0.3% each. I refer to this diet as SY10. SY30 was identical except it contained 30% yeast (w/v). One-third of the required yeast was added in increments during heating to allow easy dissolution. Standard (cornmeal) medium is 5% cornmeal (w/v), 3% yeast (w/v), 1% agar (w/v), 7% molasses (v/v), and supplemented with proprionic acid and tegosept as above.

For experiments testing the effects of larval diet, I collected embryos overnight on apple juice agar plates covered with yeast paste. Embryos were washed off plates using 1X PBS and transferred to experimental media at a uniform density [232]. To measure adult germline effects, males raised on Standard medium were collected 1–4 days post eclosion and crossed in groups of five to *C(1)DX/Y* virgins on Standard medium. After 24 hours, the males were removed and placed on either Standard medium, or Experimental media with or without 10 μM Rapamycin (LKT Laboratories), while females were maintained on Standard medium and allowed to lay eggs. Progeny from these females served as the baseline for subsequent comparisons. Males were transferred to fresh experimental media every 3 days for 20 days, after which they were again crossed to virgin *C(1)DX* females on Standard medium. Additional adult males fed in the above manner were crossed to $y^{1}\ w^{67c23}$ females to establish stocks for subsequent analysis.

RNA Analyses

Those wishing to analyze rRNA expression face at least three problems. First, it is very stable (by RNA standards), with a half-life of at least two days

[233], so steady-state rRNA levels are insufficient to detect changes in rRNA transcription. Second, it is very abundant, accounting for approximately 50% of transcription and 80–90% of steady-state RNA [111], so small differences in loading result in large variance in apparent rRNA concentration. Additionally, selection of any mRNA as a comparison ("denominator" in relative-abundance calculations) introduces even more variance as the quantification of differences in rRNA is more sensitive than differences in mRNAs with lower abundance (i.e., a 10% difference of 1000 is easier to detect than a 10% difference of 10). Third, the a priori assumption that any mRNA may not change in conditions in which rRNA expression changes may not be valid. For these reasons, I measured active pre-rRNA by detecting cDNAs derived from the *ETS-18S* junction, using reverse transcription primer GGAGGACGAGAAAATTGACAGACGCTTTGAGACAAGCATATAA. This primer was designed to be complementary to the *18S* at the junction of *ETS* and *18S*, and have a novel sequence at the 5' end for use in subsequent real time PCR. RNA and DNA were co-purified to satisfy the need for a stable (non-regulable) denominator for rRNA transcription levels. Total nucleic acids were purified from pools of either one hundred second instar larvae (for Figure 2.2), ten dissected and everted third instar larvae (for measuring effects of rapamycin and actinomycin D) (Chapter II), or pools of ten adult flies (Figure 3.8). Tissue was homogenized in a solution containing 50 mM EDTA, 100 mM Tris pH 7.0, and 1% SDS. Homogenate was extracted twice in equal volumes

phenol:chloroform:isoamyl alcohol (25:24:1, buffered with 1 M Tris pH 7.0). Under these conditions, all nucleic acids partition to the aqueous phase [234], which was further extracted with chloroform followed by diethyl ether. Nucleic acids were precipitated with 0.8 volumes propanol, washed with RNAse-free 70% ethanol, and resuspended in DNAse- and RNAse-free water. Reverse transcription was performed in 20 µL reactions with 2 µg nucleic acid, 2 units M-MuLV Reverse Transcriptase (New England Biolabs), 1X Reverse Transcriptase Buffer, 2 µM of each dNTP, and 4 µM primer for 1 hour at 42°C followed by 10 minutes at 90°C. Samples were diluted 1:250 for subsequent real time PCR reactions using primers GGAGGACGAGAAAATTGACAGACG and AAAATTTTTACCCAAAGGCAAAATATTGAATTACATTC to detect the *ETS-18S* derived cDNA. RT-PCR is further discussed below and in [132,152]. Melt-curve analysis was used to assure single melt peaks, and reaction efficiencies were determined using LinRegPCR [227] (average efficiency for the tRNA gene was 92% and for the *ETS-18S* was 82%). Efficiency correction and fold changes were calculated as in [235].

For northern blot analysis (Figure 2.2) RNA was extracted as previously described [236] from 3 pools of 20 male wandering 3rd instar larvae. 5 µg of RNA was separated on a 1.5% agarose formaldehyde gel and transferred to a charged nylon membrane [237]. Hybridization was performed using QuikHyb (Agilent) according to manufacturer's instructions using an anti-sense riboprobe complementary to the *35S* rRNA external transcribed spacer (ETS). The

ribopobe was synthesized in the presence of α-32P UTP using a Maxiscript T7 kit (Ambion) according to the manufacturer's instructions. The *in vitro* transcription template was generated via PCR using the following oligonucleotides (5'-3'):CAGTTCTTTTTGAACACGGGAC and GAAATTAATACGACTCACTATAGGCATAAAACCGAGCGCACATG. Autoradiographs were scanned using an Epson Perfection 4870 scanner and densitometry analysis was performed using Image-J v. 1.46r (NIH). Ethidium bromide stained mature rRNA served as a loading control to normalize each sample, and fold changes were calculated relative to SY10-fed larvae.

RT-PCR Quantification of Genomic DNA Copy Number

To measure rDNA copy number, RT-PCR analysis was performed as described [152]. DNA was extracted from newly eclosed adult flies in pools of three or more and quantified using a NanoDrop ND-1000. RT-PCR was performed with a StepOne Real-time PCR system and Power SYBR Green reagents (Applied Biosciences) using 4 ng DNA per reaction, unless otherwise stated. *18S* rDNA was amplified using primers AGCCTGAGAAACGGCTACCA and AGCTGGGAGTGGGTAATTTAC, while the endogenous loading control, $tRNA^{K\text{-}CTT}$, was amplified using CTAGCTCAGTCGGTAGAGCATGA and CCAACGTGGGGCTCGAAC.

For satellite measurements in Chapter IV, DNA was extracted from adult flies homogenized in pools of ten and quantified using a NanoDrop ND-1000. 10 ng was used for each reaction (except where indicated). RT-PCR was

performed was above, except with the following cycling conditions: 40 cycles: 95ºC for 3 s; 50ºC for 15 s; 60ºC for 30 s. Satellites were amplified using primers designed according to [224]. AACAC: GGTTTACACTACACATCACAAGACAACTCAACACAGCA and ACTCCAGTTG-TATTGTGATGTGTGGTGTTATGTTGTGC; AAGAC: GGTTTTAGCCAAGAGAA-GACCAGACACGACAACACAAGACTA and ACTCCATCTTGCCTTGTTTTGTCCTGTCTCGTCTTTTCTTGCCTTGTCTA; AAGAG: GGTTTTAGAAGTGAAGAT-AAGAGTAGAGATGAGAAGACAA and ACTCCATCTCTACTCTCTTGTCTTCACTTCTGTTCTCTT. $tRNA^{K\text{-}CTT}$, was used as the endogenous control. Primers were used at a concentration of 0.5 mM.

Unless otherwise noted, relative differences were calculated using the "ΔΔCT" method. Each data point presented consists of at least three independent biological samples quantified in triplicated or quadruplicated technical replicates. Error bars generally represent the standard deviation of biological replicates or standard error of tem mean of multiple technical replicates, as justified in the text [238]; error bars are generally asymmetric around the mean ("+" values are higher than "-"values") because I calculate error values prior to exponential transformation ($2^{-\Delta\Delta CT}$) for presentation. P-values were calculated from ΔΔCT values prior to exponential transformation (to get "fold" values) using StatPlus:mac v. 5.8 (AnalystSoft), Apple Numbers, or Microsoft Excel.

Immunofluorescence Microscopy

Whole mount salivary gland immunofluorescence (Figure 2.3 and 2.5) was performed as in [110]. Glands were dissected from 3rd instar larvae in 1X PBS and were then transferred to PBT (PBS containing 0.1% Triton X-100) and fixed in PBT containing 3.7% formaldehyde and blocked for 1 hour in PBT supplemented with 1% BSA. Glands were washed and incubated overnight at 4°C with a mouse anti-Fibrillarin primary antibody (Abcam) diluted 1:1000 in PNBT (PBT containing 1% BSA and 500 mM NaCl). Goat anti-mouse conjugated to rhodamine was used as a secondary antibody (1:1000). DNA was counterstained with 1 ng/mL DAPI (MP Biomedicals). All images were obtained using a Zeiss Axioskop 2 epifluorescence microscope running AxioVision (v. 4.6.3.0) with a 20X objective (numerical aperature = 0.5). Sequential excitation was performed at 543 nm (for Rhodamine and RFP) and 405 nm (for DAPI).

***In vitro* Salivary Gland Culture**

Larvae containing the homozygous *P{w^{+mC}= UAS-mRFP-Fib}* were reared in bottles and aged to the early wandering stage of the third instar. Males were dissected in 1X PBS and salivary glands were moved to Schneider media supplemented with 50 mg/mL streptomycin, 50 mg/mL penicillin, and 10% heat-inactivated fetal bovine serum (Gibco). Five to ten glands per condition were co-cultured and treated with 5 μM human insulin (Sigma) for 22–24 hours, then stained with 1 ng/mL DAPI for one hour prior to visualization. In some cases (noted in text) salivary glands were treated with 10 μM rapamycin or 0.6 μM

actinomycin D (Acros) for two hours prior to insulin addition. Images were taken of all stained salivary gland lobes near the anterior end at 20X, post-processed for bright/contrast, and scored for nucleolar structure. A nucleus was determined to contain “multiple” nucleoli if more than one separate focus of fibrillarin fluorescence was discernible. Glands that had no or poor DAPI staining were assumed to be damaged by treatment and excluded from analysis. The entire dissection and culture experiment was performed eight times over the course of two weeks and all data pooled to calculate frequencies presented in Figure 2.5.

Fluorescent in situ hybridization (FISH)

Fluorescence probe was made by end-labeling oligonucleotides of the respective satellite repeat (e.g., AACACAACACAACACAACACAACACAACAC) with digoxigenin-conjugated dUTP, and visualized with a mouse anti-digoxigenin antibody conjugated to rhodamine. Dissections, tissue preparation, and hybridizations were performed as described in [239] DNA was counterstained with 1 ng/mL DAPI (MP Biomedicals). All images were obtained using a Zeiss Axioskop 2 epifluorescence microscope running AxioVision (v. 4.6.3.0) with a 20X objective (numerical aperature = 0.5). Sequential excitation was performed at 543 nm (for Rhodamine) and 405 nm (for DAPI).

Starvation Resistance Assay

Adult males were collected 1-4 days post eclosion and allowed to feed on standard cormeal-molasses media for five days, after which they were transferred (10 flies per vial) to starvation media containing 1X PBS and 1%

agar. Vials were transferred every three days, and number of deaths was monitored daily. Flies escaping during transfer were not considered in analysis and I made no effort to distinguish between starved-to-death flies versus flies dying of some other cause (stuck to the side of the vial, for instance). Survival curves depict pooled data from at least sixty flies per genotype per experiment. Mean survival times were calculated using the Kaplan-Meier method and significance testing was performed using the log-rank test [240].

Lipid Analysis via TLC

Triacylglycerides were quantified according to [241]. Five flies per replicate were homogenized in 100 µl 2:1 chloroform:methanol and debris was pelleted via centrifugation at 8 krpm for 10 minutes. 7.5 µl of this solution was spotted (2.5 µl at a time) onto high performance thin layer chromatography (HPTLC) plates (Merck) and allowed to dry completely. 2.5µl Triolien (1mg/ml) was used as a TAG size marker and as a quantification standard. 100 ml 70:30:1 hexane:diethylether:acetic acid was used as the mobile phase and the tank was allowed to equilibrate 30 min prior to the addition of the TLC plate. Plate were developed by spraying with a general oxidizing stain composed of 8% (w/v) H_3PO_4 containing 10% (w/v) copper (II) sulfate pentahydrate then and baking at 100ºC for 10 min. Plates were scanned using an Epson Perfection 4870 scanner and densitometry analysis was performed using Image-J v. 1.46r (NIH). At least three biological replicates were performed per experiment, and each one was run on a separate TLC plate.

CHAPTER VI

SUMMARY AND DISCUSSION*

The Regulation of rDNA Instability

Genomic instability is a conserved feature of eukaryotic rDNA arrays [138,144,145,147,242,243]. “Instability,” in this case, refers to not only copy number hyper-variability, but also to the relatively rapid rate at which sequence is gained or lost—be it over a single generation [145] or even just a few somatic cell divisions [132]. This type of genomic instability is thought to be a direct consequence of the repetitive nature of rDNA. Homologous recombination between or within arrays can result in a variety of outcomes including loss or magnification events [136,140].

While instability at some rate is a natural occurrence, a number of factors are known to influence its frequency. Work performed primarily in *S. cerevisiae* using an rDNA-embedded reporter construct has identified numerous mutations which either increase or decrease this natural rDNA loss rate [138,140,174,176]. These studies underlie the generally accepted idea that rDNA hyper-instability is

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a transcriptionally-dependent phenomenon (Figure 6.1). Work in *Drosophila* has added to this view by identifying many heterochromatin components—themselves important regulators of transcription—as factors required for maintaining normal rDNA integrity (Figure 6.1) [131-133].

Apart from a few notable exceptions [182,196,242,244], most of the work concerning rDNA instability has dealt with the effects of various mutations. Previous work in our lab found measurable differences in rDNA copy number between somatic cells within the same tissue [132] suggesting, once again, that instability is natural and that fluctuations in some factor(s) or condition(s) can variably influence its occurrence. This suggested an interesting mechanism through with the environment might affect an organism's genotype. In the work presented in Chapter II, I demonstrate that diet—an established regulator of rRNA transcription [124,153,182]—is one environmental factor that can modulate rDNA copy number.

I found that flies reared on media containing elevated dietary yeast concentrations displayed increased nucleolar fragmentation as larvae (Figure 2.3) and fewer rDNA copies as adults (Figure 2.4). Although it is unknown what specific pathways mediate this effect, nucleolar instability was phenocopied by the expression of a hypermorphic insulin receptor allele as well as the administration of recombinant insulin to cultured tissue (Figure 2.5). The latter, was mitigated by treatment with drugs known to inhibit RNA polymerase I.

rRNA expression is a downstream target of many nutrient-sensing pathways [119-121], and a fairly straight-forward speculation would be that one or more of these mediate the diet-induced loss of rDNA—an explanation that would be consistent with most current models. Other possibilities include the various cellular responses to stress which have recently emerged as regulators of nucleolar integrity [173,175,179]. Given that the media on which a fly is maintained largely defines its environment, one could easily imagine a three-fold increase of dietary yeast constituting a “stressful” condition.

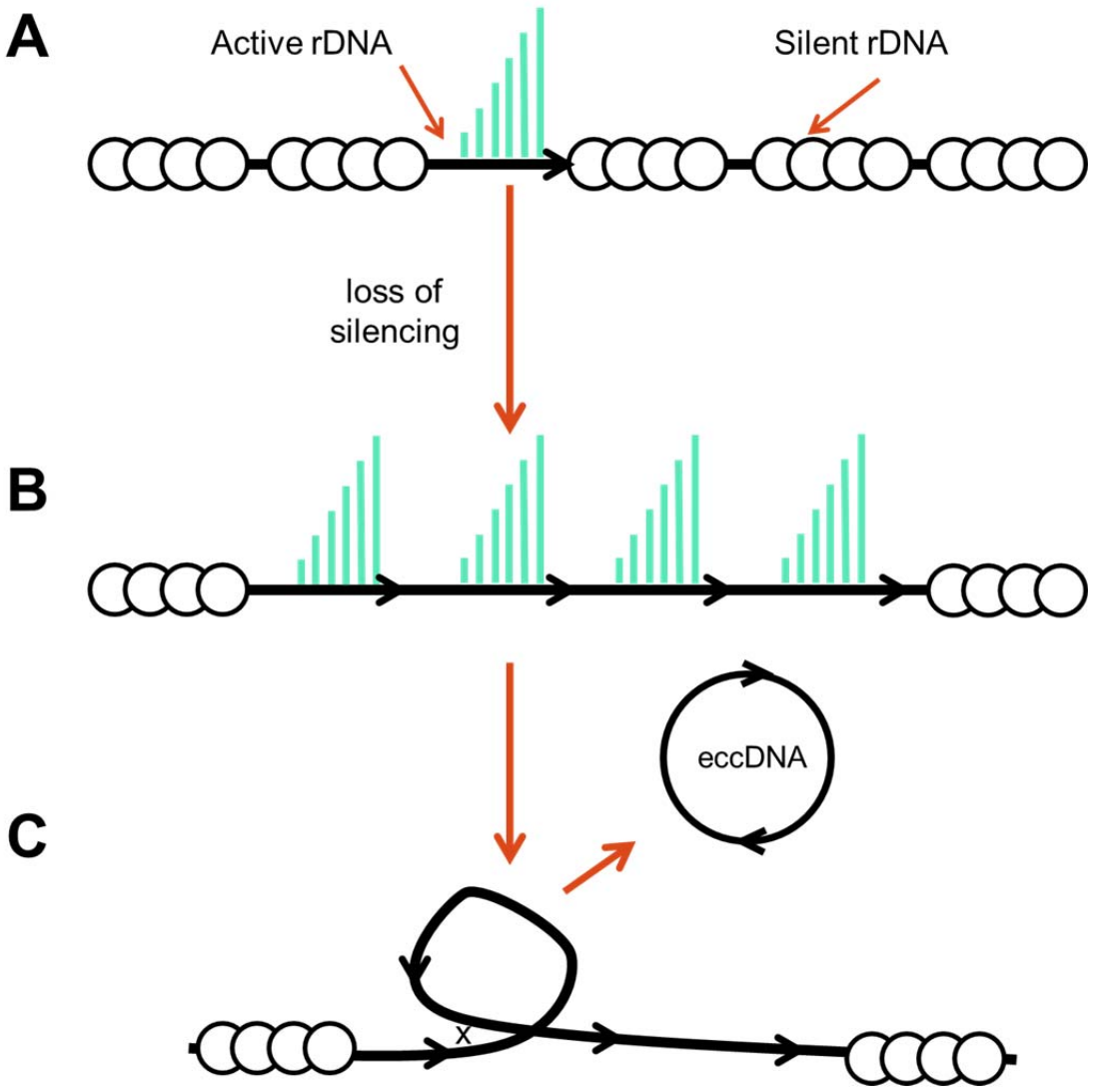

Figure 6.1 Model for transcriptionally-dependent rDNA instability. Redundant rDNA cistrons (black arrows) are found in repetitive arrays. (A) In order to maintain optimal rRNA levels, some copies are actively transcribed (turquoise lines) while others are silenced via heterochromatin (white circles). When silencing is removed—either through mutation or via some "natural" regulatory process—additional cistrons become active (B) and the array becomes susceptible to aberrant homologous recombination (C).

Dietary variation likely accounts for some rDNA loss, but some factor must be responsible for establishing a limit to the loss in nature. Although the mechanism for this maintenance is unknown, it could be an as-yet unobserved intentional regulated processes that assures minimal rDNA copy number, or it could be by normal selective pressures exerted by the *Minute* or *bobbed* phenotypes that result from very low ribosome number [146]. Alternatively, loss may be balanced by gain of rDNA through unequal sister chromatid exchange, gene conversion, re-replication, or cycles of excision, rolling-circle replication, and reintegration. Meiotic magnification and somatic pseudo-magnification at the rDNA have long been known in *Drosophila*, although the identification of a mechanism has eluded researchers for over 40 years [137,144]. Part of the loss limit may be the natural ecology of *Drosophila*, wherein older males—who have presumably accumulated more damage—may simply be less likely to mate, produce fewer offspring, or produce an altered sex ratio; ecological experiments would be needed to address these possible contributions.

While my work has clarified the relationship between rDNA instability, transcriptional output, and environmental stimuli, there are a number of unresolved questions, the largest of which is that it is unclear what actually produces the DNA damage necessary for aberrant homologous recombination. Regardless of the upstream mode of destabilization, rDNA loss or magnification requires homologous recombination which requires free DNA ends. Promising damage-inducing candidates include the various helicases that accompany the

transcriptional machinery [245,246]. Enzymes such as type I and II topoisomerases are required for transcriptional initiation and elongation and work by relaxing DNA coiling via the creation of double or single-strand cuts in the phosphor-diester backbone which are subsequently healed by the same enzyme [247-249]. Failure to repair the DNA backbone would produce the DSBs necessary for hyper-recombination and instability and the association of these enzymes with transcription would resolve the “transcriptional dependence” aspect of rDNA instability. That being said, the mechanism of helicase activity complicates the matter. The catalytic cycle of the breaking and rejoining DNA by helicases is such that the enzyme becomes covalently linked to the 5’ phosphate prior to the repair step [250]. Due to the absence of an exposed end, it is unlikely that DNA repair machinery would recognize this DNA/helicase intermediate. However, the recent discovery of incorporation of ribonucleotides in genomic dsDNA offers a potential solution to this problem since the DNA/helicase intermediate can be resolved via the linkage of the 5’ phosphate to the 2’ hydroxyl group present in ribonucleotides. Removal of the modified ribonucleotide by RNAse H leaves exposed DNA ends which are capable of serving as substrates for homologous recombination [251-253].

Instability at Other Repetitive Loci

I do not expect that induced changes to the genome are limited to the rDNA, in fact satellite sequences show copy number polymorphisms that are only now being investigated [97,98]. Extrachromosomal circles originating from

loci including stellate, microsatellites, and the histone cluster have been identified, suggesting that genomic instability may be a general feature of repetitive sequences. Furthermore, recent work in *Drosophila* has demonstrated that heterochromatin proteins are required to prevent damage to the underlying sequences and can be important mediators of DNA damage response pathway [91,141].

Using a novel RT-PCR technique, I demonstrate in Chapter IV that geographically diverse *Y* chromosomes vary in satellite sequence copy number (Figure 4.5). These chromosomes have previously been shown to differentially affect genome-wide transcription patterns [105] and it was speculated that satellite sequence polymorphisms may underlie some of those effects [92,107]. Furthermore, I found that a mutation in the gene encoding heterochromatin protein 1a (*Su(var)205*) induced satellite sequence polymorphisms on the *Y* chromosome (Figure 4.7). HP1a is also required for rDNA stability [98,107,131], and thus may be a common mediator of repetitive genome stability (Figure 4.8).

Phenotypic Consequences of Repetitive Sequence Variation

Y chromosomes isolated from geographically diverse populations differentially affect genome with expression patterns—a phenomenon termed *Y*-linked regulatory variation (YRV) [105,106,254]. Given the almost entirely heterochromatin nature of the *Y* chromosome [17,103], this finding suggest that previously unmapped variation in repetitive sequence may be phenotypically consequential. Work presented here (Figure 4.5) and previously [107],

demonstrates that copy-number variation exists in at least two classes of repeats (rDNA and satellites) and that rDNA variation in particular is partially responsible for the YRV phenomenon.

The work presented in Chapter III found that variation within *Y*-linked rDNA influences starvation resistance and lipid metabolism in *Drosophila*. These findings are consistent with previous work showing that genes related to metabolic function are differentially transcribed in *Y*-rDNA deletion strains [107]. As of now, the extent to which satellite sequence variation might have similar effects remains unknown. rDNA variation was estimated to account for only a portion of YRV and I speculate that satellite sequences may contribute to the rest. Without a method to magnify or delete such sequences, that hypothesis will be difficult to test. Like rDNA copy number [132], *Y* chromosome dose is known to be a powerful modifier of PEV [104], but it remains to be seen if such gross chromosome-wide manipulations are related to the mild copy number fluctuations we observe.

At this point it is unknown how variation within the rDNA is “sensed” by the cell and transduced to a particular phenotypic outcome (PEV, YRV, SR, lipid metabolism, etc.). Paredes proposed a mechanism in which various unknown activating and repressing factors are bound to both rDNA and other loci throughout the nucleus [26]. Deleting rDNA copies frees these factors, causing their relative concentration at other loci to increase. Depending on their binding specificity as well as the complexity of the targeted gene networks, this would

result in both the increases and decreases in transcription identified in the microarray study. Findings in this work support the idea that rDNA is an important modulator of phenotypic regulation (Chapter III), but suggest that copy number may not be the only form of consequential variation housed within the rDNA. An unbiased screen for starvation resistant *Y*-rDNA variants uncovered an array that actually had fewer rDNA copies than *Y, 10B* (Figure 3.8). Even in a phenotype as well understood as *bobbed*, it is clear that in some cases sequence quality can be at least as important as copy number [132].

It is unclear to what extent these various phenomena are related—apart from being genetically linked to the *Y*-rDNA locus. The differential transcription of various genes seems like an obvious outcome of the suppression of heterochromatic silencing (PEV) [132], yet these genes showed no location bias in terms of proximity to heterochromatin [107]. Likewise, one might expect the misregulation of lipase genes to lead to defects in lipid metabolism and sensitivity to starvation, but it is equally plausible that differential lipase expression is a regulatory response to an altered metabolic rate.

Although it is much too early to establish any specific causal relationships, variation in rRNA transcription levels (Figure 3.9) may be a unifying feature of some of these phenomena. I propose that **(1)** rRNA transcription levels vary between individual strains and that this variation is **(2)** related to some feature of rDNA sequence (copy number, arrangement, promoter strength, R1/R2 insertions, etc.). This as of yet unidentified feature

may control the recruitment rate or RNA polymerase I, or may have something to do with the overall "regulability" of a given array (i.e. how efficiently transcription from it can be turned up/down/on/off). Since rRNA production is a significant energetic cost, variation in transcription leads to **(3)** variation in metabolic rate. Under fully fed conditions, **(4a)** genes related to various metabolic and cellular processes are differentially expressed in order to compensate for the altered energy requirements of variably transcribed rRNA and maintain metabolic homeostasis. If variation in rRNA transcription persists under starvation conditions, then I would expect **(4b)** energy stores to be depleted more rapidly and death to occur earlier. This model suggests rRNA transcription as a common source for the effects of rDNA variation on genome wide transcription patterns, TAG metabolism, and starvation resistance. It is unclear at this point how rRNA transcriptional variation might modify the strength of heterochromatic silencing. Having identified an rDNA deletion with decreased rRNA expression (*Y, SSR*), it might now be possible to distinguish between the effects of transcriptional activity and copy number.

Induced Repetitive Sequence Variation as a Non-Epigenetic Mechanism of "Epigenetic" Inheritance

The environment can induce heritable genomic regulatory changes through both genetic and epigenetic means. Genetic mutations—induced by ionizing radiation or toxins—are transgenerationally stable, consequential, and random. Epigenetic models suggest a nonrandom, directed manner in which the

environment might impact specific genes [155-162]—for example, a gene in one generation becomes activated due to some environmental condition and remains activated for multiple generations despite the absence of the initiating stimulus. As previously discussed (Chapter I), the actual stability of most epigenetic modifications is questionable and the extent to which they serve as any sort of transcriptional memory remains unclear [56,164-166].

In this work (Chapter II), I demonstrate that Drosophila *Y*-rDNA stability and copy number are sensitive to environmental influence—in this case diet—and that the effects are transgenerationally heritable. Given the known consequences of rDNA variation [107,132], these findings suggest that induced instability represents both a stable and nonrandom mechanism through which environmental stimuli can heritably influence the genome. This mechanism shares many of the hallmarks of epigenetics in that it is environmentally influenced, transgenerationally heritable, and mediated by chromatin factors. A recent *Drosophila* study published in *Cell* [255] illustrates this concept and represents an interesting parallel to the work presented here. Ost and colleagues found that males fed a high sugar diet produced offspring with altered TAG levels, suppressed PEV, and differentially expressed metabolic genes—all of which are phenotypes that are now known to be linked to variation in *Y*-rDNA. Since these transgenerational alterations were accompanied by chromatin changes, it was reasonably assumed that the effects must be epigenetic in origin. Although the specifics differ, my work suggests diet-induced

rDNA instability as a testable alternative hypothesis. As of yet, it is unclear if the stabilities of other repetitive sequence classes are similarly sensitive to environmental stimuli such as diet, but the integrity of satellite sequences is known to require many of the same factors involved in epigenetic silencing [91,141], and thus represents another potential source of transgenerational inheritance. Despite ultimately being of genetic origin, the phenomena I describe in this work are of particular importance for our understanding of epigenetics since they suggest that conditions affecting chromatin may destabilize more than just the “epigenetic state” of a particular locus, but—in some cases—the underlying DNA sequences.

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APPENDIX A

SUPPLEMENTAL DATA

	% rDNA	+ SEM	- SEM
Control	100	113.5	88.1
SY30.1	106.9.9	119.9	95.3
SY30.2	60.9	67.9	54.6
SY30.3	84.5	91.0	77.7

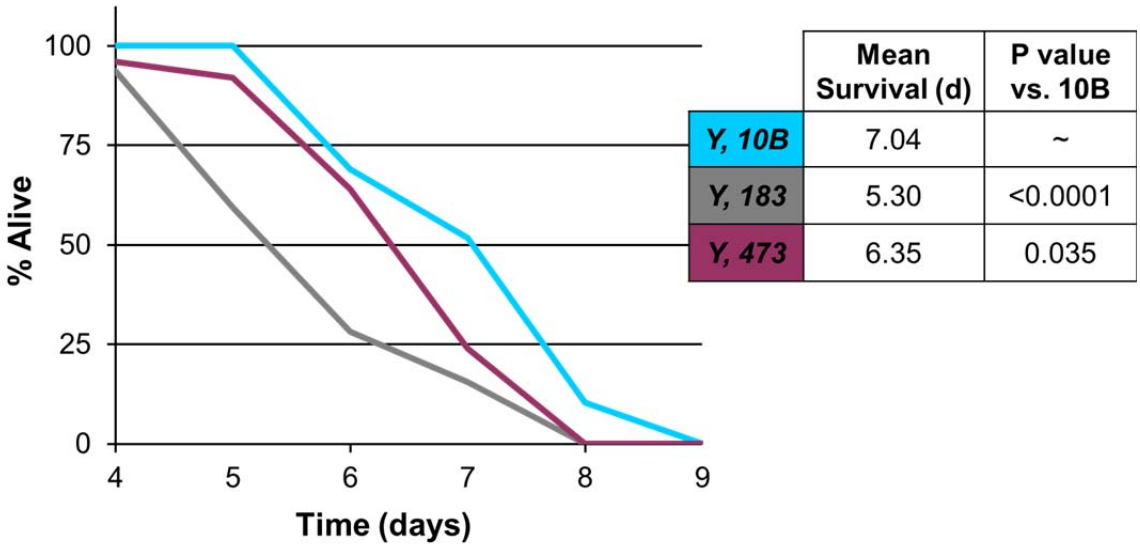
Table A.1 The effect of diet on head rDNA copy number. Real-time PCR analysis of *35S* rDNA copy number of isolated heads of adult males raised on SY30 as larvae. Genomic DNA was purified from twenty heads per sample using methods discussed in chapter VI. rDNA levels are normalized against genomic copies of $tRNA^{K\text{-}CTT}$. % rDNA was calculated relative to isogenic flies raised on standard food (defined as 100% and labeled "Control"). Standard error of the mean (SEM) was calculated from triplicate RT-PCR reactions.

	% Y-rDNA	+ SD	- SD	P value
Control	100	118.12	84.66	N/A
SY2.5	70.14	91.74	53.62	0.124

Table A.2 Transgenerational effect of dietary restriction on Y-rDNA copy number. RT-PCR was used to quantify Y-rDNA copy number of the progeny of 10B males maintained on either control medium or SY2.5 (10% sucrose, 2.5% yeast). % Y-rDNA was calculated relative to control progeny collected prior to dietary treatment, which is defined as "100%." Standard deviation (SD) was calculated from at least three independent DNA samples. P-value was calculated using Student's t-test.

Paternal Genotype	% Y-rDNA	+ SD	- SD	P value
nos-Gal4/+	100	129.33	77.32	N/A
nos-Gal4/UAS-InR.R418P	139.51	197.12	98.73	0.854

Table A.3 The effect of germline expression of constitutively active Insulin receptor on Y-rDNA copy number. Constitutively active insulin receptor (*InR.R418P*) was expressed in adult males using *nos-Gal4.VP16*, which is known to drive expression from *UAS* promoters in the female germline. % Y-rDNA was calculated relative to *the C(1)DX/Y* progeny of *nos-Gal4/+* control males, which were defined as "100%." Standard deviation (SD) was calculated from at least three independent DNA samples. P-value was calculated using Student's t-test.



	Mean Survival (d)	P value vs. 10B
Y, 10B	7.04	~
Y, 183	5.30	<0.0001
Y, 473	6.35	0.035

Figure A.1 Starvation resistance assayed via Drosophila activity monitor. Flies of the indicated genotype (n = 29, 32, 25 respectively) were placed individually into activity monitor tubes containing starvation media. Monitoring was performed in the dark following two complete 12 hour day/12 hour night cycles (48 hours total). Time of death was noted as the first day in which an individual fly displayed no activity. Mean survival was calculated using the Kaplan-Meier method and P values were calculated using the log-rank test.

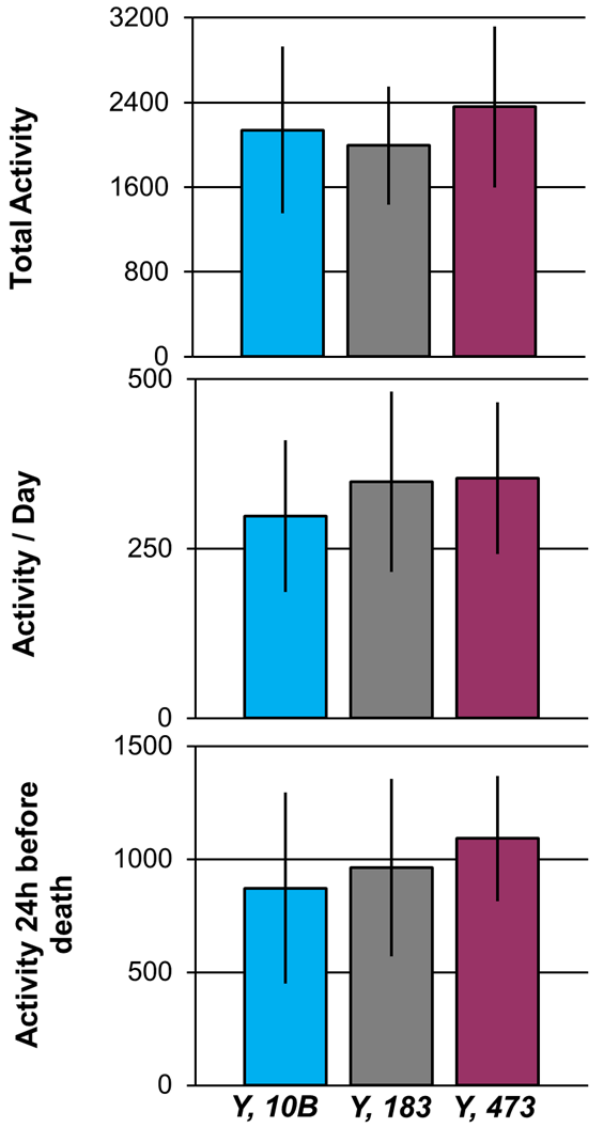


Figure A.2 Summary of activity monitor data. Flies of the indicated genotype were placed individually into activity monitor tubes containing starvation media. Monitoring was performed in the dark following two complete 12 hour day/12 hour night cycles (48 hours total). The monitor records each instance that an individual fly interrupts an infrared beam. Average total activity is presented in the top panel. The middle panel shows average total activity divided by survival time (in days) while the bottom graph indicates to average total activity occurring in the 24 hours preceding death. Note that activity was elevated in all genotypes 24 hours prior to death and that *Y, 473* was significantly more active than *Y, 10B* (P=0.0314, Student's t-test). No other statistically significant relationships were observed. All error bars represent standard deviation of 25-32 individual flies.

APPENDIX B

DETAILED EXPERIMENTAL PROTOCOLS*

B.1 pre-rRNA Quantification via RT-PCR

Nucleic Acid Isolation

1. Homogenize tissue (10 adults/3rd instar larvae or ~100 second instars) in 200 µl squish buffer

2. Add 200 µl PCI and vortex for 30 sec.

3. Microfuge 5 min. @ 13krpm and transfer supernatant to fresh tube

4. Repeat steps 2-3.

5. Add equal volumes chloroform to supernatant and vortex for 30 sec.

6. Microfuge 5 min. @ 13krpm and transfer supernatant to fresh tube

7. Precipitate nucleic acids with 0.8 volumes isopropanol—gently invert several times to mix and store on ice or at -20ºC for 20 min.

8. Microfuge 10 min. @ 13krpm

9. Discard supernatant and wash pellet with 70% ethanol

10. Discard ethanol and allow pellet to dry for <10 min. (overly dried pellets will be difficult to resuspend)

11. Resuspend pellet with ~50 µl DNase/RNase-free water—heat to 70ºC for better resuspension

12. Quantify RNA with nanodrop and dilute to 20 µg/µl

* Adapted from references 132, 152, 248, and 249 and summarized in Chapter VI

Reverse Transcription

1. For triplicate RT reactions, assemble the following on ice and mix by pipetting up and down:

35 µl	RNA
14 µl	dNTPs mix
7 µl	RT-Buffer (10X)
7 µl	RT primer

3. Heat for 5 min. at 70ºC, then place on ice for 5 additional min.

4. Add 3.5 µl M-MuLV RT and 3.5 µl RNase Inhibitor

5. Mix thoroughly via pipetting and transfer in 20 µl aliquots to three 200 µl PCR tubes

6. Incubate reactions for 60 min. @ 42ºC on a thermocycler

7. Inactivate reaction by raising temperature to 90ºC for 10 min.

8. Dilute with 80 µl water or TE and store @ 4ºC—freezing greatly reduces the quality of the cDNA whereas storing it in the refrigerator for ~1 week doesn't seem to have an effect

9. This cDNA solution is typically further diluted ~ 1:250 prior to Real-Time PCR analysis, but the precise dilution must be determined empirically (a given dilution should be relatively reproducible in the PCR reaction and have a quantification cycle of around 17 to 25)

Real-Time PCR

1. For each cDNA sample, assemble the following reactions on ice:

   pre-rRNA reaction

14 µl	cDNA
7 µl	F/R pre-rRNA primer
21 µl	SYBr Green Master mix

   tRNA reaction

14 µl	cDNA
7 µl	F/R tRNA primer
21 µl	SYBR Green Master mix

3. Divide each reaction into triplicate 12 µl RT-PCR reactions

4. Perform RT-PCR using default cycling conditions (~2 hours)—T_a=60ºC

5. C_T values for each reaction are averaged and expression levels can be quantified using either the $\Delta\Delta C_T$ method (A) or efficiency correction (B)

(A) Average $C_T^{pre\text{-}rRNA}$ – Average C_T^{tRNA} = ΔC_T

$\Delta C_T^{sample} - \Delta C_T^{ctrl} = \Delta\Delta C_T$

$2^{-\Delta\Delta CT}$ = fold change

(B) $1.8^{\Delta CTpre\text{-}rRNA} \div 1.92^{\Delta CTtRNA}$ = Expression Ratio (E)

$E^{sample} \div E^{ctrl}$ = Relative expression ratio

Reagents

Squish Buffer
50 mM EDTA
100 mM Tris (pH 7)
1% SDS

PCI
Phenol/Chloroform/Isoamyl alcohol 25:24:1 (pH 7)

TE
10 mM Tris (pH 8)
1 mM EDTA

Other Reagents
dNTP mix (10 µM each)
10X RT Buffer (NEB)
M-MuLV RT (200 U/µl) (NEB)
RNase Inhibitor (NEB)
2X *Power* SYBR Green Master Mix (Invitrogen)

RT Primer (40 µM)

5'-GGAGGACGAGAAAATTGACAGACGCTTTGAGACAAGCATATAAC-3'

pre-rRNA F/R primer (3 μM each)

5’-AAAATTTTTACCCAAAGGCAAAATATTGAATTACATTC-3’

5'-GGAGGACGAGAAAATTGACAGACG-3’

tRNA F/R primer (3 μM each)

5’-CTAGCTCAGTCGGTAGAGCATGA-3’

5’-CCAACGTGGGGCTCGAAC-3’

B.2 Lipid quantification via Thin Layer Chromatography (TLC)*

1. Homogenize 5 adult flies in 100 μl 2:1 chloroform/methanol

2. Microfuge 10 min. @ 8krpm to pellet debris

3. Store lipid solution @ -20ºC

4. Prepare the TLC tank by adding 70 ml hexane, 30 ml diethyl ether, and 1 ml glacial acetic acid along with a piece of filter paper approximately 8” X 5” placed vertically along the back wall of the tank. Close the tank and allow it to equilibrate for 30 min.

5. Meanwhile, mark the origin on the HPTLC plate by lightly drawing a horizontal line (with pencil) approximately 2 cm from the bottom of the plate

6. Demarcate individual lanes by lightly etching a ~2 cm vertical lines perpendicular to the origin using a razor blade—typically, around 10 lanes spaced 1.75 cm apart can fit on a plate

7. Spot lipid solution 2.5 μl at a time in the center of the origin, letting each spot dry completely before applying another—I typically use 7.5 μl total for each sample and 2.5 μl for the standard, but this should be determined empirically

* Adapted from reference 255 and summarized in Chapter VI

8. Once fully dry, place the HPTLC plate into the TLC tank, with the lid on, until the dye front is approximately 2 cm from the top of the plate (~15+ min.)

9. Remove plate and allow it to dry completely

10. Lightly spray the plate with the oxidizing stain, saturating the entire surface of the plate

11. Allow the plate to completely dry and then bake at 100ºC for ~10 min. until it develops

12. Developed plates can be scanned and quantified using densitometry and stored in a plastic bag indefinitely

Reagents

Extraction solvent
2 parts Chloroform
1 part Methanol

Running solvent
70 ml Hexane
30 ml diethyl ether
1 ml Glacial acetic acid

Oxidizing stain
8% (w/v) H_3PO_4
10% (w/v) copper (II) sulfate pentahydrate
in water

Triacylglyceride Standard
Triolien (1 mg/ml)