THE RIBOSOMAL DNA GENES INFLUENCE GENOME-WIDE GENE EXPRESSION IN *Drosophila melanogaster*

A Dissertation

by

SILVANA PAREDES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2011

Major Subject: Biology

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Approved by:

Chair of Committee,	Keith Maggert
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ABSTRACT

The Ribosomal DNA Genes Influence Genome-Wide Gene Expression in *Drosophila melanogaster*.

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Chair of Advisory Committee: Dr. Keith Maggert

Chromatin structure is a fundamental determinant of eukaryotic gene expression and it is composed of two chromatin environments, euchromatin and heterochromatin. Euchromatin provides an accessible platform for transcription factors; hence it is permissive for gene expression. Heterochromatin on the other hand is highly compacted and inaccessible, which in most cases leads to transcriptional repression. A locus that is composed of both of these environments is the ribosomal DNA (*rDNA*). In eukaryotes the *rDNA* is composed of hundreds to thousands of tandemly repeated genes, and it is known that maintaining both silent and active copies is fundamental for the stability of the genome. The aim of my research was to investigate the role of the *rDNA* in gene expression in *Drosophila melanogaster*.

In *D. melanogaster*, the *rDNA* loci are present on the *X* and *Y* chromosomes. I used the *Y*-linked *rDNA* array to investigate the role of this locus on gene expression.

I designed a genetic and molecular strategy to create and quantify specific, graded and isogenic *Y*- linked *rDNA* deletions. I then used the deletions to address the effect of *rDNA* deletions on gene expression using reporter genes sensitive to Position Effect Variegation. In addition, I tested the effect of the deletions in nucleolus size and structure as well as the effect of spontaneous *rDNA* deletions on gene expression. I found that changes in *rDNA* size change the chromatin balance, which resulted in increased expression of the reporter genes, decreased nucleolus volume, and altered nucleolus structure. This prompted me to ask whether this effect on gene expression occured globally in the genome. To address this we performed microarray analysis and found that *rDNA* deletions affect about half of the genes on the genome. I present here evidence that suggest a novel role for the *rDNA* as a global modulator of gene expression and also as a contributor to the gene expression variance observed in natural populations.

DEDICATION

This dissertation is dedicated to the memory of my father and to my mother for unconditional love and support and for showing me that the real purpose of life is to find happiness. To my brothers for believing in me and for helping me to become a stronger person. To my husband, Robert J. Pratt, for his unconditional love and support, and invaluable scientific input to my work. To my friends here and there for being with me during the good and the bad times.

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CHAPTER I

INTRODUCTION

Chromatin

The eukaryotic genome is packed in a limited space in the nucleus. In a mammalian cell, 1.7 meters of DNA are packed in a 5-micrometer nucleus in a way that allows for it to be replicated and transcribed properly (1). The genetic material is organized at different structural levels in order to confer the compaction that is required to fit all the information into the nucleus. Histone proteins bind to the DNA to help to create the different levels of compaction in what it is known to be the basic unit of chromatin: the nucleosome. Nucleosomes are composed of 146 bp of DNA wrapped around a histone octamer, which contains two units of each histone H2A, H2B, H3 and H4. Furthermore, the linker histone H1 and 50–60 bp of linker DNA connect the core histones to form a chromatosome (2). The main core of the histone is in direct contact with the DNA and the histone N-terminal tails are facing outwards, where they are exposed to become the target of different posttranscriptional modifications such as acetylation, methylation, ubiquitinylation, ADP ribosylation and biotinylation (3). These modifications change the physical interactions

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between the DNA and the histones and additionally create an appropriate platform for the binding of chromatin modifiers and chromatin components to establish the chromatin structure. The result of these chromatin modifications is the formation of an environment for gene expression that can be either repressive or permissive (4).

When permissive the chromatin is relaxed, thus transcription factors and chromatin remodelers have easy access to the DNA and transcription can take place. This environment is known as euchromatin (eu=good). In contrast, a transcriptional repressive environment is obtained when the chromatin is tightly compacted; hence accessibility to the DNA by transcription factors is more difficult in most of the cases (5). This repressive environment is know as heterochromatin (hetero=different relative to euchromatin) (3). When the DNA is stained with a DNA binding compound such as Hoechst, in an interphase nucleus the two chromatin environments are easily observed, heterochromatin is visualized as dark stained regions while euchromatin is observed as light and more abundant regions. In prophase, heterochromatin stains deeply and maintains a compacted organization during all the stages of the mitotic cycle (6). In *Drosophila*, heterochromatic regions are visible at the centromere and nearby it (referred to as pericentric heterochromatin), at telomeres, in the fourth chromosome, throughout the *Y* chromosome and spread among chromosome arms (referred to as intercalary heterochromatin) (7).

Heterochromatin can be constitutive or facultative. Facultative

heterochromatin refers to regions that are tightly packed, but that are present in a cell-specific manner, which can be clonally inherited. Examples of this are the inactive *X* chromosome in female mammalian somatic cells, imprinted autosomal genes and developmentally regulated *Hox* genes (8, 9). On the other hand, constitutive heterochromatin is found at the telomeres and centromeres, and it is found in all somatic cell types in an organism. Constitutive heterochromatin is a common characteristic of the eukaryote genome, composes 5% of the genome in *Arabidopsis thaliana* and 30% in both humans and *Drosophila*. In *Drosophila* the *Y* and the fourth chromosomes are largely composed of constitutive heterochromatin. This type of heterochromatin is characterized by low gene density, reduced meiotic recombination, late replication during S phase, enrichment in highly repeated DNA sequences such as transposons and satellite DNA, and very low levels of transcription (10).

For many years it was believed that constitutive heterochromatin was only genomic waste, but this idea has been modified based on genetic, cytological and molecular studies done primarily in the model organism *D. melanogaster*. These studies have shown that constitutive heterochromatin performs important cellular functions such as serving as a repressor and activator of transcription (11), ensuring proper achiasmate disjunction in female meiosis I (12, 13), and carrying essential genes for viability and fertility (8, 14). In addition, about 600 predicted genes have been identified by the annotation of the heterochromatin sequence (15). Therefore perfect assembly of heterochromatin is an essential

step for the cells.

The presence of coding genes in heterochromatin is not an exclusive characteristic of *Drosophila*, but it seems to be a conserved trait in the evolution of eukaryotic genomes. Heterochromatic genes have been also found in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Arabidopsis thaliana*, *Oryza sativa* and in humans (16-20).

There is not a clear group of characteristics that can be used to discriminate euchromatin from heterochromatin. The two chromatin environments share basic characteristics, such as DNA, transcription factor binding, transcriptional corepressors, chromatin remodelers, histone variants, chromatin-modifying enzymes and DNA modifications. In addition, the histones of constitutive heterochromatin can be distinguishable from euchromatin in that they are remarkably under acetylated (21). However, there are some unique features required for proper establishment of heterochromatin such as: very long stretches of repeated DNA sequences that do not have enhancers and promoters and that mostly encode aberrant RNAs transcripts. Furthermore, there are different marks that are specific for each heterochromatic region. These marks can be histone variants and post-translational histone modifications.

Histone variants also help to mark regions of silencing from regions of transcription in different eukaryotes. For example the histone H2A variant, H2A.Z marks regions of active transcription, however it was recently found to be

localized in pericentric heterochromatin, which means that even though it is mostly enriched in euchromatin it is not restricted to it. Similarly, the histone H3 variant H3.3 is tightly related with active transcription. On the other hand, heterochromatic regions are marked with the histone H2 variant macro H2A and the histone H3 variants H3.2 and CenH3 (*CENP-A* in humans, *Cse4* in *Saccharomyces cerevisiae,* and CID in *Drosophila melanogaster*), which is present exclusively at the centromeric regions (9, 22). Similarly, there are some euchromatic marks that are conserved from yeast to humans, such as hyperacetylation of histones H3 and H4 and methylation of histone H3 at the lysine 4 residue (H3K4) (4, 23). Acetylation reduces the binding strength of the DNA with the lysine residue on the histone tail, and the weakening of this interaction makes the DNA more accessible for transcription factors (24). In addition to this, lysine acetylation also provides a specific target for several transcriptional activators and chromatin remodeler proteins, which bind to the targets through their bromodomain (25). Acetylation of different lysines, for instance H4K12, has also been implicated with heterochromatin. Therefore, acetylation is a mark that can be used to obtain specific effects on gene expression but is mostly associated with transcriptional activation (26). Histone methylation is another mark that has been associated with both euchromatin and heterochromatin. Methylation in histone H3K4, H3K36 and H3K79 are found on actively transcribed regions, while methylation in histones H3K9, H3K27 and H4K20 are prominent markers of silencing (27).

A prominent mark of heterochromatic regions is Heterochromatin Protein 1 (HP1), which was identified biochemically using monoclonal antibodies against nuclear proteins tightly bound to DNA in *Drosophila*. This protein was observed to be mostly present in pericentric chromatin and other heterochromatic regions in polytene chromosomes (28). HP1 has an N-terminal chromo domain (29) which binds the histone H3 tail when it is dimethylated on lysine 9, and a C-terminal chromo shadow domain which is known to mediate protein-protein interactions such as the homodimerization of HP1, and HP1 binding to several other proteins to form a higher order chromatin structure (30-32). The binding characteristics of HP1 have suggested a model for heterochromatin formation in which two HP1 proteins interact through their chromo shadow domains while interacting with different nucleosomes that are methylated at the residue lysine 9 of the histone H3 tail through their chromo domain. This interaction locks the nucleosomes and converts the chromatin into a sturdy structure that is much less accessible to transcription factors (33). The chromo shadow domain of HP1 also recruits H3K9 histone methyltransferase (HMT), which helps to propagate the methylation mark and consequently more HP1 proteins. Together this will spread the heterochromatic state to the neighboring regions. This spreading model is thought to be conserved from fission yeast to humans (5). However, recent studies have challenged the view of HP1- mediated heterochromatin formation as an immobile platform. It has been shown that the binding of HP1 is transient and dynamic, and that its localization is observed in heterochromatin

and euchromatin meaning that the interactions between this protein and H3K9 methylation might be weak enough to allow HP1 to be mobilized to other targets by competition (34-36). Localization of HP1 in euchromatin has a transcriptional activator effect which is not dependent on H3K9 methylation. Although this has been observed to occur in several euchromatic genes, the mechanism of activation is still unclear but it seems that it might involve interaction of HP1 with transcription factors (37). Variations to the HP1-H3K9 methylation model are observed in *Neurospora crassa* and mammals, where DNA-methyltransferases are known to interact with HP1 to silence DNA regions (38, 39).

DNA methylation is a shared feature of silent regions that is present in animals, plants, fungi and *Drosophila* (40, 41). Cytosine bases that are next to guanine (CpG) are converted in 5-methylcytosine by DNA-methyltransferases, and this alteration is a mark of gene silencing and genome integrity (40). An important addition to the heterochromatin formation model came from studies done in fission yeast, which found a connection between RNAi and heterochromatin formation. In fission yeast, heterochromatin contains tandem arrays of a repeated unit. Using transgenes inserted on these arrays and mutations in genes involved in RNAi, it was demonstrated that the mutations of RNAi genes resulted in derepression of the array repeats and the transgenes present on them (42). The transcriptional activation obtained coincided with the loss of H3K9 methylation and the redistribution of SWI6 (HP1 homologue in yeast) on the genome. Likely due to the role of heterochromatin in centromere

structure, an abnormal chromosomal segregation also result from the disruption of RNAi (43). This data suggested that the tandem repeats present in heterochromatin generate RNAs that can be processed into double stranded RNA and later into siRNAs by the RNAi pathway. The siRNAs, associated with the RNAi-induced transcriptional silencing (RITS) complex, guide the targeting of H3 methyltransferase to the chromatin and after H3K9 has been methylated, HP1 binds to set up the repressive transcriptional environment (42).

In *Drosophila*, a very similar mechanism was shown to occur. The heterochromatic chromocenter of polytene chromosomes is rich in H3K9 methylation (44), and enriched with HP1 (45). Mutations in components of the RNAi pathway generate loss of gene silencing coincident with a decrease in the level of methylation of H3K9 and a redistribution of HP1 from the chromocenter (46). In addition, centromeric and pericentromeric transcripts have been detected throughout several stages of mammalian development. Although many of the factors involved in heterochromatin formation are conserved from yeast to mammals, it is not clear yet if these transcripts are involved in heterochromatin formation or maintenance (47).

Position Effect Variegation

In 1930 H. J. Muller discovered a phenomenon known as Position Effect Variegation (PEV) that changed the view of chromosome structure (48). He created a series of X-ray induced *Drosophila* mutants that affected the

expression of the *white*$^{+}$ gene (which confers the red pigmentation to the eyes). He observed that in some of the recovered mutants the red pigment of the eye did not change to a different shade or to white, but instead they generated a mottled phenotype characterized by the presence of red and white patches in the eye as a consequence of differential clonal *white*$^{+}$ expression (49). The common characteristic of these mutants is that they had chromosomal rearrangements that displaced the *white*$^{+}$ gene, normally found in euchromatin, to within or nearby heterochromatin. It was later suggested that the inactivation of the *white*$^{+}$ gene in some patches was caused by spreading of the heterochromatin inducing its silencing (50). Likewise, genes that are endogenously located in heterochromatin become silenced when they are translocated within or nearby euchromatin. An example of this is the *light* gene, which is actively transcribed from its location within pericentric heterochromatin (51). The finding that chromatin influences gene expression has been extensively used to study factors that are involved in chromatin formation and its role in regulation of gene expression.

PEV can be observed as a *cis*-inactivation effect in chromosomal rearrangements (as first discovered), and in transposon insertions, when a transposable element that carries a euchromatic gene is inserted into heterochromatin. In addition PEV can be observed as a *trans*-inactivation effect. The *cis*-inactivation PEV gives recessive phenotypes, hence in the presence of a wild type allele the PEV phenotype is lost (52). In contrast, the *trans*-

inactivation PEV gives a dominant phenotype and is not very common. Rearrangements that affect the *brown*$^{+}$ (*bw*) gene are the most studied example of PEV caused by *trans*-inactivation. These rearrangements give a PEV phenotype in the presence of a chromosome that carries a wild type *brown*$^{+}$ allele, suggesting that the variegating *brown* gene has a trans-inactivating effect over the wild type allele. The most studied *brown* allele is the *brown*^{Dominant} (*bw*^{D}), which is not a chromosomal rearrangement but instead it has a block of centromeric heterochromatin inserted within the *brown* gene making it non functional (53). Flies that are heterozygous for this locus (*bw*$^{+}$/*bw*^{D}) exhibit a PEV phenotype that responds to modifiers of PEV (54). Furthermore, it has been shown that the effect is pairing dependent, since insertions of the *brown*$^{+}$ allele at chromosomal locations that do not pair with the *bw*^{D} allele, are able to restore the wild type phenotype (55). The proposed hypothesis is that during chromosome pairing the heterochromatin block inserted into the *bw*^{D} allele is able to drag the two alleles into a nucleus compartment where the centromeric regions localize, therefore silencing the expression of the *brown* wild type allele (56).

Modifiers of PEV

The PEV phenotype can be enhanced or suppress by mutations on genes that encode chromatin components or modifiers. Indeed, the study of these mutations has increased our knowledge on chromatin structure and its effects on

gene expression. When the PEV phenotype is enhanced by a mutation, the silencing of the reporter gene increases indicating that the absent gene product is involved in creating a chromatin environment accessible to transcription or euchromatin. Genes that increase gene silencing on PEV are known as enhancers of variegation (E(var)s). In contrast, when the PEV phenotype is suppressed by a mutation, the silencing of the reporter gene is decreased indicating that the mutated gene is involved in heterochromatin formation. Genes that decrease gene silencing on PEV are known as suppressors of variegation (Su(var)s) (50, 52, 57).

On the other hand, genes that are normally expressed within heterochromatin (e.g. *light*$^{+}$) have an opposite response to the action of Su(var)s and E(var)s. Therefore, mutations that act as Su(var)s for euchromatic genes will act as E(var)s for heterochromatic genes, and the same is truth for E(var)s (58, 59). Many of the alelles found to affect PEV are dominant mutations and some of them are known to be haplo suppressor/triplo enhancers, such as Heterochromatin protein 1(HP1) (*Su(var)205*) and Histone H3K9 methyltransferase (*Su(var)3-9*), which suggests that they have to be exactly at the wild type dose in order to maintain balanced chromatin (60, 61). PEV has been observed in mouse (62) and fission yeast (63). In addition, many of the PEV modifiers discovered in *Drosophila* are conserved in those organisms, plants, and *S. cerevisiae* (21).

There are additional factors that can modify the PEV phenotype such as

temperature: high temperatures (29ºC) suppress the phenotype while lower temperatures (~16ºC) enhance it (52). Changes in heterochromatic content in the nucleus can also alter the PEV phenotype. It has been shown that the addition of an extra *X* or *Y* chromosome suppresses the PEV phenotype and the subtraction enhances it (50, 64). Likewise, addition of centromeric heterochromatin have an effect on PEV in the same way as the *Y* chromosome (52). It has been proposed that the presence of extra sequences with heterochromatic potential recruit heterochromatic factors titrating them away from other regions on the genome, leading to a more euchromatic environment of the endogenous sequences that are adjacent to heterochromatin (65).

The In(1)w^{m4} allele

The chromosomal rearrangement most commonly used to study PEV and modifiers of PEV is the *In(1)w^{m4}* (*w^{m4}* for short = white mottled). This rearrangement is an inversion of the *X* chromosome that juxtaposes the normally euchromatic *white*$^{+}$ gene to heterochromatin (Figure 1.1). The proximity to heterochromatin makes the *white*$^{+}$ gene become inactive in some eye cells, leading to the variegating phenotype known as mottled (66). The nature of the decision of whether cells silence the gene or not is unknown.

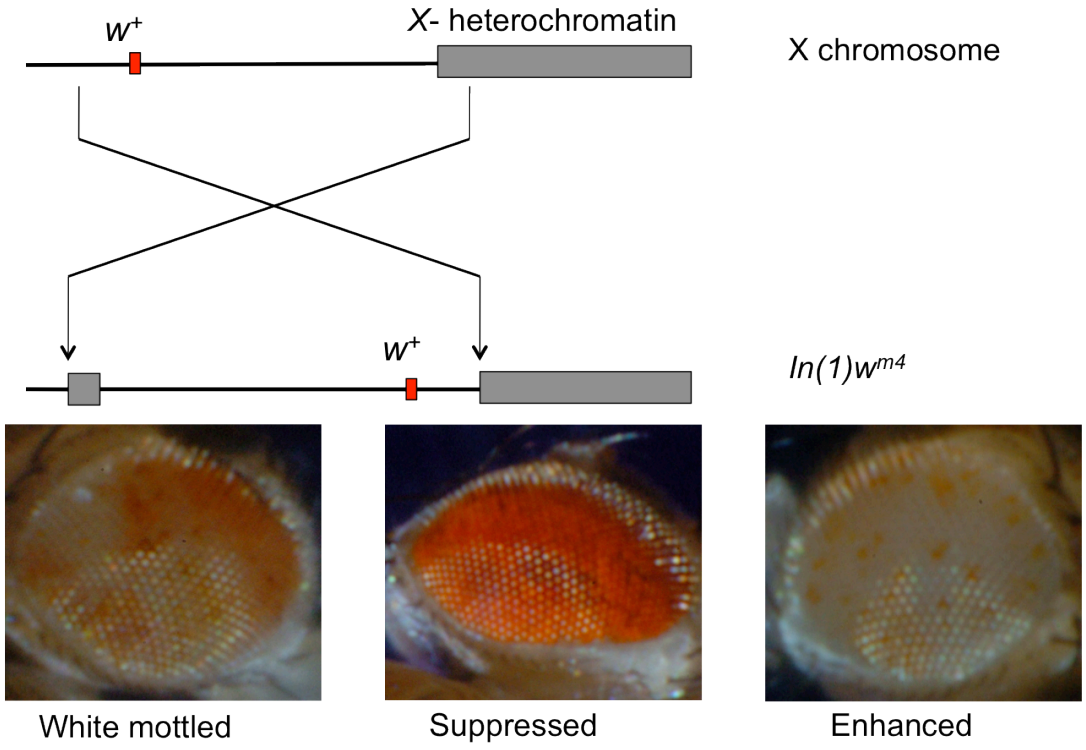


Figure 1.1 Schematic representation of the chromosomal rearrangement *In(1)w*^{m4}. Top: X chromosome showing the two breakpoints that generated the inversion that places the *white*$^{+}$ gene nearby heterochromatin. Bottom: Pictures of eyes showing the white mottled phenotype *In(1)w*^{m4}*/Y,10B* (left), suppression of silencing *In(1)w*^{m4}*/YrDNADef* (middle) and enhancement of silencing *In(1)w*^{m4}*/Y,10B*; *mod(mdg4)*/TM3, *Sb Ser* (right).

The first genes that were identified to be susceptible to PEV effects were genes that have a visible phenotype. Later studies showed that other genes, whose expression is not as easily observed, could be affected by PEV as well. It was then suggested that all genes had the potential to exhibit PEV as long as they are located in an appropriate rearrangement (50). The frequency with which a gene is inactivated is negatively correlated to the distance to the chromosomal breakpoint. When the strength of PEV is measured, genes that are closer to the

chromosomal breakpoint have stronger mutant phenotypes and are more frequently inactivated. This observation has suggested that the PEV is the result of physical heterochromatin spreading from a chromosomal breakpoint towards euchromatin (52). The proposed mechanism by which this spreading is stopped is related to the presence of antagonizing chromatin components and modifiers in a dose-sensitivity fashion. According to this model, the spreading could be mediated until the available heterochromatin factors present are depleted (67). A euchromatic mark that is believed to stop heterochromatin spreading is phosphorylation of histone H3S10 by JIL-1 kinase. This mark is found in euchromatin interbands (regions of low compaction in polytene chromosomes) and antagonizes heterochromatin spreading (68).

Ribosomal DNA genes (*rDNA*)

The *rDNA* genes are highly conserved among eukayotes. They are found as tandem repeats within arrays that can range from less than 100 to more than 10.000 repeated units in one or more chromosomes (69). Each repeat encodes the rRNA precursor, namely, the 35S pre-rRNA in *Drosophila* and 45S pre-RNA in mammals (70, 71). These are transcribed by RNA Polymerase I and then posttranscriptionally modified to generate three of the four ribosomal RNAs the 18S, 5.8S and 28S rRNA. Interestingly, in most organisms the 5S rRNA is transcribed by RNA Polymerase III and is clustered elsewhere on the genome. However, in a few organisms, including *S. cerevisiae,* the 5S is found on the

same *rDNA* array with the other RNA genes, but is transcribed from the opposite strand by RNA Polymerase III (72).

The number of chromosomes that harbor an *rDNA* array is variable among different eukaryotes. They are present in five chromosomes in humans, six chromosomes in mouse, two chromosomes in *Arabidopsis*, and only one chromosome in budding yeast, fission yeast and *N. crassa* (73-75). The total number of rRNA repeats differs greatly among eukaryotes and is been found to be positively correlated with the size of the genome (76).

rDNA in Drosophila

In *Drosophila*, the *rDNA* arrays are localized on the proximal heterochromatin of the *X* chromosome and on the short arm of the *Y* chromosome. Each array contains about 150-250 copies of the repeats, however copy number is known to be quite variable among populations (77, 78). It has been shown that about 110 copies are required for viability, which is much lower than the total number of copies present; hence the arrays and the copies within the arrays are redundant (79, 80).

Each *rDNA* repeat is about 11 kb and contains the rRNA encoding genes and some additional sequences such as intergenic spacers and retrotansposon-like elements R1 and R2 (Figure 1.2) (81, 82). Importantly, within the intergenic spacers are 240 bp repeats which serve as the autonomous pairing sites of *X*-*Y* chromosomes in male meiosis, since no rRNA encoding sequence is necessary

for the intergenic spacer to serve as pairing sites (83). The repeats in the array are imperfect in that some contain *28S* genes that have been interrupted and rendered non-functional by insertion of R1 or R2 sequences. The insertion sites of these elements are conserved among repeats and only 74 bp apart from each other within the *28S* gene (84). The level of R1 and R2 insertion can vary from only a small percentage to >70% of the total *rDNA* units among different populations of *Drosophila* (79, 85, 86). R1 inserts are found predominantly on the *X* chromosome, while R2 are found commonly in both *X* and *Y* but more predominately on the *Y* (79).

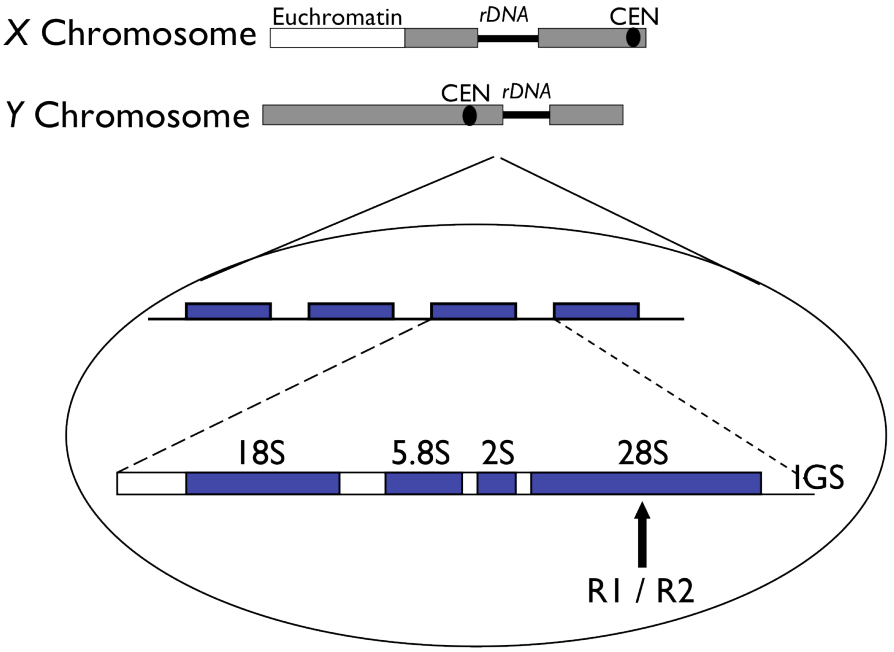


Figure 1.2 Schematic representation of the *rDNA* arrays in *Drosophila melanogaster*. Top, the Y chromosome. Black oval represents the centromere. Gray rectangles are heterochromatic blocks. Bottom, array repeated units enhancing a single 35S gene, which encodes the 18S, 5.8S, 2S and 28S. Arrow indicates the gene where the R1 and R2 insertion sequences have landing sites.

The *rDNA* array is also known as the *bobbed* locus because the phenotype produced by deletions on the array produce the bobbed phenotype. This phenotype is likely the consequence of the decreased ability to produce enough proteins and it is characterized mostly by small bristles, etching of the abdominal tergites and delayed development (87, 88) (Figure 1.3). This phenotype is known to be negatively correlated to the copy number of the *rDNA* repeats, that is, the less copies of *rDNA* the more severe the phenotype is. Additionally, it has been shown that bristle length increases with the dosage of the *rDNA* copies accordingly, and when alleles of different strengths are combined the effect on bristle length is additive (87). The range of *rDNA* copies that produce a bobbed phenotype has been shown to be from ~60-80% of the wild type copy number, where as ~50% or less causes lethality (80).

Figure 1.3 The bobbed phenotype. Female flies from the genotype *C(1)X, rDNA*$^{-}$ /Y. Left, female carrying a *Y rDNA*$^{+}$ chromosome which confers a wild type phenotype. *bobbed* pictures are from females that carry a *Y* chromosome with different *rDNA* copy numbers.

Many of the lab stocks that have been used to carry out experiments to understand the bobbed phenotype have been known to decrease in severity of the phenotype or have total reversion to wild type over time. This reversion occurs through a process called “magnification”, where the number of *rDNA* copies on the array is increased. This mechanism of magnification has been the subject of many studies (79, 89). Currently the most accepted mechanism to explain *rDNA* magnification is the one proposed by K. D. Tartof in 1974, which proposes Unequal Sister Chromatid Exchange (USCE) as the means by which the *rDNA* array can reestablish its size. USCE can both increase and decrease array size (90). *rDNA* magnification occurs both somatically, known as “pseudo magnification”, and pre-meiotically in the germline in which case is heritable and is known as “magnification” (78, 79, 88-90).

Most of the studies in magnification have been done on *X*-linked *rDNA* arrays and they have shown that magnification on the *X* requires the presence of a *Y* chromosome that lacks some or all of the copies of *rDNA*. In addition, the two chromosomes were required to be together for several generations in order for the *X*-linked array to magnify (88-92). However, exceptions to these observations are: 1) it has been shown that *X*-linked *rDNA* that was originally homozygous *bobbed lethal* (bb^{l}) magnified in one generation at a low frequency generating an *X* chromosome homozygous viable (93), and 2) it has been shown that *rDNA* arrays on *Y* chromosomes are able to magnify without exposure to another chromosome with an *rDNA* deletion (94).

Magnification is not the only spontaneous alteration in the *rDNA* array size. Spontaneous deletions resulting in the formation of extra chromosomal circular DNA (eccDNA) is an interesting phenomena that has been observed in many eukaryotes from yeast to humans including *Drosophila (95).* The mechanism for eccDNA formation is thought to be the looping out of copies by intrachromosomal homologous recombination. Interestingly, the presence of eccDNA is not unique to the *rDNA* array, but also result from other tandemly arrayed repeated genes, such as histones, *Stellate* and *Supressor of Stellate* and occur throughout the life cycle in *Drosophila*. The plasticity of the genome is a characteristic of many eukaryotes and it is observed in tandem repeated genes, however the functional advantage of the formation of eccDNA has not been elucidated yet (95-98).

Growing cells require continuous rRNA synthesis, which can be controlled at different levels: 1) the amount of *rDNA* copies present, 2) the *rDNA* transcription rate per gene by its devoted polymerase RNA Pol I, and 3) the epigenetic state of the copies present on the *rDNA* array (75, 99). This epigenetic regulation results in the occurrence of both active and inactive copies on the arrays and is a general characteristic the eukaryotic genome. The mechanism of silencing has been well characterized in mammalian cells and budding yeast (70, 100-103); however, paucity of studies on the silencing mechanism in *Drosophila* has left the knowledge of the mechanism largely incomplete in this system. Within the arrays only a subset of units are

transcriptionally active (~60% of the total number of *rDNA* copies among all the arrays), and transcription of this fraction of the repeats is enough to provide the cell with the essential amount of ribosomes for adequate protein synthesis. Transcription of the active *rDNA* copies makes more than 80% of the total cellular RNA in the cell (70, 100, 104). On the other hand, the remaining copies on the array (~40% of the array) are inactive copies that have been epigenetically silenced (75). An exception to the presence of both active and silent *rDNA* copies on the same array is a phenomenon known as nucleolar dominance, which been observed in plants such as *Arabidopsis* and also in *Drosophila*. In some genetic hybrids, the entire array that has been inherited by one of the parents is silenced. In other words, the array of one of the parents dominates the nucleolus by being the only array able to maintain active *rDNA* genes. This is a reversible epigenetic phenomena and it is still unclear how the parental sets are discriminated (105).

In mammals, the silencing of the copies occurs by epigenetic mechanisms such as DNA methylation and histone modification, which mark the chromatin state as open or closed for transcription by RNA Pol I (102). The silenced state, which is established by the nucleolar chromatin remodeling complex (NoRC), is maintained throughout the cell cycle and is inherited from cell to cell (75, 106). A non-coding RNA transcribed from the intergenic spacer binds a subunit of the NoRC complex and is required for silencing. The proposed hypothesis is that the non-coding RNA guides the complex towards

the *rDNA* copies for subsequent silencing (75). For establishment, NoRC is recruited to the *rDNA* promoter where it interacts with other factors to deacetylate histones H3 and H4 and methylate H3K9, H3K20 and H3K27. These steps lead to DNA methylation, which impairs the assembly of the transcription initiation complex. Another silent *rDNA* regulator is the mammalian homolog of Sir2, SIRT1, which has been also implicated with the silencing of the *rDNA*. SIRT1 is known to belong to the silencing complex known as eNoSC (energy-dependent nucleolar silencing complex), where it serves to sense the energy levels present in the cell and upon glucose starvation becomes activated leading to deacetylation of SL1, an *rDNA* transcription initiation factor, impairing the transcription initiation complex assembly on the *rDNA*. Additionally, the eNoSC complex is activated to save energy, which leads to establishment of heterochromatin at the *rDNA* by histone deacetylation, H3K9 methylation and transcriptional repression. These findings have linked the cellular energy balance with the epigenetic state of the *rDNA* locus (107).

The role of Sir2 in *rDNA* silencing has been more extensively studied in the budding yeast *S. cerevisiae.* Here the Sir2 histone deacetylase (HDAC) protein is the main silencing component not only of the *rDNA* genes but the telomeres and the silent mating type loci (108). Sir2 belongs to the family of HDAC known as Sirtuins, which are widely conserved from archaea to humans and have vital functions among them (109). Due to its link with aging, Sir2 has

been in the scope of many studies, which have shown that Sir2-mediated silencing extends the life span of budding yeast (110).

The Nucleolus

Transcription of the *rDNA* copies by RNA Polymerase I forms the nucleolus, hence the *rDNA* is also known as the Nucleolus Organizer Region (NOR). The nucleolus is a dynamic membrane free structure that is formed inside the nucleus at the beginning of G1 and it is disassembled when transcription is turned off as cells enter mitosis (72). The main function of this structure is ribosome biogenesis, but it has been shown to perform additional important tasks such as, cell cycle regulation, control of aging, modification of small nuclear RNP's, nuclear export pathways and telomerase function (111). For instance, in cell cycle regulation the nucleolus accumulates and dissociates different proteins at specific times during cell cycle. Some of these proteins are BLM, WRN (112), PTHrP (113), and CDC14A (114) and have functions such as phosphorylation and sumoylation, which are known to be important regulators of a wide variety of cellular processes. Another example is telomerase, which is sequestered in the nucleolus until it is released at the late stages of the S phase, when the telomeres need to be replicated (115).

An additional important process attributed to the nucleolus is as a sensor and responder to cellular stress. An example of this is the role in the stabilization of the tumor suppressor protein p53. The p53 protein is commonly very

unstable, but it is stabilized when the cell experiences stress conditions, which leads to a series of events that end with cell cycle arrest or apoptosis. The protein HDM2 constantly destabilizes p53. Under stress conditions, a nucleolar protein named p14ARF leaves the nucleolus to capture HDM2 and physically sequester it into the nucleolus, promoting p53 stabilization (116).

Moreover, aging, cancer and human disease are also tightly linked to alterations of the nucleolus. Changes in nucleolus size and structure have been recognized for a long time as markers of proliferating cancerous cells as is the increased presence of active NORs (AgNORs), which is used for cancer prognosis (117). In addition, deregulation of ribosome biosynthesis has been correlated with disease raising the suggestion that over expression of rRNA is one of the steps towards tumorogenesis (118).

Interestingly, the nucleolus structure and function are sensitive to changes in dosage of chromatin modifiers and heterochromatic components. In *Drosophila* it was recently shown that nucleolar stability is affected in cells that lack of histone H3K9 methyltransferase and HP1 as well as several components of the RNA interference pathway, such as *Ago2*, *Aubergine, dicer-2*, Piwi, etc. It was proposed that the loss of chromatin compaction at the *rDNA* as a consequence of these mutations stimulates intra chromosomal recombination of the repeats on the array. The *rDNA* eccDNA nucleates extra chromosomal nucleoli which are observed as the formation of macro and micro extra chromosomal nucleoli (macro and micro nucleoli) (119). This hypothesis agrees

with previous reports in *Drosophila* that shown that a single *rDNA* repeat inserted at an ectopic location was able to nucleate a nucleolus (120).

The emerging findings about the nucleolus and the identification of its proteome in humans, plants and budding yeast (115) is tracing the path towards a better understanding of the many different roles that the nucleolus has in the cell. Studying the Nucleolus Organizer Region is a step towards deciphering this magnificent and important nuclear compartment.

Research Aims

Drosophila has *rDNA* arrays located exclusively in both the *X* and *Y* chromosomes. Spontaneous *rDNA* deletions within these arrays occur from yeast to humans, but the functional relevance of these polymorphisms in *rDNA* array size is still unclear. Polymorphisms linked to the *Drosophila Y* chromosome affect global gene expression, but the specific regions within the *Y* chromosome responsible for these effects are unknown. Importantly, aging and cancer cells show both altered *rDNA* array sizes and exhibit global changes in gene expression patterns. The long-term goal of my study was to investigate the role of the *rDNA* genes and the nucleolus on gene expression. The specific objectives of the research I present here were to: 1) Design a strategy to obtain isogenic fly lines with specific and graded *rDNA* deletions. 2) Test the effects of those *rDNA* deletions on variegated gene expression and 3) Analyze the extent of the effect of the *rDNA* deletions on global gene expression.

CHAPTER II

CREATING *Y*-LINKED *rDNA* DELETIONS BY *I-CREI* ENDONUCLEASE EXPRESSION

INTRODUCTION

Over the past six decades, hundreds of studies have investigated the roles of the *rDNA*. Mainly biochemical approaches have been used to study this locus from yeast to humans (121). Studies focused on investigating characteristics related to the multiplicity of the *rDNA* in *Drosophila* have required the use of fly lines that have variations in the size of the *rDNA* arrays. Nevertheless, due to its repetitive nature, it has been difficult to create specific deletions since it is hard to screen for them unless they are extreme enough to produce a phenotype. Therefore, despite the advances in the molecular biology techniques available to create targeted gene deletions, there are no studies that have generated specific, graded, and targeted *rDNA* gene deletions until present. With the exception of spontaneous *rDNA* deletions (87), invasive mutagenic methods like ethyl-methane-sulfonate (EMS) and X-rays were the only available techniques to induce damage to the DNA for many years (122-124). Another common method utilized chromosomal inversions and translocations to vary copy numbers of *rDNA* cistrons. Even though these methods were effective in altering *rDNA* copy number, they were also time consuming since they required large screenings and were also unspecific because they mobilized chromosomal blocks to non endogenous sites. Since

the methods often generated genetic changes in addition to varying *rDNA* copy number, correlating phenotype to genotype was complicated (78, 125, 126).

The first approach to create specific *rDNA* deletions came in 1981 when L.G. Robbins showed that the *Rex* mutation, which is a maternal inducer of mitotic exchange between *rDNA* cistrons, could create deletions to the *rDNA* under very specific conditions. This technique was limited in that the Rex mutation had to be maternally transmitted and the mitotic exchange occurs only in compound *XY* chromosomes (127). This method generates free *Y* chromosomes that harbor *rDNA* deletions. However, it is unknown whether the *rDNA* cistrons remaining are from the *X*, *Y* or both chromosomes.

In 2005, Maggert and Golic showed that the *I-CreI* endonuclease specifically recognizes a sequence of the *28S* gene from *Drosophila melanogaster*. *I-CreI* is a homing endonuclease from *Chlamydomonas reinhardtii* that recognizes a highly conserved 24-bp sequence within its 23S gene of the *rDNA* (128). By inducing the expression of an *I-CreI* transgene in *Drosophila*, the only interchromosomal exchanges recovered involved the *X* and *Y* chromosomes indicating specificity to the *rDNA* locus (129).

I wanted to study the role of the *rDNA* loci in gene expression. To do this, I generated deletions within the *rDNA* loci then tested the effects of the deletions on gene expression. Hence, I needed a method that allowed me to obtain specific, graded and isogenic *rDNA* array deletions. The use of *I-Cre*I was key to developing this strategy because: 1) It is specific to the *rDNA*, which eliminates

the background caused by the modification of other sequences on the genome, 2) Since it recognizes the *28S* genes that are present in all the repeats of the array, this allows the creation of graded deletions when *I-Cre*I is expressed in a conservative fashion. Therefore, I can estimate ranges of *rDNA* that are required to cause certain phenotypes, and 3) It allows genetic manipulation to obtain isogenic fly lines with *rDNA* deletions for a proper comparison within the lines. Thus, using *I-Cre*I I developed a genetic strategy to generate and study targeted *rDNA* deletions.

I also needed a technique that allowed the reliable estimation of the number of *rDNA* copies removed. For that purpose, I developed a strategy based on Quantitative Real-Time PCR to molecularly quantify the number of *rDNA* copies on the array.

RESULTS AND DISCUSSION

Genetic approach to create the *Y*-linked *rDNA* deletions

The *Y*-linked *rDNA* array is almost unexplored because most of the studies about *rDNA* have been done on *X*-linked arrays. However, it is known that the *X* and *Y* chromosomal arrays differ in many ways. For instance, the presence of R1 and R2 insertion sequences is not uniform for both arrays (130, 131). In addition, at least one base difference has been found between *X*-linked and *Y*-linked *18S* RNA transcripts and the 5' end of the nontranscribed spacer. Furthermore, the size and frequency of the intergenic spacers varies between

the two arrays, and those in the *X*-linked arrays are more sequence homogeneous than those in the *Y*-linked arrays (79). Therefore, the *rDNA* information that has been obtained with the *X*-linked arrays might not properly be extrapolated to the *Y*-linked array.

On the other hand, the *Y* chromosome is very easy to manipulate and monitor through crosses because even though it does not determine the sex, the sex on the fly reports its presence or absence. For all those reasons I used the *Y*-linked *rDNA* array to create the *rDNA* deletions.

In my initial experiments, I used the *Y* chromosome *Y,10A* (Figure 2.1) (129). This chromosome has a *P* element transposon containing a *white*$^{+}$ gene flanked by recombination targets (FRTs) (132) inserted at the tip of the short arm, and a translocation of the tip of the *X* chromosome containing the *yellow*$^{+}$ at the end of the long arm. The latter can be used to monitor the *Y* chromosome in genetic crosses. The related *Y,10B*, is identical in sequence save for FLP-mediated loss of the *white*$^{+}$ gene. Loss of the *white*$^{+}$ in *10B* allowed me to use the *white*$^{+}$ gene as a reporter for effects on gene expression. For simplicity, *Y10* will be used throughout the text to refer to *Y,10A* and *Y,10B* unless a distinction needs to be made.

Figure 2.1 Schematic representation of the *Y,10A* chromosome. Grey rectangles denote heterochromatic blocks. Thin line at the left end represents the piece of the X chromosome that was translocated with the marker gene *yellow* (y^+). Blue oval represents the centromere. Red box represents the *white*$^+$ marker gene.

To combine the marked *Y* chromosome with the *I-Cre*I transgene, I crossed homozygous *XX* females that have the *I-CreI* transgene on the *X* chromosome to males that had the marked *Y* chromosome (*10A* or *10B*) (Figure 2.2, Generation 0). Progeny from this cross were heat shocked as larvae to induce the expression of *I-CreI* and deletion of the *rDNA*. In order to recover individual *Y* chromosomes ($YrDNA^{Def}$, where *Def* = deficiency) from chimeric males and to remove *I-Cre*I, adult male progeny (*I-Cre*I / *Y10*) were selected and crossed *en masse* to female virgins homozygous for the mutations *y w* on the *X* chromosome (*y w / y w*) (Figure 2.2, Generation 1). These males were viable regardless of the size of the *Y*-linked *rDNA* deletion because they were complemented by the wild type *rDNA* array present on the *X*-chromosome.

In order to keep individual *Y*-linked *rDNA* deletions, these males were individually crossed to two different females: *y w / y w* females and $C(1)DXrDNA^-$ / *YrDNA* females. Crosses to the *y w* females established an

isogenic stock for each individual *Y* chromosome and by crossing to unmated females each generation, spontaneous mutations in the background were minimized (Figure 2.2, Generation 2).

Crosses to *C(1)DXrDNA*$^{-}$ / *YrDNA* females generated females where the only source of *rDNA* was present on the *Y* chromosome that was exposed to *I-Cre*I. Females of genotype *C(1)DXrDNA*$^{-}$ / *YrDNA* harbor a compound *X* chromosome *C(1)DX* (i.e. two attached *X* chromosomes) that lack *rDNA* and a *Y* chromosome with a wild type *rDNA* array (*YrDNA*). Analysis of the progeny of this cross (Figure 2.2, Generation 3) both phenotypically and molecularly allowed characterization of the effect of *rDNA* copy number on *Drosophila* biology.

Phenotypically the *Y*-linked *rDNA* deletions were characterized by their influence on male:female ratios in Generation 3, and the lethality or relative severity of the bobbed phenotype (Table 2.1). The frequency of chromosome translocations *T(X;Y)* was also monitored for comparison to previous frequency using these methods (a frequency of 17.9% has been previously reported (129)) (Table 2.1).

If the strategy produces a gradient of deletions, then I expect a gradient of phenotypes among the females. At one of the extremes of the gradient would be wild type females, where relatively little or no *Y*-linked *rDNA* deletion occurred, and at the other extreme dead females, where the remaining *Y*-linked *rDNA* is insufficient for survival. Somewhere in the phenotypic middle is the

bobbed phenotype characterized by small bristles and late development (Described on Chapter I). The severity of this phenotype is negatively correlated to the *rDNA* cistron copy number. Since loss of the *rDNA* affects viability of the females in Generation 3, I could also expect that the males containing large deletions would give rise to fewer viable females thereby skewing the male:female sex ratio of Generation 3.

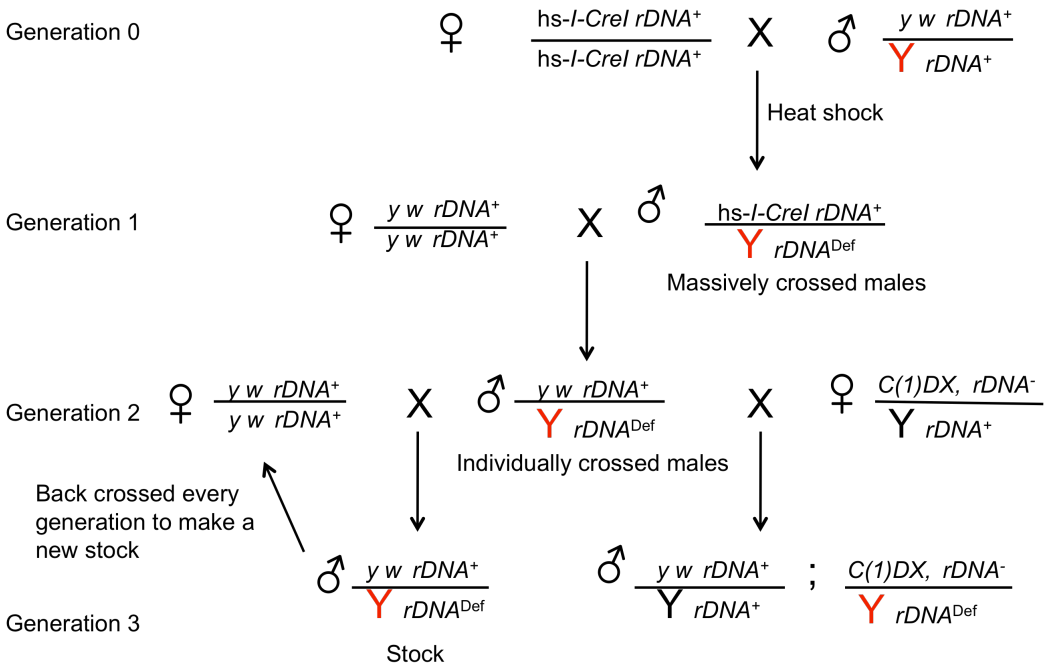


Figure 2.2 Crosses to generate *rDNA* deletions. Generation 0. Females harboring a heat shock inducible *I-CreI* transgene were crossed to males that harbor the *Y* marked chromosome (red). Generation 1. Heat shock was done as larvae and adult males were crossed en masse to females *y w*, which have wild type *rDNA* arrays in both *X* chromosomes, to recover the *Y* chromosome that now likely contains an *rDNA* deficiency ($rDNA^{Def}$). Generation 2. Males harboring the Y $rDNA^{Def}$ were crossed to y w females (left) to produced a stable stock, and to *C(1)DX/Y* females (right) to qualitatively and quantitatively analyze the size of the deletion.

I did two screens for *Y*-linked *rDNA* deletions. The first one used the *Y,10A* chromosome and the second one *Y,10B*. A total of 1160 individual chromosomes were tested for male:female ratio (Table 2.1). Phenotypically bobbed and lethal were the only lines that were pursued in further studies because I could visually detect that they had undergone an *rDNA* deletion, which

facilitated the screening and also increased the likelihood of observing other phenotypic *rDNA* related changes, for instance in gene expression.

Screen	Y10 chromosome	Chromosomes screened	Altered sex ratio	*T(X;Y)*	Bobbed Phenotype	Lethal phenotype
1	10A	560	32	12	0	7
2	10B	600	60	6	9	16
Total		1160	92	18	9	23

Table 2.1. Summary of independent *Y* chromosomal lines that harbor an *rDNA* deletion. Total number of screened chromosomes are shown for *10A* and *10B.* Number of lines that shown altered sex ratio, *T(X:Y)* translocations, and a bobbed or lethal phenotypes are shown.

Chromosome cytology

During mitosis active Nucleolus Organizer Regions (NORs) stay relatively undercondensed and this chromatin structure appears as a secondary constriction in methaphase chromosomes. The undercondensation causes the NORs to appear as gaps on the chromosomes (Figure 2.3, Top) (105). To investigate whether the phenotypes of my lines corresponded with cytological changes in the NORs and no other alterations in chromosome structure, I analyzed mitotic chromosome spreads. In order to visualize the effect of the *rDNA* deletions on the secondary constrictions, I prepared interphase and mitotic chromosomes derived from third instar larval neuroblasts of flies harboring *Y*-linked *rDNA* deletions *lethal* and *bobbed*, and the *wild type* progenitor *Y* (Figure

2.3, Bottom). The arrows are pointing to the *rDNA* region of the *Y* chromosomes, while arrow heads point to *X* chromosomes. Lines *bb-465* and *bb-76* are lines that genetically showed a bobbed phenotype and cytologically the show a mild deletion that is enough to create a reduction on the size of the constriction. Lines *l-480*, *l-498*, *l-481*, *l-510* and *l-473* genetically showed a lethal phenotype, and a cytological reduction in the size of the constriction even smaller than for the bobbed lines. There was no evidence of damage in the other chromosomes on the spread.

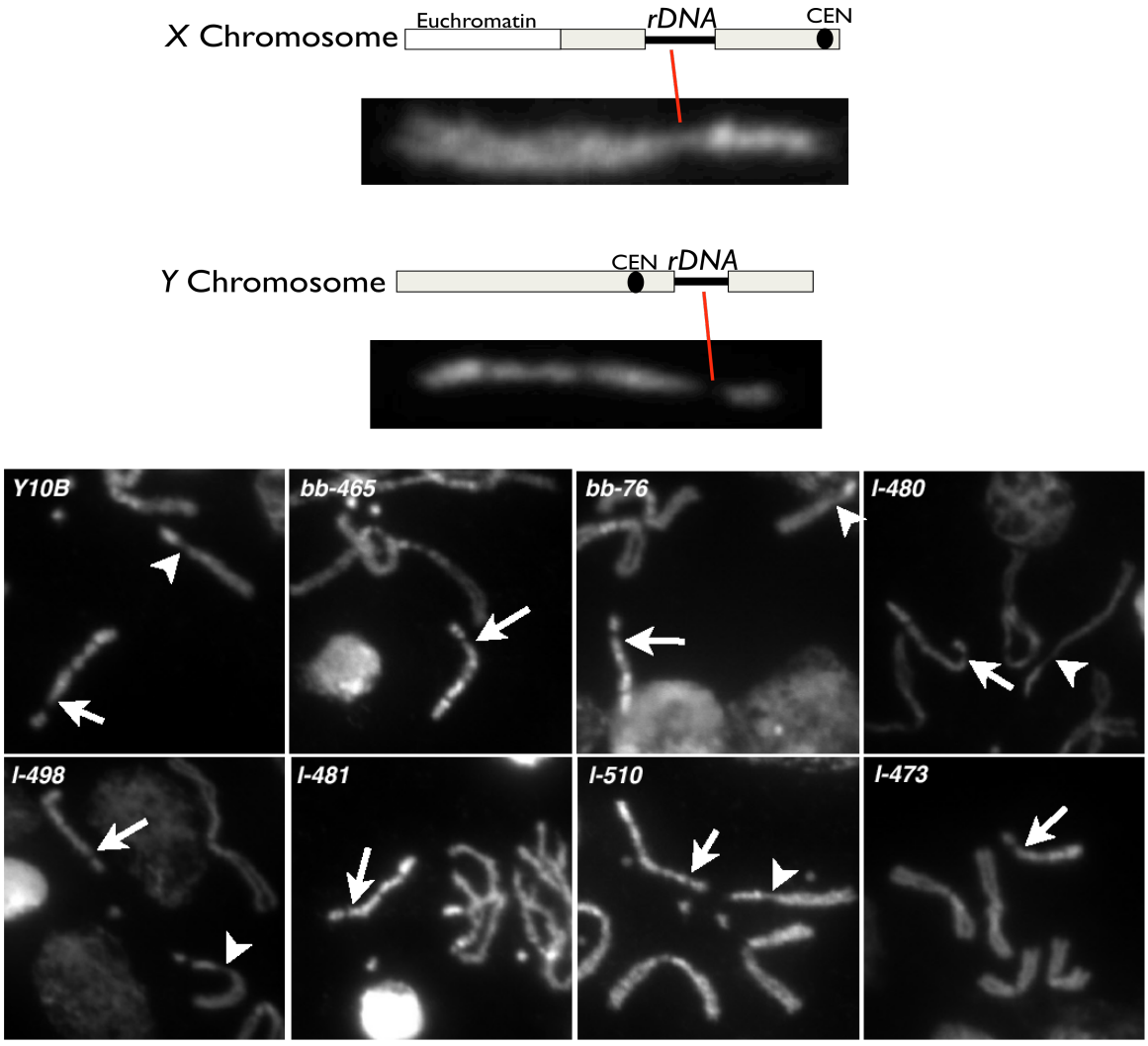


Figure 2.3 Chromosome spreads of *bobbed* and *lethal* lines. Top: Schematic representation of *X* and *Y* chromosomes showing the localization of the *rDNA* in comparison to a metaphase *X* and *Y* chromosomes stained with DAPI. Bottom: Prometaphase chromosomes stained with DAPI. Arrows are pointing to constriction created by the *Y*-linked *rDNA* array. Arrowheads point towards the constriction created by *X*-linked *rDNA* arrays.

The constriction size was a visual but not quantifiable indication of how much *rDNA* was removed, and the size of the constriction matched the estimated size of the array determined by genetic crosses. However, it is imprecise and unreliable to estimate the deletion size by measuring the constriction because it is unknown if the size of a transcriptionally active copy is cytologically the same size as an inactive copy. Therefore, two arrays might have the same number of copies but could appear of different sizes cytologically depending on the relative proportion of active versus inactive copies.

Quantitative Real-time PCR to measure *rDNA* array size

Previous studies used *rDNA* quantification techniques that were cumbersome and not as precise as modern molecular techniques (i.e. slot blot, Southern blot) (80). Those techniques required the use of high amounts of DNA, for which dozens of flies were needed. Since it is known that the *rDNA* array is very volatile (95, 96, 98), I needed a method that allowed me to quantify *rDNA* from individual flies and also reliably detect small changes in *rDNA* copy number. In order to quantify the *rDNA* deletions, I developed an assay to measure the *rDNA* copy number using Real-Time PCR.

I needed to select two genes for PCR amplification, the target and the normalizer. The target gene is the gene that I want to quantify, and the normalizer is a gene that is constant and will serve as internal control. As the target gene I choose the *18S* because it is a gene that remains intact in all the

copies of the array, and it is long enough to allow testing of primers on different regions for standardization purposes. As the normalizer I choose the *tRNA*^{Lys} gene because it is multicopy (30 copies per genome) but scattered through the genome, which makes it likely more stable than tandemly repeated genes, which can be variable in number due to natural variance or interchromatid recombination (133). I used the comparative C_T method to obtain a relative quantification of the copy number of *rDNA* cistrons. The comparative C_T method is based on the difference of the C_T values (C_T=cycle threshold) from the target gene and normalizer, which are determined by the point during the exponential phase of the PCR reaction where the fluorescent intensity of the reaction is above background, and the cycle number of the PCR in which this happens (134). When using this approach the method needs to be validated to ensure the reliability of the result. The way to validate this method is to ensure that the amplification efficiency of the target gene is approximately equal to the amplification efficiency of the normalizer gene (134). To address this issue it was necessary to observe how the difference between the two amplified genes (ΔC_T) varies with template dilution. I validated this method using six different concentrations of gDNA (Table 2.2).

Input Amount ng gDNA	18S Average C_T	tRNA Average C_T	ΔC_T 18S-tRNA
15	19.18±0.11	22.59±0.06	-3.41±0.12
10	19.79±0.07	23.13±0.15	-3.34±0.17
7	20.62±0.11	23.92±0.11	-3.30±0.16
5	21.25±0.40	24.30±0.07	-3.05±0.12
2.5	22.15±0.20	25.31±0.09	-3.15±0.12
1	23.91±0.06	27.43±0.20	-3.52±0.12

Table 2.2 Average C_T value for *18S* and *tRNA* at different gDNA input amounts. Plus/Minus are SD of three replicates. DNA used was extracted from *C(1)DX/Y10B* females. C_T refers to the number of amplification cycles on the PCR at which a threshold has been set.

The change in amplification cycles between the target and normalizer gene (ΔC_T) for different DNA concentrations was within a tight range (S.D. = 0.17). The data were plotted as a linear regression of the log of the input amount of gDNA versus the ΔC_T, and found the slope was 0.0503, well within the acceptable absolute value of the slope of the linear regression of <0.1 (134) (Figure 2.4). Additionally, the assay could reliably detect *rDNA* copy number at very low concentrations of DNA (1,5 pg = ~5 genome equivalents).

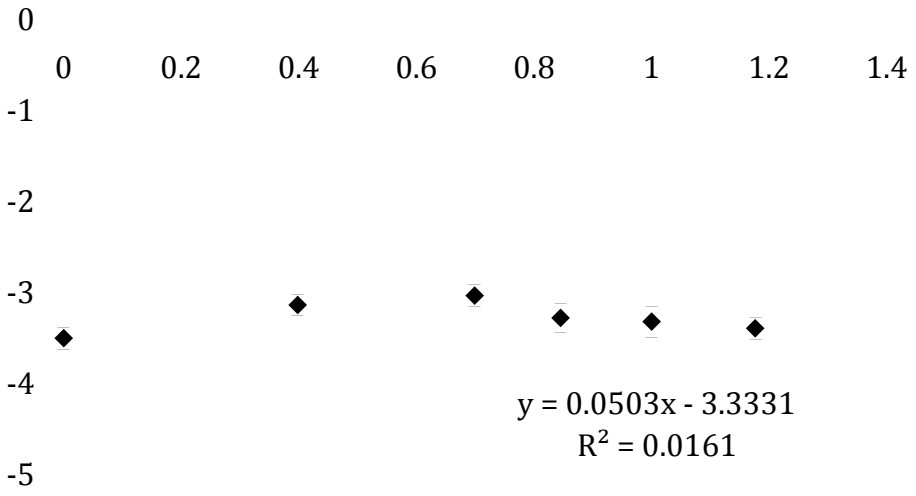


Figure 2.4 Plot of the log input amount of DNA versus ΔC_T taken from Table 2.2. The slope is 0.050 indicating that the assay is valid.

In order to estimate the *rDNA* copy number present in an array, I used the ΔC_T value and performed the following equation:

rDNA copies = $(2^{-(\Delta C_T)})$ X 30,

where the number 30 is a constant value given by the number of *tRNA*^{Lys} copies present on the genome and ΔC_T is elevated to 2 because each cycle represents two-fold template change.

Allelic series of *rDNA* deletions

rDNA arrays can be variable, hence the presence of a wild type array in the same genetic background with a *YrDNA*^{Def} could introduce variability when measuring the *rDNA* copy number. Therefore, it is ideal to quantify the *rDNA*

copy number in flies where the *YrDNA*^{Def} is the sole source of *rDNA*. For this purpose, I used females of the genotype *C(1)DX,rDNA*$^{-}$*/YrDNA*^{Def} (Figure 2.2, Generation 3) to estimate the *rDNA* copy number remaining in the *YrDNA*^{Def} after the deletion. This analysis was done only for chromosomal lines that showed a bobbed or lethal phenotype. From these progeny two classes of females could have arisen. First, triplo-*X* metafemales can be obtained by the inheritance of the patroclinous *X* chromosome marked with *y w.* However, these progeny were expected to be rare because these females are known to die in late stages of development. Rare escapers have a characteristic yellow phenotype that made them easy to exclude from my studies (135). Alternatively, females can inherit the *Y* chromosome *YrDNA*^{Def}. In order to survive, these females rely on the amount of *rDNA* that is still present in the *YrDNA*^{Def} chromosome plus the rRNA and ribosomes that have been maternally loaded into the egg. Early studies investigating the *X*-linked *rDNA* array shown that a mild deletion (leaving ~90-110 *rDNA* copies as the sole source of *rDNA*) yielded *bobbed* females and stronger deletions (leaving less than 90 *rDNA* copies) yielded lethal females (79).

I generated a series of twenty-five deletions, of which nine expressed a bobbed phenotype and sixteen were lethal (Figure 2.5). I could analyze the lethal females because they survive until the pupal stage due to the rRNA that is maternally loaded into the egg. Adult females were used for *rDNA* quantification from those with bobbed phenotype. The developmental stage at which the *rDNA*

was quantified did not affect the outcome of the quantification, since I performed quantification tests in larvae, pharates (lethal females that survive until pupae) and adults, and the results were consistent for all of them (data not shown).

I established ranges of *rDNA* copy number for each chromosomal line based on the quantified *rDNA* from three to seven individual siblings female flies of genotype *C(1)DX*, *rDNA^{-}/Y10BrDNA^{Def}*. For each fly I did three replicated measurements for both the target gene *18S* and the normalizer gene *tRNA^{Lys}*. This allowed me to have a reliable amplification cycle value (C_T), which was obtained as an average of the three replicates. In some cases I obtained a replicate value that was out of a set deviation range from the other two replicate values, therefore this point of data was discarded from my analysis. The criteria of exclusion was C_T values that were deviated >0.5 cycle difference from the other two C_T values. The frequency of this was approximate 0.5% of the total number of reactions.

Since the *YrDNA^{Def}* chromosomes were derived from the *Y,10B* chromosome, the latter was used as reference to estimate percentage of *rDNA* copy number on the deletions relative to wild type. Previous reports about copy number have been done with other techniques and using different chromosomes which vary in copy number, thus in addition to copy number I also present the quantification results as a percentage of the wild type *Y10* chromosome.

As expected, I observed a correlation between the degree of the deletion and the severity of the phenotype. Fly lines that had the large deletions were

lethal while flies with mild deletions were bobbed, and the expressivity of the bobbed phenotype correlated with the size of the array. These series of deletions defined the amount of relative *rDNA* at two phenotypic transitions.

The first transition is from *wild-type* to *bobbed*: phenotypically bobbed flies starting to appear when the *rDNA* was reduced to ~90% of the wild type copy number, which is ~260 copies as the sole source of *rDNA* in the fly. This value is higher than in previous observations, which reported this transition at ~150-200 *rDNA* copies (80, 88). The difference between previous observations and my observations might be due to the use of *X* chromosomes in previous reports, and the quantification techniques that were different and not as precise as our technique (i.e. Southern blot). The second phenotypic transition defined was from *bobbed* to *lethal*: lethality started to be appear when the *rDNA* was reduced to ~65% of the wild type copy number, which is ~190 *rDNA* copies. Again, our values for this transition were also higher than the previously reported for *X*-linked arrays, where ~97 copies are enough for viability (80) (Figure 2.5).

Since the *rDNA* arrays have epigenetically silent copies and differences in insertion sequences R1/R2, these might also explain why our observations differ from previous reports. These features influence the activity of the copies; hence it is likely that other differences in the copy number for each phenotypic transition are observed when using a different chromosome than *10A*.

Although most chromosome deletions showed similar correlation, *Y10B* $rDNA^{l\text{-}539}$ did not. This chromosome is phenotypically lethal but has *rDNA* levels

in a range similar and above the wild type level (Figure 2.5). I hypothesize that many of the functional copies may have been damaged by other nucleases prior to being re-ligated into the array. The requirement for *rDNA* copies might have induced magnification, which in this case might have occurred using the now non-functional copies and/or R2 interrupted copies, making an almost intact array in terms of size yet containing mostly non functional copies. An additional hypothesis is that most of the copies that were left on the array after *I-Cre*I exposure were epigenetically silenced, and after magnification this state was maintained yielding a large and inactive array.

rDNA magnification is observed in the *YrDNA*^{Def} lines

rDNA magnification has been observed to happen even in wild type *rDNA* arrays (136). However, most of the previous studies have shown that *Y*-linked *rDNA* deletions are very stable or need of special conditions to magnify, and *X*-linked deletions need to be in the presence of a *Y bobbed* chromosome to be magnified (88-92, 127, 137). In my preliminary screens using the *Y10A* chromosome, I surprisingly noticed that after seven generations of establishing stable stocks and without selective pressure, I observed the reversion of the lethal phenotype to bobbed. To elucidate the mechanism of this phenotypic reversion I first genetically confirmed that this reversion mapped to the *Y* chromosome and not to a suppressor accumulated on the *X* chromosome or on an autosome. For this purpose, I crossed males harboring the *YrDNA*^{Def}

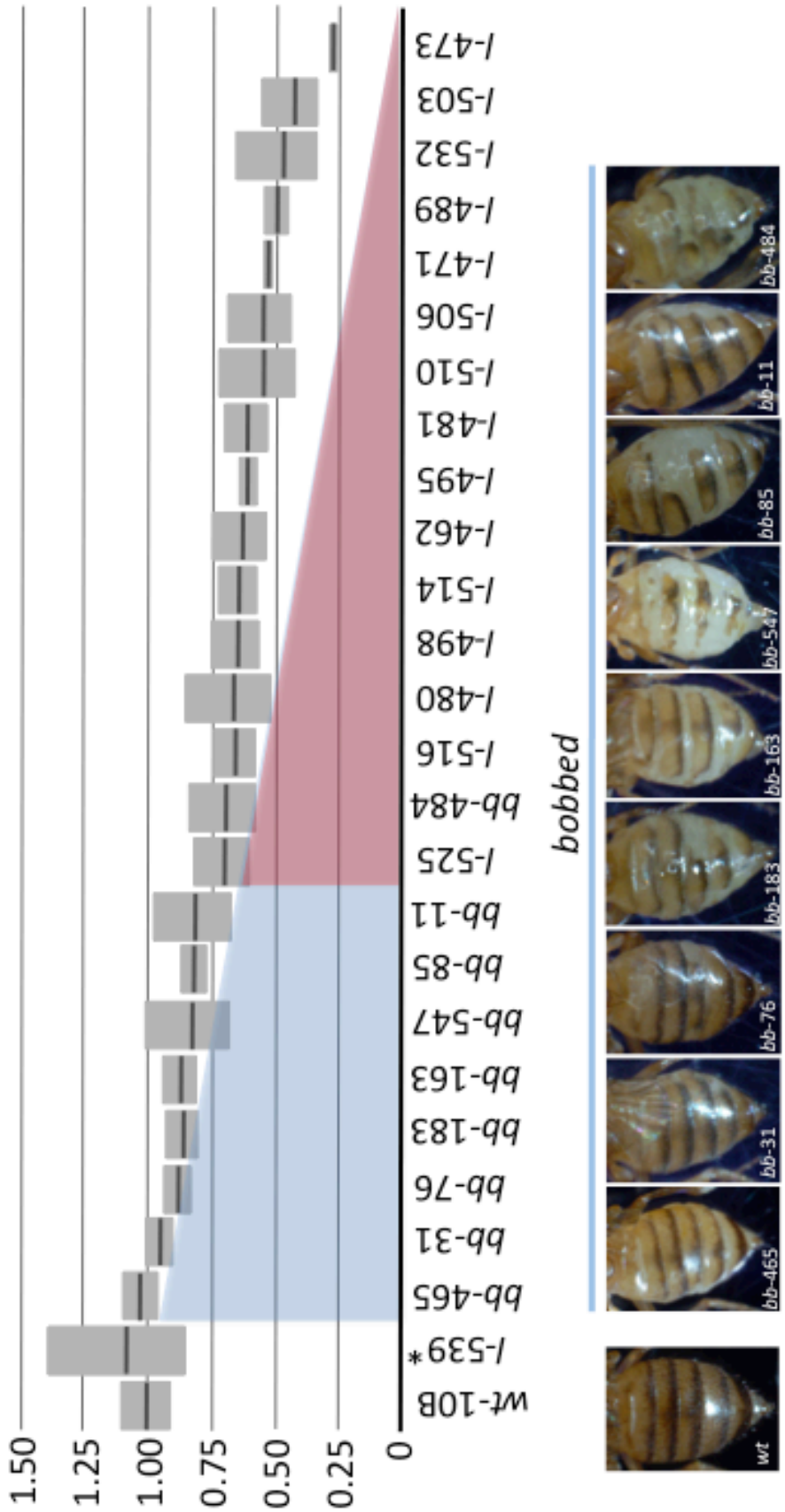

1.50
1.25
1.00
0.75
0.50
0.25
0
wt-10B
l-539*
bb-465
bb-31
bb-76
bb-183
bb-163
bb-547
bb-85
bb-11
l-525
bb-484
l-516
l-480
l-498
l-514
l-462
l-495
l-481
l-510
l-506
l-471
l-489
l-532
l-503
l-473
bobbed
wt
bb-465
bb-31
bb-76
bb-183
bb-163
bb-547
bb-85
bb-11
bb-484

Figure 2.5 Allelic series of *Y*-linked *rDNA* deletions**.** Ranges of *rDNA* percentages relative to wild type are shown (Y axe). Each range was obtained from the *rDNA* quantification of 5-8 individual female *C(1)DX/Y* flies. The ranges are sorted by the mean and include experimental error and standard deviation of the population analyzed. Pictures are from females bobbed and wild type (left) for comparison. Blue area marks where the bobbed phenotype starts to be observed (~90% of the wild type) and where the transition to lethal starts (~65% of the wild type), which is denoted by the red area.

chromosome to females that had marker genes on the autosomes. The progeny of this cross was back crossed to the same females to obtain males with genetic markers in the autosomes and *X* chromosome but mantaining the *YrDNA*^{Def} chromosome. In other words, the autosomes and *X* chromosome were removed from the background. Then I crossed these males to the *C(1)DXrDNA*$^{-}$ */Y* females again and from this cross I obtained viable female progeny that were phenotypically bobbed, which means that neither the *X* or the autosomes had a suppressor for the lethality, hence the *Y* chromosome was responsible for the bobbed phenotype. In addition, *rDNA* quantification showed an increase in the array size suggesting that *rDNA* magnification was the cause of the reversion of the phenotype. In order to follow the kinetics of magnification of the *YrDNA*^{Def} chromosomes I decided to monitor the *rDNA* array size every generation in the second screen I did using the *Y,10B*.

I selected six *Y rDNA*^{Def} chromosomes to follow magnification for at least four generations. Four chromosomes were lethal and two were bobbed. Every generation I analyzed the *rDNA* amount of 4-10 individual pupae or adult females. As expected, I observed a progressive increase of *rDNA* copy number every generation. The kinetics of magnification is variable within the lines, but all lines showed an overall increase in size through the generations. With the exception of the flies that exhibited large increases or decreases (i.e. when there is a change in the phenotype), on average the four generations showed an increase of ~5% of the wild type the array size, which is equivalent to an

increase of ~15 cistron copies each generation, similar to previous observations on the *X* chromosome (88). I found that in line *I-498* magnification initially increased after the first generation ~11.6% of the wild type array size (from 46% to 57% of wild type), which is equivalent to ~33 *rDNA* copies (Figure 2.6), and the following generations the array stayed almost steady with a slight increased of ~0.5% (~2 *rDNA* copies).

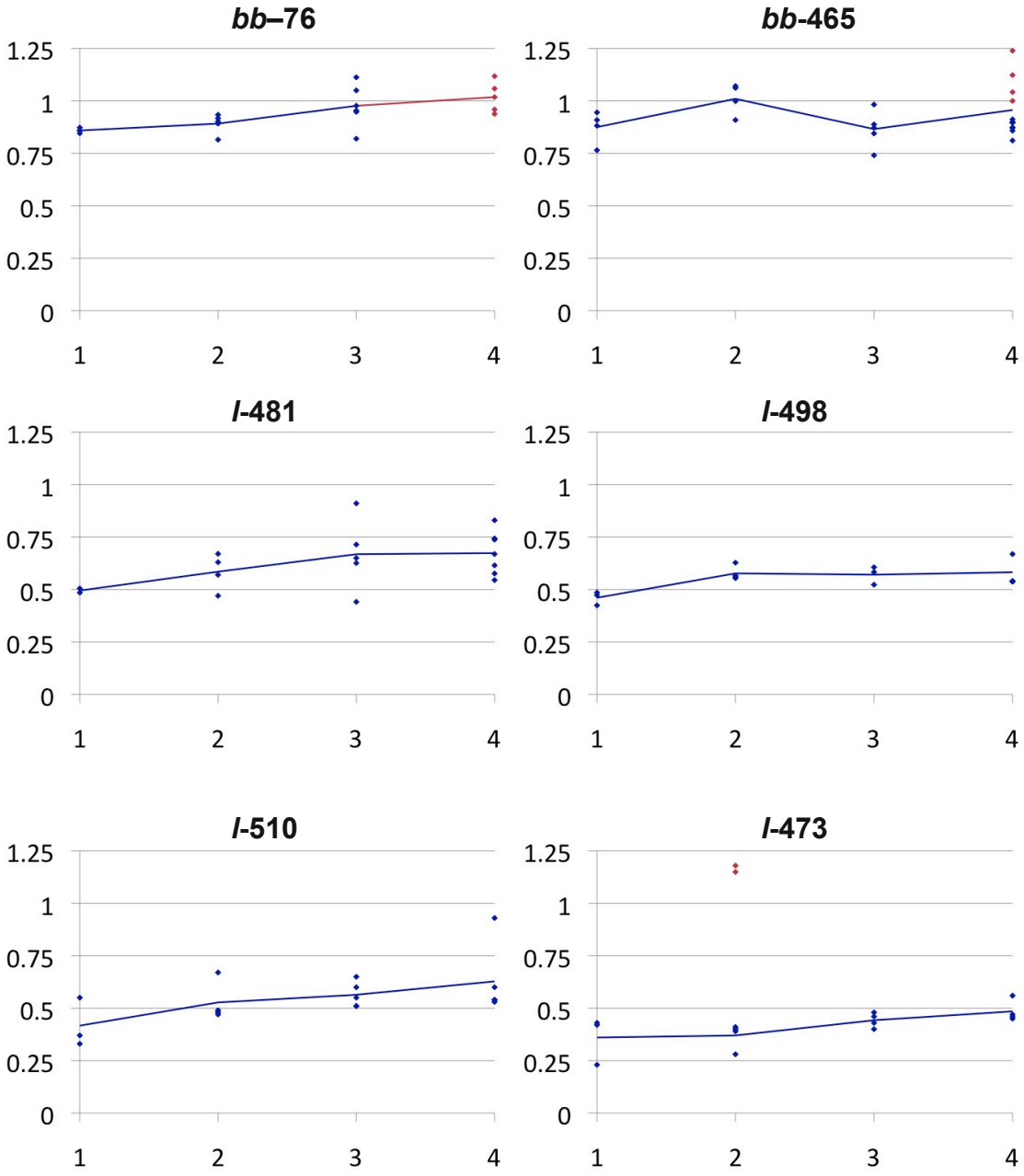


Figure 2.6 *rDNA* magnification occurs in the *Y* chromosome. Six chromosomes were monitored throughout 4 generations. Y axis show percentage of *rDNA* relative to wild type. X axis denote generations 1 to 4. Blue points are individuals with the same phenotype as denoted by the name of the line (lethal or bobbed), and red points are individuals that change to the next phenotype: from lethal to bobbed, or from bobbed to wild type. The lines connecting the generations are marking the average in *rDNA* amount within the individuals at each generation.

In contrast to this chromosome, the averages of the other three lethal lines analyzed, *l-481*, *l-510* and *l-473*, showed more noticeable magnification each generation, although the total amount of magnification in these lines varied it has consistent small increases each generation. The almost steady state levels that I observed with line *l-498* could be due to small sample size, which was common for all the analyzed lines. The magnification in line *l-498* is happening indeed but by taking small groups of individuals in which the larger events are underrepresented I might be diluting the real magnified state at each generation.

An interesting case of magnification is observed in line *l-473* from the first to the second generation. I observed two individuals that had an increased of ~80% the size of the original chromosome, giving rise to a chromosomes that contained in average ~116% of the wild type array (~335 *rDNA* copies) (Figure 2.6). The total increased was 3.2 times greater than the array size found in the *Y* chromosome from the first generation. Since a single USCE event can only theoretically double the array size, either more than one event of USCE occurred and/or there is an alternative mechanism that also contributes to the magnification of the array. Furthermore, even though the two individuals harbored arrays larger than wild type, they were phenotypically bobbed. This could hypothetically be due to a larger fraction of interrupted or damaged copies within the template used for magnification.

The *bobbed* line *bb-465* had an initial increase of ~13% of the wild type array (~37 *rDNA* copies) from the first to the second generation, and then it decreased about the same percentage from the second to the third generation. This reduction could be explained by having this progeny derived from a single father in whom magnification did not occurred in first to second generation (Figure 2.2, Generation 3, left). The transition from third to fourth generation gives two kinds of progeny: Some that retain the same array size and some that underwent magnification at the level of wild type or higher and consequently changed the phenotype from bobbed to wild type (Figure 2.6).

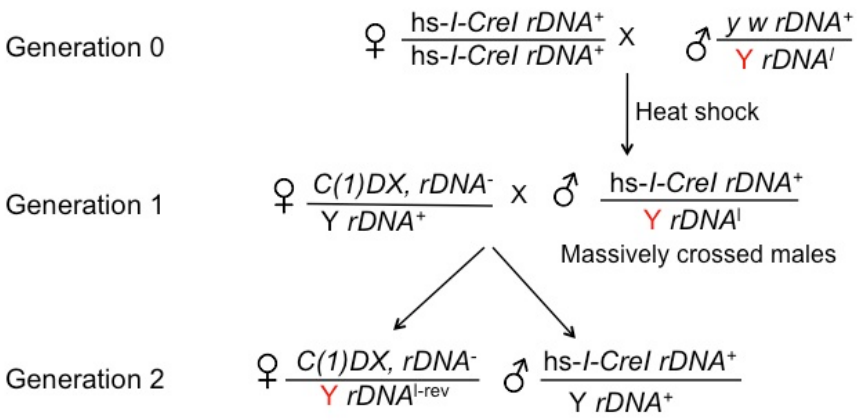
Line *bb-76* shows a clear trend of magnification from first to the fourth generation, maintaining the bobbed phenotype for the first three generations and switching to wild type on the last generation where the average array size reaches wild type levels (~101% of wild type = ~291 *rDNA* copies). For all the six analyzed chromosomes, the levels of *rDNA* copy number agree with the two phenotype transition ranges previously described, ~190 *Y*-linked *rDNA* copies is the transition from viable to lethal and ~260 *Y*-linked *rDNA* copies is from wild type to bobbed (Figure 2.5).

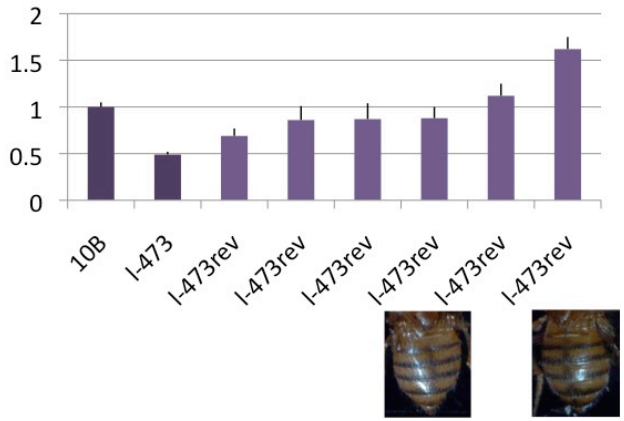
My data shows that the *Y*-linked *rDNA* arrays are able of magnify even in the absence of a special inducing chromosome as previously observed (91). Since previous studies have shown that *Y*-linked *rDNA* deletions are very stable (138), it is reasonable to think that a magnifying element was present in the *Y10*

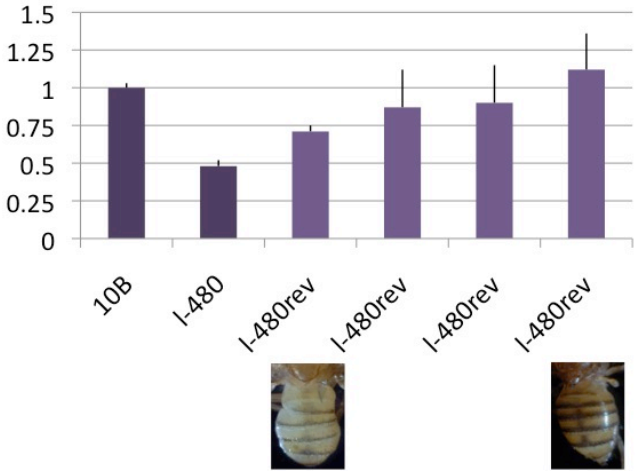
chromosome, or that the induction of *I-Cre*I has itself induced the magnification potential of this chromosome.

Magnification can be induced by the expression of *I-Cre*I

The process of Unequal Sister Chromatide Exchange involves unequal pairing of sister chromatides, followed by chromatid breakage and rejoining (90). It has been observed that mutations in genes involved in DNA double-strand break repair inhibit magnification (i.e. *mus-101* and *mei-41*). In addition mutants in *mus-108* have been observed to be defective in magnification and reduction, suggesting that these two processes might have a general component (79). I wanted to test whether double strand breaks induced by *I-Cre*I, could induce magnification in the *rDNA* array. To assay this I took advantage of the skewed male:female ratios among progeny of a male containing a lethal $YrDNA^{Def}$ crossed to *C(1)DX,* $rDNA^{-}$/*Y* females. I used three of the lethal lines as a starting point of *rDNA* copy number (*I-473*, *I-480* and *I-481*), and crossed the male flies to females that contained the *I-CreI* transgene in both *X* chromosomes. Then, I induced the expression of the endonuclease on these lethal $YrDNA^{def}$ ($YrDNA^{I}$) chromosomes by heat shock (Figure 2.7, Generation 0). The male progeny from this cross were outcrossed *en masse* to females *C(1)DXrDNA*$^{-}$*/YrDNA* (Figure 2.7, Generation 1). As control I performed the same crosses using lines that did not contain the *I-CreI* transgene on the *X* chromosome. It is expected that if *I-Cre*I induces magnification of the array on the $YrDNA^{I}$ then the proportion of


Generation 0
♀ hs-I-CreI rDNA+ / hs-I-CreI rDNA+ x ♂ y w rDNA+ / Y rDNAI
Heat shock
Generation 1
♀ C(1)DX, rDNA- / Y rDNA+ x ♂ hs-I-CreI rDNA+ / Y rDNAI
Massively crossed males
Generation 2
♀ C(1)DX, rDNA- / Y rDNAI-rev ♂ hs-I-CreI rDNA+ / Y rDNA+


2
1.5
1
0.5
0
10B
I-473
I-473rev
I-473rev
I-473rev
I-473rev
I-473rev
I-473rev


1.5
1.25
1
0.75
0.5
0.25
0
10B
I-480
I-480rev
I-480rev
I-480rev
I-480rev

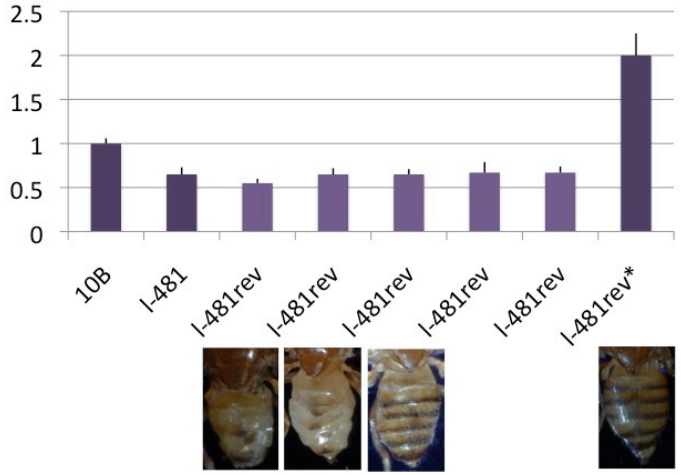

2.5
2
1.5
1
0.5
0
10B
I-481
I-481rev
I-481rev
I-481rev
I-481rev
I-481rev
I-481rev*

Figure 2.7 Induction of magnification by *I-CreI* expression. Top left panel shows the genetic strategy to induce *I-Cre*I expression in *Y* lethal chromosomes. Top right panel and bottom panels show the quantification of individual female flies revertant. *rDNA* quantification in shown as percentage relative to wild type (*10B*) defined as 1.00. For each line the *rDNA* amount before the exposure to *I-Cre*I and wild type are shown as dark purple bars. Light purple bars are quantification of revertant female flies. Graph bars are averages of replicate qPCR reactions and error bars are standard error of the mean. Dark purple bar I-481* is from the control cross without *I-Cre*I expression, which is a NDJ event.

viable females will increase among the progeny only when the *YrDNA^{I}* was exposed to the *I-Cre*I endonuclease (Figure 2.7, Generation 2). On the other hand only male progeny were expected if there was not magnification of the array. The results are summarized in Table 2.3.

I recovered viable females *C(1)DX*, *rDNA^{-}/ YrDNA$^{I\text{-rev}}$* for each of the three lethal lines that we analyzed. Some females exhibited a very strong bobbed phenotype, meaning that although there was increase in array size, it was just enough to provide RNA for survival. Additionally, females with a wild type phenotype (*bb^{+}*) were recovered. I molecularly confirmed the increase in *rDNA* copy number by qPCR on the revertant females for the three chromosomal lines and in the original *Y* chromosomes before the *I-CreI* treatment (Figure 2.7). For two of the lines, *I-473* and *I-480*, the amount of *rDNA* on each chromosome after the induction of *I-CreI* correlates with the expressivity of the phenotype for each female fly (Figure 2.7). An exceptional case was revertants from *I-481,* which did not contain increases in *rDNA* size. A hypothesis for this is that the few copies present on the array could be epigenetically silenced and induction of *I-Cre*I could have stimulated the activation of some or all of them, yielding viable females with no increase in array size.

I also recovered two females phenotypically wild type (*bb^{+}*) from the control crosses, *I-480* and *I-481*. These females could have been derived from non-disjunction events, such that they contained the *Y rDNA^{+}* chromosome from the mothers along with the *Y rDNA^{I}* chromosome from the fathers. Molecular

quantification of the *rDNA* array in these females confirmed this hypothesis since the amount of *rDNA* present on the *Y* chromosomes of these females was ~200% of the wild type 10B chromosome (Figure 2.7, I-481 rev*).

Together these data suggest that *I-CreI*-induced double stranded DNA breaks stimulate the process of magnification. This agrees with a previous hypothesis that suggested that reductions in the size of the *rDNA* array result in DNA breaks in the ribosomal genes, which triggers the recombinational DNA repair pathway that causes sister chromatid exchange and further magnification (139).

X Chromosome	***Y10B rDNA^{I}* Chromosome**	**X/Y male progeny (Generation 2)**	***C(1)DX/Y10B, rDNA$^{I\text{-}rev}$* female progeny (Generation 2) and phenotypes**
*I-Cre*I	*I-473*	149	3 bb (1.9%), 3 bb^{+} (1.9%)
*I-Cre*I	*I-480*	196	1 bb (0.5%), 3 bb^{+} (1.5%)
*I-Cre*I	*I-481*	126	6 bb (4.5%)
X	*I-473*	111	-
X	*I-480*	116	1 bb^{+} (NDJ) (0.8%)
X	*I-481*	62	1 bb^{+} (NDJ) (1.6%)

Table 2.3 Magnification induced by *I-Cre*I expression in *YrDNA^{I}* chromosomes. *bb*$^{+}$ means wild type phenotype. Females *bb*$^{+}$ from non disjunction events (NDJ) are present only in control crosses. Percentages of the presence of *bb* and *bb*$^{+}$ females are shown in parenthesis next to the number.

CONCLUSION

The *rDNA* is an extensively studied locus among different eukaryotes. However because of its multiplicity, it has been difficult to genetically study this locus by creating specific deletions. It has been shown that the endonuclease *I-Cre*I creates specific cuts within the *rDNA* of *Drosophila melanogaster* (129). But a sensitive technique to reliably quantify the amount of *rDNA* genes in an array had not been described.

Here I designed a genetic strategy to create specific, isogenic and graded deletions to the *rDNA* using *I-Cre*I endonuclease. In addition, I developed a molecular strategy based on Real-time PCR, to measure the amount of *rDNA* copies. I created a series of twenty five *Y*-linked *rDNA* deficient lines, in which I observed a magnification rate of ~15 *rDNA* copies per generation. In addition, I showed that magnification of the *rDNA* could be induced by exposure to *I-Cre*I.

Together my data show a relatively easy method to create specific *rDNA* deletions in the *Y*-linked *rDNA* array of *Drosophila melanogaster*. This technique could be applied to study *X*-linked *rDNA* arrays as well. The $YrDNA^{Del}$ lines that I created are useful to further study the biology of the *rDNA*.

CHAPTER III

THE *rDNA* IS A REGULATOR OF CHROMATIN BALANCE

INTRODUCTION

Chromatin structure has a fundamental role in the regulation of gene expression. Understanding how chromatin is formed and how its structure is regulated is an essential step towards understanding regulation of gene expression. Chromatin encompasses two contrasting environments, repressive and permissive, which are known as heterochromatin and euchromatin respectively. Hence, a good locus to study chromatin is the *rDNA* array, as it is composed of both of these two environments (85, 100). The epigenetic regulation of the *rDNA* has been intensely studied in some organisms such as plants, mammals and yeast (140), but in *Drosophila* not much is known about this mechanism. This locus nucleates the nucleolus, an important compartment formed inside the nucleus during cellular interphase, in which several essential cellular processes take place, but most notably ribosome biogenesis (141). The nucleolus structure is sensitive to changes in dosage of some heterochromatin components such as Heterochromatin Protein 1 (*Su(var)2-5*), Histone H3K9 methyltransferase (*Su(var)3-9*), and also some members of the RNAi pathway which act as Su(var)s such as *dicer- 2, Aub*, and *Spn-E* (119). As a consequence of these mutations, defects in nucleolus structure are observed as the formation of multiple extra chromosomal nucleoli. Since it is known that a single *rDNA* repeat is able to form a small nucleolus by recruitment of and active

transcription by RNA Polymerase I (120), it is proposed that changes in the dosage of heterochromatin components causes chromatin relaxation. This relaxed chromatin state then facilitates recombination between the repeats followed by formation of *rDNA* extra chromosomal circles and consequent formation of extra chromosomal nucleoli (119). Interestingly, fluctuations in *rDNA* array size happen spontaneously throughout the life cycle of many organisms (95, 98, 133), and have been observed to occur both somatically and meiotically (79, 91). Additionally, the *rDNA* locus has been shown to affect and induced gene variegation (125, 142, 143). Notably, the formation of multiple extra chromosomal nucleoli and misregulation of genes are also phenotypes observed in aging and cancerous cells (99, 144).

I wanted to study the effect of the *rDNA* locus on gene expression. In order to do that, I introgressed the *Y* chromosomes of the $YrDNA^{Def}$ lines into different genetic backgrounds that contained a reporter gene. The reporter genes I used were in a chromosomal rearrangement sensitive to PEV. Since I observed *rDNA* magnification in my preliminary experiments, I decided to monitored *rDNA* array size and reporter gene expression for several generations. The evidence of spontaneous *rDNA* size fluctuation occurring throughout the life cycle of *Drosophila* pointed me to extend my analysis to the expression of a second reporter gene in otherwise wild type flies, to address whether this natural process also influences gene expression. In addition, the evidence of changes in nucleolus structure as a consequence of changes in

rDNA chromatin composition led me to examine the effect of the *YrDNA*^{Def} lines on nucleolus structure.

RESULTS AND DISCUSSION

rDNA deletions affect gene expression

The *rDNA* array is composed of euchromatic and heterochromatic tandem repeats, which determine the transcriptional state of the copies on the array (85, 100). Hence, it is an ideal locus to study chromatin structure and its role on gene expression. Furthermore, the correlation between *rDNA* copy number and genome size across many plants and animals (76), and the role of its high copy number on stability of the genome (145) suggest that this locus has an additional essential role other than ribosome biogenesis. The question I wanted to answer was: does the *rDNA* have a role in the regulation of gene expression?

To address this question I used the *YrDNA*^{Def} fly lines (Chapter II) to study the effect of *rDNA* copy number on the expression of reporter genes. I needed a sensitive test that allowed me to visually detect changes in gene expression. These changes are easier to detect visually when the gene is on a PEV rearrangement as opposed to its endogenous location, where slight increase or decrease might not be visually detectable. In addition, PEV is the classical model to study changes in chromatin composition, which has given rise to most of our knowledge regarding the role of chromatin structure in gene

expression. In my first genetic approach I used as a reporter for gene expression, the classical PEV allele *In(1)w*^{m4} (66) (Described in Chapter I). This reporter allowed me to determine if *rDNA* alterations have a positive (Su(var)) or negative (E(var)) effect on gene expression by testing whether there is a change in the red pigment of the eyes of the flies compared to wild type.

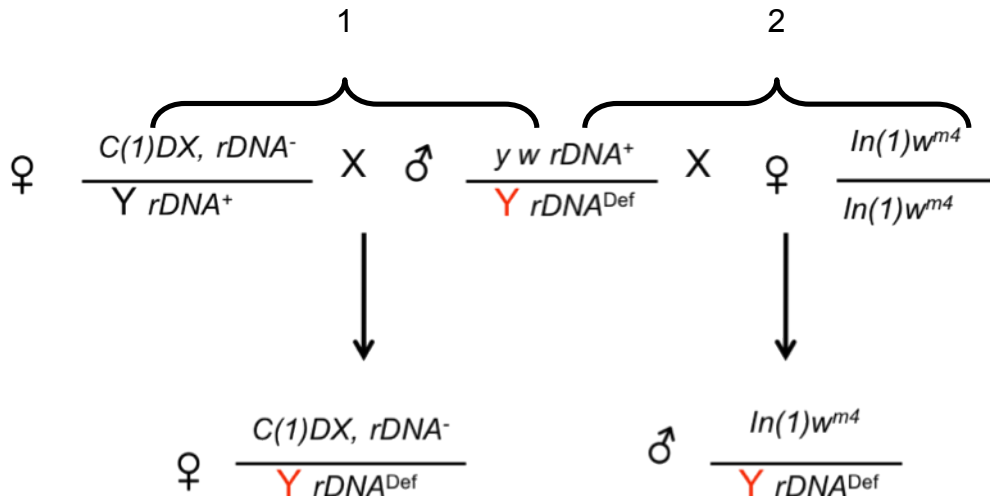


Figure 3.1. Crosses to measure *rDNA* deletions and test the effect on gene expression. 1) genetic approach to estimate the amount of *rDNA* present on the *YrDNA*^{Def} array (red Y). *C(1)DX,rDNA*$^{-}$*/Y* females were used to leave the *YrDNA*^{Def} as sole source of *rDNA* on the individual. 2) is the genetic approach to test effect on gene expression. Only male progeny was analyzed by eye pigment.

I performed two crosses: 1) to obtain a quantification of the *Y*-linked *rDNA* array and 2) to test the effect of the *rDNA* deletion on gene expression. The first cross used the same genetic strategy as when creating the deletions (Figure

3.1, cross #1). The use of *C(1)DX,rDNA⁻/Y* females was a convenient way to isolate the *Y*-linked *rDNA* for molecular quantification and also phenotypically corroborate the level of *rDNA* in each generation in a single generation. The second cross tested the expression of the *white*$^{+}$ gene present on the *X* chromosomal rearrangement *In(1)w*^{m4} (Figure 3.1, cross 2). I analyzed the pigmentation of the eyes of the male *In(1)w*^{m4}/*YrDNA*^{Def} progeny from this second cross to *In(1)w*^{m4} / *In(1)w*^{m4} females. Thus, the two crosses allowed estimation of both *rDNA* copy number and effects on gene expression in one generation, and allowed the investigation of whether these were correlated. However, a drawback of this strategy was that to ensure the success of the cross I could not used the same male for both crosses, instead I had to use different males for each one of the crosses. Due to the volatility of the *rDNA* array (133), this might have added some variability to the progeny of both crosses, because the flies that I analyzed for gene expression necessarily came from a different progenitor than the flies that I use to measure *rDNA* copy number.

From the twenty five *YrDNA*^{Def} lines that I created, I selected a subset of *Y* chromosomes for further analysis based on two criteria: 1) that there was representation of the *bobbed* and *lethal* deletions, and 2) that there was representation of the different rates of *rDNA* magnification. Therefore I chose the six *YrDNA*^{Def} lines analyzed for magnification in Chapter II, two with mild deletions that produce bobbed flies (*bb-465* and *bb-76*) and four large deletions

that are lethal (*l-481*, *l-498*, *l-510* and *l-473*). As a control I used the parental chromosome *Y,10B* that contains a full *rDNA* array (*Ywt*). For the sake of simplicity I will refer to these chromosomes based on the percentage of *rDNA* that they have left relative to wild type (*Y,10B*) as follows: *bb-465* = *bb*-0.87, *bb-76* = *bb*-0.85, *l-481* = *l*-0.49, *l-498* = *l*-0.46, *l-510* = *l*-0.41and *l-473* = *l*-0.36. The control *Y,10B* will be referred as *Ywt*.

I observed that the *Y* chromosome control (*Ywt*) had a neutral effect, that is, the eye pigmentation remained similar to the eye pigmentation on the *In(1)w^{m4}* stock. In contrast, *Y* chromosomes that had undergone an *rDNA* deletion showed strong expression of *white*$^{+}$. In other words the *YrDNA^{Def}* behaved as classical Su(var) mutations (e.g. HP1, *Su(var)3-9*) (Figure 3.2).

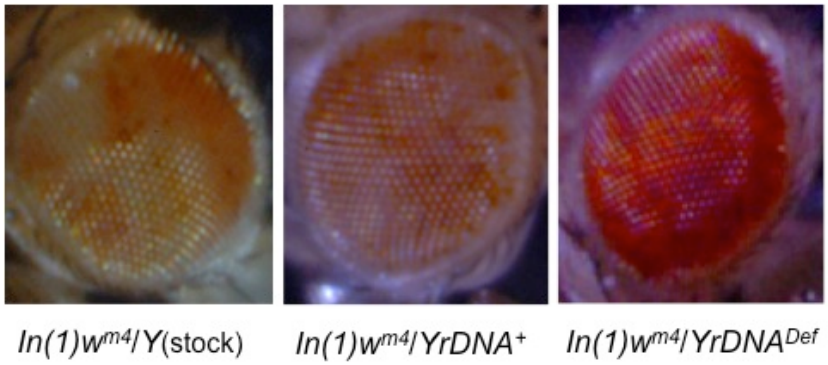


Figure 3.2. Su(var) effect of *rDNA* deletion on the *white*$^{+}$ gene present on the *In(1)w^{m4}* chromosome. Left, picture of an eye from a male fly taken directly from the fly stock. Middle, picture of an eye from a male fly that harbors the *Ywt* chromosome (*Y,10B*). Right, picture of an eye from a male fly that harbors a *Y* chromosome with an *rDNA* deletion (*YrDNA^{Def}, l-473*).

Further inspection reveled three categories of *white*$^{+}$ expression based on visualization, which I confirmed by pigment extraction and further spectrophotometer measurement (Figure 3.3). Henceforth, I measured the degree of reporter expression for the *Y* chromosomes in each generation as the percentage of male progeny in each category.

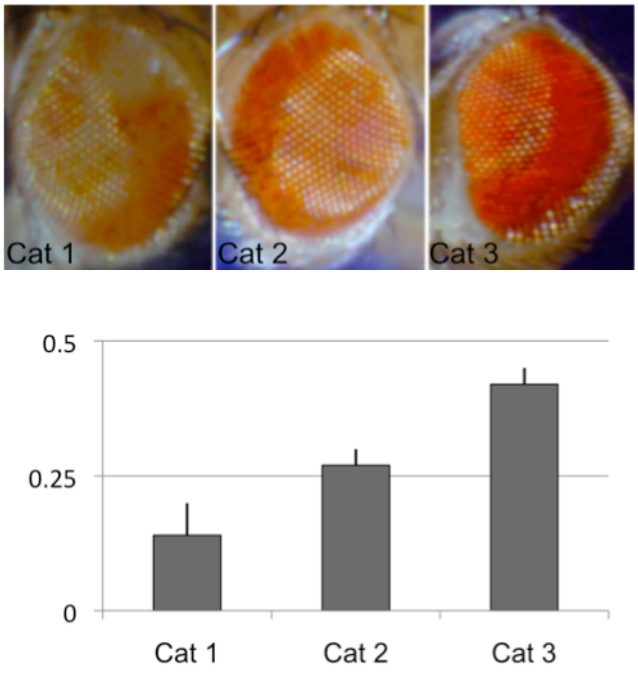


Figure 3.3 Categories of gene expression according to pigment extraction. Top, pictures of eyes displaying the three categories. Cat 1 denotes the lowest expression and Cat 3 the highest. Bottom, spectrophotometer measurements for each category taken at an absorbance of 480 nm (Y axis). Error bars are + standard deviation of 5 replicate measurements.

After several generations of follow up, I compared the categories of gene expression versus the estimated *rDNA* copy number on the *Y*-linked array and observed a inverse correlation across the seven lines. In other words small *Y*-linked *rDNA* arrays generated a high percentage of individuals with high expression of *white*$^{+}$, while bigger *rDNA* arrays have a lower percentage of individuals with high expression (Figure 3.4).

One exception to this observation was the lethal line *l-539* (Chapter II). Since this is lethal despite the *rDNA* quantification revealing that it harbored a *Y*-linked *rDNA* array that was larger than wild type (~115%), I hypothesize that the array is composed of damaged and/or interrupted copies, which are unable to provide functional rRNA. Interestingly, despite the larger array size this line increased the expression of the *white*$^{+}$ gene. This suggests that the functionality of the copies rather than the physical number may be what actually influences gene expression. But how? Perhaps loss of *rDNA* copies, either by deletion or interruption, results in a compensatory activation of *X*-linked *rDNA* copies. By altering the percentage of active copies in the genome, loss of functional *rDNA* may alter the heterochromatin/euchromatin balance and thereby alter the *white*$^{+}$ expression. This will be discussed in Chapter V.

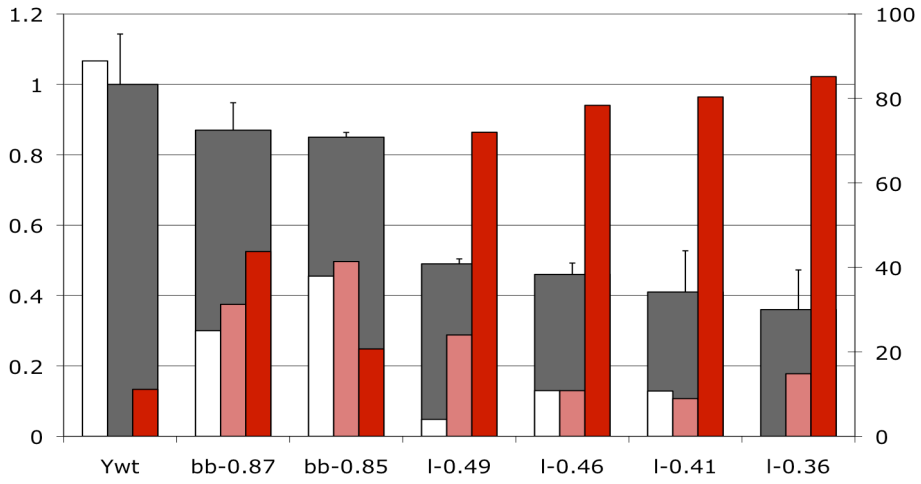


Figure 3.4 Inverse correlation of *rDNA* amount versus *white*$^{+}$ expression for *In(1)w*^{m4}. Grey bars represent percentage of *rDNA* amount relative to wild type, indicated in the left Y-axis. Right Y-axis indicates percentage of male flies found on each category of *white*$^{+}$ expression. Bright red bars are cat 3 of expression. Medium red bars are cat 2 of expression. White bars are cat 1 of expression. X-axis shows the seven genotypes analyzed. Error bars are + standard deviation of the mean, which was derived from the measurement of 5-8 individual flies. Student's t-test was performed for significant differences between the amounts of *rDNA* among the lines and *P* values obtained are as follows: *Ywt* vs. *bb-0.87* (P = 0.036), *Ywt* vs. *bb-0.85* (P = 0.025), *bb-0.87* vs. *bb-0.85* (P = 0.950), *bb-0.85* vs. *l-0.49* (P < 0.001), *l-0.49* vs. *l-0.46* (P = 0.01), *l-0.46* vs. *l-0.41* (P = 0.23), *l-0.46* vs. *l-0.36* (P = 0.027), *l-41* vs. *l-0.36* (P = 0.36).

Effects on PEV can vary depending on the location of the gene tested in the rearrangement (50). Hence, not all the chromosomal rearrangements will respond in the same way to changes in chromatin caused by Su(var)s and E(var)s. I wanted to test whether the increased *white*$^{+}$ expression effect of the *YrDNA*^{Def} was general or simply an allele-specific effect of the *In(1)w*^{m4} allele. In addition, I wanted to rule out a possible parental influence on the increased expression of *white*$^{+}$ observed. There are several alleles derived from the

In(1)w^{m4} such as: *w^{m454l}, w^{m451b}, w^{m4d}*, *w^{m4h}*. The former one, *In(1)w^{m4h}*, is a stronger variegating allele since it contains only ~5% of the red eye pigments compared to wild type (57). This allele has been shown to be more responsive to Su(var)s (146). Therefore, a strong effect of suppressor of variegation should be noticeable in most or all the progeny. In addition, this line has different chromosomal breakpoints than *In(1)w^{m4}* and previous observations have shown that this allele is insensitive to the parental source of the rearrangement. For instance, exposure of parents to temperature or inheritance of the allele from different genetic backgrounds has less of an effect relative to other alleles (57). I performed the same analysis as above using the variegating *In(1)w^{m4h}* allele, and following the same genetic strategy and pigment categorization as for the *In(1)w^{m4}* previously explained.

I obtained a similar inverse correlation as with the *In(1)w^{m4}* allele (Figure 3.5). However the higher category of gene expression was present in 100% of the individuals that harbored the smaller arrays (*l-0.49, l-0.41* and *l-0.36*), while only up to 80% was on the test with *In(1)w^{m4}*. This agrees with the stronger sensitivity of this allele to the presence of strong Su(var)s. In addition, it rules out a possible maternal contribution to the phenotype observed with the *In(1)w^{m4}*. Together this data show that *rDNA* array size affects variegated gene expression at least in the *X*-chromosome.

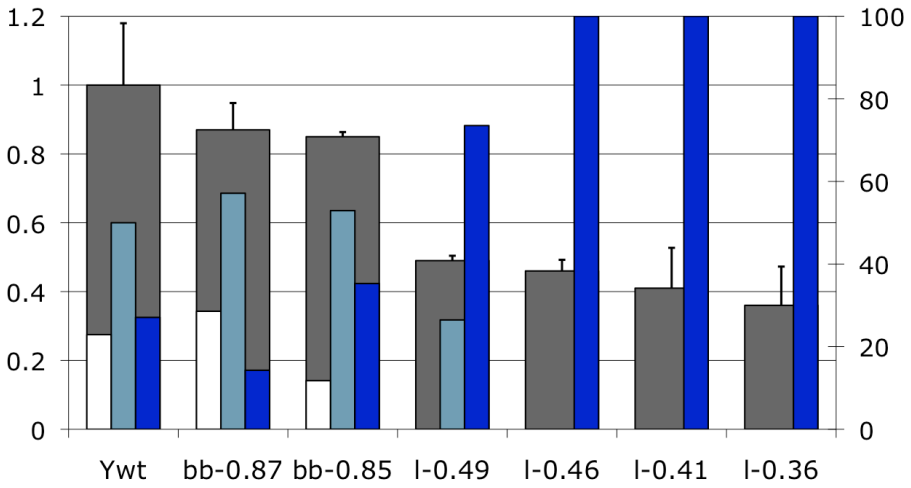


Figure 3.5 Inverse correlation of *rDNA* amount versus *white*$^{+}$ expression for *In(1)w*^{m4h}. Grey bars represent percentage of *rDNA* amount relative to wild type, indicated in the left Y-axis. Right Y-axis indicates percentage of male flies found on each category of *white*$^{+}$ expression. Intense blue bars are cat 3 of expression. Medium blue bars are cat 2 of expression. White bars are cat 1 of expression. X-axis shows the seven genotypes analyzed. Error bars are + standard deviation of the mean, which was derived from the measurement of 5 - 8 individual flies. Student's t-test was performed for significant differences between the amounts of *rDNA* among the lines and *P* values are the same as in Figure 3.4.

In addition, to test that the effect was not *X* chromosome-specific, I analyzed the effect of the *rDNA* deletions on a euchromatic gene located in an autosome. I used the *Stubble-variegator* (*Stubble*^{V}) as gene reporter. *Stubble* is a gene that is located in the third chromosome and when mutated produces short bristles. *Stubble*^{V} places a dominant *Stubble* mutant gene nearby heterochromatin, hence heterochromatic gene silencing will inactivate the mutated gene producing wild type bristles, and Su(var)s will make this mutated

gene more active resulting in a larger number of mutant, short bristles. I tested two medium deletions (*bb*-0.87 and *bb*-0.85), one large deletion (*l*-0.36) and the wild type array (*Ywt*). I measured five kinds of bristles among the male population and found that the *rDNA* deletions affected the expression of *Stubble*, but not in the same way in terms of magnitude, direction, and level of expression for all the kinds of bristles tested (Figure 3.6). In other words, *rDNA* deletions act as Su(var) for some bristles and as E(var) for others.

This agrees with previous observations showing that *Stubble* does not respond in the same way to some modifiers of variegation compared to other genes tested that were present on the same genetic background. For instance, C.P. Bishop tested the response of two variegating genes present in the same genetic background to different modifiers of variegation. He used the *yellow*$^{+}$ gene and *Stubble*^{v}, and found that in some cases the two genes responded in the same way, but for some modifiers of variegation only one of the two was affected (147). This suggests that the response of different genes to the same modifier of variegation can vary, as it was observed with the response to the *rDNA* deletions.

Arguably the shortened bristles characteristic of the bobbed phenotype (148) could have complicated the analysis of *Stubble*^{v}. However, two observations suggest otherwise: 1) the bobbed phenotype is recessive, and should be fully complemented by the wild type array on the *X*-chromosome in these flies (79), and 2) I measured the the rRNA levels in flies that harbor the

YrDNA^{Def} and a wild type *X*-linked array and found that the rRNA levels are similar to wild type (see below). However, I did not measure the rRNA levels in this specific genetic background, and even so this analysis requires using whole flies, which would have only shown the average level of rRNA for all the tissues. Since the fraction of active *rDNA* genes varies between different cell types (149), it is complicated quantify the degree to which the *bobbed* mutation contributes to the bristle phenotype.

This analysis suggests that *rDNA* deletions influence the chromatin balance generally on all chromosomes. Furthermore, the direction of the effect on gene expression is not biased and can vary in different regions of the organism.

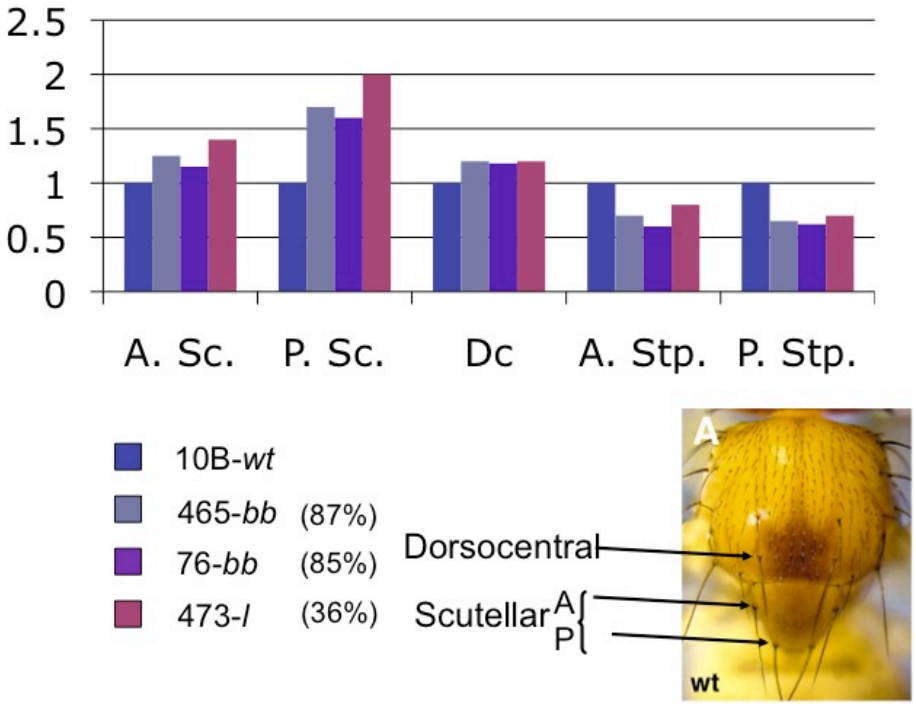


Figure 3.6 *rDNA* deletions affect the expression of *Stubble*^{V}. Top, percentages of length for five different bristles relative to wild type (Y10B). X axis shows the five kinds of bristles measured. A. Sc. is anterior scutellar, P.Sc. is posterior scutellar, Dc is dorsocentral, A. Stp. is anterior sternoplural, P.Stp. is posterior sternoplural. Picture on the bottom right displays some of the analyzed bristles.

Until this point, my data had shown that *rDNA* deletions have an effect on gene expression for the *In(1)w*^{m4}, *In(1)wm*^{4h} and some of the bristles in the *Stubble*^{V} similar to the effect of many genes that are involved in heterochromatin formation, such as HP1, Su(var)3-9, Modulo, Su(var)3-7, etc (52). This suggested that creating a deletion within the *rDNA* caused an alteration on the heterochromatin environment of the nucleus, leading to differential gene expression.

This hypothesis predicts that if I affect the heterochromatic environment then genes normally found in heterochromatin, even in autosomes, will also

have an alteration in expression. To address this issue I tested the *light*$^{+}$ gene. This is a gene required for normal levels of pigmentation in larval and adult tissues including the eye, and it is found in the pericentric heterochromatin on the second chromosome, where the generally repressive environment is actually required for normal expression (52). I used a chromosomal rearrangement that makes this gene variegate. Eye cells that silence this gene will have a less intense pigmentation as the wild type cells in which the gene is active. Since the effect of heterochromatin on *light*$^{+}$ expression is opposite to that of *white*$^{+}$, I expected an opposite effect of *rDNA* deletion on the expression of *light*$^{+}$. In other words, just as others Su(var)s mutations (i.e. HP1) (150), *rDNA* deletion should act as an E(var) on *light*$^{+}$.

I tested three chromosomes, two harboring mild deletions (*bb*-0.87 and *bb*-0.85) and one with a large deletion (*l*-0.36). As with the *white*$^{+}$ gene, I obtained three categories of *light*$^{+}$ expression, from low variegation (low gene silencing) to high variegation (high gene silencing). Consistent with the *rDNA* deletions generating a generally transcriptionally permissive environment, I observed that the *light*$^{+}$ gene became less active as the *rDNA* deletion became larger (Figure 3.7). This data suggested that deletions to the *rDNA* caused changes in chromatin structure, and this effect could influence the entire genome.

Some Su(var) mutations, such as *Su(var)205* (HP1), can enhance variegation of heterochromatic genes such as *light*$^{+}$ (151). This suggests a

double role for these proteins: as inducers of the silencing of euchromatic genes and as promoters of normal expression of heterochromatic genes. Since deletions to the *rDNA* have the same effect in euchromatic and heterochromatic genes, this points to the *rDNA* as a balancer of the two environments, possibly in cooperation with some of these known chromatin proteins and modifiers.

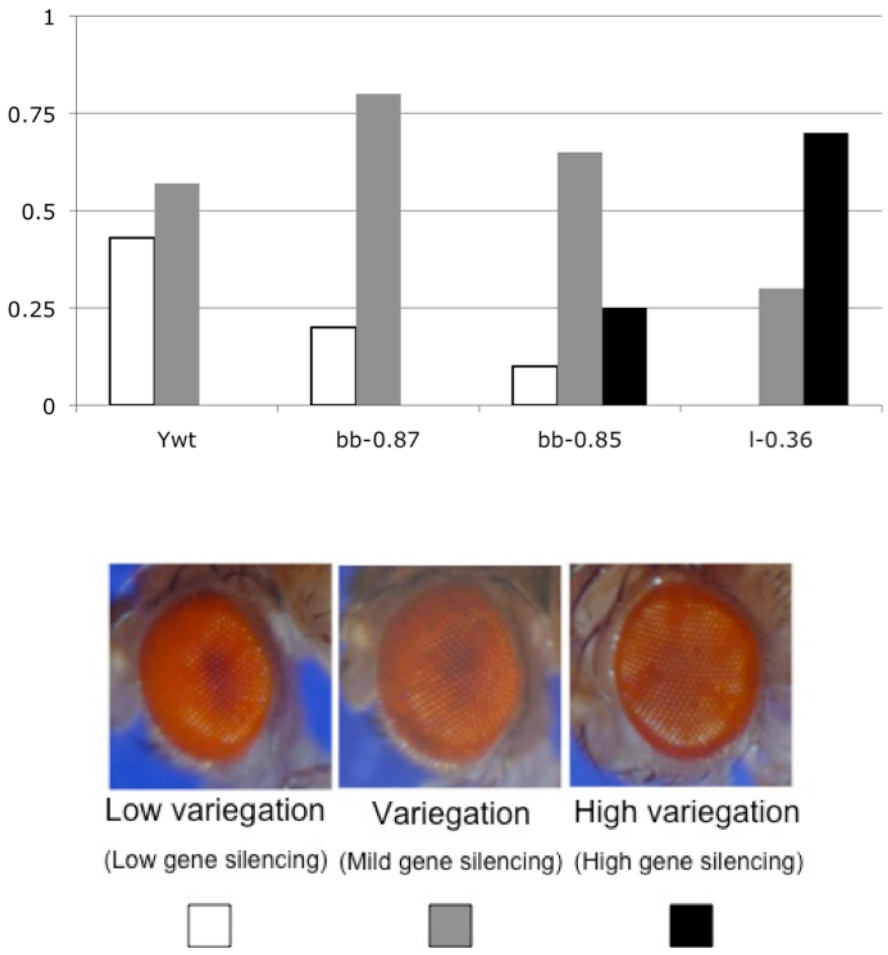


Figure 3.7 *rDNA* deletions act as an E(var) on the *light*$^{+}$ gene. Top panel, percentage of male flies located on the three different categories of *light*$^{+}$ expression. Bottom panel, pictures of eyes from the 3 categories of expression. Notice the disappearance of the pseudo pupil on the eye exhibiting high variegation compared to the eye with low variegation as a consequence of *light+* gene silencing.

Together this data shows that deletions within the *Y*-linked *rDNA* array of *Drosophila melanogaster* alter the balance between heterochromatin and euchromatin in the nucleus, and as a consequence of this alteration gene expression changes occur in different regions of the genome. The investigations using the PEV marker genes w^{m4}, w^{m4h} and $light^{var}$ as reporters of gene expression found that *rDNA* deletions act as classical suppressors of variegation. That finding suggested that the environment of the nucleus becomes more heterochromatic or silent due to the *rDNA* deletion. However, a somewhat more complex story was suggested by the marker gene $Stubble^{v}$. Here, changes in gene expression were still observed, however the direction and magnitude of the change varied depending on the location of the bristles on the fly. This suggested two characteristics of the role of the *rDNA* on gene expression: 1) that the effect on chromatin balance was not biased towards changes in any specific direction of gene expression, and 2) that there were differences in the effect according to the group of cells or tissue analyzed. This was not surprising as it is known that different tissues exhibit unique gene expression profiles conferred by different chromatin structures (152, 153). In other words, the level of heterochromatic gene silencing occurs in a tissue specific manner. Hence, the effects on chromatin balance caused by *rDNA* deletions can also be expected to be tissue specific depending on the heterochromatin/euchromatin ratio present in certain group of cells.

This data suggests an additional important role for the *rDNA*, as a regulator of chromatin structure. *rDNA* deletions behave as classical Su(var) mutations (52, 57, 66), which means that just as those chromatin components, the presence of *rDNA* repeats are important to maintain the balance of the chromatin in the nucleus.

rDNA magnification reverts the changes in gene expression

The best proof that a mutation is causing a phenotype is given by a complementation assay. The natural increase in *rDNA* copies, known as *rDNA* magnification, has been very well documented in *Drosophila* (90, 93, 137, 139). Since I observed in my preliminary experiments that the *rDNA* deletions increased in size, I realized that *rDNA* magnification would provide the best complementation assay. For that reason, I decided to monitor the *rDNA* deletion lines each generation for over five generations analyzing the *rDNA* deletion phenotype (using the $In(1)w^{m4}$ allele as reporter) as well as the *rDNA* copy number. Consistent with my expectations, I observed an increase in array size every generation (about 15 copies each generation on average, Chapter II), and a gradual reversion of the Su(var) phenotype each generation (Figure 3.8). In other words, the complementation assay provided by *rDNA* magnification confirmed that the *rDNA* deletion was the cause of the changes in chromatin balance that led to increased gene expression.

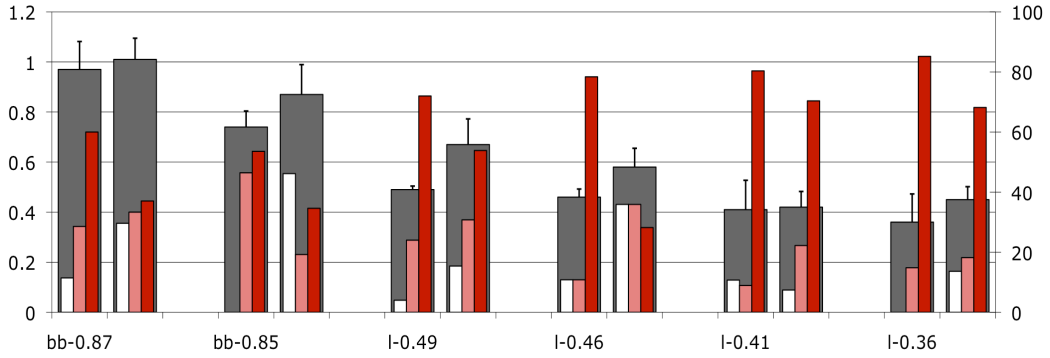
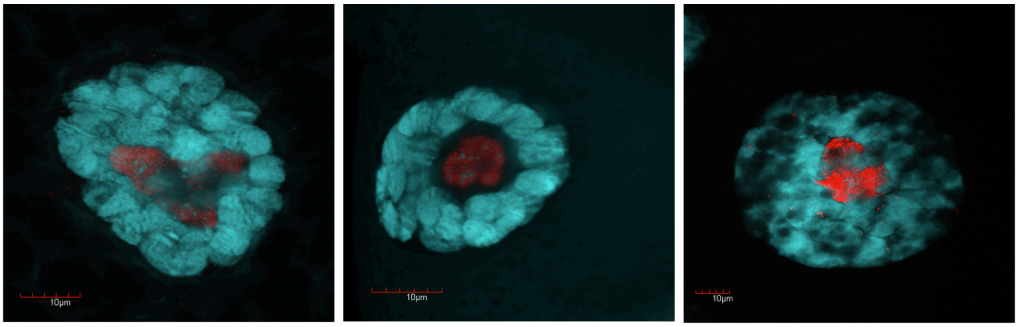


Figure 3.8 *rDNA* magnification reverts the phenotype. Shown six $YrDNA^{Def}$ chromosomes in two subsequent generations. Left Y axis, estimated percentage of *rDNA* relative to wild type represented by grey bars. Each grey bar is a subsequent generation for the same line. Right Y axis, percentage of male flies found on each category of $white^{+}$ expression. Bright red bars are Cat 3 of expression. Medium red bars are Cat 2 of expression. White bars are Cat 1 of expression. Error bars are + standard deviation of the mean derived from 5-8 individual measurements. The analysis was done using the $In(1)w^{m4}$ allele as reporter. Magnification tests were analyzed with a Student's t-test and the following *P* values were obtained for each line: *bb-0.87* generations (P = 0.393), *bb-0.85* (P = 0.016), *l-0.49* (P < 0.001), *l-0.46* (P = 0.002), *l-0.41* (P = 0.818), *l-0.36* (P = 0.051).

rDNA deletion affects nucleolus size and structure

It is known that heterochromatin structure is important for nucleolus structure, as it has been shown that mutations in heterochromatin components cause nucleolus instability (119). Hence I wanted to test whether causing a deletion to the *rDNA* would have an effect on the nucleolus in terms of size and structure. In order to address this question, I decided to study the nucleolus of polytene chromosomes in salivary glands, since they are in a tissue known for having high levels of gene expression, thus the nucleolus is constantly formed in

these cells as opposed to mitotic tissue. I choose to do this experiment in female flies *C(1)DX/YrDNA*^{Def} because the only source of *rDNA* comes from the *Y* chromosome, which makes easier the detection of any changes. This experiment was done with three *Y* chromosomes: one mildly reduced (*bb*-0.87), one largely reduced (*bb*-0.36) and the control *Ywt*. I immunostained whole mount salivary glands using a antibody against fibrillarin, which is a protein involved in post transcriptional rRNA regulatory processes that is found exclusive in the nucleolus (115). Additionally, I stained the DNA using DAPI to have a measurement of the size of the nucleus. Using confocal microscopy and three-dimensional reconstruction, I obtained the volume of the nucleolus relative to the volume of the nucleus. Not surprisingly, I observed that the volume of the nucleolus was reduced in the lines with *rDNA* deletions compared to wild type. In addition, the reduction in volume was correlated with the size of the deletion; the larger deletion formed the smaller nucleolus. The average volume from thirty different nuclei (ten per individual) from the same chromosomal line are shown (Figure 3.9).



Ywt	*bb* – 0.87	*l* – 0.36
Nucleolus volume relative to nucleus		
19.7%	12.5%	9.6%

Figure 3.9 Nucleolus size is affected by the *rDNA* deletion. Top pictures are slices of confocal imaging representing each analyzed line. Nucleolus volume as a percentage relative to the nucleus volume is shown for each line as an average of 30 different nuclei (10 per individual) for each analyzed line.

Interestingly, not only size was affected but also structure. I observed the presence of mini and micronucleoli, which are small extra chromosomal nucleoli, only in the lines that harbor the deletions (Figure 3.10). Because mutations in heterochromatin components induce the formation of extra chromosomal nucleoli (154) and a single *rDNA* repeat is able to nucleate the nucleolus (120), it is possible that this is the consequence of the combination of two processes: magnification and over activation of the remaining copies on the *rDNA* array as a compensatory mechanism for rRNA production, which could lead to the formation of extra chromosomal *rDNA* circles.

Interestingly these observations resemble the changes in nucleolus size and structure that are observed in aging and diseases such as cancer and Down Syndrome (110, 117, 155), where a common characteristic is the increased transcription of the *rDNA* copies. For instance, mutations on the *rDNA* silencer protein Sir2 decreases the life span in yeast while increases in the dosage extended it (156). In addition, increased levels of rRNA, changes in nucleolus volume, and increase in the number of *rDNA* arrays that are active in the cell (known as AgNORs) are common in cancerous cells (117) and in patients with Down syndrome (155). Furthermore, it is known that maintaining a subset of inactive *rDNA* copies is essential for the stability of the genome (76). Together these suggest that increases in the activity of the *rDNA* copies are related to changes in cell programming that lead to disease.

I did not observed formation of extra chromosomal nucleoli in the wild type cells analyzed, which suggests that the deletions in the *rDNA* cause nucleolar instability and further formation of extra chromosomal nucleoli. These observations might help to understand the connection between nucleolus and disease.

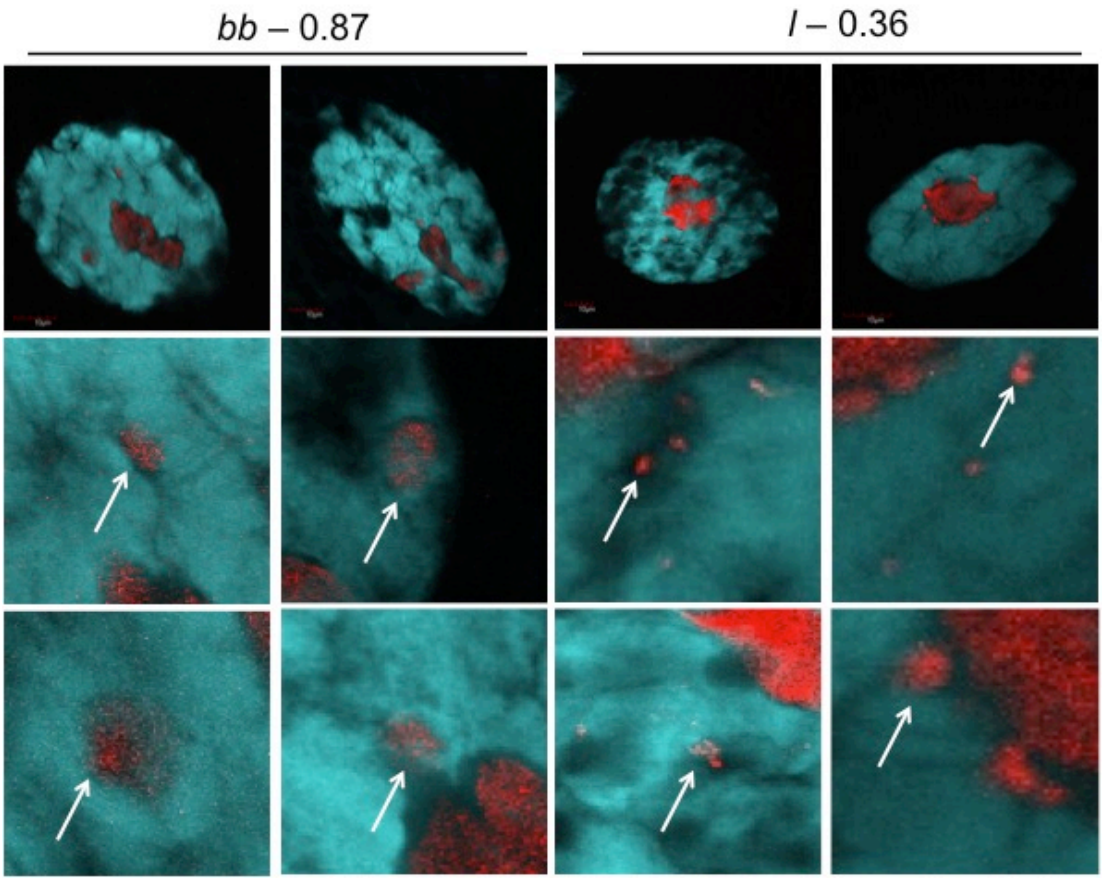


Figure 3.10 Nucleolus structure is affected by *rDNA* deletions. Pictures of nucleolus from two lines of *YrDNA*^{Def} are shown. Formation of mini and micronucleoli (white arrows) was observed exclusively on the lines that contain an *rDNA* deletion. Top panel, whole nucleus. Middle and bottom panels, zoom in pictures to highlight the extrachromosomal nucleoli.

Translational capacity is maintained as in wild type conditions

The genetic approach that I used to analyze the effect of *rDNA* deletions on gene expression uses a genetic background that contains a wild type *X*-linked *rDNA* array present on the *In(1)w*^{m4} chromosome. This array is able to supply the cell with enough rRNA. Indeed, I quantified the *rDNA* copy number for

this chromosome and found that it contained twice the amount of the parental *Y,10B* chromosome (~400 *rDNA* copies). However, *rDNA* transcription is regulated at different levels: epigenetically, transcriptionally and by *rDNA* copy number (75, 99). In addition, it is known that heterochromatin components and modifiers (i.e. HP1, Histone H3K9 methytransferase) are dose sensitive, meaning that subtle changes in dosage could generate a phenotype (52). Thus, there is still the possibility that the observed effect of increased *white*$^{+}$ expression is being caused by an insufficient amount of steady state rRNA, which decreases the translational capacity of the cell, reducing the amount of proteins required to build up heterochromatin. In order to investigate this possibility, I decided to measure the rRNA levels present on the same fly population that were used to analyze the eye phenotype (*In(1)w*^{m4}*/YrDNA*^{Def}*)*. In order to have a measurement of intact 28S and 18S rRNA, excluding the possibility that one or both of these rRNAs were incompletely transcribed in the mutants, I used gel quantification. This method allows the detection of full-length rRNAs as opposed to other methods such as qPCR, which can pick up fragmented products. I extracted total RNA and did gel quantification of three lines: a mildly deleted (*bb*-0.87), a largely deleted (*bb*-0.36) and wild type (*Ywt*). Quantification values relative to wild type showed that there is a slight but not significant increase in the rRNA amount produced by the *YrDNA*^{Def} lines (Figure 3.11). This indicates that the wild type array on the *X* chromosome is providing

enough rRNA to the cell, hence the phenotype is not caused by a limitation on the translational capacity.

It is known that the number of active *rDNA* copies varies between different cell types, meaning that the fraction of *rDNA* genes that are actively transcribed changes during development and differentiation (149). Hence, an aspect to consider here is that by using whole flies for rRNA measurement I am averaging the amount of rRNA in all the tissues. An ideal experiment to rule out translational capacity would be to correlate gene expression vs. rRNA amount in the same tissue. However the method used to estimate full-length rRNA makes it difficult to perform this experiment in such small pieces of tissue.

Overall the rRNA quantification suggests that the effect of increased *white*$^{+}$ expression is not a by-product of a reduction in the amount of rRNA that produces a limitation in the translational capacity of the cell.

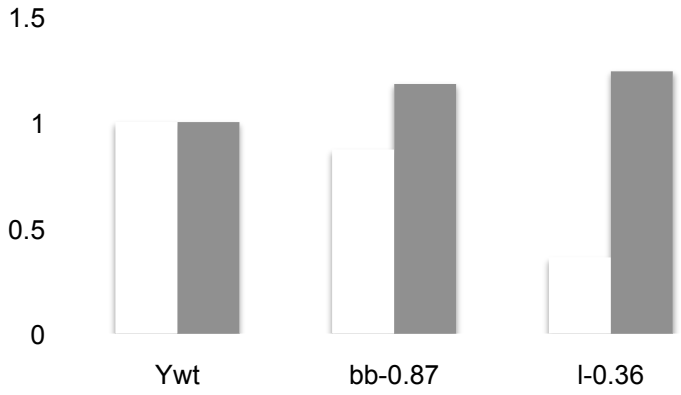


Figure 3.11 Comparison of rRNA content among three lines. Y-axis is percentage of rRNA relative to wild type (which is defined as 100%). The genotype of the adult flies used to estimate rRNA amount is $w^{m4}/YrDNA^{Def}$ and w^{m4}/Ywt. Grey bars are the mean of five independent replicated, error bars are + standard deviation of the mean. Slightly high values are not statistically significant (Student's t-test). White bars are expected rRNA amount relative to the amount of *rDNA* present in each line.

Spontaneous *rDNA* deletions affect gene expression somatically

It is been shown that reduction in *rDNA* copy number by extra chromosomal circle formation occurs throughout the life cycle of different organisms including *Drosophila* (95-97). Additionally in *Drosophila*, during embryogenesis, heterochromatic silencing is relieved in differentiated tissues but is kept in cells that contain precursor cells for adult tissues (152). Therefore, I wanted to test whether the spontaneous *rDNA* variation that is observed in somatic tissue could influence gene expression. In order to address that question, I used as a reporter gene a variegated GFP transgene inserted in the heterochromatic *Y* chromosome in an otherwise wild type fly line (Figure 3.12). I decided to look at larval brains because of the relatively high rate of division,

which increased the likelihood of observing the mitotic intrachromatide recombination events leading to spontaneous *rDNA* deletions. Furthermore, the visually detectable dynamic range of GFP expression in these tissues was relatively better compared to other larval tissues. I dissected neuroblasts according to the level of GFP expression and measured the *rDNA* genes present in each fragment of the tissue. As expected, reductions in *rDNA* amount correlated with increased expression of the GFP transgene (a Su(var) phenotype), and the patches of tissue that had lost more *rDNA* had the higher GFP expression (Figure 3.12). That suggests that the spontaneous changes in *rDNA* that occur during mitosis have an effect on gene expression.

The *rDNA* quantification assay is highly sensitivity, since it allows us to reliable quantify *rDNA* from DNA concentrations equivalent to ~5 genomes (Chapter II), therefore this technique was adequate to estimate *rDNA* amount in small fragments of tissue. While this represents a significant improvement over analyzing whole flies, the data still represents an average of *rDNA* copy number and a qualitative estimate of the GFP expression among the dissected cells. A true understanding of the linearity of the relationship between *rDNA* copy number and the gene-expression response of the cell will require techniques that allow more accurate measure of expression and *rDNA* copy number on individual cells.

The biological relevance of spontaneous *rDNA* loss through extra chromosomal DNA circles is unknown. However, since this is (52) a ubiquitous

process in eukaryotic organisms including humans (95, 96), it might have a very important role for the genome. Given that more sequences have been identified in *Drosophila* other than the *rDNA* to form extra chromosomal DNA circles throughout the life cycle, such as 5S, *Stellate*, *Suppressor of Stellate* and Histone cluster (97), it is a reasonable hypothesis that the plasticity of these other regions could also contribute to the chromatin balance. Differences in the size of repeated genomic sequences have also been observed in humans. For instance, striking variability in the 45S and 5S *rDNA* gene cluster size (50kb->6Mb) was observed among healthy humans (157), and formation of extra chromosomal DNA circles has also been observed from the 5S and satellite repeats in human cells (96). Spontaneous and environmentally induced changes in *rDNA* array size in different tissues throughout development could create a mosaic in chromatin structure that could have implications in development and cell differentiation. Furthermore, the differential gene expression generated could influence the predisposition of certain tissues and certain individuals to disease.

Lu and colleagues showed that heterochromatic gene silencing starts during embryogenesis, but it is suppressed in differentiated cells (152). This suggests that it is important in early development to maintain a differential regulation of gene expression, therefore extreme changes in *rDNA* could lead to developmental abnormalities. For instance, the phenotype of the Down syndrome individuals cannot be fully explained by the extra gene dosage that is

provided by the additional chromosome 21 (158, 159). A recent study proposed that the presence of an extra nucleolus organizer (present on chromosome 21) accounts for a major part of the Down Syndrome phenotype. It was observed to be transcriptionally active, causing an increase in the rRNA levels and ribosomal proteins compared to wild type cells (155, 160). It is possible then that the functional importance of the ubiquitous presence of ecc *rDNA* and other sequences is to confer plasticity to the genome so it will have the ability to modulate the genome in response to changes in the environment.

Since the *rDNA* is constantly changing, differences from cell to cell, programmed or spontaneous, can lead to different patches of gene expression in the same tissue. This could be a mechanism that contributes to the determination of cellular fate and differentiation, either intentionally during development, or unintentionally during aging and carcinogenesis.

Together this data suggest that natural *rDNA* loss influences gene expression in the same way as induced *rDNA* deletions, where larger *rDNA* losses induce stronger gene expression.

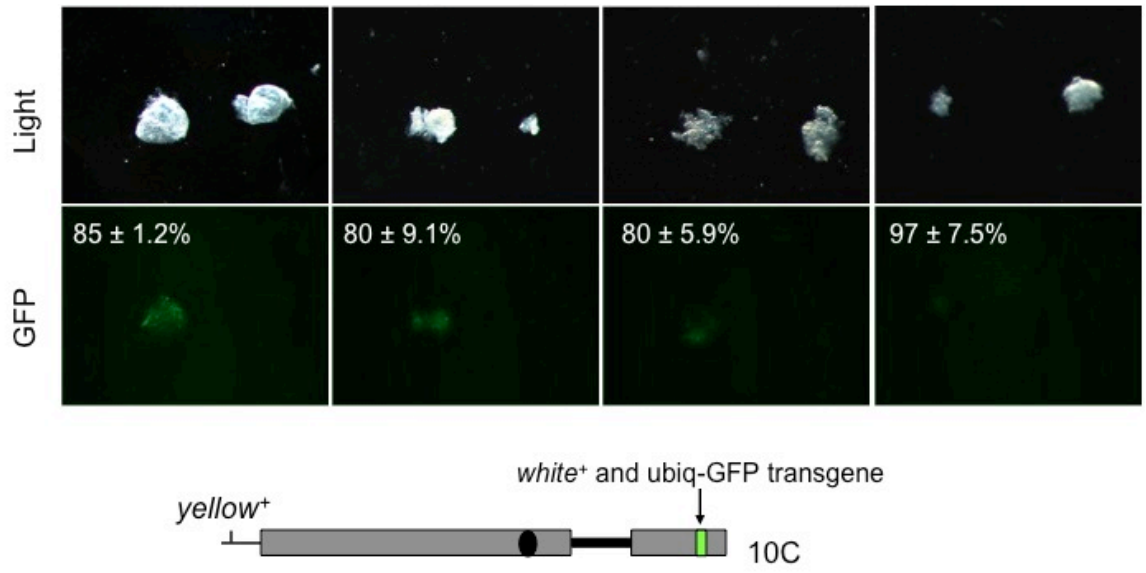


Figure 3.12 Spontaneous *rDNA* deletion influences gene expression. Pictures of brain tissue dissected according to GFP expression are shown. Brains were derived from male flies that have a *Y* chromosome that harbors a variegating *GFP* transgene. *rDNA* quantification of brain tissues revealed fewer *rDNA* copies in the pieces of tissue that have a higher expression of the *GFP* transgene. Percentages of *rDNA* present in GFP-expressing tissue relative to non-expressing tissues are shown ± S.E.M. Schematic representation of the *Y* chromosome used for this analysis is shown.

Mutants of heterochromatin components have small *rDNA* arrays

An intriguing point is that our observed effects caused by *rDNA* copy number are similar to those observed by mutations involved in chromatin compaction (52). Since mutations in heterochromatin components destabilized the *rDNA* array inducing the formation of extra chromosomal nucleoli (119), I wanted to test whether the *rDNA* arrays found in *Y* chromosomes from heterochromatin mutant backgrounds have differences in *rDNA* array size. To test this, I measured the array size of *Y* chromosomes isolated from different

chromatin modifiers stocks. Using the same genetic strategy as for my *YrDNA*^{Def} chromosomes, I made this *Y* chromosomes the sole source of *rDNA* on the fly. It is known that *Y* chromosomes isolated from different regions have differences in *rDNA* array size (161), and consistent with this I observed an entire series of lengths among the different *Y* chromosomes that were tested. Interestingly, the smallest *Y*-linked *rDNA* arrays were found on the *Y* chromosomes that were isolated from two mutants involved in heterochromatin formation: *Su(var)2-1*, and *Su(var)3-9* (Figure 3.13, top). Additionally, in the fly stocks of these mutants (before outcrossing them to the *C(1)DX* line) I found females that were phenotypically bobbed (Figure 3.13, bottom).

Despite the fact that extensive studies have provided conclusive evidence that connects many of the Su(var) proteins to heterochromatin formation (66), this findings suggest that the Su(var) phenotypes observed in those mutants could be in part due to a reduction in the size of the *rDNA* array. A supporting observation for this hypothesis was obtained from a study that correlated microarray gene expression analysis with physical localization of HP1 using cytology and chromatin immunoprecipitation (ChIP) in *Drosophila*. This study showed that mutants in HP1 exhibit differential expression of hundreds of genes throughout the genome without any bias in the direction of expression. This demonstrates that despite the known characteristics of the role of HP1 in heterochromatic silencing, it is also involved in silencing and activation of euchromatic genes. Interestingly, within the cytological region 31 (left arm,

second chromosome), which is known to be a high spot for HP1 localization according to cytological reports, is found the gene CG13135 that has been consistently shown to be up regulated in HP1 mutants. However neither by ChIP nor cytology, was HP1 enrichment found on any region of this gene in salivary glands in a wild type background (162). This data suggested that the alteration on expression of CG13135 in the HP1 mutant is due to an indirect effect, or that there is association of HP1 with this gene in tissues other than the salivary glands. Together this study and the evidence that showed that mutations in HP1 and other chromatin modifiers cause nucleolus instability (119) support the hypothesis that the effect of chromatin proteins and modifiers could be partially obtained throughout changes in the *rDNA* array size and possibly some other repetitive sequences in the genome.

Another compelling observation consistent with this hypothesis is the case of the modifier of variegation *E(var) 3-93D,* which exhibited an array at the bigger end of the measured chromosomes (Figure 3.13, top), suggesting that the large size could mediate its effect as an E(var). This E(var) is a very mysterious gene because it has an imprinting–like effect on the *Y* chromosome. Flies exposed to the *E(var) 3-93D* mutation (on chromosome III) can continue passing the E(var) phenotype (mapped to chromosome *Y*) for many generations after the mutation has been removed from the background (163). Interestingly, even after 11 generations of being without the presence of the mutation, the *Y* chromosome is still able to cause the E(var) phenotype. The explanation for this

imprinting effect has not been found yet, but my findings suggest that the gene product of *E(var) 3-93D* could mediate an increase in the size of the *rDNA*. This larger array would be maintained for many generations, hence passing along the E(var) phenotype by altering the chromatin balance in the nucleus. I showed that *I-Cre*I-induced double stranded DNA breaks induces magnification of the *rDNA* array (Chapter II). Using this approach we could create enlargement of a wild type *rDNA* array to test whether it acquires the ability to produce an E(var) phenotype.

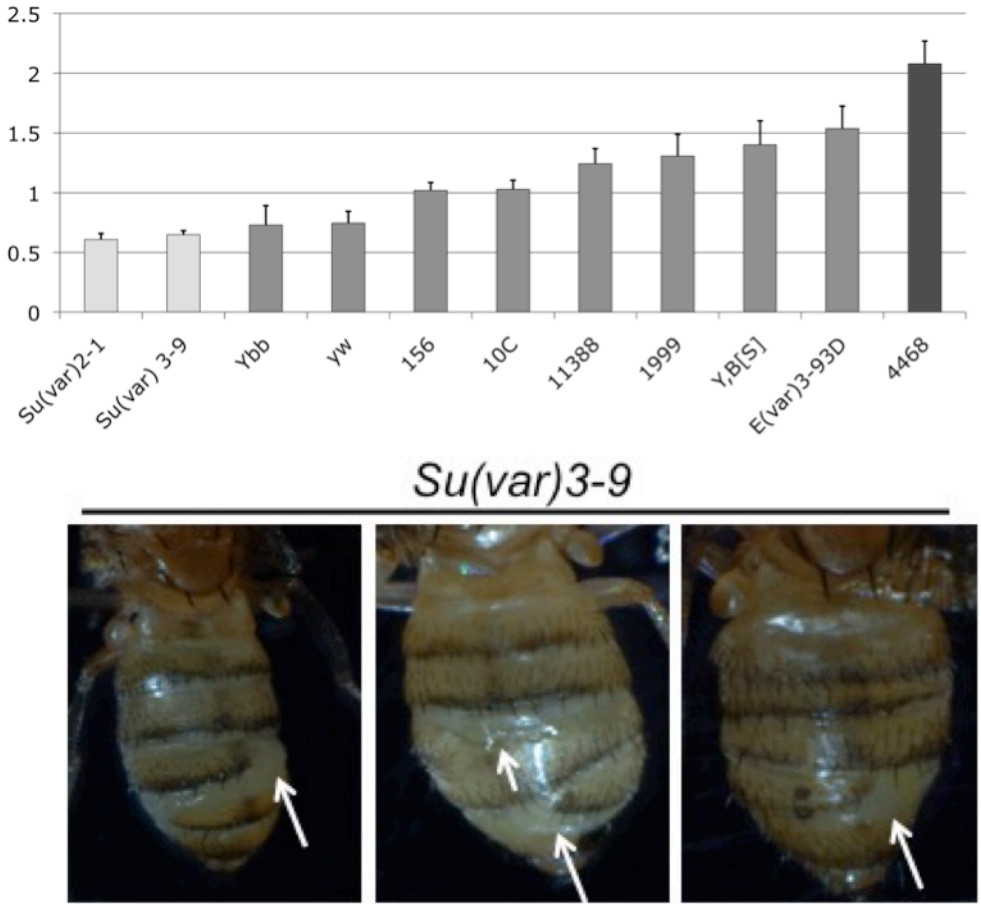


Figure 3.13 *Y*-linked *rDNA* arrays from mutants in heterochromatin formation are small. Top, plot displaying *Y*-linked *rDNA* quantification from different genetic backgrounds. *Y* chromosomes were introgressed into female flies from the genotype *C(1)DX/Y*. Y-axis indicates percentage of *rDNA* relative to our chromosome control *Ywt*. Error bars are + standard deviations of the mean derived from *rDNA* measurement of 5-7 individuals. Chromosome 4468 contains an *rDNA* duplication. Bottom, pictures of female flies X/X from the original stock of the mutant background *Su(var)3-9*. White arrows point to the cuticle formation defects, which are characteristic of the bobbed phenotype.

Y-linked *rDNA* arrays present in Su(var) backgrounds have a reduction in size compared to other chromosomes tested. However, since *Y*-linked *rDNA* can be variable among populations (77), it is possible that these chromosomes have always had an small array. In addition, if the effect of the Su(var)s on *rDNA* arrays is a general effect, it is expected that this reduction would be observed if a different *Y* chromosome is introgressed into this background. In order to address these two issues, I measured *rDNA* arrays from two different *Y* chromosomes before and after they were introgressed into several modifier of variegation backgrounds for several generations (about 1-2 years after the introgression) (Figure 3.14). As a control, the *Y* chromosomes tested were isolated from natural populations and were kept in the original background (164).

I found that in all the tested mutant backgrounds, there was alteration in the *rDNA* array size for both *Y* chromosomes. Interestingly, some modifiers have a positive effect for one chromosomes while a negative effect for the other one (i.e. Armitage, *Su(var)2-10*). I hypothesize that these differences could be chromosome specific, meaning that perhaps the polymorphic differences in the two *Y* chromosome tested can influence the response of the array to modifiers of variegation. It is possible that differences in other repetitive sequences present in these chromosomes (i.e. *Suppressor of Stellate*, satellite sequences, transposable elements) act also as targets of modification and can bias the action of modifiers of variegation on the *rDNA*. In addition, Su(var)s are known to cause instability of the nucleolus possibly by relaxing the chromatin at the *rDNA*

loci followed by extra chromosomal circle formation by intrachromatid recombination (119). The process of intrachromatid recombination requires double stranded DNA breaks (DSB), and I previously showed that induction of DSB by exposure to *I-Cre*I endonuclease induces magnification (Chapter II). Hence, it is possible that in some cases the effect of Su(var)s mutations lead to increases in *rDNA* array size.

The fact that all modifiers exerted an effect in the *rDNA* array size suggests that this might be a general effect. However the mechanism of action for each modifier might be different. A comparison between microarray expression profiles of modifiers of variegation vs. $YrDNA^{Def}$ lines could reveal if there is a significant overlap between the two groups and possibly suggest the pathways that each modifier take to affect gene expression by the modification of the *rDNA*. A summary of the absolute effect of the mutants tested for both chromosomes is shown (Figure 3.14).

Together these data suggest that the *rDNA* plays an important role in chromatin structure, which influences gene expression in the same way as heterochromatin components (44, 66), and part of the effect of some of these components on gene expression could be mediated throughout the alteration of the *rDNA* array size.

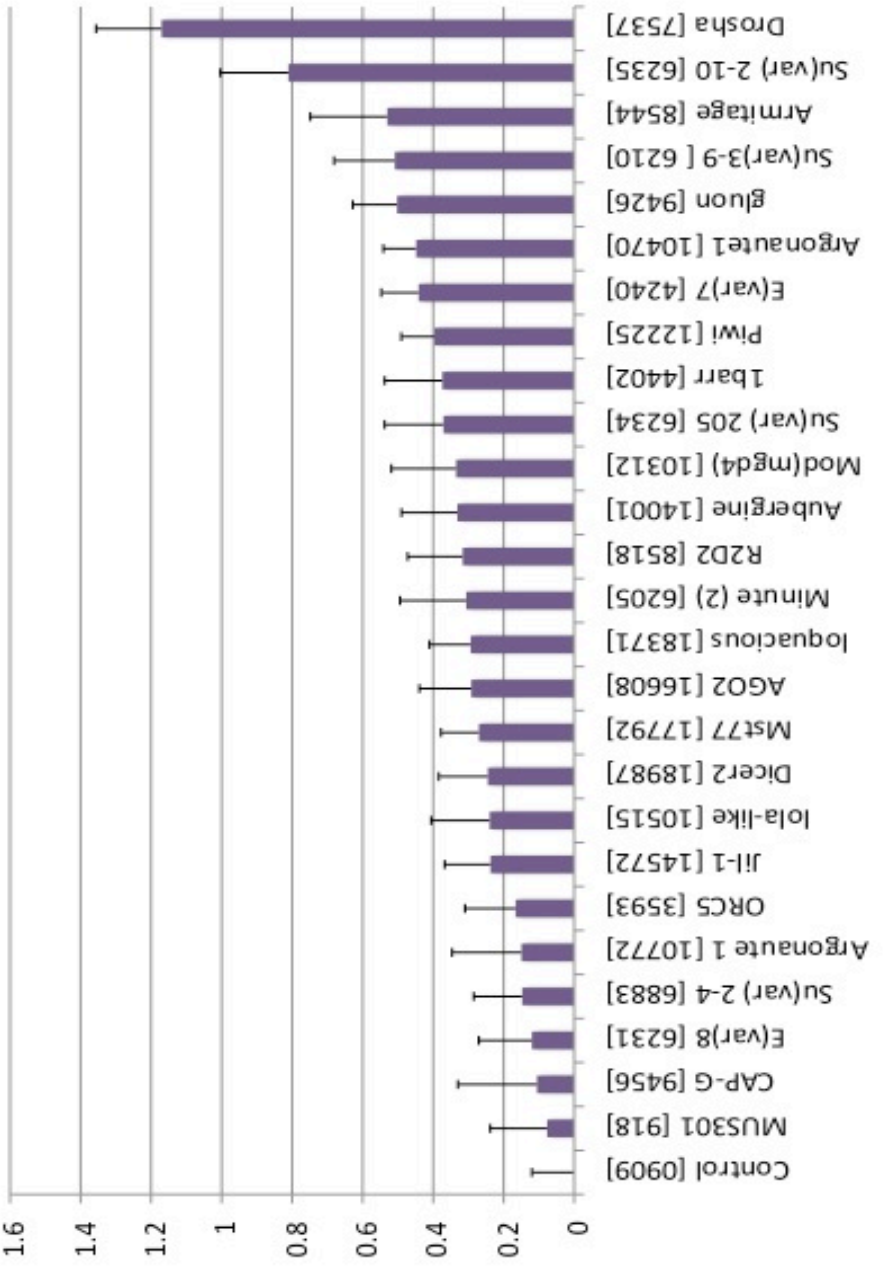


Figure 3.14 Modifiers of variegation affect the *rDNA* array size of two independent *Y* chromosomes. Plot displays the absolute value of the size difference of the array in the mutant background relative to the control for the two chromosomes tested. *Y*-axis is the ratio of *rDNA* amount relative to the control chromosome. *X*-axis shows twenty six mutations tested. Error bars are + standard deviations of the mean calculated from 5 individual flies measured.

CONCLUSION

Nothing is known about the role of the *rDNA* as a modulator of chromatin balance. However, evidence about the importance of maintaining a large number of *rDNA* copies in order to maintain genome integrity (145) and the positive correlation between genome size and *rDNA* copy number in many eukaryotes (76), suggests that the *rDNA* might have an additional important role besides ribosome biogenesis. In addition, the observed natural *rDNA* loss in several eukaryotes through development has suggested a role for this mechanism (95), but the functional advantage of this process remains unknown.

Here I present evidence that induced and spontaneous *rDNA* deletions have an effect on gene expression. In addition, the deletions affect nucleolus size and structure in a similar way as these changes are observed in aging and diseased cells. The effects on gene expression resemble the effects of mutations in heterochromatin components and the analysis of *rDNA* arrays in these mutant backgrounds revealed reduction in *rDNA* size. My data connects the *rDNA* deletions with the effects caused by heterochromatin components, which suggests that these proteins might mediate their effect in part through the *rDNA*. This points to a novel view of how some chromatin components could regulate the genome.

Together my data shows that the *rDNA* act as a modulator of chromatin, which suggests that the effect of this modulation could affect chromatin and gene expression across the entire genome.

CHAPTER IV

THE *rDNA* IS A POLYMORPHIC LOCUS THAT MODULATES GLOBAL GENE REGULATION

INTRODUCTION

The *rDNA* locus has a remarkable plasticity that allows it to be in constant lose and gain of cistron copies both meiotically and mitotically throughout development. In eukaryotes the size of the genome is positively correlated with the number of *rDNA* copies present and this number can oscillate from around 50 to 25,000 *rDNA* copies (76). This variability can also be observed within individuals of the same population and even within cells of the same tissue (Chapters II and III). A functional advantage for having the ability to increase and decrease in copy number has not yet been demonstrated, although it has been shown that having excessive cistron copy numbers (more than that required for sufficient rRNA in the cell) is beneficial for maintenance of genome integrity (145). In *Drosophila*, this characteristic of *rDNA* reduction and expansion could be responsible for the variability in *Y*-linked *rDNA* array size observed among thirty four different *D. melanogaster Y* chromosomes (77). Recently a similar study showed that polymorphisms on *Y* chromosomes from natural *D. melanogaster* populations have an effect on the regulation of gene expression of thousands of genes. The regions on the *Y* chromosome that have the effect on gene expression have not been mapped. However, because the *Y* chromosome has several sources of potential variation immersed within heterochromatic

blocks such as repetitive sequences and transposable elements (165), it was suggested that these factors could contribute to the observed effects (164). Here we asked if the PEV effects observed for our *rDNA* deletions ($YrDNA^{Def}$) have a broader impact on global gene expression and if the global gene expression changes linked to natural *Y* chromosome polymorphisms correlates with changes in *rDNA* array size. To address these questions we performed genome-wide gene expression analysis of some of our *rDNA* deleted lines, quantified the *rDNA* arrays from the natural isolates, and then perform a comparative analysis using the microarray expression data from these *Y* chromosomes.

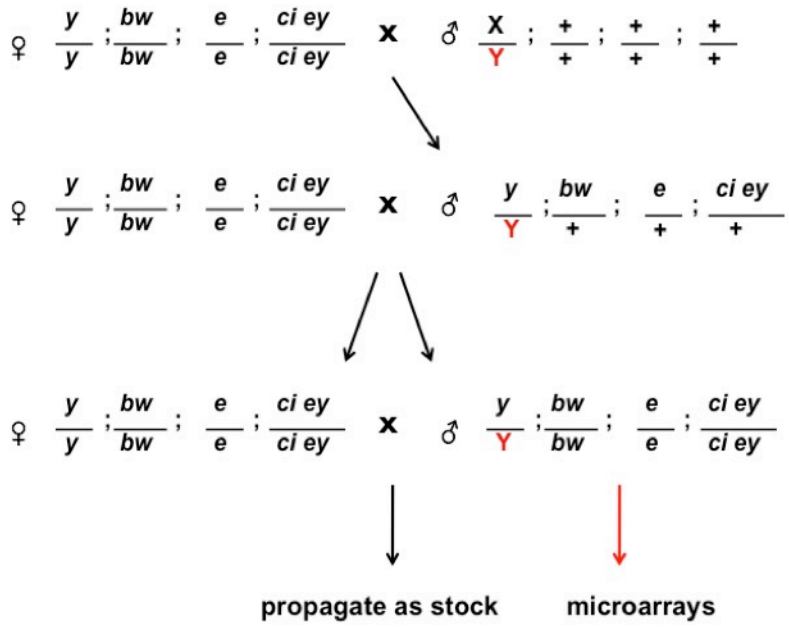
RESULTS AND DISCUSSION

Induced *rDNA* deletions affect global gene expression

In order to address the hypothesis that changes in *rDNA* copy number may modulate genome-wide gene expression we used three of our $YrDNA^{Def}$ chromosomes: two harboring mild deletions *bb*-0.87 and *bb*-0.85, one harboring a large deletion *l*-0.46 and the wild type control *Ywt* (Chapter II and III). Since we wanted to compared our results to the previous study on natural populations, the *Y* chromosomes were introgressed into an isogenic background of autosomes and *X* chromosome by repeated backcrossing of males $YrDNA^{Def}$ to females from the same fly stock that was previously used in the natural populations study (164) (Figure 4.1A). From this cross adult male flies were used as the source of RNA extraction for microarrays, which were done by

comparative hybridization of the three *YrDNA*^{Def} chromosomes to the *Ywt* and to each other (Figure 4.1B).

A.



B.

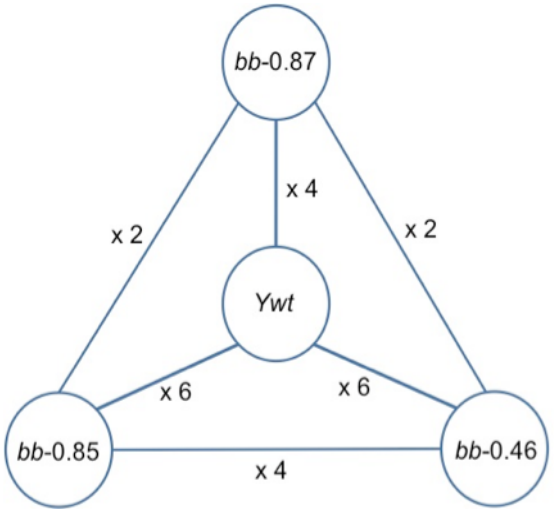


Figure 4.1 Microarray crosses and design. **A.** Crossing scheme to introgress *Y* chromosomes to a common and isogenic genetic background. *y* (*yellow*), *bw* (*brown*), *e* (*ebony*), *ci* (*cubitus interruptus*), *ey* (*eyeless*) were used as recessive genetic markers. **B.** Array design. Lines are direct comparisons and numbers are replicates per comparison.

We observed substantial gene expression variation among these strains as compared to the random expectation (obtained with Bayesian posterior probabilities) across a range of *P*-value thresholds (Figure 4.2).

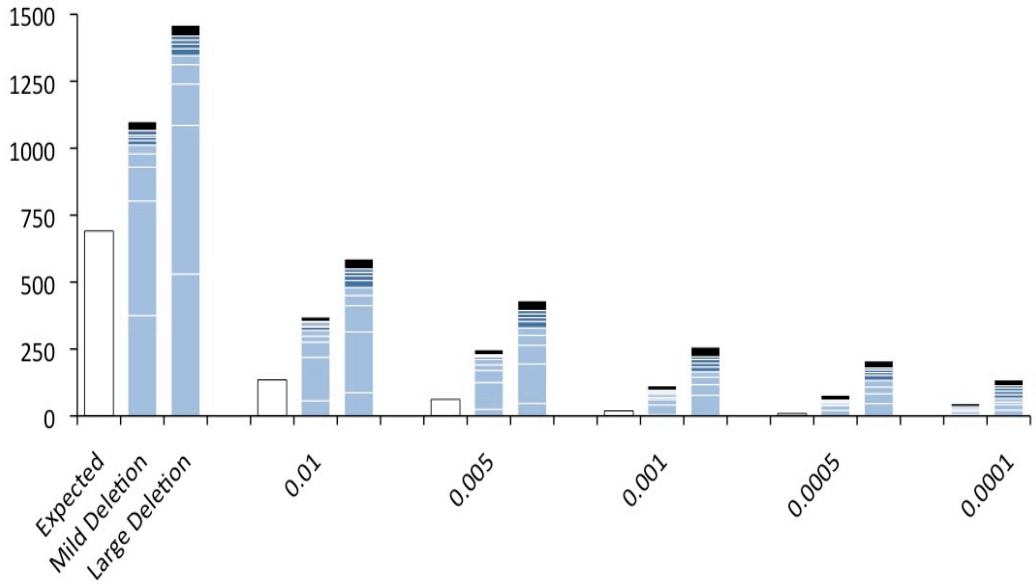


Figure 4.2. Number of genes differentially expressed for *Y* chromosomes bearing deletions within the ribosomal DNA. Y-axis is number of genes. Mild Deletion is averages of *bb*-0.87 and *bb*-0.85. Large Deletion is *l*-0.46. Data are given at $P < 0.05$ and other indicated Bayesian posterior probabilities. White bars are expected values. Numbers of differentially expressed genes are broke down into deciles of fold expression. Light blue indicate differentially expressed genes with changes less than 1.5-fold, dark blue indicate >1.5-fold and ≤2 -fold, black indicate changes greater than 2-fold.

According to our previous observations (Chapter III), we expected that the deletions in the *rDNA* array would result in gene expression modulation that would not only be replicated in each independently generated mutant, but would

also be more pronounced in mutants lacking a larger proportion of the original locus. In agreement with this expectation, the numbers of differentially expressed genes are positively correlated with the *rDNA* deletion size (Figure 4.2 and 4.3A).

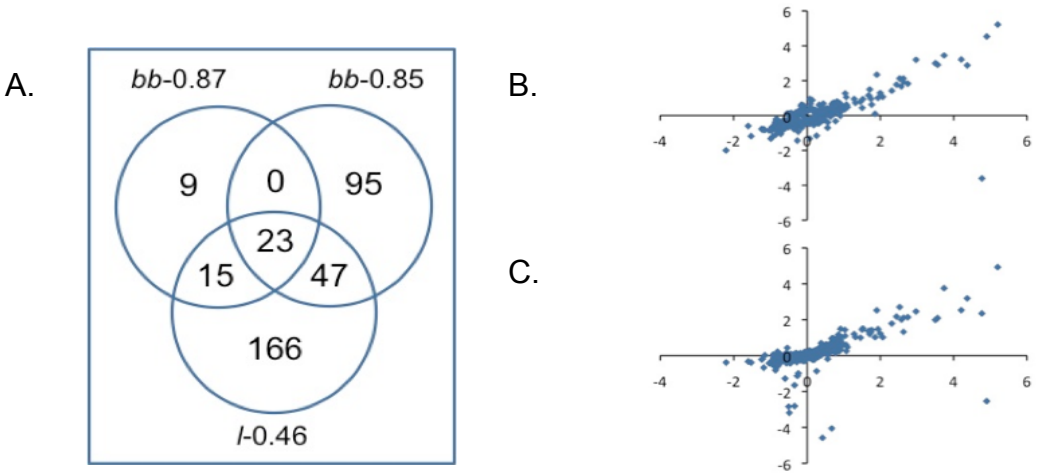


Figure 4.3 Overlapped genes among the *YrDNA*^{Def} chromosomes. **A.** Venn diagrams representing total number of genes expressed differentially relative to the wild-type chromosome, either uniquely differential or shared by multiple chromosomes (at $P < 0.001$, FDR < 0.05). **B.** Correlation of log-fold-changes comparing differentially expressed genes between *l*-0.46 and *Ywt* (X axis) to those differentially expressed between *bb*-0.87 and *Ywt* (Y axis), Rho = 0.84. **C.** Correlation of log-fold-changes comparing differentially expressed genes between *l*-0.46 and *Ywt* (X axis) to those differentially expressed between *bb*-0.85 (Y axis), Rho = 0.78.

We observed that the *Y* chromosome with the lowest *rDNA* copy number (*bb*-0.46) induced the highest number of expression changes, whereas the two *Y* chromosomes with mildly-deleted arrays resulted in smaller numbers of differentially expressed genes (Figures 4.2 and 4.3A). This finding

corroborates our previous observations that chromosomes with fewer *rDNA* copies have a stronger effect on position effect variegation (Chapter III).

Furthermore, the genes identified as differentially expressed in the mild deletions were a subset of those induced in the strain with largely deleted *rDNA* (Figure 4.3A). Accordingly, 44 - 59% ($P < 0.001$, FDR < 0.05) of the genes identified by mild deletions were also identified by the *Y* chromosome with the largest *rDNA* deletion. In support of the reproducibility of the gene expression modulation 24% of differentially expressed genes were shared by at least two chromosomes with reduced *rDNA* arrays, whereas fewer than 0.2% were to be expected by chance ($P < 0.001$). In addition, the direction and magnitudes of changes in expression were significantly correlated (correlation coefficient rho = 0.78 - 0.84, $P <$ 10E-16) between *Y* chromosomes harboring *rDNA* deletions (Figure 4.3B,C). These data unequivocally establish the relevance of *rDNA* copy number variation to modulation of genome-wide gene expression.

Since natural polymorphisms and induced deletions of the *rDNA* show no overt dominant phenotype (77), and even *X0* males, other than being sterile due to the loss of *Y*-linked fertility genes, are phenotypically normal despite having no *Y*-linked *rDNA* (166), we expected that induced *rDNA* deletion would have impacts on individual gene expression that were generally small. Indeed, we found that 85.1% of genes whose expression differed significantly from wild-type had changes in expression level of no more than 50% (Figure 4.4).

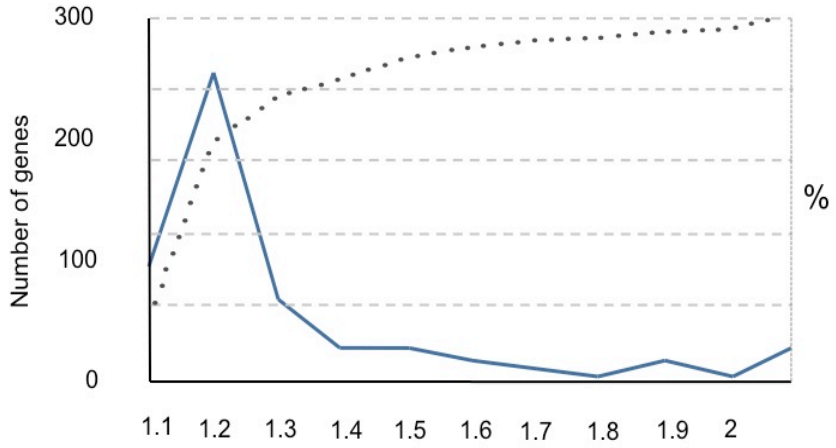


Figure 4.4 Number of differentially expressed genes at *P* < 0.01 for large deleted chromosome from Figure 4.2. Number of genes presented as absolute counts (solid lines, left Y axis) and cumulative percentage (dotted lines at 20% increments, right Y axe). X axe indicates deciles of gene expression fold change.

We found unexpected and unlikely that the number of genes showing only a 10% change in relative expression would be less than those showing a 20% change. This likely results in an underestimation in our count of the number of differentially expressed genes exhibiting the small changes. Using linear regression to extrapolate a corrected values for the first decile, we estimate that as many as 1200 - 1700 differentially expressed genes at P < 0.05 and 360 - 435 at P < 0.01 might escape statistical detection despite our high level of replication (Figure 4.5A-C). These estimates suggest that as much as 45% of the genome might be subtly affected by partial *rDNA* deletion, supporting the hypothesis that the *rDNA* is a global genome modulator.

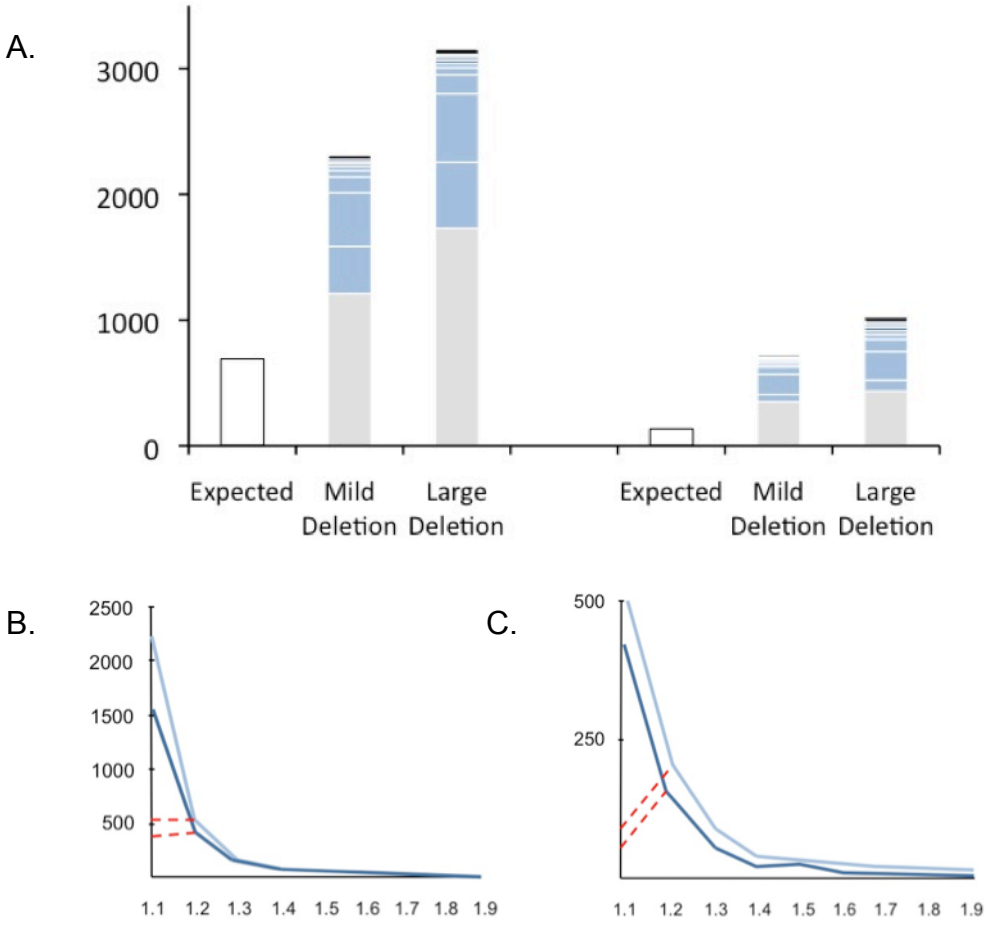


Figure 4.5 Estimation of the number of differentially expressed genes expected at ≤1.1 fold. **A.** Number of differentially expressed genes (data from Figure 4.2) with estimated number of genes whose expression was modulated by less than 10% and missed due to limited statistical power (grey). Data were generated from linear regression of subsequent five deciles. Projections are shown for $P < 0.05$ and $P < 0.01$. **B.** Data from A graphed as separate deciles (X axis) to show quality of estimation for $P < 0.05$. Red lines indicate first decile without extrapolation for comparison. **C.** Data from A graphed as separate deciles (X axis) to show quality of estimation for $P < 0.01$. Red lines indicate first decile without extrapolation for comparison.

Effects on gene expression are common to both sexes

To determine the generality of *rDNA* induced expression changes, we investigated the differential expression induced by the *Y* chromosome *rDNA*-deleted *l-0.46* in the *XXY* female genotype. We crossed males harboring either the *l*-0.46 or the *Ywt* chromosomes to females *XXY* and the unmated female progeny was used as the source of RNA extraction for the microarrays (Figure 4.6). We performed eight microarray replicates comparing the two mentioned chromosomes in the *XXY* background.

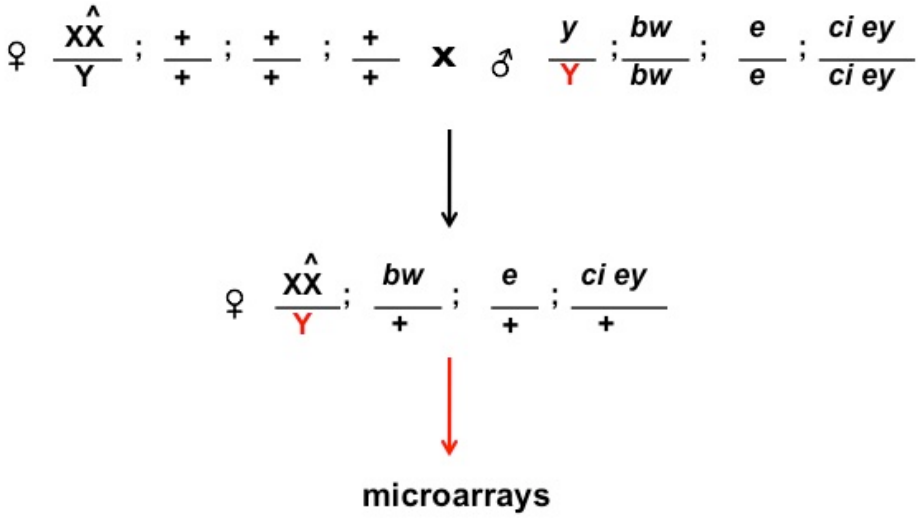


Figure 4.6 Crossing scheme to generate *XX*/*Y* aneuploid females. Females harbor a compound X chromosome which is represented by the ^ symbol linking the two X chromosomes. *y (yellow), bw (brown), e (ebony), ci (cubitus interruptus), ey (eyeless).*

We observed hundreds of gene expression differences between *XXY-0.46* and isogenic *XXY* females bearing a wild-type *Y* chromosome (*Ywt*). This

observation agrees with a recent study done by our collaborators (Bernardo Lemos and Dan Hartl) showing that natural isolated *Y* chromosomes affect gene expression in *XXY* females, even though in this genetic background *Y*-linked genes are not transcribed (167). However, the number of differentially expressed genes was about half in *XXY* females than in males (Figure 4.7). This could be because the additional heterochromatin provided by the extra *X* chromosome buffers the heterochromatin/euchromatin ratio so that gene expression is no longer as responsive to *rDNA* changes in the *Y* chromosome. Another possibility is that the presence of an extra *rDNA* array on the females could essentially partially complement for the loss on the *Y*-linked *rDNA*.

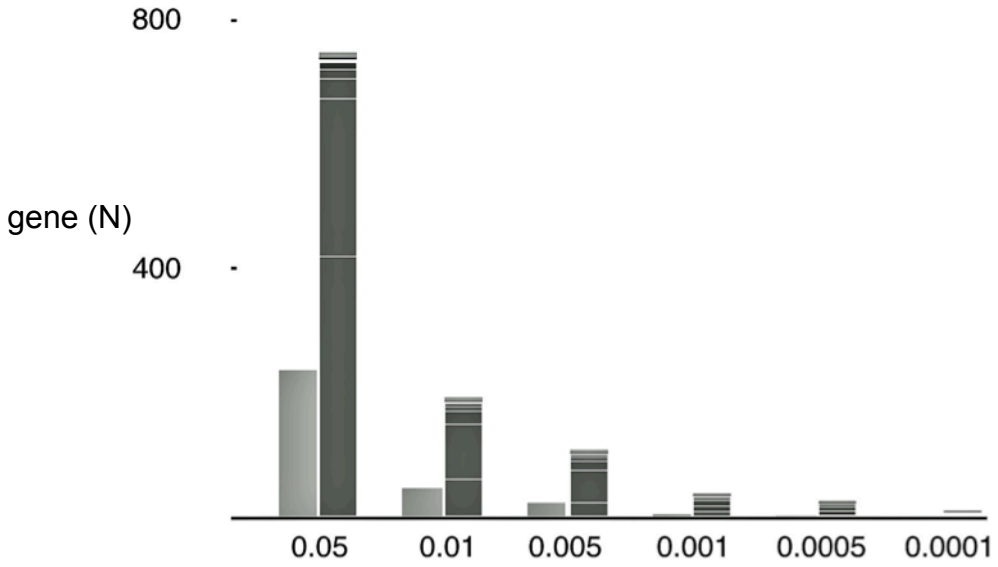
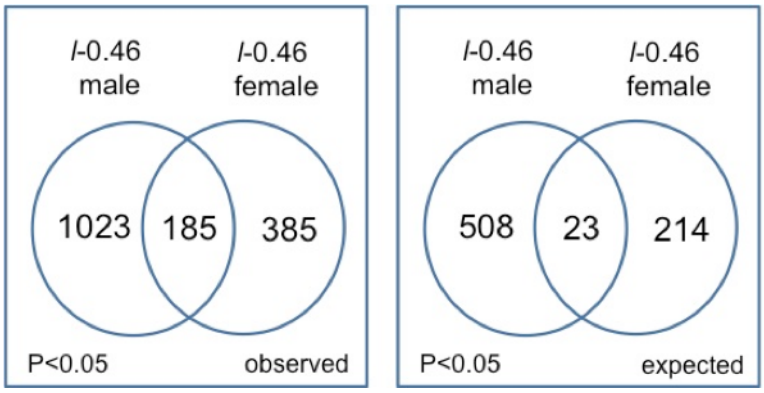


Figure 4.7 Differentially expressed genes in *XXY* females harboring the *I-0.46* chromosome (relative to *XXY* females harboring *Ywt*). Data is presented as in Figure 4.2.

Nevertheless, we observed a significant enrichment of differentially expressed genes shared between males and females harboring the same *Y* chromosome; at $P < 0.05$, 185 genes were shared between the sexes whereas only 23 shared genes were expected by chance alone (Figure 4.8A). While the number of affected genes differed between the sexes, the magnitude of the effect of *rDNA* deletion in gene expression was similar with a significant correlation in fold-changes between the sexes (rho = 0.45, $P < 10E-16$) (Figure 4.8C) and a significant association between down-regulated genes (Figure 4.8B).

A.



B.

l - 0.46		female	
		up	down
male	up	30	37
	down	17	101

C.

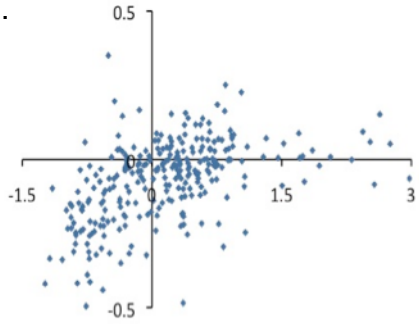
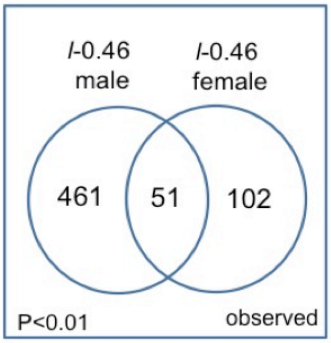
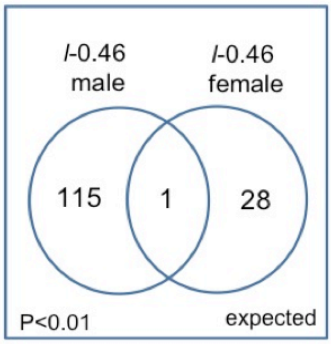


Figure 4.8 Differentially expressed genes overlapped between males and females. **A.** Venn diagrams representing number of differentially expressed genes unique or common to *X*/*Y l*-0.46 males and *XX*/*Y l*-0.46 females (at $P < 0.05$). Left, observed values. Right, expected by chance values. **B.** Breakdown of overlapping genes from observed values on A, separately categorizing genes whose expression was increased (up) or decreased (down) relative to the wild-type *Ywt* chromosome in the same genetic background. **C.** Correlation of log-fold-changes comparing differentially expressed genes between *l*-0.46 and *Ywt* in males (X axis) to those differentially expressed between *l*-0.46 and *Ywt* in females (Y axis); rho = 0.45.

A similar trend of up- and down-regulation and commonly shared genes remained across a range of *P*-values used for ascertaining differential expression (Figure 4.9). Together, our data show that *rDNA* copy number variation commonly affects male and female transcription and identifies a similar set of "*rDNA*-sensitive" genes. These data suggest that the response of a gene to *rDNA* deletion is an attribute of the gene structure and/or its regulation, rather than a sex-dependent effect.

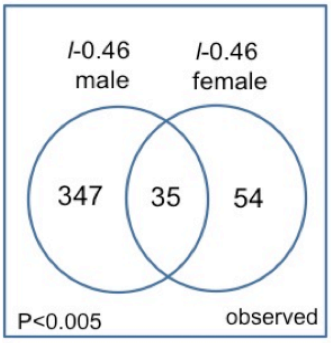
A.

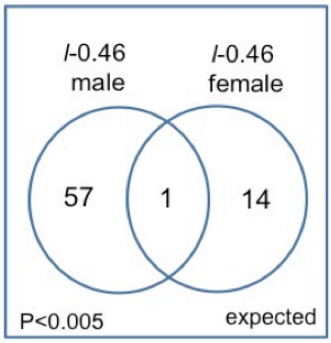




P< 0.01		female	
		up	down
male	up	3	6
	down	0	42

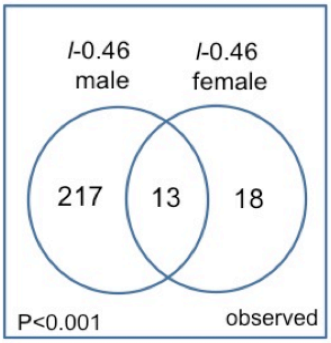
B.

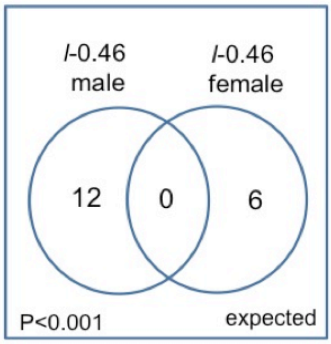




P<0.005		female	
		up	down
male	up	1	4
	down	0	30

C.





P<0.001		female	
		up	down
male	up	0	1
	down	0	12

Figure 4.9 Observed and expected data at different P values for overlapping genes between males and females. Data from Figure 4.8A,B at different P values. Values observed and expected by chance are shown. **A.** For $P < 0.01$. **B.** For $P < 0.005$. **C.** For $P < 0.001$.

Eukaryotic genomes posses a hundred to thousands of *rDNA* copies (76) and they have evolved mechanisms to restore the copy number in case of *rDNA* loss (i.e. *rDNA* magnification) (90). However, they maintain the number within a certain range as opposed to have unlimited expansion. Based on the hypothesis that an extra *rDNA* array is what causes the diluted effect in females, it is reasonable to suggest that the controlled expansion in copy number could be a mechanism to keep the number of *rDNA* copies within the range of sensibility that the cell requires to sense when there are changes in copy number and respond to it. It is possible then that the conserved mechanism of natural *rDNA* loss by extra chromosomal circle formation (95) has evolved to ensure that the cell will keep the number of *rDNA* copies at the size that is required for the specific tissue or developmental stage that it belongs to.

Effects on gene expression are genome wide and not biased

One mechanism for the wide-ranging effects of *rDNA* copy number on gene expression might arise if deletions of the *rDNA* compromised global heterochromatin structure and limited its spreading to euchromatin (168, 169). Indeed, this is suggested by our observation that four heterochromatin-induced variegating alleles were affected by *rDNA* deletion (Chapter III). To address the issue we tested two strong predictions of such a heterochromatin spreading model: 1) that genes residing in the proximity of heterochromatin will be more

strongly affected by *rDNA* copy number changes, and 2) that the majority of gene expression changes will be seen as increase in expression as repressive heterochromatin is reduced.

We therefore tested for a statistically significant enrichment of differentially expressed genes according to their cytological location. The cytological distribution of differentially expressed genes was visualized by plotting according to their genomic location by cytological band, the number of differentially expressed genes from all three *Y*-chromosomes (Figure 4.10A, black bars; Figure 4.10B, black line) and the number of analyzed genes from the microarray (Figure 4.10B, grey line and bars). No cytological band showed a significant enrichment of differentially expressed genes. Furthermore, in agreement with my previous observations of the effects on the *Stubble*^{V} gene (Chapter III), the effects were not biased towards any specific direction of regulation.

For instance, while we found that the number of differentially expressed genes drops near the cytological bands juxtaposed with centric heterochromatic blocks (bands: 20, 40/41, 80/81) (Figure 4.10A), this is indistinguishable from our expectation based on lower gene densities in these regions (Figure 4.10B).

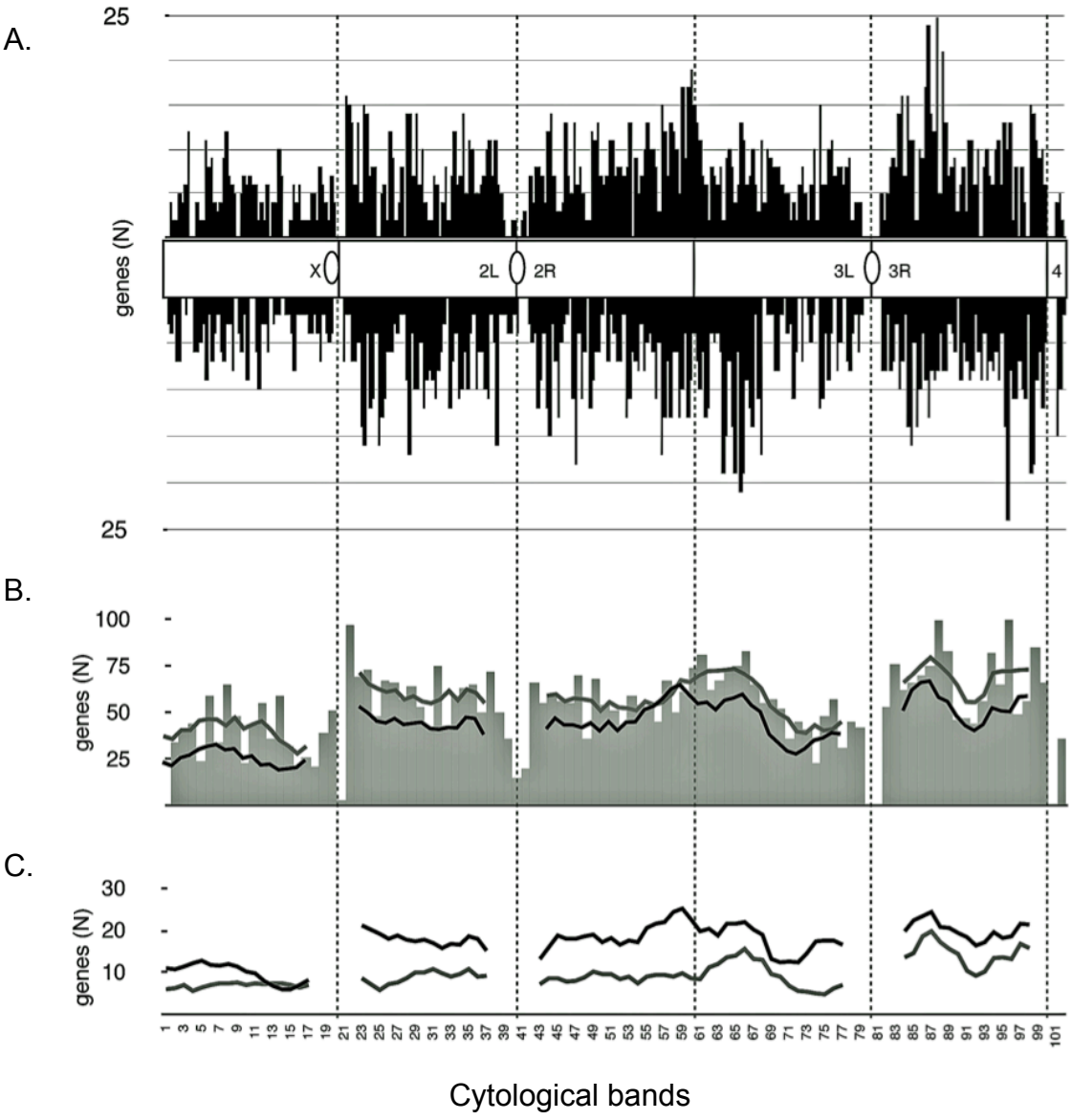

A.
25
genes (N)
X
2L
2R
3L
3R
4
25
B.
100
75
50
25
genes (N)
C.
30
20
10
genes (N)
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59 61 63 65 67 69 71 73 75 77 79 81 83 85 87 89 91 93 95 97 99 101
Cytological bands

Figure 4.10 Dispersion of differentially expressed genes on the genome. **A.** Number of differentially expressed genes either up-regulated (above stylized chromosome map) or down-regulated (below chromosome map) as a function of cytological location. Each cytological division shows grouped data for each of the three *Y* chromosomes (*bb*-0.87, *bb*-0.85, and *l*-0.46) relative to the wild-type *Ywt* at $P < 0.05$. Map represents euchromatic regions of the genome and location of centric heterochromatin (ovals). **B.** Distribution of microarray spots yielding usable data for this study (gray bars) with scanning 5-division average (upper lines, gaps are intended to avoid centromeric regions). Overlaid scanning 5-division average (lower lines) of data from all *rDNA* deletion chromosomes (taken from A) are compared at same scale. **C.** Scanning 5-division average of number of differentially expressed genes from males (upper lines) and females (lower lines) bearing *l*-0.46, relative to individuals bearing the wild-type *Ywt* chromosome (at $P < 0.05$). Cytological divisions are aligned across entire figure (dotted vertical lines).

Furthermore, we could discern no general trend for genes near the telomeres (Bands: 1, 21, and 60/61). Finally, while loci on the heterochromatin rich fourth chromosome and *X* chromosome were less affected than were chromosome *2*- and *3*-linked genes (40 - 68% the frequency, Figure 4.10B, black lines), these trends are also indistinguishable from that expected given the distribution of genes analyzed (Figure 4.10B, gray bars and gray lines). This analysis indicated that the genes affected by *rDNA* deletion are evenly distributed in the genome, and are neither influenced by proximity to heterochromatin nor chromosome linkage. There was also no preponderant increase or decrease of differential expression.

Corroborating our conclusion that *rDNA* deletion affects the same set of genes in males and females, we saw that the distribution of affected genes in males and females was coincident (Figure 4.10C). Taken together these results argue against a simple heterochromatin spreading model but instead suggest that loci with sensitivity to *rDNA* copy number variation are scattered through the genome.

One aspect to consider here is that since we used whole flies for RNA extraction for this analysis, the data obtained represents averaged gene expression profiles from several types of cells. It is known that different tissues exhibit unique gene expression profiles conferred by different chromatin structures (152, 153), hence the effect of the *rDNA* deletions on gene expression can vary according to the type of tissue analyzed. Thus, it is reasonable to

hypothesize that if we could obtain microarray expression profiles from small pieces of tissue, for instance just a piece of brain, the outcome would possibly be differential gene expression biased towards a specific direction of expression, and perhaps also exhibit chromosomal clustering of the differentially expressed genes. Since gene expression changes in a specific cell type are known to occur in diseases like cancer (170), the analysis of expression profiles in a tissue specific manner would reveal if there are cell types that are more tolerant to changes in the *rDNA* copy number, which could be useful for therapeutic applications.

rDNA **variation greatly contributes to the *Y* chromosome natural polymorphisms that cause differential gene expression**

An intriguing possibility is that polymorphisms of the *rDNA* copy number in naturally occurring *Y* chromosomes could account in part for the differential genome-wide modulation of gene expression exerted by these chromosomes. One indication that such *rDNA*-driven *Y*-linked variation is relevant came from categorizing the differentially expressed genes by Gene Ontogeny (GO) category. These analyses pointed to five categories that overlapped with those discovered by comparing differential gene expression due to natural *Y* chromosome polymorphisms (Table 4.1) (164). Interestingly, we found that two of these categories are linked to energy metabolism: mitochondrial membrane and electron transport, for both males and females. It is known that ribosome

production is a high energy consuming cellular activity. Hence, ribosome biosynthesis must rapidly adapt to changes in intracellular energy status. Balancing the intracellular energy is critical for cell survival, indeed energy metabolism has been implicated in aging and aging-related diseases (171). Therefore, it is important to understand the alterations in mitochondrial energy metabolism. Aging studies in *C. elegans*, *Drosophila*, mice and humans have identified misregulation of genes involved in ATP synthesis and mitochondrial respiration but it is not known if the changes in gene expression are a cause or consequence of the aging process (172, 173). Finding enrichment in these two groups of genes suggests that *rDNA* copy number could influence the regulation of expression of genes involved in energy metabolism. Aging studies of flies carrying the $YrDNA^{Def}$ chromosomes could help to discern whether the expression of those genes is a cause or a consequence of the aging process.

These data suggest that *rDNA* copy number itself might play a central role regulating energy metabolism through modulation of gene expression.

An additional hypothesis from these data is the following: our data showed that alterations in *rDNA* copy number cause differential expression of almost half of the genome. These changes in gene expression could be interpreted by the cell as a signal of stress, which could trigger the apoptosis pathways. It is known that stress-induced apoptosis is caused by a perturbation of the mitochondrial membrane (174). The mechanism of the membrane perturbation is unknown, but it is known that after the perturbation occurs

several pro-apoptotic proteins and cytochromes are released from the mitochrondria to the cytosol. The release of these proteins leads to the activation of the caspase pathway that directs the cell to apoptosis (175). We do not observe a phenotype in the male flies that were used for microarray analysis. Thus, if the gene expression alterations caused by the *rDNA* deletions are signaling the apoptotic pathway, there must also be a response mechanism counteracting this pathway. I hypothesize that the enrichment in differentially expressed genes involved in mitochondrial membrane and electron transport could be due to the counteracting response mechanism, perhaps by preventing mitochondrial membrane perturbation. Consistent with this hypothesis, I also found that several genes involved in proteolysis were down regulated; some of them were members of caspase pathways and apoptosis regulators (data not shown). Disruption of apoptosis is known to be another marker of cancerous cells (175). Together this data with the additional observations that are common between cancerous cells and *rDNA* deletions, such as increased rRNA transcription and changes in nucleolus structure (99), suggest that changes in *rDNA* copy number could be a major determinant in the change of cellular reprogramming that leads to tumorogenesis.

Category	Description	N	P-value
GO:0031966	mitochondrial membrane	46	2.1E-10
GO:0006118	electron transport	44	2.3E-05
GO:0016298	lipase activity	16	1.1E-03
GO:0006629	lipid metabolic process	53	1.4E-03
GO:0006631	fatty acid metabolism	9	1.8E-02
GO:0031966	mitochondrial membrane	38	7.2E-12
GO:0006118	electron transport	38	1.4E-09

Table 4.1 Gene Ontogeny (GO) categories for which differential gene expression was significantly enriched. These categories overlap with those observed in the *Y* natural isolated chromosomes study. Blue cells display categories for males. Red cells display categories for females.

Finally, to address the relevance of *rDNA* copy number variation in natural populations directly, we integrated data from gene expression variation due to natural *Y*-linked polymorphisms and our induced changes in *rDNA* copy number (*YrDNA*^{Def}). We found a striking level of overlap in the identity of differentially expressed genes compared to the level expected by chance in pair wise comparisons (Figure 4.11), as well as when comparing the total number of differentially expressed genes between the natural isolated *Y* chromosomes and our *YrDNA*^{Def} chromosomes (Figure 4.12).

A.

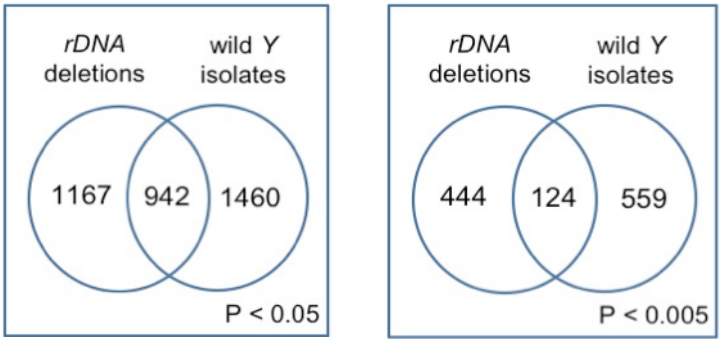
P<0.05 white cells P<0.005 grey cells Sample sizes on diagonal	*YCongo - YZimb.*	*YZimb. - YOhio*	*YOhio - YCongo*	*bb-0.87 – Ywt*	*bb-0.85 – Ywt*	*l-0.46 – Ywt*
l-0.46 – Ywt	263	323	220	278	441	1165/335
bb-0.85 – Ywt	389	343	300	211	1163/307	100
bb-0.87 – Ywt	122	130	122	574/96	42	63
YOhio - YCongo	700	397	1272/240	6	26	13
YZimb. - YOhio	473	1233/321	36	10	61	59
YCongo - YZimb.	1345/346	90	104	8	49	28

B.

P<0.05 white cells P<0.005 grey cells Sample sizes on diagonal Expected values	*YCongo - YZimb.*	*YZimb. - YOhio*	*YOhio - YCongo*	*bb-0.87 – Ywt*	*bb-0.85 – Ywt*	*l-0.46 – Ywt*
l-0.46 – Ywt	50	44	53	110	150	568/57
bb-0.85 – Ywt	41	42	53	109	572/43	9
bb-0.87 – Ywt	56	44	56	532/45	7	8
YOhio - YCongo	253	117	554/53	0	3	1
YZimb. - YOhio	114	573/53	7	0	0	1
YCongo - YZimb.	543/46	5	14	0	0	1

Figure 4.11. Pair wise overlaps of differentially expressed genes among *YrDNA*^{Def} chromosomes and *Y* natural isolates. **A.** Observed number of shared genes differentially expressed across pairwise chromosome comparisons. $P < 0.05$ data are shown above the diagonal (white cells), $P < 0.005$ are shown below the diagonal (grey cells), and total number (shared plus unique) of differentially expressed genes ($P < 0.05$ / $P < 0.005$) are shown on the diagonal (blue cells). **B.** Expected numbers for A calculated from randomized datasets. Data presented as in A.

A.



B.

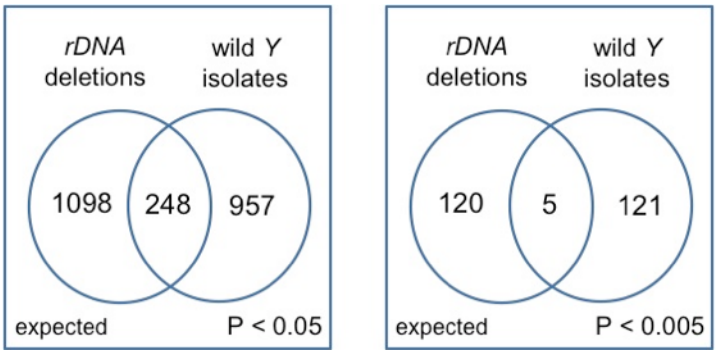


Figure 4.12 Overlapped genes between the two groups of *Y* chromosomes. **A.** Venn diagrams representing shared and unique differentially expressed genes encompassing the three *YrDNA*^{Def} chromosomes (*rDNA* deletions) relative to the wild-type *Ywt* and the three natural isolated chromosomes (wild *Y* isolates) at $P < 0.05$ and $P < 0.005$. **B.** Expected overlapping and differentially expressed genes for the data on A.

Using real-time PCR we confirmed that the natural *Y* chromosomes possessed polymorphisms in *rDNA* copy number, the range of which included the two more mild *rDNA* deletions used in this study: *bb*-0.85 and *bb*-0.87 (Figure 4.13A). Moreover, the effect of naturally occurring *Y* chromosomes from *Y*Ohio and *Y*Zimb on PEV are consistent with effects seen with our induced *rDNA* deletions, which showed that small *rDNA* arrays act as Suppressors of PEV (Figure 4.13B) (Chapter III) (167).

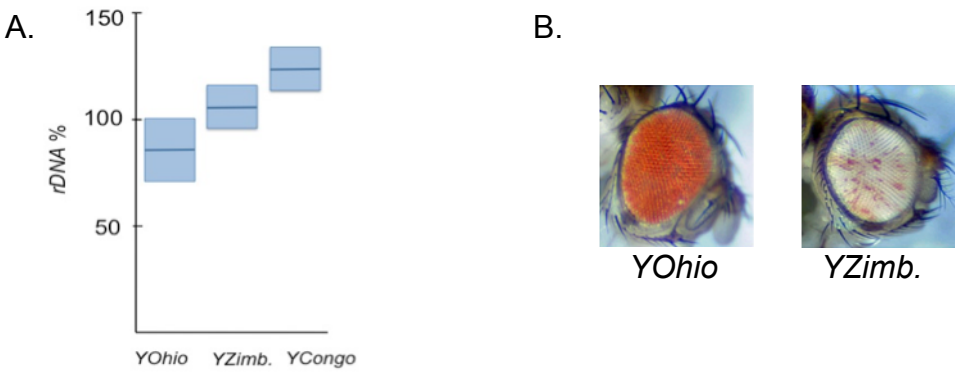
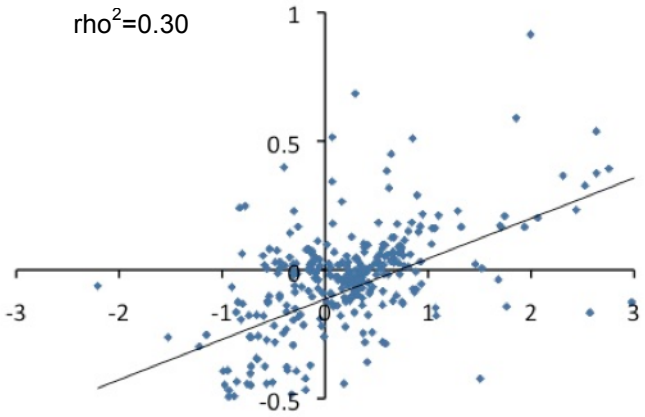


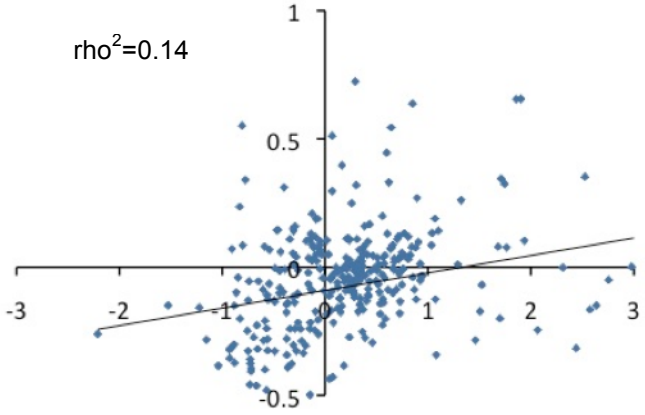
Figure 4.13 *rDNA* array size for the natural isolated *Y* chromosomes. **A.** Copy numbers of *rDNA* arrays for natural *Y* chromosomes used in this study. Copy numbers are reported as a percentage of the *Ywt* chromosome. Plots show average ± 1 S.D. **B.** Pictures of eyes from males that harbor the *In(w^{m4h})* chromosome, where the origin of the *Y* chromosome is the only source of genetic variation. Pictures taken from Lemos *et. al.* 2010 (167).

Finally, fold-changes estimated between natural *Y* chromosome comparisons and induced *rDNA* deletion chromosome relative to the wild-type chromosome are significantly correlated (rho = 0.25 - 0.55, $P < 10E-12$). Since the square of the correlation coefficient rho (ρ^2) estimates the fraction of the variance in the Y-axis that is explained by the X-axis in a linear regression analysis (176), our analyses indicate that approximately 5 - 30% of gene expression variation detected on natural *Y* chromosomes might be due to polymorphisms in the *rDNA* alone (Figure 4.14).

A.


rho^2=0.30
1
0.5
0
-0.5
-3
-2
-1
0
1
2
3

B.


rho^2=0.14
1
0.5
0
-0.5
-3
-2
-1
0
1
2
3

C.

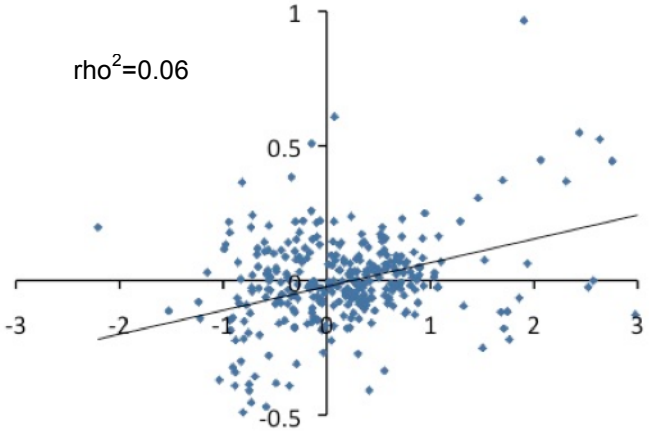

rho^2=0.06
1
0.5
0
-0.5
-3
-2
-1
0
1
2
3

Figure 4.14 Estimated contribution of *rDNA* deletions to the differential gene expression conferred by the *Y* chromosome in natural populations. **A.** Correlation of log-fold-changes comparing differentially expressed genes between *I*-0.46 and *Ywt* (X axis) to those differentially expressed between *YZimb*. and *YOhio* (Y axis); rho = 0.55, *P* < 10E-16. **B.** Correlation of log-fold-changes for *I*-0.46 versus *Ywt* (X axis) compared to *YCongo* versus *YZimb* (Y axis); rho = 0.38, *P* < 10E-12. **C.** Correlation of log-fold-changes for *I*-0.46 versus *Ywt* (X axis) compared to *YCongo* versus *YOhio* (Y axis); rho = 0.25, *P* < 10E-6.

An aspect to consider here is that even though the two analyses were done in the same genetic background, the *Y* chromosomes that were compared (from natural isolates and $YrDNA^{Def}$) are different. This means that they can vary in several regions throughout the chromosomes (i.e. satellite repeats, transposable elements, and other heterochromatic blocks). This suggests that the estimated 5-30% contribution of the *rDNA* to the differential gene expression observed in natural isolates might come as well from the variation in these sequences. However, the absence of tools to investigate these other polymorphic regions on the *Y* chromosome impedes our ability to further differentiate the individual contributions of these regions.

The ability to modulate the genome represents an important feature to thrive in hostile environments. Our data suggests that the differential gene expression induced by *Y* chromosomes isolated from natural populations is caused in part by alterations in *rDNA* array size. An interesting observation suggesting that the changes in gene expression caused by alterations in *rDNA* array size are important for the adaptation to environmental changes came from a Gene Ontology (GO) analysis that I performed on the group of genes that were differentially expressed in response to both the *Y* natural isolates and my $YrDNA^{Def}$ chromosomes (data not shown). This analysis revealed that most of the statistically significant enriched genes that are common for the two groups analyzed, are involved in immune defense processes against pathogens such as: antibacterial humoral response, humoral immune response, response to

other organism, antimicrobial humoral response, etc. In *Drosophila*, it is known that the interaction between host and parasites initiates an immune response that is characterized by specific gene expression patterns. While these genes responding to the parasite are characterized, the genes responsible for the initiation of the response are poorly understood. Importantly this initiating response, like the *rDNA* arrays, varies both within and among populations (177). Therefore, we can hypothesize that variations in *rDNA* array size could mediate the adjustment of the genome in response to differences in natural environments such as presence and diversity of pathogens, either directly by inducing changes in the expression of immune response genes or indirectly by inducing changes in the genes that initiate the immune response.

The observed effects on gene expression are probably not unique to *Drosophila*, as phenotypic changes in response to environmental alterations are known to happen in plants such as flax and *Arabidopsis*, which have been attributed to genome alterations that map to the *rDNA* (171, 178). In *Arabidopsis* differences in methylation of the *45S rDNA* gene have been observed in different natural isolates. In flax, environmental changes generate stable phenotypic alterations that mapped to changes in the *rDNA* array size principally. In addition, variation in *45S* and *5S rDNA* arrays have been observed among healthy humans (157), and a positive correlation between *rDNA* copy number and genome size has been found in different species of plants and animals (76), suggesting that the variation in *rDNA* size could be mediating changes in gene

expression in several organisms. Together these suggest that changes in *rDNA* copy number are a common characteristic of the eukaryotic genome, which influences gene expression and has possibly evolved to modulate the genome in response to environmental changes for the adaptation and survival.

In addition, Lyckegaard and Clark showed that there is variation among natural isolated *Y* chromosomes in the *rDNA* arrays and also in another repeated array, the *Suppressor of Stellate* (77). This suggests that changes in repeated sequences other than the *rDNA* could also influence gene expression. I have developed the tools to study the *rDNA* array, but it would be also interesting to develop a strategy to manipulate other repetitive sequences on the genome in order to determine whether they have a role and to what degree they contribute to the differential gene expression observed in natural populations.

CONCLUSION

We had previously shown that the *rDNA* has an effect on chromatin balance, which influences gene expression. Our collaborators had shown that polymorphisms in natural isolated *Y* chromosomes induce differential gene expression of thousands of genes. However, two important questions were left unanswered from these two observations: 1) to what extent does the *rDNA* affect gene expression in the genome?, and 2) which are the polymorphic regions in the natural isolated *Y* chromosomes responsible for the effects on gene expression?.

Here I report data that shows that the effects of *rDNA* deletions in gene expression occur globally in about half of the genome, without clustering or bias ib the direction of change. In addition, these effects are not sex specific, as they can be observed in males and females harboring an *rDNA* deletion. Furthermore, by comparing our analysis with the previous analysis on natural isolated *Y* chromosomes, we found that the *rDNA* contributes ~5-30% to the differential gene expression caused by the natural isolated *Y* chromosomes.

Together this data shows that the *rDNA* is a modulator of global gene expression. This modulation might have important implications such as adaptability of the species to environmental changes and evolution. Since there are other repetitive sequences present in the *Y* chromosome (i.e. S*uppressor of Stellate,* satellite repeats), these sequences might represent part of the unknown 70% contribution to the differential gene expression observed in natural populations.

This chapter was co written with Keith Maggert and Bernardo Lemos.

CHAPTER V

SUMMARY AND DISCUSSION

SUMMARY

I developed a genetic strategy to specifically remove *rDNA* cistron copies by the induction of the *I-Cre*I endonuclease and to molecularly estimate the *rDNA* cistron copy number by Quantitative Real-Time PCR. The strategy allows one to obtain graded deletions; hence I created an allelic series of twenty-five *rDNA* deletions. I found that deletions of the *rDNA* magnify an average of fifteen cistron copies per generation and that *I-Cre*I-induced double-stranded breaks can cause magnification (Chapter II). I tested the effect of these *rDNA* deletions on gene expression using different reporter genes that are localized in chromosomal rearrangements that exhibit Position Effect Variegation (PEV). I observed that the *rDNA* deletions strongly modify the expression of the reporter genes and that the size of the *rDNA* array is inversely correlated with the effect on gene expression. Consistent with these results, I found that spontaneously occurring *rDNA* deletions have the same effect on gene expression. Furthermore, I found that *rDNA* deletions affect the nucleolus size and structure (Chapter III). With our collaborators, we tested the extent of these effects on gene expression by performing microarray hybridization analysis. We found that *rDNA* deletions affect the expression of about half the genes on the genome, that this effect is subtle (mostly < 1.5 fold expression), and that the affected genes do not cluster in any particular region of the genome. In addition, the

effect can be observed in both sexes; as we detect a set of "*rDNA* sensitive" genes that change regardless of the sex of the individuals analyzed. In addition, we compared previous data of differential gene expression caused by *Y* chromosomes isolated from natural environments with the data from the *Y* chromosomes with induced *rDNA* deletions. We found that there is a significant overlap within the sets of differentially expressed genes from both studies, suggesting that the *rDNA* is a major contributor to the gene expression differences caused by *Y* chromosomes isolated from natural environments (Chapter IV). Together the data I presented here show a previously undescribed role of the *rDNA* as a major regulator of gene expression, which has important implications for different areas of biology.

DISCUSSION

For many years heterochromatin was underestimated, misunderstood and oversimplified. It was considered as "junk" DNA because of its enriched composition in repetitive sequences and transposable elements (179). This erroneous view changed when important characteristics were attributed to it, such as the presence of essential protein coding genes and crucial chromosome functions like: centromeres, telomeres and meiotic chromosome pairing (12, 180). Through genetic and biochemical approaches several characteristics of the heterochromatin composition have been discovered, such as histone modifications and associated non-histone proteins. Furthermore, most of the

heterochromatin related proteins are dose sensitive, which means that they are maintained at specific levels. This suggests that the balance between heterochromatin and euchromatin is very sensitive, as it can be altered just by increasing or decreasing the dosage of a single protein such as HP1, histone methyltransferases, histone deacetylases, histones acetyltransferases, etc. In natural environments, organisms are constantly exposed to short and long term changes that can have an impact on gene expression such as nutrient availability, radiation, temperature, light, chemicals and others (52, 181). Adaptation to environmental changes requires the fine tuning of gene expression in order to survive (182). Hence it is reasonable to think that there could be conserved mechanisms that are able to fine-tune gene expression in order to find a proper balance that adapts the organism to these changes.

The Model

The model I present here suggests that the *X* and *Y rDNA* arrays keep the balance between heterochromatin and euchromatin by maintaining the array size within a range that allows the coexistence of active and inactive copies, which I called "*rDNA* equilibrium" (Figure 5.1). Mutations in heterochromatin components that affect nucleolar structure (119) or spontaneous deletions to the *rDNA* array cause alterations in the *rDNA* equilibrium by forcing the inactive copies to become active. This forced activation leads to chromatin imbalance by displacing heterochromatin components, which can be redistributed in the

genome by binding to other sequences (Figure 5.1). The silencing factors are released to find new targets in the genome causing activation and repression. Some sequences might be more susceptible to recruitment of the newly released heterochromatic factors making them "*rDNA* sensitive genes".

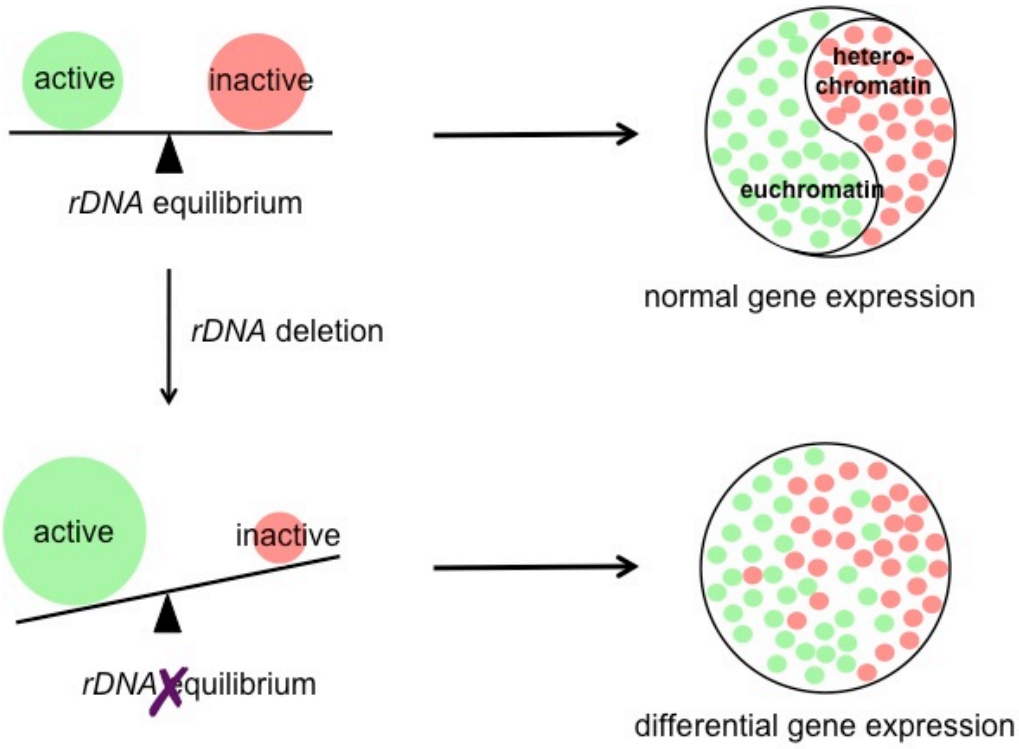


Figure 5.1 Model for *rDNA*-mediated chromatin balancing. The *rDNA* maintains an equilibrium by having certain ratio of copies epigenetically silenced / active copies. This equilibrium helps to maintain the global balance of euchromatin / heterochromatin. Deletions to the *rDNA* drive the activation of silent copies, which disrupt the equilibrium and as a consequence of this, the chromatin balance is changed.

Thus it is reasonable to hypothesize that the cell could balance the system by getting rid of any block of heterochromatin that is not essential (e.g. that contains satellite repeats, transposable elements). However, instead by using an essential sequence the system guarantees the stability of the genome by ensuring a lower limit to the copy number. In addition, because of the repetitive nature, the ability to magnify and the multiple levels of transcriptional regulation, the *rDNA* is a convenient locus to use for this purpose. Furthermore there might be more repetitive sequences that contribute to this regulation and possibly serve as backup mechanisms (i.e. *Suppressor of Stellate*, satellite sequences).

This provides an explanation for why the *rDNA* arrays have a much higher copy number than what the cell requires to survive, and supports recent data suggesting the high copy number as a means to maintain genome integrity (145). Previous studies have suggested that deletions to the *X*-linked *rDNA* array act as enhancer of variegation (124, 125). The *X* and *Y*-linked *rDNA* arrays differ in some aspects (79) but both have shown to exert effects on gene expression. Hence, interplay between the two *rDNA* arrays could be a mechanism that modulates chromatin, an additional role for the *rDNA* that until now has been unseen.

Together my data suggest that the *rDNA* is a major regulator of global gene expression. Since chromatin structure is the main determinant of gene expression, the *rDNA* acts as an architect of this structure by balancing the

euchromatin/heterochromatin ratio. In *Drosophila* it is not known what factors are involved in silencing the *rDNA*, but it is known that the silencing of the *rDNA* can be altered by changes in classical heterochromatin components and modifiers (119). The silent copies of the *rDNA* are a recruiting point for heterochromatin components such as HP1 and Sir2. In addition to the *rDNA*, these two proteins are known act on other heterochromatic and euchromatic regions to mediate heterochromatin silencing, euchromatic gene silencing. HP1 is also known to mediate euchromatic gene activation (162, 183, 184). These and several other chromatin modifiers are present in the nucleus in a specific dosage, such that an increase or decrease yields a phenotype (52). It has been shown in budding yeast that delocalization of SIR factors from the telomeres causes its redistribution over all chromosomes producing misregulation of gene expression (185). Hence, it is reasonable to hypothesize that as a consequence of the induced activation of the silent copies on the *X*-linked *rDNA* array, the displacement of heterochromatin components away from the *rDNA* could lead to redistribution of these factors on all the chromosomes producing both gene activation and silencing.

In addition, there is evidence that suggests RNAi as a regulator of another multiple tandem array in *Drosophila* called the *Stellate* gene (186). Since the rRNA is the most abundant in the cell (75) it is challenging to find small RNAs that could be involved in the silencing of the repeats, since it could be interpreted as degradation products. Components of the RNAi pathway

contribute to the stability of the nucleolus, suggesting that this pathway could be mediating the silencing of the *rDNA* inactive copies (119). Consistent with this finding, I detected alterations in the array sizes of two *Y*-linked *rDNA* arrays of different origin, after they were introduced to mutants for several components of the RNAi pathway such as Piwi, Ago1, Ago2, Armitage, Drosha, loqs, Dicer2, etc (Chapter III). In mammals, binding of the NoRC subunit Tip5 to a noncoding RNA from the intergenic spacer is crucial for stabilization of the complex and heterochromatin formation. The noncoding RNA shares sequence identity with the 45S promoter, which could serve to direct the NoRC to the promoter for silencing (75). The roles of the RNAi silencing machinery in heterochromatin formation in *Drosophila* have not yet been elucidated to the same extent as in fission yeast, but it is reasonable to hypothesize that RNAi could be the mediator of silencing of the *rDNA* repeats and probably could mediate silencing of other genes in the genome by chromatin silencing and post transcriptional silencing when delocalized from the *rDNA* repeats. There is still so much to learn about this phenomenon to elucidate a plausible mechanism for the pathways through which the *rDNA* deletions alter global gene expression.

Copy number vs. rDNA transcription

My data showed a positive correlation between the size of the *rDNA* deletion and the magnitude of the effect on gene expression. However a question that still remains is whether the *rDNA* copy number causes the effects

on gene expression directly or indirectly. For instance, are the effects a consequence of the loss of *rDNA* copies or the transcriptional activation of the silent *rDNA* copies as response to the loss? Evidence that follows suggests that *rDNA* transcriptional activity correlates with gene expression changes. I found similar levels of rRNA in the lines that harbor the deletions compared to wild type (Chapter III). This indicates that to compensate for the deletion, there is either an increase in rRNA transcription per gene, activation of silent copies on the *X*-linked array or both. In addition, activation of these silent copies would be predicted to open the chromatin structure and promote the formation of extra chromosomal nucleolus, as observed for mutations in heterochromatin components (119) and as observed in my $YrDNA^{Def}$ lines (Chapter III). Conversely, when *rDNA* transcription was reduced by treatment with the RNA polymerase I inhibitor rapamycin, I observed enhancement of variegation of the w^{m4} allele, and an increase in the nucleolus volume (data not shown). Together this data suggest that the *rDNA* transcriptional activity determines the changes in gene expression. A similar interesting test would be to measure effects on gene expression when inducing the activation of the silent *rDNA* copies in a wild type array. Enhancement of variegation would be expected as would be predicted by this model.

In agreement with this hypothesis, additional evidence suggests that transcription of *rDNA* copies has an additional role to the cell other than rRNA production. For instance, a recent hypothesis has postulated a major role of the

rDNA copy number in maintenance of the genome integrity, suggesting that maintenance of a high copy number allows the genome to keep a subset of copies silenced. This in turn enables efficient replication-coupled recombination-DNA repair by allowing condensin to associate to the silent copies and mediate sister chromatid cohesion. In other words, activation of most of the *rDNA* copies at the same time is deleterious for the cell (145). Furthermore, it is known that the number of active *rDNA* copies varies between different cell types, which means that the fraction of *rDNA* genes that are actively transcribed changes during development and differentiation (149). This suggests that *rDNA* activity could mediate the differential gene expression that is observed in cell determination. In addition, Laferte *et. al.* showed that Pol I transcriptional activity induces the transcriptional activity of Pol II and Pol III (187, 188). How Pol I transcription affects Pol II and Pol III transcription remains elusive, but is it important to understand how this interplay occurs given that deregulation of ribosome biogenesis and subsequent changes in nucleolus structure and gene expression are associated with the alterations in cell cycle and cell growth that are linked to cancer and aging (155, 189). My data might help to understand the connection of the *rDNA* to some of these phenotypes.

The clonal nature of Position Effect Variegation

The clonal patches that exhibit PEV in the eye of a fly which harbors the *In(1)w*^{m4} rearrangement, occur due to the ability of the heterochromatin to

spread over the *white*$^{+}$ gene. It is known that inactivation of the *white*$^{+}$ gene is determined at the end of the first larval instar, and it is based on cell lineage when around 20 eye precursor cells are present (65). However, it is unknown what confers the ability of some cells to silence (Figure 5.2, black arrow) while other cells are unable to do so (Figure 5.2, white arrow). In other words, it is unknown what is the cause of somaclonal variation.

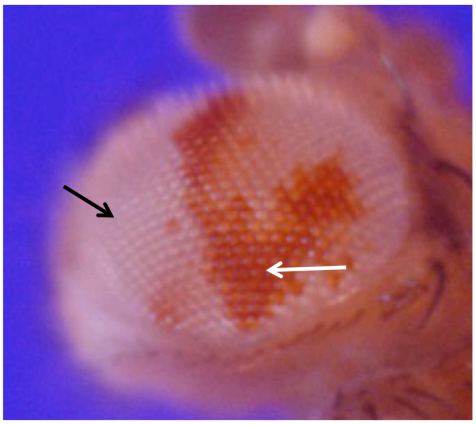


Figure 5.2 Eye with differential expression of the *white*$^{+}$ gene. Black arrow is showing the patches, where *white*$^{+}$ is silent. White arrow points to the patches where *white*$^{+}$ is active.

My data suggest that this ability is conferred by the amount of *rDNA* copies found in the progenitor cells of the eye. Likely there are two ranges of *rDNA* levels that determine if the gene is heterochromatically silenced or not (Figure 5.3, green and red bar). The progenitor cell has a high number of *rDNA* copies such that the *white*$^{+}$ gene should be off. After mitotic divisions, the *rDNA* could be lost at different degrees such that the daughter cells will have less *rDNA* than the progenitor but some will still be within the range of *rDNA* copy

number that is required to promote *white*$^{+}$ gene silencing. On the other hand, other daughter cells might have lost enough *rDNA* to shift the chromatin balance towards the activation of the *white*$^{+}$ gene (Figure 5.3).

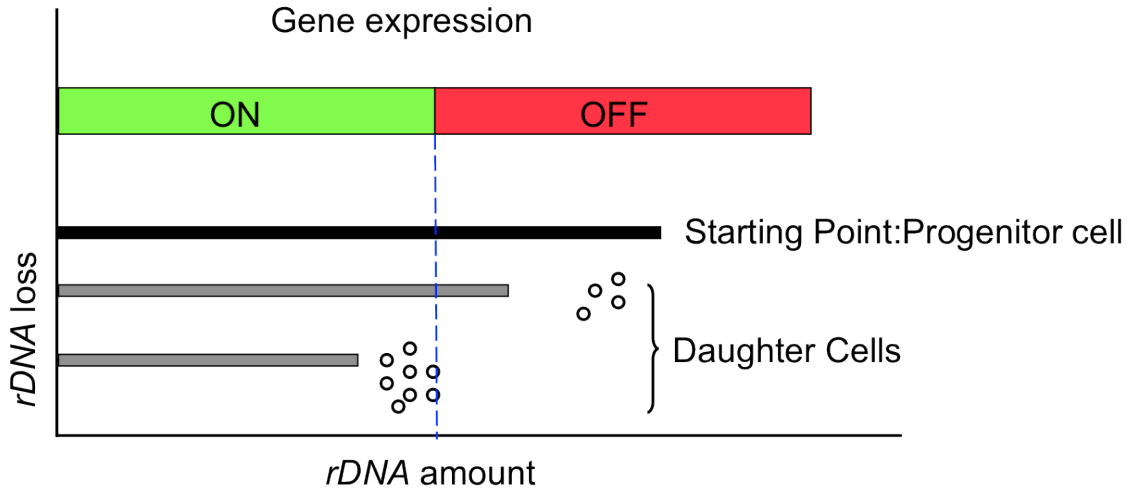


Figure 5.3 Model to explain differences of *white*$^{+}$ expression in the eye. Eye precursor cells start with a high level of *rDNA* copies that promote the gene silencing. After several divisions, some daughter cells will lose *rDNA* at a low degree, such that the *white*$^{+}$ gene will remain inactive. In contrast, in some daughter cells the levels of *rDNA* loss are higher so that the *white*$^{+}$ gene can be actively transcribed.

This interpretation of the data can also be used to describe the differences in expression of the *white*$^{+}$ gene observed between isogenic sibling flies. Accordingly, the parental fly has a high level of *rDNA* copies, which is enough to establish heterocromatin-mediated silencing of the *white*$^{+}$ gene (Figure 5.4, Starting point). The progeny could have different degrees of *rDNA* loss, hence some could have eye progenitor cells starting with a high copy

number, which might eventually lost more *rDNA* in the daughter cells, but most of them would remain within the level that keeps the *white*$^{+}$ gene silenced (Figure 5.4, white eye). In contrast, some progeny might have undergone higher rates of *rDNA* loss in early development, such that the progenitor cells might start with already low levels that are unable to maintain the heterochromatic silence (Figure 5.4, red eye).

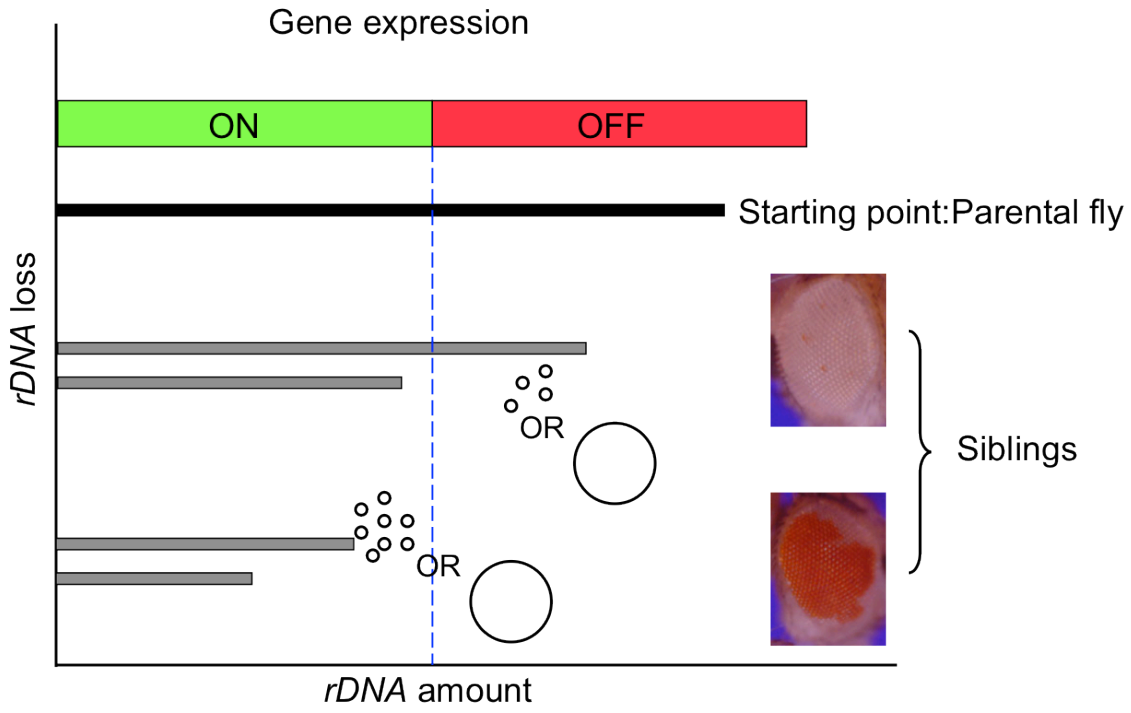


Figure 5.4 Model to explain differences of *white*+ expression between siblings. The parental fly has high levels of *rDNA* to maintain silenced the *white*$^{+}$ gene. The progeny inherits this level of *rDNA* but after several mitotic divisions some progeny maintains the high levels of *rDNA* copy number that silence the *white*$^{+}$ gene, while others have a higher *rDNA* loss rate that activates the gene.

A possible mechanism

The observed changes in the volume of the nucleolus could be the mechanism of how the heterochromatin factors are redistributed in the genome. This is based on two characteristics of the nucleolus: 1) the role in maintenance of chromosomal territories, and 2) the ability to sequester hundreds of proteins.

The nucleus is a very complex environment that contains several nuclear bodies such as the nucleolus, histone locus bodies, splicing factor compartments, Cajal bodies, promyelocytic leukemia bodies, Gemini bodies, and several others (190). In addition to this the nucleus contains the chromosomes and recent studies have proposed that the chromosomes preferentially occupy a specific volume within the nucleus, which is known as chromosomal territories (191). The positioning of the chromosomes in the nucleus is a major determinant of gene expression, hence nuclear organization has been proposed to be a novel type of epigenetic regulation. In agreement with this, differences in how the chromosomes are distributed in the nucleus have been observed among different cell types and in cancerous cells (191, 192). In addition, a recent study in the yeast *S. cerevisiae* found that all the genes are not randomly positioning relative to the nuclear envelope and the nucleolus, hence it was proposed that the nucleolus has a very important role in organization of the chromosomal territories (193). Since I observed that changes in *rDNA* copy number affect the nucleolus size and structure, it is plausible to think that these alterations, or similar alterations in any other nuclear body, could

disrupt the chromosomal territories leading to misregulation of gene expression. This resembles an observation in budding yeast, where the telomere sub compartment was disrupted, but the SIR factors (telomere silencing proteins) remained unaffected. The effect of this disruption was alteration in genes throughout the entire genome, suggesting that the SIR factors were redistributed to other sequences different than the telomeres (185). This hypothesis predicts that another mechanism for altering global gene expression would be the alteration of other nuclear bodies. Possibly because the nucleolus is the most prominent nuclear body in the nucleus, the effects of the alterations on this compartment could be the most extreme.

The nucleolus contains hundreds of proteins involved in multiple nuclear processes such as ribosome biogenesis, chromatin structure, translation, chaperones, etc (194). Many of these proteins are released from the nucleolus when they are required to carry out a specific function (115). For example, the protein Modulo acts as a suppressor of variegation and it is found in the nucleolus where it is believed to be regulated by this localization (195). Therefore, it is reasonable to hypothesize that an alteration in the volume of the nucleolus could alter the physical capacity of the nucleolus to maintain the proteins it contains. These alterations in volume could also affect the role of the nucleolus to response to certain cues from the cell cycle and external conditions, by affecting its ability to regulate the interchange of proteins in and out of this compartment. Changes in nucleolus are commonly related to cancer, aging and

disease, which are cellular processes that also have alterations in transcription by RNA Polymerase I, II and III (155, 196, 197).

Placing my model into perspective takes me to the very first definition of “epigenetics” that was coined by Conrad Waddington in 1942 and when he described the “epigenetic landscape” (198, 199). His hypothesis proposes that development occurs similarly to a ball rolling in a landscape that contains multiple hills and valleys. During development, cells are “balls” that take different paths on the landscape of valleys. Once they land in a valley, the landing determines cell fate. The “hills” of the landscape are the barriers that avoid uncontrolled differentiation. Cell determination is protected by these “hills”, which can change if there is enough perturbation (199). The current view of epigenetics refers to heritable modifications of genes that are unrelated to the DNA sequence, which exert changes in gene expression and can be variable among tissues of the same organism (200). The model that I propose ties these two hypothesis together in a way that the “hills” that protect the “valleys” or cell state, are in a major part determined by the *rDNA* copy number. Activation of silent *rDNA* copies changes the chromatin state of different genes on the genome, and this can be heritably maintained and variable in different tissues of the same organism. Since the state of a cell is determined by its gene expression profile, my data suggest that the *rDNA* could be a major designer of this gene expression profile that maintains cell identity.

MATERIALS AND METHODS

Fly stocks for induced *I-Cre*I *rDNA* deletions

The *Y10A* chromosome is y^+ Yw^+, *Dp*(*1;Y*) y^+, P{w^+=*RSw*}*10A* (129). The first exon of the *white*$^+$ gene in *RSw* is flanked by FRT sequences (201). A chromosome with *FLP*-induced loss of *white*$^+$ is referred to as *Y10B*. Prior to using either *Y10A* or *Y10B* for these experiments, we crossed single males to females for three generations prior to our experiments. The *X* chromosome is y^+ w^{67c23}. The *I-Cre*I expressing line is *P{v*$^{+t1.8}$*=hs-I-CreI.R}2A*, v^1/*Y; Sb*/*TM6b, Ubx* (202), obtained from the Bloomington *Drosophila* Stock Center. The attached-*X* chromosome is *C(1)DX*, y^1 f^1 bb^0 (138). White-mottled stocks are *In(1)*w^{m4} or *In(1)*w^{m4h}, light-variegator stock is lt^{x13}/*SM1*, *Cy lt*. Deleted *Y* chromosome-bearing males were backcrossed every generation to an isogenic stock. The fly strain variegating for green fluorescence protein, *Y10C*, is *y* Y^+, $rDNA^+$, *P{X97, ubiq-GFP,* w^+*}10C*, generated using *FLP/FRT*-mediated replacement (203) of a $GFP^{S65T.Ubi-p63E}$ transgene (cloned from y^1 *w**; *In(2LR)Gla*, wg^{Gla-1} Bc^1/*CyO*, *P*{$w^{+mW.hs=Ubi-GFP.S65T}$}*PAD1*) at the *Y10B P*-element insertion site (129). For Chapter III and IV the names of the stocks used were changed to a name that includes the percentage of *rDNA* content left on the *Y* chromosome relative to the parental *Y* chromosome as follows: *Ywt* is Y10B, *YrDNA*-0.87 is *bb*–465, *YrDNA*-0.85 is *bb–76*, *YrDNA*-0.49 is *I– 481*, *YrDNA*-0.46 is *I*–498, *rDNA*-0.41 is *I*-510, and *YrDNA*-0.36 is *I*-473. Flies were raised on cornmeal molasses agar at

25°C and 80% humidity.

Induction and screen for deletions

Flies were allowed to lay eggs for 2–3 days, and larvae to develop for 1 more day. Second and third instar larvae were heat-shocked in circulating water baths at 36°C. In experiments involving *Y10A*, larvae were heat-shocked on 2 successive days, each treatment lasting 45 min. In experiments involving *Y10B*, larvae were heat-shocked on 1 day for 45 min. Heat-shock-induced expression was monitored by underrepresentation of *I-Cre*I bearing male progeny in relation to P{$v^{+t1.8}$=*hs-I-CreI.R*}*2A*, v^{1} / y^{1} w^{67c23} siblings and by cuticle or eye defects indicating expression-induced cell lethality (129). *X*–*Y* translocation chromosomes were identified as sterile yellow males and yellow+ females and were excluded from analysis.

Real-time polymerase chain reaction

Primers AGCCTGAGAAACGGCTACCA and AGCTGGGAGTGGGTAATTTACG amplify 63 nucleotides of the *18S* gene in the *35S rDNA*. After confirming single melting curve kinetics using an ABI Step-One real-time polymerase chain reaction machine (Applied Biosystems) running Step-One v1.0 software, we used the Power SYBR Green master mix (Applied Biosystems) reagent, 500 nm primers, and 10 ng nucleic acid with 40 cycles alternating between 95° for 3 sec and 60° for 30 sec. DNA samples were

prepared using a modified procedure from K. Dobie (204, 205). The organic extractions were followed with ether extraction, rather than ethanol precipitation, which produced 1–2 mg total nucleic acid/fly. DNA was quantified using a Nanodrop and diluted to 10 ng/ L. Amplification data were processed by determining the point at which fluorescence first crossed a threshold of 10 standard deviations above the average of all previous cycles (‘‘no amplification’’) of fluorescence from each extract, as determined by the Step-One software. Extracts were run in triplicate (occasionally quadruplicate) identical samples with 10 ng of template. Samples in discordance with the other samples (a threshold cycle with a difference of >2 standard errors of the mean) were interpreted as errors in reaction or reaction preparation and were excluded. Fewer than fifty of 5000 total samples were discarded using this criterion. *tRNA*$^{K\text{-}CTT}$ genes were amplified using primers CTAGCTCAGTCGGTAGAGCATGA and CCAACGTGGGGCTCGAAC to generate a 63-nucleotide product. Cycle differences between *rDNA* and *tRNA* genes (‘‘ΔC_T’’) were compared to the same measurement from DNA pooled from a large population (~200) of adult flies or larvae bearing chromosome *Y10B* (‘‘$\Delta\Delta C_T$’’), generating the percentage of wild-type *rDNA* quantity. Adult DNA was used for *rDNA*^{bb} lines, and larval DNA was used for *rDNA*$^{bb\text{-}l}$ lines. The same pooled *Y10B* preparations of DNA were used for all experiments. We present either standard deviation (with pooled root-sum errors) if individuals are compared to other individuals or standard errors of the mean (with pooled root-

squared-sum errors) if array size from individuals is shown.

Cytology and photography

Photographs of adult flies were taken using a Nikon D2H camera attached to a Nikon SMZ- 1500 microscope. Neuroblast spreads were prepared following the protocol of S. Pimpinelli, S. Bonaccorsi, L. Fanti, and M. Gatti (206).

Dissection

Larvae were raised on standard cornmeal molasses fly food supplemented with baker's yeast and raised at 18°C. Salivary glands or brains from wandering third instar larvae where dissected in PBS. Tissues destined for immunofluorescence were processed immediately. Tissues destined for real-time PCR were frozen at 70°C.

Immunofluorescence/Confocal Microscopy

For immunofluorescence, salivary glands were washed in PBT (PBS supplemented with 0.1% Tween-80), blocked for 2 h in PBT with 10% BSA, and incubated with antibodies overnight at 4°C in PBT supplemented with 1% BSA and 500 mM NaCl. Mouse anti-fibrillarin antibody (Abcam) was used at a 1:200 dilution, and goat anti-mouse conjugated to TRITC (Jackson ImmunoResearch Laboratories) was used at 1:200 as secondary antibody. Confocal fluorescent images were obtained on a Olympus FV1000 confocal microscope with a 100

immersion oil objective. Sequential excitation with lasers was done at 405 nm and 543 nm to observe DAPI staining and rhodamine, respectively, and were analyzed with FV10-ASW 1.7 Viewer software. Three dimensional reconstruction of nucleoli and nucleus was done using ImageJ with the LOCI and Voxel-Counter plug-ins. Nucleolus volume was determined relative to the total nucleus. Ten nucleoli were analyzed in each of three different salivary glands for each fly line analyzed.

Brain tissue DNA Preparations

Frozen tissue was sonicated in 200μL PBS using a Misonix XL-2000 with three 10-s pulses and 20-s intervals. One microliter from the sonicated sample was used in each of triplicate real-time PCR reactions.

RNA Analyses

RNA was extracted according to Bogart and Andrews (207). Pupae were *C(1)DX*/*YrDNA*-deletion, identified using the *Y*-linked *yellow*$^{+}$ gene of *Ywt* (129), and adult flies were w^{m4}/*YrDNA*-deletion. RNA was electrophoretically separated at 100 V for 215 min in 1.5% agarose with running buffer 400 mM Mops (3-morpholinopropanesulfonic acid, 3-(N-morpholino)propanesulfonic acid), pH 7.0, 100 mM sodium acetate, and 10 mM EDTA (EDTA) supplemented with 18% formaldehyde. RNA was stained with ethidium bromide and quantified relative to tRNA using a Typhoon TRIO Variable Mode Imager (GE Healthcare) running

ImageQuant 5.2. RNA was isolated from five pools of 10 flies each for comparison.

Pigment Extraction

Fly heads were removed by banging frozen flies, and incubated in 8% NaOH, 66% ethanol (50 μL per head) in the dark for 24h at 37°C. Pigment quantification was done using a BioRad SmartSpec3000 spectrophotometer at 320 nm (208) and 480 nm (46).

Fly stocks for RNA extraction for microarrays

The *Y* chromosomes with targeted deletions in the *rDNA* locus were introgressed into an isogenic (*X* chromosome, autosomes, and mitochondrial genome) laboratory stock as previously described *(164)*. This isogenic stock is expected to contain very little genetic variation, and upon receipt was subjected to no fewer than eight additional generations of brother-sister mating to reinforce homozygosity of the genetic background. Four *Y* chromosomes were analyzed: The original *Y* chromosome that contains a wild type *rDNA* array (100%), two derived chromosomes with mild deletions 87% (*YrDNA*-0.87) and 85% (*YrDNA*-0.85) of wild-type, and one grossly reduced derived chromosome that contains 46% (*YrDNA*-0.46) of wild-type. Flies were grown under 24h light at constant temperature (25°C) and humidity (80%). *XXY* female flies were obtained by crossing males from the isogenic *Y* chromosome substitution lines described

above to females from a laboratory stock containing a compound (attached) *X* chromosome, *C*(*1*)*M4*, *y*.

Gene expression analyses.

Microarrays were approximately 18,000-feature cDNA arrays spotted with *Drosophila melanogaster* cDNA PCR products. For RNA extraction, newly emerged male flies were collected and aged for three days at 25°C, after which they were flash frozen in liquid nitrogen and stored at -80°C. When females were analyzed, they were collected within 7 hours of eclosion to assure they were unmated prior to aging under the same conditions as were males. Total RNA was extracted from whole flies using TRIZOL (Gibco-BRL, Life Technologies, Gaithersburg, Maryland). cDNA synthesis, labeling with fluorescent dyes (Cy3 and Cy5) and hybridization reactions were carried out using 3DNA protocols and reagents (Genisphere Inc., Hatfield, Pennsylvania). Slides were scanned using AXON 4000B scanner (Axon Instruments, Foster City, California) and the GenePix Pro 6.0 software. Stringent quality-control criteria were used to ensure reliability of foreground intensity reads for both Cy3 and Cy5 channels. Foreground fluorescence of dye intensities was normalized by the Loess method in the library Limma (209, 210) of the software R. Significance of variation in gene expression due to *Y* chromosome origin was assessed with linear models and empirical Bayes moderated F statistics in Limma (209, 210). *P* values were

adjusted for multiple testing by using the method of Benjamini and Hochberg to control the false discovery rate (211). Test results were considered to be significant if the adjusted *P* values were less than 0.05, nominally controlling the expected false discovery rate to no more than 5%. Differential expression was also assessed using the Bayesian Analysis of Gene Expression Levels (BAGEL) model (212). False discovery rates were estimated based on the variation observed when randomized versions of the original dataset were analyzed. Similarly, expected values for the overlap between independent datasets were estimated by considering permuted versions of the datasets. Results were robust to choice of linear models in Limma or BAGEL. Enrichment in gene ontology categories was assessed using a modified Bonferroni correction with GeneMerge (213). Microarray gene expression data will be placed in the GEO database following publication.

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APPENDIX A

Table A.1: List of differentially expressed genes at different *P*-values for the *l-0.46 rDNA* deleted line

P<0.0001	P<0.0005	P<0.001	P<0.005		P<0.01	
FBgn0003321	FBgn0003356	FBgn0013672	FBgn0001263	FBgn0041579	FBgn0032472	FBgn0027095
FBgn0004431	FBgn0031663	FBgn0020508	FBgn0015371	FBgn0053269	FBgn0000083	FBgn0027329
FBgn0010038	FBgn0036833	FBgn0027073	FBgn0023211	FBgn0052548	FBgn0039774	FBgn0038243
FBgn0011704	FBgn0040582	FBgn0028482	FBgn0010425	FBgn0029826	FBgn0000079	FBgn0004429
FBgn0015586	FBgn0051288	FBgn0028940	FBgn0029876	FBgn0031001	FBgn0034573	FBgn0011016
FBgn0024986	FBgn0002868	FBgn0032897	FBgn0032412	FBgn0034474	FBgn0050151	FBgn0040362
FBgn0028380	FBgn0029990	FBgn0053173	FBgn0032618	FBgn0035517	FBgn0036262	FBgn0010453
FBgn0028381	FBgn0031563	FBgn0036623	FBgn0039471	FBgn0029094	FBgn0036547	FBgn0040529
FBgn0030040	FBgn0031930	FBgn0020506	FBgn0050503	FBgn0039366	FBgn0015033	FBgn0033190
FBgn0030929	FBgn0032068	FBgn0029785	FBgn0030357	FBgn0083167	FBgn0015034	FBgn0031562
FBgn0030955	FBgn0032947	FBgn0013988	FBgn0033821	FBgn0003886	FBgn0029838	FBgn0032265
FBgn0031263	FBgn0034328	FBgn0039169	FBgn0037678	FBgn0032054	FBgn0034140	FBgn0003514
FBgn0031317	FBgn0034512	FBgn0039930	FBgn0038343	FBgn0037102	FBgn0051974	FBgn0016701
FBgn0031489	FBgn0046763	FBgn0034402	FBgn0038966	FBgn0031315	FBgn0040491	FBgn0038369
FBgn0031520	FBgn0025592	FBgn0039311	FBgn0053209	FBgn0033452	FBgn0028433	FBgn0043532
FBgn0032002	FBgn0032947	FBgn0037664	FBgn0004922	FBgn0010041	FBgn0029006	FBgn0035743
FBgn0032321	FBgn0034796	FBgn0042188	FBgn0035300	FBgn0010426	FBgn0039570	FBgn0029665
FBgn0033302	FBgn0043470	FBgn0014020	FBgn0000120	FBgn0051344	FBgn0038482	FBgn0029827
FBgn0033659	FBgn0051300	FBgn0034647	FBgn0013767	FBgn0033124	FBgn0040958	FBgn0039112
FBgn0033787	FBgn0022073	FBgn0036279	FBgn0019949	FBgn0013762	FBgn0032420	FBgn0019624
FBgn0033788	FBgn0034089	FBgn0051063	FBgn0034331	FBgn0014865	FBgn0051217	FBgn0013348
FBgn0033789	FBgn0034582	FBgn0011726	FBgn0036211	FBgn0037038	FBgn0037354	FBgn0036695
FBgn0033857	FBgn0003732	FBgn0035094	FBgn0037752	FBgn0039354	FBgn0031434	FBgn0030177
FBgn0033869	FBgn0005664	FBgn0037568	FBgn0039790	FBgn0031801	FBgn0038930	FBgn0037433
FBgn0034296	FBgn0013987	FBgn0038830	FBgn0051954	FBgn0024997	FBgn0030802	FBgn0032511
FBgn0034758	FBgn0015031	FBgn0040097	FBgn0010651	FBgn0026084	FBgn0037877	FBgn0034392
FBgn0035378	FBgn0026741	FBgn0040318	FBgn0034335	FBgn0033327	FBgn0004597	FBgn0027053

Table A.1: Continued

P<0.0001	P<0.0005	P<0.001	P<0.005		P<0.01	
FBgn0035583	FBgn0028886	FBgn0010044	FBgn0035941	FBgn0042179	FBgn0034680	FBgn0029507
FBgn0035862	FBgn0034741	FBgn0011670	FBgn0036556	FBgn0001112	FBgn0053969	FBgn0033089
FBgn0036320	FBgn0035176	FBgn0028518	FBgn0032322	FBgn0028872	FBgn0010114	FBgn0033610
FBgn0036357	FBgn0036993	FBgn0032888	FBgn0026061	FBgn0051380	FBgn0028704	FBgn0035710
FBgn0036732	FBgn0037308	FBgn0034258	FBgn0036091	FBgn0028541	FBgn0035154	FBgn0051111
FBgn0036766	FBgn0038098	FBgn0032235	FBgn0051233	FBgn0037885	FBgn0033317	FBgn0015774
FBgn0036929	FBgn0038346	FBgn0034577	FBgn0036738	FBgn0024238	FBgn0011305	FBgn0022935
FBgn0037065	FBgn0038980	FBgn0040837	FBgn0035674	FBgn0029684	FBgn0030928	
FBgn0037562	FBgn0051087		FBgn0039768	FBgn0040793	FBgn0041630	
FBgn0038292	FBgn0051757		FBgn0063449	FBgn0011760	FBgn0015286	
FBgn0038353	FBgn0004240		FBgn0030484	FBgn0042134	FBgn0015801	
FBgn0038918	FBgn0011227		FBgn0031741	FBgn0039752	FBgn0013307	
FBgn0039670	FBgn0011668		FBgn0035664	FBgn0051155	FBgn0024957	
FBgn0039714	FBgn0015000		FBgn0051777	FBgn0000277	FBgn0038964	
FBgn0039776	FBgn0025692		FBgn0034527	FBgn0001149	FBgn0037302	
FBgn0039869	FBgn0028987		FBgn0034883	FBgn0036277	FBgn0037071	
FBgn0041205	FBgn0030832		FBgn0037305	FBgn0051678	FBgn0000114	
FBgn0050269	FBgn0031412		FBgn0037874	FBgn0030262	FBgn0027657	
FBgn0051086	FBgn0034760		FBgn0005672	FBgn0033468	FBgn0033294	
FBgn0051901	FBgn0036372		FBgn0036374	FBgn0039094	FBgn0038484	
FBgn0052054	FBgn0038961		FBgn0014857	FBgn0039788	FBgn0030272	
FBgn0052548	FBgn0051469		FBgn0032981	FBgn0000150	FBgn0000394	
FBgn0053926	FBgn0026208		FBgn0034147	FBgn0003390	FBgn0024939	
FBgn0053926	FBgn0030958		FBgn0039849	FBgn0053179	FBgn0050196	
FBgn0065032	FBgn0034152		FBgn0035619	FBgn0015584	FBgn0031873	
FBgn0052407	FBgn0040723		FBgn0002939		FBgn0037297	
FBgn0000078	FBgn0011554		FBgn0005630		FBgn0038172	
FBgn0000121	FBgn0027932		FBgn0032666		FBgn0050000	
FBgn0001285	FBgn0032706		FBgn0037347		FBgn0034808	
FBgn0002571	FBgn0033453		FBgn0040211		FBgn0022936	

Table A.1: Continued

P<0.0001	P<0.0005	P<0.001	P<0.005		P<0.01	
FBgn0003357	FBgn0033478		FBgn0028526		FBgn0025803	
FBgn0003358	FBgn0033786		FBgn0036198		FBgn0037899	
FBgn0003863			FBgn0033613		FBgn0052068	
FBgn0011555			FBgn0053514		FBgn0031974	
FBgn0015001			FBgn0034628		FBgn0035620	
FBgn0019940			FBgn0035670		FBgn0031449	
FBgn0020906			FBgn0035779		FBgn0037146	
FBgn0023197			FBgn0039697		FBgn0033296	
FBgn0023541			FBgn0042185		FBgn0032820	
FBgn0027525			FBgn0053178		FBgn0052441	
FBgn0028945			FBgn0052600		FBgn0052177	
FBgn0030098			FBgn0028955		FBgn0083938	
FBgn0031533			FBgn0036015		FBgn0024432	
FBgn0031653			FBgn0051150		FBgn0031633	
FBgn0031654			FBgn0033216		FBgn0032275	
FBgn0031968			FBgn0039769		FBgn0046297	
FBgn0031971			FBgn0017558		FBgn0016693	
FBgn0032066			FBgn0036422		FBgn0028833	
FBgn0032144			FBgn0028572		FBgn0050163	
FBgn0033250			FBgn0033774		FBgn0026380	
FBgn0033639			FBgn0036888		FBgn0040475	
FBgn0033999			FBgn0038912		FBgn0010621	
FBgn0035665			FBgn0036746		FBgn0036334	
FBgn0035666			FBgn0050011		FBgn0033582	
FBgn0035667			FBgn0036825		FBgn0031535	
FBgn0035781			FBgn0037537		FBgn0033222	
FBgn0035886			FBgn0051357		FBgn0030449	
FBgn0035887			FBgn0011361		FBgn0031546	
FBgn0035968			FBgn0028894		FBgn0030347	
FBgn0036769			FBgn0030480		FBgn0021967	

Table A.1: Continued

P<0.0001	P<0.0005	P<0.001	P<0.005		P<0.01	
FBgn0036831			FBgn0033268		FBgn0031942	
FBgn0038516			FBgn0054034		FBgn0037039	
FBgn0039154			FBgn0000044		FBgn0028372	
FBgn0039342			FBgn0035043		FBgn0033734	
FBgn0039472			FBgn0044030		FBgn0038400	
FBgn0039474			FBgn0024833		FBgn0020907	
FBgn0039476			FBgn0033167		FBgn0000276	
FBgn0039628			FBgn0038455		FBgn0033907	
FBgn0039629			FBgn0000042		FBgn0036909	
FBgn0039760			FBgn0001085		FBgn0040496	
FBgn0039761			FBgn0035089		FBgn0011570	
FBgn0039777			FBgn0032805		FBgn0029522	
FBgn0039905			FBgn0036210		FBgn0033760	
FBgn0040104			FBgn0036563		FBgn0051072	
FBgn0040383			FBgn0037632		FBgn0002855	
FBgn0040827			FBgn0038347		FBgn0037279	
FBgn0040959			FBgn0032819		FBgn0051450	
FBgn0051104			FBgn0035929		FBgn0001291	
FBgn0051343			FBgn0042206		FBgn0032704	

Table A.2: List of differentially expressed genes at P<0.05 for the *l-0.46 rDNA* deleted line

FBgn0027572	FBgn0003250	FBgn0022361	FBgn0037763	FBgn0024891	FBgn0039505	FBgn0037718
FBgn0034490	FBgn0029804	FBgn0027334	FBgn0033196	FBgn0034198	FBgn0039405	FBgn0033177
FBgn0037053	FBgn0033649	FBgn0033065	FBgn0035151	FBgn0038345	FBgn0038526	FBgn0039065
FBgn0003249	FBgn0032250	FBgn0035976	FBgn0063491	FBgn0036967	FBgn0037464	FBgn0030545
FBgn0031589	FBgn0031216	FBgn0013981	FBgn0030607	FBgn0001404	FBgn0033267	FBgn0029174
FBgn0030011	FBgn0032258	FBgn0036498	FBgn0052843	FBgn0039598	FBgn0037749	FBgn0000392
FBgn0031636	FBgn0037634	FBgn0032264	FBgn0000464	FBgn0010269	FBgn0033357	FBgn0031004
FBgn0035540	FBgn0039312	FBgn0033362	FBgn0029608	FBgn0035449	FBgn0014374	FBgn0031304
FBgn0037701	FBgn0003279	FBgn0031937	FBgn0036389	FBgn0036767	FBgn0034468	FBgn0034362
FBgn0040673	FBgn0037933	FBgn0037705	FBgn0031816	FBgn0032013	FBgn0001297	FBgn0034599
FBgn0031373	FBgn0039494	FBgn0025286	FBgn0003275	FBgn0038680	FBgn0038719	FBgn0034110
FBgn0032773	FBgn0039600	FBgn0030264	FBgn0032772	FBgn0038206	FBgn0025865	FBgn0038587
FBgn0034247	FBgn0029927	FBgn0031913	FBgn0050126	FBgn0038043	FBgn0032446	FBgn0033574
FBgn0036973	FBgn0034484	FBgn0033373	FBgn0037171	FBgn0036787	FBgn0037498	FBgn0003676
FBgn0003638	FBgn0033842	FBgn0033226	FBgn0011768	FBgn0038763	FBgn0039025	FBgn0031043
FBgn0033232	FBgn0034162	FBgn0051163	FBgn0032422	FBgn0035708	FBgn0034664	FBgn0037215
FBgn0033904	FBgn0035529	FBgn0032770	FBgn0034118	FBgn0038474	FBgn0036340	FBgn0037168
FBgn0033995	FBgn0040918	FBgn0038481	FBgn0040282	FBgn0032775	FBgn0037249	FBgn0028375
FBgn0039555	FBgn0040322	FBgn0038846	FBgn0010053	FBgn0004117	FBgn0014023	FBgn0035244
FBgn0030990	FBgn0052700	FBgn0038957	FBgn0003141	FBgn0012036	FBgn0038428	FBgn0050185
FBgn0014026	FBgn0032911	FBgn0033602	FBgn0043576	FBgn0031312	FBgn0035109	FBgn0019952
FBgn0040993	FBgn0040715	FBgn0037955	FBgn0033460	FBgn0032087	FBgn0002772	FBgn0039310
FBgn0032336	FBgn0053252	FBgn0035692	FBgn0052484	FBgn0050497	FBgn0020018	FBgn0039145
FBgn0035489	FBgn0004666	FBgn0040732	FBgn0082585	FBgn0001218	FBgn0033607	FBgn0010288
FBgn0032115	FBgn0030013	FBgn0031865	FBgn0029002	FBgn0053988	FBgn0028985	FBgn0052306
FBgn0036030	FBgn0033060	FBgn0033246	FBgn0038181	FBgn0000592	FBgn0035318	FBgn0030828
FBgn0011296	FBgn0000114	FBgn0016715	FBgn0032444	FBgn0034827	FBgn0035957	FBgn0031399
FBgn0030485	FBgn0038465	FBgn0050004	FBgn0000142	FBgn0037163	FBgn0039565	FBgn0041102
FBgn0034052	FBgn0031889	FBgn0030903	FBgn0010638	FBgn0029882	FBgn0052109	FBgn0032399
FBgn0036992	FBgn0039857	FBgn0035780	FBgn0039184	FBgn0030827	FBgn0034361	FBgn0030088

Table A.2: Continued

FBgn0002521	FBgn0001229	FBgn0025454	FBgn0053205	FBgn0011672	FBgn0034407	FBgn0034158
FBgn0033776	FBgn0038321	FBgn0024939	FBgn0034718	FBgn0031910	FBgn0039599	FBgn0038752
FBgn0052705	FBgn0040805	FBgn0031143	FBgn0002641	FBgn0028388	FBgn0052383	FBgn0029594
FBgn0033261	FBgn0023215	FBgn0016013	FBgn0035415	FBgn0032680	FBgn0064766	FBgn0038584
FBgn0024321	FBgn0032997	FBgn0014000	FBgn0030787	FBgn0037303	FBgn0037715	FBgn0031398
FBgn0028536	FBgn0035252	FBgn0002926	FBgn0052687	FBgn0015019	FBgn0030305	FBgn0003206
FBgn0027601	FBgn0051266	FBgn0033047	FBgn0083990	FBgn0034697	FBgn0051713	FBgn0016031
FBgn0039767	FBgn0038681	FBgn0036918	FBgn0038052	FBgn0036813	FBgn0034645	FBgn0037323
FBgn0027560	FBgn0030503	FBgn0037954	FBgn0000114	FBgn0045866	FBgn0038876	FBgn0037051
FBgn0034290	FBgn0035359	FBgn0027507	FBgn0039637	FBgn0036990	FBgn0021795	FBgn0030050
FBgn0040735	FBgn0035541	FBgn0031929	FBgn0063499	FBgn0050183	FBgn0038199	FBgn0028944
FBgn0052695	FBgn0034725	FBgn0039298	FBgn0033957	FBgn0035139	FBgn0001197	FBgn0034181
FBgn0028491	FBgn0037697	FBgn0004381	FBgn0039007	FBgn0021768	FBgn0040575	FBgn0051272
FBgn0020513	FBgn0032791	FBgn0040260	FBgn0000047	FBgn0028932	FBgn0003721	FBgn0011743
FBgn0035979	FBgn0052076	FBgn0010287	FBgn0053526	FBgn0037016	FBgn0031228	FBgn0031529
FBgn0024184	FBgn0037971	FBgn0032237	FBgn0030873	FBgn0034217	FBgn0032075	FBgn0004432
FBgn0021856	FBgn0015544	FBgn0039098	FBgn0038466	FBgn0030007	FBgn0030612	
FBgn0036221	FBgn0028844	FBgn0052245	FBgn0039402	FBgn0033208	FBgn0036659	
FBgn0040384	FBgn0037046	FBgn0037934	FBgn0028411	FBgn0032222	FBgn0031026	
FBgn0051674	FBgn0015035	FBgn0036760	FBgn0033613	FBgn0034157	FBgn0024196	
FBgn0036381	FBgn0030696	FBgn0037739	FBgn0000464	FBgn0026315	FBgn0035713	
FBgn0030581	FBgn0033383	FBgn0019643	FBgn0032938	FBgn0037468	FBgn0039291	
FBgn0037478	FBgn0015829	FBgn0026582	FBgn0051004	FBgn0004554	FBgn0034155	
FBgn0027348	FBgn0031645	FBgn0038983	FBgn0037594	FBgn0037819	FBgn0015372	
FBgn0032885	FBgn0036249	FBgn0000454	FBgn0034646	FBgn0001104	FBgn0031954	
FBgn0000715	FBgn0030482	FBgn0037110	FBgn0035499	FBgn0035049	FBgn0015571	
FBgn0019830	FBgn0064225	FBgn0051645	FBgn0032055	FBgn0003867	FBgn0030092	
FBgn0032507	FBgn0035996	FBgn0037199	FBgn0015583	FBgn0038787	FBgn0063495	
FBgn0032707	FBgn0032601	FBgn0052705	FBgn0030528	FBgn0039068	FBgn0037098	
FBgn0010497	FBgn0038749	FBgn0028428	FBgn0034515	FBgn0024227	FBgn0000253	
FBgn0014455	FBgn0053080	FBgn0052506	FBgn0013467	FBgn0034485	FBgn0027582	

Table A.2: Continued

FBgn0040239	FBgn0028561	FBgn0032330	FBgn0015576	FBgn0001233	FBgn0033691	
FBgn0031495	FBgn0040256	FBgn0053196	FBgn0034245	FBgn0030035	FBgn0038195	
FBgn0036267	FBgn0029687	FBgn0034887	FBgn0035906	FBgn0036298	FBgn0037312	
FBgn0036774	FBgn0039453	FBgn0034194	FBgn0034277	FBgn0020250	FBgn0031692	
FBgn0015924	FBgn0016126	FBgn0032661	FBgn0037022	FBgn0033366	FBgn0031094	
FBgn0038211	FBgn0039226	FBgn0000472	FBgn0038039	FBgn0038208	FBgn0033800	
FBgn0022085	FBgn0032404	FBgn0020497	FBgn0029643	FBgn0038654	FBgn0015299	
FBgn0032515	FBgn0034739	FBgn0033170	FBgn0030048	FBgn0000046	FBgn0031049	
FBgn0034638	FBgn0030101	FBgn0016694	FBgn0004228	FBgn0000153	FBgn0036662	
FBgn0058006	FBgn0040002	FBgn0024689	FBgn0004244	FBgn0035782	FBgn0031774	
FBgn0003248	FBgn0010620	FBgn0032167	FBgn0035194	FBgn0031320	FBgn0034716	
FBgn0052085	FBgn0029828	FBgn0035092	FBgn0037440	FBgn0017448	FBgn0017567	
FBgn0034761	FBgn0035845	FBgn0037680	FBgn0028926	FBgn0031408	FBgn0028984	
FBgn0032919	FBgn0029606	FBgn0033820	FBgn0033652	FBgn0051446	FBgn0083938	
FBgn0038275	FBgn0004179	FBgn0037973	FBgn0002773	FBgn0035983	FBgn0034753	
FBgn0028919	FBgn0013718	FBgn0039889	FBgn0030991	FBgn0010774	FBgn0010039	
FBgn0002989	FBgn0035986	FBgn0033265	FBgn0031882	FBgn0042198	FBgn0023525	
FBgn0033871	FBgn0036232	FBgn0002940	FBgn0034723	FBgn0037174	FBgn0000045	
FBgn0011274	FBgn0032413	FBgn0037560	FBgn0020369	FBgn0051644	FBgn0040070	
FBgn0010223	FBgn0028563	FBgn0015268	FBgn0028986	FBgn0039625	FBgn0038908	
FBgn0035632	FBgn0050393	FBgn0031132	FBgn0029082	FBgn0034724	FBgn0030306	
FBgn0038037	FBgn0063492	FBgn0039360	FBgn0004574	FBgn0034877	FBgn0027594	
FBgn0040007	FBgn0032960	FBgn0050296	FBgn0032495	FBgn0041337	FBgn0039665	
FBgn0002590	FBgn0052483	FBgn0028540	FBgn0026737	FBgn0000279	FBgn0037482	
FBgn0021875	FBgn0035921	FBgn0032389	FBgn0039254	FBgn0016120	FBgn0050387	
FBgn0037872	FBgn0021872	FBgn0035416	FBgn0031381	FBgn0029907	FBgn0033269	
FBgn0029176	FBgn0032682	FBgn0001145	FBgn0034903	FBgn0051688	FBgn0053158	
FBgn0000038	FBgn0038858	FBgn0019890	FBgn0053258	FBgn0013726	FBgn0052298	
FBgn0028644	FBgn0000100	FBgn0032518	FBgn0033570	FBgn0034986	FBgn0023423	
FBgn0029830	FBgn0033983	FBgn0015794	FBgn0032005	FBgn0036495	FBgn0031450	
FBgn0034897	FBgn0039316	FBgn0039091	FBgn0035930	FBgn0023479	FBgn0034649	

Table A.2: Continued

FBgn0032834	FBgn0052486	FBgn0030258	FBgn0050502	FBgn0037443	FBgn0037614	
FBgn0042712	FBgn0030571	FBgn0025743	FBgn0037147	FBgn0050502	FBgn0026630	
FBgn0050005	FBgn0040228	FBgn0026179	FBgn0003274	FBgn0031323	FBgn0033608	
FBgn0000566	FBgn0011744	FBgn0041342	FBgn0035355	FBgn0025637	FBgn0038200	
FBgn0032509	FBgn0036501	FBgn0051265	FBgn0013305	FBgn0043578	FBgn0038893	
FBgn0025628	FBgn0035258	FBgn0043841	FBgn0040308	FBgn0033837	FBgn0039303	
FBgn0030608	FBgn0015509	FBgn0035917	FBgn0002741	FBgn0000278	FBgn0030999	
FBgn0052130	FBgn0033139	FBgn0020224	FBgn0010612	FBgn0038658	FBgn0036115	
FBgn0026749	FBgn0039873	FBgn0031611	FBgn0047038	FBgn0040634	FBgn0045038	
FBgn0035839	FBgn0010622	FBgn0032156	FBgn0038029	FBgn0042112	FBgn0035871	
FBgn0037943	FBgn0026753	FBgn0037643	FBgn0030883	FBgn0031691	FBgn0032464	
FBgn0029714	FBgn0013749	FBgn0038056	FBgn0031050	FBgn0039672	FBgn0034694	
FBgn0033079	FBgn0039541	FBgn0038115	FBgn0050217	FBgn0029501	FBgn0034819	
FBgn0036068	FBgn0054035	FBgn0053715	FBgn0005655	FBgn0052944	FBgn0032074	
FBgn0015781	FBgn0014163	FBgn0022984	FBgn0041711	FBgn0030245	FBgn0026738	

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