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Teflon (Tef) is a gene required for paired autosomes to maintain connections until Anaphase I in male *Drosophila melanogaster*. The protein contains three canonical zinc finger motifs, which are typically involved in sequence-specific DNA binding, suggesting that Tef binds DNA directly. Towards identifying Tef binding sequences, we have overexpressed Tef fused to Green Fluorescent Protein (GFP) in salivary glands, and have used anti-GFP antibodies to localize Tef-GFP to 62 different cytogenetic regions. Using duplications we mapped one of these sites to a 27 kb region on the X chromosome. Through chromatin immunoprecipitation (ChIP), we have further defined Tef binding sequences within this region. It has been proposed that Tef may function as either a transcriptional regulator or as a physical bridging complex that holds homologs together. Transcriptional analysis by quantitative real time PCR (qRT-PCR) of salivary gland and testis cDNA has revealed that Tef does not alter gene expression in the proximity of a Tef binding site. Nor did we observe any ability of Tef binding regions to enhance segregation of X duplications from their X homolog at meiosis I. Independent of the presence or absence of Tef binding sites, however, we found that a subset of X duplications segregate from an rDNA-deficient X chromosome in males. This suggests X sequences other than the rDNA have the ability to pair in male meiosis.

IDENTIFYING THE BINDING SEQUENCE OF TEFLON, A PROTEIN REQUIRED FOR AUTOSOMAL HOMOLOG CONJUNCTION DURING MEIOSIS I IN *DROSOPHILA MELANOGASTER* MALES

By

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Approved by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

For diploid organisms, sexual reproduction is dependent on meiosis to produce haploid gametes containing equal numbers of chromosomes. This is dependent on three related processes during meiosis I: homolog recognition and pairing, homolog conjunction, and separation of homologs at anaphase I. During pairing, homologous chromosomes must somehow identify their partners and align. Conjunction ensures that homologs remain attached until the metaphase-anaphase transition and is necessary to establish proper orientation of bivalents on the metaphase plate. Lastly, at anaphase the previously established connections between chromosomes are dissolved so that chromosomes can move to opposite poles of the cell prior to division. While the goal is the same from all meiotic systems—proper pairing and segregation of homologs is necessary to produce haploid gametes—various mechanisms have evolved to accomplish this task.

In many organisms, the pairing of chromosomes takes place as they undergo homologous recombination, a process that involves the reciprocal exchange of genetic material that contributes to diversity among gametes. In recombination-proficient organisms, the synaptonemal complex (SC) forms during prophase I between paired chromosomes. The SC, which is found in most meiotic systems, is a protein lattice that connects paired homologs while they recombine and may aid in the completion of

meiotic recombination (PAGE and HAWLEY 2004). Upon completion of recombination, the SC is disassembled and bivalent pairing is maintained by a combination of chiasmata—structures that assemble where reciprocal DNA exchange takes place—and sister chromatid cohesion proteins (cohesins) that prevent the resolution of crossover events until anaphase I. Before bivalents form, however, homologs must identify their partners. How this is accomplished differs greatly between organisms.

In some organisms, the gathering of telomeres on the inner nuclear envelope aids in the recognition of homologs by bringing chromosomes into close proximity with one another. This organizing structure, called a "bouquet formation," limits the nuclear space between chromosomes and allows for partner testing as well. Examples of bouquet formation in chromosome pairing during meiosis have been found in *Schizosaccharomyces pombe* (CHIKASHIGE *et al.* 1994) and *zea maize*, and mutants that disrupt bouquet formation in these organisms have decreased levels of homolog pairing (COOPER *et al.* 1998; GOLUBOVSKAYA *et al.* 2002).

In *Caenorhabditis elegans*, specialized sites along the chromosomes establish pairing. These homolog recognition sites, or pairing centers (PC), act in *cis* to stabilize homolog pairing and encourage the formation of SC between the paired chromosomes (MACQUEEN *et al.* 2005). Different zinc-finger proteins are recruited to different PCs, thereby stabilizing connections between homologs and initiating synapsis. HIM-8 is a protein that binds to a pairing center on the X chromosome (PHILLIPS *et al.* 2005), and the ZIM proteins bind to their respective pairing centers on the autosomes (PHILLIPS and DERNBURG 2006). The localizations of the ZIM/HIM-8 proteins do not overlap, and the

proteins effect pairing and synapsis only for the chromosomes that they bind. Each of these DNA-binding proteins is recruited to their binding sites by short sequence elements that are enriched at specific sites (PHILLIPS *et al.* 2009). In addition to their ability to bind DNA, the ZIM/HIM-8 family proteins establish connections between the chromosomes and the nuclear envelope by interacting with the SUN/KASH domain proteins SUN-1 and ZYG-12 during early meiotic prophase (SATO *et al.* 2009). ZYG-12 is required to localize dynein to the nuclear envelope, which then moves the attached chromosomes along the nuclear envelope with microtubule forces. Similar to bouquet formation, chromosome movements around the nuclear envelope facilitated by these interactions are thought to promote homolog recognition and synapsis.

Some organisms establish homologous pairing in a recombination-independent manner, and the mechanisms by which meiotic chromosome pairing and conjunction occurs in such cases are not completely understood. The best studied of these systems is male *Drosophila melanogaster*. Whereas recombination occurs in female *Drosophila*, it is completely absent in males. Furthermore, an achiasmate system utilized by females to segregate non-crossover chromosomes is also absent in males. Rather, it is believed that male fruit flies evolved a separate mechanism for ensuring chromosome conjunction and regulating the onset of anaphase I in the absence of chiasmata.

Males may have, in fact, evolved two systems for segregating their chromosomes: one for the autosomes and another for the sex chromosomes. It has been demonstrated that autosomal pairing and/or conjunction depends on euchromatic homology, and that heterochromatin is not sufficient for pairing. Chromosomes containing heterochromatin

from chromosome 2 and euchromatin from chromosome 3 are capable of pairing with a normal chromosome 3, yet free duplications containing only heterochromatin from chromosome 2 are unable to pair with a complete normal chromosome 2 or which each other (YAMAMOTO 1979). In addition, a Y chromosome containing chromosome 2 euchromatin is able to pair and segregate from a normal 2, while T(2,Y) translocations involving heterochromatic regions of chromosome 2 are not able to establish pairing between the Y and the normal 2 (MCKEE *et al.* 1993). The observation that any region of chromosome 2 euchromatin was able to direct pairing and segregation in this study suggests that there are multiple discrete pairing and/or conjunction sites distributed along the euchromatic arms of autosomes.

The importance of euchromatin in autosomal pairing, however, has been challenged by observations on the pairing behavior of fluorescently-marked loci during meiotic prophase. In males homozygous for any of twelve lacO array insertions at different euchromatic loci on chromosome 2, LacI-GFP signals are widely dispersed in late-prophase spermatocytes, indicating that these loci are unpaired. (VAZQUEZ *et al.* 2002). Homologs appear to enter meiosis already paired at the euchromatin during early interphase in roughly 50% of spermatogonia. During early prophase (stage S3), homologs form separate nuclear domains along the nuclear envelope. Soon after the formation of these domains, four distinct LacI-GFP signals are observed in late-prophase, indicating that at this stage both homologs and sister chromatids are unpaired. While the homologs remain in their own nuclear territories, they no longer appear to be paired at euchromatic loci. Homologs are also unpaired at their centromeres, as indicated by

monitoring the number of spots of GFP-labeled CID, a constitutive centromere protein. Vazquez *et al*. suggest that the formation of chromosome territories allows for association of homologs in the absence of physical pairing. While the mechanism of this chromosome sorting is unknown, the localization of chromosomes near the nuclear envelope (NE) suggests that chromosomes may be tethered to the nuclear periphery by physical interactions with the NE. During chromosome condensation in prometaphase, homologs are again physically associated with their partners until the metaphase-anaphase transition. At this stage, euchromatic pairing does not seem to be responsible for homolog interactions, as LacI-GFP signals are not associated as tightly as they were upon entering meiosis. These findings propose an alternative to what McKee *et al.* and Yamamoto found by suggesting that euchromatic associations are unlikely to provide the physical linkage between homologs required for the proper segregation of chromosomes in meiosis I.

Unlike autosomes, the sex chromosomes do not share euchromatic homology, and XY pairing has been demonstrated to take place at the heterochromatin. Sex chromosome pairing in male *D. melanogaster* occurs specifically at the 240 bp intergenic repeats of the ribosomal DNA (rDNA) (MCKEE *et al.* 1992). The rDNA, which contains roughly 200 genes, is embedded in the centric X heterochromatin and at the base of the short arm of the Y, is the nucleolus organizer region and is responsible for synthesis of ribosomal RNA. rDNA transgenes are sufficient to restore X-Y pairing and disjunction when inserted into an X lacking the heterochromatin (MCKEE and KARPEN 1990).

Some aspects of sex chromosome conjunction appear to be shared with autosomal conjunction, as two proteins have been identified that are required for both. Stromalin in Meiosis (SNM) and Mod(mdg4) in Meiosis (MNM) both bind the rDNA, and are necessary for the conjunction and segregation of both autosomes and sex chromosomes in meiosis I (THOMAS *et al.* 2005). Mutations in *mnm* and *snm* lead to the presence of univalents at prometaphase I and metaphase I, which causes high levels of nondisjunction of all chromosomes (THOMAS *et al.* 2005). As for paired sex chromosomes, MNM has also been demonstrated to bind to autosomal bivalents. These observations suggest that there are some similarities in the mechanisms of conjunction of both autosomes and sex chromosomes.

Other observations, however, indicate that some aspects of these mechanisms differ. Specifically, mutations in *teflon* (*tef)* disrupt autosomal conjunction but do not affect the segregation of the sex chromosomes. In *tef* mutants, autosomal homologs are unpaired and are often displaced from the metaphase plate, whereas sex chromosomes remain paired and aligned as in wildtype spermatocytes (TOMKIEL *et al.* 2001). Elucidating Tef's role in chromosome pairing may help uncover the differences and/or similarities between sex and autosomal conjunction.

The molecular characterization of Tef revealed that the protein contains three canonical C2H2 zinc finger domains, one at the amino terminus and two at the carboxyl terminus (ARYA *et al.* 2006). Zinc finger protein domains are characterized by an antiparallel β-sheet containing two cysteine residues that form a loop, and an α-helix that contains two histidine residues that comprise another loop (MILLER *et al.* 1985). These

two structural units coordinate the binding of a zinc ion, which in turn stabilizes the domain's folded structure. The α-helix loop is thought to contain residues that make contact with the major groove of the DNA double helix through hydrogen bonds with three successive bases. The presence of zinc finger domains within Tef suggests that the protein is capable of binding directly to DNA—possibly at specific sequences along the chromosomes—and that this interaction may be involved in mediating homolog associations during meiosis I. Mutational analysis has shown that the zinc finger domains at either end of Tef are required for its function (ARYA *et al.* 2006).

One model for Tef's involvement in maintaining homolog adhesion in meiosis I posits that the protein forms a physical connection between chromosomes (ARYA *et al.* 2006). The zinc fingers at one of the proteins' termini may bind directly to DNA while its other terminus interacts with either itself or another complex of proteins, most likely SNM and MNM, thus creating a bridge that holds the chromosomes together. This model is supported by the observation that MNM localization to autosomal bivalents is Tef-dependent (THOMAS *et al.* 2005). Alternatively, Tef may act as a transcription factor that regulates other genes involved in maintaining homolog connections. The identification of *cis*-acting DNA sequences recognized by Tef may help discern between these two models.

Here, we provide direct evidence that Tef can bind to chromosomes, and our studies report on the mapping of Tef-binding sequences using a cytogenetic approach combined with chromatin immunoprecipitation (ChIP). The influence of Tef on

expression of genes associated with these sites is examined, as well as the ability of these sites to direct pairing and segregation.

CHAPTER II

MATERIALS AND METHODS

Drosophila culture and stocks

Stocks were acquired from the Bloomington Stock Center (www.flybase.org). All crosses and stocks were maintained on standard cornmeal, molasses, yeast, agar medium at 25°C. The wildtype strain used was Canton-S.

Localization of Tef to Salivary gland chromosomes

Salivary glands were dissected out of third instar larvae in Phosphate Buffered Saline (PBS: 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) + 0.1% Triton X-100 and then fixed in 3.7% formaldehyde + 10% Triton X-100 for 15 – 30 seconds. Glands were then placed in 8 µL of 50% acetic acid in water + 3.7% formaldehyde on a silanized coverslip and squashed on a microscope slide. Using a needle, chromosomes were spread by zigzagging diagonally over the coverslip, followed by gentle tapping with a pencil eraser. Tissue was snap-frozen in liquid nitrogen, and the coverslip was removed. Slides were fixed in cold methanol for five minutes and washed three times in1X PBS for three minutes each. Tissues were incubated overnight at 4°C with either rabbit anti-GFP antibodies or rabbit anti-Tef antibodies diluted 1:500 in PBS + 1% Bovine Serum Albumin (BSA). Three consecutive three minute washes in 1X PBS were then repeated, and tissues were incubated for 2 hours at room temperature with goat

anti-rabbit Alexafluor 546 secondary antibodies diluted 1:500 in PBS + 1% BSA. Two ten minute washes in 1X PBS were done, followed by a one minute wash in 0.1μM 4'6-diamidino-2-phenylindole (DAPI), and finally a one minute 1X PBS wash. Slides were mounted in 50% glycerol in PBS. Antibody staining was examined by confocal microscopy using an Olympus Fluoview FV500 and image acquisition software. Alternatively, GFP signal was viewed directly in salivary glands that had been fixed one minute in methanol.

Localization of Tef to an X duplication on chromosome 3

Flies were obtained that had insertions of the X chromosome cytogenetic region 11B region into chromosome 3L at position 65B (Figure 1). These duplications were genetically introduced into flies expressing the Tef-GFP transgene by crossing males from the duplication stocks with virgin females from the Tef-GFP stock. Larvae from these crosses were heat-shocked for at least one hour, and salivary gland spreads were prepared and incubated with antibodies as described above. Antibody staining was examined as above by confocal microscopy. At least eight chromosome spreads from at least two different individuals were examined for each line.

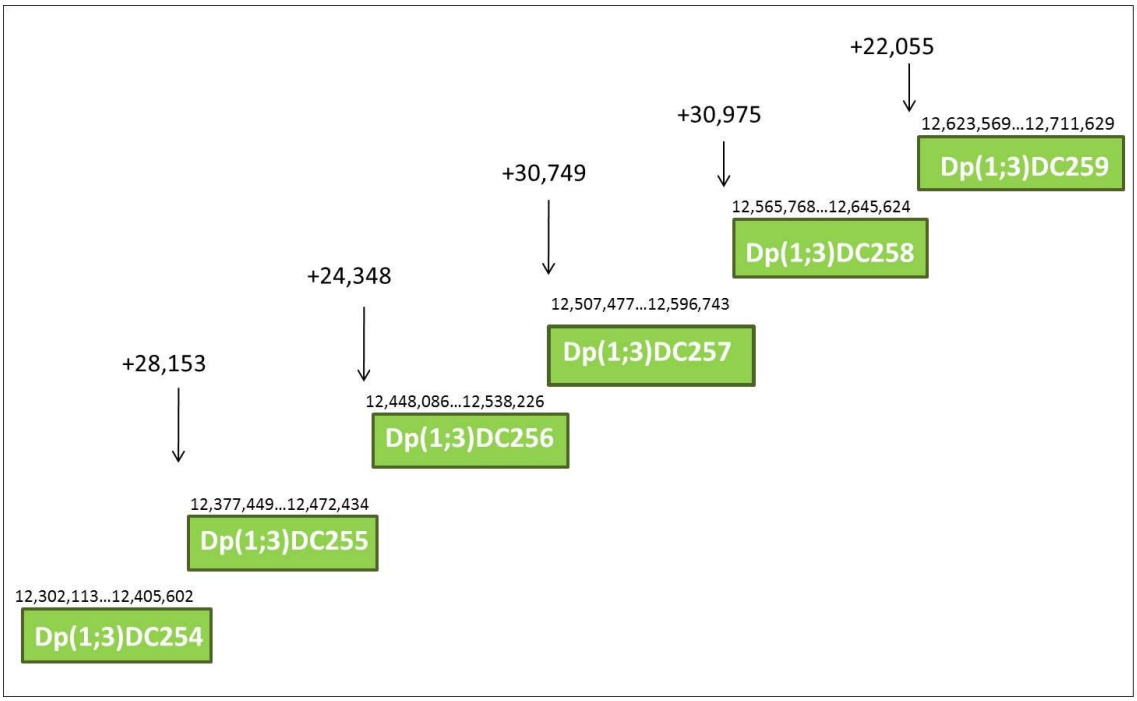

Figure 1: Duplications of 11B sequences at 65B. The X chromosome material in each duplication is indicated by the number above each box, and the number of base pairs of overlap between duplications is indicated by the numbers above the arrows.

Testing the ability of a chromosome 3 duplication on the X to segregate from a normal 3

w^{1118} females were crossed to w^{1118}; *Dp(1;3)DC257/+* males to test for segregation of the duplication away from an intact X. The duplication is marked with w^{+}, so w^{+} males and *w* females will result when the X segregates away from the 3 carrying the *Dp(1;3)*. w^{1118}; *Dp(1;3)DC257/+* females were crossed to w^{1118} to control for the effect of the duplication on viability.

Testing the ability of X duplications to segregate from an intact X in males

A collection of Y chromosomes containing duplicated regions of the X were tested for their ability to segregate away from an intact X in males bearing an X chromosome lacking the rDNA heterochromatin *(In (1)sc^{4L}sc^{8R}*). Test and control males, which contain a y^{+} Y without additional X material, were mated to 10 *ywsn; c(4)ciey* virgin females, and progeny were scored on days 13, 15, and 18 (Figure 2).

The paternal X chromosome contains a *y* allele, and the paternal Y chromosome is marked with *y+* and *w+* genes as well as the dominant allele *B^{S}*. The maternal X chromosomes are homozygous for *y*, *w*, and *sn*. Therefore, paternal nondisjunction of the sex chromosomes can be monitored by the phenotypes of the progeny, such that paternal nondisjunction events will produce *B^{S}* females (from *diplo-XY* sperm) or *ywsn* males (from *nullo-XY* sperm), and normal disjunction will produce *y* females or *snB^{S}* males (Figure 3). Comparisons of nondisjunction from Dp-bearing versus control males were made using a two by two contingency table and a chi-squared analysis.

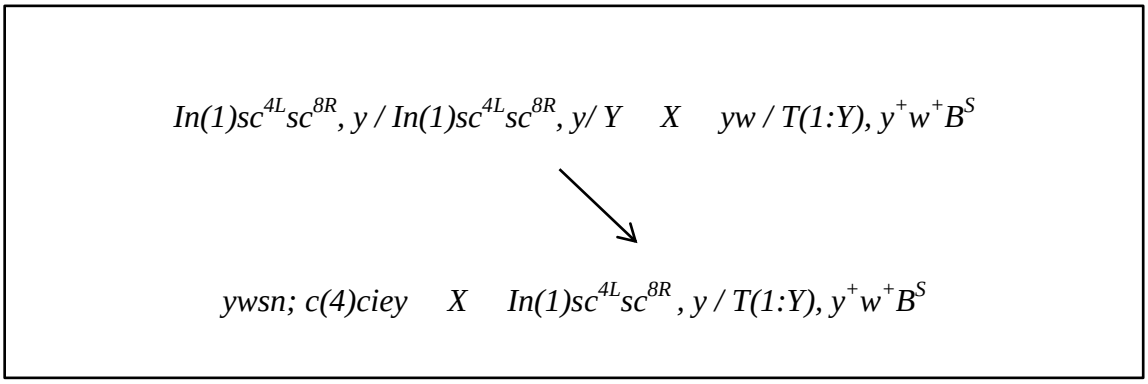

Figure 2: Crosses used to generate males to test the effects of T(1;Y)s on sex chromosome pairing and disjunction.

♀ ♂	*In(1)sc^{4L}sc^{8R}, y*	*T(1:Y),* y^{+}B^{s}w^{+}	$\frac{In(1)sc^{4L}sc^{8R}, y}{T(1:Y),\ y^{+}B^{s}w^{+}}$	0
ywsn	y ♀	sn B^{s} ♂	B^{s} ♀	y w sn ♂

Figure 3: Phenotypes of progeny of *In(1)sc^{4L}sc^{8R} , y / T(1:Y), y^{+}w^{+}B^{S}* males

RNA isolation

Salivary glands from 50 third instar larvae were dissected Schneider's Drosophila medium (GIBCO BRL, Gaithersburg, MD) after a one-hour heat shock at 37˚C. Approximately 5 µg of RNA was isolated using the RNeasy mini protocol for isolation of total RNA from animal tissues (Quiagen, Valencia, CA). Alternatively, RNA was isolated from 20-50 testis dissected out of 1-5 day old males.

First strand cDNA synthesis

To synthesize cDNA, 2 µg of total RNA was added to 10 ng/µL of each of the following reverse primers, designed to anneal to the 3' ends of *Tis11*, *Tomosyn*, *CK1alpha*, *CR33963*, and *Actin 5C*, and the volume was brought up to 40 µL in water: *Tis11* 5'AGAATGCAAGTACGGCGAGA3', *Tomosyn* 5'CCAATTTGGAGCAGCTCGGC3', *CK1alpha* 5'TGCTGAAGCAGAAGACCCAT3', CR33963 5'TTCCTTTGCACATGTGCCTG3', and *Actin 5C* 5'GTTCTTGGGAATGGAGGCTT3'. The primers were annealed to the RNA in a thermocycler at 70˚C for 10 minutes then held at 25˚C or another 10 minutes. 40 µL of the following enzyme mix was added and held at 25˚C for ten minutes: 2 µL Superscript II (Invitrogen, Carlsbad, CA) or Powerscript (Clontech, Mountain View, CA) reverse transcriptase, 8 µL DDT, 4 µL dNTPs (10 mM each), 16 µL 5x First Strand Buffer, 10 µL water. cDNA was synthesized in a thermocycler at 42˚C for 50 minutes. The reaction was terminated by incubation at 70˚C for ten minutes. The cDNA was then used as template in an RT-PCR reaction.

qRT-PCR

The following forward primers were paired with the corresponding reverse primers listed above, and used at a concentration of 2pmol/µL: *Tis11* 5'AGAACCTGGGCAACATGAAC3', *Tomosyn* 5'GTACACGAAATGCCCGAACA3', CK1alpha 5'CCGAGTTCTCCATGTATCTG3', *CR33963* 5'GCAAACAACGCACACACGTA3', and *Actin 5C*

5’AAGCTGTGCTATGTTGCCCT3’. Approximately 1µg cDNA was used as template for qRT-PCR with Power SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA), performed according to the manufacturer’s protocol. Reactions were carried out in the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Samples were held for ten minutes at 95˚C, followed by 40 cycles of 30 seconds at 95˚C, 30 seconds at 58˚C, 30 seconds at 72˚C, and fifteen seconds at 75˚C. To determine relative ratios of transcripts, the values for *tef* and *tef+* were divided by the corresponding *actin 5C* control values (SCHMITTGEN and LIVAK 2008).

ChIP

Chromatin preparation: Salivary glands were dissected out of third instar larvae in Schneider’s Drosophila medium and washed three times in PBS + 1.0% TX-100. Salivary glands were rinsed in 800 uL of fixing solution (50 mM HEPES at pH 7.6, 100 mM NaCL, 0.1 mM EDTA at pH 8, 0.5 mM EGTA at pH 8, 3.7 % formaldehyde). The cross-linking reaction was stopped by washing the glands with 1X PBS + 0.125 M glycine + 0.1% TX-100, followed by two washes in 1X PBS. Glands were resuspended in 1 mL of sonication buffer (10 mM Tris at pH 8, 1 mM EDTA at pH 8, 0.5 mM EGTA at pH 8) and sonicated for a total of 100 pulses while kept on ice. Samples were adjusted in 0.5 % sarcosyl and dialyzed overnight at 4˚C against dialysis buffer (5% glycerol, 10 mM Tris at pH 8, 1 mM EDTA at pH 8, 0.5 mM EGTA at pH 8). Chromatin was harvested from dialysis tubing and centrifuged at 13,600 RPM for 10 minutes to remove insoluble material.

Determination of Chromatin Fragment Size: A sample of sonicated chromatin was end-labeled with [α-P^{32}]dTTP using recombinant Terminal Deoxynucleotide transferase (Fisher Scientific, Hanover Park, IL). Reactions were incubated overnight at 37˚C. Radioactive chromatin was spun through sephadex G50 columns, rinsed with dialysis buffer and then RNAse A- and proteinase K- treated as described below (see: *Removal of crosslinks and DNA purification*). Samples were then phenol-chloroform extracted and DNA was separated on an agarose gel overnight. A molecular weight ladder was run next to the chromatin sample. The gel was cut into pieces 1 cm in length, and levels of radioactivity were counted for each gel segment. The majority of chromatin fragments were found to be between 180 bp and 1000 bp in length (Figure 4).

Immunoprecipitation: 5 µL of rabbit anti-Tef antibody was diluted into 200 µL of sonication buffer and 1 µL of 10% sodium azide and preabsorbed by incubation overnight at 4˚C with formaldehyde-fixed salivary glands from wildtype Canton-S flies. 100 µL of Protein A Agarose beads (Invitrogen, Carlsbad, CA) were washed two times in sonication buffer and then incubated overnight at 4˚C with the chromatin. Beads were pelleted, and 100 µL of unbound chromatin was set aside to serve as input DNA, while the rest of the chromatin was incubated with shaking overnight at 4˚C with the preabsorbed rabbit anti-Tef antibody. 100 µL of Protein A Agarose beads were washed twice with sonication buffer then added to the chromatin-antibody solution, and the mixture was incubated with shaking overnight at 4˚C. Beads, antibodies, and bound chromatin were centrifuged at 2,300 RPMs for two minutes. The supernatant was removed, and the pellet was washed three times for 30 minutes. The first wash was in 10

mM Tris (pH 8), 1 mM EDTA, 1 mM EGTA, and 0.5% NP40. Subsequent washes were done in 10 mM Tris (pH 8), 1 mM EDTA, and 1 mM EGTA. The pellet was resuspended in 400 mL in 10 mM Tris (pH 8), 1 mM EDTA, and 1 mM EGTA.

Removal of crosslinks and DNA purification: RNAse A (50 µg/mL) was added to the immunoprecipitated chromatin and to the input chromatin and incubated for 30 minutes at 37˚C. Samples were then adjusted to 0.5% SDS, 500 µg/mL Proteinase K (Life Technologies, Carlsbad, CA) and incubated overnight at 37˚C, followed by a six hour incubation at 65˚C. A phenol-chloroform extraction was performed, followed by ethanol precipitation.

qRT-PCR: 37 sets of primer pairs were used at a concentration of 2 pmol/µL (Table 1). Immunoprecipitated DNA and input DNA were used as template for qRT-PCR using the primer pairs and reaction conditions described above. The qRT-PCR data was analyzed by the comparative C_T method in which experimental chromatin was normalized to input chromatin (SCHMITTGEN and LIVAK 2008).

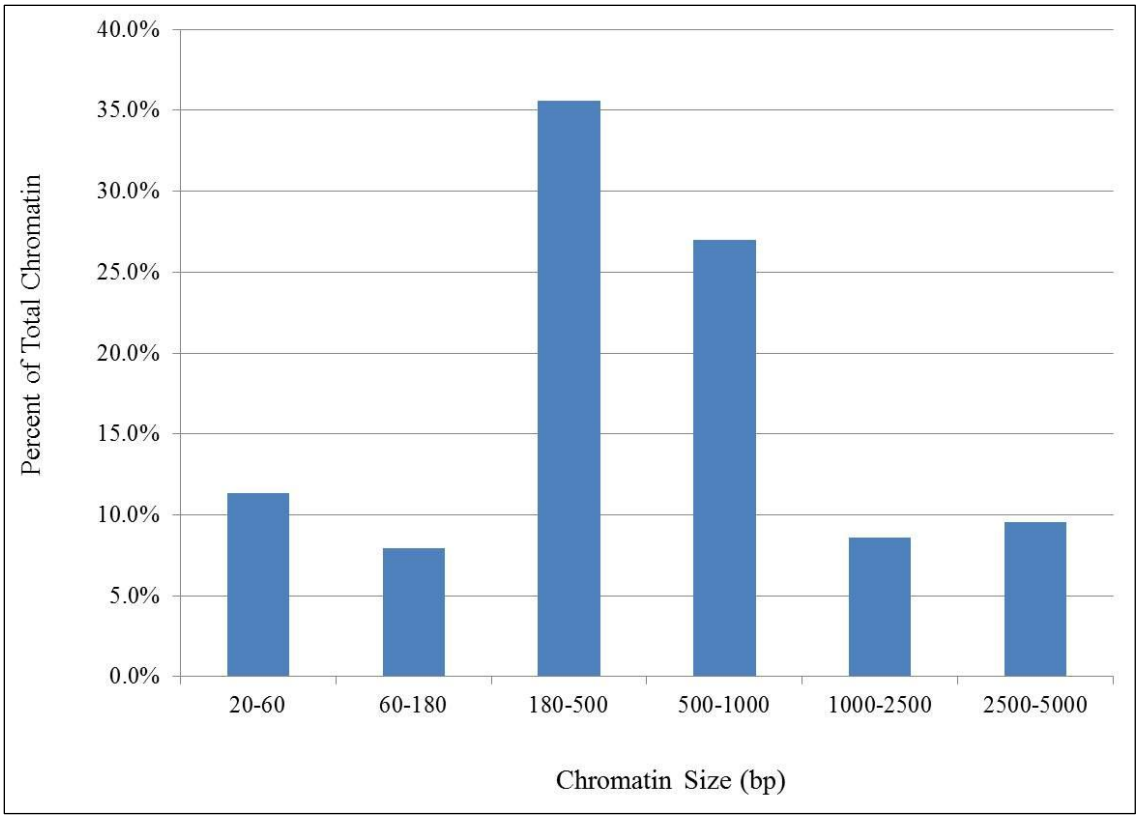

Figure 4: Size distribution of chromatin fragments generated by sonication.

Name	Sequence	Name	Sequence
F12538226	CGGCGGCCTGAATGTCAA	R12552317	AGATGATTAACGCTGCGC
R12538434	TGAGCGAAATTTGCCACG	F12552944	GAGCTGAAGTGACTGGAA
F12539048	GGTGTTCTGTGGATTCTG	R12553143	ACCTGTGCAAACTCGACT
R12539245	AGAGCGGATGTACTGCGT	F12553647	GTGCGATCCAAATCGAGT
F12539820	CATACGCACCCAACCTAA	R12553858	AGAAATTCTCGTTGCGCC
R12540046	GGTGAGTAGTGGTAGTGA	F12554391	GCACATGTGCCTGGAAAA
F12540630	GTCTCCCCAATGTTCCAA	R12554553	GAGAGCGTTTGATTAGCG
R12540860	TTGTGAGGTTGGGCAAAG	F12555156	GGTATTTGGGCTTCCAAG
F12541472	CTTGCACTGATTACCGAC	R12555366	AAATGCGGCGCAAAATGC
R12541645	GCAAGCGATCACTGTAGT	F12555816	CAGCAATAACACCACTCG
F12542276	CGCACACCAACACAGATT	R12556070	CTTAACTCATCCGAGAGC
R12542579	TGCCGTTGATCTGAGCAA	F12556523	GGCACCACAAACACTTTC
F12543168	AGCAGTCAGTGACATCGT	R2556824	ACATTCCCGAACGAAGTG
R12543385	GACTAGAGGTGCAATCTG	F12557379	CGGTATTGCGTTTCAGCA
F12543970	GTCTTTCTCTAGCCCTAG	R12557541	CGTAAAAAGTAGTCGCCG
R12544149	AGAGCGAAAGAGACAGAC	F12558000	GAGCCCATATACCCATTC
F12544609	ATCGCTGAAGAATGGCTC	R12558216	GTATATGTGCACAGTCCG
R12544785	CTAAAGTACCGCTAGGCT	F12558729	AGGATCACCGCTACTGAT
F12545417	CGCAAATCTCAGCCAGTA	R12558984	GCTTCGCACGCAAATAAC
R12545605	CGCCAGGTCTCAAACGTA	F12559555	CTCCCACACAATCATCTG
F12546066	AGTCTTCGATGTCGCCAA	R12559760	GGCACTGAGAGCAAAATC
R12546288	CTAAGCAAGAGCCATCTG	F12560336	GATGGTGCTGTCTCTGTT
F12546831	CGAAGGAGGGTTTCTTCA	R12560536	GTGTTGAACACGTGGTTG
R12547013	ACCGATCGATTCGGTTTC	F12561001	CCGCAATATCATCCCTTC
F12547651	CATAACAGCAGCACACGA	R12561216	TCAACAGCTTCGAAGGCA
R12547842	CACCTGATATCGTGGAAC	F12561867	GGCCTGCACTTGGTTTAA
F12548446	TGTAAGGTGCAACTGCAG	R12562050	TCTCTGCTTATCAGTCGG
R12548653	GGGCTGAAGGGAAATTCT	F12562668	GTTCTCGGTTAGCAGCTA
F12549270	GATGGACAAGATGCGGAT	R12562844	CTCCGATTGTTTTGGCGT
R12549437	GGTAAATGTCGCCAAACG	F12563338	GTATTGCGTGAGGGCAAA
F12549957	CGCTTGGATACGTGATGA	R12563600	CCCCATGTTTGACCACAT
R12550163	TGTAATCTGGCTGCTCCT	F12564150	GACGGAACTCTCTTGGAT
F12550746	CAAGCAACGGCATCATCA	R12564341	CAGAGGTTAACTGCAAGC
R12550918	TGTGTGGGTGTGTTATGC	F12564727	CGTCGTTTATCCACCAAC
F12551419	CAAAGCCAACTGAGATGG	R12564913	CATCCAAGCCATCGATTG
R12551650	ATGCAGATGTGAACAGCG	F12565586	GTTCGATTCGCAGGCGTT
F12552157	GCATAGCTGGTAGTTAGC	R12565783	TCTAGAACAGACGATCCC

Table 1: Primer pairs (5’ to 3’) used in qRT-PCR for ChIP analysis. Primers amplify approximately 200 bp fragments spanning the 27,542 bp Tef binding region on 11B.

CHAPTER III

RESULTS

A Tef-GFP fusion protein expressed in salivary glands binds to chromosomes

Tef was localized to salivary gland chromosomes in flies overexpressing a heat shock-inducible Tef-GFP fusion protein. GFP signal was detected on salivary gland chromosomes using anti-GFP antibodies, and was shown to overlap with the signal obtained using anti-Tef antibodies by indirect immunofluorescence (IIF). No signal was observed on similarly stained chromosomes from flies lacking the *tef-gfp* transgene. This demonstrates that the anti-Tef antibodies are specific for the overexpressed Tef-GFP protein in this tissue (Figure 5). GFP antibodies were then used to localize Tef-GFP binding sites on salivary gland chromosomes that had been fixed and spread. Following antibody hybridization, examination by confocal microscopy revealed that Tef binds to specific regions on salivary gland polytene chromosomes (Figure 6). Sixty-two specific Tef binding sites were identified on polytenes (Table 2). On the X chromosome, a particularly strong and consistent Tef signal was found at cytogenetic interval 11B, which spans a region of ~ 200,400 bp, suggesting that this region contains one or more Tef binding sequence.

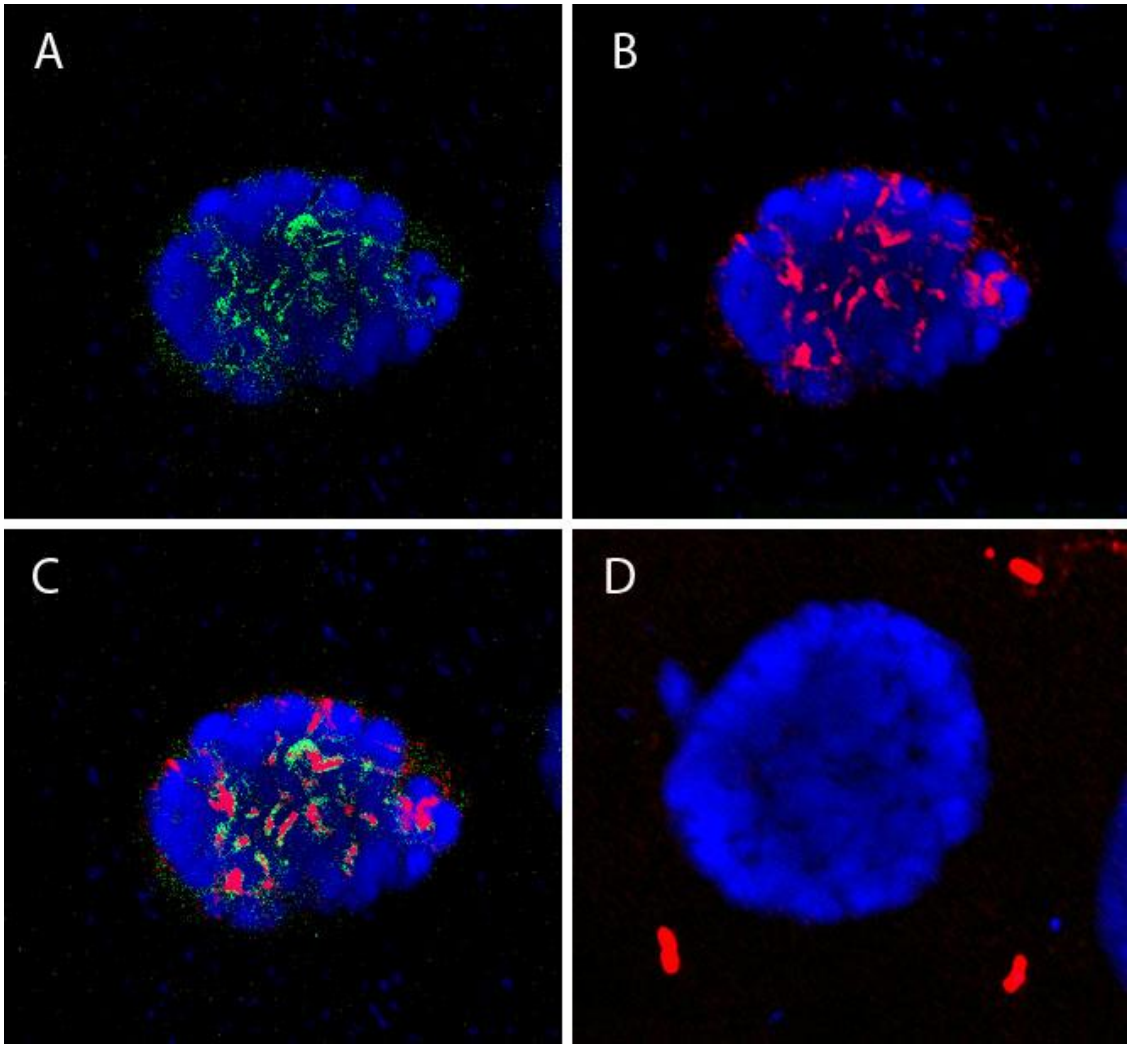

Figure 5. A Tef-GFP fusion protein overexpressed in salivary glands binds to salivary gland chromosomes. A. GFP signal. B. Anti-Tef C. Overlay of GFP and anti- Tef signals. D. Anti-Tef antibody staining of Canton S flies lacking the *tef-gfp* transgene.

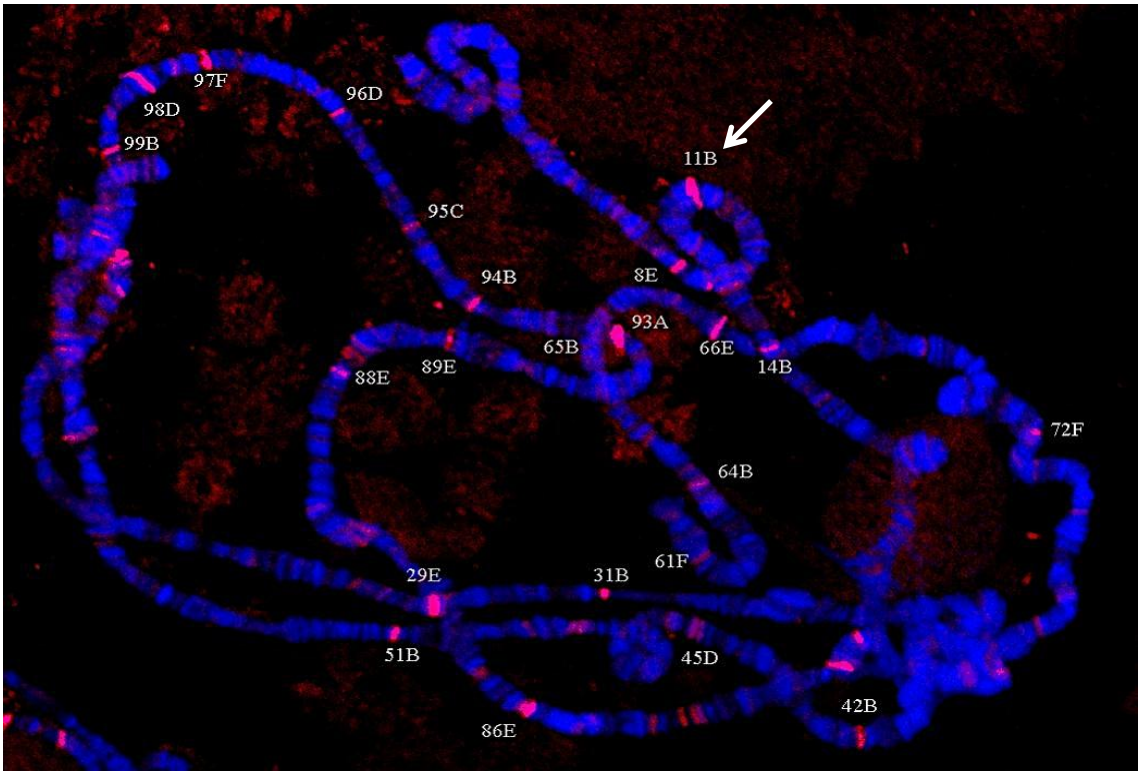

Figure 6. Localization of a Tef-GFP fusion protein to salivary gland polytene chromosomes. Anti-GFP hybridization is shown in red. The arrow shows 11B on the X chromosome, where anti-GFP signal was consistently seen on salivary gland chromosome squashes.

Chromosome Arm	Cytolocation
X	2C
	8E
	9A
	11B
	13B
	13D
	14B
	18D
2L	21E
	22A
	29E
	31B
	38D
	38F
	39F
	39B
	39E
2R	42B
	44B
	45D
	46A
	47D
	49B
	49F
	51B
	56F
	57C
	57F
	59C
	59D
	60C

Chromosome Arm	Cytolocation
2R	60E
	60D
	60F
	60A
3L	61B
	61F
	63A
	64B
	64C
	66E
	72F
	74A
	77D
	78E
3R	84F
	86A
	86E
	87D
	88E
	89A
	89E
	90F
	93A
	94B
	95C
	96D
	97F
	98A
	98B
	98D
	99B

Table 2: Location of Tef-GFP binding sites on salivary gland chromosomes. Shaded boxes represent locations where Tef-GFP binding was observed at least twice.

Tef-GFP binds within a 27,542 bp region of the X chromosome duplicated onto chromosome 3

To map Tef binding site(s) within 11B, a collection of fly lines were obtained that had insertions of overlapping duplications of sub-regions of 11B region on chromosome 3L at cytogenetic region 65B (Figure 1). Tef-GFP localization was not observed at 65B in our previous localizations. Each duplication was genetically introduced into flies bearing the Tef-GFP transgene. Larvae were heat-shocked for at least one hour to induce Tef-GFP expression and salivary glands were then dissected out and fixed. Chromosomes spreads were made, and IIF and confocal microscopy were then used to visualize the binding of Tef-GFP to region 65B. Anti-GFP hybridization at 65B was observed in only one of the six lines containing 11B duplications, (Dp(1;3)DC257), suggesting that one or more Tef binding sites is/are located somewhere within this duplication (Figure 7). As there is overlap between this region and the two adjacent duplicated regions—neither of which showed anti-GFP hybridization at 65B—the region of interest was narrowed down to 27,542 base pairs unique to Dp(1;3)DC257.

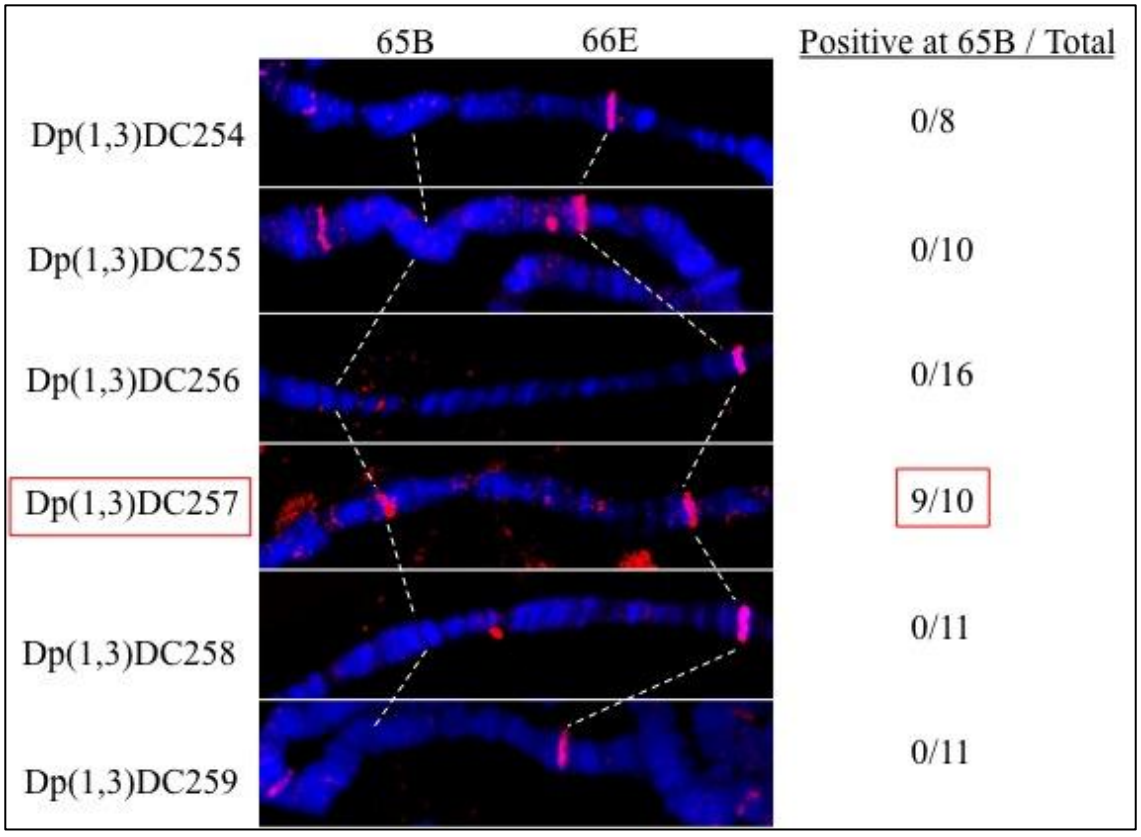

Figure 7. The Tef-GFP fusion protein binds to position 65B on chromosome 3 only when Dp(1;3)DC257 is inserted at that region. Anti-GFP signal was localized to position 65B for this line in nine out of the ten salivary gland chromosome squashes viewed, whereas none of the other lines showed anti-GFP signal at this region. Position 66E is a region with consistently strong anti-GFP hybridization and is shown as a positive control.

Mapping the 11B Tef binding site by ChIP

ChIP was performed to further define the Tef binding site within the 27, 542 11B sequence. Salivary glands were dissected out of flies overexpressing the Tef-GFP fusion protein, were fixed with formaldehyde to crosslink proteins associated with the DNA, and the chromatin was sheared by sonication. The average fragment size obtained was between 500-1000 bp in size (Figure 4). This chromatin was incubated with rabbit anti-Tef antibodies which had been preabsorbed to wildtype salivary glands, then immunoprecipitated with Protein A Agarose beads. The precipitated immune complexes were purified and the protein-DNA crosslinks removed. The resulting chromatin preparation was then used as template for qRT-PCR. Primers were designed to span the 27,542 bp region on the X that Tef binds (Table 1). These primers amplified roughly 200 bp fragments spaced approximately 500 bp apart. We expected Tef-binding sites within the 27,542 bp region to be preferentially precipitated, and enriched in qRT-PCR relative to input chromatin.

ChIP revealed five fragments that contain putative Tef-binding sites (Figure 8). Fragment 18, which is amplified by primers F12551419 and R12551650, showed over a five-fold enrichment, suggesting that Tef may bind somewhere within the 2000 bp region surrounding this primer set. In addition, fragments 2, 29, 34, and 37 were enriched in qRT-PCR relative to the input chromatin, although to a lesser extent.

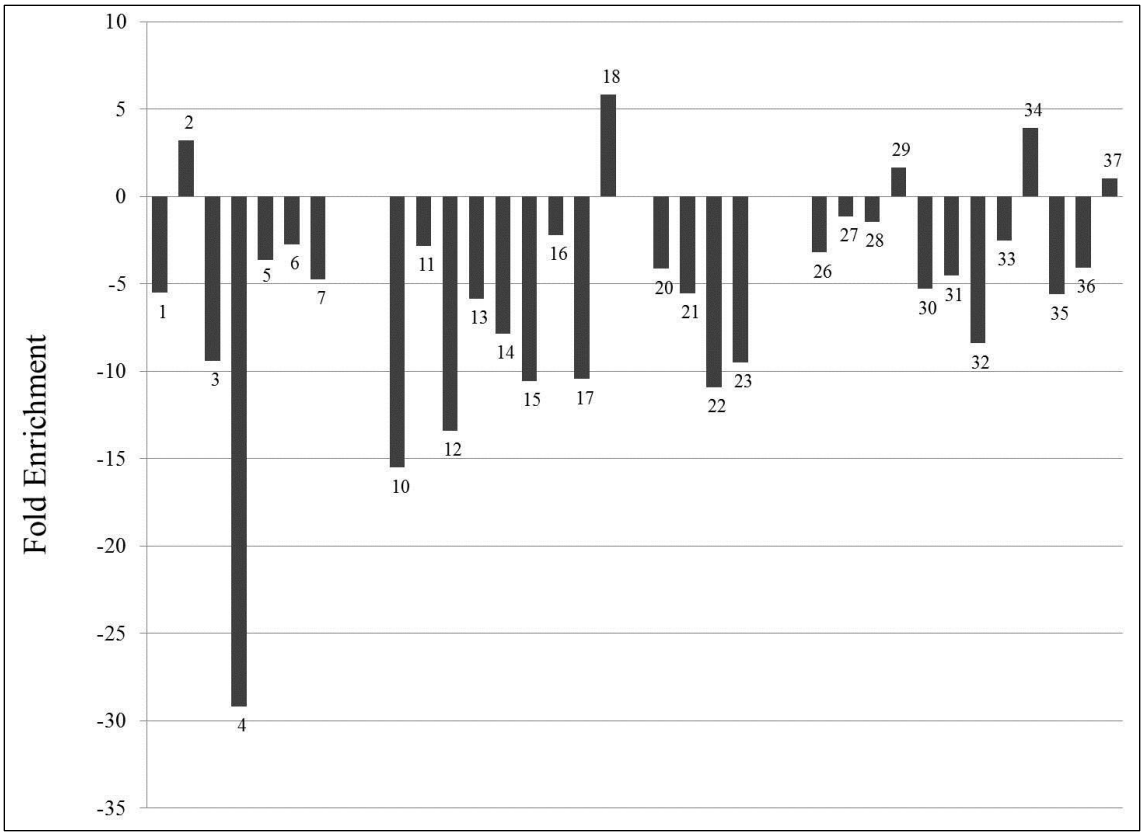

Figure 8. Fold enrichment of DNA sequences recovered from anti-Tef ChIP relative to input chromatin. Fragments are shown in distal to proximal order spanning the 11B Tef binding region. Five fragments showed significant enrichment (2, 18, 29, 34, and 37) suggesting the presence of Tef-binding sequences in or adjacent to these fragments.

Tef does not alter the transcript levels of genes at 11B on the X chromosome

One of the proposed models for Tef's involvement in meiosis I is that it regulates genes needed for homolog conjunction or pairing (ARYA *et al.* 2006). Four genes lie within the 11B X chromosome duplication that binds Tef: *Tis11, Tomosyn, CK1alpha,* and *CR33963* (Figure 9). None of these are known to play a role in male meiosis, but this possibility has not been ruled out. Tis11 is a DNA binding protein that may be a component of the RNAi pathway in *Drosophila* (DORNER *et al.* 2006). *Tomosyn* was

identified in a genetic screen in *Drosophila* for homologs of mammalian genes associated with exocytosis of synaptic vesicles (LLOYD *et al.* 2000). *CKIalpha* encodes a protein kinase that regulates the Wnt receptor signaling pathway (POLAKIS 2002). Finally, *CR33963* is a non-protein coding gene with unknown function. We wanted to examine whether or not Tef altered the transcription of any of these four genes.

To explore this idea, cDNA was made from salivary gland RNA isolated from both w^{1118} flies and flies carrying the Tef::GFP transgene. qRT-PCR was then used to look for altered expressions of four genes that lie within the known Tef binding site on the X in both tissues types: *tis11, tomosyn, CKIalpha,* and *CR33963*. There was no meaningful change in gene expression between salivary gland cells that do not express Tef and those that overexpress the Tef-GFP fusion protein. To confirm these results in tissue where Tef ordinarily functions, qRT-PCR was performed using testis cDNA isolated from flies carrying four different *tef* alleles, as well as cDNA from *wild type* flies bearing the progenitor *cn bw* chromosome on which these *tef* alleles were induced (Figure 10). The four *tef* alleles act as null alleles with respect to their effects on autosome segregation in meiosis, and each is predicted to encode a nonfunctional protein. We failed to detect a consistent effect of *tef* on gene expression for any of the potential target genes (Figure 11).

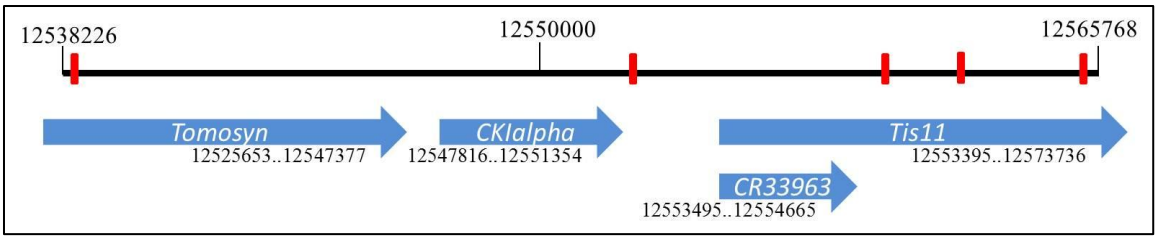

Figure 9: Location of the Tef binding sites relative to genes in 11B. Genes are shown as blue arrows, and the exact location of the genes on the X are indicated below. Red hatch-marks indicate Tef binding sites determined by ChIP.

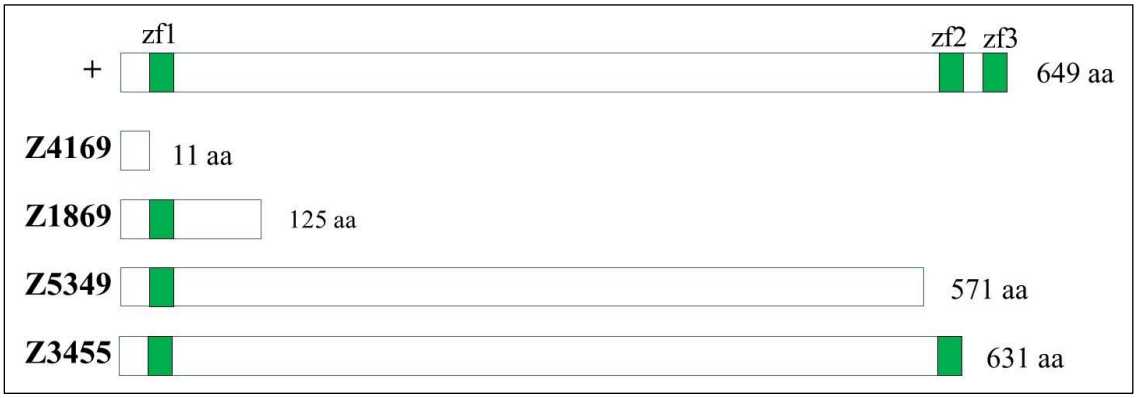

Figure 10: Predicted proteins encoded by the *tef* alleles used for qRT-PCR analysis. Wildtype (+) Tef is 649 amino acids in length and contains three zinc finger domains, shown as green boxes.

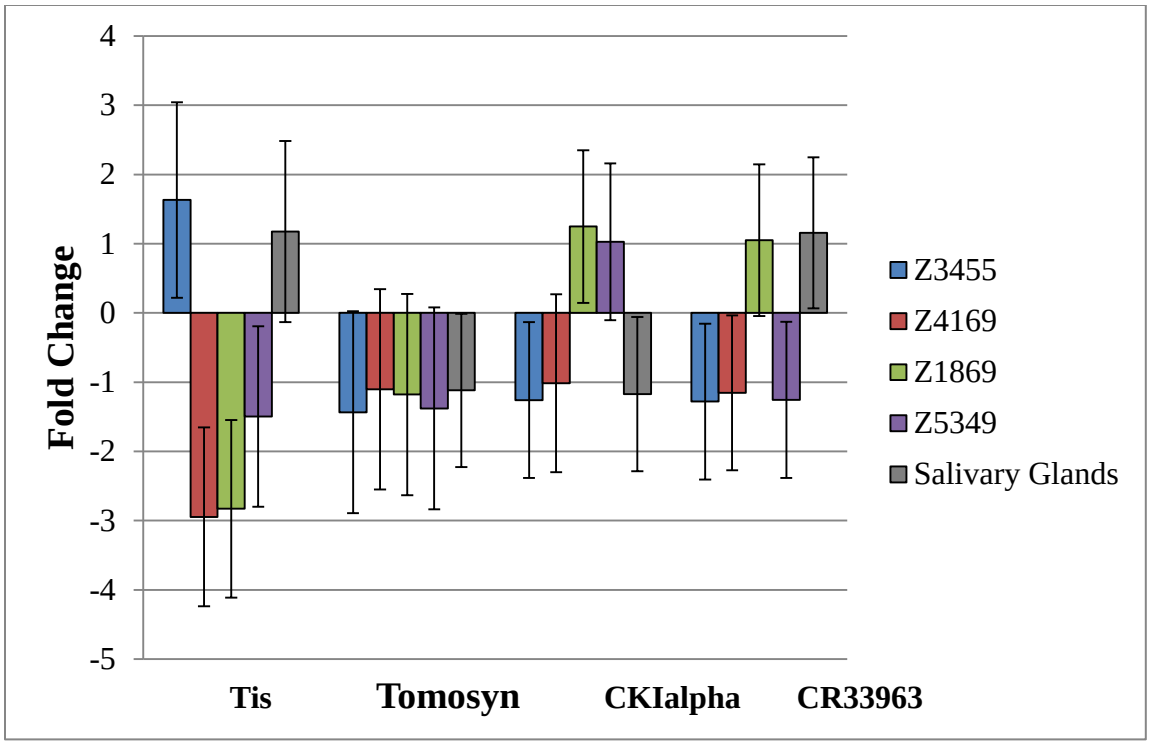

Figure 11: No meaningful changes in expression of *Tis11, Tomosyn, CK1alpha,* and *CR33963* are observed in testis isolated from four different *tef* mutants or in salivary glands overexpressing Tef. The fold change for each gene is shown for each of the *tef* alleles and for salivary gland cDNA. Error bars represent standard deviation.

An X duplication on chromosome 3 does not direct segregation of the X and the 3

A second model for Tef's involvement in meiosis I is that the protein maintains pairing between autosomes by physically binding them together as part of a conjunction complex (ARYA *et al.* 2006). Given that *tef* mutations do not affect sex chromosome pairing, it was somewhat surprising to find Tef binding sites on the X chromosome. Our results raised the possibility that Tef might be able to facilitate conjunction between X chromosomes. As males are normally hemizygous for the X, there is not normally an opportunity to observe X-X interactions in this sex. To test this idea, we obtained flies that carried a duplication of the 27,542 11B sequence on chromosome 3. If Tef's role in

meiosis I is to physically join homologs together, perhaps the presence of the X duplication containing a Tef binding site on chromosome 3 would be sufficient to establish novel pairing between the X and the 3. To test this, w^{1118} males heterozygous for Dp(1,3)DC257—which contains a w^{+} marker—were crossed to w^{1118} females. As pairing in male meiosis has been shown to be non-competitive (McKee *et al.* 1993), we expected that if chromosome 3 is also able to pair with a piece of the X euchromatin and direct segregation, then the Y and the 3 carrying the X duplication will preferentially segregate together, producing w^{+} males and *w* females.

To ensure that the introduction of X euchromatin onto chromosome 3 did not result in a decrease in viability, control crosses were set up in which females carrying the duplication were crossed to w^{1118} males. No viability differences were observed between control and experimental crosses, as *w* females and w^{+} males were recovered at close to the same ratio (Table 3). Experimental crosses revealed that X segregation from the 3 occurs 49.7% of the time, indicating that segregation is random. This suggests that segregation is not being directed by the X duplication on the 3, and that this shared homology is not sufficient for pairing.

F1 Phenotype	*w* ♂	*w* ♀	w^+ ♂	w^+ ♀	X ↔ Dp
Sperm Genotype	Y; 3	X; 3	Y; Dp(1;3)	X; Dp(1;3)	-----
Experimental	1547	1487	1729	1702	0.497
Control	1061	1105	1148	1099	0.510

Table 3: Segregation of the X from Dp(1;3)DC257. X ↔ Dp is calculated as (w ♀ + w^+ ♂) / (w ♂ + w^+ ♀).

X duplications onto the Y are capable of segregating from an intact X lacking rDNA repeats in males

Our results have revealed that Tef binding sites exist on the X chromosome. If Tef is only involved in the segregation of autosomes, it is unknown why Tef binding sites would be conserved on the X. One model for Tef function is that it acts as a transcription factor. However, our transcriptional analysis of genes on the X suggests failed to support this model. The alternative model—that Tef physically holds homologs together during meiosis I in males—was not supported by segregation tests using X material duplicated on chromosome 3. In these latter tests, however, the amount of homology was limited, and perhaps was not great enough to influence segregation patterns. To further examine the role of Tef binding sequences in homolog pairing and conjunction, we examined a collection of T(1;Y) translocations, in which larger fragments of the X were inserted into the Y chromosome.

Normally, males do not possess two X chromosomes, so in order to determine whether or not X euchromatin is able to pair with itself, a collection of Y chromosomes

containing duplicated regions of the X (COOK *et al.* 2010) were tested for their ability to segregate away from an intact X. We chose an X homolog that lacked the rDNA, a heterochromatic region that has been shown to be necessary for the proper pairing and segregation of the sex chromosomes (MCKEE and KARPEN 1990). Thus, any segregation of the T(1;Y) form the X could be attributed to pairing between the X and the duplicated X material on the Y. All of the X duplications examined were derived from the same attached XY chromosome and are located at the same position on the Y. Therefore, the only difference between each T(1,Y) is the piece of the X euchromatin it contains. We also wanted to examine whether or not the ability to direct segregation was dependent on the presence of a Tef-binding site. These duplications span various regions of the X chromosome and are of varying size, which allowed us to test if different regions of the X euchromatin are sufficient to direct segregation (Figure 12). Two of these duplications contain Tef-binding sites, while four of them do not. The *In(1)sc*^{4L}*sc*^{8R}*, y* X chromosome used is lacking almost all of the X heterochromatin, which abolishes normal pairing between the X and the Y due to the absence of rDNA repeats. If the duplication of the X on the Y reestablishes segregation, then the incidence of sex chromosome nondisjunction from progeny carrying the duplication should decrease relative to the progeny of control males carrying a normal y^+ Y and an X lacking rDNA.

To eliminate the influence of viability differences of progeny bearing the different T(1;Y)s, only progeny classes that were genotypically identical from all crosses were considered for this comparison. The numbers of progeny produced from nullo-XY sperm were divided by the sum of the progeny produced from nullo-XY sperm plus X sperm.

These results are confounded by meiotic drive, which skews the recovery of sperm classes according to chromatin content. In this case, the recovery of X sperm is decreased relative to nullo-XY sperm. As the ratio of nullo-XY/(X + nullo-XY) would be similarly decreased by a decrease in meiotic drive or nondisjunction, it is impossible to discern between these two possibilities. However, because drive is decreased when XY pairing is increased, (MCKEE and LINDSLEY 1987), our results likely reflect pairing of the X chromosome duplications with the intact X.

Among the progeny of control males, the incidence of nondisjunction is 0.41. This is the ratio obtained when the sex chromosomes do not pair (Table 4). However, the incidence of sex chromosome nondisjunction is lowered among progeny of males bearing any one of five X duplications tested. A sixth duplication failed to alter nondisjunction. This duplication, from line 29736, is the most proximal T(1,Y) tested. While most of the duplications had effects on the segregation of chromosomes, these effects were not equal. For example, while the duplication contained in line 29791 was able to significantly decrease the incidence of nondisjunction, this duplication was still not as effective at directing segregation as those more distal to it.

There was not a strong correlation between the presence of Tef binding sites on the ability of duplications to decrease the incidence of nondisjunction. Our results show a Tef binding site at 8E, which is found within line 32128. In addition, line 32529 contains the 14B Tef binding site. None of the other duplication lines that decrease the incidence of nondisjunction, however, contain Tef binding sites. Furthermore, the ability of the duplications to pair with the X does not seem to correlate with the amount of

sequence homology ($R^2 = 0.0346$) (Figure 13). The size of the duplications able to significantly decrease the incidence of nondisjunction ranges from approximately 870,852 bp to 1,513,945 bp in length, while the duplication from line 29736, which was unable to significantly direct segregation of the chromosomes, is 1,092,550 bp in length.

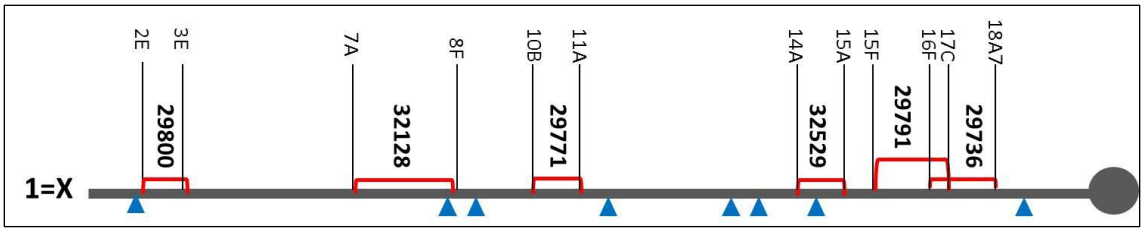

Figure 12: Cytological locations on the X of the T(1,Y) duplications used for segregation studies. Line numbers are indicated, as are the cytological locations on the X. Progeny from lines 29800, 32128, 29771, 32529, and 29791 significantly decrease the incidence of nondisjunction relative progeny from y^+ Y controls. Blue triangles indicate sites where Tef-GFP binds in salivary gland chromosomes.

		Sperm Class					
Line	**X duplications**	**X**	**Y**	**XY**	**0**	**X0/ (X0+XX)**	***p***
29800	2E1-2E2;3E4	107	23	0	9	0.08	<.001
32128*	7A3-7B1;8F9	195	33	0	23	0.11	<.001
29771	10B3;11A1	93	9	0	13	0.12	<.001
32529*	14A1-14A5;15A8	65	2	1	4	0.06	<.001
29791	15F4-15F9;17C1	164	10	1	65	0.28	<.001
29736	16F6-17A1;18A7	40	1	1	21	0.34	>.3
y+ control	------	266	124	11	187	0.41	---

Table 4: Nondisjunction incidences among progeny from T(1,Y) x *In(1)sc*^{4L}*sc*^{8R} crosses. Each line number is specified along with the cytogenetic limits of the X chromosome duplications. XO/(XO+XX) is a measure of nondisjunction that eliminates viability differences of the various T(1;Y) chromosomes. Asterisks indicate duplications that contain Tef binding sites.

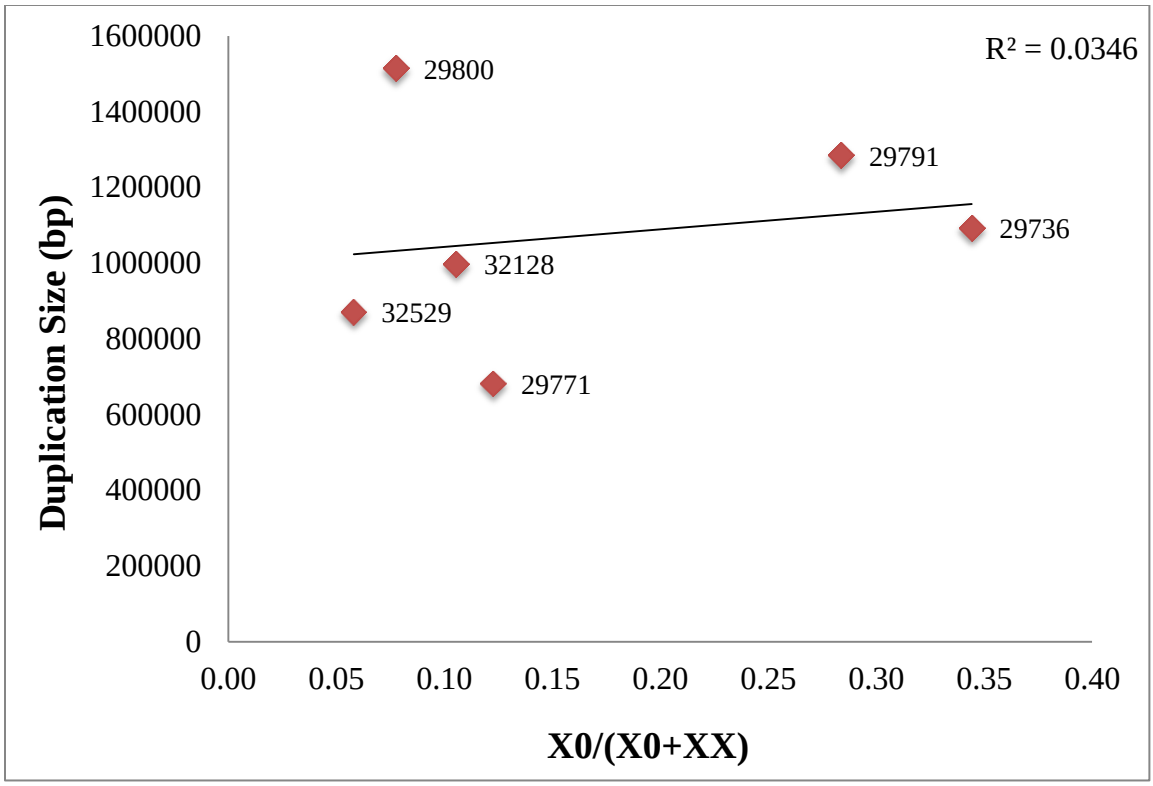

Figure 13: T(1;Y) size versus the incidence of nondisjunction. The size of X duplications on the Y do not correlate with their ability to direct segregation of the X and Y.

CHAPTER IV

DISCUSSION

Using *in situ* hybridization to Tef-GFP on polytene chromosome spreads, we were able to identify 62 different sites that the Tef-GFP protein binds. Thirty-six of these sites had strong signals in almost all spreads, whereas others were weaker or less consistently detected (Table 2). These differences could be due to some regions having more Tef binding sites than others, which could contribute to differences in GFP-hybridization intensities. Alternatively, cell-to-cell or larvae-to-larvae variations in protein expression may account for detection differences, as increased protein abundance may have resulted in binding to lower affinity sites. The 11B polytene chromosome band on the X chromosome showed consistently strong hybridization with the GFP antibody, and through use of a series of duplications of this region placed at a novel site on chromosome 3, we were able to refine the mapping of Tef-GFP binding sites to a 27,542 bp region on the X.

Salivary glands were a useful tool because they contain polytene chromosomes, which are generated by successive rounds of endomitosis, that is, DNA replication without cell division. Each polytene chromosome has a characteristic light and dark banding pattern, a feature that can be used to identify where on the chromosome proteins are bound. While this makes cytology easier, there are, of course, limitations to this experiment.

One caveat to these results is that Tef is a meiotic protein that functions in testis, and here we have mapped binding sites in a somatic tissue that may not express proteins that determine binding specificity. Thus, it is possible that the localization of Tef to salivary gland polytene chromosomes differs from its localization to chromosomes in the testis. The Tef binding sites defined in salivary glands have provided us with potential targets, but not necessarily all of these will be bound *in vivo* in meiosis. We cannot rule out the possibility that Tef binds only a subset of these sites in meiotic tissue, where its chromosome localization may be refined by the presence of other factors such as other proteins or chromatin conformation. Zinc fingers do, however, have specificity for DNA sequences, and their binding sites are routinely defined *in vitro* by gel-shift or filter binding assays. Our assay has the advantage over many of these techniques in that through cytological observations, we are able to detect binding *in vivo.*

To further map the Tef binding site within the 11B region on the X, ChIP was performed using anti-Tef antibodies on chromatin from salivary glands of flies overexpressing a Tef-GFP fusion protein. Five different regions were preferentially immunoprecipitated, suggesting that there may be multiple Tef binding sites within 11B. This would be consistent with the observation that the anti-GFP signal at 11B is relatively robust. In particular, fragment 18 was enriched almost five fold relative to the input chromatin, and will be the focus of future experiments conducted to further define a specific Tef binding sequence. It will be important to first verify these results by an independent method, as the anti-Tef antibody could have some cross-reactivities to other antigens which were not removed by preabsorption. Repeating the ChIP with anti-GFP

antibodies, for example, would be useful to see if the same fragments are enriched. It will also be important to repeat this experiment in the future using chromatin isolated from testis to determine which of these sites are relevant to Tef's meiotic function.

To verify the ChIP results, we performed a BLAST analysis to search for sequence homology between five cytogenetic intervals that showed consistently strong Tef binding sites (11B, 21E, 29E, 31B, and 66E). This analysis identified TATATG as a sequence that was present in all five regions. Comparison to the roughly 2000 bp of sequence surrounding each ChIP-enriched fragment revealed that this sequence is also present in fragments 18 and 29, making this sequence a strong candidate for the Tef binding sequence. Although there is no definitive code for C2H2 zinc finger binding sites, a computer algorithm for predicting zinc finger protein binding sites also identified TATATG as a potential binding site for Tef (PERSIKOV *et al.* 2009).

One way to confirm this sequence or to identify the true Tef binding sequence would be to create smaller primer sets that span these fragments and again perform a ChIP experiment to see if any smaller sequences are enriched within these regions. In addition, an electromobility shift assay could be performed using cell extracts expressing Tef. Another way to further define the Tef binding site would be to create new transgenes containing different segments of the putative Tef binding region in tandem arrays and insert them into a novel site in the genome. Our IIF assay could then be used to confirm Tef binding. Although we cannot presently detect Tef in meiotic cells, such tandem arrays might result in a concentration of bound Tef that would be detectable in meiosis

The finding that Tef binds to the X chromosome was not predicted. Tef has thus far only been implicated in the pairing or conjunction of autosomes, as *tef* mutations do not affect sex chromosome segregation (TOMKIEL *et al.* 2001). There must be some reason why Tef binding sites on the X are conserved, however. One possibility is that Tef regulates the transcription of genes on the X needed for proper chromosome pairing and segregation of the autosomes. To explore this idea, we wanted to look at changes in gene expression in salivary glands from flies overexpressing Tef-GFP relative to flies that do not express the protein. qRT-PCR was performed to look for altered expressions of the four genes—*tis11 , tomosyn, CKIalpha,* and *CR33963*—that lie within the 11B Tef-binding region. No meaningful changes in gene expression were found between salivary gland cells that do not express Tef and those that overexpress the Tef-GFP fusion protein.

This analysis was extended to testis, and qRT-PCR was performed using cDNA from either wildtype flies or flies carrying one of four different *tef* alleles, all of which function as null alleles (ARYA *et al.* 2006). While two of the alleles, Z4169 and Z1869, showed a nearly three-fold decrease in expression of *Tis11,* it is unlikely that the changes in gene expression are significant. We would expect all alleles to exhibit the same effect on transcription. Instead, these findings could possibly be the result of differences in the genetic background of the chromosomes carrying each allele. It thus seems most likely that Tef does not play a role in regulating genes in the 11B region of the X chromosome.

Since Tef binding sites were found on the X, we wondered if Tef could facilitate pairing of X sequences if two copies were present in males. This was examined by asking if an X duplication on chromosome 3 could direct segregation of the X and from

the 3. To do this, we utilized the 11B X duplication on chromosome 3 that contains the 27,542 bp Tef binding region (Dp(1;3)DC257). We expected that if the X was able to pair with its euchromatic duplication on chromosome 3, then this 3 would segregate away from the X. This was not found to be the case, however: we found no evidence for segregation of the X from the 11B duplication, suggesting that this piece of X euchromatin on an autosome is not sufficient for pairing and/or directing segregation. Because of the small size of this duplication (89,266 bp) there simply may not have been sufficient homology to cause observable pairing between the X and chromosome 3. This notion is supported by previous observations on the ability of chromosome 2 duplications to pair with an intact 2.

It has been shown that euchromatic 2-Y transpositions can cause quadrivalent formation between the X, normal 2, Df(2,Y), and Dp(2,Y) during prophase 1 (McKee *et al.* 1993), indicating that a normal 2 can pair with its euchromatic homology translocated onto the Y. In addition, the frequency of quadrivalent formation is directly proportional to the length of the transposition, such that the greater the length of the homology, the more likely that it will pair. For example, a translocation containing 961 polytene bands formed a quadrivalent 100% of the time, demonstrating that the normal chromosome 2 always pairs with its duplicated homology on the Y. Quadrivalent formation was only observed 17% of the time, however, when a translocation containing only three polytene bands was placed on the Y (McKee *et al.* 1993). Thus, it is not too surprising that our Dp(1;3) containing less than a single polytene band would not be capable of pairing back with the normal X in meiosis I. Unfortunately, testing larger X fragments for pairing

ability in males is limited by their effects on sex determination; diploid males carrying large X duplications develop intersexual characteristics (DOBZHANSKY 1934). It might be useful to examine the pairing of the X and Dp(1;3) cytologically, as we only inferred the pairing of these chromosomes from their segregation. It is possible that the X duplication on the third chromosome could be interacting with the X, but that this pairing is limited and thus insufficient for directing segregation.

The role of Tef in sex chromosome pairing was also examined by asking if X duplications on the Y could facilitate XY pairing in males. The pairing of the X with the Y is normally mediated through rDNA pairing sites in the heterochromatin, and the X euchromatin is not normally involved in pairing in males. We wondered if X euchromatin would pair if we created a situation in males where additional X sequences were present. This might alter the current model of Tef's function, as it would suggest that Tef's autosome-specificity actually reflects specificity for euchromatin. Furthermore, this test would allow us to ask if there is a correlation between X pairing ability and the presence of Tef binding sites. To examine this possibility, we studied the capability of an X chromosome deleted for the rDNA to pair with a collection of Y chromosomes containing duplicated regions of the X euchromatin in males. Because the rDNA heterochromatin is needed for sex chromosome pairing (MCKEE and KARPEN 1990), this allowed us to assess the capability of X euchromatin to pair with itself. Six T(1;Y) chromosomes, each bearing a different X duplication, were tested for segregation from an rDNA-deficient X homolog. Five were capable of directing sex chromosome segregation as evidenced by the decreased incidence of nondisjunction among progeny

relative to controls. Only two of the six duplications, however, contain strong Tef binding sites as defined by our previous analysis (Figure 12). Thus, our observations suggest that the ability of X euchromatin to pair and conjoin may not depend on Tef. There are limitations to this interpretation, however. For example, conceivably there are more Tef binding sites on the X then we were able to detect through IIF, or perhaps only a subset of the Tef binding sites identified in salivary glands are also present in meiotic cells. A requirement for Tef could be directly tested by asking if the T(1,Y)s retain their ability to segregate from a heterochromatin-deficient X in a *tef* background.

Whether or not Tef is involved, this experiment raises interesting questions about homolog pairing in *Drosophila*. The ability of X fragments to pair in males suggests that euchromatic pairing may simply rely on homology, and that discrete pairing sites may not be involved. It is otherwise difficult to explain how or why pairing sites would be conserved in a system where they are never utilized. It is possible, however, that pairing sites could be conserved for some other reason; perhaps these regions are the same as those used in the somatic pairing of chromosomes which is observed in Drosophila, or they may be involved in XX pairing in females. If pairing were simply based on sequence homology, one might expect that pairing ability might be proportional to length of sequence homology. Our results suggest that this is not the case: no correlation was found between the size of the duplication on the Y and its ability to pair back with a heterochromatic-deficient X (Figure 13). Rather, our data suggest that some euchromatic sequences are inherently better at promoting pairing and conjunction than others. This segregation assay could potentially be used to map these sequences.

While Tef's role in sex chromosome pairing in males remains unclear, the results of our experiments suggest that Tef is capable of binding directly to chromosomes, and that this process is most likely sequence-dependent. The presence of Tef binding sites on the X was unexpected, as *tef* mutations do not affect XY pairing in males. Whether or not Tef plays a role in the conjunction of sex chromosomes needs further examination. Our results do suggest the possibility that at least on the X, general euchromatic homology may be sufficient for pairing regardless of the presence of Tef binding sites on the X. Future studies to define the Tef binding site will certainly aid in uncovering the role of Tef in homolog pairing in male meiosis.

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