

CTCF CONTRIBUTES TO THE REGULATION OF THE RIBOSOMAL DNA IN

Drosophila melanogaster

A Dissertation

by

PAOLA ANDREA GUERRERO

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

December 2011

Major Subject: Biochemistry

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Approved by:

Co-Chairs of Committee,	Keith Maggert Geoffrey Kapler
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ABSTRACT

CTCF Contributes to the Regulation of the Ribosomal DNA in *Drosophila melanogaster*. (December 2011)

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The 35S rDNA gene clusters on the X and Y chromosomes of *Drosophila melanogaster* are repeats of approximately 150 to 225 copies. Each are transcribed as a single unit by RNA Polymerase I and modified into the 18S, 5.8S, 2S and 28S ribosomal rRNAs. Reduction in the array copy number results in a bobbed phenotype, characterized by truncated bristles and herniations of abdominal cuticle, due to a decrease in protein production. In some copies within the arrays, R1 and R2 retrotransposable elements are inserted in a conserved region of the 28S gene which represses the transcription of a functional rRNA. Inserted arrays are transcribed at very low levels, but it is not clear how they are identified for repression. Similarly, a subset of uninserted arrays are silenced, and the epigenetic mechanism controlling how this decision is made it is also unknown. The CCCTC binding factor (CTCF) is a boundary element binding protein and a transcriptional regulator found in the nucleolus of

differentiated mammalian cells, whose localization requires poly (ADP-ribose)ylation. We investigated whether CTCF might be involved in the regulation of rDNA expression in *Drosophila*. Our data show that CTCF is found at the nucleolus of both polytene and diploid nuclei, and we have identified binding sites in the 28S gene, R1 and R2 elements by a bioinformatic approach. ChIP data indicate that CTCF binds only to the site in the R1 retrotransposon. Reduction of CTCF or members of the poly(ADP-ribose)ylation pathway by RNAi in S2 cells causes an increase in the amount of 35S rDNA gene, R1, and R2 transcripts. In flies, CTCF and PARG mutant alleles show disrupted nucleoli and increased rRNA transcripts. Mutant alleles of CTCF suppress variegation of a P-element inserted in a 35S *rDNA* array, but not of elements inserted elsewhere in the genome. Consistent with a role for CTCF in rRNA regulation, we found that during oogenesis CTCF is recruited to the nucleolus of nurse cells at early stages when the demand of ribosomes is low and it leaves this compartment in later stages when the cell increases rRNA production. We conclude from these studies that CTCF acts as a regulation of rDNA transcription by RNA polymerase I.

DEDICATION

To my family and my dearest Selçuk

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CHAPTER I

INTRODUCTION

Problem Statement

Ribosomal DNA transcription regulation is crucial for cell survival. Different mechanisms are involved in finding a balance between transcription rate and rDNA copy number. This dissertation studies the role of a specific protein, CTCF, in the regulation of *Drosophila* ribosomal DNA.

Significance of This Work

CTCF is a protein that has multiple functions in the regulation of gene expression in non-nucleolar chromatin, this work shows the role of CTCF at the nucleolus and more specifically at the ribosomal DNA loci. Similarly, we identified a novel mechanism for rDNA transcription repression and the components involved in this process. Lastly, we were able to identify how CTCF is recruited and maintained at this subnuclear compartment.

Chromatin Compaction

The genome in a higher eukaryote is packaged in a complex called

This dissertation follows the style and format of *Proceedings of the National Academy of Sciences*.

chromatin which is formed by DNA, RNA, histones and non-histone proteins [1]. The most basic level of chromatin compaction is the nucleosome which consists of approximately 146 base pairs wrapped around a histone octamer, formed by two copies each of the four core histone proteins (H2A, H2B, H3, and H4) (Fig 1.1). The structure of core histones consists in three alpha helices separated by two loops and an unstructured and flexible N-terminus. The organized part of the structure is found inside the nucleosome while the N-terminus faces out. The N-terminus is subject to post-translational modifications such as acetylation, methylation, ubiquitination, biotinylation, phosphorylation, ADP ribosylation and sumoylation [2]. In the next level of chromatin organization, based on in vitro studies, binding of histone H1 helps to stabilize the 30nm chromatin fiber [3]. However, in reality, this structure seems to be rarely found, since advance electron microscopy has been unsuccessful to identify significant amounts of the 30nm chromatin fiber.

Alternatively, Bas van Steensel and colleagues have postulated three mechanism involved in chromatin folding [4]. The first one is the *local chromatin compaction* which, in electron micrographs, looks like patches of densely stained chromatin. These areas are composed of aggregated nucleosomes stabilized by histone H1 and Polycomb proteins. The second mechanism is *long-range interactions* between loci that are distant on the same chromosome or that are located in different chromosomes. This follows a distinct pattern; active

chromatin areas interact with themselves and inactive chromatin areas interact with themselves.

Long range interactions are maintained by factors such as, insulator proteins (e.g. CTCF), polycomb proteins, cohesins, and transcription regulators. The third mechanism is the *attachment to nuclear landmarks* such as nuclear laminas, nuclear pore complexes, and the nucleolus. Nuclear laminas are found at the inner nuclear membrane, forming lamina-associated domains (LAD). Most genes found at these domains are silent.

Nuclear pore complexes (NPC) bind genomic regions that have been identified in flies, yeast and mammals; these DNA sequences do not overlap with LAD targets and their binding is transient since they are often found at the nucleoplasm. Nucleolus associated domains (NAD) anchors gene families involved in specific processes, such as development and odor reception. NAD and LAD targets overlap which predicts that these genomic regions interact with both nucleolus and nuclear lamina [5].

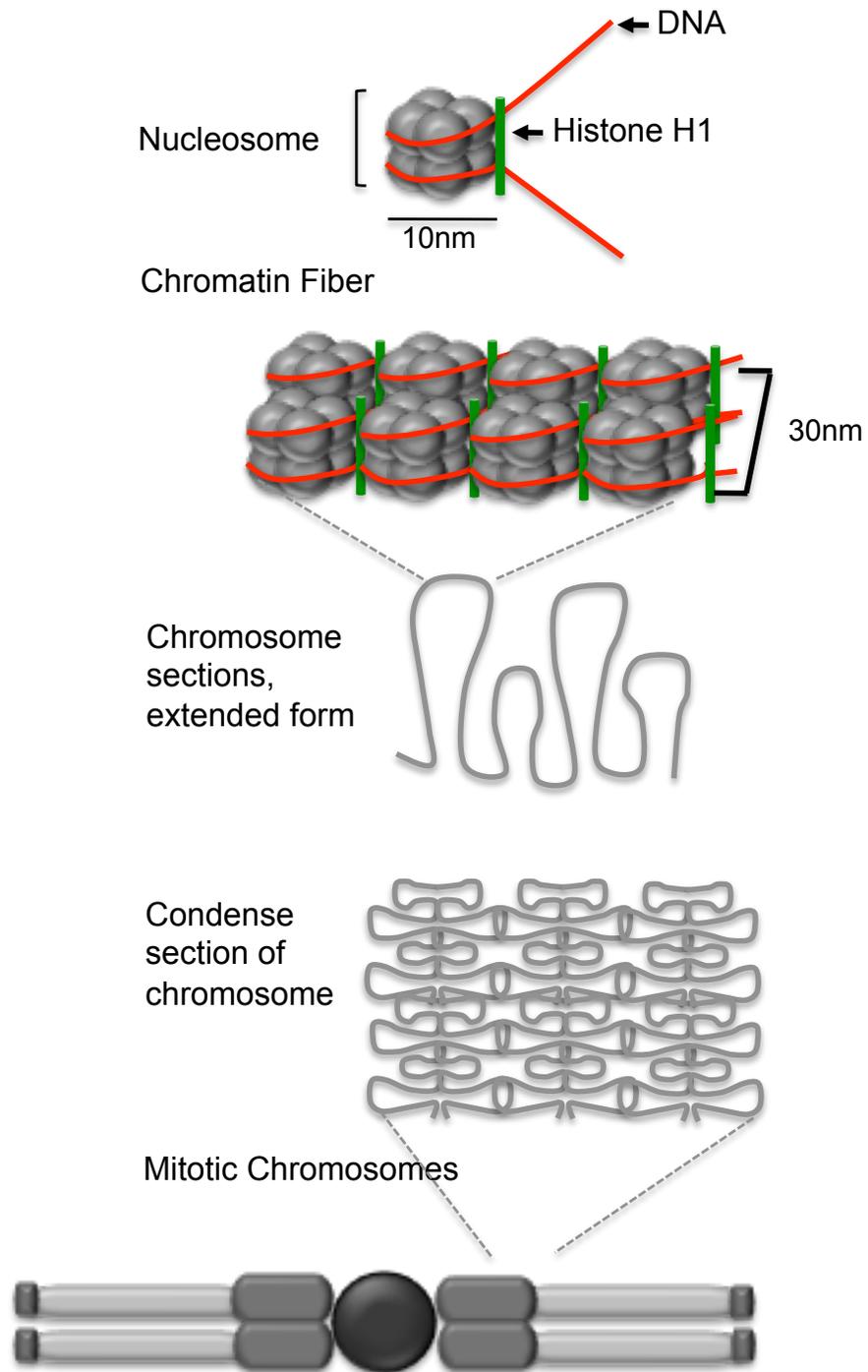


Figure 1.1 Levels of chromatin compaction. Adapted from [6]

Chromatin Types

Heterochromatin and euchromatin are the most recognized chromatin classification. Heterochromatin, is the most condensed type and it is found at the nucleolus organizer regions, pericentromeric regions, telomeres, and mating loci in yeast. It is characterized for having few transcribed genes, with the exception of the ribosomal DNA (rDNA) loci, and being enriched in repetitive sequences. It is also known to be late replicated, and to have reduced accessibility to DNase treatment. Similarly, it has regularly spaced nucleosomes, which contain hypoacetylated histones, and are enriched in methylated Lysine 9 of histone H3 [7,8]. In contrast, euchromatin, is the less condensed form, is gene rich, and early replicated. It is also sensitive to DNases, and have nucleosomes placed loosely which contain acetylated histones H3 and H4, and are enriched in histone H3 Lysine 4 methylation [7].

In a broad sense, heterochromatin and euchromatin are the two main chromatin states; however, a more detailed view divides euchromatin into constitutive euchromatin and facultative heterochromatin. Constitutive euchromatin is the place for housekeeping genes which are ubiquitously expressed and are required for processes such as, replication, cell cycle control and transcription. These genes are found in R bands, obtained by staining metaphase chromosomes with trypsin or acid-base-warm salt treatments, while tissue-specific genes are localized in G-bands and constitute facultative heterochromatin. Heterochromatin is also divided into β and α heterochromatin

both of which reside in the C-bands. β -heterochromatin, includes areas rich in repetitive sequences and where few genes are found, while α -heterochromatin describes genes that require the heterochromatic state to be transcribed such as, the ribosomal DNA. It also includes retrotransposons and genes with very large introns [7].

Bas van Steensel and colleagues introduced a different view of chromatin classification based on the mapping of binding sites for 53 chromatin proteins in *Drosophila* Kc culture cells [9]. Five chromatin types were identified: yellow, red, blue, black and green. Yellow and red chromatin are areas with high gene occupancy. Yellow has ubiquitously transcribed genes while red constitutes tissue-specific genes. Blue chromatin is characterized by the binding of polycomb group proteins, involved in gene silencing during development, and it is enriched in histone H3 lysine 27 trimethylation, a known histone mark required for the recruitment of these proteins. CTCF is found in red and blue chromatin. Black chromatin includes silent tissue-specific genes and represents around two-thirds of *Drosophila* silent genes. Green chromatin is bound by HP1 and histone H3 lysine 9 methylation which used to be called heterochromatin. However, recent studies have shown that HP1 bound chromatin is mostly active [10] which makes green chromatin a more neutral term than heterochromatin.

Nucleolar Structure

The nucleolus is a non-membrane-bound nuclear body whose major function is to synthesize ribosomal components. In 1781, Felice Fontana first described the nucleolus as “an specific spot inside the nucleus” [11]. Many years later, looking at thin sections of amphibian oocytes, Oscar Miller observed that this compartment was subdivided in a fibrous cortex which was surrounded by a granular cortex [12]. In 1973, L. R. Orrick identified Nucleolin, which was later found to be localized at the fibrillar component of the nucleolus [13]. In the following years, electron microscopic studies helped to disentangle the three major nucleolar components: the fibrillar center (FC), dense fibrillar component (DFC), and the granular component (GC) (Fig 1.2). rDNA transcription seems to take place at the fibrillar center [14,15,16,17] or at the boundary between FC and DFC [18,19]. Once the rDNA is transcribed, the RNAs are post-transcriptionally modified at the DFC by small nucleolar ribonuclear proteins (snoRNPs) and processing factors, leading to the generation of individual 18S, 5.8S, 2S and 28S. In the granular component, ribosomal subunits are assembled and almost completely mature ribosomes are exported to the cytoplasm. The mechanism used to export these ribosomes is not completely known, but there is evidence that the 60S subunit is transported to the cytoplasm by simple diffusion [20]. On the other hand, motor proteins such as Actin and Myosin have been found at the nucleolus, and they may also be involved in this process [21].

The function of ribosomal genes is not restricted to building ribosomes. Recent studies have shown that these genes are involved in the regulation of non-nucleolar chromatin. For instance, reduction in rDNA copy number is involved in the attenuation of silencing of a marker gene inserted somewhere in the genome and mutants of heterochromatin proteins are known to have short rDNA arrays [22,23]. Similarly, alterations in rDNA transcription is a common characteristic of cancer cells. For instance, increase in nucleolar size indicates upregulation in rDNA transcription and correlates with the uncontrolled growth of cancer cells. In addition, in *S. cerevisiae*, repression of rDNA homologous recombination by Sir2 increases longevity [24].

We observed CTCF, a protein that has multiple roles in regulation of gene expression, at the nucleolus of salivary glands polytene nuclei and we wondered whether it had a role in rDNA transcription regulation. Work done in mammalian cell culture by Torrano and colleagues [25] showed that after inhibiting RNA polymerase I transcription, CTCF was no longer found at the nucleolus and that after tethering CTCF to the nucleolus, rRNA transcripts decreased. These initial observations showed that CTCF was involved in repressing rDNA transcription. However, direct rDNA binding and a loss-of-function phenotype needed to be showed to confirm this result. Initially, we studied the roles CTCF plays in gene expression regulation.

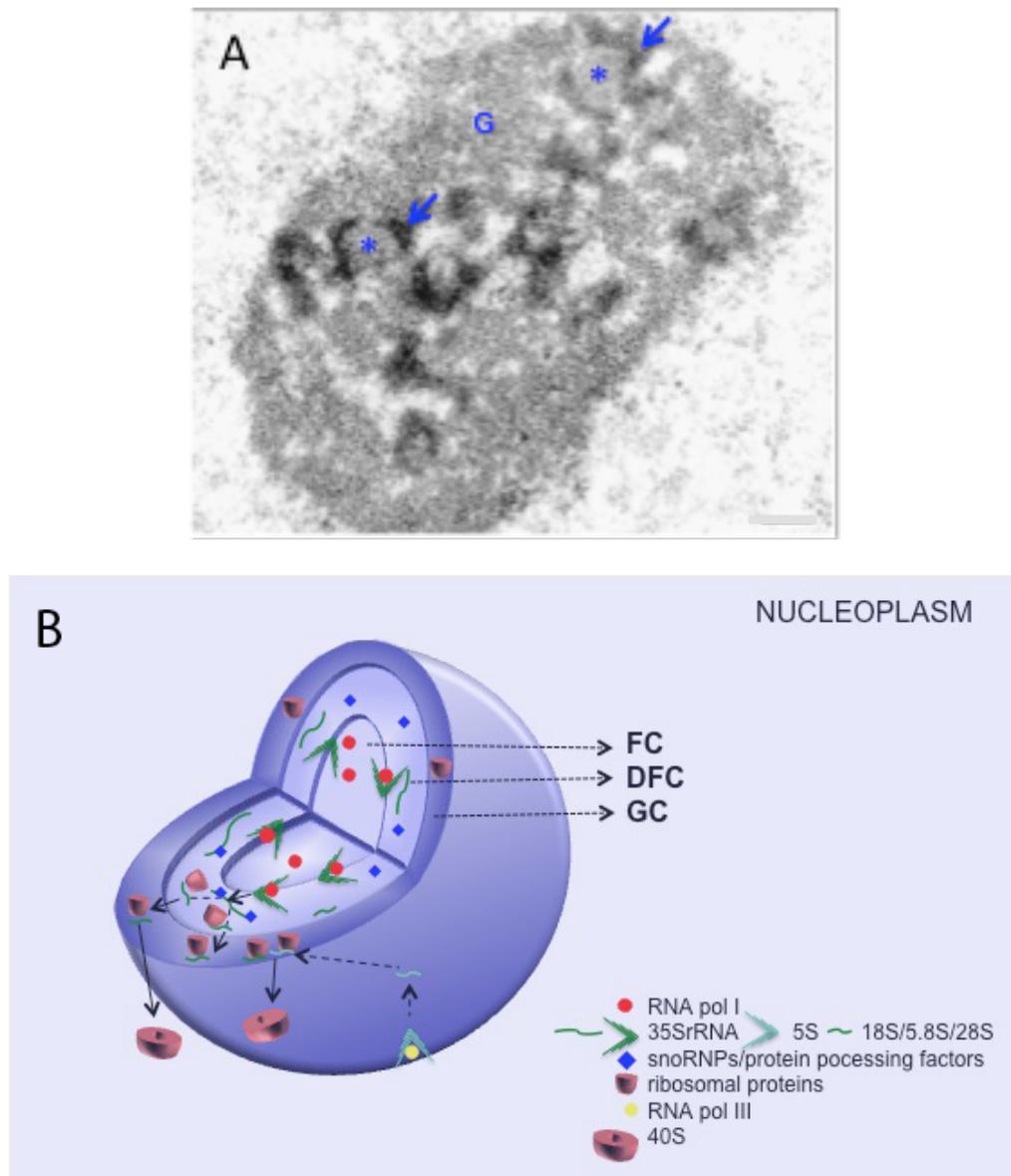


Figure 1.2 The nucleolus. A) Electron microscopy of HeLa cells, fibrillar center (asterisks), dense fibrillar component (arrow), granular component (G) [26]. B) RNA polymerase I is enriched at the FC, snoRNPs are accumulated at the DFC. Adapter from [27].

The CCCTC Binding Factor (CTCF)

CTCF is a 93.13 kDa (flies) and 82.8 kDa (humans, chickens, mice and frogs) 11 zinc finger nuclear protein (Fig 1.3) that has multiple roles in gene expression and utilizes different zinc finger combinations to bind its target sequences [28]. It can be phosphorylated, poly(ADP-ribosyl)ated and SUMOylated. Phosphorylation of its C-terminal is related to transcriptional repression and cell growth inhibition [29]. Poly(ADP-ribosyl)ation of its N-terminal keeps CTCF at the nucleolus [30], it is involved in maintaining imprinting of around 140 mouse CTCF targets [31] and absence of CTCF poly(ADP-ribosyl)ation is associated with breast tumor and cell proliferation [32]. SUMOylation of its amino and carboxyl terminal contributes to its repressive function at the *c-myc* P2 promoter since mutations in the amino (MKTE) and carboxyl (VKKE) motifs showed a decrease in repression of a luciferase reporter construct [33].

CTCF was discovered in 1990 by Lobanenko and colleagues as a repressive binding factor of the chicken *c-myc* gene promoter [34,35]. Later in 1996, Filippova and colleagues obtained a similar result while studying the promoters of human and mouse *c-myc* genes [28]. At the same time, a work published in 1990 by Baniahmad and colleagues described a negative protein 1 (NeP1) that binds a silencer module found -2.4 kb upstream of the transcription start site (TSS) of the chicken lysozyme gene [36]. NeP1 was later cloned and characterized and it is unveiled to be CTCF [37]. In addition, Vostrov and

Quitschke in 1997 showed that CTCF was a transcription activator of the amyloid β -protein precursor [38]. These two discoveries would have indicated that CTCF was just a classical transcription factor. However, in 1999 Bell and colleagues found a new role for CTCF. Upstream of the chicken β -globin gene promoter there was a 42 bp fragment which was responsible for preventing enhancer-promoter interaction and binding of CTCF was required for this enhancer-blocking activity [39]. Similarly, CTCF was reported to be involved in the maintenance of X chromosome inactivation and in the expression of genes that escape X inactivation in mammals [40].

Apart from its role as transcriptional repressor, activator and enhancer blocker, CTCF can also buffer genes from repressive chromatin. Genome mapping of CTCF binding sites in vertebrates yielded around 26,000 sites. A subset of them were identified at the transition zones between active and repressive chromatin. A different subset were flanking genes that are co-transcribed such as; the 5S rDNA gene cluster, and at the promoter of loci involve in metabolism, apoptosis, neurogenesis and many other cellular processes [41,42,43]. Similarly, CTCF mediates chromatin-chromatin interactions in *cis* and in *trans* and it associates with nucleophosmin at two nuclear landmarks: the nucleolus and the nuclear lamina. These intra- and inter-chromosomal interactions have been detected by techniques such as, fluorescence in situ hybridization (FISH) and chromosome conformation capture (3C) [44].

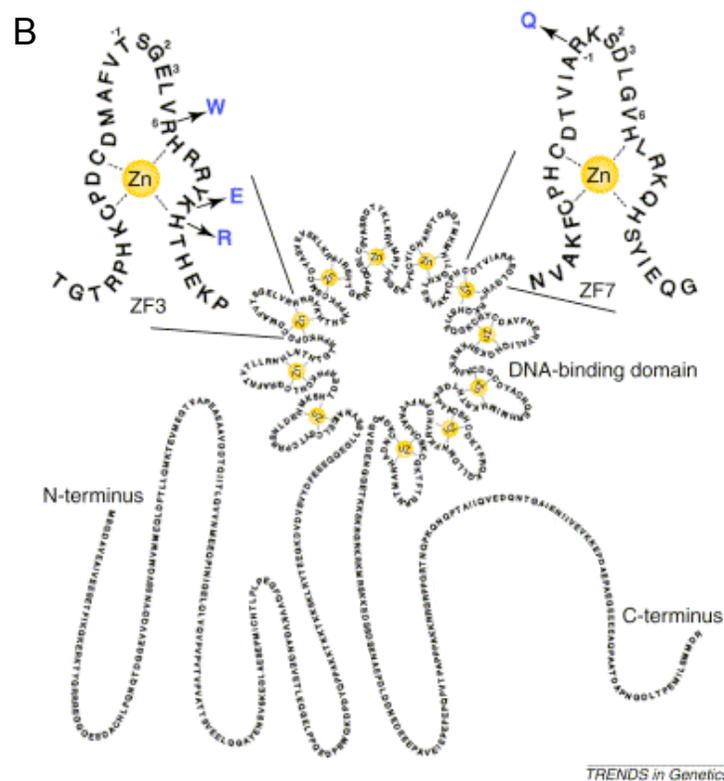
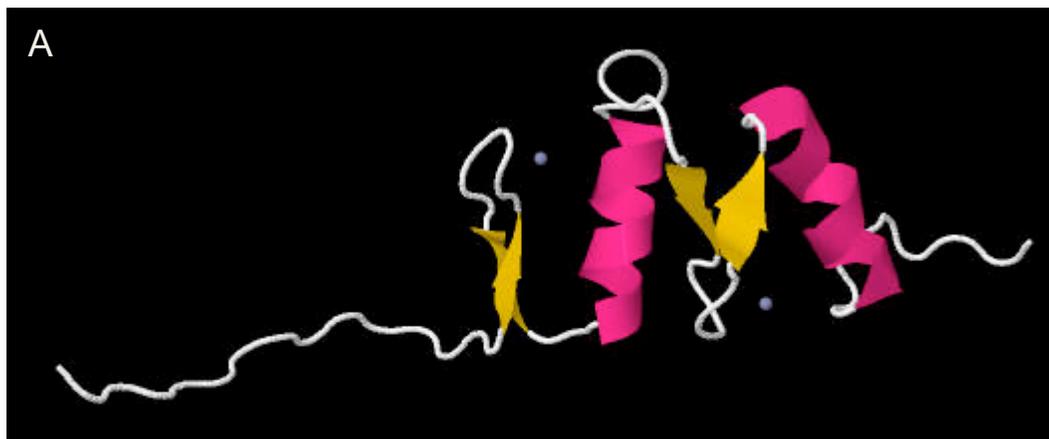


Figure 1.3 CTCF. A) Structure of Zinc Finger Domain of CTCF by Solution NMR, Protein Data Base ID: 2CT1, 2 zinc atoms are shown. This domain is characterized by one alpha helix, two beta strands and a hairpin loop. B) Representation of the 11 Zinc fingers domain, blue amino acids represent residue substitutions involved in tumorigenesis, DNA binding domain (KKRRGRP) is also shown [45].

One of the regions where CTCF is involved in maintaining intra-chromosomal interactions is at the *Igf2-H19* locus (Fig 1.4 A), where the paternally expressed *insulin-like growth factor 2 (Igf2)* gene is located upstream of the maternally expressed *H19*, a noncoding RNA gene. In this locus, a differentially methylated region (DMR) binds CTCF in the maternal allele, when DMR is unmethylated, and it is absent in the paternal allele, when DMR is methylated. Feil and colleagues inserted three binding sites for Gal4 (UAS), a transcriptional regulated protein from yeast, into the DMR H19. In a different construct, Gal4 was fused to a unique tag (MYC). By using anti-MYC antibodies in chromatin immunoprecipitation, they were able to identify regions from the *Igf2-H19* locus that associated with the DMR H19.

They found that DMR H19 region contacted DMR1 of the *Igf2* gene in the maternal chromosome whereas a different region (DMR2) was found to be associated with DMR in the paternal chromosome [46]. From these results, they proposed a model in which in the maternal allele unmethylated DMR binds DMR1 leaving *Igf2* inside a “silent loop” and bringing H19 and the downstream enhancers together (Fig 1.4 B). The DMR-DMR1 complex is maintained by CTCF, which is known to bind nucleophosmin, a protein that is bound to the nuclear matrix. On the paternal allele, DMR associates with DMR2 which brings *Igf2* and the downstream enhancers close, in this allele DMR is methylated; therefore, another protein different than CTCF is perhaps binding and stabilizing the complex (Fig 1.4 C). A study done by Weber and colleagues in 2003,

showed that DMR2 associates with the nuclear matrix in cells where *Igf2* is expressed [47]. These experiments showed that in this imprinted locus, looping is isolating undesired enhancer-promoter interactions and facilitating desired associations, and perhaps nuclear matrix interactions are maintaining the integrity of these loops.

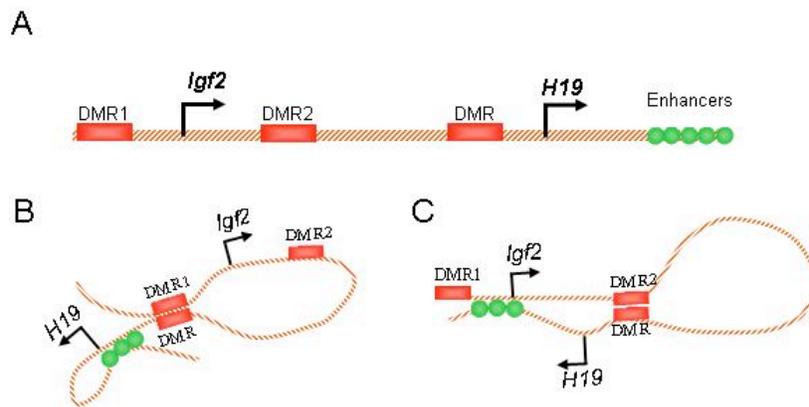


Figure 1.4 Looping at the *Igf2-H19* locus. A) The *Igf2-H19* imprinted locus, DMR: Differentially methylated regions. B) Maternal allele. C) Paternal allele. (Adapted from [48])

In mammalian cell culture, CTCF has been cytologically localized at the nucleolus, in most of the cells. In undifferentiated cells, it is excluded from the nucleolus. In mitosis, it is associated with chromosome arms, centrosome and midbody [49]. However, it is unknown whether in other organisms this protein can be recruited earlier in development. In *Drosophila melanogaster* CTCF was characterized by Moon and colleagues in 2005 [50]. Their sequence alignment showed the expected 11 zinc fingers which were very similar in sequence to human CTCF. They also observed *in vitro* and *in vivo* binding of CTCF to a sequence that possesses enhancer-blocking activity, Front abdominal 8 (Fab-8). This sequence was tested in vertebrate cells and flies and in both cases CTCF acted as an enhancer blocker. The majority of work done in *Drosophila* CTCF has been concentrated in its function as boundary element at euchromatic areas. We explored its role at the nucleolus and more specifically in rDNA transcription.

Ribosomal DNA Transcription

The first images of rDNA transcription came from the work done by Oscar Miller. He showed individual 35S units and their associated transcripts (Fig 1.5) [12]. In *Drosophila melanogaster*, the 35S rDNA gene clusters or NOR (nucleolus organizer regions) are located at the proximal heterochromatin of the X chromosome (heterochromatic band H29) and on the short arm of the Y chromosome (heterochromatic band H20) (Fig 1.6). They are repeats of

approximately 150 to 225 copies and each 35S rDNA contains 8120 base pairs. Each single unit is transcribed by RNA polymerase I (pol I) and processed and modified into 18S, 5.8S, 2S and 28S gene products (Fig 1.6). The recombination rate between the rDNA cluster in the X chromosome and the one on the Y is low due to the differences in their sequence. For instance, the non-transcribed spacer is different in size and frequency, there are base substitutions in the 18S gene and the X chromosome array is more enriched in R1 elements (explained in more detail later) [51].

In *Drosophila melanogaster*, the 5S rDNA gene cluster, which is transcribed by RNA pol III, is found outside of the 35S rDNA array and it is located at the right arm of chromosome two (euchromatic band 56E2). In higher eukaryotes, the 5S rDNA is found in a different location than the 35S rDNA. In contrast, in lower eukaryotes such as: *Saccharomyces cerevisiae*, *Torulopsis utilis*, *Dictyostelium discoideum* and in bacteria, this gene is intercalated with the 35S rDNA [52,53].

In mammalian cells, transcription by RNA polymerase I requires the binding of an upstream binding factor (UBF) and a promoter selective factor (TIF-IB/SL1) which yields the formation of the pre-initiation complex [54]. TIF-IB/SL1 is a protein complex that contains the TATA binding protein and five associated factors. UBF recruits RNA polymerase I, stabilizes TIF-1B/SL1 binding and removes histone H1 which leads to rDNA transcription [55,56]. It is

also involved in RNA polymerase I elongation [57] and its binding through the entire rDNA unit implies its involvement in keeping an open chromatin state [54].

rDNA transcription is not constant through the cell cycle; the highest rate of transcription is found in S and G2 phase. During mitosis rDNA transcription stops and the nucleolus is disassembled. rDNA transcription is later reactivated in G1[54]. At prophase, cyclin B levels increases, causing the phosphorylation of pol I initiation complex, and leading to the dissociation of several pol I subunits. Then, rRNA processing factors, such as Nucleophosmin and Fibrillarin, leave the nucleolus. UBF is one of the few factors known to stay bound to rDNA during mitosis [27].

rDNA Transcription Regulation

There is a redundancy in the number of copies found at the rDNA array and only the transcription of a subset of them is required. Maintaining a balance between active and silent copies is critical for cell survival, changes in rRNA synthesis is characteristic of tumor cells; hence, new cancer therapeutics are focus in inhibiting RNA polymerase I transcription [54].

A



B

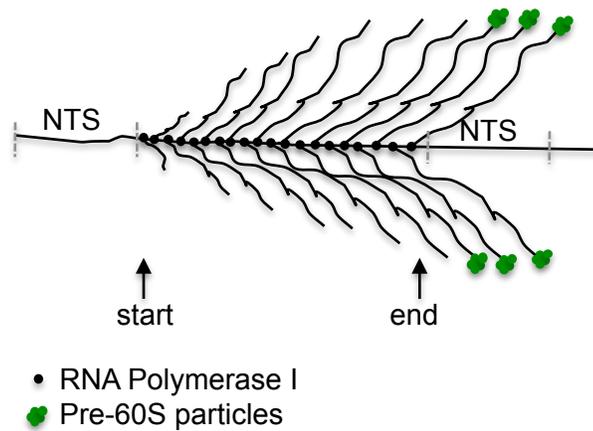


Figure 1.5 The 35S rDNA. A) Miller spread. B) Components of a Miller Spread: showing start and end of transcription of a single rDNA unit transcribed multiple times by different RNA polymerase I molecules and as a result many pre-rRNA transcripts are visualized at any time [58].

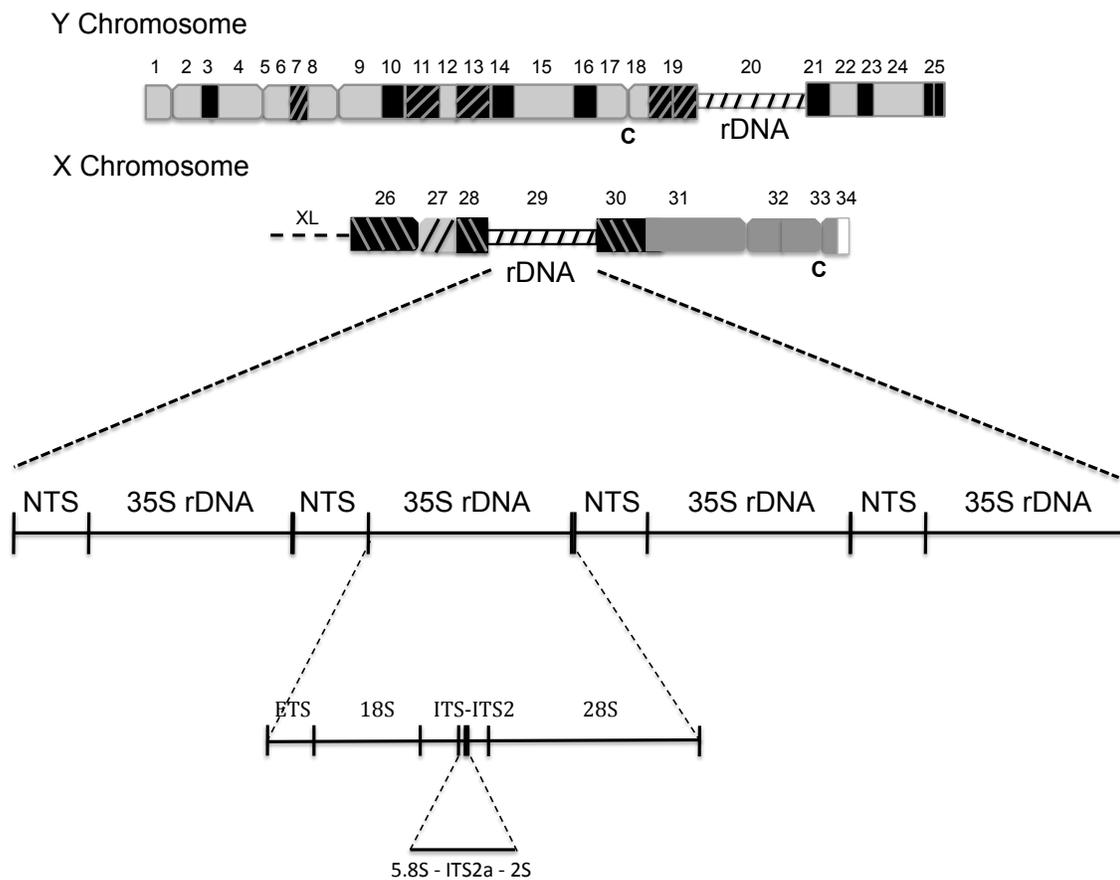


Figure 1.6 *Drosophila melanogaster* 35S rDNA arrays are located in X and Y chromosomes. Y and X chromosomes heterochromatic bands are depicted and numbered, centromeres (C) and rDNA loci location are shown. A 35S rDNA repeat is also shown. NTS: non-transcribed spacer, ETS: external transcribed spacer and ITS: internal transcribed spacer. X and Y chromosomes were adapted from [59].

rDNA transcription can be controlled in two ways: by decreasing the number of rDNA copies or by controlling the number of copies that are actively transcribed. Binding of UBF, NoRC and RENT affect rDNA transcription. Phosphorylation of the Upstream Binding Factor (UBF) plays an important role in rDNA transcription. Hence, its hypophosphorylation indicates transcription silencing in quiescent cells, and its phosphorylation corresponds to transcription activation [60,61]. Similarly, acetylation of UBF is related to rDNA transcription.

The Nucleolar Remodeling Complex (NoRC) is involved in the epigenetic silencing of the rDNA. It is formed by TIP5 (TTF-I interacting protein 5) and SNF2h. TTF-I is a key player in re-establishing RNA polymerase I transcription after replication. It is involved in transcription termination of the rDNA by binding to downstream termination sequences, and it mediates arrest of the replication fork. Once DNA replication occurs, its binding influences Pol I transcription [62].

The Regulator of Nucleolar Silencing and Telophase Exit (RENT) complex is formed by Sir2, Net1, Nan1 and Cdc14. Sir2, originally described in the silencing of yeast sub-telomeric regions, is involved in the silencing at the rDNA locus and it requires Net1 for its recruitment. Net1 also associates with Cdc14 and Nan1 [63]. Cdc14 is a phosphatase involved in the regulation of mitosis exit by dephosphorylating Cdk1 [64]. Work done by Huang and Moazed demonstrated that Net1 and Sir2 bind two sites at the *S. cerevisiae* 35S rDNA; the first one lays at the non-transcribed spacer 1 (NTS1), the second one is located at the 35S rDNA promoter and goes inside the 35S gene. They also

showed that these two proteins interact with RNA polymerase I and that this pathway seems to be independent of the binding of the RENT complex to FOB1, a protein required for rDNA recombination and therefore array size regulation [65].

In mammals and plants, DNA methylation affects rDNA transcription. Previous studies done in mouse and rat showed that the proportion of active/inactive *rDNA* genes compared to the proportion of methylated/unmethylated *rDNA* genes was the same [66,67]. Likewise, methylation of CpG at position -133 of the rDNA gene prevented binding of UBF and as a consequence, transcription was impaired [68]. In human cell lines, chromatin immunoprecipitation against pol I followed by bisulphite sequencing identified a fraction of active unmethylated promoters bound by RNA polymerase I and UBF; while a different fraction of unmethylated promoters were bound by UBF only [69]. On the other hand, work done by Caburet and colleagues in 2005 showed that one-third of rDNA genes in humans are not arranged in the regular 18S to 28S direction, instead they form palindromes (18S-28S-28S-18S). Although their methylation status is unknown, it is possible that this particular conformation makes them more susceptible to methylation [70].

Histone modifications also influence rDNA transcription. Active rDNA promoters are enriched in histone H3 with methylated lysine 4 (H3K4) and in trimethylated histone H3 lysine 36. Similarly, acetylated H4K16, H3K27, and H3K9 are also present at active rDNA promoters. On the contrary, silent rDNA

promoters are enriched in methylated H3K9, H4K20, and H3K27. Studies performed at the rDNA promoters of four different human cell types indicated that active marks are more conserved among the four cell types while repressive marks have a low degree of correlation [71,72].

Recent studies, indicated that tumor suppressors play a role in down regulating rRNA synthesis [54]. The retinoblastoma susceptibility (Rb) gene, pocket protein 130 (p130), TP53, phosphatase and tensin homolog deleted in chromosome ten (PTEN) and p14ARF, are some of the genes involved in this process. Likewise, CTCF has been postulated as a tumor suppressor gene because of its role in cell differentiation, cell cycle arrest and apoptosis [73].

An alternative mechanism to indirectly regulate rDNA transcription is the variation in rDNA array size. In *Drosophila*, reduction of rDNA copy number causes the bobbed phenotype due to a decrease in protein production. Mutants in factors involved in maintaining the silent state of the rDNA such as, Su(var)3-9, showed a decrease in rDNA copy number [22,23]. This phenotype can be alleviated by rDNA magnification which occurs during mitosis and meiosis when the rDNA of sister chromatids is unequally exchanged. rDNA reduction and magnification will be discussed in detail in Chapter V.

In *Drosophila melanogaster*, except for DNA methylation, rDNA transcription regulation involves all these pathways. However, it has an additional mechanism, it is inserted by R1 and R2 retrotransposable elements.

R elements

In 1977 four different laboratories identified intervening sequences interrupting the coding region of the ribosomal DNA in *Drosophila melanogaster* [74,75,76,77]. Similarly, Oscar Miller showed that there were active rDNA copies that were separated by long stretches of inactive chromatin, which based on size would correspond to a rDNA unit inserted by a 5 kb sequence (Fig 1.7) [78]. His work and other's showed that inserted copies are inactive or transcribed at very low levels [78,79,80]. In *Drosophila*, these intervening sequences were initially called type I and type II insertions and eventually were renamed R (for ribosomal) 1 and R2 elements. R elements are non-long terminal repeats retrotransposons found in at least five animal taxa (Fig 1.8) and inserted at different regions of the 35S rDNA, but mostly at the 28S rDNA. In *Drosophila melanogaster*, R1 and R2 are inserted in a conserved region at the 28S (approximately two thirds downstream from 28S 5' end) and R2 elements are inserted 74 base pairs upstream from R1. The size of R1 is 5356 base pairs and for R2 is 3607 base pairs. Although many 5' truncated versions of both elements exist, R2 elements are only found inserted at the 28S rDNA. In contrast, R1 elements are found at the 28S, centromeric chromatin and at chromosome 4 [81,82]. rDNA units that are inserted by R1 or R2 elements cannot produce a functional 28S rDNA. The rDNA locus at the X-chromosome possesses more R1 copies than the array on the Y-chromosome and approximately 60% of X-linked rDNA copies are inserted with R1 elements. R2 elements are found at lower

frequency in both rDNA arrays (15%) and double insertions are 5% on average [83]. While R1 elements have two open reading frames (ORF), R2 have only one. However, in both cases their ORF share the same transcription direction as the rDNA unit they are inserted on. Similarly, their genes code for reverse transcriptases (RT) [84]. R2 element sequences do not contain a promoter and they are co-transcribed with the 35S rDNA. Additionally, R2 encodes a self-cleaving ribozyme that is formed by the first 200 bp at the 5' end of the retrotransposon [85]. This enzyme has the ability to synthesize DNA at low dNTP concentrations and it has a high error rate; these two enzymatic activities makes it very similar to the human immunodeficiency virus type 1 (HIV-1) RT [86].

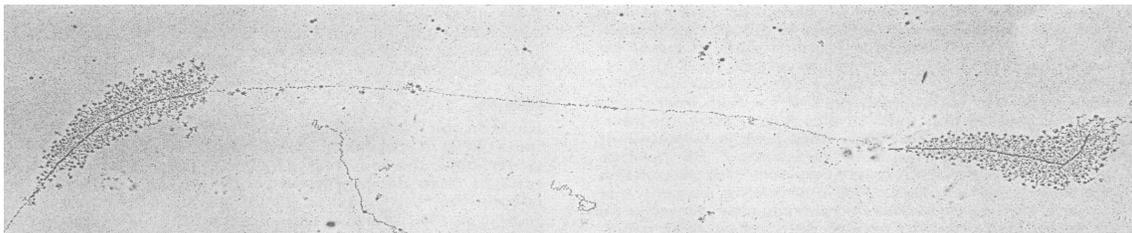
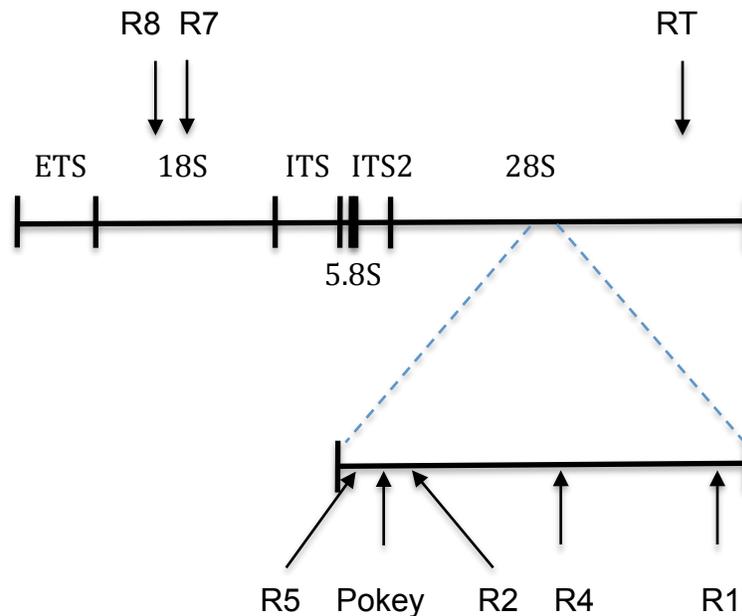


Figure 1.7 Electron micrograph published by Oscar Miller. Two active rDNA copies of about 8.3 kb in length separated by a long chromatin region of about 13.5 kb which could correspond to a silent rDNA unit inserted by a R1 element. [78].



- R1:** Arthropods.
R2: Arthropods, Cnidaria, Platyhelminthes, Tunicates, Vertebrates.
R4: Nematodes.
R5: Platyhelminthes.
R7: Arthropods.
R8: Cnidaria.
RT: Arthropods.
Pokey: Arthropods.

Figure 1.8 Distribution of R elements and other mobile elements in the 35S rDNA. Adapted from [87].

R elements are integrated into *Drosophila* 35S rDNA by using the target primed reverse transcription (TPRT) mechanism (Fig 1.9). It starts with the cleavage of the bottom strand, releasing a 3' hydroxyl followed by polymerization of cDNA by the R element reverse transcriptase (step 2, Fig 1.9); the next step is the cleavage of the top strand nine base pairs upstream from the bottom strand

cleaved site, and the release of a 3' hydroxyl (step 3, Fig 1.9); the final step is the polymerization of the second DNA strand (step 4, Fig 1.9). The result of this process is the integration of an R element and the deletion of nine base pairs from the insertion site (step 5, Fig 1.9) [88,89].

Previous work done by Eickbush and colleagues showed that inserted R1 and R2 rDNA copies can be lost from the rDNA array, presumably by recombination [83,90,91] and that transcription level of these inserted elements is directly proportional to their retro-transposition rate. Likewise, when crossing a line that has actively transcribed R2 inserted copies with a line that has low transcribed R2 inserted copies, the result is the silencing of the entire rDNA array coming from the parent with the active R2 inserted copies, causing a phenomenon described as nucleolar dominance [92] and originally observed in interspecific hybrids of amphibians, plants and flies.

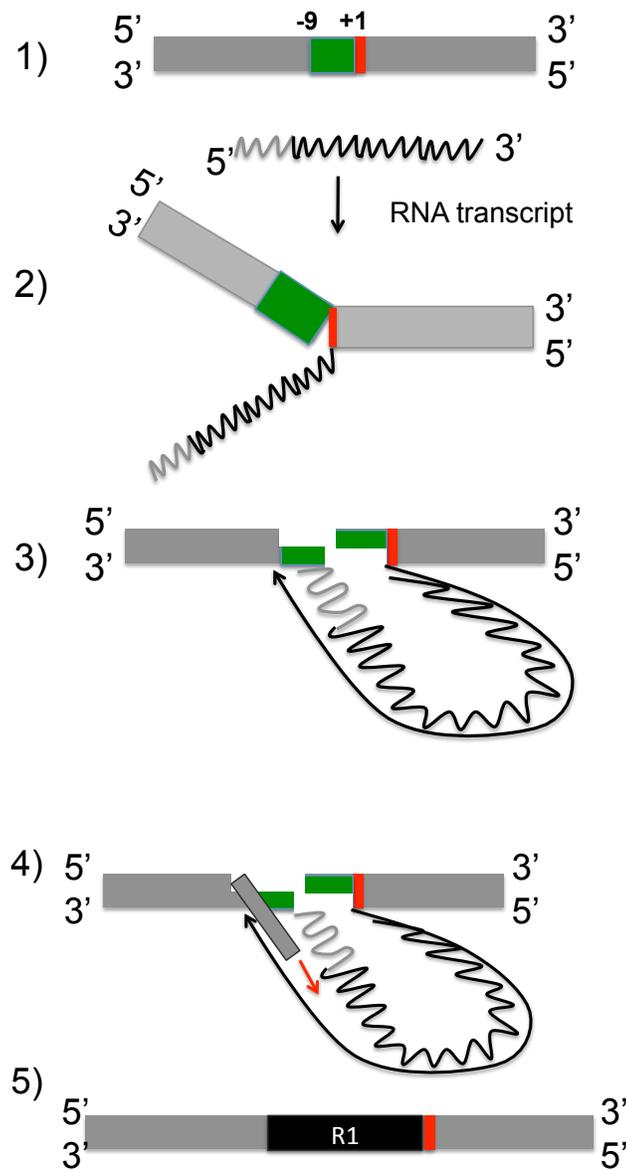


Figure 1.9 R elements integration into the 35S rDNA. 1) 28S rDNA fragment where R element will be integrated. +1 indicates the insertion site. 2) Bottom strand is cleaved 3) First DNA strand is synthesized by the R element Reverse Transcriptase and top strand is cleaved . 4) Synthesis of second DNA strand. 5) Final integration of the R element which generates deletion of nine base pairs (green region). Adapted from [89].

These findings showed how R-elements transcription can influence non-inserted units at the point of altering chromatin structure of the entire rDNA array. However, it is still unknown how the transcription of these R elements is regulated and which factors may be involved in the process.

Work done by Labrador and colleagues showed that in lines with silent R1 and R2 retrotransposons, these elements were mostly found at the bright DAPI areas of the nucleolus. These areas are thought to be highly condense and transcriptionally silent which would predict they will be enriched in inactive chromatin marks such as, histone H3 lysine 9 and histone H3 lysine 27 methylation. However, work done by Labrador and colleagues show almost complete absence of H3K9me at the nucleolus and partial staining of H3K27me at the condense nucleolar areas [93]. Therefore, it was unclear how R1 and R2 elements remained silent.

Aims of This Study

There is a clear connection between CTCF and nucleolar transcription. Likewise, previous observations done in mammals started disentangle the details of this relationship; however, the direct role of CTCF in the regulation of the ribosomal DNA and in nucleolar structure is still unclear. We would like to shed more light into this issue using *Drosophila melanogaster* as our model system. I pursue the following aims in this dissertation: 1) Determine if CTCF is generally involved in nucleolar regulation by determining its location in multiple

cell types, 2) Investigate the binding of CTCF to the rDNA, 3) Analyze the effects of CTCF loss-of-function mutations in rDNA transcription, rDNA array copy number, and nucleolar architecture; 4) Determine whether factors that are known to affect CTCF nucleolar recruitment, such as post-translational modifications, can also affect indirectly rDNA transcription. 5) Study whether mechanisms involved in rDNA silencing, such as nucleolar dominance, can be affected in CTCF loss-of function mutants.

CHAPTER II

CELLULAR LOCALIZATION OF CTCF

Introduction

CTCF plays multiple roles in the regulation of gene expression. It is known for having several interacting partners; it binds transcription enzymes (RNA polymerase II), transcription regulatory factors (Class II transactivator, Kaiso, Oct4, Regulatory factor X, YB1, and YY1), chromatin constituents (CHD8, Sin3A, Taf-1/Set, and H2A/H2A.Z), proteins involved in genome integrity (Poly(ADP-ribose) polymerase I), and nuclear architectural proteins (Nucleophosmin/B23, Cohesins, Lamin A/C and Topoisomerase II) [94]. The insulator role of CTCF has been extensively studied. However, more recent findings identified a fraction of CTCF at the nucleolus which has brought a different prospective of the role of CTCF in gene expression.

Yusufzai and colleagues studied factors that co-purified with CTCF using nuclear extracts from HeLa cells and identified nucleophosmin, a nuclear matrix protein which is abundant at the nucleolus, as the main interacting partner [95]. Similarly, using a transgene carrying a 5.8 Kb of the chicken HS4 insulator, they showed that in the presence of CTCF, this sequence is tethered to the nucleolar periphery. This work was the first to show CTCF at the nucleolus; however, their interpretations were limited to its role as insulator and in the formation of chromatin loops to regulate gene expression. Later, work done by Torrano and

colleagues [25] showed how upon cell differentiation CTCF was targeted to the nucleolus and that the zinc-finger domain was involved in its localization. They refined the sub-nucleolar location of CTCF to the dense fibrillar and granular components. Likewise, they observed that inhibition of pol I transcription removed CTCF from the nucleolus and overexpression of CTCF inhibited nucleolar transcription [25]. Although this work established a connection between CTCF and the rDNA, it lacked a mutant phenotype and did not clarify the role of this protein on the regulation of the rDNA.

Recent work done by Nobelen and colleagues has shown that CTCF binds upstream to the ribosomal spacer promoter and interacts with the Upstream Binding Factor (UBF), which is responsible for the formation of RNA polymerase I pre-initiation complex. Depletion of CTCF caused a reduction in transcription from the spacer region but it did not affect 18S rDNA transcription. The authors explained that CTCF may be involved in keeping rDNA copies poised for transcription [96].

In *Drosophila melanogaster* CTCF was found in approximately 300 to 400 sites on polytene chromosomes. These chromosomes come from cells where many rounds of endoreplication occur. In these cells, the centric heterochromatin do not endoreplicate and it bundles together forming the chromocenter. Polytene chromosomes have a particular banding pattern. They have bright compact bands and more loose dim areas forming the interbands, which are enriched on actively transcribed genes. CTCF is completely excluded from the

heterochromatic chromocenter and enriched at interbands [97]. The majority of work done in *Drosophila* CTCF has been concentrated in its function as boundary element at euchromatic areas. We initially observed CTCF at the nucleolus of polytene cells nucleoli and based on the previous work done by Torrano and colleagues [25], we decided to investigate the role of CTCF in the regulation of the rDNA in *Drosophila* to identify whether CTCF was acting as a repressor of rDNA transcription.

Results and Discussion

Cytological location of CTCF in *Drosophila melanogaster*

Previous work showed that differentiation was required for CTCF recruitment to the nucleolus, and so its regulation of the rDNA during development was unknown. We assessed multiple tissues and cell types in order to determine whether CTCF required differentiation to be visibly recruited to the nucleolus. Initially, we stained differentiated salivary gland polytene chromosomes with an antibody raised against the C-terminal of CTCF and kindly provided by Dr. Jumin Zhou.

We observed CTCF binding at euchromatic arms, as it was described before [97], and at the nucleolus (Fig 2.1). The nucleolus is composed of multiple chromatin structures, leading to two cytologically different DAPI-stained chromatin morphologies: condensed brighter threads and loose dim areas. This distinction is different from the fibrils and granules observed by electron

microscopy (ER); in order to distinguish the Fibrillar, Dense Fibrillar, and the Granular Component, immunofluorescence needs to be performed. For example, antibodies against Fibrillarin indicate the location of the dense fibrillarin component and antibodies against nucleophosmin/B23 identify the granular component.

Previous work done by the Labrador and colleagues showed that condensed bright areas represent inactive rDNA copies which are unbound by UBF, and partially bound by Heterochromatin Protein One 1 (HP1) and mono-methylated Histone H3 at lysine 27 (H3K27), a mark for repressive chromatin [93]. The loose dim areas and areas with non-DAPI staining represent actively transcribed rDNA copies which are bound by UBF, and enriched in open chromatin marks such as, acetylated H4K12, H4K8, H3K14 and phosphorylated H3S10. Similarly, R1 and R2 retrotransposons, inserted at the rDNA, partially colocalized with the brighter areas. These elements are known to be silent or transcribed at very low levels.

Drosophila CTCF was absent from the condensed DNA threads and associated with the lighter areas and with non-DAPI stained areas. Similarly, it colocalized with Fibrillarin, a protein involved in rRNA processing (Fig 2.2). These observations showed that the binding of CTCF in nucleolar and non-nucleolar chromatin seems to follow the same trend; it preferentially binds the loose dim and non-DAPI stained areas which in polytene chromosomes correspond to interbands. In non-nucleolar chromatin these areas are where

most genes are found and CTCF binding is required to insulate them from heterochromatin or to prevent enhancer-promoter communication. This predicts that the function of CTCF at the nucleolus would be also to act as an insulator between active and inactive rDNA units. If that is the case we would expect dCTCT binding at the nucleolus at the transition between active and silent chromatin marks as it has been reported in non-nucleolar chromatin. Therefore, we decided to look at the distribution of active or silent marks at the nucleolus. Labrador and colleagues have shown that histone H3 lysine 9 di-methylation, a common landmark for inactive transcription, was almost absent from the nucleolus. We wanted to explore whether any other type of H3K9 methylation was present by using an antibody that recognizes the total level of all three forms. However, H3K9 methylation was only found at the chromocenter and no signal was found at the nucleolus. On the other hand, di- and tri- methylated histone H3K4, an active chromatin mark, was found at the nucleolus, partially colocalized with CTCF and absent from the condensed DNA areas (Fig 2.3 B).

The nucleolus is a very complex compartment that not only contains rDNA genes but also around 700 proteins (based on humans studies). The fact that CTCF colocalizes with Fibrillarin and H3K4me indicates that perhaps this protein is involved in rDNA transcription and/or rRNA processing; however, its broad binding at the nucleolus anticipates multiple roles. In order to determine whether CTCF was involved in rDNA regulation we first needed to see it present

at the rDNA loci; hence, we looked at a stage of the cell cycle when the nucleolus disassembles: during mitosis.

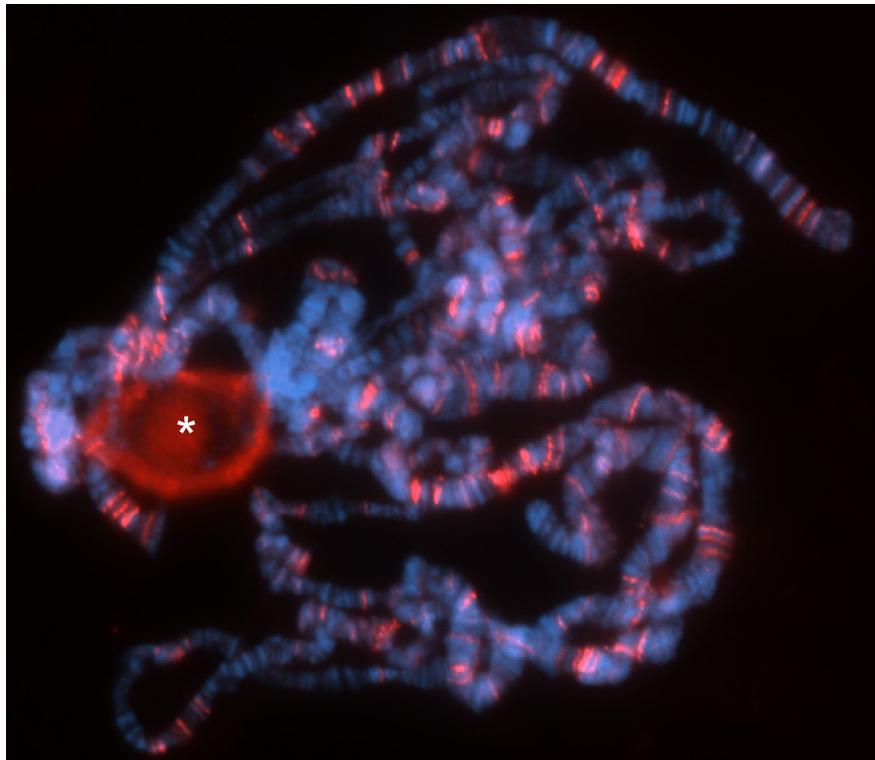


Figure 2.1 Epifluorescence microscopy of a salivary gland nucleus spread. Red shows CTCF staining in euchromatic arms and nucleolus (asterisk). DNA is stained with DAPI (4',6-diamidino-2-phenylindole) and shown in blue.

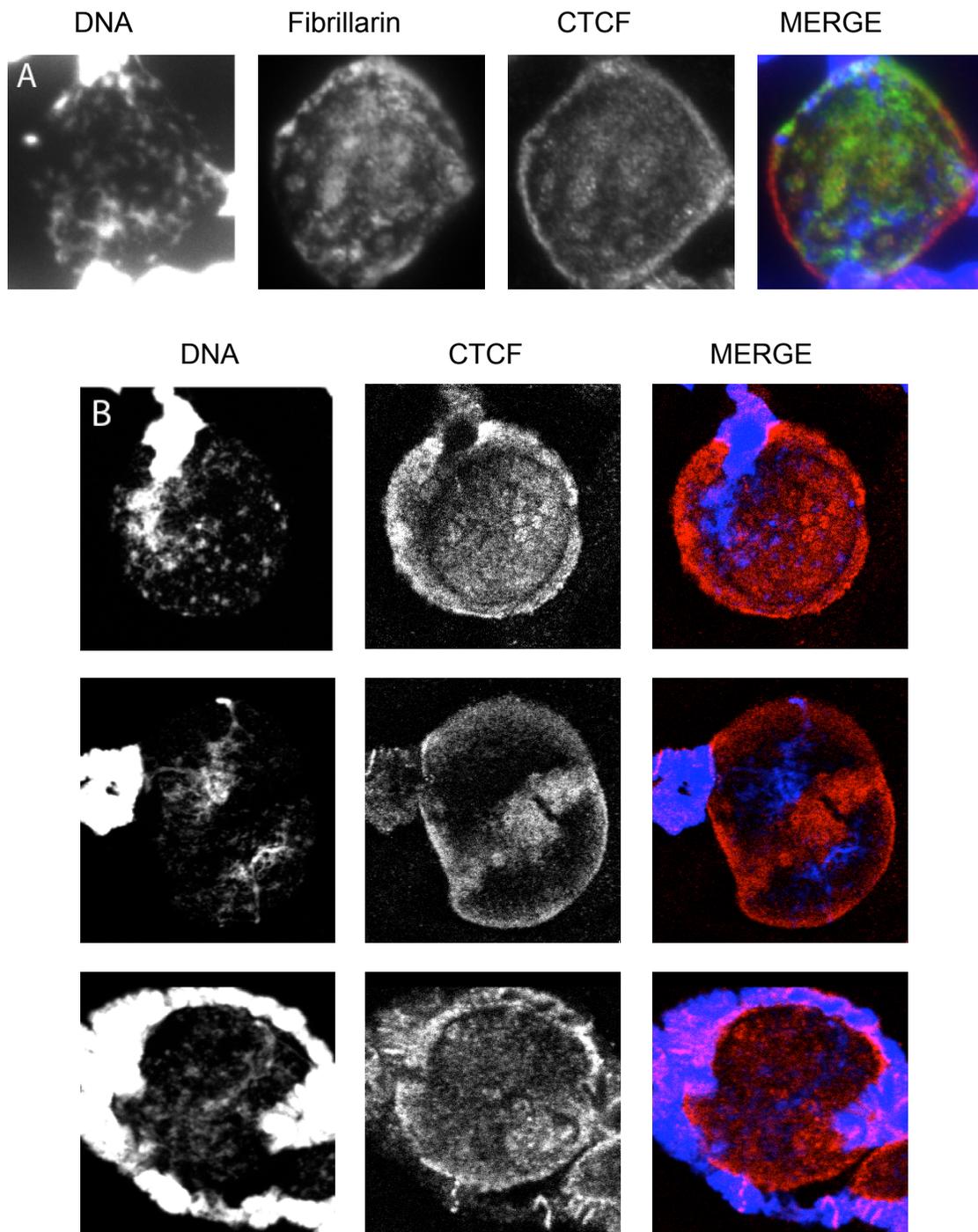


Figure 2.2 *Drosophila* CTCF localization at the nucleolus of polytene nuclei. A) Epifluorescence microscopy. Nucleolus was immunostained with anti-CTCF (red) and anti-Fibrillarlin (green). B) confocal imaging of nuclei stained with CTCF. Independent channels and merge are shown.

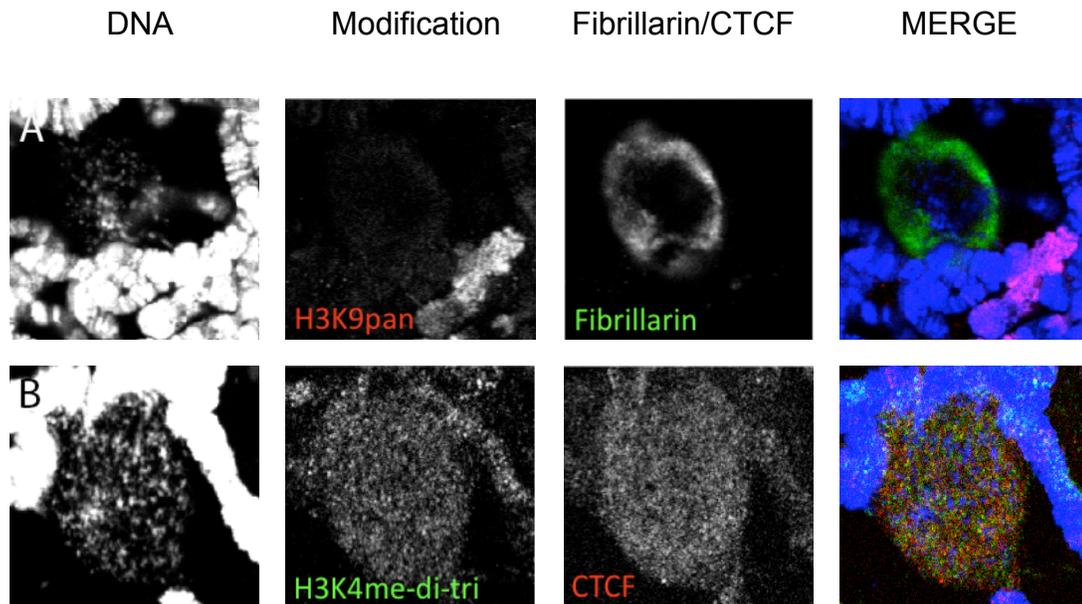


Figure 2.3 Histone modifications at the nucleolus. Epifluorescence microscopy showing A) repressed histone marks (pan methylated H3K9) and confocal microscopy showing B) active histone marks (di- and tri- methylated H3K4) at the nucleolus of polytene cells.

In mitotic chromosomes CTCF was found at euchromatic arms and it was absent from the heterochromatic centromere and from the compacted small fourth chromosome (Fig 2.4 A). In the X chromosome, CTCF was localized at the secondary constriction or NOR (nucleolar organizing region, the site of the rDNA arrays) and at the euchromatic arm (Fig 2.4 A). In the Y chromosome, CTCF was only found at the NOR (Fig 2.4 B). The only heterochromatic area where we were able to detect CTCF cytologically was the rDNA loci.

During mitosis rDNA transcription stops and the nucleolus is disassembled and is later reactivated in G1 [54]. At prophase, cyclin B levels increase, causing the phosphorylation of RNA polymerase I initiation complex,

and leading to the dissociation of several RNA polymerase I subunits. rRNA processing factors, such as Nucleophosmin and Fibrillarin, leave the nucleolus. UBF is one of the few factors that stays bound to rDNA during mitosis [27]. The fact that CTCF persist at this stage, presumably indicates that the binding of CTCF with the rDNA is probably direct or through UBF. Similarly, it shows that CTCF is not only present as a component of a mature nucleolus, and it is not retained in the nucleolus as is p53, without having an active role. Also, it refutes the possibility of being degraded at the nucleolus or being part of mature ribosomes.

In order to get more insights into the requirements for nucleolar CTCF recruitment, we examined binding at undifferentiated neuroblast and Schneider 2 (S2) cells and found CTCF at the nucleolus of these cells and colocalized with Fibrillarin (Fig 2.5). In neuroblasts, CTCF was enriched at the nucleolus, while in S2 cells CTCF was homogeneously distributed at the nucleus, including the nucleolus. These results showed that in *Drosophila melanogaster*, CTCF does not require cell differentiation in order to be recruited to the nucleolus and perhaps other factors such as ribosomal demand dictate its recruitment.

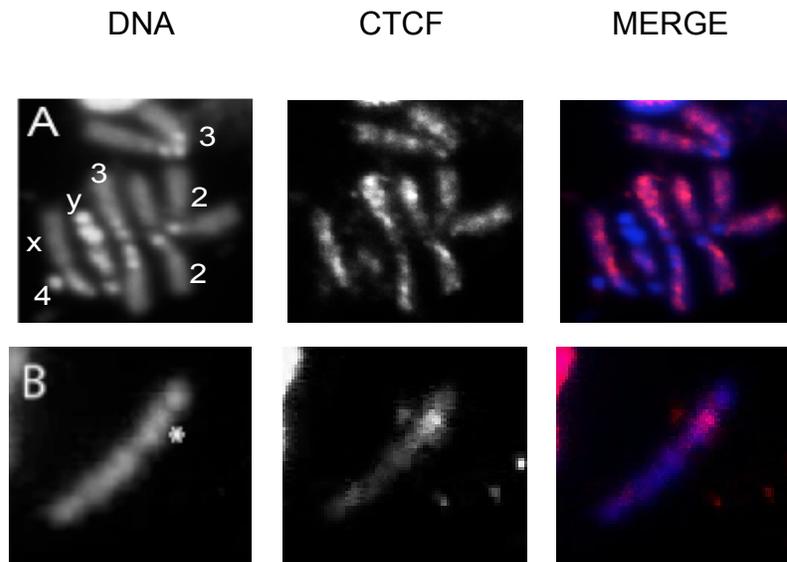


Figure 2.4 *Drosophila* CTCF localization at mitotic chromosomes. Epifluorescence microscopy. A) Spread of labeled neuroblast mitotic chromosomes. CTCF, shown in red, is enriched at euchromatic arms of second, third and X chromosomes, it is also present at the rDNA locus (*) of the X chromosome and it is absent from chromosome four. Y chromosome is partially stained. The level of fluorescence at euchromatic areas makes it harder to visualize Y chromosome staining. B) Y chromosome, CTCF localizes at the rDNA locus (*).

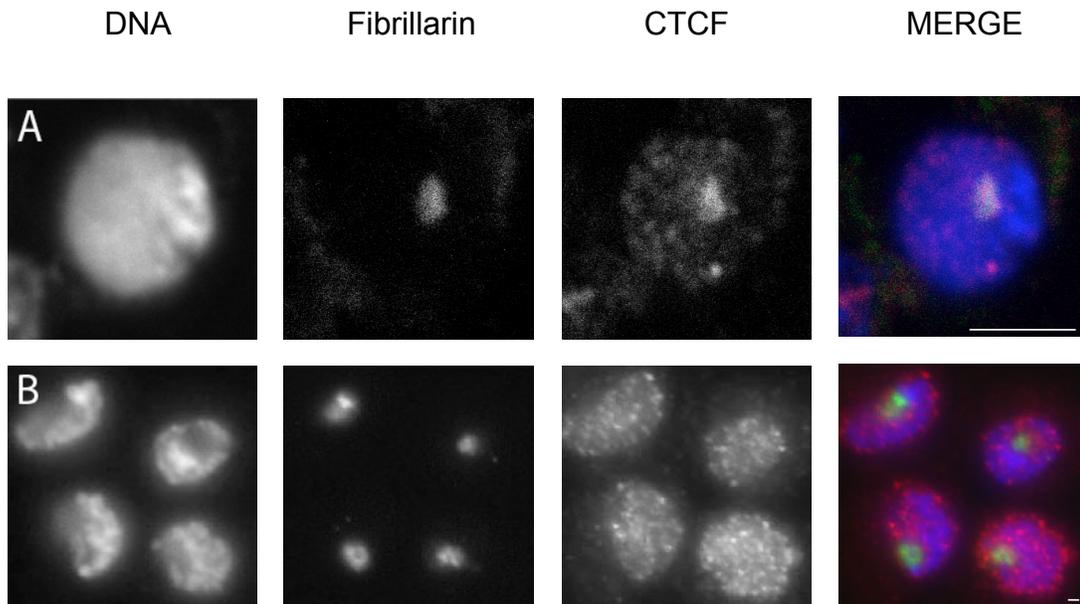


Figure 2.5 *Drosophila* CTCF localization in undifferentiated cells. A) Confocal microscopy of a neuroblast nucleus. B) Epifluorescence microscopy of S2 cell nucleus. Scale bar = 1 micrometer

CTCF binding at the 35S rDNA

The major component at the nucleolus is the ribosomal DNA and using immunofluorescence, we observed CTCF at the nucleolus of differentiated and undifferentiated cells. Likewise, CTCF was present at the NOR of the X and Y mitotic chromosomes at the time when nucleolar proteins are not present which indicates that CTCF is directly interacting with the rDNA and perhaps regulating its expression. This anticipates that potential binding sites for CTCF will be found at the 35S rDNA.

Computational methods combined with chromatin immunoprecipitation (ChIP) on ChIP or ChIP-Seq have identified CTCF binding consensus sites in

humans, mice and flies. Although many discrepancies exist among the consensus, even within the same species, these consensus have been useful to identify potential unknown sites not picked by genome-wide studies. In humans, CTCF is mostly found intergenic (43%), but it is also at introns (22%), promoters (20%) and exons (12%) [42]. Over 75% of these sites identified experimentally contained the predicted CTCF consensus site. On the other hand, the opposite case has also been observed; there are predicted CTCF binding sites that experimentally do not show any binding [98].

In *Drosophila*, using ChIP followed by microarrays, two different groups have studied genome-wide binding of CTCF [98,99] and found a similar consensus binding site (Fig 2.6). CTCF was mostly found at intergenic (42.18%), intronic (27.69%) and transcription start sites (19.94%) [99]. Even though human and *Drosophila* CTCF share only 53% identity, their binding profile is very similar in both organisms. This is expected since the central region, where the zinc finger domain is located, is very conserved while their differences are found in the amino and carboxy termini.

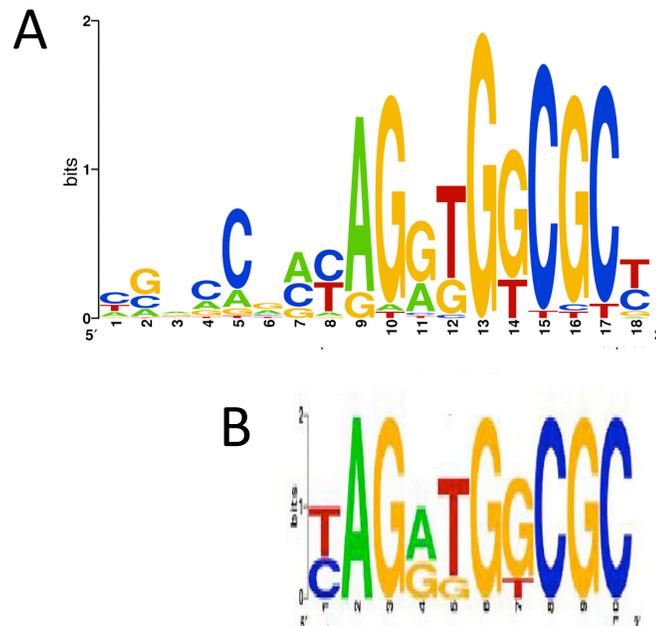


Figure 2.6 *Drosophila* CTCF consensus. A) Holohan [98] , B) Smith [99]

In mammalian cell culture the non-transcribed spacer (NTS) of the 35S rDNA contains one CTCF binding which has been confirmed by CHIP [96]. I used Patser, a software developed in the Stormo Lab at Washington University [100], and the two DNA consensuses from Holohan [98] and Smith [99] to map potential CTCF binding sites at the 35S rDNA, R1 and R2 elements. I analyzed the R1 and R2 sequences because it was known that insertion of these elements caused silencing of the inserted unit; therefore, I reasoned CTCF could be involved in rDNA transcription regulation through binding the R1 and R2 elements and maintaining the silent state.

Patser identified no sites in the NTS. This sequence is very polymorphic, its size can range from 2.1 to 5 Kb; however, it is made of repetitive units. I

designed primers to amplify all repeat types which made us believe we were able to test all possible polymorphisms. In contrast, the program identified two sites in the 28S gene, three in R1 and one in R2 sequence (Fig 2.7 A). Consequently, I performed ChIP on third instar larvae and tested those potential sites for occupancy. I used as positive control Fab-8, a well characterized CTCF site which contains a predicted binding site, experimentally it has been shown to bind CTCF and it acts as an enhancer blocker upon CTCF binding. I used two negative controls; an already published genomic sequence that has a predicted binding site but showed no binding by CTCF [98] and the 5S rDNA gene. Fab8 showed a 40 fold enrichment over background while both negative controls did not show enrichment for CTCF. When tested the predicted binding sites, no binding was found to the 28S rDNA or to the R2 element; only the R1 was immunoprecipitated by antiserum directed to CTCF (Fig 2.7 B).

The binding of CTCF to the R1 element is not as strong as the binding to Fab-8 which is possibly due to the R1 copies found outside the 35S rDNA that are not bound by CTCF. Similarly, it is likely that in a given cell, CTCF binds to some R1-inserted rDNA copies stronger than to others because of their distribution in the rDNA array. Hence, R1 elements that are clustered together will recruit CTCF much more effectively than dispersed R1 inserted copies, because it will be harder to tether and maintain CTCF at these units that are surrounded by actively transcribed uninserted copies.

R1 copies also exist at the centric heterochromatin and truncated forms are found on chromosome four [93,101,102]. However, in *Drosophila* CTCF was undetected in the centric heterochromatin and fourth chromosome ([97] and Fig 2.4 A) which indicated that the binding signals we observed by CHIP came from the R1 inserted at the rDNA. On the other hand, CTCF could be binding the R1 elements outside the rDNA in quantities below our detection level. However, previous studies have shown that the amount of R1 inside and outside the rDNA array is very similar which will result in a detectable CTCF binding.

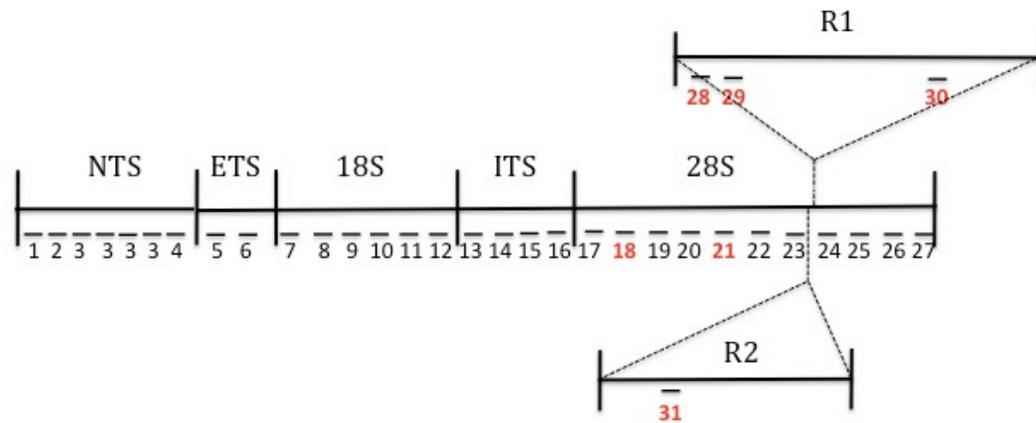
I considered that CTCF may bind to rDNA at an alternative consensus, or perhaps indirectly through protein-protein interactions. To rule out this possibility, I created primer sets that could assay the entire 35S rDNA transcription unit in approximately 350 bp steps. However, I was unable to find any more binding. Three independent CHIP experiments were conducted and in all cases the same result was obtained: CTCF was bound only at the R1 element (sites 28 and 29 on figure 2.7C). The fact that CTCF did not bind the predicted sites at the 35S rDNA and R2 element indicated that these were not true CTCF binding sites. Lack of enrichment in predicted CTCF binding sites have also been observed previously [98]. We also considered the possibility of CTCF binding to rRNA directly and perhaps being involved in rRNA processing which I will describe in Chapter III.

It was not surprising we could not detect CTCF binding at the NTS because unlike in mammals, *Drosophila* non-transcribed spacer does not

contain predicted CTCF binding sites. In the same way, alignment of *Drosophila* and mammalian NTS sequences did not show similarities. Although CTCF may be involved in the transcription regulation of the rDNA in both organisms, the mechanism used by this protein is perhaps different. There are two major differences between these arrays. First, in mammals DNA methylation silences rDNA copies and CTCF binding depends on the methylation status of the rDNA units, while in *Drosophila* DNA methylation has not been identified. Second, in arthropods rDNA units are inserted with R elements which causes silencing of inserted units. The fact that CTCF binds the rDNA indirectly through R1 binding indicates that other factors may be involved in this process.

R1 elements are not unique for *Drosophila*, they are also found in other arthropods such as; *Ixodes scapularis*, *Bombyx mori*, *Papilio dardanus*, *Anopheles gambiae*, and *Tribolium castaneum*. I looked for potential CTCF binding sites at the R1 elements of these organisms and only *Anopheles* and *Tribolium* contained a single potential CTCF binding site. Similarly, I searched for binding sites in other mobile elements that are inserted in the 35S rDNA and are found in different animal taxa (Chapter I, Figure 1.7). Except for R7Ag1, all other mobile elements tested contained CTCF binding sites (Table 2.1). These results indicated that perhaps CTCF could regulate rDNA transcription in other organisms by binding to these elements and that in the absence of a CTCF binding site at the rDNA NTS, *Drosophila* and apparently other animals have used transposable elements to regulate rDNA transcription by recruiting CTCF.

A)



B)

18 TAGAGGGTGCG
 21 TGGATGGCGCT
 28 CAGATGGCTCC
 29 CATGGGTCGTC
 30 CATGGGTCGTC
 31 AGGGCGGCGCC

C)

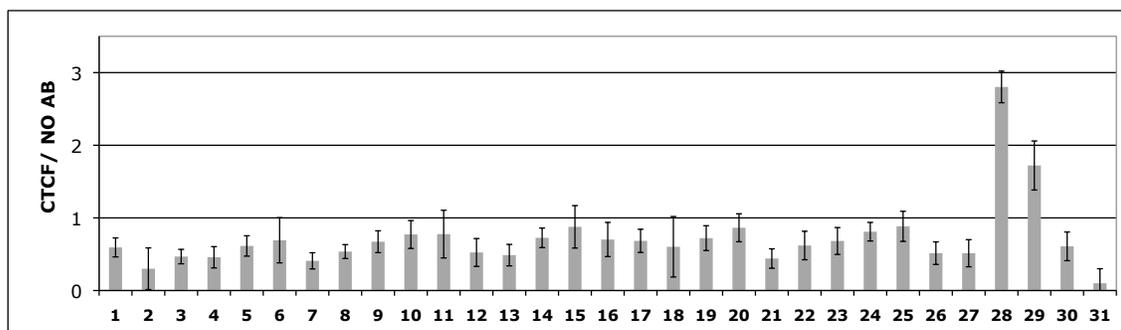


Figure 2.7 CTCF binding sites at the 35S rDNA gene, R1 and R2 elements. A) 31 fragments amplified by real-time PCR are depicted by a grey bar. Red numbers indicate CTCF sites identified by patser. B) List of potential binding sites. C) Chromatin Immunoprecipitation of CTCF at the 35S rDNA, R1 and R2 elements. Data represent the average of three independent ChIP experiments.

Table 2.1 CTCF binding sites in rDNA-linked transposable elements. The location of these elements in the 35S rDNA unit is shown in Figure 1.7.

Mobile Element	CTCF consensus sites
R4	3
R5	1
R6Ag1	2
R6Ag2	2
R6Ag3	1
R7Ag1	0
R7Ag2	3
RT1	2
RT2	4
Pokey	1

Conclusion

We initially observed CTCF at the nucleolus of differentiated salivary glands nucleoli and we wondered which possible roles it may play. The nucleolus is the place where the ribosomal DNA is found and it is also the place where hundreds of proteins have been identified. Previous studies conducted in mammals showed CTCF binding to two nucleolar proteins: PARP-1 and Nucleophosmin/B23. In mammals Parp-1 recruits CTCF to the nucleolus and Nucleophosmin/B23 tethers CTCF to the nucleolar periphery. Inhibition of RNA polymerase I, responsible for transcribing the rDNA, prevents CTCF nucleolar

recruitment. Thus, we decided to investigate the role of CTCF in rDNA regulation.

CTCF immunostaining of the entire nucleolus did not show any obvious substructural localization; it colocalized with Fibrillarin which made us think in a possible role in rRNA processing. Similarly, it partially colocalized with H3K4me, a histone mark known to be enriched at active loci. On the other hand, we observed that unlike mammals, cell differentiation was not a requirement for CTCF nucleolar recruitment. We tested two different cell types; in interphase neuroblast CTCF was enriched at the nucleolus and in S2 cells CTCF was present at the nucleolus but it was homogeneously distributed in nucleolar and non-nucleolar chromatin.

I looked at mitotic spreads and observed CTCF at the X and Y chromosomes rDNA loci suggesting that CTCF was binding directly to the rDNA. To test this hypothesis, we searched for potential bindings sites, using two published consensus binding sites and performed chromatin immunoprecipitation. I detected binding at the R1 element and although we cannot assure based only on ChIP that it corresponds to the R1 elements inserted in the rDNA, we did not observe CTCF immunostaining at R1 located outside the rDNA loci.

A recent study showed CTCF binding at the NTS of mammalian rDNA; hence, we explored this possibility and looked at potential binding sites at *Drosophila* NTS. No potential sites were found and experimentally ChIP did not detect CTCF signal at the NTS.

CTCF directly binds the rDNA, both in interphase and condensed chromosomes. It does so in humans and *Drosophila*, yet its not clear what its role as a boundary element binding protein could contribute to rDNA regulation. To address this, it is critical to investigate the loss-of-function consequence on rDNA expression.

CHAPTER III

ROLE OF CTCF IN RIBOSOMAL DNA TRANSCRIPTION

Introduction

In a cell, the demand for protein translation dictates the rate of rDNA transcription. However, in a growing cell not all rDNA copies are actively transcribed. In 1976, using transmission electron microscopy, Oscar Miller showed how in *Xenopus* early embryogenesis, only about half of the rDNA genes generated nascent transcripts. These genes were spread through the cluster, hence, active and inactive arrays were intercalated [51]. It is not well understood how in a single rDNA cluster, in which the promoter for every single unit is identical, some units are silenced and some are not. Similarly, another subset of rDNA copies are silent or transcribed at very low levels by the insertion of R1 and R2 elements. These elements are inserted in a conserved site at the 28S gene and are co-transcribed with the 35S rDNA [85,93].

A key to understanding how different rDNA units are regulated may come from our observation that R1 linked rDNA units are bound by CTCF. We wanted to explore whether CTCF was involved in the regulation of the rDNA transcription and in nucleolar architecture. Loss-of-function alleles of CTCF have been generated in adult flies by Mohan and colleagues [97]. Using transposable element (P-element) imprecise excision, they created five CTCF amorphic alleles. They also generated a deficiency of 16kb by FRT (FLP recognition

target) recombination. Trans-heterozygotes (null/hypomorph) showed no protein by western blot (pupa and larva) and immunofluorescence. Phenotypically, mutants die as pharate adults; that is they develop until late pupae stages but do not eclose out of the pupal case. Similarly, mutants showed homeotic transformation and defects in enhancer blocking activity of the Fab-8 insulator. We obtained these alleles from the Geyer and Renkawitz laboratories and used them to study the role of CTCF in rDNA transcription, rDNA array size and nucleolar morphology.

CTCF post-translational modifications are well known in mammals. It is acetylated, phosphorylated, sumoylated and poly(ADP-ribosyl)ated [30,103]. Poly(ADP-ribose) Polymerase 1 (PARP-1) is the best characterized member of the PARP family and is responsible for adding long branched poly(ADP-ribose) polymers (pADPr) to a wide variety of proteins, one of them CTCF, and to itself. It is also responsible for 90% of pADPr. PARP-1 is mostly found unmodified in their inactive state and upon auto poly(ADP-ribosyl)ation it is activate. Poly(ADP ribose) glycohydrolase (PARG) completes the cycle by removing ADP ribose units and restoring PARP to its inactive state. Metabolism of pADPr is involved in processes such as; DNA replication, transcription and repair, necrosis, apoptosis, centrosome duplication, chromatin decondensation and regulation of telomere function [103,104]. PARP members are found in different cellular compartments; for instance, PARP-1 is found at centromeres, nucleus and

centrosome, PARP-2 is found at telomeres, centromere and nucleus, and PARP-3 is found at the daughter centriole, nucleus and cytoplasm.

Inhibition of poly(ADP-ribose) polymerase (PARP) by adding 3-aminobenzamide (ABA) removes CTCF from the nucleolus [25] and it also compromises insulator function [31]. This indicates that PARP-1 participates in nucleolar recruitment of CTCF and it may cooperate with its function inside and outside the nucleolus. PARP-1 and PARP-2 accumulation at the nucleolus requires RNA polymerase I transcription and both proteins bind to B23/nucleophosmin. Post-translational modifications of *Drosophila* CTCF have not been studied yet. However, in *Drosophila* PARP is localized at the nucleolus of polytene salivary gland nuclei, in PARP mutants the nucleolus is not formed, and ABA treatment removes PARP from the nucleolus [105]. PARG has also been identified in *Drosophila*. It is found mostly in the nucleoplasm and in lesser amount at the nucleolus and chromosomes. In PARG mutant flies, the nucleolus is fragmented and PARP is removed from the nucleolus [106]. These observations indicate that the PARP-PARG cycle is involved in nucleolar stability most probably by tethering CTCF to this compartment. In order to understand how CTCF is regulated at the nucleolus, we decided to investigate whether altering the PARP-PARG cycle would cause an effect in CTCF localization and as a result in rDNA transcription.

Results and Discussion

Role of CTCF in maintaining nucleolar structure

Torrano and colleagues initially identified the subnuclear localization of CTCF by over-expressing a GFP-CTCF construct in mammalian cells, they also showed that the zinc finger region was responsible for nucleolar localization, and that inhibition of PARP removed CTCF from the nucleolus [25]. Although the findings of this work are of paramount importance, they did not have loss-of-function alleles which will add valuable information in understanding nucleolar regulation by CTCF. We were interested in knowing whether nucleolar architecture, rDNA transcription, and rDNA processing were affected and how this would affect an entire organism.

First, we looked at Mohan's CTCF mutant alleles (Fig 3.1). Using confocal microscopy of whole-mount salivary glands stained with CTCF and Fibrillarin antibodies, I observed fragmented nucleoli (by Fibrillarin staining) and very low amounts of CTCF at the nucleolus (Fig 3.2 B-C-D compared to A). In a homozygous CTCF^{p35.2}/CTCF^{p35.2} background, CTCF was highly reduced from nucleolar and non-nucleolar chromatin (Fig 3.2B) while in a heterozygous CTCF^{p30.6}/+ and Df(3L)0463/+ background, CTCF was mostly reduced at the nucleolus and partially reduced at the non-nucleolar chromatin (Fig 3.2C-D). This phenotype resembles what was observed before by Peng and Karpen [23]. They were interested in studying how heterochromatin and associated proteins regulate nucleolar architecture. The authors stained whole mount salivary glands

nuclei of mutants in heterochromatin proteins such as histone methyl transferases of histone H3K9 (Su(var)3-9 HMTase) and Heterochromatin protein 1 (Su(var)205) with Fibrillarin, and observed ectopic Fibrillarin staining. They predicted these ectopic areas would correspond to mislocalized rDNA copies. In order to test this hypothesis, the authors performed fluorescence in situ hybridization (FISH) of ribosomal DNA combined with Fibrillarin immunostaining and observed a clear correspondence between the ectopic Fibrillarin staining and dispersed rDNAs; hence, these areas corresponded to ectopic nucleoli. In these mutants, heterochromatin formation was defective and the authors predicted that this would cause instability of repeated DNAs which would generate the formation of extrachromosomal circles (ecc). To test this, they quantified the amount of extrachromosomal circles in a Su(var)3-9 mutant background compared to wild type and they obtained a major increase in the former one.

Based on these findings, we inferred that the ectopic Fibrillarin areas we observed in the different CTCF mutant backgrounds would correspond to rDNA extrachromosomal circles. I hypothesized that, in time, these ectopic rDNA units will be lost and the rDNA array will become shorter. Therefore, I measured rDNA array copy number in three CTCF mutants background. We did not know which rDNA array, the one in the X or in the Y chromosome, will be affected the most so we isolated the Y chromosome by crossing males from the CTCF mutant stocks (*Df(3L)0463*, *CTCF^{p30.6}*, *CTCF^{p35.2}*) to females (*C(1)DX,y^{1f1bb0}/Y*)

carrying a compound X chromosome ($C(1)DX,y^1f^1bb^0$) in which both X chromosomes lack the entire rDNA array; therefore, the sole rDNA source comes from the Y chromosome. In the next generation, I collected $C(1)DX,y^1f^1bb^0/Y$ females carrying the Y chromosome from the CTCF mutant stock (cross shown in Fig 3.3 A) and measured the array size of the Y-linked 35S rDNA array. In the three mutants examined, the rDNA copy number was reduced compared to wild type which for comparison purposes is shown as 100%; hence, *Df(3L)0463* was 66.5%, *CTCF^{p30.6}* 61.9%, and *CTCF^{p35.2}* 49.6%.

Similarly, I measured the array size of their siblings ($C(1)DX,y^1f^1bb^0/Y; + / TM6BTb$) to see whether the reduction caused by CTCF could be reversed in a single generation. However, I obtained the same array copy number as their CTCF counterpart. These results indicate that reduction in CTCF gene product caused the loss of rDNA units and this effect could not be reversed in a single generation, which agrees with early studies showing that rDNA magnification is a slow process that takes between three to seven generations [107]. Therefore, it is not surprising to have similar array sizes between CTCF mutant and wild type siblings. On the other hand, it is possible that the Y-linked rDNA array copies in the CTCF stocks were small before the CTCF mutant alleles were introduced to the stock. Similarly, it is possible that the effect we are observing was not due to CTCF at all.

In order to discard these last two interpretations we will have to follow an isolated Y-linked rDNA array from the CTCF stock, for many generations and see whether in the absence of the CTCF mutation, rDNA magnification restores the rDNA array to its original size.

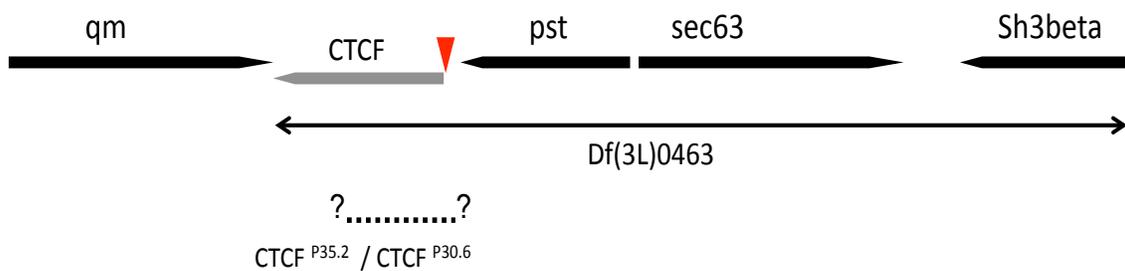


Figure 3.1 Representation of the genomic region where CTCF is found (chromosome 3L, cytological band 65F6). Red inverted triangle represents the location of the P-element used by Mohan and colleagues to generate alleles *CTCF^{P35.2}* and *CTCF^{P30.6}*. Question marks flanking dotted line indicate that the breakpoints for these two alleles are unknown. *Df(3L)0463* uncovers CTCF and three more neighboring genes.

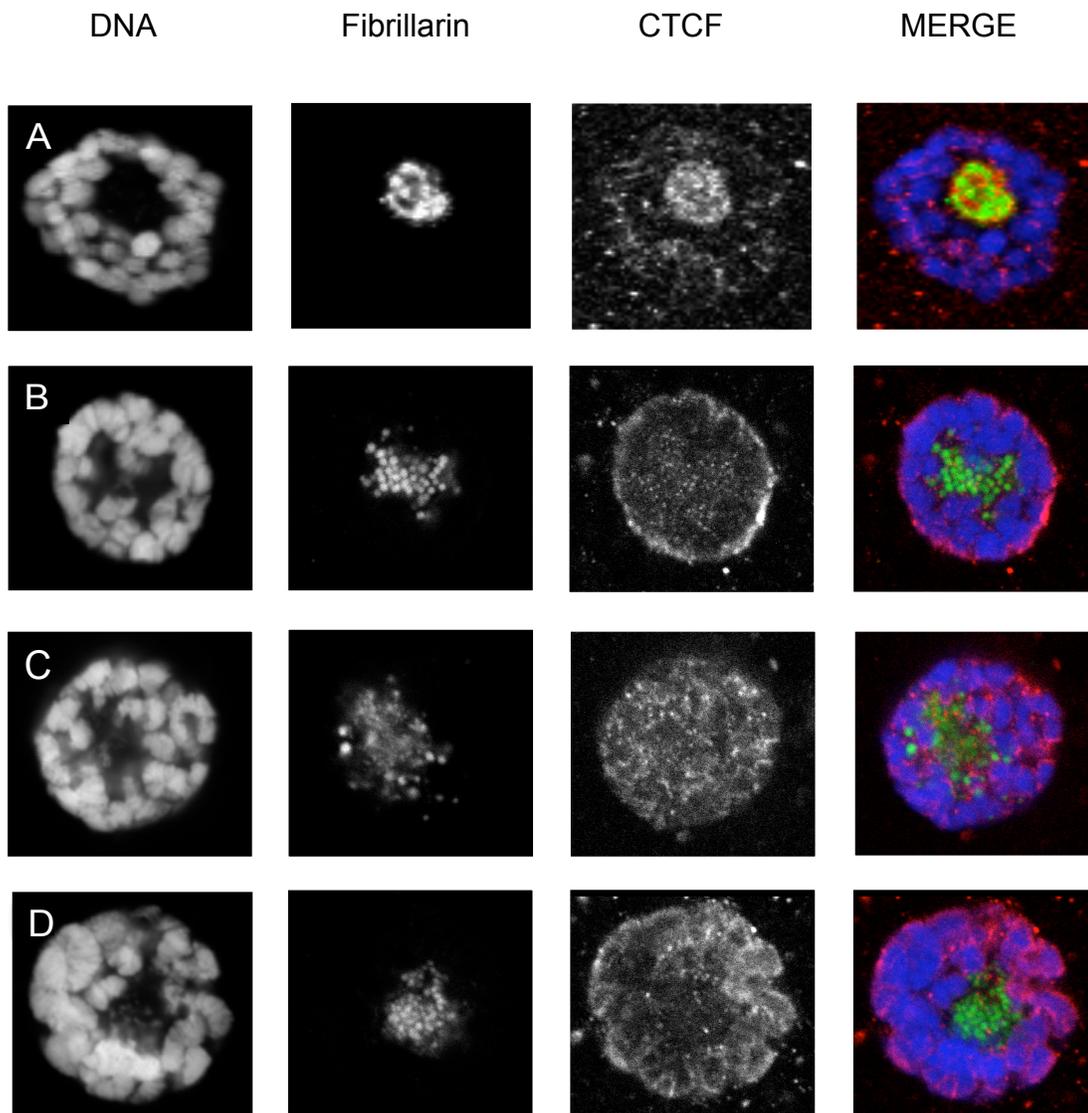


Figure 3.2 Confocal imaging of whole-mount salivary glands nuclei. CTCF (red), Fibrillarin (green). A) Wild type (yw), B) CTCF^{p35.2}/CTCF^{p35.2}, C) CTCF^{p30.6}/+, D) Df(3L)0463/+.

A)

Generation 0:

♀ $C(1)DX,y^1 f^1,bb^0/Y ; + / +$ X ♂ $y^1w^{67c23}/Y ; CTCF^{p35.2}/TM6BTb$

Generation 1:

♀ $C(1)DX,y^1 f^1,bb^0/Y ; CTCF^{p35.2} / +$

B)

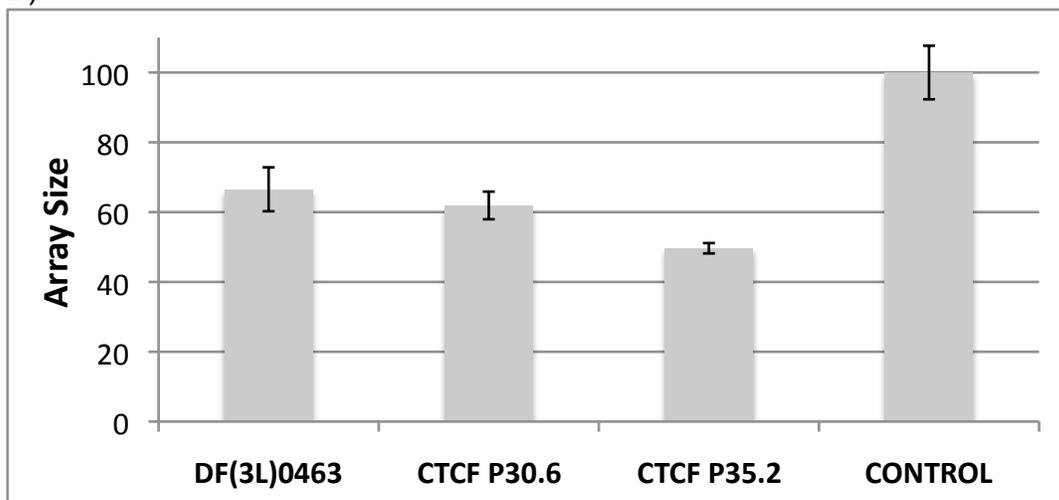


Figure 3.3 Y-linked 35S rDNA array size. A) Isolation of the Y chromosome from CTCF mutant stocks. $C(1)DX,y^1 f^1,bb^0$ is a compound X chromosome lacking rDNA. B) 35S rDNA array size of isolated Y chromosomes from each stock compared to a Y chromosome isolated from control.

Reduction in the rDNA array copy number results in the bobbed phenotype, characterized by truncated bristles and lack of abdominal cuticle due to a global decrease in protein production. Although these two tissues seem to be more affected, perhaps because of their higher protein demand, it is

expected a drop in rRNA production in other tissues as well but not enough to cause a phenotype. We observed the bobbed phenotype in C(1)DX,y^{1f1bb}⁰ flies carrying the Y chromosomes from Df(3L)0463 and CTCF^{p30.6} mutant stocks, which confirmed a functional reduction in rRNA level, consistent with a decrease in the rDNA array copy number (Fig 3.4). I did not observe a bobbed phenotype in C(1)DX,y^{1f1bb}⁰; CTCF^{p35.2} females even though the reduction in rDNA copy number was half of wild type (49.6%). This probably indicated that rRNA transcripts in this stock increased in order to compensate the loss in rDNA copies. This low correlation between copy number and transcripts level had been seen before in *D. melanogaster* and *D. hydei*, where flies with low rDNA content phenotypically looked wild type, and flies with enough rDNA copies for wild type expression showed bobbed phenotype [108].

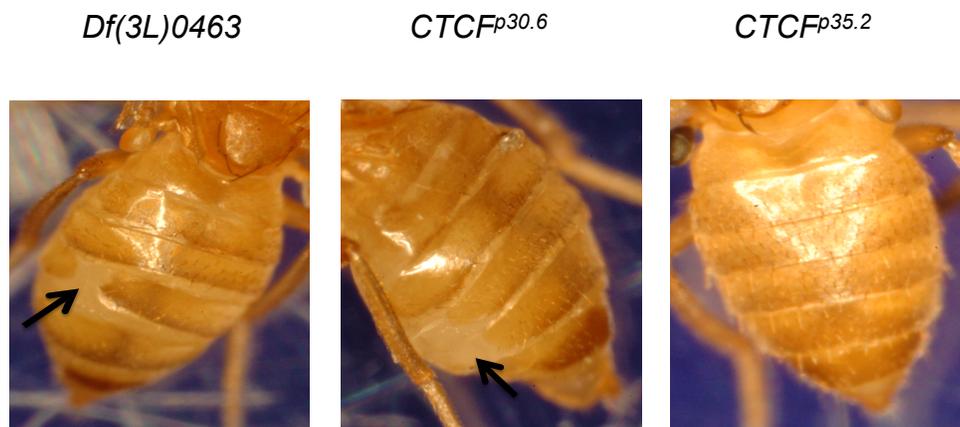


Figure 3.4 Bobbed phenotype in CTCF mutant flies. Black arrows point bobbed areas in the abdominal area. CTCF^{p35.2} did not show a bobbed phenotype.

Role of CTCF in rDNA transcription

We have observed strong immunofluorescence signal by CTCF at the nucleolus of different cell types and more specifically at the NOR of both X and Y chromosomes. Similarly we observed reduction in rDNA array copy number and the corresponding bobbed phenotype. However, we did not see direct CTCF binding at the 35S DNA. In order to demonstrate that CTCF is involved in the regulation of the rDNA transcription, I compared transcript amounts of 18S rRNA, non-inserted copies, R1 and R2 inserted copies in *CTCF^{p35.2}* homozygous pharate and heterozygous pupae. To quantify rRNA transcripts I used reverse transcriptase real time PCR (RT-Q PCR) instead of Northern blots because small differences in total 35S rRNA transcripts are less-accurately quantified by the latter technique. Q-RT-PCR also allowed me to unambiguously identify transcripts from R1 elements inserted and co-transcribed with the rDNA, and avoid those transcripts derived from the extra R1 copies found elsewhere in the genome. I isolated rRNA from female pupae and converted RNA to cDNA using antisense DNA primers that would convert non-inserted 35S, R1-, and R2-containing transcripts. I measured non-inserted R1 and R2 copies by using a forward primer located upstream of the R2 insertion site and a reverse primer located downstream of the R1 insertion site (Fig 3.5). To measure R1 inserted copies as co-transcribed with the 35S rDNA, real time PCR was performed using as forward primer a sequence from the 28S, which lays upstream of the R1 insertion site.

I observed a small but significant increase in RNA amounts of total 35S rDNA, by measuring 18S rRNA, and non-inserted R1 and R2 copies in homozygous compared to heterozygous mutant flies (Fig 3.6). Even though CTCF may act as a regulator of all rDNA transcription, the relatively small effect caused by mutation was expected because of the large pool of rRNA in the nucleus and cytoplasm generated from the large number of active rRNA genes. However, my results indicated that CTCF acts to repress rRNA transcription, consistent with the gain-of-function studies performed by Torrano and colleagues.

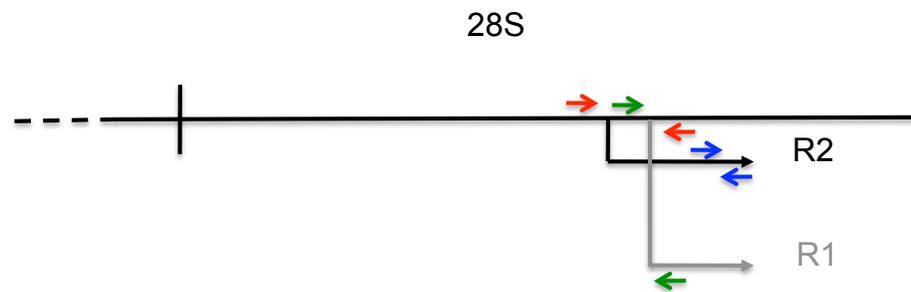


Figure 3.5 Primers used to measure transcript levels. Non-inserted copies (red), R1 inserted (green) and R2 inserted (blue) copies primer sets are shown. Total 35S rRNA transcripts were measured by amplification of the 18S rDNA.

Since the R1 and R2 elements are co-transcribed with the rDNA and are generally silent or transcribed at very low level, they were an ideal way to measure the increase in RNA transcripts caused by the CTCF mutation. R1 and R2 transcripts were low copy-number, indicating that they were transcriptionally

silent, and so small increases in R1 and R2 transcripts were expected to be more pronounced than increases in 35S rRNAs. R1 and R2 transcripts were higher in a homozygous mutant background (Fig 3.7). I noted that R2 transcripts were six fold higher than R1 despite ChIP indicating that CTCF binds in the R1 and not the R2. Three explanations may account for this discrepancy.

First, in a CTCF mutant background, the R1 inserted copies are perhaps more prompted to be lost from the rDNA array. These copies are normally silent and this would generate the formation of local heterochromatic foci; however, once CTCF is greatly reduced, the R1 copies will be activated which would cause destabilization of local heterochromatin, resulting in the formation of extrachromosomal circles and eventual loss of the R1 copies.

Second, the Y chromosome from the *CTCF^{p35.2}* mutant stock has suffered reduction in the rDNA array copy as shown in Figure 3.3. Therefore, it probably contains less R1 than R2 copies which would explain why we saw a moderate effect in R1 transcripts between homozygous and heterozygous individuals; however, this effect was more pronounced in R2 copies.

Third, R1 and R2 inserted copies are known to be grouped together within the rDNA array as it has been shown by Labrador and Eickbush laboratories; therefore, any alteration in R1 transcripts will cause an effect in R2 inserted and non-inserted units that are in the vicinity.

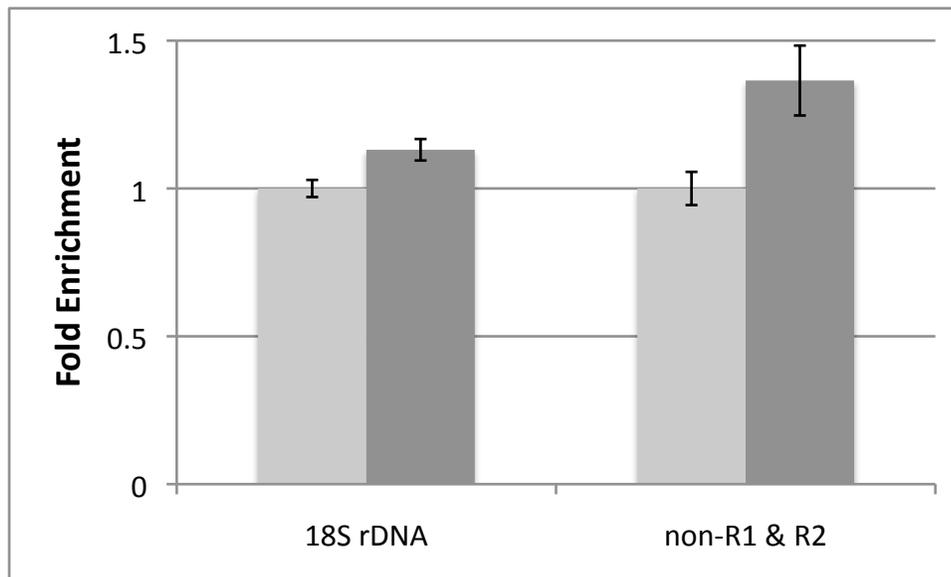


Figure 3.6 Transcript levels of the 18S rDNA and non-inserted R1 & R2 copies. *CTCF^{p35.2}/+* (light gray), *CTCF^{p35.2}/CTCF^{p35.2}* (dark gray). Y axis shows 18S rDNA and non-inserted copies expression.

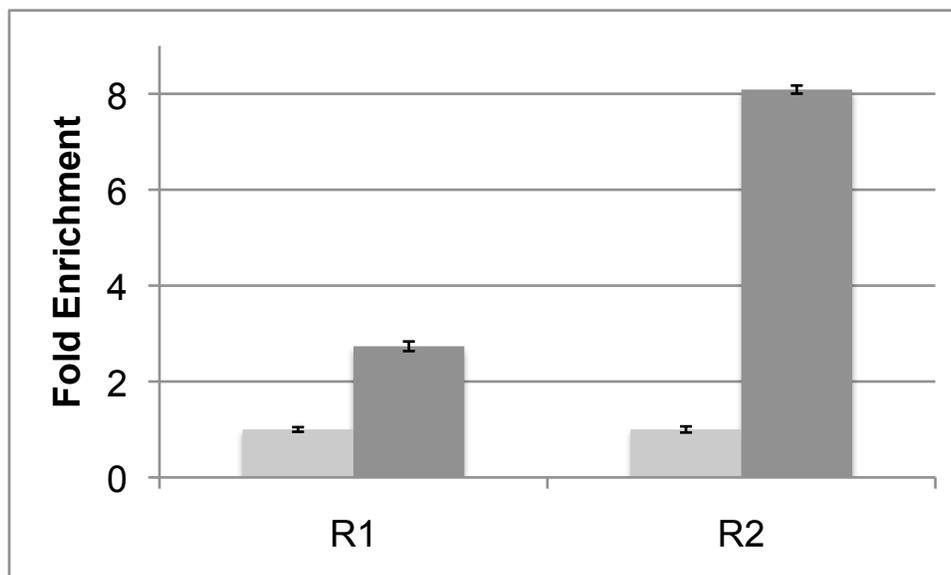


Figure 3.7 Transcript levels of R1 and R2 inserted copies. *CTCF^{p35.2}/+* (light gray), *CTCF^{p35.2}/CTCF^{p35.2}* (dark gray). Female pupae were used. Y axis shows R1 and R2 expression.

The 35S rDNA is transcribed as an entire unit by RNA polymerase I and the primary transcript is processed into 18S, 5.8S and 28S. Since the rRNA transcripts are increased in CTCF mutants, we wanted to know whether this corresponded to steady-state or nascent transcripts. We suspected they were nascent because R1 and R2 transcripts are normally degraded; however, we were able to quantify them. In order to test for nascent transcripts, I measured pre-rRNA unprocessed junctions in a homozygous and heterozygous CTCF^{p35.2} female larvae and observed more unprocessed junctions in the homozygous mutant background as shown in Figure 3.8: 18S-ITS (1.24 fold), 5.8S-ITS2 (1.75 fold), 2S-ITS2 (2.4 fold), ITS2-28S (1.41 fold).

Increase of unprocessed junctions could also indicate that CTCF is involved in rRNA processing, and in its absence processing is reduced, leading to longer-lived unprocessed or incompletely-processed rRNA species. It has not been reported yet whether CTCF is involved in this mechanism or if it binds RNA. To shed some light onto this matter, I treated third instar larvae salivary glands with 50 µg/ml of RNase and immunostained with Fibrillarin and CTCF. I observed a dramatic decrease in CTCF staining at the nucleolus without altering non-nucleolar chromatin binding (Fig 3.9 A-B).

Quantification of fluorescence intensity showed a decrease of 80% in the signal compared to untreated nuclei (Fig 3.9 C). These results added a higher level of complexity to the role of CTCF at the nucleolus. CTCF is known to bind nucleophosmin, a protein involved in rRNA processing that binds RNA [109]. Therefore, CTCF is perhaps not only inhibiting rDNA transcription by binding the rDNA-linked R1 element, but also through binding to nucleophosmin/rRNA complexes or directly to RNA. Further studies need to be done to investigate whether CTCF binds directly rRNA. On the other hand, Fibrillarin staining was not visibly affected by the RNase treatment; this was very surprising because this protein is involved in rRNA processing and it associates with small RNAs such as U3, U8, and U13. Therefore, it is possible that although RNAs are depleted from the nucleolus, Fibrillarin remains because of binding to nucleolar proteins. For instance, Fibrillarin interacts with Nop56, a protein required to assemble 60S and also involved in pre-RNA processing, and this interaction does not required RNA to be maintained [110].

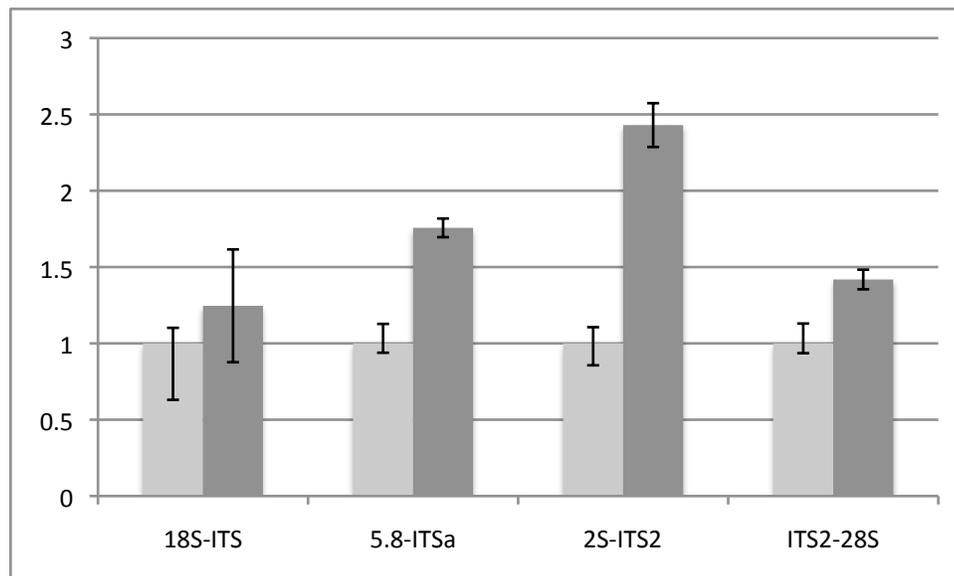
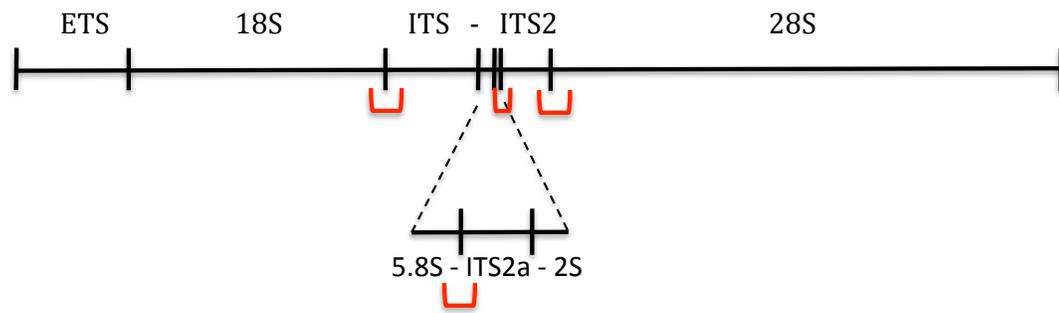
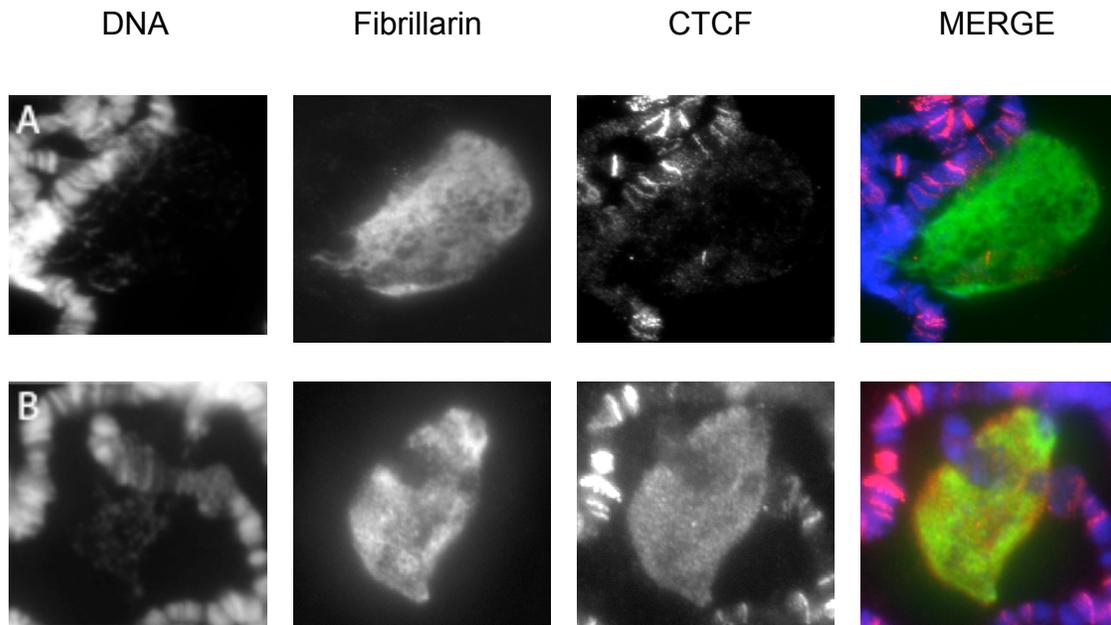


Figure 3.8 Pre-rRNA unprocessed junctions in a CTCF mutant. *Upper part*, representation of 35S rDNA unit showing in red brackets, the junctions amplify by Reverse Transcriptase Real-Time PCR. *Lower part*, quantification of unprocessed junctions in CTCF^{p35.2/+} (light gray) and CTCF^{p35.2/CTCF^{p35.2}} (dark gray).



C)

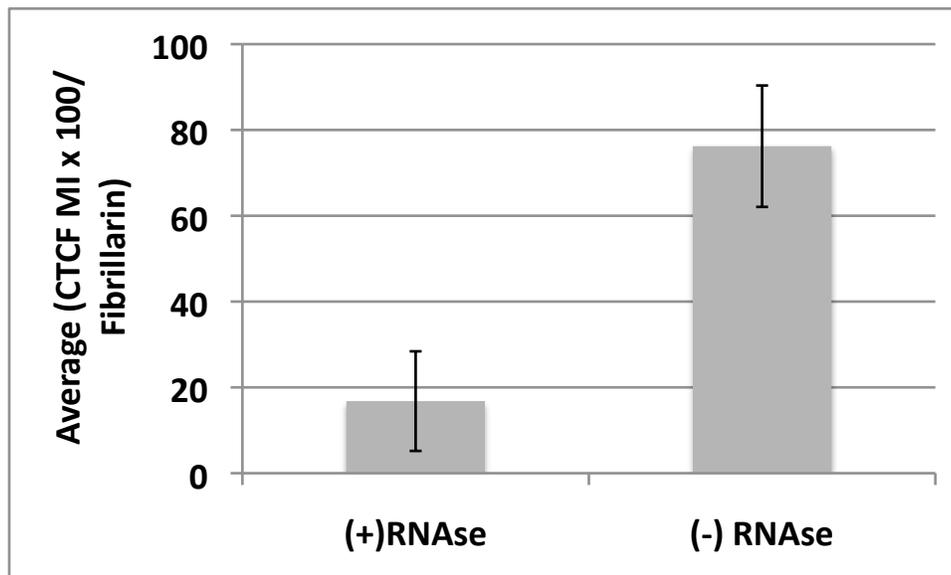


Figure 3.9 CTCF binding after RNase treatment. Epifluorescence microscopy of polytene nuclei stained with CTCF (red) and Fibrillarin (green). A) RNase treated cells. B) Control. C) Average of fluorescence intensity quantification of CTCF shown as percentage relative to the Fibrillarin signal. MI: Mean intensity

CTCF knockdown

To confirm the effect of CTCF reduction in rDNA transcription, I performed RNAi experiments in Schneider 2 (S2) cells using double stranded RNA (dsRNA) directed at CTCF. After a three day treatment using 15 μ g of double-stranded RNA, I analyzed the efficacy of knockdown by measuring reduction of mRNA level and loss of protein by decrease of immunofluorescence signal. As a consequence of CTCF reduction, I measured changes in rRNA levels using primers specific for 18S, R1, R2.

Using immunofluorescence, I observed that CTCF and Fibrillarin were greatly depleted from the nucleolus and nucleoplasm (Fig 3.10 C compared to A-B). Similarly, the nucleolus appeared fragmented and diffused (Fig 3.11). I speculated that this perturbation of nucleolar integrity was caused by loss of silent chromatin and the generation of extrachromosomal circles. On the other hand, LacZ and control did not show alterations in nucleolar structure or CTCF staining, which showed that the phenotype observed was caused by CTCF depletion.

Similarly, I measured the fluorescence intensity of CTCF and Fibrillarin signals from 112 treated and untreated S2 cells (Fig 3.12). The ratio CTCF:Fibrillarin was very similar between treated ($R^2=0.395$) and untreated cells ($R^2=0.3187$), which indicates that upon CTCF dsRNA treatment, Fibrillarin is also affected to a similar degree. This effect could be directly through binding to CTCF or indirectly by altering nucleolar architecture and as a resulting loosening

Fibrillarin binding. CTCF protein level was reduced to 31.7% (± 19.1 SD) of wild type which agrees with the little fluorescence observed in both nucleolus and non-nucleolar chromatin and indicates that a majority of the protein was depleted by treatment.

A reduction of 70% approximates the reduction expected from using heterozygous animals, in which I observed reliable nucleolar fragmentation phenotypes (Figure 3.2). This observation favors the idea that CTCF is not just sequestered at the nucleolus because of protein excess but instead is there because it is required. In addition, quantification of CTCF mRNA levels showed a 40% reduction after RNAi treatment (Fig 3.13). Although CTCF protein depletion was more dramatic, those cells were only an aliquot of all the population of cells treated with dsRNA.

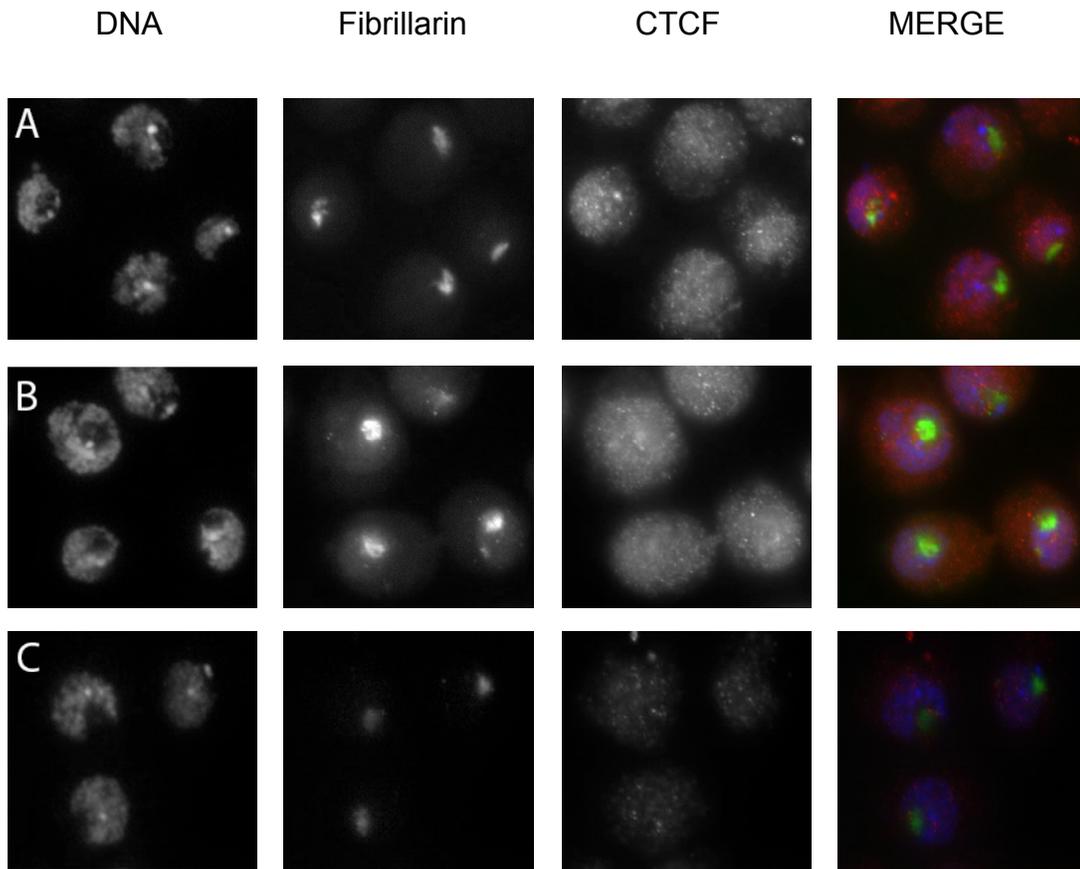


Figure 3.10 Epifluorescence microscopy of S2 cells after dsRNA treatment. CTCF is shown in red and Fibrillarin in green A) Control, B) dsRNA directed at LacZ and C) dsRNA directed at CTCF.

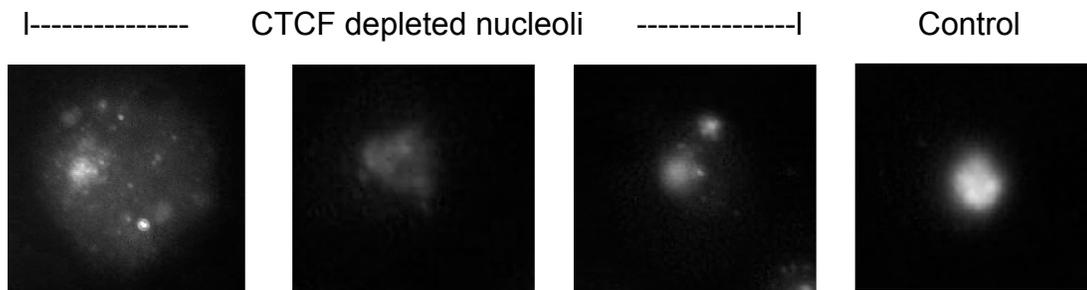


Figure 3.11 Epifluorescence microscopy. Nucleoli of cells treated with dsRNA directed at CTCF and control. Fibrillarin staining is shown only.

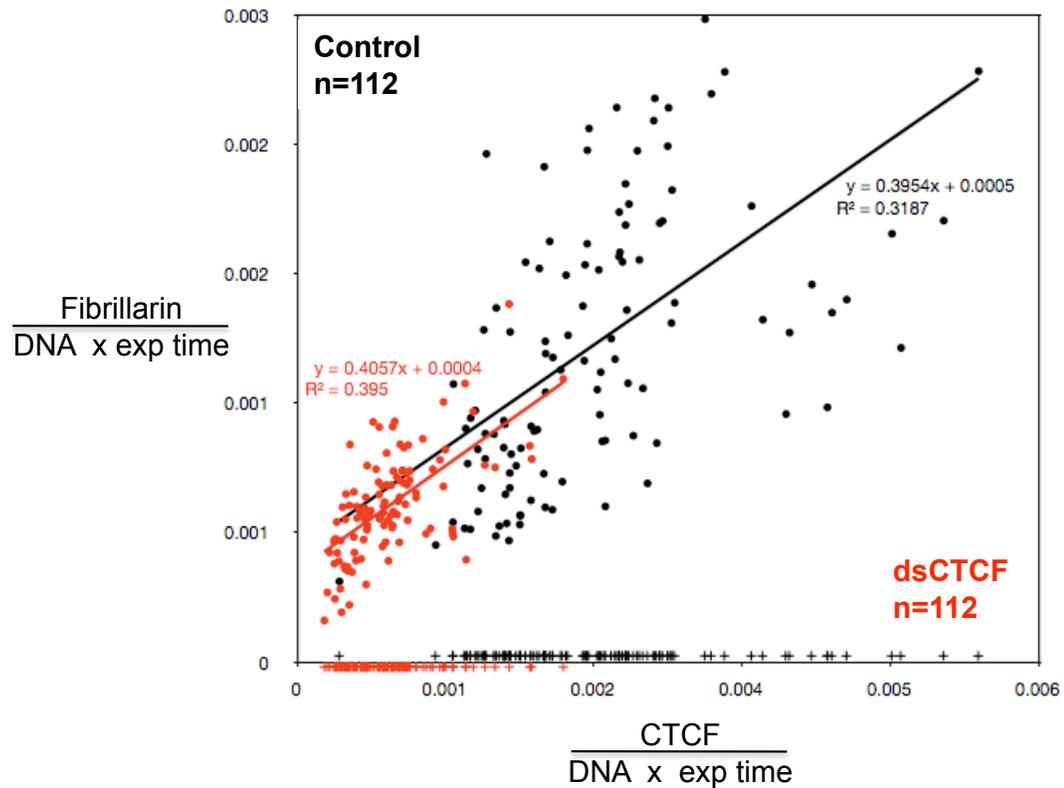


Figure 3.12 Measurement of fluorescence intensity of CTCF and Fibrillar staining using Image J software. RNAi treated cells are shown in red while untreated cells are in black. Y axis shows Fibrillar data compared to DNA signal (DAPI staining) and to exposure time. X axis shows CTCF signal. All data points were also plotted on the X axis for easy interpretation.

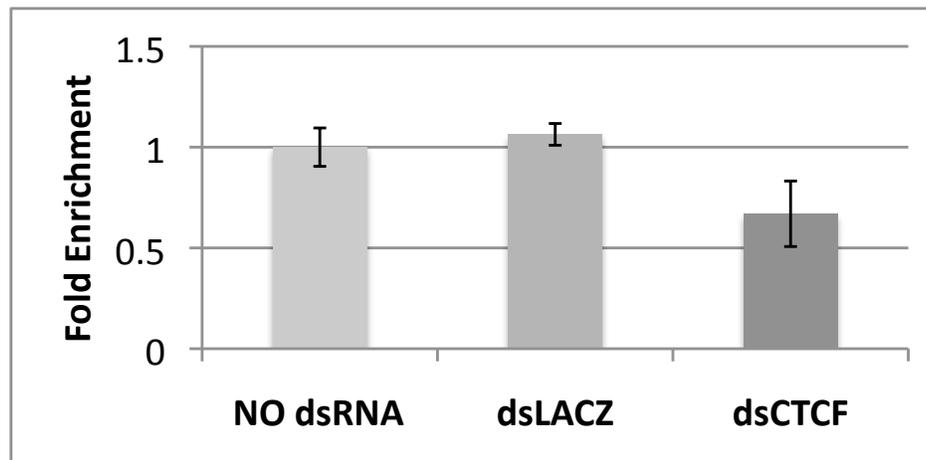


Figure 3.13 CTCF mRNA level after RNAi treatment. Y axis shows CTCF expression.

35S rDNA transcripts were measured in CTCF RNAi treated cells and an increase (30%, Fig 3.14) was observed. This confirmed our previous results obtained with the *CTCF^{p35.2}* stock. Therefore, with double stranded RNA directed at CTCF we were not only able to recapitulate the phenotype but also to observe the same effects in rDNA transcription. The small increase in 35S rDNA transcripts was expected because of the large pool of RNAs in the cell.

To observe a more dramatic effect on rDNA and confirm our previous results, R1 and R2 transcript levels were measured, since in regular conditions these elements are silent or transcribe at very low levels. R1 transcript amounts were 4.5 fold enriched in CTCF depleted cells (Fig. 3.15) while R2 transcripts increased 2.5 fold (Fig 3.16). These results were in agreement with what is expected as an immediate respond follow CTCF depletion. R1 elements, which are directly bound by CTCF, will have an increase in transcript amounts, and R2

located in the vicinity would also be affected. However, this perturbation of local heterochromatin would generate instability in the rDNA array, causing the loss of rDNA copies and shortening of the array. This was the case of the Y-linked rDNA array in the CTCF^{p35.2} stock (Figures 3.3 and 3.7). Therefore, I speculated that in a longer term the effects on R1 transcripts observed in S2 cells will be less because of the loss in R1 inserted copies. I believe rDNA array size reduction does not occur very fast in CTCF mutants. When measuring Y-linked rDNA array size in a C(1)DX,y^{1f1}bb⁰ background both CTCF^{p35.2/+} and +/TM6BTb had the same rDNA content. Therefore, a single generation did not cause much effect in rDNA copy number, but it did so on rDNA transcripts.

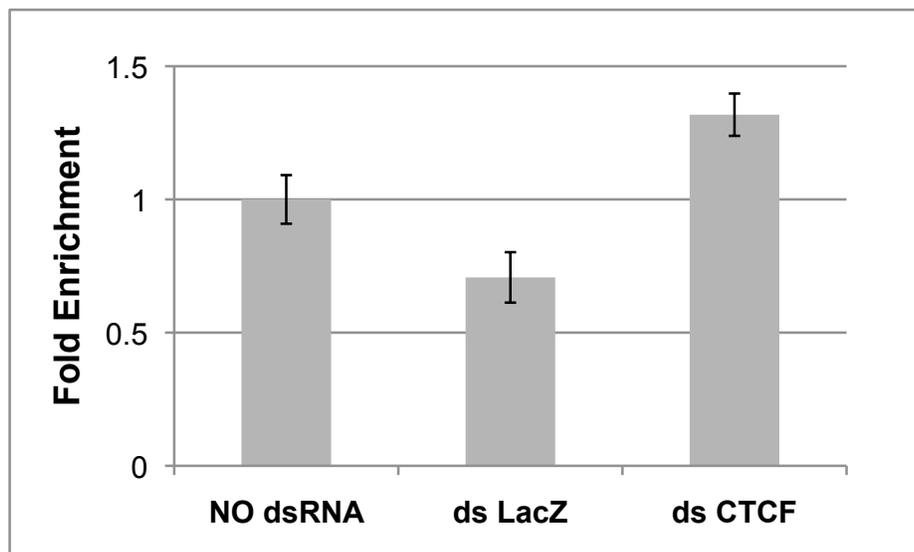


Figure 3.14 rDNA transcripts in CTCF mutant cells. Overall 35S rDNA transcripts level in untreated cells (NO dsRNA), cells treated with dsRNA directed at LacZ, and cells treated with dsRNA directed at CTCF. Y axis shows 35S rDNA expression.

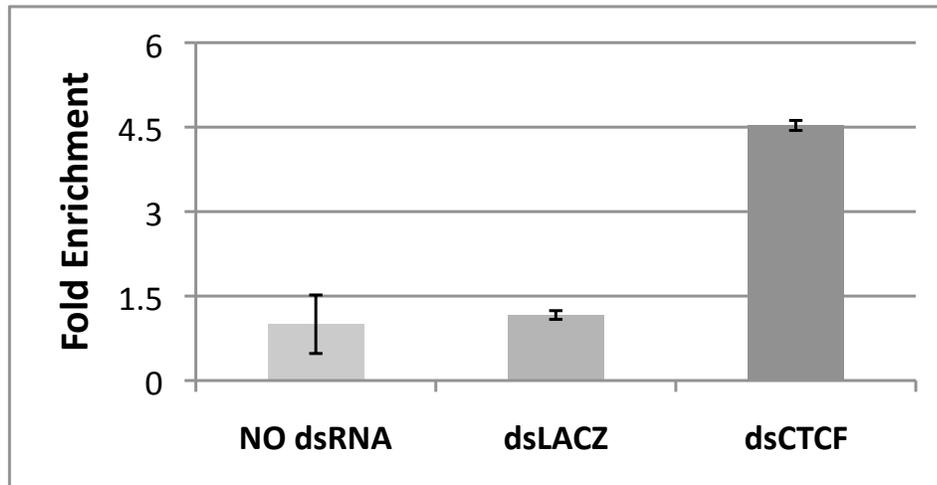


Figure 3.15 Transcripts level of R1 elements in dsRNA treated cells and controls. Y axis shows R1 expression.

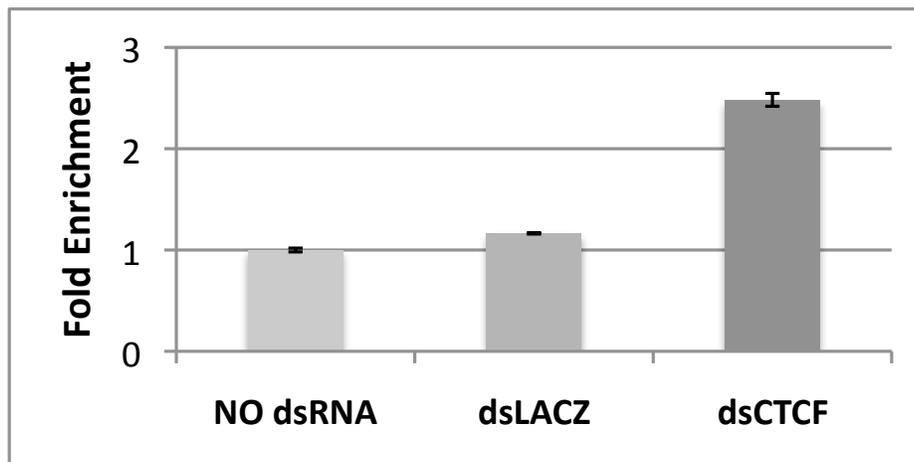


Figure 3.16 Transcripts level of R2 elements in dsRNA treated cells and controls. Y axis shows R2 expression.

Effect of PARP and PARG in rDNA transcripts

Poly (ADP-ribosyl)ation of CTCF in mammalian cell culture was required for CTCF nucleolar localization and RNA polymerase I transcription. Although

this post-translational modification has not been shown in *Drosophila* CTCF, the localization of PARP and PARG in the nucleolus and nucleoplasm respectively, and the alterations in nucleolar morphology observed in their mutants (as described earlier), led us to investigate how these two proteins were involved in nucleolar regulation and how CTCF was part of this mechanism. We predicted that if PARP and PARG were contributing to the regulation of rDNA transcription through CTCF, in a PARP and PARG mutant we would observe mislocalization of CTCF, alterations in nucleolar integrity and effects in rDNA transcription.

After a three day treatment with dsRNA directed at PARP and PARG in S2 cells, I did not observe a visible decrease of CTCF signal from the nucleolus by immunostaining (Fig 3.17 C-D). However, I noticed that CTCF was mislocalized at the cytoplasm. On the other hand, Fibrillar staining showed a less compacted and fragmented nucleolus (Fig 3.17 C-D compared to A-B). Torrano and colleagues had shown that inhibition of PARP impaired CTCF nucleolar localization and we observed a similar phenotype. Although I could not see a visible reduction of CTCF at the nucleolus, the mislocalization of the protein at the cytoplasm in PARP and PARG dsRNA treated cells revealed that probably CTCF found at the cytoplasm was not poly(ADP-ribose). Similarly, the phenotype observed with Fibrillar resembles what seen before in CTCF depleted cells; therefore, in a PARP and PARG mutant background nucleolar integrity is affected when the CTCF Poly(ADP-ribose)ation cycle is compromised.

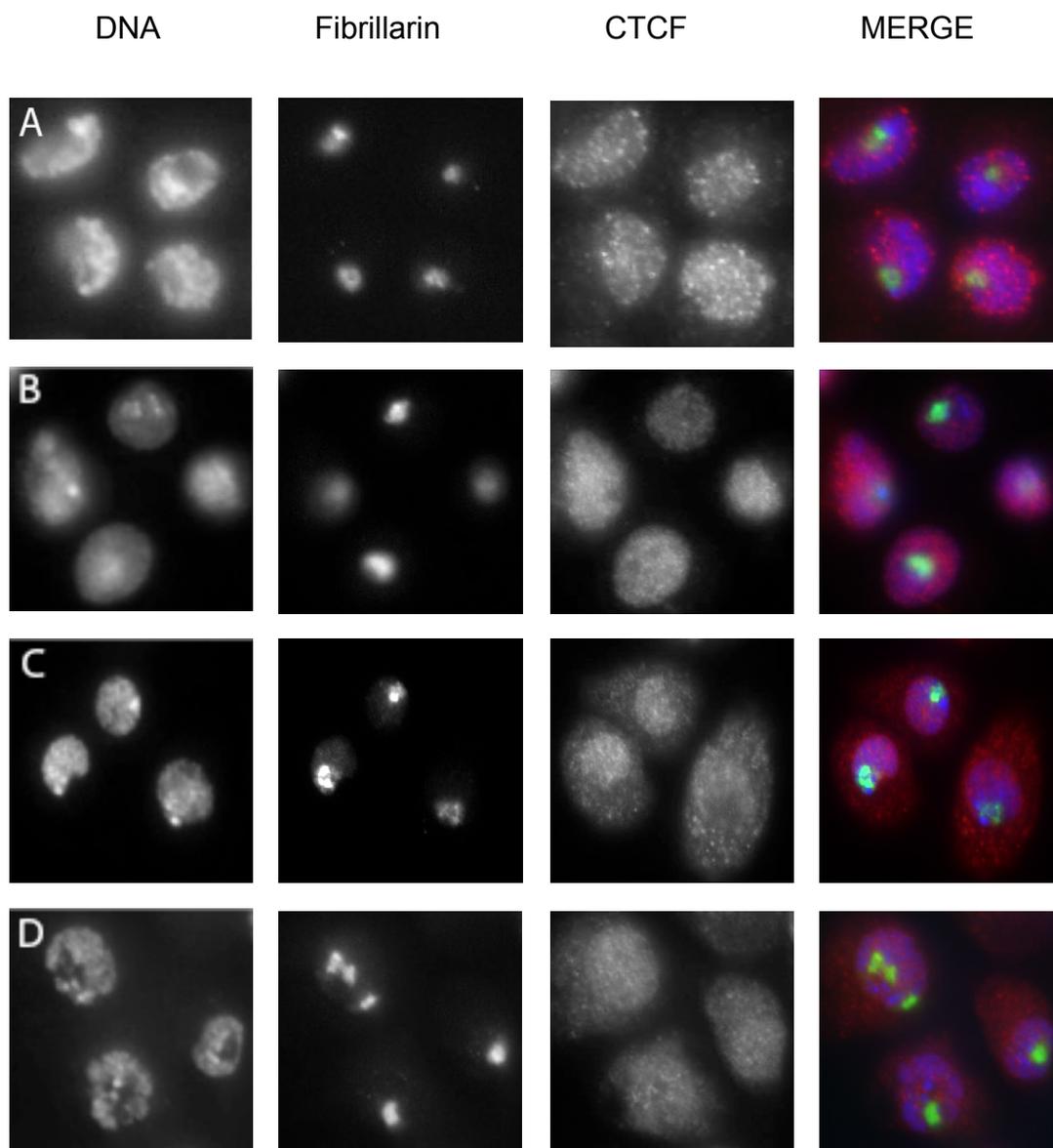


Figure 3.17 Epifluorescence microscopy of S2 cells. CTCF (red), Fibrillarin (green). A) No dsRNA. dsRNA directed at B) LacZ , C) PARP and D) PARG.

PARP and PARG mRNA levels were dramatically decreased after dsRNA treatment (Figs 3.18 & 3.19 respectively), PARP was reduced to 11.4% of wild type level (Fig 3.18) and PARG was reduced to 34.8% (Fig 3.19). In cells treated

with RNAi directed toward PARP, R1 transcripts increased 1.8 fold (Fig 3.20) while R2 transcripts did not increase (Fig 3.21). In a PARG depleted cells, R1 transcripts increased 2.8 fold (Fig 3.22) and R2 transcripts increased 1.8 fold (Fig 23). The reason we observed a greater effect in R1 inserted copies is perhaps attribute to having more R1 elements in the rDNA array and R2 being dispersed and away from R1 inserted copies. Together these data show that depletion of PARP and PARG partially removed CTCF from the nucleolus, disrupted nucleolar integrity and affected transcripts level of the ribosomal DNA. These observations were very similar to that seen in CTCF depleted cells which indicates that PARP and PARG were also involved in maintaining nucleolar integrity and regulating rDNA transcript levels; however, we cannot assure at this point whether this is a indirect or direct effect. It is very likely that in *Drosophila*, CTCF is poly(ADP-ribosyl)ate as it has been observed in mammals and this post-translational modification directs CTCF to the nucleolus; therefore, the PARP-PARG cycle indirectly would regulate rDNA transcription. Non-nucleolar chromatin was not affected which predicts that poly(ADP-ribosyl)ation of CTCF exclusively regulates nucleolar CTCF.

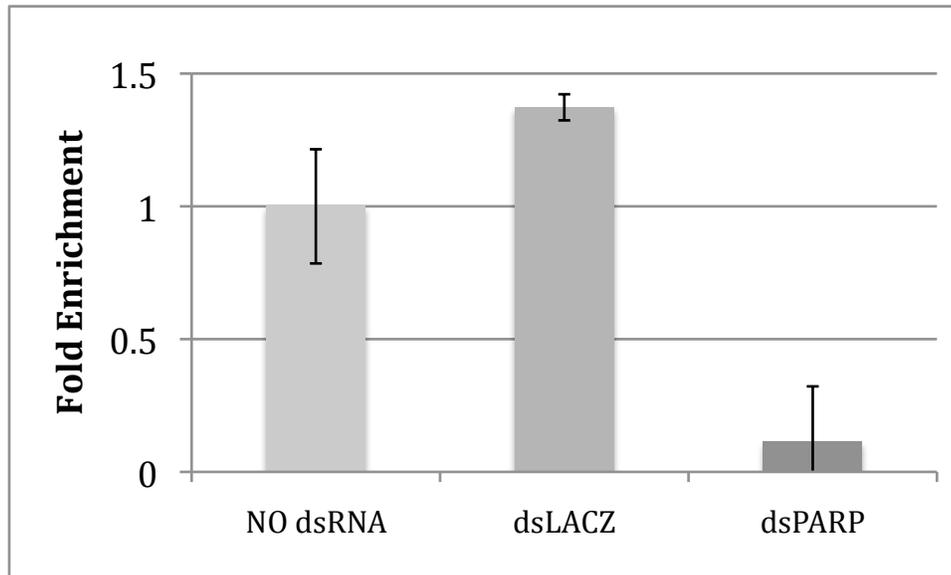


Figure 3.18: PARP mRNA level after RNAi treatment. Y axis shows PARP expression.

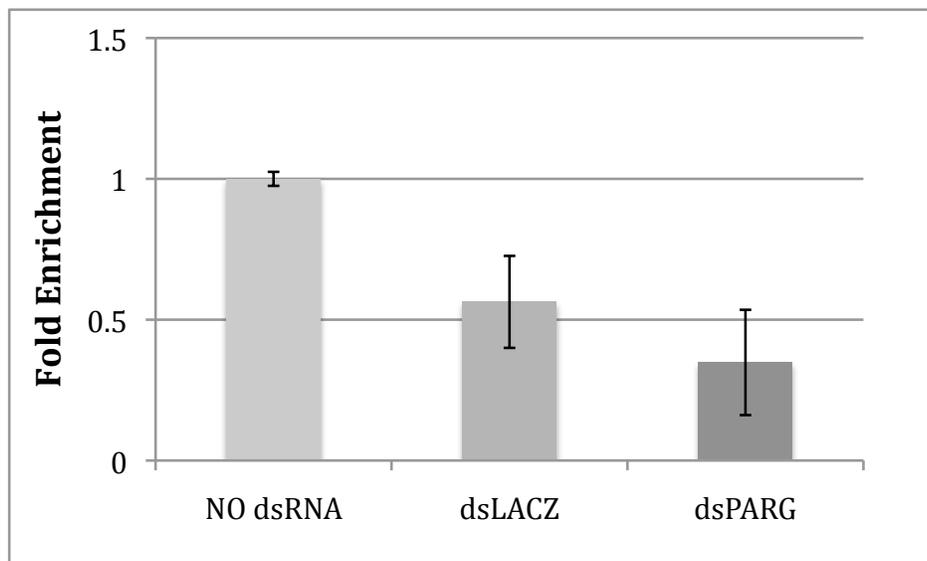


Figure 3.19 PARG mRNA level after RNAi treatment. Y axis shows PARG expression.

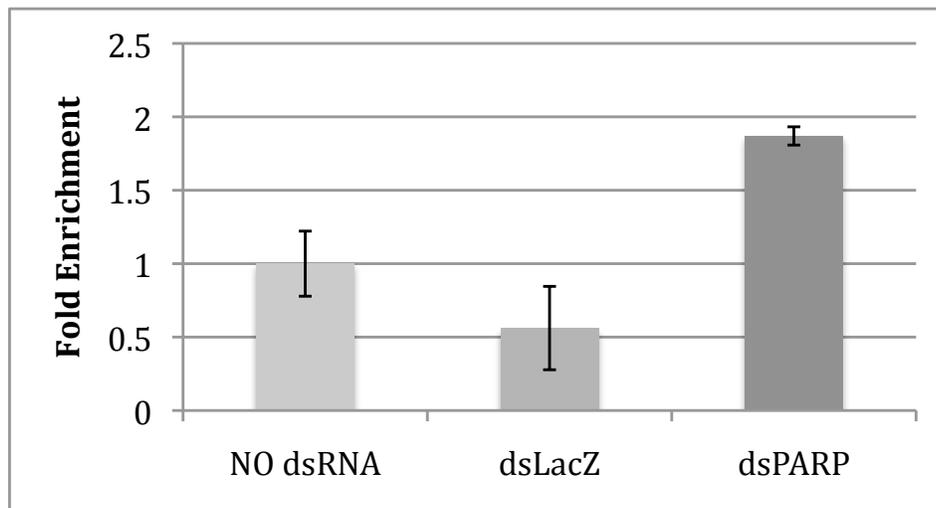


Figure 3.20 Transcripts level of R1 elements in PARP dsRNA treated cells and controls. Y axis is showing R1 expression

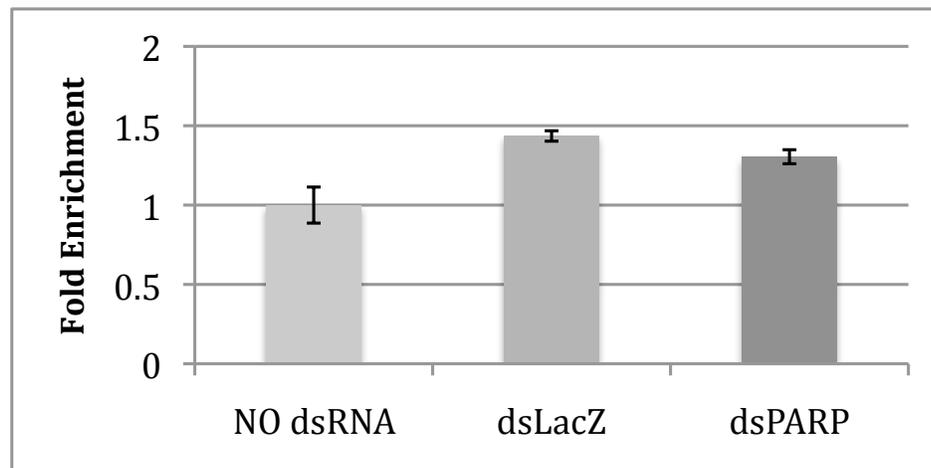


Figure 3.21 Transcripts level of R2 elements in PARP dsRNA treated cells and controls. Y axis shows R2 expression.

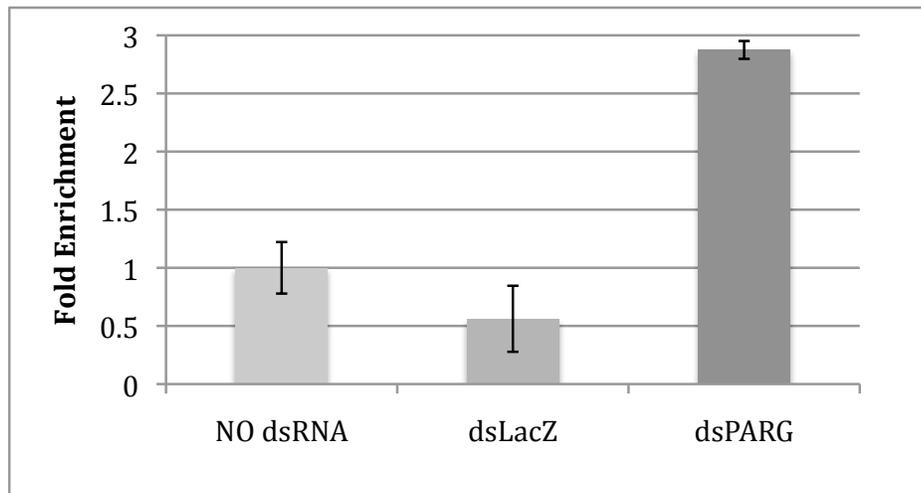


Figure 3.22 Transcripts level of R1 elements in PARG dsRNA treated cells and controls. Y axis shows R1 expression.

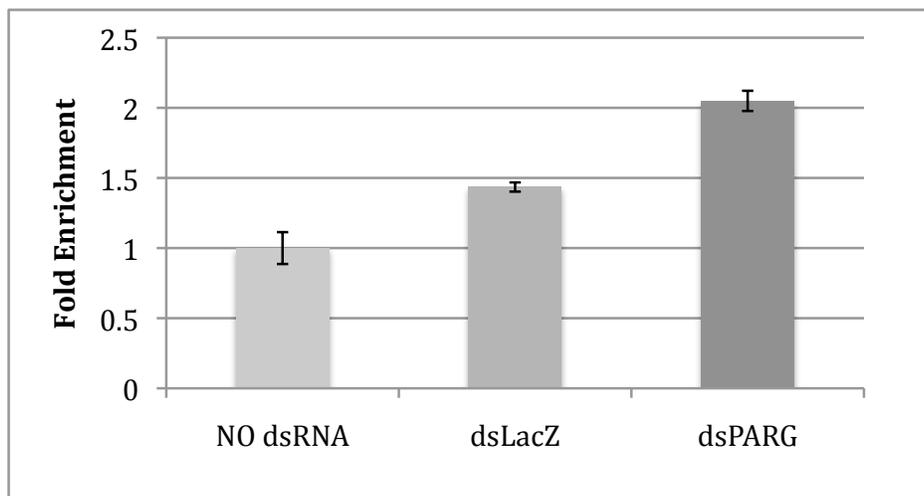


Figure 3.23 Transcripts level of R2 elements in PARG dsRNA treated cells and controls. Y axis shows R2 expression.

RNAi experiments were a very important tool to observed the immediate response in rRNA levels after PARP and PARG depletion. However, we could

not observe complete depletion of CTCF from the nucleolus regardless of the effectiveness of both PARP (11.4%) and PARG (34.8%) knockdowns. As it was mentioned before PARP and PARG mutant flies showed no nucleoli or very fragmented nucleoli, respectively. Therefore, we wanted to see whether under these conditions CTCF remained at the nucleolus and how rRNA transcripts were affected. We obtained a Parg mutant allele (*Parg*^{27.1}), originally created by Hanai and colleagues in 2004 [111], and stained whole-mount salivary glands nuclei with Fibrillarin and CTCF antibodies (Fig 3.24). Staining with Fibrillarin showed a disrupted nucleolus and complete absence of CTCF from the nucleolus which confirmed the results obtained with S2 cells. We wanted to measure 18 rDNA, R1 and R2 transcripts; however, this was not possible because in *Drosophila*, *Parg* gene is located at the X chromosome so it makes impossible to compare the same rDNA array with and without *Parg* because the rDNA is linked to the same chromosome, thus; I could not look at a matched control.

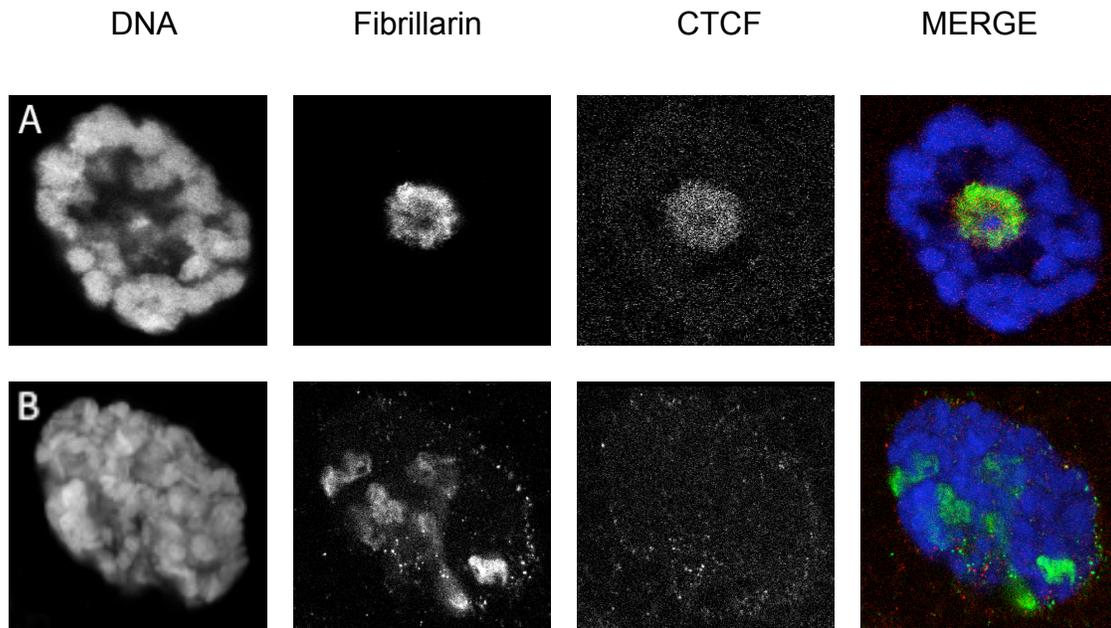


Figure 3.24 Epifluorescence microscopy of whole-mount salivary gland nuclei. A) control, B) *Parg^{27.1}*. Fibrillar is shown in green and CTCF in red.

Conclusion

Using gain-of-function CTCF alleles, Torrano and colleagues showed important initial observations in the role of nucleolar CTCF. However, this work lacked a loss-of-function phenotype. We recently observed CTCF at the rDNA loci of the X and Y chromosomes and chromatin immunoprecipitation showed binding at the R1 element. Based on these observations and combined with Torrano results we predicted that CTCF was involved in the rDNA transcription regulation.

We looked at previously characterized CTCF mutant alleles and observed dramatic fragmented nucleoli, reduction in rDNA array copy number, bobbed

phenotype, and increased in 18S rRNA transcripts. These results indicated that CTCF was acting as a repressor of rDNA transcription and in a mutant background, the disturbance of local heterochromatin by the activation of silent copies, was affecting rDNA chromatin and as a result nucleolar architecture was compromised.

The effects on 18S rRNA transcripts were small; therefore, we looked the R1 and R2 elements inserted in the rDNA because: 1) R1 was bound by CTCF and 2) activation of silent copies was a more sensitive method. We observed an increase in transcripts level of both R1 and R2 elements. We suspected we were looking at nascent transcripts instead of steady-state levels because normally R1 and R2 transcripts are degraded; therefore, I measured unprocessed rRNA junctions and they in fact increased in a homozygous mutant background. We confirmed these results by treating S2 cells with dsRNA directed at CTCF and measuring the same parameters: CTCF immunofluorescence, 18S, R1 and R2 transcripts.

The increase in unprocessed junctions, led us to investigate whether CTCF was involved in rRNA processing. Similarly, we were only able to identify CTCF binding at the R1 element and by immunofluorescence, CTCF was enriched at the nucleolus. Therefore, apart for binding rDNA, CTCF could be also binding rRNA. After RNase treatment, CTCF was reduced to a 20% compared to untreated cells. This shows that 80% of nucleolar CTCF required RNA to be maintained and/or recruited to this compartment. However, further

studies need to be performed to examine whether CTCF binds directly RNA and the requirements for this interaction (e.g. zinc finger combination used and consensus binding site).

Torrano and colleagues work also showed another characteristic of nucleolar CTCF; it was post-translationally modified by PARP and this was necessary for nucleolar recruitment. We wondered whether Poly(ADP-ribosylation) of CTCF had a contribution in its role as repressor of rDNA transcription, specially because in flies, PARP and PARG mutants have shown nucleolar fragmentation and impediment to form nucleoli. Therefore, I knocked down PARP and PARG in S2 and I observed nucleolar fragmentation, increase in 18S, R1 and R2 transcripts, and CTCF mislocalization. These results showed that in flies, as well as in mammals, CTCF requires the poly(ADP-ribosylation) cycle to function at the nucleolus.

We gained crucial information in the role of CTCF in rDNA transcription. However, we would like to move one step further and see how other aspects of rDNA transcription control, such as nucleolar dominance, are affected in CTCF mutants and which other factors, besides the PARP-PARG cycle, brings CTCF to the nucleolus.

CHAPTER IV

REGULATION OF rDNA TRANSCRIPTION IN CTCF MUTANTS

Introduction

Protein demand dictates the rate of rDNA transcription; therefore, rDNA transcription is directly linked to cell growth and proliferation. For instance, oxidative stress, nutrient starvation, cell confluence and protein synthesis inhibition, decrease rDNA transcription. On the other hand, cell growth and proliferation factors increase rDNA transcription [54]. As discussed in Chapter I, control of rDNA transcription involves many mechanisms such as; UBF post-translational modifications, rDNA methylation, recruitment of remodeling complex NorC, and the regulator of nucleolar silencing and telomerase exit (RENT). Apart from these mechanisms that control the epigenetic state of individual rDNA copies, there is a process that involves the silencing of an entire rDNA array: nucleolar dominance.

In interspecific hybrids, the rDNA array coming from one of the parents is silenced by a phenomenon called nucleolar dominance (ND). It was initially described in plants [112] and later it was found in other organisms such as, amphibians and flies [113]. When chromosomes condensed during mitosis, the rDNA locus is not condensed in the same way as the rest of the chromosome due to the persistence of UBF at the active copies [114]. As a result, a constriction is formed and since it does resemble the centromeric constriction, it

is therefore called “secondary constriction”. Cytologically, nucleolar dominance can be visualized by the absence of the secondary constriction at the rDNA locus derived from one of the parents [115]. The first insights in understanding ND came from plants where inhibition of cytosine methylation and histone deacetylation led to the loss of nucleolar dominance [116]. It was shown later by Chromatin Immunoprecipitation (ChIP) that the promoters of the inactive rDNA array were enriched in H3K9me2, while the active array was a mix of H3K9me2 and H3K4me3. Likewise, a variation of the ChIP technique (ChIP-chop) demonstrated that DNA associated with methylated H3K9 was also hypermethylated at CG dinucleotides, while DNA associated with methylated H3K4 was hypomethylated and enriched in RNA Polymerase I [117].

In *Drosophila* interspecific hybrids, when polytene chromosomes are formed, one of the rDNA arrays is preferentially replicated while the other array is under-replicated, a phenomenon known as replicative dominance. However, if the dominant chromosome loses rDNA copies, replicative dominance is abolished [118]. Nucleolar and replicative dominance seem to share a common regulatory mechanism since they were observed to occur simultaneously in the same hybrid [113].

rDNA transcriptional regulation in *Drosophila* has many of the mechanisms described above. However, it has an additional component. It contains intervening sequences interrupting the transcribed region of the ribosomal DNA and characterized as R1 and R2 retrotransposable elements.

Previous work showed that inserted copies are silent or transcribed at very low levels. Similarly, it showed that inserted R1 and R2 rDNA copies can be lost from the array presumably by recombination [83,90,91] and that the transcription level of these R-elements is directly proportional to their retrotransposition rate. Likewise, this work showed that if a line which has active R2 transcription is crossed with a line that has low or no R2 transcription, in the heterozygous females (it has one X chromosome from the mother and one X chromosome from the father) of the next generation, R2 expression was low. They interpreted this as the low R2 transcripts being dominant over high R2 transcripts. They stained mitotic chromosomes from those heterozygous females with DAPI and noticed that while one X chromosome formed a secondary constriction (active rDNA array) the other one did not (inactive rDNA array) which has been described as nucleolar dominance. Therefore, the R2 transcription level was reflecting the activity of the entire rDNA array. Finally, they studied the distribution of the R1 and R2 elements of inactive and active lines and noticed that inactive lines had R elements clustered together, leaving regions of uninserted rDNA units, while in active lines R elements were spread throughout the rDNA array. They proposed that in the active lines because uninserted and R-inserted units are intercalated and silencing of the entire array will be inviable, some R2 inserted units are also transcribed. However, in the heterozygous females of the next generation, the rDNA array with the clustered R elements is

preferentially transcribed because the region of uninserted rDNA units supplies enough rRNA for survival; therefore, the active line rDNA array is silenced [92].

The discovery of nucleolar dominance helped to understand how different organisms control the redundancy on rDNA copies and NORs, allowing only the transcription of the number of copies required for wild type expression. Our studies showed that in CTCF mutants rDNA transcription increases and leads to the loss of rDNA units; therefore, we wondered whether CTCF mutants could alleviate nucleolar and replicative dominance.

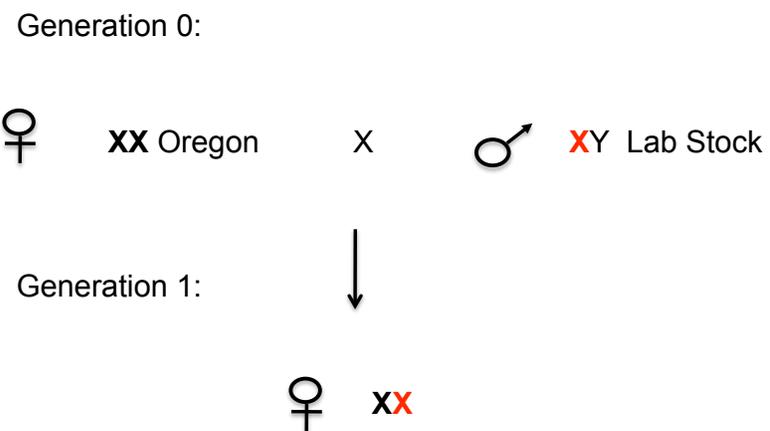
Results and Discussion

Nucleolar Dominance

Nucleolar dominance in interspecific *Drosophila melanogaster* / *simulans* hybrids has shown that *D. melanogaster* was dominant over *simulans* [119]. These studies were done crossing *D. melanogaster* females to *D. simulans* males because in the reciprocal cross the predominant progeny is males. Similarly, intra-species nucleolar dominance has also been reported. Oregon R and Canton S are well known *D. melanogaster* wild type strains and studies performed by Sharyn Endow showed that Canton S was dominant over Oregon R [118]. In order to know whether CTCF mutants were able to abolish nucleolar dominance, firstly, we needed to establish whether nucleolar dominance existed between our *D. melanogaster* laboratory strain and wild type strains from the Bloomington Stock Collection Center. I tested our laboratory strain against:

Canton S, Oregon R, Harwich and Bogota wild type strains and I observed nucleolar dominance only with Oregon R (Fig 4.1). We could not determine which strain was dominant because the X chromosomes were not physically distinguishable. We crossed Oregon R to four *CTCF* mutants stocks. *Df(3L)0463* and *CTCF^{p35.2}* were previously described in Chapter IV, *TCF¹* and *CTCF⁹* were deleted using homologous recombination in our laboratory according to the method described by Maggert, Gong, and Golic [120]. I did reciprocal crosses between *CTCF* mutants and Oregon R as shown in Figures 4.2 and 4.3. *CTCF¹* did not show alleviation of nucleolar dominance in both crosses (Fig 4.2 A-1-2 and Fig 4.3 A-3-4). On the other hand, *CTCF⁹* and *Df(3L)0463* showed ND alleviation when *CTCF* mutation was brought maternally and paternally (Fig 4.2 B-C and Fig 4.3 B-C). Lastly, *CTCF^{p35.2}* showed release from ND only when *CTCF* came from the father (Fig 4.3 C3-4), but not in the reciprocal cross (Fig 4.2 C1-2). Therefore, we could not determine whether *CTCF* mutant could alleviate nucleolar dominance because the lack of consistency among the different mutant alleles.

A)



B)

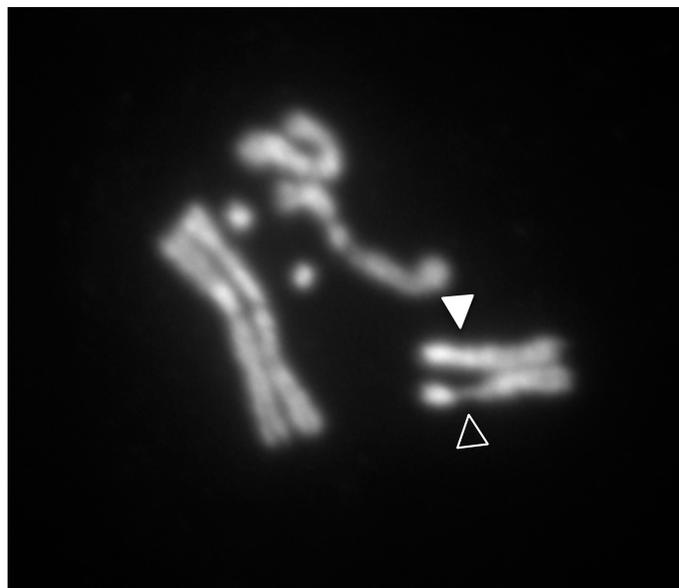


Figure 4.1 Nucleolar dominance. A) Cross to detect nucleolar dominance. B) Mitotic spread of a neuroblast nucleus. X chromosome forming secondary constriction is pointed by an open triangle while X chromosome with inactive NOR is pointed by a filled triangle.

A)

Generation 0:

$$\text{♀ } X^{\text{Lab}} / X^{\text{Lab}} ; \text{CTCF}^{\text{p35.2}} / \text{TM6BTb} \quad \times \quad \text{♂ } X^{\text{Ore}} / Y$$


Generation 1:

$$\text{♀ } X^{\text{OR}} X^{\text{Lab}} ; \text{CTCF}^{\text{p35.2}} / + \quad \text{vs} \quad \text{♀ } X^{\text{Ore}} X^{\text{Lab}} ; + / \text{TM6BTb}$$

B)

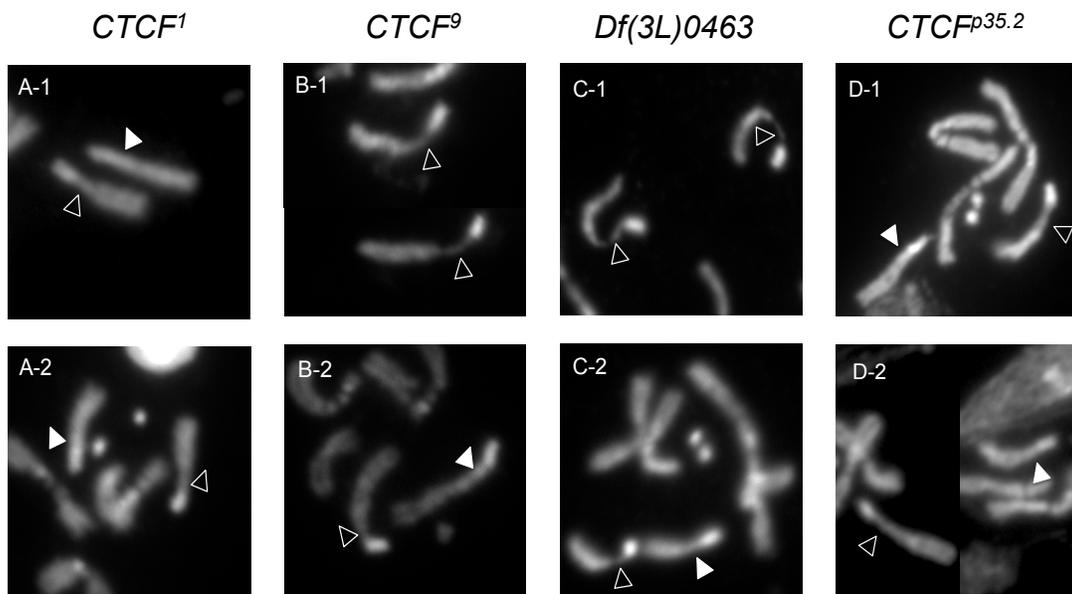


Figure 4.2 Nucleolar dominance in CTCF mutants. A) CTCF mutants females were crossed to Oregon males. $\text{CTCF}^{\text{p35.2}}$ is shown to exemplify all four CTCF mutants shown in part B. B) Upper Row: G1 females from cross A) carrying the CTCF mutant chromosome. Lower row: Control, TM6BTb chromosome. X^{Lab} : laboratory strain, X^{Ore} : Oregon strain. Open triangle shows secondary constriction while filled triangle indicates lack of secondary constriction.

A)

Generation 0:

$$\text{♀ } X^{\text{Ore}} / X^{\text{Ore}} \quad \times \quad \text{♂ } X^{\text{Lab}} / Y ; \text{CTCF}^{p35.2} / \text{TM6BTb}$$

Generation 1:

$$\text{♀ } X^{\text{OR}} X^{\text{Lab}} ; \text{CTCF}^{p35.2} / + \quad \text{vs} \quad \text{♀ } X^{\text{Ore}} X^{\text{Lab}} ; + / \text{TM6BTb}$$

B)

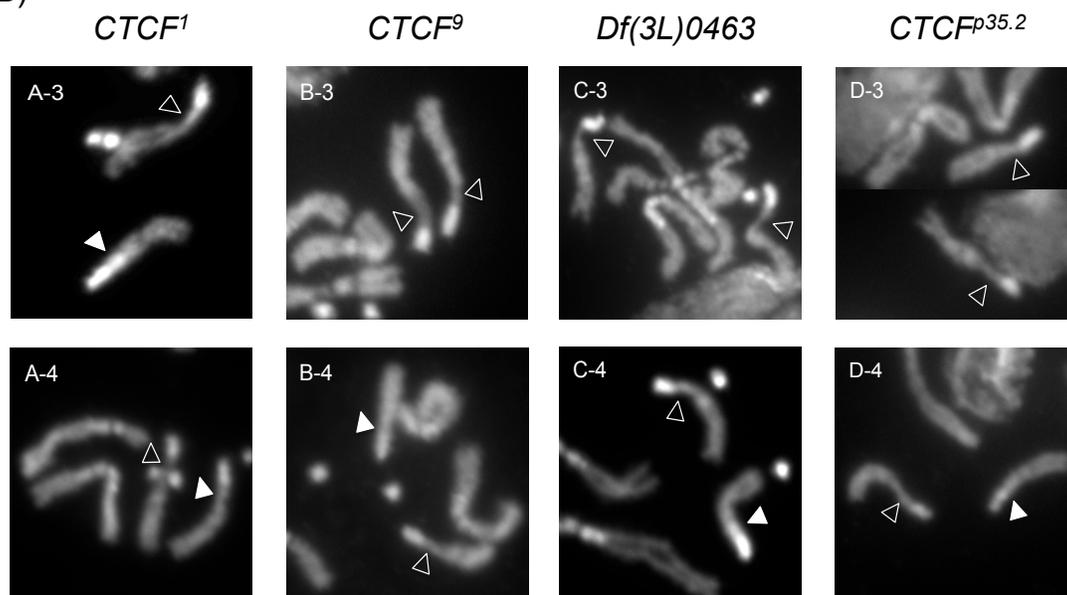


Figure 4.3 Nucleolar dominance in CTCF mutants. A) Oregon females were crossed to CTCF mutant males. B) Upper row: G1 females from cross A) carrying the CTCF mutant chromosome. Lower row: Control. X^{Lab} : laboratory strain, X^{Ore} : Oregon strain. Open triangle shows secondary constriction while filled triangle indicates lack of secondary constriction.

We then looked at the effects of CTCF on replicative dominance. We wondered whether rDNA replication would be affected in CTCF mutants and would be more consistent that the effects in nucleolar dominance. We examined whether rDNA replication would increase in a CTCF mutant background. I crossed males from CTCF mutant stocks *CTCF⁹*, *Df(3L)0463*, and *CTCF^{p35.2}* to control females as shown in Figure 4.4 A, and collected three brains from third instar larvae *X/Y; CTCF^{p35.2}* and *X/Y; + /TM6BTb* males. After sonicating each brain separately in phosphate buffered saline (PBS) solution, I used 1µl for real time PCR (RT-PCR). I quantified the number of uninserted, R1 and R2 inserted rDNA copies in males carrying the CTCF mutant chromosome and in males carrying the balancer chromosome. Figure 4.4 B shows the average of three RT-PCRs performed to CTCF mutant brains and controls, each reaction run in triplicate. I observed that in both *CTCF⁹*, *Df(3L)0463*, the uninserted copies were under-replicated in a CTCF mutant background compared to the control (Fig 4.4 B). In *CTCF^{p35.2}*, on the other hand, I observed the opposite effect, the mutant chromosome was dominant over the control. Therefore, while the replication of R1 and R2 inserted copies was not significantly affected in any CTCF mutant background, the replication of the uninserted copies was affected; however, the trend was not consistent among the three mutant background studied. It is worth to notice that the two alleles that strongly showed nucleolar dominance alleviation (*CTCF⁹*, *Df(3L)0463*), showed under-replication of uninserted copies. This could indicate that possibly active transcription of both rDNA assays is

required to balance under-replication in the of mutant background. In contrast, in *CTCF^{p35.2}*, nucleolar dominance was not abolished because the number of rDNA copies in the mutant chromosome was higher compared to the control.

We did not obtain consistent results in the CTCF mutants tested in both nucleolar and replicative dominance. Although the CTCF mutation is the main source of variation among the stocks, additional mutations could have been introduced when the mutant alleles were created and may have been the source of the dissimilarities observed. We initially thought that the reduction of rDNA copies in the CTCF mutant alleles would impede nucleolar dominance. Deletion of rDNA copies is known to abolish replicative dominance, which seems to be closely linked to nucleolar dominance. In *CTCF^{p35.2}* in spite of observing reduction to 49.6% of wild type, nucleolar dominance was not abolished when the CTCF mutation was brought maternally. On the other hand, the reduction in the rDNA copy number of *Df(3L)0463* was considerably less (66.55%), however, this seemed to be enough to remove nucleolar dominance.

A)

Generation 0:

♀ $X/X; +/+$ × ♂ $X/Y; CTCF^{p35.2} / TM6BTb$

Generation 1:

♂ $X/Y; CTCF^{p35.2} / +$ vs ♂ $X/Y; + / TM6BTb$

B)

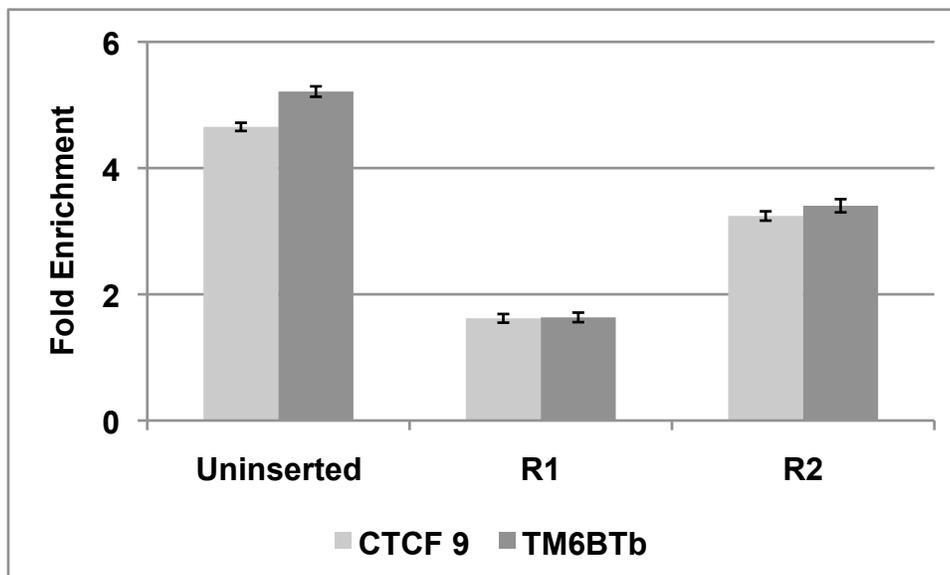


Figure 4.4 Replicative dominance. A) Males from $CTCF^0$, $Df(3L)0463$, and $CTCF^{p35.2}$ stocks were crossed to isogenic females and G1 third instar larvae brains were collected. B) Comparative Real Time PCR of mutant versus control brains. Each bar represents the average of three independent experiments. Y axis represents the fold enrichment ($2^{-\Delta C_t}$) normalized to an endogenous target ($tRNA^{K-CTT}$).

B)

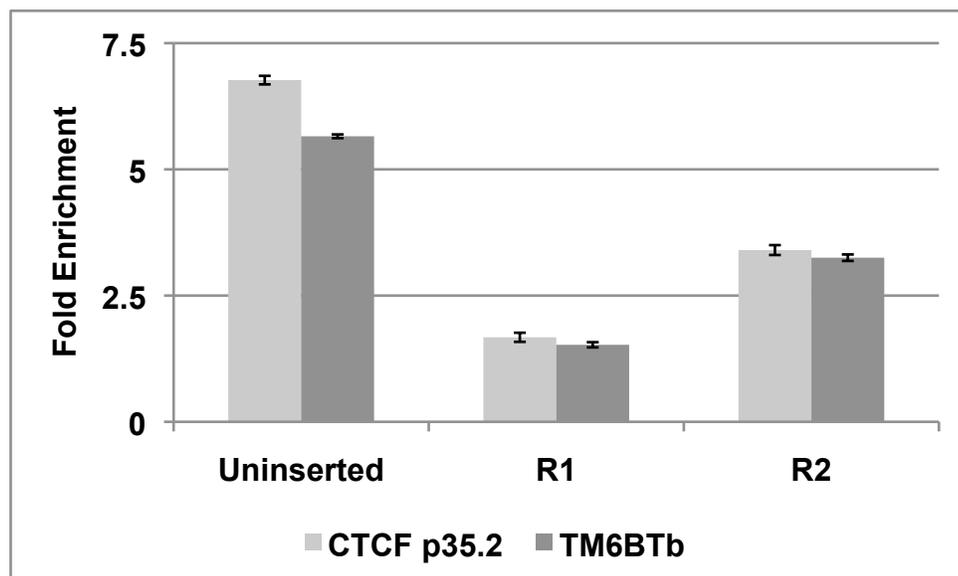
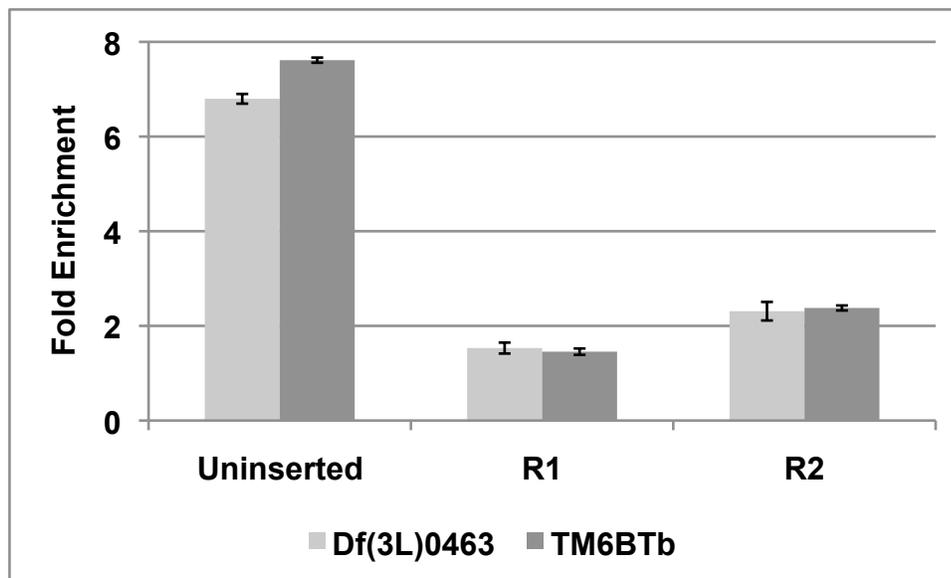


Figure 4.4 Continued.

Considering R1 and R2 distribution in the rDNA array, a possible reason for this discrepancy could be that if for instance, in the *CTCF^{p35.2}* background R-elements are spread out in the array while in the Oregon stock they are clustered together. In the hybrid Oregon/*CTCF^{p35.2}*, uninserted clustered rDNA units from the Oregon X chromosome will provide enough rRNA for survival and as a result the rDNA in *CTCF^{p35.2}* background would remain silenced. In the opposite case, if in the *Df(3L)0463* the R units are clustered together, transcription from both rDNA arrays may occur. In order to better understand how CTCF affects nucleolar and replicative dominance, further studies need to be conducted to measure R elements distribution throughout the rDNA array in the Oregon strain and CTCF mutant stocks.

Extrachromosomal Circles and RNA polymerase I transcription

Activation of the silent inserted and uninserted rDNA copies causes the loss of some of these units from the array by the formation of extrachromosomal circles. It is not known whether extrachromosomal circles are generated during RNA polymerase I transcription or by DNA replication. We used rapamycin, a RNA polymerase I inhibitor, to study this phenomenon. If in CTCF mutants, the generation of extrachromosomal circles is a consequence of an increase in rRNA transcripts, and this resulted in the formation of the ectopic nucleoli observed by fibrillarin; by inhibiting RNA polymerase I transcription, we expected

that extrachromosomal circles would not form and the nucleolus would appear compacted.

CTCF^{p35.2} and control flies were fed with Rapamycin to a final concentration of 1 μ M. G1 female third instar larvae were collected and whole-mount salivary glands were processed for immunofluorescence to detect Fibrillarin. As shown in Chapter III (Fig 3.2), CTCF mutants nucleoli are less compacted and fragmented than wild type. We believed that these ectopic nucleoli observed correspond to rDNA units lost from the array, since we measured rDNA copy number and noticed rDNA copy number reduction in all CTCF mutants tested (Fig 3.3). Similarly, earlier studies done by Peng and Karpen, described an increase in rDNA extrachromosomal circles from heterochromatin proteins that had the same fragmented nucleolus phenotype [23]. We reasoned that if rDNA transcription was involved in this process; we could alleviate the mutant phenotype by inhibiting RNA polymerase I. Rapamycin treatment of *CTCF^{p35.2}* resulted in a more compacted nucleolus while the *yw* control appeared unchanged (Fig 4.5). Therefore, in a CTCF mutant background inhibition of RNA polymerase I transcription prevented nucleolar fragmentation which indicated that rDNA copies are lost from the array presumably by the formation of extrachromosomal circles during rDNA transcription. However, this does not discard the possibility of DNA replication being also the source of extrachromosomal circles formation. Further studies

need to be conducted to test whether rDNA units are also lost during DNA replication.

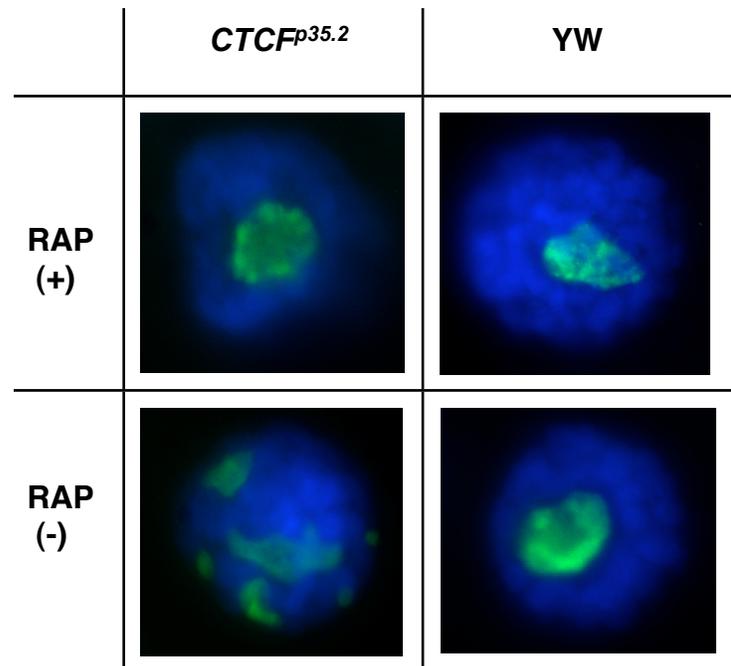


Figure 4.5 Fragmented nucleoli alleviation by Rapamycin. Whole-mount salivary glands of *CTCF^{p35.2}* and yw control flies fed (+) or not (-) with 1uM Rapamycin. Fibrillaritin is shown in green and DNA in blue.

CTCF recruitment to the nucleolus

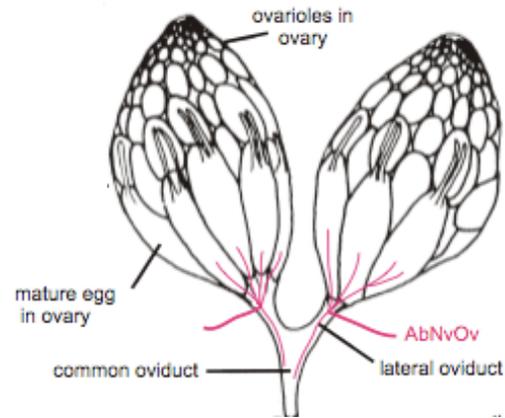
Previous work done in mammalian cell culture showed that RNA polymerase I transcription was required to keep CTCF at the nucleolus [25]. Likewise, showed that CTCF repressed rDNA transcription. We wanted to know whether CTCF was recruited to the nucleolus at all stages through development or if during those stages when the translational demand was high, CTCF would

be removed from the nucleolus. We looked at the developing ovarioles where protein demand increases progressively. *Drosophila* possesses two ovaries (Fig 4.6A) and each contains between 12 to 18 ovarioles, where oogenesis occurs. This process starts at the anterior end of the ovariole (left in Fig 4.6B-C) and progresses towards the posterior end. Oogenesis is divided in 14 stages based on morphology as shown in Figure 4.6B-C. At the anterior end of the ovariole, the germarium (Fig 4.6B) consecutively divides giving rise to egg chambers or cystoblast that contain nurse cells, follicle cells and the oocyte. These egg chambers increase in size and mature progressively towards the posterior end of the ovariole. Nurse cells transfer their content to the oocyte and then die. After this, the oocyte is released from the oviduct (Fig 4.6 A) [121].

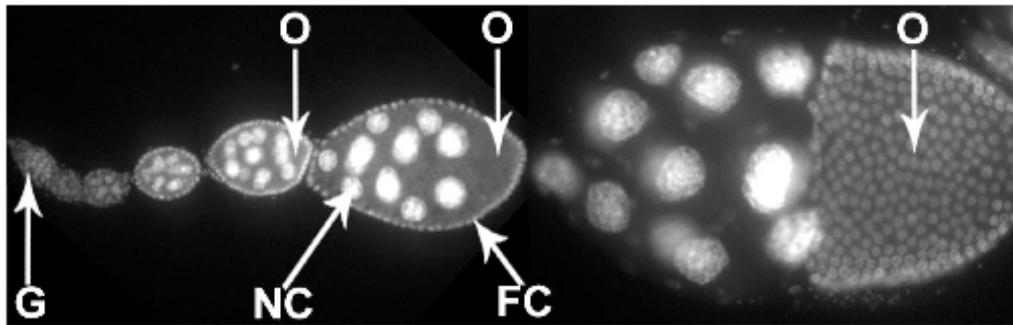
Work done by Dapples and King in 1970 showed that during oogenesis, the volume of nurse cells nucleoli increases [122]. Similarly, Mermod and colleagues [123] showed that the rRNA synthesis in each egg chamber increases gradually, which would indicate that in stages 1 to 4, the demand of ribosomes is low compared to more advanced 8 to 10 stages (Fig 4.6 D).

Figure 4.6 Progression of oogenesis at the ovarioles. A) *Drosophila* ovaries composed of 12 to 18 ovarioles. AbNvOv (abdominal nerve to the ovary). Adapted from [121]. B) Oogenesis progression in a single ovariole stained with DAPI. G: germarium, O: oocyte, NC: nurse cells, FC: follicle cells [124]. C) Different stages of oogenesis. Staufen (green) and Actin (red) are highly expressed during oogenesis [125]. D) rRNA production in egg chambers. Y axis shows developmental class and X axis shows incorporation of radioactive precursors. Red arrows are pointing specific incorporation which corresponds to the stages flanked by dotted lines [123].

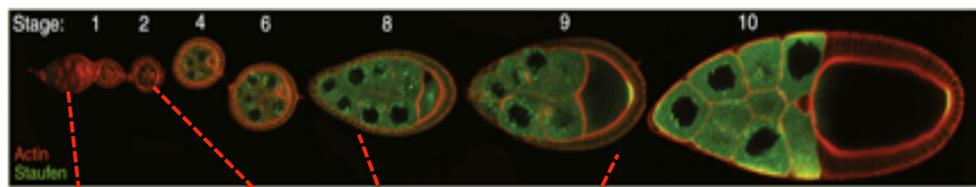
A)



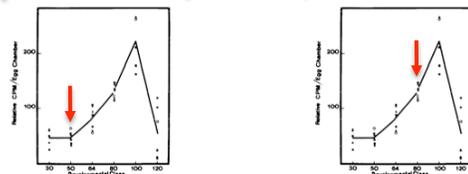
B)



C)



D)



We reasoned that in early cystoblasts (stages 2-3), when translation demand is low, CTCF would be recruited at the nucleolus and in later stages (9-10) we anticipated CTCF would be removed from the nucleolus due to a higher demand in protein production. We dissected ovaries and stained whole-mounts ovarioles with Fibrillarin and CTCF. We observed that in nurse cells of young cystoblasts (Stage 2 in Fig 4.7A), CTCF was found at the nucleolus. At stage 8, the nucleolus of nurse cells appeared very fragmented and CTCF was absent (Fig 4.7B). Finally, in later stages, CTCF was enriched at the nucleoplasm and almost completely absent from the nucleolus (Stage 10 Fig 4.7C). These results indicate that CTCF is present at the nucleolus when the demand of ribosomes is low, and it leaves this compartment when the cell requires a higher amount of ribosomes to overcome protein demand. Therefore, CTCF not only acts as repressor of rDNA transcription to maintain the right amount of rRNAs required for wild type transcription, but also it allows the expression of silent copies when the cell protein demand increases.

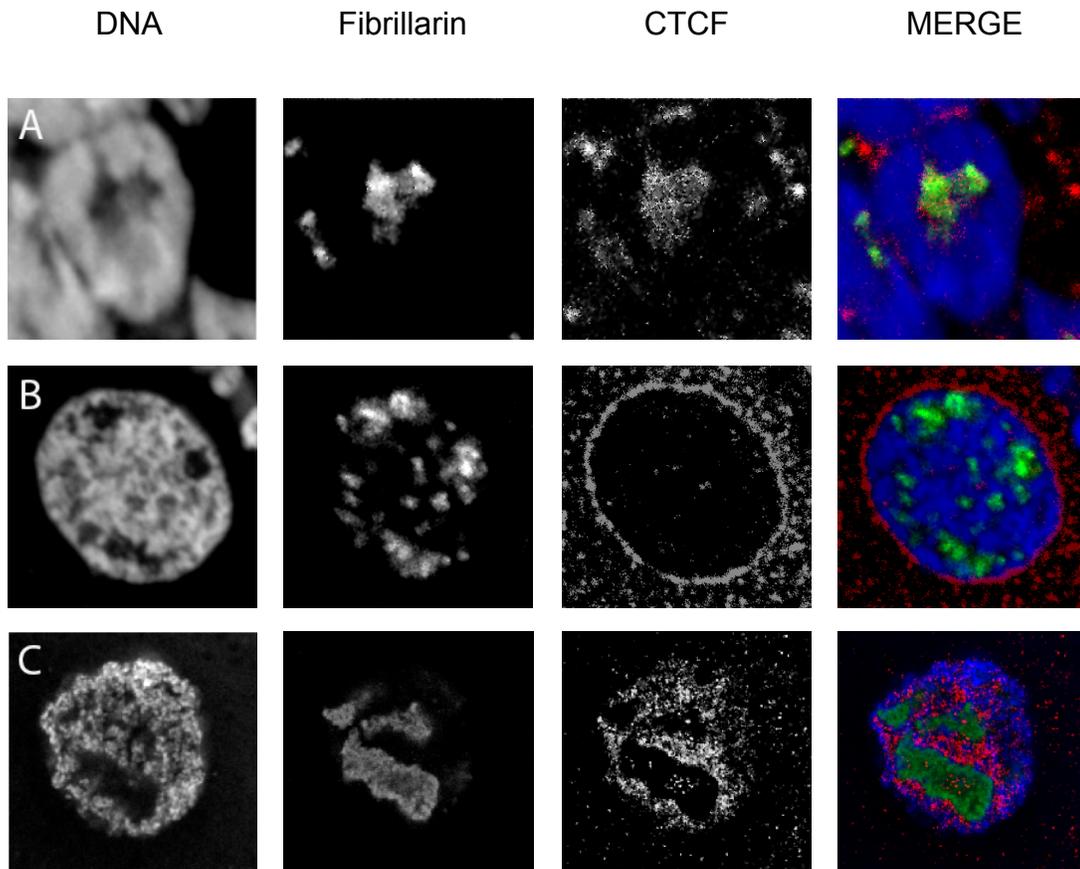


Figure 4.7 CTCF recruitment during oogenesis. Confocal imaging of whole-mount ovarioles. CTCF is shown in red, Fibrillarin in green and DNA in blue. Stages A) 2, B) 8 and C) 10 are shown.

Conclusions

We wanted to explore whether CTCF as a result of being a rDNA transcriptional repressor, could also have an effect in other processes involved in the control of rDNA transcription such as, nucleolar dominance and in a related mechanism called replicative dominance. Similarly, we investigated the origin of the ectopic nucleoli observed in CTCF mutants and we wondered

whether rDNA transcription and/or replication was the process through which the extrachromosomal circles were generated. Finally, we studied how development and in concrete cellular protein demand dictates CTCF nucleolar recruitment.

Nucleolar dominance was established in hybrids Oregon/Lab strain. Subsequently, we tested this dominance in a CTCF mutant background hoping that because of the reduction in rDNA copy number we had observed in these mutants, we would be able to activate transcription of the silent array and cause the release of nucleolar dominance. However, we could not establish whether CTCF affected nucleolar dominance because of the lack of consistence using different mutant alleles.

Replicative dominance was tested by quantifying the number of uninserted, R1 and R2 inserted copies in a CTCF mutant and control backgrounds. We were expecting an increase in rDNA replication in the mutant background to balance the loss of copies measured previously. However, replication of R-inserted copies was unaffected in both backgrounds. Replication of uninserted copies was affected but the result was not consistent. In *CTCF⁹* and *Df(3L)0463* backgrounds rDNA uninserted copies were under-replicated while in the *CTCF^{p35.2}* the opposite effect was observed. Therefore, the lack of consistency in the results indicates that CTCF probably does not play a major role in nucleolar and replicative dominance.

To determine whether the ectopic nucleoli observed in CTCF mutants were caused during rDNA transcription, RNA polymerase I was inhibited. After

staining whole-mounts salivary glands with Fibrillarin, a more compacted nucleolus was observed. This indicated that perhaps the local open chromatin observed during rDNA transcription was one of the sources for extrachromosomal circles. However, the involvement of rDNA replication need to be studied.

Lastly, we tested the implications of translational demand in CTCF recruitment to the nucleolus of nurse cells during oogenesis. In early stages, when the demand is low, CTCF was found at the nucleolus; however, once rRNA synthesis increases, CTCF was not recruited at the nucleolus. Therefore, rDNA transcription output determined CTCF presence at the nucleolus.

CHAPTER V

EFFECTS OF CTCF IN PEV AND BOBBED PHENOTYPE

Introduction

CTCF alleles did not have an effect in nucleolar and replicative dominance, despite observing an increase in RNA transcripts level and decrease in rDNA copy number in all mutant backgrounds tested (*CTCF¹*, *CTCF⁹*, *Df(3L)0463*, *CTCF^{p35.2}*). Therefore, to obtain a better understanding of rDNA transcription regulation by CTCF, we looked at two processes that could be affected by rDNA transcription: Position effect variegation (PEV) and the bobbed phenotype.

Position effect variegation

PEV refers to the variable expression of a gene in clonal cells depending upon the chromatin environment. It was discovered in 1930 by Muller who detected X-rays induced mutations that caused alterations in *white⁺* expression [126]. The best chromosomal rearrangement studied in *Drosophila* is *white mottled 4* (*w^{m4}*) in which the *white⁺* gene, that is normally found at the tip of the X chromosome, is moved near heterochromatin. As a result, the *white⁺* gene, responsible for red eye pigmentation, is variably expressed in different cells of the eye (Fig 5.1). Mutants that enhance (white eye) or suppress (red eye) this phenotype have been extensively studied. Enhancers of variegations (E(var)s)

include chromatin modifying enzymes (JIL-1, *trx*, *ash1*, *Lid*, *Hdac1*), chromatin binders (*mod(mdg4)*, *spt16*), nucleosome remodeling factors (*brm*, *lswi^c*), DNA binders (*E2f*, *E(var)3-9*, *Trl*, *BEAF-32*, *zeste*), proteins involved in RNA processing (*Hel25E*, *vig2*), and in DNA replication (*wapl*). Suppressors of variegation (*Su(var)s*) includes histone variants (*H1*, *His2Av*), chromatin-modifying enzymes (*Su(var)3-9*, *Suv4-20*, HDACs, *lal*), Chromatin binders (*Su(var)2-5* or *HP1*, *D1*, *Pc*, *Sce*, *Psc*), nucleosome remodeling factors (*dAtrx*, *Acf1*, *Actr13E*), DNA binders (*salm*, *slbo*, *pho*, *sens*), proteins involved in chromatin replication (*Orc1*, *Orc2*, *mus209*, *Cdc6*) and in chromosome segregation (*Incnp*) [127]. *Su(var)* gene products are mostly found in heterochromatin and mutations in *Su(var)* genes affect heterochromatin formation and maintenance. In contrast, *E(var)s* gene products are mainly found at euchromatin and mutations in *E(var)* genes make euchromatin weaker or permits heterochromatin spreading [127].

Drosophila was the initial model organism where PEV was studied. Apart from pericentric heterochromatin, position effect is also generated by retrotransposons, telomeres, chromosome 4, and tandem repeats. RNAi machinery is known to be involved in tandem repeats and retrotransposon silencing [2]. CTCF is found in heterochromatin, at the rDNA locus where it represses rDNA transcription; therefore, we wondered whether it would affect heterochromatin elsewhere and as a result, modify *w^{m4}* expression. We predicted that it will act as a *Su(var)* because of its repressive role.

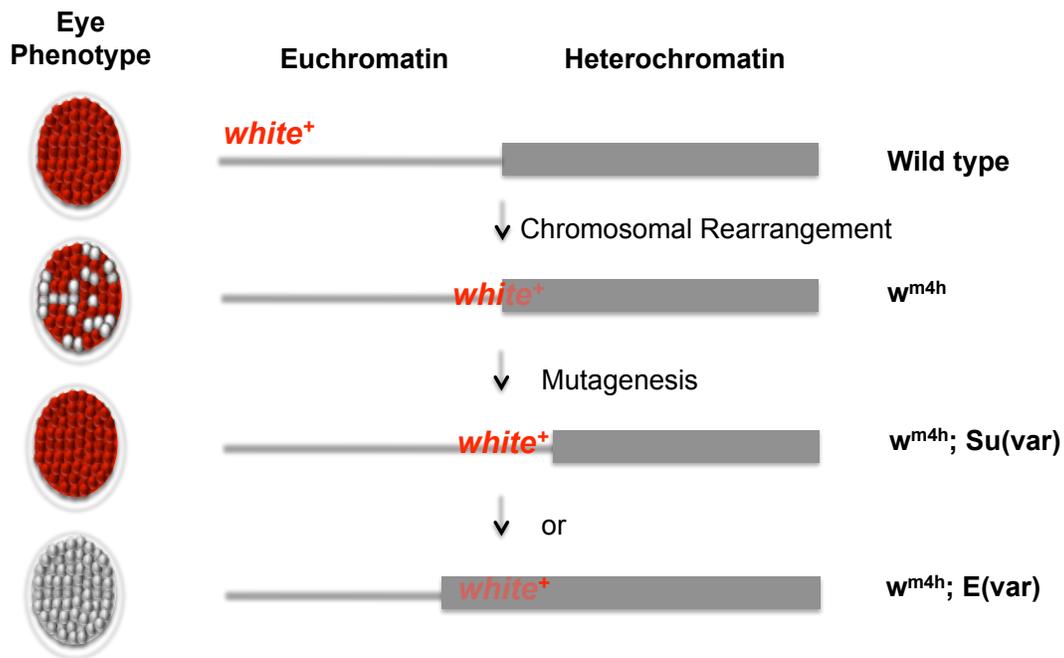


Figure 5.1 Position effect variegation. Representation of the heterochromatic and euchromatic regions in the X chromosome of *D. melanogaster*. The $white^+$ gene that is normally found in euchromatin is moved near by heterochromatin causing PEV which is observed as patchy areas of $white^+$ expression on the eye. Genes that suppress (Su(var)s) or enhance (E(var)s) PEV have been extensively studied. Adapted from [127].

Bobbed phenotype

As shown in Chapter III (Fig 3.3) in CTCF mutants we observed reduction in 35S rDNA copy number. Partial deletion of the rDNA array gives rise to the bobbed phenotype (also observed in CTCF mutants) which is characterized by the shortening of bristles, delay in development and etching of abdominal tergites due to a decrease in protein production. This phenotype is alleviated by rDNA magnification and two models have been proposed to explain this process.

The first model, proposed by Ritossa [51], states that in bobbed flies extra rDNA copies are produced and they can be reintegrated in the germ line rDNA. It also states that the integration process occurs in more than one generation; therefore, the fragment that is not integrated “goes along” to the next generation and it is later incorporated. This model was critical for the initial studies of rDNA magnification but it failed to explain most of the published data; for instance, it did not clarify why the rDNA of ring chromosomes cannot be magnified. The second model, which is generally accepted, was proposed by Tartof and establishes that rDNA magnification and reduction occurs during mitosis and meiosis when the rDNA of sister chromatids is unequally exchanged [51]. This results in having one chromatid with a reduced rDNA array and the other chromatid with a magnified rDNA array. This model explains that magnification does not occur in ring chromosomes because it would generate dicentric chromatids, which was tested by Tartof. Similarly, this model predicts that rDNA magnification and reduction will have the same frequency as long as magnification does not produce a selective advantage for the germ cell [51].

Work done in 1986 by Marcus and colleagues showed that recessive autosomal suppressors, which are found to accumulate in certain bobbed stocks, can revert the bobbed phenotype without increasing rDNA copy number [128]. However, the majority of studies have shown rDNA magnification as the mechanism used to revert the bobbed phenotype.

During the initial steps of rDNA magnification, rDNA transcription increases [129] which would predict that increasing rDNA transcription without rDNA magnification could also revert the phenotype. However, work done by Mohan and Rissota in 1970, showed that in ovaries of bobbed flies, the rRNA content was the same as in wild type, but the rRNA transcription rate was lower. They also observed retardation in egg deposition in the bobbed flies which they interpreted as the time required for the egg to accumulate enough rRNA before it was deposited. Similarly, they proposed that in bobbed flies, wild type levels of rRNA would be produced in tissues that are crucial for development. On the other hand, in tissues that are less critical for development, rRNA levels would be only enough for survival and the bobbed phenotype would be observed [130].

Although rDNA magnification and recessive autosomal suppressors are the two main processes that have been described to alleviate the bobbed phenotype, in CTCF mutants we have observed an increase in rRNA nascent transcripts. This could indicate that the rDNA transcription rate was higher. If that was the case, we could presumably observe alleviation of bobbed phenotype in a CTCF mutant background.

Results and Discussion

Position effect variegation of CTCF

In order to test whether CTCF would have an effect on position effect variegation, we looked at w^{m4} expression in a CTCF mutant background. A

different allele previously characterized by Mohan and colleagues was used, *CTCF^{p30.6}*, which was created by P-element imprecise excision (Fig 3.1) [97]. We were expecting that as a result of CTCF localization in the heterochromatic rDNA loci and its repressive role in rDNA transcription, *CTCF^{p30.6}* would suppress *w^{m4}* expression; however, no effect was observed (Fig 5.2). We reasoned that perhaps CTCF will affect PEV only if the source of variegation was the rDNA since it is the only heterochromatic region where its gene product is found (Chapter II, Fig 2.4). The Karpen laboratory has generated a vast collection of P-element insertions that were mapped to the Y chromosome and autosomes [131,132]. These transposable elements contain two marker genes, *white⁺* and *yellow⁺*, the former controls eye pigmentation and the latter controls body and wing pigmentation. One of these P-element inserted lines (D285) has transposed to the rDNA locus. Hence, we decided to compare the *white⁺* variegating expression of D285 in a CTCF mutant and wild type backgrounds. As controls, we used another Y-linked P-element from the Karpen collection, B486 which has a very low *white⁺* variegating expression, and ROMA, a line generated by Dr. Sergio Pimpinelli which contains the same P-element as in D285 and B486 (P{SUPorP}) and it also maps to the Y chromosome [133]. ROMA is characterized for having a very high *white⁺* variegating expression.

We looked at the expression of D285, B486 and ROMA in heterozygous, trans-heterozygous, and homozygous pharate adults (Table 5.1). Heterozygous

were obtained by crossing CTCF mutant females to males carrying the Y-linked P-element (Fig 5.3A). Males were scored in the next generation.

To obtain trans-heterozygous and pharate adults, one of the CTCF mutant alleles was first introduced to males from the D285, B486 and ROMA stocks and then crossed to females from the CTCF stocks (Fig 5.3B). In all cases the same trend was observed. In mutants heterozygous for CTCF, expression of the *white*⁺ inserted in the rDNA (D285) was suppressed. Moreover, expression was suppressed to a greater degree in trans-heterozygous combinations that reduced CTCF gene activity more (Fig 5.3C-D and Table 5.1). The greatest decrease in CTCF gene product is in homozygous *CTCF^{p35.2} / CTCF^{p35.2}* individuals, which die as pharate adults. However, suppression was not higher than in heterozygous siblings. This result may be due to pigmentation in pharate individuals being not fully complete since they die earlier in development. In contrast to D285, *white*⁺ expression in the ROMA line was not affected in any mutant combination of CTCF, *white*⁺ expression from B486 was slightly increased only in the *CTCF^{p35.2} / Df(3L)0463* background, indicating that effect may be due to other factors in those stocks. Thus, the D285 line alone showed consistent suppression of rDNA-induced PEV by mutation in CTCF, indicating a role for CTCF in rDNA-induced PEV.

These results showed how CTCF mutants affect exclusively the silent state of the rDNA and as a result, the expression of a variegating *white*⁺ inserted in the rDNA was affected. However, they did not alter PEV of the common model

w^{m4} allele. Consistent with our findings, CTCF gene product is not found in heterochromatin, except for the rDNA loci. Similarly, this confirms our observations showing CTCF as a repressor of rDNA transcription.

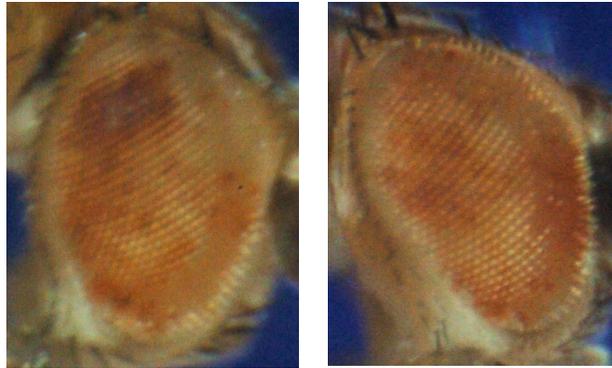


Figure 5.2 Position effect variegation of w^{m4} in a CTCF mutant background. $white^+$ expression in control (left) and $CTCF^{p30.6}$ (right).

A)

Generation 0:

 $\text{♀ } Y^{1W^{67c23}}/y^{1W^{67c23}}; CTCF^{p35.2}/TM3Sb \quad \times \quad \text{♂ } y^{1W^{67c23}}/Y [SUPor-P, y^+w^+]; + / +$

Generation 1:

 $\text{♂ } y^{1W^{67c23}}/Y [SUPor-P, y^+w^+]; CTCF^{p35.2}/+ \quad \text{vs} \quad \text{♂ } y^{1W^{67c23}}/Y [SUPor-P, y^+w^+]; + /TM3Sb$

B)

Generation 0:

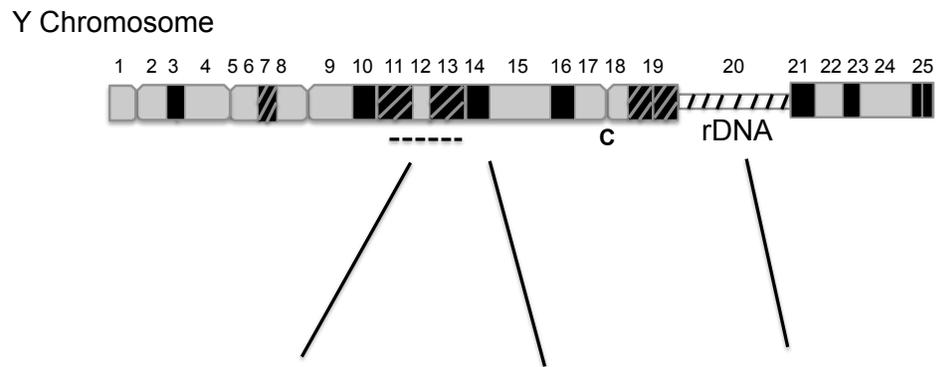
 $\text{♀ } y^{1W^{67c23}}/y^{1W^{67c23}}; CTCF^{p35.2}/TM3Sb \quad \times \quad \text{♂ } y^{1W^{67c23}}/Y [SUPor-P, y^+w^+]; CTCF^{p35.2} /TM6BTb$

Generation 1:

 $\text{♂ } Y^{1W^{67c23}}/Y [SUPor-P, y^+w^+]; CTCF^{p35.2}/CTCF^{p35.2} \quad \text{vs} \quad \text{♂ } Y^{1W^{67c23}}/Y [SUPor-P, y^+w^+]; + /TM3Sb$

Figure 5.3 Suppressor effect of CTCF mutants. A) Heterozygous females *CTCF^{p30.6}/+*, *CTCF^{p35.2}/+*, *Df(3L)0463/+* were crossed to three set of males carrying P-elements (P{SUPorP}) inserted in the Y chromosome: B486, ROMA and D285. Heterozygous males were scored and expression was compared to their siblings carrying the balancer chromosome (TM3Sb) B) Same as A) but males also carry a CTCF mutant allele; therefore, trans-heterozygous and homozygous pharate (*CTCF^{p35.2}/CTCF^{p35.2}*) can be scored in G1. C) Representation of the Y chromosome showing the location of ROMA, B486 and D285. D) Expression of these three lines in wild type (upper row), heterozygous (middle row) and trans-heterozygous (bottom row) CTCF mutant backgrounds.

C)



D)

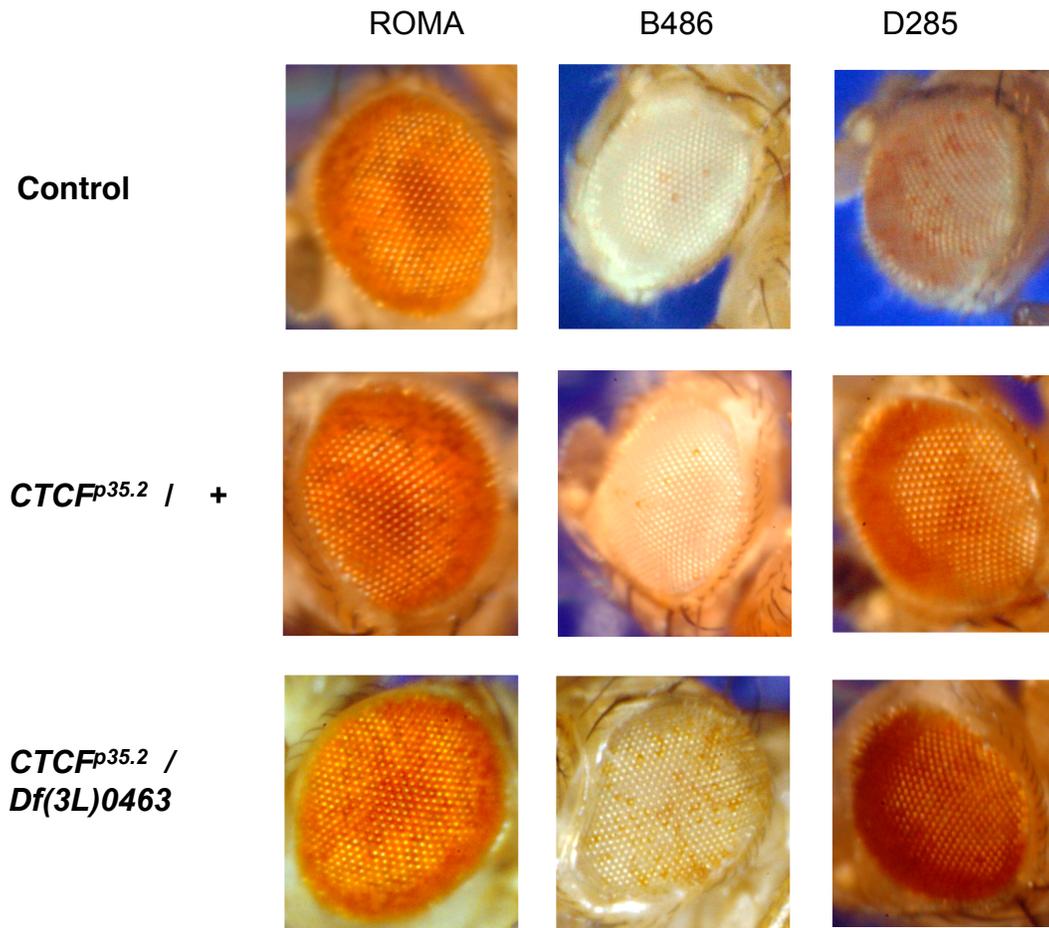


Figure 5.3 Continued.

Table 5.1: *white*⁺ variegation expression of P-elements inserted in the Y chromosome. ROMA and B486 linked *white*⁺ expression was not affected in different CTCF mutant background. D285 expression was affected. Expression was scored from white (0) to red (4).

Phenotype	<i>white</i> ⁺ expression		
	ROMA	B486	D285
Wild Type	3	0	2
<i>CTCF^{p30.6} / +</i>	3	0	3
<i>CTCF^{p35.2} / +</i>	3	0	3
<i>Df(3L)0463 / +</i>	3	0	3
<i>CTCF^{p30.6} / Df(3L)0463</i>	3	0	4
<i>CTCF^{p35.2} / Df(3L)0463</i>	3	0	4
<i>CTCF^{p35.2} / CTCF^{p35.2}</i>	3	0	3

rDNA allelic series

In *Drosophila*, the rDNA array contains a restriction site for the homing endonuclease I-Crel. Previous work done in our laboratory used this tool to create an allelic series of Y-linked rDNA deletions [134]. Using RT-PCR, the rDNA array size of the Y chromosome was determined in a setting where the X-linked array was entirely removed. The change from wild type to bobbed occurred when ~10% of the rDNA copies were lost and the change from bobbed to lethal occurred when ~35% of the rDNA copies were lost (Fig 5.4).

We predicted that if CTCF acts as a negative regulator of rDNA transcription, then CTCF mutants may increase expression and act to suppress

the bobbed and bobbed-lethal phenotypes that result from shortened rDNA arrays. We predicted that in a CTCF mutant background, the expression of the bobbed and lethal alleles would be suppressed and this would result in a shift from bobbed to wild type and from lethal to bobbed. To test this hypothesis, as shown in Figure 5.4, I crossed $C(1)DX, y^1 f^1 bb^0/Y; +/+$ females to $X/y^+Y10B, rDNA^{bb}; CTCF^{p35.2}/TM6B, Tb$ males, and compared bobbed expression in G1 $C(1)DX, y^1 f^1 bb^0/y^+Y10B, rDNA^{bb};$ females (Fig 5.5 A). I categorized bobbed severity into low, medium and high (Fig 5.5B). Generally, no G1 females are observed in the lethal lines and few are seen in the bobbed lines; therefore, to obtain reliable data, several crosses need to be set up. On the other hand, CTCF mutations reduce rDNA copy number which would decrease viability of the $C(1)DX, y^1 f^1 bb^0$ females. The following results are preliminary; however, they seem to show a particular trend.

Four bobbed (bb-465, bb-31, bb-76 and bb-11) and six lethal (l-516, l-480, l-514, l-462, l-481, and l-473) alleles were crossed to $C(1)DX$, and G1 females were scored into wild type, bobbed (low, middle and high, Fig 5.5B) and pharate adults. Although the number of G1 females generated was low, in a $CTCF^{p35.2}$ background all four bobbed alleles tested showed a shift from bobbed to wild type compared to the control (Fig 5.6 A). For instance, in the bb-465 line, more wild type and bobbed individual were observed in the $CTCF^{p35.2}$ background (5 wild type and 12 bobbed) compared to the control (1 wild type and 5 bobbed). The same was observed for the bb-31, bb-76, and bb-11 lines. We also

observed an effect in the lethal alleles; however, the transition was not from lethal to bobbed but instead from lethal to pharate or to wild type. This was the case in four (I-516, I-480, I-514, and I-481) out of six alleles tested (Fig 5.6 B). For instance, in the I-516 allele, wild type and pharate G1 females were observed (2 wild type and 4 pharate) while no G1 females were observed in the control.

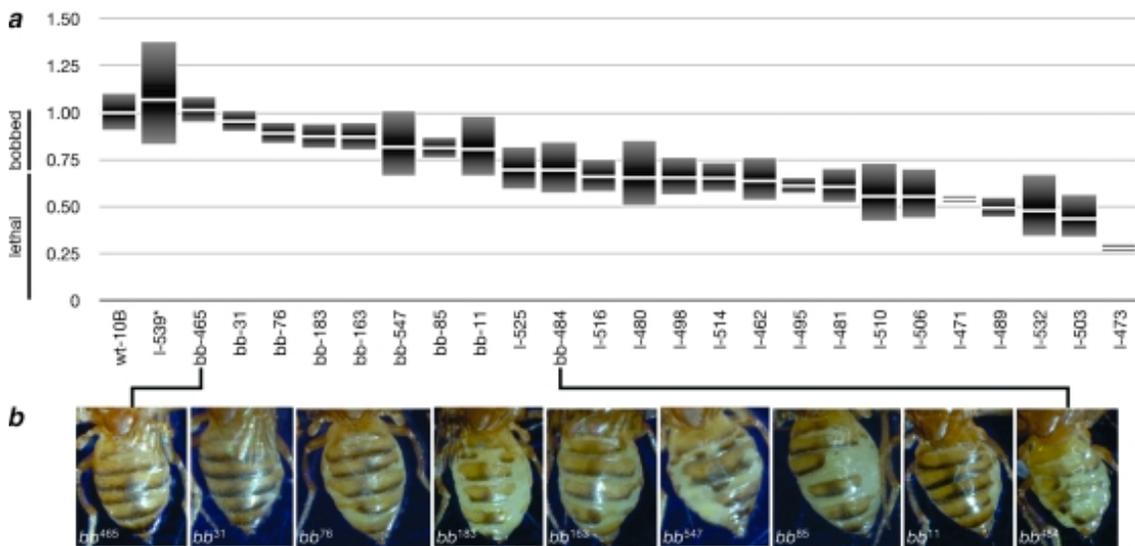


Figure 5.4 Y-linked rDNA allelic series. A) Y-rDNA array size of wild type (wt), bobbed (bb), and lethal (I) alleles. Y axis shows the ratio compared to wild type. B) Abdomens of bobbed lines. The sole rDNA source for these experiments was the Y chromosome. Copied from [134].

A)

Generation 0:

$$\text{♀ } C(1)DX, y^1 f^1, bb^0 / Y ; + / + \quad \times \quad \text{♂ } y^1 w^{67c23} / y^+ Y10B, rDNA^{bb} ; CTCF^{p35.2} / TM6BTb$$

Generation 1:

$$\text{♀ } C(1)DX, y^1 f^1, bb^0 / y^+ Y10B, rDNA^{bb} ; CTCF^{p35.2} / +$$

$$\text{♀ } C(1)DX, y^1 f^1, bb^0 / y^+ Y10B, rDNA^{bb} ; + / TM6BTb$$

B)

Low

Medium

High



Figure 5.5 Alleviation of bobbed phenotype. A) $C(1)DX, y^1 f^1, bb^0 / Y ; + / +$ females, in which the compound X chromosome lacks rDNA, were crossed to males carrying Y-linked rDNA alleles and $CTCF^{p35.2}$. Bobbed phenotype was scored in G1 females carrying the $CTCF^{p35.2}$ or balancer chromosomes. B) Example of abdomens categorized as low, medium and high bobbed phenotype.

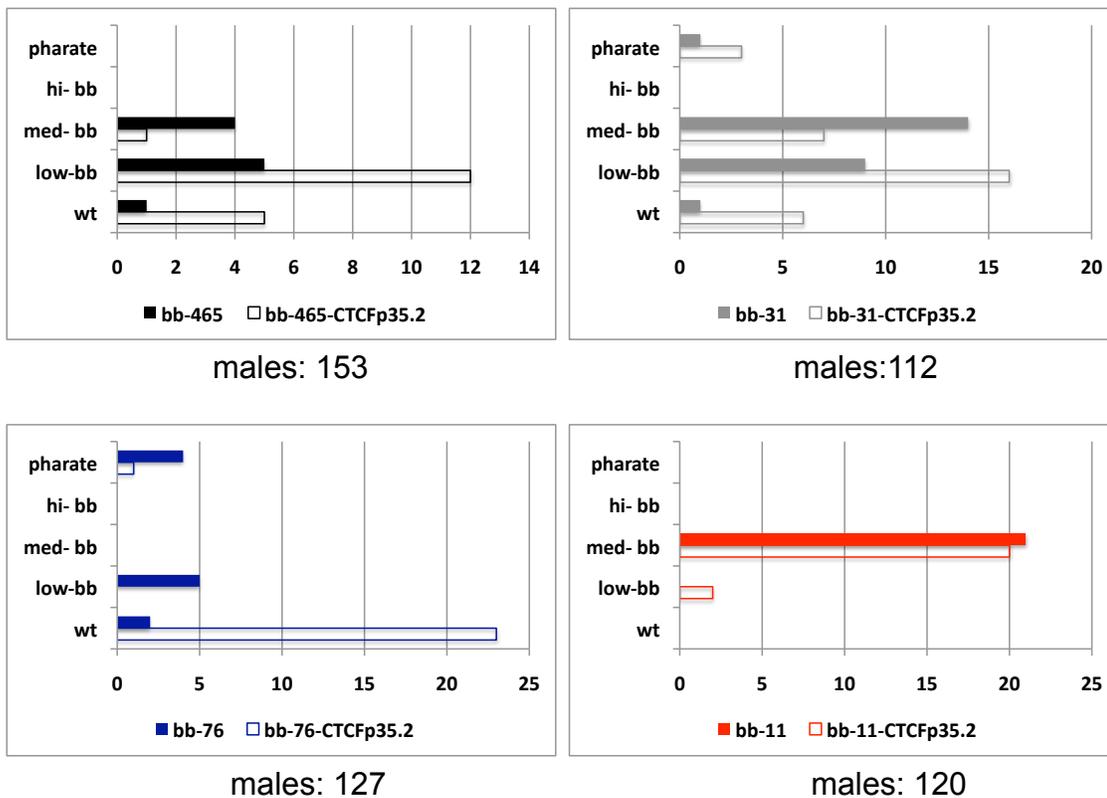
A) Bobbed Alleles

Figure 5.6 Alleviation of bobbed and lethal phenotypes in *CTCF^{p35.2}* flies. A) Four bobbed alleles: bb-465, bb-31, bb-76, bb-11 and B) six lethal alleles: l-516, l-480, l-514, l-462, l-481, l-473 were tested. Number of individuals are shown on X axis; bobbed categories on Y axis correspond to classification shown in figure 5.5B. Filled bars represent control and empty bars show mutant background. Total number of males obtained in G1 are shown below each graph.

B) Lethal Alleles

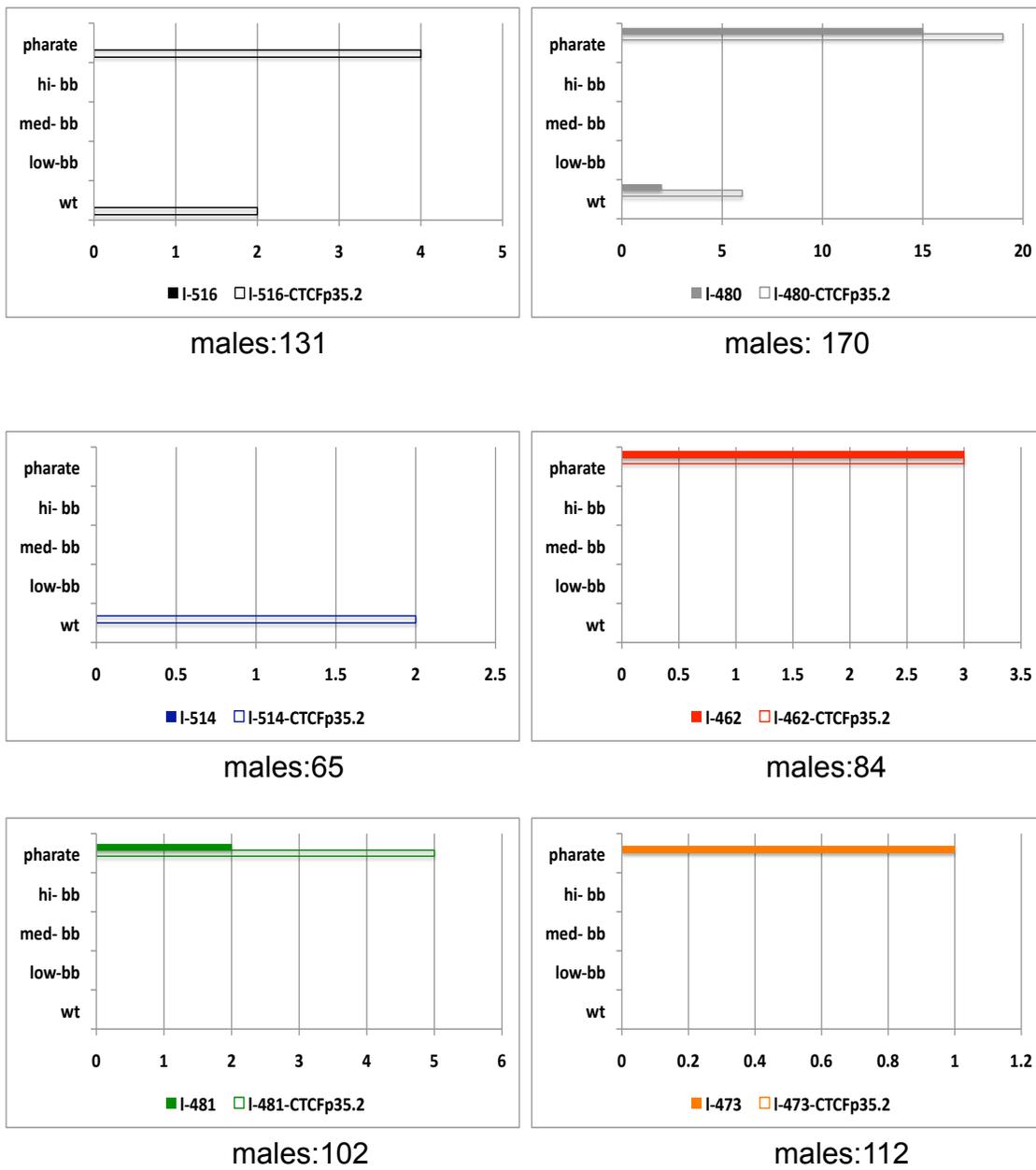
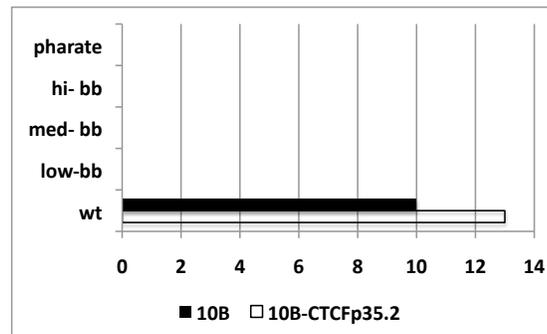


Figure 5.6 Continued.



males:181

Figure 5.6 Continued.

These results indicated that increase in rRNA transcripts and presumably rDNA transcription rate alleviated the bobbed phenotype. The fact that a transition from lethal to bobbed was not observed, but instead pharate and wild type females were seen, indicated that possibly as it was postulated by Mohan and Rissota in 1970, in the lethal alleles egg deposition occurred after rRNA were accumulated to wild type levels. However, it is unclear why bobbed and lethal alleles would behave differently.

To confirm that the alleviation of the bobbed and lethal phenotype were caused by CTCF and not by an unrelated mutation present in the *CTCF^{p35.2}* stock, I repeated the same experiments using the amorphic allele, *CTCF⁹*.

I crossed *C(1)DX,y¹fl^{bb0}/Y ; + / +* females to males *X/ y⁺Y10B, rDNA^{bb}; CTCF⁹ / TM6BTb*, and compared bobbed expression in G1 females (Fig 5.7). Four bobbed (bb-465, bb-31, bb-76 and bb-11) and five lethal lines (l-516, l-480, l-514, l-481, and l-473) were tested (Fig 5.8). Three bobbed lines (bb-465,

bb-76 and bb-11) showed a bobbed to wild type shift compared to the control.. Three lethal lines showed lethal to wild type transition (I-480, I-514, and I-473). Therefore, the results obtained with the *CTCF*⁹ recapitulated what was obtained with *CTCF*^{p35.2} and confirmed our original hypothesis.

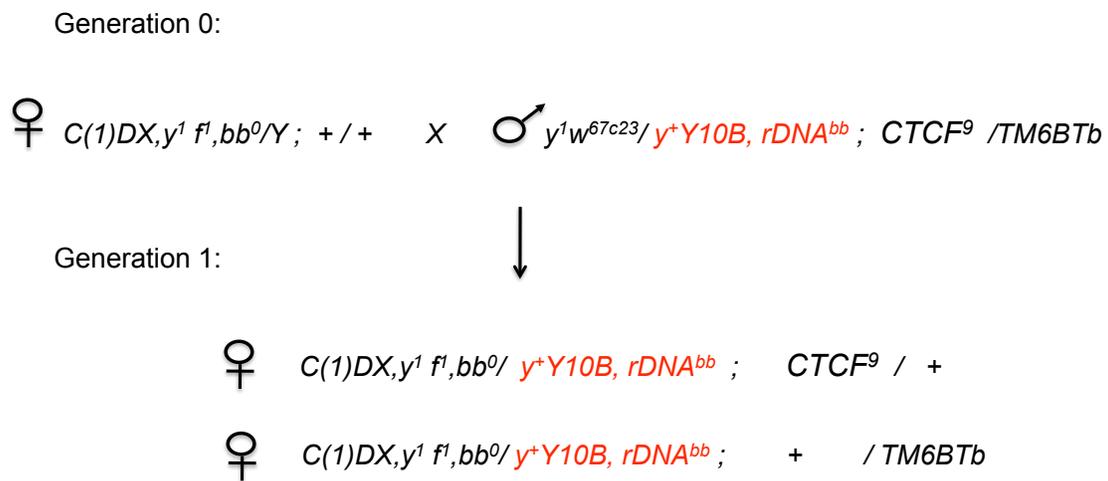


Figure 5.7 Cross used to test alleviation of bobbed phenotype. A) *C(1)DX, y¹f¹bb⁰ / Y ; + / +* females, in which the compound X chromosome lacks rDNA, were crossed to males carrying Y-linked rDNA alleles and *CTCF*⁹. Bobbed phenotype was scored in G1 females carrying the *CTCF*⁹ or balancer chromosomes.

A) Bobbed Alleles

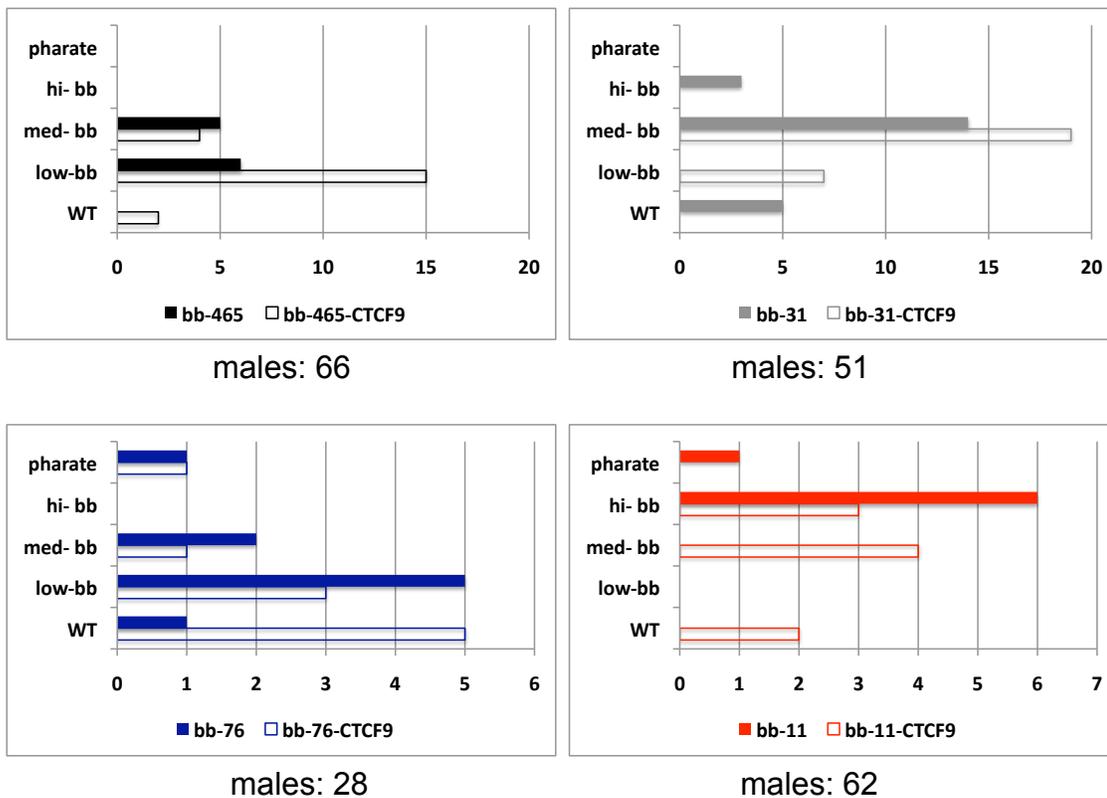


Figure 5.8 Alleviation of bobbed and lethal phenotypes in *CTCF⁹* flies. A) Four bobbed alleles: bb-465, bb-31, bb-76 and bb-11 and B) six lethal alleles: l-516, l-480, l-514, l-462, l-481, and l-473 were tested. Number of individuals are shown on X axis; bobbed categories on Y axis correspond to classification shown in Figure 5.5B. Filled bars represent control and empty bars show mutant background. Total number of males obtained in G1 are shown below each graph.

B) Lethal Alleles

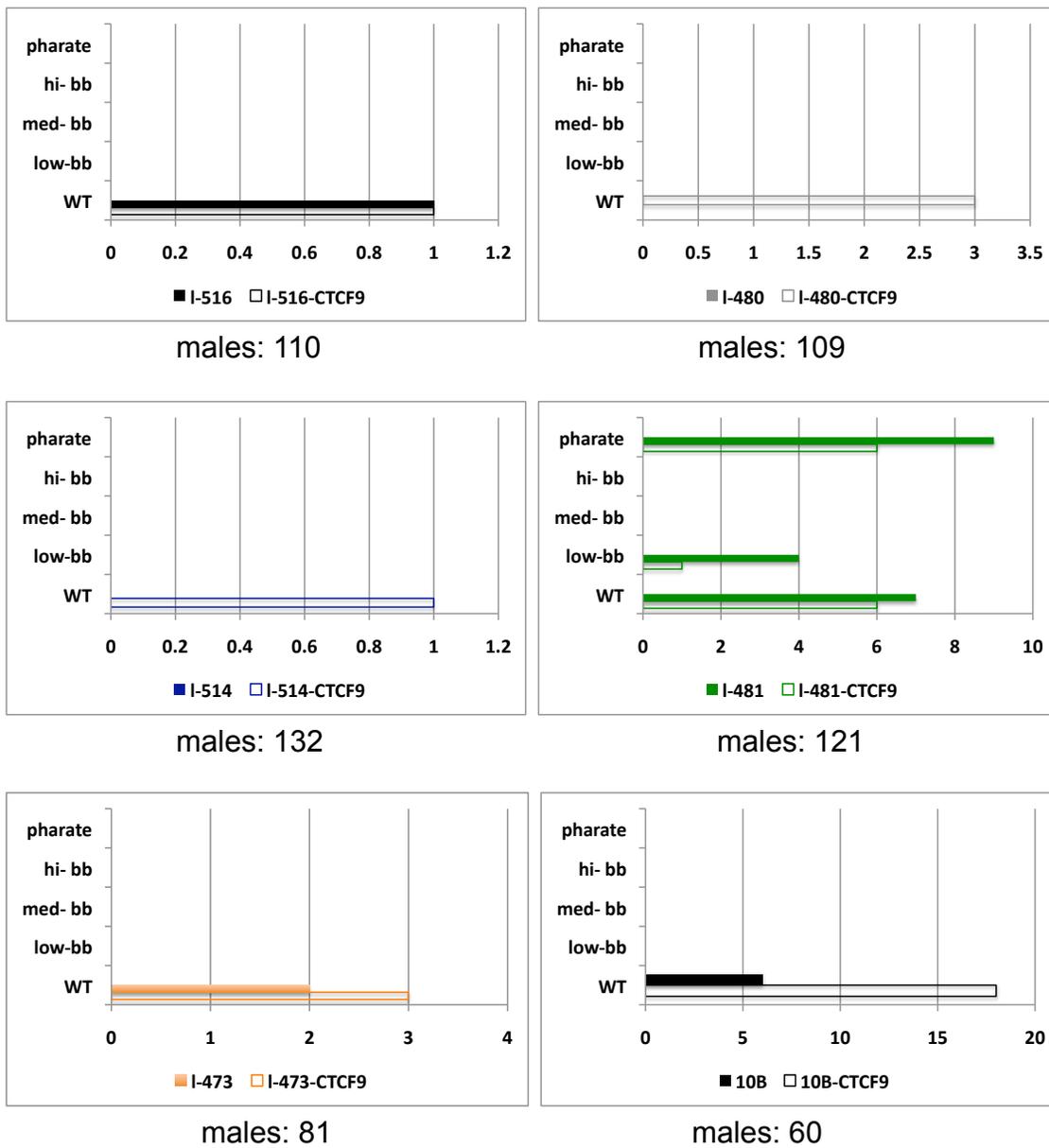


Figure 5.8 Continued.

Conclusions

We wanted to explore whether CTCF mutants could affect position effect variegation and revert the bobbed phenotype, since both mechanisms can be affected by rDNA transcription. Su(var) gene products are found in heterochromatin, CTCF is mostly found in euchromatin except for the rDNA loci where it represses RNA polymerase I transcription. Therefore, we tested whether CTCF mutants could suppress w^{m4} variegating expression; however, that was not the case. We reasoned that since CTCF, outside of the rDNA, is not found in heterochromatin, it is not expected to affect the w^{m4} expression. Consequently, we studied the expression of a variegating $white^+$ inserted in the rDNA loci in different CTCF mutant backgrounds. The expression of $white^+$ was suppressed in a heterozygous background and this effect was even higher in a trans-heterozygous background. This showed that destabilization of heterochromatin at the 35S rDNA loci caused by CTCF mutants, increased the expression of a marker gene inserted in the array, but it did not affect the variegation of a gene inserted somewhere else in heterochromatin. This is quite interesting because the 35S rDNA regulation shares components involved in controlling repressive chromatin elsewhere such as, Su(var) 3-9. However, the mechanism used by CTCF seems to be independent, since in its absence only rDNA imposed PEV can be affected. We are currently following the expression of D285 in a $CTCF^{p35.2}$ background. We are studying whether additional

reductions in rDNA copy number will cause the loss of this marker from the rDNA array.

We used bobbed and lethal allelic series created in the laboratory to study whether the increase in nascent transcripts was reflecting 1) the activation of silent copies and 2) an increase in RNA polymerase transcription rate which we expected it would cause alleviation of the bobbed and lethal phenotypes. In the bobbed lines, we observed more wild type and low-bobbed females in both *CTCF^{p35.2}* and *CTCF⁹* backgrounds compared to the control and in lethal lines we observed more pharate and wild type individuals than in the mutant background. We were expecting a transition from lethal to bobbed rather than lethal to wild type. It is possible that: 1) the transcription rate was even higher in CTCF mutants-lethal background and that is why a lethal-bobbed transition is not observed, or 2) that in the absence of CTCF, magnification was faster, or 3) that in these lines rRNAs accumulated to wild type levels before egg deposition.

As shown in Chapter III, reduction in rDNA array size in CTCF mutants affected the abdominal cuticle causing the bobbed phenotype. Therefore, using this tissue to evaluate alleviation of bobbed and lethal phenotype is a reasonable option. However, the data set showed was very small; therefore, future studies should be conducted to obtain a reliable number of G1 females and thus confirm these preliminary results.

CHAPTER VI

SUMMARY AND MODEL

Summary

CTCF has been studied extensively for its multiple roles in the regulation of non-nucleolar chromatin; however, more recent studies have showed its involvement in rDNA transcription regulation. Mammalian studies indicated that it was possibly involved in the repression of rDNA transcription; however, this was not directly tested due to lack of loss-of-function alleles. We observed CTCF at the nucleolus of salivary glands polytene chromosomes in *Drosophila melanogaster*. CTCF was found at the nucleoli of both differentiate and undifferentiated cells. Mitotic spreads showed CTCF immunofluorescence signal at the nucleolar organizer (or rDNA locus) of both X and Y chromosomes. Potential binding sites were then identified at the 35S rDNA R1 and R2 genes; however, binding was only found at the R1 element. Using chromatin immunoprecipitation we could not distinguish between CTCF signal from the R1 element inserted at the 35S rDNA and the R1s found outside the nucleolus. However, we did not observed CTCF immunostaining at R1s located outside the rDNA loci and as our results showed later, in CTCF mutants the transcription of the 35S-linked R1 elements is directly affected.

We obtained previously published amorphic alleles and analyzed their nucleolar integrity by immunofluorescence staining with Fibrillarin and CTCF. In

these mutants the nucleolus was fragmented and CTCF was absent from this compartment but its non-nucleolar binding did not seem to be affected. Immunostaining signal from *Drosophila* CTCF in wild type salivary glands nuclei showed a stronger signal from the non-nucleolar chromatin than from the nucleolus; thus, it is expected that reduction in CTCF gene product would affect nucleolar signal in a greater degree. rDNA copy number was decreased in these mutants, and two of them showed the bobbed phenotype (*Df(3L)0463*, *CTCF^{p30.6}*), consistent with the loss in rDNA units.

Transcripts level of 18S, R1 and R2 increased in homozygous *CTCF^{p35.2}* compared to heterozygous which indicated that CTCF was directly repressing rDNA transcription. Quantification of unprocessed junctions showed that increased transcripts corresponded to nascent transcription. To confirm our results, CTCF was knocked down in S2 cells and nucleolar fragmentation was observed. Likewise, Fibrillarin signal was diminished and diffused. CTCF signal was reduced at the nucleus including the nucleolus. CTCF mRNA levels showed a 40% reduction and 35S, R1 and R2 RNA amounts increased. rDNA transcriptional repression is a novel mechanism for CTCF since previous work has focused on its role as boundary element in non-nucleolar chromatin. Apart from binding the 35S-linked R1 element and having a role in rDNA transcription, we observed that 80% of CTCF present at the nucleolus was dependent on RNA binding. Since we observed an increase in unprocessed rRNA junctions

and CTCF is known to bind nucleophosmin, it would be interesting to explore whether CTCF is involved in rRNA processing and whether it binds RNA directly.

Previous work showed that poly(ADP-ribosyl)ation was required to recruit CTCF to the nucleolus. PARP and PARG were knocked down in S2 cells and fragmented nucleolus, and increase in 35S, and R1 transcripts was observed, recapitulating the results obtained with CTCF knock down. R2 transcripts amount was not affected in PARP mutants and it was slightly increased in PARG mutants. CTCF was present at the nucleoli of PARP and PARG knocked out S2 cells, however, a fraction of it was mislocalized at the cytoplasm. In a PARG mutant fly stock, we noticed absent of CTCF from the nucleolus and nucleolar fragmentation. These results showed that in *Drosophila*, this post-translational modification was not only required to maintain CTCF at the nucleolus but it was also necessary to silent rDNA copies.

I tested whether CTCF mutants could have an impact in processes affected by rDNA transcription. Nucleolar dominance and replicative dominance did not appear to be disturbed, indicating that CTCF probably does not play a major role in these processes. Position effect variegation was affected only when the source of PEV was the rDNA, consistent with finding CTCF mostly in euchromatin except for the rDNA loci. I also tested alleviation of the bobbed and lethal phenotypes caused by rDNA copies deletion, and we observed a partial alleviation in both lethal and bobbed lines. Finally, we observed that protein demand dictated CTCF recruitment to the nucleolus of nurse cells at the

ovarioles; CTCF was present at the nucleolus at earlier stages when the translational demand was low and once the demand for rRNA increased, CTCF left this compartment. The results presented in this work introduced a new mechanism for the regulation of rDNA transcription in *Drosophila melanogaster*.

Model

Our results showed that CTCF is recruited to the nucleolus, specifically to the R1 inserted rDNA units and it represses transcription of R1 inserted, and R2 and uninserted neighboring copies. Although, the distribution of R-inserted copies in the CTCF mutant stocks was not measured, it has been shown before that in rDNA arrays with silent R2 inserted units, these, together with R1 inserted copies, are clustered. Similarly, we observed activation of R2 copies in a CTCF mutant background consistent with having R2 near by R1 inserted rDNA units. Hence, if CTCF is depleted from the nucleolus, the transcript levels of R1, R2 and uninserted copies will increase. This instability of previously silent copies will cause the formation of extrachromosomal circles and the shortening of the rDNA array (Fig 6.1) which will result in a bobbed phenotype. Indirectly, we observed a similar effect by reducing factors involved in CTCF nucleolar recruitment. Decreasing the levels of PARP and PARG affected CTCF recruitment and as a result rRNA transcripts level increased.

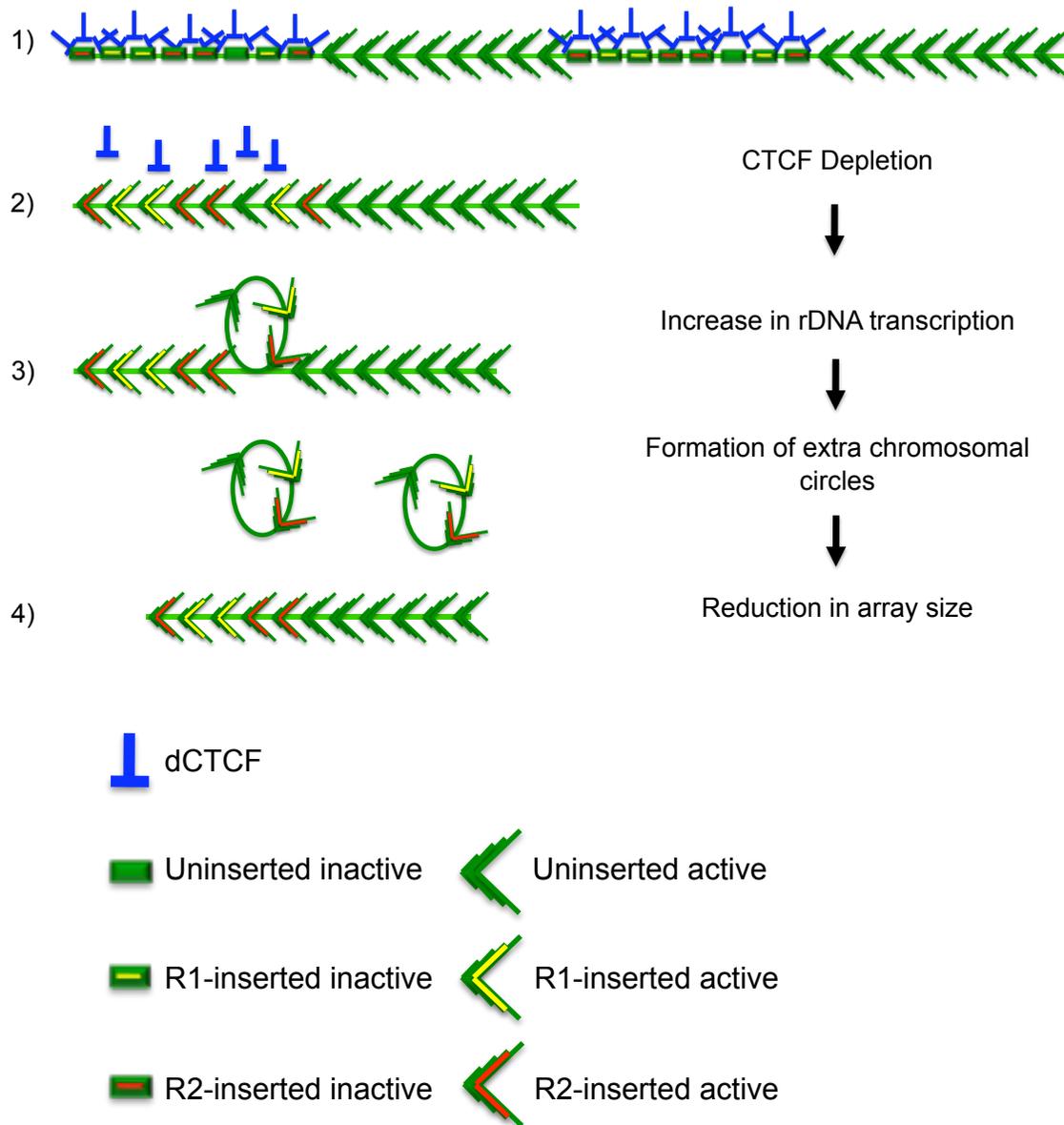


Figure 6.1 CTCF represses rDNA transcription. 1) rDNA locus with R1 and R2 inserted copies clustered together. CTCF binds to R1, silences it as well as R2 and uninserted copies in the vicinity. 2) CTCF depletion activates clustered and silent R1, R2 and uninserted copies. 3) rDNA copies are lost from the rDNA array by the generation of extrachromosomal circles. 4) rDNA array becomes shorter.

Implications of the Model

CTCF was initially described as a transcriptional repressor, a transcription activator and a boundary element, having both enhancing blocking and heterochromatin barrier activities. Later, Ohlsson and colleagues performed sequential deletion of CTCF zinc fingers and noticed that some zinc fingers were required for binding certain DNA sequences but not to others; therefore, they proposed a model in which CTCF employs different zinc finger combinations to bind specific DNA sequences and presumably interact with different partners which would generate a “CTCF-code” [45].

Genome wide studies have demonstrated that CTCF binds approximately 26,000 sites in mammals [73] and 12,433 sites in *Drosophila* [99]. Similarly, chromosome conformation capture and fluorescence in situ hybridization techniques showed that CTCF can mediate long distance interactions in *cis* and in *trans*. This generated a second model in which CTCF is a genome wide organizer of chromatin structure [135]. This model is based on the importance of the spatial organization of chromosomes in a cell nucleus and how this influences gene activity. It also implies that CTCF mediates the formation of chromatin loops and that mechanisms in which CTCF is involved such as, transcription repression and boundary activity, are secondary effects of its primary role as organizer of chromatin structure.

Due to the variety of DNA sequences and proteins CTCF interacts with, an alternative mechanism combines these two models and suggests that

perhaps some of these *cis* and *trans* associations require different zinc finger combinations and as a result different proteins are recruited.

A recent study postulated CTCF as a tumor suppressor gene because its ectopic expression inhibited cell division. Similarly, CTCF is also known to regulate other tumor suppressor genes that downregulate rRNA synthesis such as, retinoblastoma protein (pRb) and p53 [54]. One of the characteristics of tumor cells is the increase in rRNA amounts [54] and our model shows clearly how CTCF is indeed a tumor suppressor gene not only by repressing RNA polymerase I transcription but also by giving stability to the rDNA array, since in the absence of CTCF the nucleolus falls apart. This nucleolar stability also gives balance to the entire nucleus; therefore, the *cis* and *trans* interactions in non-nucleolar chromatin, postulated in model II, are maintained. Depletion of CTCF in S2 cells showed a decrease in protein signal not only at the nucleolus but in the rest of the nucleus which indicated that perhaps other regions apart from the rDNA were misregulated. In whole mount salivary glands we did not observe alterations in non-nucleolar chromatin by immunofluorescence signal. CTCF is provided maternally and CTCF mutants are phenotypically lethal; therefore, in salivary glands, maternal CTCF was still present at the non-nucleolar chromatin, while nucleolar CTCF seemed to be depleted. It is possible that because there are more CTCF binding sites outside than inside the nucleolus, in a CTCF mutant background the nucleolar chromatin was more affected.

In mammals, one predicted CTCF binding site was found in the non-transcribed spacer (NTS) at the 35S rDNA locus, and chromatin immunoprecipitation showed CTCF binding. In *Drosophila*, we could not find potential CTCF binding sites at the rDNA NTS and chromatin immunoprecipitation did not show binding at this region. However, CTCF was bound at the R1 element inserted at the rDNA. We also found potential CTCF binding sites in other transposable elements that are inserted at the 35S rDNA in different animal taxa (Fig 2.1) which suggests that apart from mammals, the genome in other organism uses retrotransposable elements to recruit CTCF at the nucleolus and this perhaps is the result of lacking a binding site at the NTS.

Future Directions

One of the findings of this work was the RNA dependence of CTCF at the nucleolus since it has not been shown whether CTCF can bind RNA directly. Nucleophosmin, a protein involved in rRNA processing that binds RNA, interacts with CTCF. This would predict that CTCF binds indirectly to RNA through Nucleophosmin. However, if on the other hand CTCF binds RNA directly, the region of the protein involved in this binding needs to be determined. Zinc finger domains are known to bind RNA; thus, this region would be a reasonable place to start. Apart from pre-rRNA processing, CTCF could be binding to R1 RNAs

that are co-transcribed with the 35S rDNA and target them for degradation as part of its role as rDNA transcriptional repressor.

Our results indicated that CTCF is poly(ADP-ribosyl)ate in *Drosophila*. We certainly observed the same effects when CTCF, PARP or PARG were knock down. However, it is important to investigate whether the increase in rRNA, R1 and R2 transcripts observed in PARP and PARG mutants was indirectly as consequence of removing CTCF from the nucleolus.

Previous studies showed that rRNA content in bobbed and wild type lines was the same; however, the rDNA transcription rate was lower in bobbed lines. I observed alleviation of the bobbed and lethal phenotypes in CTCF mutants and I suspected this was due to an increase in the rate of rDNA transcription. However, the data set obtained was very low. Therefore, future studies need to be conducted in large-scale to produce enough reliable data. Similarly, it should be established whether the alleviation of bobbed and lethal phenotypes were the result of an increase in RNA polymerase I transcription rate.

Our current model assumes that R1 and R2 inserted rDNA units are clustered. This is based on previous work showing that in rDNA arrays with silent R1 and R2 elements, these rDNA inserted units are found together, allowing the transcription of uninserted units. We believe that is the case for the CTCF stocks used in this study; however, this needs to be tested.

Regulation of rDNA copy number and rDNA transcription is crucial for cell survival. Reduction in rDNA copy number in yeast, increases the sensitivity to

agents causing DNA damage such as, UV radiation and methyl methanesulfonate (MMS). It also induces rDNA replication defects, decreases sister chromatid cohesion and thus the repair of double-strand breaks is compromised [136]. On the other hand, increase in rDNA transcription is a characteristic of tumor cells. Our results indicate that rDNA transcription is tightly involved in the regulation of rDNA copy number and CTCF is one the factors controlling rDNA transcription regulation.

CHAPTER VII

MATERIALS AND METHODS

Fly Strains

Fly stocks were maintained at 25°C on standard cornmeal, supplemented with yeast and tegosept. Stock w^[1118] Parg^[27.1]/FM7i, P{w[+mC]=ActGFP}JMR3 was obtained from the Kyoto Stock Center. CTCF^{p35.2}/TM6BTb, CTCF^{p30.6}/TM6BTb, and Df(3L)0463/TM6BTb were kindly provided by Dr. Pamela Geyer at the University of Iowa and it was characterized in a previous study [97]. D285, B486 and ROMA are stocks containing P{SUPorP} transposons at different locations in the Y chromosome [133].

Immunofluorescence and Confocal Microscopy

Salivary glands were dissected in phosphate-buffered saline (PBS), incubated for 20 seconds in PBS containing 1% Triton X-100 and 3.7% formaldehyde, then transferred to a solution of 3.7% formaldehyde and 50% acetic acid for 2 minutes and immediately squashed. Slides were washed twice in PBS for 10 minutes, transferred to PBS containing 0.1% Triton X-100 for 10 minutes and blocked with PBS containing 1% BSA for one hour at room temperature. Primary antibody was added and slides were kept at 4°C overnight. Rabbit anti-CTCF antibody was kindly given by Dr Jumin Zhou and was used at 1/500 dilution. Mouse anti-Fibrillarin antibody was purchased from Abcam and was used at 1/200 dilution. Slides were washed twice with PBS containing 1%

BSA and incubated with secondary antibody at room temperature for two hours. Goat anti-rabbit conjugated to rhodamine and goat anti-mouse conjugated to DL488 (Jackson ImmunoResearch) were each used at 1/200 dilution. Slides were immersed in 4',6-diamidino-2-phenylindole (DAPI at 1ng/mL) for 5 minutes, washed, and mounted in vectashield (Vector Laboratories).

Brains were dissected in 0.07% (w/v) Sodium Chloride, incubated for 7 minutes in 0.05% (w/v) Sodium Citrate, transferred to a solution of Methanol/Formaldehyde/water (11/11/2 ratio) for 30 seconds, then transferred and squashed in 45% acetic acid. Thereafter, the slides were treated as above.

For whole mount salivary glands and ovaries, dissection was performed in PBS and 1% Triton X-100, then transferred to PBT (PBS containing 0.1% Tween-80) and fixed in PBS containing 1% Triton X-100 and 3.7% formaldehyde. The tissue was blocked for 1 hour with PBT supplemented with 1% BSA. Primary antibodies were diluted in PNBT (PBT containing 1% BSA and 500mM NaCl) and incubated overnight at 4°C. The tissue was then washed in PNBT and incubated in secondary antibody for 2 hours, then washed and mounted in 70% glycerol.

For confocal microscopy, sequential excitation was performed at 488nm (for DL488), 543nm (for Rhodamine) and 405nm (for DAPI) in an Olympus FV1000 confocal microscope. The images were processed using FV19-ASW 1.7 viewer.

S2 cells immunofluorescence was performed as described [137]. Quantification of CTCF and fibrillarin immunofluorescence signal was done by independently capturing DAPI (for DNA), and rhodamine and fluorescein (for protein epitopes) channels and exporting to NIH Image-J. Entire fluorescence signals were integrated and divided by exposure time to determine intensity/time in arbitrary units. Individual nucleus measurements were normalized to DAPI signals to create datasets amenable to graphical and statistical comparison.

Chromatin Immunoprecipitation

ChIP experiments were carried out as described [138], with some modifications. Briefly, 200 μ L of third instar larvae were used per immunoprecipitation reaction; chromatin was cross-linked for 10 minutes at room temperature with 1% formaldehyde. Sonication was performed for 8 minutes, with 20 second pulses followed by 40 seconds “cooling off” period. After confirming fragment size averaging approximately 500 base pairs, protein concentration was estimated using the Bradford assay. 500 μ g of chromatin was incubated with 3-4 μ L of rabbit anti-CTCF antibody. 50 μ g of chromatin was set apart as input. For all buffers, PMSF and Complete Protease Inhibitor Cocktail tablets (Roche) were used as protease inhibitors. DNA was diluted in 1/20 for antibody and no antibody samples and 1/300 for input. Real Time PCR was used for quantification of precipitated DNA.

Primers used to amplify regions shown in Figure 2.7 are: 1 GGTTGCCAAACAGCTCGTCATC and CGAGGTGTTTGGCTACTCTTG, 2

GCCAAACACCTCGTCATCAA and GAGAGGTCTGGCAACCAC, 3
GAGTAGCCAAACACCTCGTC and GAGAGGTCTGGCAACCAC, 4
GCTGTTCTACGACAGAGGGTTC and CAATATGAGAGGTCTGGCAACCAC, 5
GGTAGGCAGTGGTTGCCG and GGAGCCAAGTCCCGTGTTTC, 6
ATTACCTGCCTGTAAAGTTGG and CCGAGCGCACATGATAATTCTTCC, 7
TTCTGGTTGATCCTGCCAGTAG and CGTGTGTACTTAGACATGCATGGC, 8
AGCCTGAGAAACGGCTACCA and AGCTGGGAGTGGGTAATTTACG, 9
GTAAGCGTATTACCGGTGGAGTTC and GTACCGGCCCAATAAACTCTCG,
10 CGACGCGAGAGGTGAAATTC and TATCTGATCGCCTTCGAACCTC, 11
CGTACCTGTTGGTTTGTCCCAT and TACTTTCATTGTAGCGCGCGTGC, 12
GCATTGATTACGTCCCTGCCC and CCG TAACACGCAAGGCG, 13
GTTAGTGTGGGGCTTGGC and CGC CGTTGTTGTAAGTACTCG, 14
GTTGTACCTGGCATCCATCAGG and CTGGTTGGTTATGGGGTTTGC, 15
GAAACTAAGACATTTTCGCAAC and CACCATTTTACTGGCATATATCAATTCC,
16 CTGTGCGTCATCGTGTGAACT and GTACATAACAGCATGGACTGCG, 17
CCTCAACTCATATGGGACTACCC and CGCTCCATACACTGCATCTCAC, 18
GTGAGATGCAGTGTATGGAGCG and GCTGCACTATCAAGCAACACG, 19
GATTCAGGATACCTTCGGGACC and GAACGCCCCGGGATTGTG, 20
GAGTATAGGGGCGAAAGACCAA and GCACCAGCTATCCTGAGGG, 21
GGAGTGTGTAACAACTCACCTGC and GGTATACAACTTAAGCGCCATCC, 22
CTAAGTTCAAGGCGAAAGCCG and CGGATACTCAACAGGTTACGG, 23
GCAGCTGGTCTCCAAGGTG and CCCAGAACGAGCACATAAACC, 24

CAAGTAAGCGCGGGTCAACGG and CCCTTGGCTGTGGTTTCGCTAG, 25
 CGGGCTTGGAATAATTAGCGG and CCGAGGTGTAATATCTCCCAC, 26
 GGACATTGCCAGGTAGGGAG and GCTGTCCCTGTGTGTACTGAAC, 27
 CCGTGCTGGACTGCAATG and CATTGGCATCACATCCATTGTCG, 28
 GGGACAGCTTAGTGCACTCTAC and CCAGCAATCGTATGCTCGCTG, 29
 GCGGAAGCAGTGCCTC and CAGTTTCGCCTGCGTTGG, 30
 CGCTTCGTGGGAGATCATGC and CCAATCTCCGTGCACTTC, 31
 CCCCAGGAGTTGCTAATCTAACC and GGGAGTGATGGAGTTGTTTCCG.
 Primers with sequence AAGTTGTGGACGAGGCCAAC and
 CGTTCTCGTCCGATCACCGA were used as endogenous control which
 amplified a fragment of the *5S rDNA*.

Reversed Transcriptase Real Time PCR (RT-Q PCR)

RNA from adult flies or S2 cells were extracted as described [139]
 Primers used for the reverse transcriptase reaction were: 35S
 GTACCGGCCACAATAACACTCG, R1 CCAGCAATCGTATGCTCGCTG, R2
 GCCAACACTGTGTGTGGTCA, uninserted R1 and R2
 CCGAGGTGTAATATCTCCCAC *Rho1* CTTAGCCGAACACTCCAAATAGG.
 Real-Time PCR: 35S AGCCTGAGAAACGGCTACCA and
 AGCTGGGAGTGGGTAATTTACG, R1-inserted (Fig 3.5, green
 arrows) GCCTCGTCATCTAATTAGTGACGCGC and
 CCACGAGCGCAACGAAAACACG, R2-inserted (Fig 3.5, blue arrows)
 GGATGTGATGCTCCCGAAAC and CAAGTCCCCGCTTGATTCTGA, uninserted

R1 and R2 (Fig 3.5, red arrows) GCCTCGTCATCTAATTAGTGACGCGC and CCCTTGGCTGTGGTTTCGCTAG, *Rho1* GTGGAGCTGGCCTTGTGGG and CTAGCGAATCGGGTGAATCCACTG, 18S-ITS1 GTAGGTGAACCTGCGGAAGG and GTTGCCAAGCCCCACACTAAC, 5.8S-ITS2a CGCATATCGCAGTCCATGCTGT and CAACCCTCAACCATATGTAGTCC, 2S-ITS2 GGACTACATATGGTTGAGGGTTG and GCTAGACATTTCTCAGTATTATTG, ITS2_28S GAATTGTCTCTTATTAATGATTCGG and GTAGTCCCATATGAGTTGAGG. Reverse Transcriptase reaction product cDNA was diluted 1:25 to 1:60 as determined empirically with test samples to optimize melting curve (single-peak) and crossing threshold (not greater than cycle 29).

The primers used to quantify *CTCF*, *PARP* and *PARG* mRNA levels were: *CTCF* ACGAGGAGGTGTTGGTCAAG and ATCATCGTCGTCCTCGAAA, *PARP* GTTTGCAGAAGAGCTCGGAATTC and either CCCCAACTACAAATACATGTGC or GCTGAACTTTGTAGTAGGAGTTC, *PARG* CGCCGCAGAGCAAGTGC and either CTTCGACATCCTGGCGCAG or GCGTTCTTGTGGTGCTTG.

Genomic DNA extraction

Females of genotype *C(1)DX*, *y¹ f¹ bb⁰* were crossed to *y¹ w¹¹¹⁸/Y*; *CTCF/TM6B*, *Tb* and genomic DNA from Tubby and non-Tubby males was extracted and subjected to Real-Time PCR [134].

RNAi in S2 cells

Drosophila Schneider 2 (S2) cells were culture in Schneider media supplemented with 50 µg/mL streptomycin, 50 µg/mL penicillin and 10% heat-inactivated fetal bovine serum (GIBCO). After reaching a density of 10⁶ cells/mL, they were washed twice in serum free medium. 15 µg of double-stranded RNA was added to 1 mL of S2 cells resuspended in serum free medium, mixed by swirling and incubated at room temperature for 1 hour. 2 mL of medium containing serum was then added, and cells were cultured at 25°C for three days. An aliquot was taken on every day for five days and samples were analyzed by immunofluorescence and RT-Q PCR. Double-stranded RNA was generated by PCR amplifying gene sequence using primers that contained the T7 RNA Polymerase promoter: *CTCF* ACTAAAGGCCCAAGCTCA and TGACAGTGCCATCTTTCTGC, *PARP* GAGTTCGACACGAGCGAGT and GCGCCTTGCTTCTCCTT, *PARG* CCGGCAGTTCTGGAGAA and CCATGAGATCCTCGCGATATT, *LacZ* TAATACGACTCACTATAGGAGGTATTCGCTG and TAATACGACTCACTATAGGCGATCGTAATCACC. RNA was transcribed using the T7 MEGAscript Kit (Ambion) without deviation from the manufacturer's instructions.

RNAse treatment

Salivary glands were dissected in PBS containing 0.1% Triton X-100, then transferred to PBS containing 0.1 % Triton and 50 µg/ml of RNase and

incubated for 10 minutes. Salivary glands were incubated 30-60 seconds in PBS containing 1% Triton X-100 and 3.7% formaldehyde, then they were treated as above.

Rapamicyn treatment

Adult flies from *CTCF^{p35.2}* and control stocks were placed on fly food containing rapamicyn to a final concentration of 1 μ M. Third instar larvae were collected and whole mount salivary glands were stained with Fibrillarlin antibody.

CTCF knockout

CTCF cloning into required vectors for knockout was performed by Silvana Paredes. I used homologous recombination to replace the *Drosophila CTCF* with *white⁺* gene as shown in Figure 7.1. After recovering *white⁺* flies and setting up 902 crosses, 172 possible targeting events were recovered, and 37 had a lethal phenotype. After testing the flanks of the insertion both by PCR and Southern analysis, two lines, *CTCF¹* and *CTCF⁹* were found to be clean knockouts (Fig 7.2).

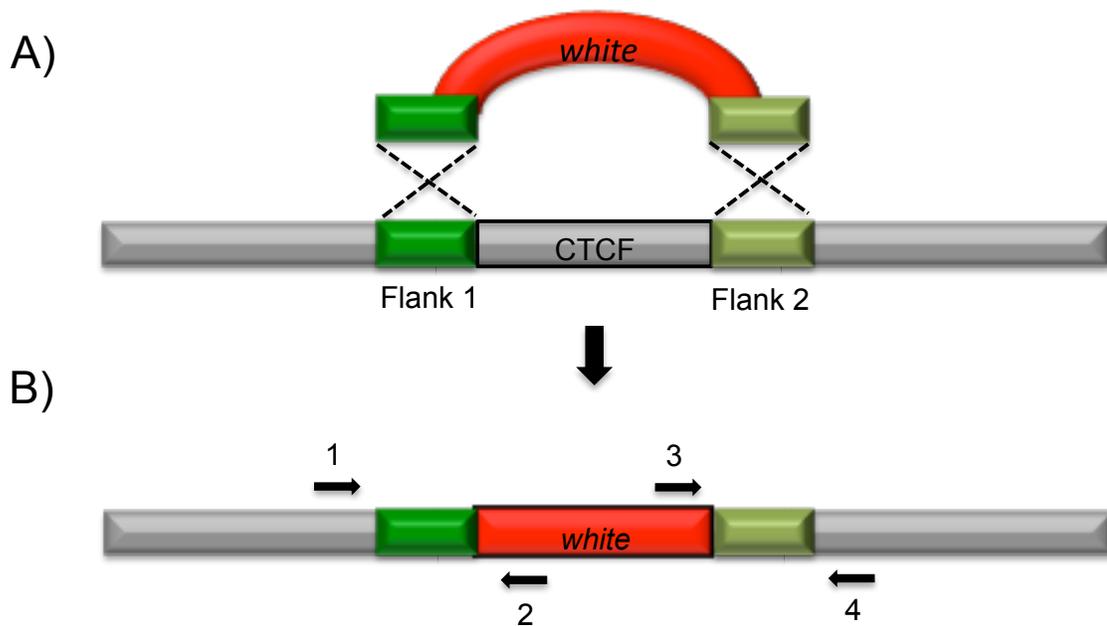


Figure 7.1 Generation of CTCF knockout. A) Homologous recombination between a transposable element carrying *white*⁺ and CTCF flanking sites and CTCF genomic flanks, black cross shows recombination sites. B) Product of homologous recombination, *white*⁺ is replacing CTCF. 1 and 2 are showing primers locations to amplified flank 1 by PCR (2.6 Kb), 3 and 4 are primers to amplified flank 2 (4.7 Kb)

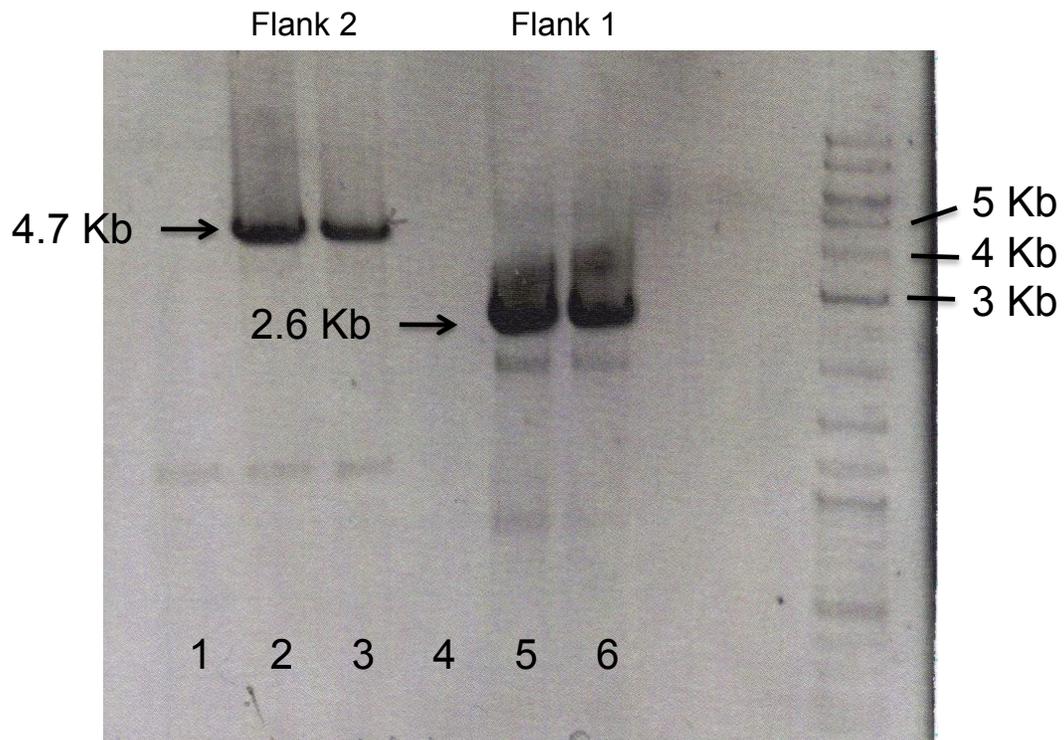


Figure 7.2 Knockout flanks tested by PCR. 1 and 4: control, 2 and 5: *CTCF*¹, 3 and 6 *CTCF*⁹. 1-3 flank 2, 4-6 flank 1.

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APPENDIX A

POSITION EFFECT VARIATION OF THE 5S RIBOSOMAL DNA

Introduction

RNA polymerase III is responsible for transcribing small non-coding RNAs. Some are involved in protein synthesis (5S rDNA and tRNA), others in post-translational processing (H1, MRP, and U6 RNAs), intracellular transport (7SL RNA), regulation of RNA polymerase II transcription elongation (7SK), and DNA replication (Y RNA). RNA polymerase III also transcribed genes with unknown function such as, SINEs, BC200, and BC1 [140]. 5S rDNA and tRNA gene promoters are characterized for having their internal control regions (ICR) within their transcribed unit. For tRNA genes, these regions are called the A and B boxes and for 5S rDNA genes, the A and C boxes. Transcription by RNA polymerase III requires the binding of transcription factor III C (TFIIIC) to these boxes, followed by the recruitment of TFIIIB and finally RNA polymerase III. Remarkable work, looking at the transcription of the 5S rDNA and tRNA genes, have shown that only a fraction of them is transcriptionally active.

Using electron microscopy, French and colleagues in 2008 showed that in *Saccharomyces cerevisiae* not all 5S rDNA genes are active and that the number of RNA polymerase III bound to a single 5S unit can range from one to three. These observations indicated that 5S rDNA transcription is controlled in two

ways: by the number of active and silent copies and by the number of RNA polymerase III loaded on each 5S rDNA unit [141].

In 2010, Barski and colleagues showed that in different cell types the percentage of *tRNA* genes occupied by RNA polymerase III was different, and only a subset of them were active. Their work also demonstrated that histone modifications at genes transcribed by RNA pol III resembled those at RNA pol II genes. For instance, histone acetylation and methylation of histone H3 lysine 4 was present at actively transcribed RNA polymerase III genes, while methylation of histone H3 lysine 9 and lysine 27 were found at inactive genes. Similarly, they showed RNA polymerase II recruitment at *tRNA* promoters in *Drosophila* S2 cells and to the 5S rDNA in HeLa cells, however; it was unknown whether RNA pol II transcripts could be generated at these loci. They used a RNA polymerase II inhibitor, α -amanitin, and observed a decrease in transcription of 5S, U6, and pre-*tRNA* transcripts [142]. These results indicated that perhaps the regulation of transcription by RNA polymerases II and III is interconnected. The authors speculated that factors that are common for both polymerases such as, TATA-binding protein and *c-myc*, may be involved in bringing RNA polymerase II to RNA pol III genes promoter. These observations anticipate that mutations in genes involved in establishing and maintaining the silent state at RNA polymerase II transcribed genes would affect RNA polymerase III genes.

In 2006, Peng and Karpen studied the effects of the histone methyltransferase, Su(var)3-9, in nucleolar integrity in *Drosophila*. Their work

showed that in a *Su(var)3-9* mutant background, acetylation of histone H3 at lysine 9 (active chromatin mark) at the 5S rDNA, increased more than 15 fold compared to wild type. However, histone H3 lysine 4 di-methylation, also an active chromatin mark, was not affected at the 5S rDNA. Similarly, they measured the formation of extrachromosomal circles in *Su(var)3-9* mutants as a result of the disruption of silent chromatin (Chapter III) in RNA polymerase I (35S rDNA), II (satellite repeats) and III (5S rDNA) transcripts. In wild type conditions, significant amounts of 5S rDNA extrachromosomal circles were normally generated. In a *Su(var)3-9* mutant background no effects were shown in the generation of extrachromosomal circles from the 5S rDNA, while in RNA polymerases I and II targets, extrachromosomal circles were observed. The authors also studied the formation of extrachromosomal circles in a *Dicer-2* mutant background, member of the RNAi machinery. They did not observe extrachromosomal circles from the RNA pol II or III targets but they did so from RNA polymerase I transcripts [23]. These results showed that some factors involved in RNA pol II transcription regulation are also shared by RNA pol III; however, the discrepancies observed predicted that additional components participate in the regulation of RNA polymerase III transcription.

In many organisms (flies, humans, frog and plants) 5S rDNA genes are located in a different cluster from the 35S rDNA array, in the yeast *Saccharomyces cerevisiae*, each 5S copy is inserted in the intergenic spacer of the 35S rDNA. In *Drosophila melanogaster*, the 5S rDNA cluster is located at the

right arm of chromosome 2, cytological band 56E2 and the transcript unit is 135 base pairs long. We obtained a fly stock containing a P-element, marked with *white*⁺, inserted in the 5S rDNA gene cluster and noticed that the expression of the *white*⁺ gene was variegating. We wanted to investigate whether this variegation could be affected by: 1) Su(var)s and E(var)s which alter the expression of RNA polymerase II genes, 2) an extra copy of the Y chromosome which is known to suppress variegation of *w^{m4}*, 3) RNA interference members that are known to affect RNA polymerase II variegation, 4) CTCF mutations, since CTCF binds at the flanks of the 5S rDNA gene cluster.

On the other hand, we wanted to see whether a connection between 35S and 5S rDNA existed; in other words, is the transcription level or rDNA copy number of one array affecting the other?. Finally, we wondered whether the inserted *white*⁺ gene, which has a RNA polymerase II type promoter, was transcribed by RNA polymerase II or III. We expected that answering these questions would help to understand the regulatory mechanism of RNA polymerase III transcription.

Results and Discussion

5S rDNA Position Effect

Stock number 205676 was obtained from the Kyoto stock center and had the genotype *y*[1] *w*[67c23]; P{w[+mC]=GSV6}GS14066/SM1. As shown in Figure A.1 it is inserted in the 5S rDNA array, which we confirmed by PCR, and it

has a variegation *white*⁺ expression. This is the first report showing RNA polymerase III transcription as the source of variegating expression. For practical reasons, this line will be referred as 5S^{PEV} in the rest of this chapter.

We wondered whether the expression of 5S^{PEV}, which contains a promoter for RNA polymerase II, would be affected by modifiers of RNA polymerase II transcription; therefore, we tested its expression in mutant background of 13 Su(var)s, 4 E(var)s and one member of RNAi machinery. For our surprise, there was no effect on 5S^{PEV} expression (Fig A.2). Then, we tested whether an extra copy of the Y chromosome, which is a strong suppressor of variegation, would alter 5S^{PEV} expression; however, it had no effect (Fig A.3). These results indicated that 5S^{PEV} is perhaps transcribed by RNA pol III, and as a result, factors affecting RNA pol II transcription had no effect on its expression.

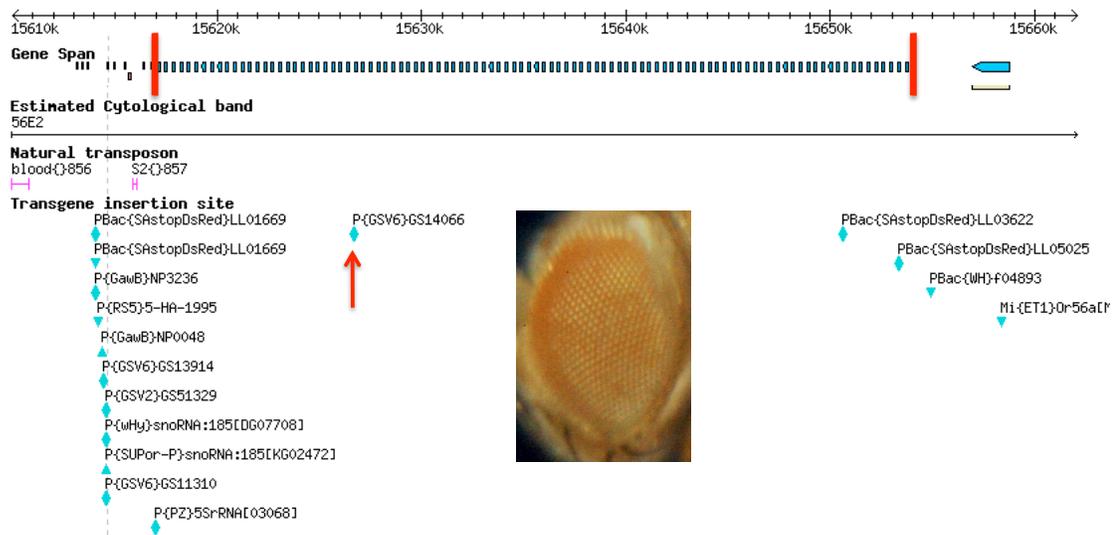
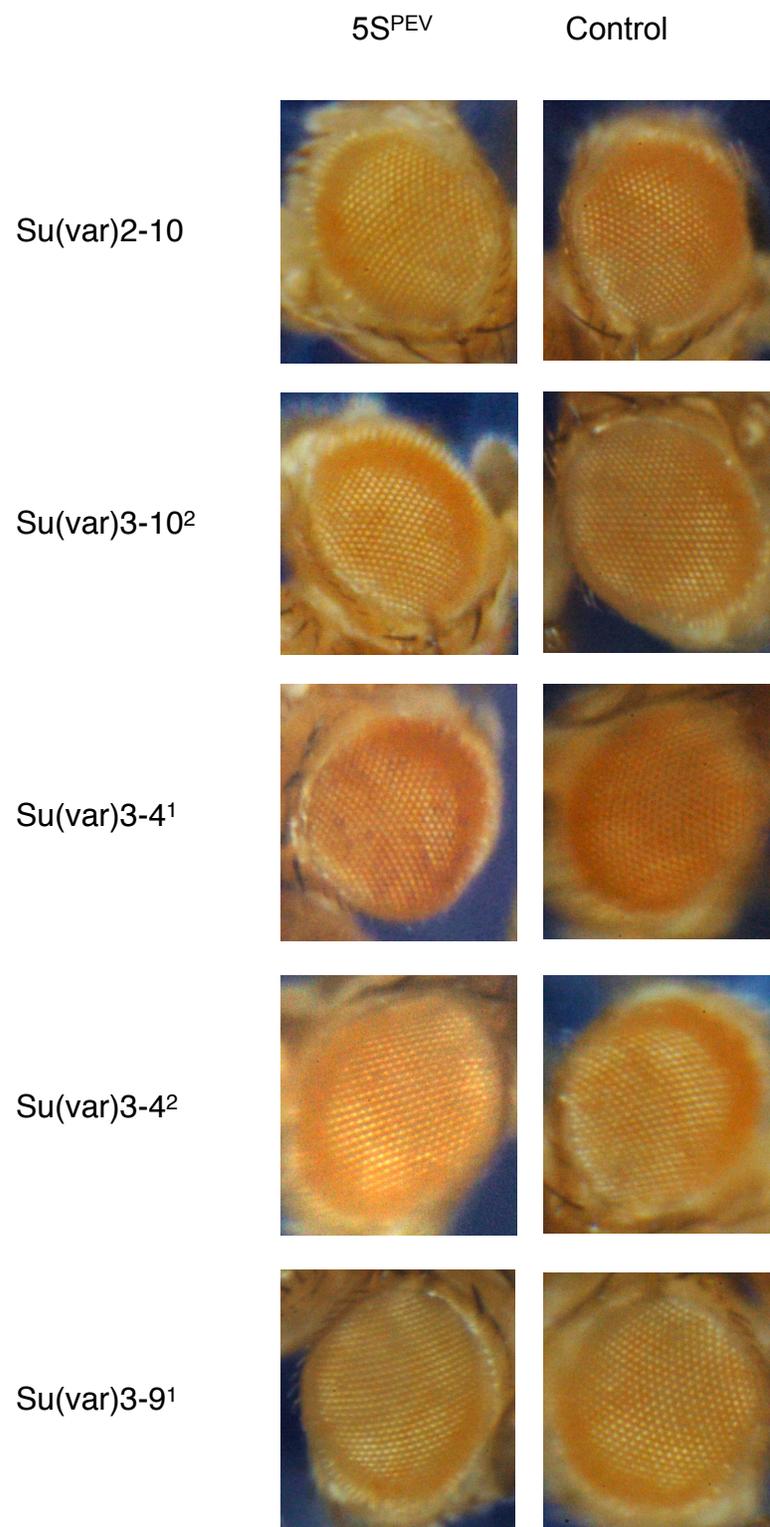
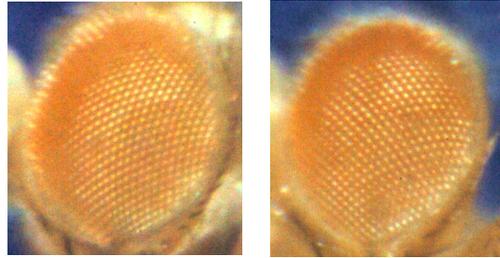


Figure A.1 The 5S rDNA cluster. The variegating line used in this study is pointed by a red arrow. Expression of the marker gene *white*⁺ is shown. Red vertical lines show the ends of the 5S rDNA array

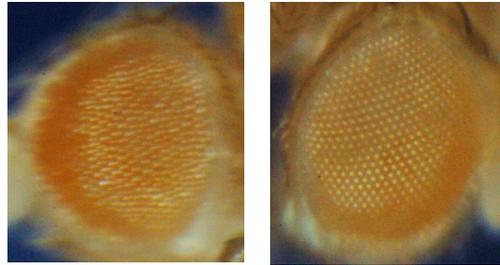
Figure A.2 5S rDNA Position Effect Variegation is not affected by RNA polymerase II variegators. Su(var), E(var), and RNAi members tested.



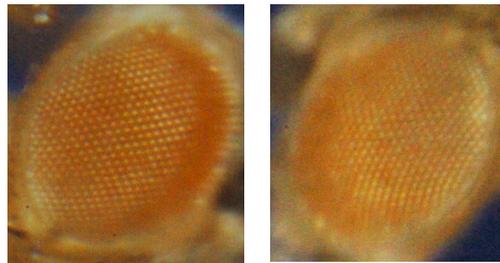
Su(var)3-9²



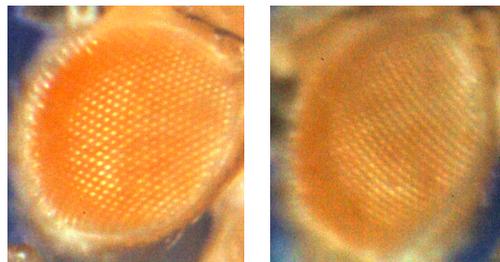
Su(var)2-1¹



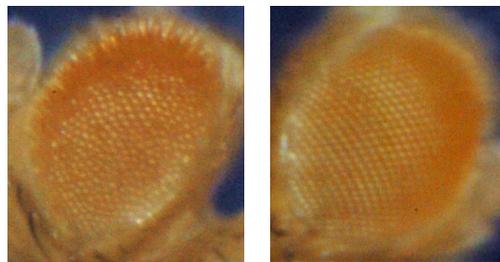
Su(var)2-1²⁻¹³

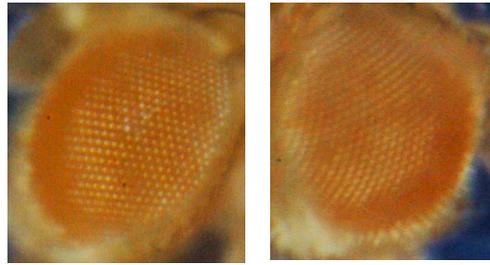
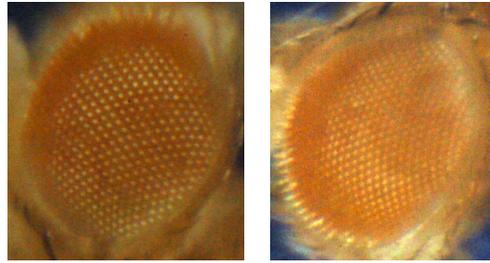


Su(var)2-5⁵

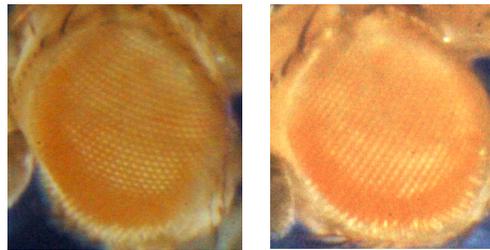
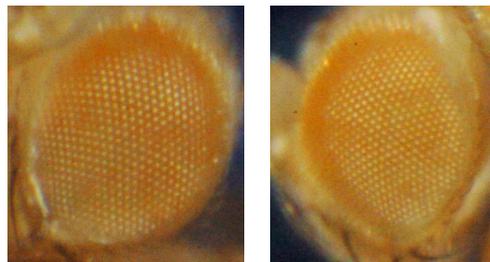
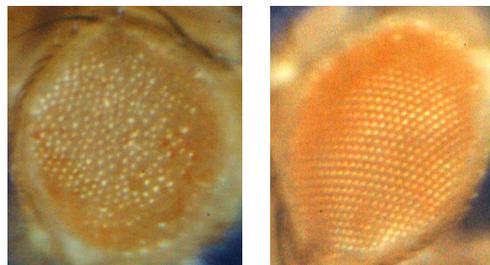


Su(var)2-10²

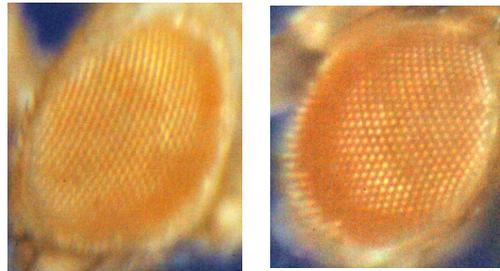


Su(var)2-10¹Su(var)2-4⁰¹

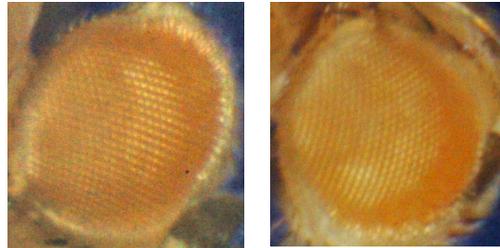
JIL-1 Su(var)3-1[3]

E(var)5¹E(var)7¹

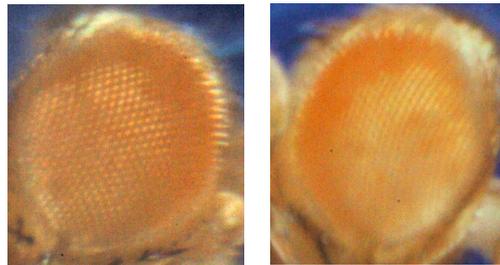
E(var)8^{48g}



mod(mgd4)⁰³⁸⁵²
(E(var)3-93D)



spn-E^{hls-03987}



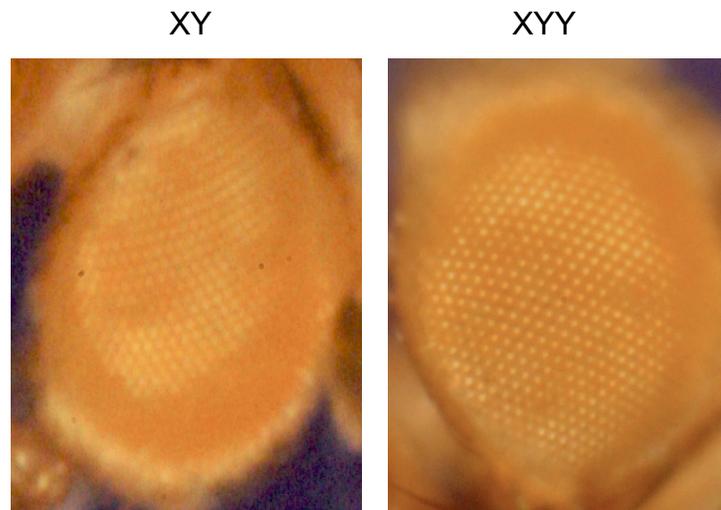


Figure A.3 5S rDNA Position Effect Variegation is not affected by an extra copy of the Y chromosome.

I stained salivary gland polytene chromosomes with CTCF and noticed it was found at the 5S rDNA, cytological band 56E (Fig A.4); however, the precise location (56E2) could not be determine. Published data from flybase.org showed that CTCF, which belongs to class I insulators (Fig A.5) binds at both flanks of the 5S rDNA array. We wondered whether CTCF was regulating rDNA transcription and as a result could affect the expression of 5S^{PEV}. We tested three trans-heterozygous *Df(3L)0463/CTCF^{p30.6}*, *Df(3L)0463/CTCF^{p35.2}*, *CTCF^{p35.2}/CTCF^{p30.6}* and one homozygous *CTCF^{p35.2}/CTCF^{p35.2}* background (described previously in Chapter III); however, 5S^{PEV} was not affected (Fig A. 6). This predicts that CTCF does not regulate 5S rDNA transcription and its location near by this cluster is possibly to regulate genes found at both flanks of the 5S array.

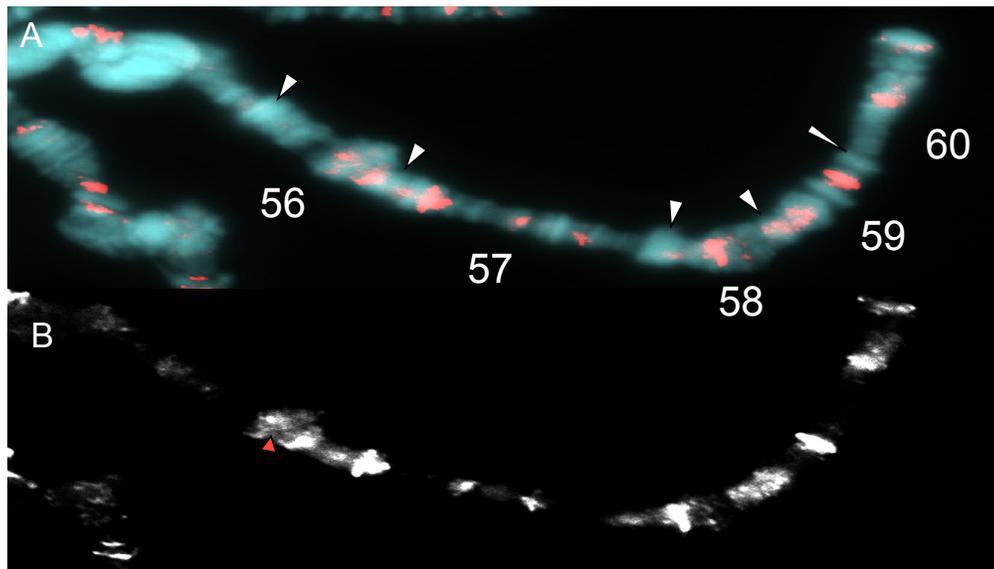
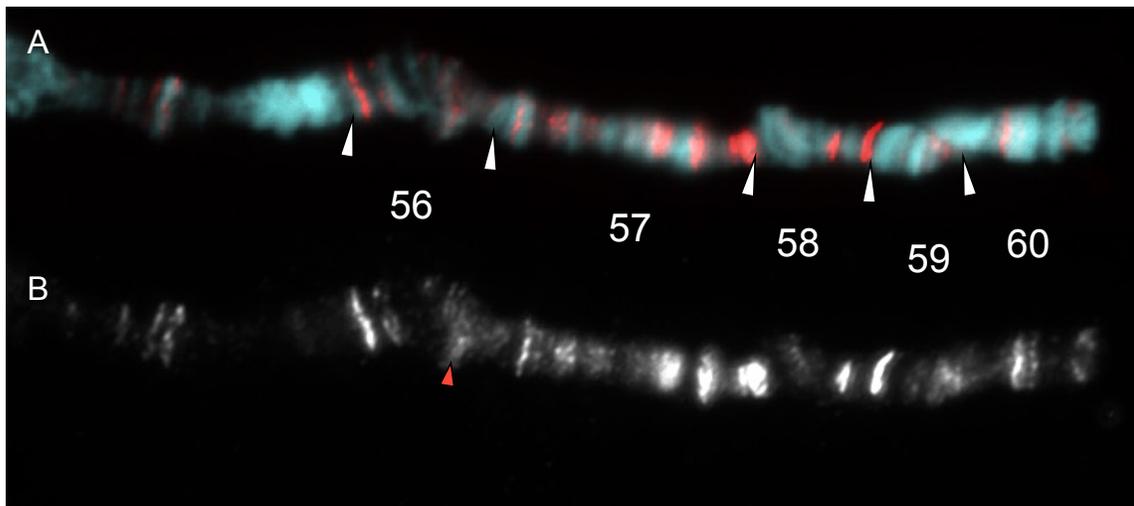
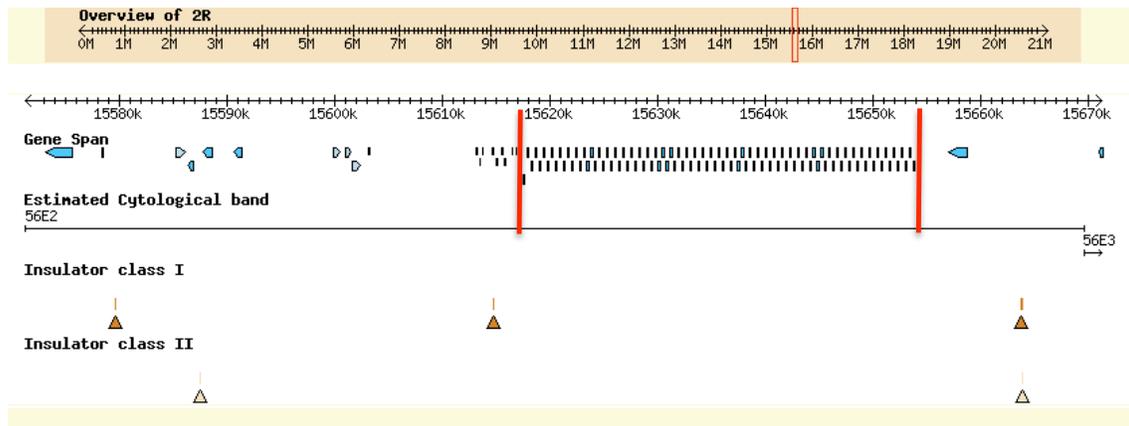


Figure A.4 CTCF is found at the same cytological band as 5S rDNA. Two second chromosome right arms are shown. CTCF is in light red and DNA in light blue. CTCF is shown as independent channel below the merge. Red arrow shows the location of the 5S rDNA locus.



9

Figure A.5 Insulators flanking the 5S rDNA array. Insulator type I: BEAF-32/CP190/CTCF and insulator type II: Su(Hw). 5S rDNA copies are flanked by red vertical lines.

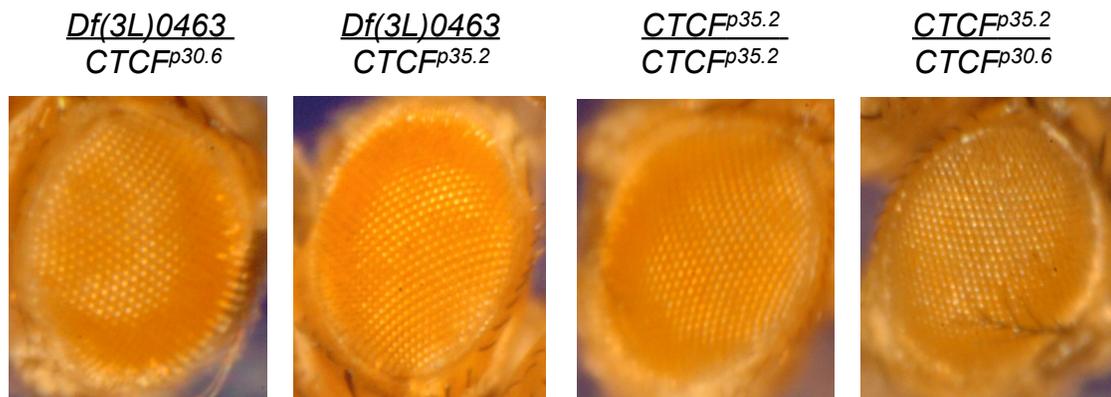


Figure A.6 5S rDNA Position Effect Variegation is not affected by CTCF mutants. The variegating line was tested in three heterozygous and one homozygous mutant background.

5S rDNA transcription level and copy number:

5S rDNA transcripts in Su(var)s and E(var)s mutants were measured indirectly through 5S^{PEV} expression and it did not appear to be affected. We

decided to measure it directly by performing RT-Q-PCR in 3 RNAi members, 5 E(var)s, 8 Su(var)s adults mutant flies and compare them to a control stock, 10B (Fig A.7). Rho gene was used as internal control for each reaction. Out of 8 Su(var)s tested, three showed 2 to 2.5 fold increase in 5S rDNA transcription (Su(var)3-4, Su(var)3-7, and Su(var)2-1), three had around 1.5 fold increase (Su(var)2-10, Su(var)3-1, and Su(var)4-20), and in two of them, transcription was not affected (Su(var)3-9 and Su(var)2-5). Three out of the 5 E(var)s tested showed an increase in 5S rDNA transcription, E(var)9 (2.7 fold), E(var)8 (1.6 fold), and E(var)7 (1.4 fold) and in two of them 5S rDNA transcription was greatly reduced; Trl (74%) and mod(mdg4) (39%) compared to the control. Only one RNAi component showed an 1.4 fold increase in 5S rDNA transcription (*snp-E*), while in the other two, transcription was reduced piwi (73%), *aub* (54%), compared to the 10B control.

In general, 5S rDNA transcription was affected in the different variegator backgrounds tested; however, the results obtained did not show what expected for a RNA polymerase II gene variegating expression: 1) Although, six Su(var)s showed an increase in 5S rDNA transcription, the classical HP1(Su(var)2-5)-Su(var)3-9 pathway did not show an effect. Su(var)3-9 is known to be present at the 5S rDNA locus; however, it is not known whether HP1 is found there. Therefore, the silencing mechanism at the 5S rDNA may require Su(var)3-9 methylation; however, additional factors (different from HP1) seem to be involved. 2) In three E(var)s tested 5S rDNA transcription increased. This

showed that the regulation at the 5S is quite different from RNA pol II genes. 3) Mod(mdg4) belongs to the Insulator type II-Su(Hw) complex and as shown in Figure 8.5, this complex binds at both sites of the 5S rDNA locus. Consequently, the decrease in 5S rDNA transcription we observed in a mod(mdg4) mutant background, indicates that perhaps the insulator activity of Su(Hw)-mod(mdg4) helps in the regulation of 5S rDNA transcription, possibly by isolating the cluster by a looping mechanism and generating a spot for active RNA polymerase III transcription. 4) Three components of the Piwi-interacting RNA (piRNA) pathway were tested, 5S rDNA transcription decreased in two of them (*piwi* and *aub*) and increased in one (*spn-e*). piRNAs function is to silence transposable elements in the germ line. The fact that these results did not show a particular trend suggested that this pathway is not involved in 5S rDNA silencing.

5S and 35S rDNA transcripts are assembled together to form mature ribosomes, this would predict that they could be regulating each other and that for instance, reduction in copy number of one array would alter the array size of the other. We decided to compare the array size of the 35S and 5S rDNA in adult flies of the different RNA pol II variegator stocks (Fig A.8). 5S rDNA array size was reduced in all mutant background tested except for Su(var)3-7. This reduction was from 60.8% (Su(var)3-9) to 92.7% (mod(mgd4)) compared to the 10B control. 18S rDNA copy number was higher than 5S rDNA in almost all backgrounds tested. In the Su(var)s mutant background, with the exception of Su(var)3-7, 5S rDNA array copies were around 50% less than 18S rDNA copies.

For E(var)s and piRNA components the trend was very similar, with the exception of *spn-E* and *E(var)-8*; however, the differences between 5S and 18S rDNA copy number were very variable. In E(var)s, the decrease in 5S rDNA copies compared to 18S ranked from 13.7% (Trl) to 86% (E(var)-7). In piRNA components, this decrease ranked from 4% (*armi*), to 50.1% (*piwi*).

These results showed that while the number of 5S rDNA copies did not vary that much in all background tested, the number of 18S rDNA was more affected by the RNA pol II variegators background, consistent with the fragmented nucleoli present on some of these mutants Chapter III and [23]). Hence, these two rDNA components did not seem to be involved in each other array size regulation. To confirm this result, 5S rDNA array size was measured in some of the 35S rDNA lines lines created previously in the lab (Chapter V and [134]), to study whether the decrease in 35S rDNA array size will cause a reduction in 5S rDNA. We did not observed an effect on the 5S rDNA array size in two bobbed line tested (*bb-465*, *bb-76*). In the lethal lines, no particular trend was followed (Fig A.9) indicating that these two rDNA arrays are independently regulated and confirming our previous results.

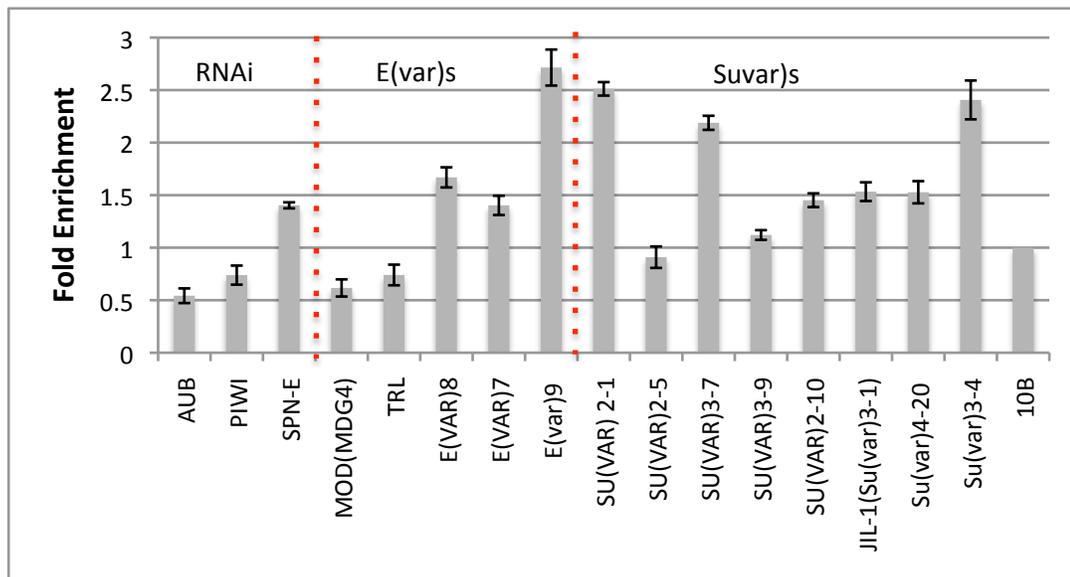


Figure A.7 5S rDNA transcription in Su(var), E(var), and RNAi mutants background.

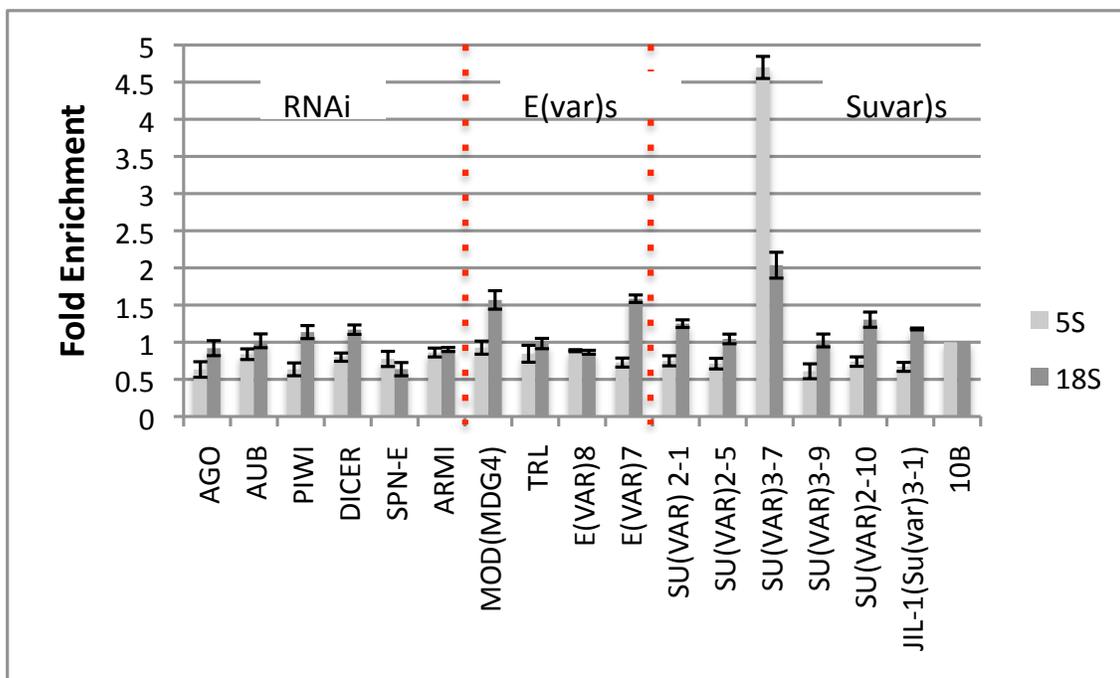


Figure A.8 5S and 18S rDNA copy number in Su(var), E(var), and RNAi mutants background.

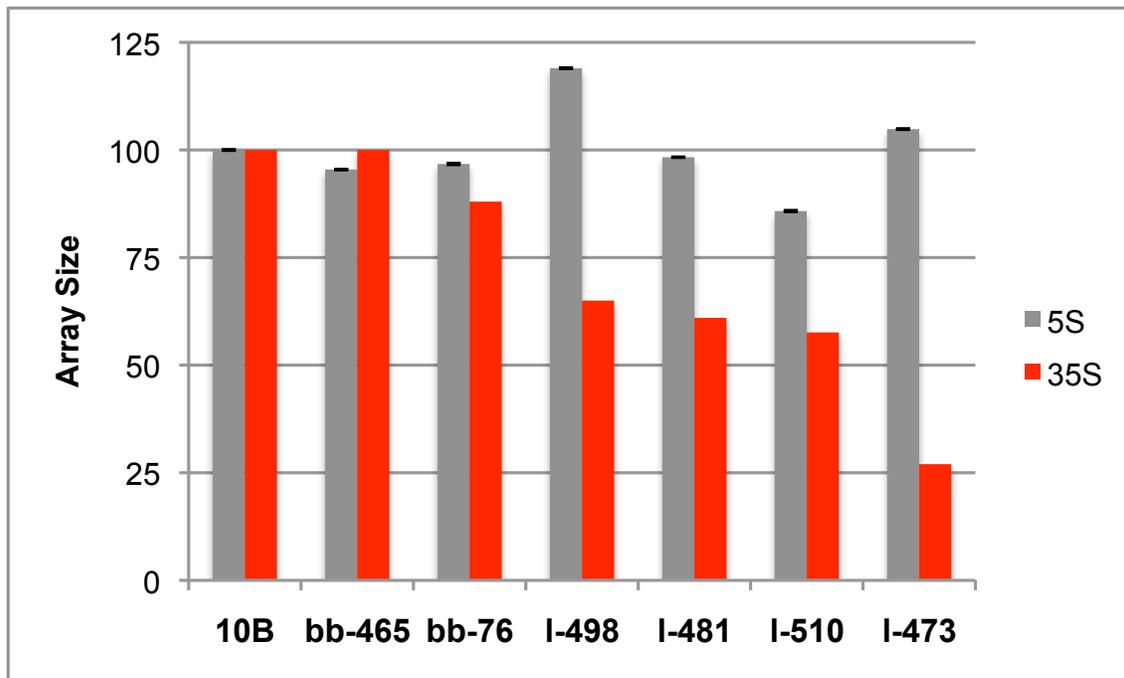


Figure A.9 5S rDNA copy number in different bobbed and lethal 35S rDNA lines. 5S rDNA copy number is represented in grey while 35S rDNA from Paredes and Maggert [134] is shown in red. I: lethal, bb-bobbed.

Transcription of 5S^{PEV}

In order to get more insights into understanding the regulation of 5S^{PEV} and consequently the 5S rDNA, we investigated whether a 5S rDNA deficiency would suppress the variegation of 5S^{PEV} line, which could indicate that RNA polymerase III was driving its expression. Fly stock 25678 (w[1118]; Df(2R)BSC594/CyO) has a deletion with break points 56E1-56F9, which includes the entire 5S rDNA array (56E2). We compared the expression of 5S^{PEV}/Df(2R)BSC594 and 5S^{PEV}/CyO, and observed a dramatic suppression effect by the deficiency (Fig A.10). This result can be interpreted in two ways: 1) Reduction in 5S rDNA copy number caused an increase in RNA polymerase III

transcription and as a result $5S^{PEV}$ expression increased as well. This assumes that $5S^{PEV}$ is transcribed by RNA polymerase III. 2) Increase in 5S rDNA transcription, creates a more open chromatin state which facilitated the recruitment of RNA polymerase II to $5S^{PEV}$, resulting in its elevated expression. To resolve this enigma, further studies need to be conducted to determine whether $5S^{PEV}$ transcripts are produced by RNA polymerase II or III transcriptional machinery.

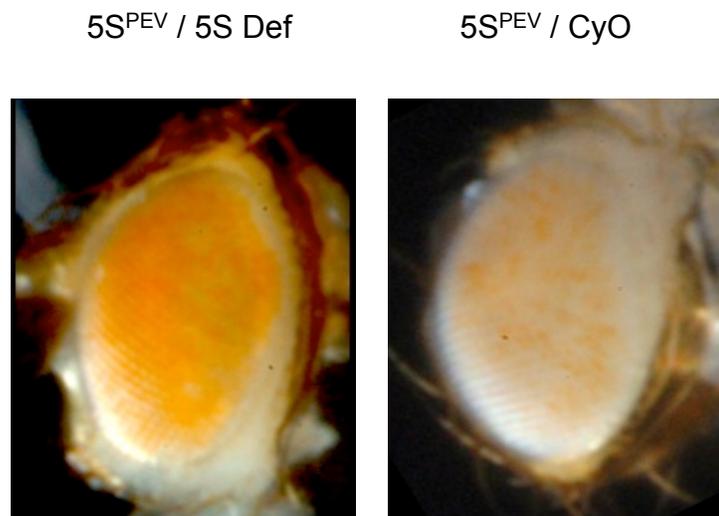


Figure A.10 A 5S rDNA deficiency suppressed the variegation of a RNA polymerase II gene inserted in the 5S rDNA gene cluster.

We decided to get some insights in determine whether $5S^{PEV}$ was a result of RNA polymerase II or III transcription. The only RNA polymerase subunit annotated in *Drosophila* is RpIII128. We noticed that the Bloomington fly stock

15840 ($y^1 w^{67c23}; P\{w^{+mC} y[+mDint2]=EPgy2\}^{EY01866}/CyO$) contained a P-element that was inserted at the promoter of this RNA polymerase III subunit (Fig A.11) and decided to use imprecise excision to generate a deficiency that removed part or the entire *RpIII128* gene as shown in figure A.12. Out of 113 *white*⁺ excisions obtained, 11 of them were lethal. These possible candidates were crossed to 5S^{PEV}, and tested by Southern blotting (Fig A.13). Two of them seemed to suppress the variegation of 5S^{PEV} (*RpIII128*¹⁷ and *RpIII128*⁶²) and Southern blot analysis showed presence of additional bands (Table A.1). Both lines phenotypically showed deletion of the *white*⁺ from P-element (P{EPgy2}). We decided to test whether the excision had left behind fragments of the P-element; thus, I designed primers for the 5' and 3' end of P{EPgy2} and tested *RpIII128*¹⁷ and *RpIII128*⁶² lines by PCR. P{EPgy2} was run in parallel as control.

PCR products showed that in both mutants, the excision was imprecise, leaving fragments of the P-element. In the case of *RpIII128*¹⁷, a 912bp fragment from the 3' end of the element was found, while for *RpIII128*⁶² a 1023bp fragment was amplified from the 5' of the element (Fig A.14).

Our results indicated that the 5S^{PEV} expression observed is the result of RNA polymerase III transcription. This brings a different prospective on the requirement for RNA pol III transcription, since the promoter of 5S^{PEV} does not have the A, B or C boxes required for the recruitment of TFIIC. This revealed that having several RNA pol III molecules around a RNA pol II gene promoter could be enough to trigger transcription by RNA polymerase III, and as a result

variegators of RNA polymerase II transcription cannot affect the expression of this gene. This also predicted that the presence of Insulator type I and II flanking the 5S rDNA locus is possibly preventing the uncontrolled activation of neighboring genes by RNA polymerase III machinery. Further studies need to be conducted to confirm that 5S^{PEV} product is not polyadenylated and therefore it is not transcribed by RNA polymerase II. Similarly, the breakpoints of the deletions in the *RpIII128*¹⁷ and *RpIII128*⁶² lines need to be mapped more precisely, to examine whether genomic regions, surrounding the P-element insertion point, were also removed. Lastly, it should be tested whether both lines are lethal over each other to discard possible unrelated mutations responsible for the lethal phenotype.

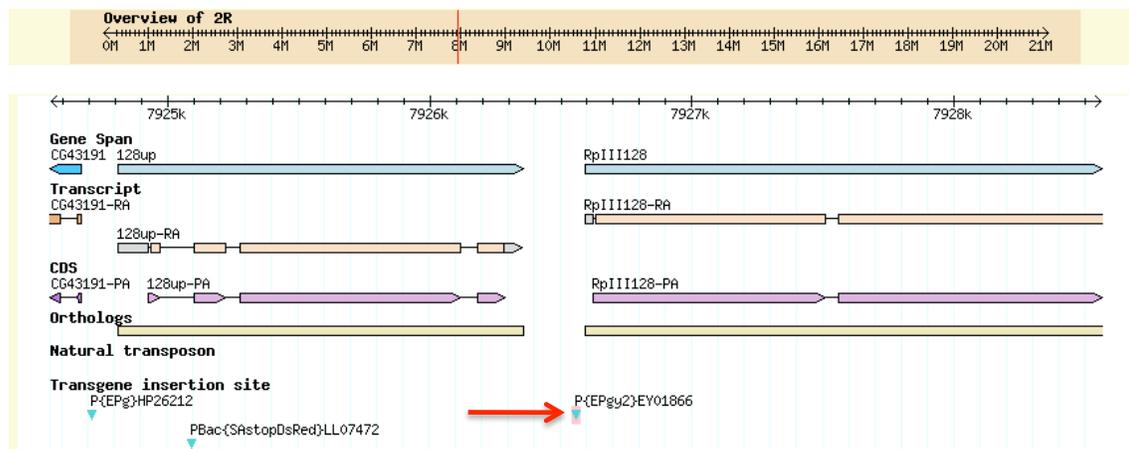


Figure A.11 *RpIII128* genomic region. P-element used for imprecise excision is pointed by a red arrow.

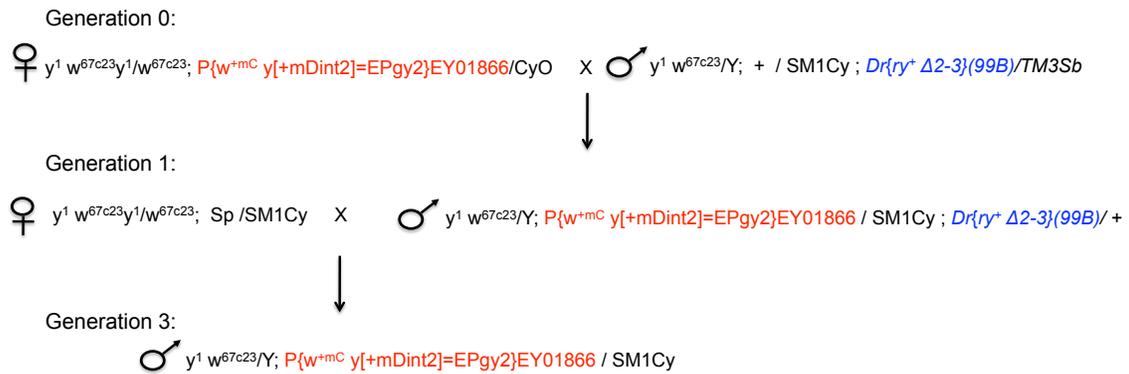


Figure A.12 Cross performed to obtain RpcIII128 mutants. P-element inserted at the RpcIII128 promoter is shown in red. Transposase source is shown in blue. In generation 3, white eye males carrying the SM1Cy balancer were picked and tested for lethality.

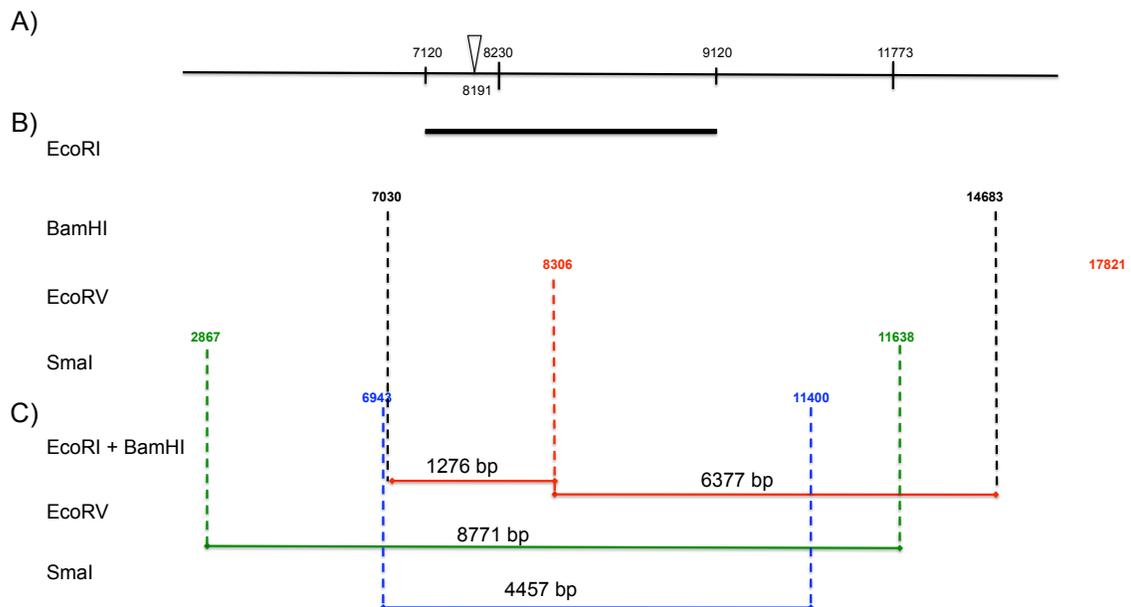
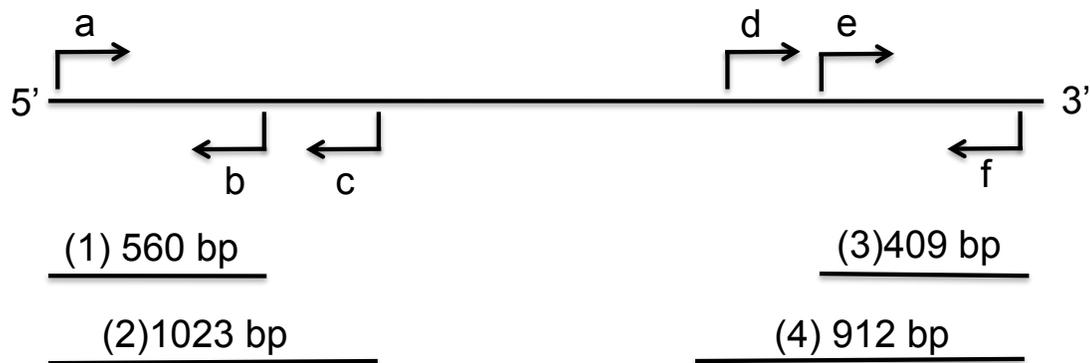


Figure A.13 RNA polymerase III subunit RpcIII128 genomic region. A) A 20 Kb genomic region including the *RpcIII128* gene was analyzed. 8230 represent the location of the transcription start site. Up side down triangle shows the location of $P\{w^{+mC} y[+mDint2]=EPgy2\}EY01866$. Solid line below the map represents the location of the probe used for Southern blot analysis. B) Enzymes used in southern blot analysis. C) Cuts expected in precise excision of the P-element.

Table A.1 Expected and observed fragments by Southern blot analysis.

Allele	Enzyme	Expected (Kb)	Observed (Kb)
RpIII128 ¹⁷	BamHI + EcoRI	1.2 + 6.3	1.2 + 6 + 8 + 2.8
RpIII128 ¹⁷	EcoRV	8.7	8.7 + 9
RpIII128 ¹⁷	SmaI	4.4	4.4 + >10
RpIII128 ⁶²	BamHI + EcoRI	1.2 + 6.3	1.2 + 6 + 1,8

A)



B)

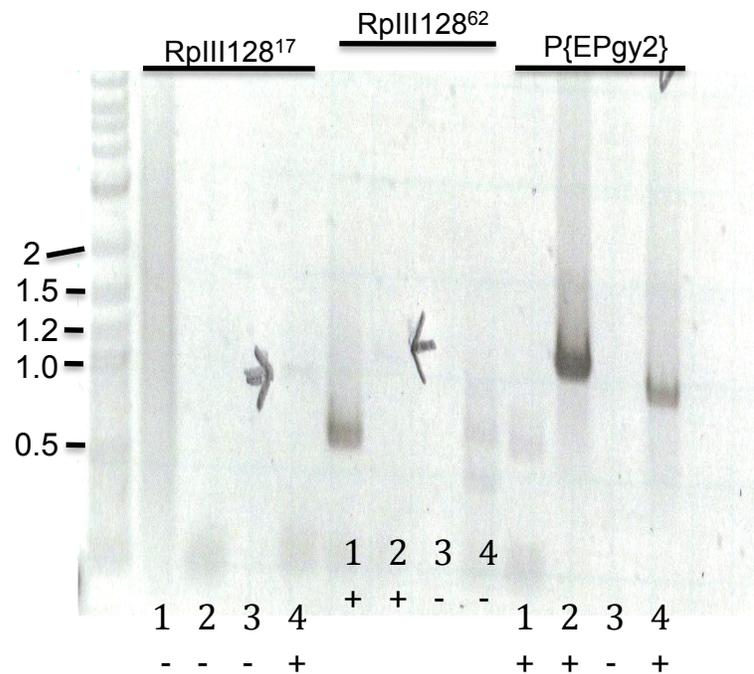


Figure A.14 Map of P{EPgy2}. A) Location of the primers used by PCR and fragment size expected. Numbers in parenthesis correspond to the lines on gel in part B. B) PCR products using primer combinations of part A in two mutant backgrounds (*RpIII128¹⁷* and *RpIII128⁶²*) and control P{EPgy2}. Arrows on gel pointed to real bands that are not that evident in the picture. + and - shows the present or absent of the PCR product.

Conclusions and Future Work

We want to understand how RNA polymerase III transcription is regulated. Recent studies have shown that factors involved in RNA polymerase II genes are also recruited to RNA polymerase III genes; however, RNA pol III transcription does not seem to respond to variegators of RNA pol II transcription. We tested mutants in Su(var)s, E(var)s, piRNA members, and an extra copy of the Y chromosome, all of these are RNA pol II variegators and none of them showed effect on 5S^{PEV} expression. Then, we tested RNA pol I variegators, CTCF mutants, but once again no effect was observed. This led us to realize that perhaps 5S^{PEV} was transcribed by RNA polymerase III. We observed an increase in 5S^{PEV} expression when this line was crossed to a 5S rDNA deficiency and to a potential RNA polymerase III mutant; therefore, our preliminary data indicated that indeed this gene was transcribed by RNA pol III. However, further studies should be performed to confirm this result.

In the short term, the RpcIII128 deficiencies location and lethality need to be precisely mapped. On the other hand, it should be confirmed whether 5S^{PEV} is not a product of RNA pol II transcription, possibly by looking at polyadenylation of the transcript. In the bobbed lethal 35S rDNA lines, only the 5S rDNA copy number was measured, I think it will be interesting to know whether 5S rDNA transcription is affected in these lines.

It was very surprising for us to observe variegation of the 5S^{PEV} which implies that 5S rDNA genes are not constantly being transcribed and possibly

only a subset of them are required for survival. Therefore, in the long term it would be very interesting to know which factors are responsible for this regulation and whether they are exclusively involved in regulating 5S rDNA, or they could also control other RNA pol III genes such as, *tRNA* genes. This could help to explain why only a percentage of these genes are transcribed in the different cell types reported by Barski and colleagues [142].

VITA

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