

DYNAMIC INTERACTION BETWEEN CHROMATIN BOUNDARIES IN THE
DROSOPHILA HOMEOTIC GENE COMPLEXES

by

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(Under the Direction of Haini Cai)

ABSTRACT

Chromatin boundaries, or insulators, partition the genome into structural and functional domains. Previous studies show that insulators can modulate enhancer-promoter interactions and therefore regulate gene expression. Interactions between chromatin boundaries could also alter chromatin structure through an unknown mechanism to regulate gene expression, indirectly. Our lab recently discovered that one *Drosophila* insulator, SF1, interacts with multiple regions within *Antennapedia* complex (ANT-C). In order to understand how boundary interactions regulate gene expressions in different tissues, we used the Chromatin Conformation Capture (3C) technique to detect the interactions between DNA segments. By comparing the interaction using tissues with different gene expression patterns or different developmental stages, we found that the chromatin boundary interactions are distinct in tissues with different gene expressions, indicating that boundary interactions play a critical role in altering gene expression in different tissues.

INDEX WORDS: *Drosophila*, transcription, chromatin boundary, Chromatin Conformation Capture, SF-1, enhancer, promoter, PRE, and FACS.

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DEDICATION

To Zhen, Crystal and my coming baby boy.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Eukaryotic gene regulation

Regulation of gene expression controls not only the amount of one gene's functional product but also the timing and location of the transcription. In eukaryotes, the most important and also the major regulatory mechanism involves regulatory elements called enhancers and promoters, which are the DNA regions containing binding sites for protein factors that control gene transcription. Enhancers can be located upstream, downstream, or even within the gene they control. Enhancer-promoter interaction does not need to be within a short distance, but can occur over a long linear distance from 10-50 kb up to several hundred kb (Ling et al., 2004; Morley et al., 1992), or even across different chromosomes (Rubtsov, Polikanov, Bondarenko, Wang, & Studitsky, 2006). In many cases, enhancer-promoter interaction is not specific, which means that enhancers can direct the expression of different genes simultaneously. Some enhancers can also direct gene expression in an orientation independent fashion, which means they can function whether they are in the endogenous orientation or in a reverse orientation; upstream or downstream to the gene (Collins, Azmi, Berru, Zhu, & Shulman, 2006).

Gene expression is also largely influenced by chromatin structure. Heterochromatin mediated gene repression is an important mechanism of the silencing of gene transcription (Grewal & Jia, 2007; Roseman, Pirrotta, & Geyer, 1993). The silencing effect of heterochromatin can spread into the surrounding region causing the chromatin to become tightly packed, therefore blocking the access of RNA polymerase II to DNA (Grewal & Jia, 2007). The

above long range, non-specific activating and repressing influences raise the question: How can an individual gene be precisely, specifically regulated?

1.2. Insulators and their functions

Insulators are regulatory DNA elements that create boundaries in chromatin, delineating the ranges over which other regulatory influences take effect. Insulator elements have been characterized in a variety of organisms, ranging from yeast to humans (Bell, West, & Felsenfeld, 2001; Elgin & Workman, 2000). Insulators are defined operationally by two characteristics: 1) they interfere with enhancer-promoter interactions when present between the two; and 2) they buffer transgenes from chromosomal position effects (Figure 1) (Gaszner and Felsenfeld 2006). The former property suggests that insulators might work together with enhancers and promoters to ensure proper temporal and spatial gene transcription. The latter attribute suggests that insulators might play a role in the organization of the chromatin into functional domains, such that genes present in one domain are not influenced by regulatory elements present outside of the domain.

Since the first insulator element was found more than ten years ago, studies on insulators have demonstrated that they function in various aspects of biology, including regulation of developmental genes such as Hox genes, gene imprinting, and protection from X-chromosome inactivation in female animals.

1.3. Insulator elements in different organisms

As the interest has grown in the past few years, insulators have been characterized in variety of organisms ranging from yeast to human. The two characteristics of insulators are used

as experimental assays to identify and characterize new insulators.

In yeast, insulators were found at telomeres and mating-type loci, where they can separate active chromatin from silenced ones (Donze, Adams, Rine, & Kamakaka, 1999).

In *Drosophila*, a variety of insulators have been described, including Mcp, Fab6, Fab7 and Fab8 elements present in the Bithorax complex (BX-C) (Barges et al., 2000; Gruzdeva, Kyrchanova, Parshikov, Kullyev, & Georgiev, 2005; Hogga, Mihaly, Barges, & Karch, 2001), the *scs* and *scs'* elements flanking the 87A7 hsp70 heat shock gene locus (Kellum & Schedl, 1991, 1992), the *gypsy* insulator present in the *gypsy* retrotransposon (Geyer & Corces, 1992; Holdridge & Dorsett, 1991), an insulator present at even-skipped promoter, which contains a binding site for the GAGA protein (Ohtsuki & Levine, 1998), and the SF1 insulator element, which is located between Hox gene *Sex combs reduced* (*Scr*) and pair-rule gene *fushi-tarazu* (*ftz*) within the Antennapedia complex (ANT-C) (Belozarov, Majumder, Shen, & Cai, 2003).

In vertebrates, the first boundary element discovered is located at the 5' end of chicken β -globin locus and was initially characterized through its ability to interfere with activation of transcription of a reporter gene by the LCR (Locus Control Region) (Chung, Whiteley, & Felsenfeld, 1993). Two different human MARs (matrix attachment regions) from the apolipoprotein B and alpha1-antitrypsin loci can work as insulators in *Drosophila* to insulate a transgene from position effects. Both elements reduced variability in transgene expression without enhancing levels of the *white* reporter gene expression (Namciu, Blochlinger, & Fournier, 1998).

1.4. Mechanisms of insulator functions

Insulators are defined by their ability to interfere with the enhancer-promoter interactions

and to prevent transgenes from chromosomal position effect (CPE). Given this broad standard, it is not surprising to see that the insulators identified so far in diverse organisms show little or no sequence similarity. Different insulators require distinct protein components, suggesting that different insulators may function via different mechanisms. Since the discovery of the first insulator, different, sometimes even conflicting models have been proposed to account for the insulator properties. In this section, current insight of the insulators' enhancer blocker and barrier function will be discussed with supporting evidence. The models are not necessarily mutually exclusive, so potentially any insulator could employ one or a combination of mechanisms in order to function properly.

1.4.1. Models of the enhancer blocker function

Based on different observations and hypotheses, several models were proposed to explain the enhancer blocker function of the insulator. The “roadblock” model, is based on the observations that enhancers can recruit RNA polymerase II machinery and then gradually open up the chromatin between the enhancer and promoter (Vieira et al., 2004). An insulator placed between the enhancer and promoter could stop the delivery of Pol II, therefore blocking the activation. Consistent with this model, insertion of the 5' HS4 insulator between the HS2 enhancer in the human *β-globin* LCR and a downstream *ε-globin* gene dramatically reduces the level of Pol II found at the promoter. The insertion also causes the Pol II accumulation in the region from the HS2 enhancer to the 5'HS4 insulator (Zhao, Kim, Song, & Dean, 2006).

The second model is the “enhancer tracking” model. In this model, the enhancer activity tracks along DNA/chromatin to the promoter in the form of physical interactions between enhancer-nucleoprotein complex and intervening sequences. Insulators present between the

enhancers and promoters would block the tracking process, and therefore block the enhancer-promoter interaction. In the *Drosophila cut* locus, an insertion of the su(Hw) insulator can block the promoter-distal enhancers of the *cut* gene but not the promoter-proximal enhancers resulting cut-wing phenotype (Dorsett, 1993). The phenotype can be further enhanced by mutations of the CHIP gene, which is known to facilitate enhancer-promoter interaction (Morcillo, Rosen, & Dorsett, 1996). By interacting with *Chip*, su(Hw) insulator stops the enhancer complex from tracking further.

A modified “enhancer-tracking” model, also called “promoter decoy” model, suggests that an insulator can trap the enhancer by mimicking a functional promoter, and therefore prevent the activation of the true promoter. In support of this model, many *Drosophila* insulators contain promoter-like sequences. However, some enhancers can activate multiple promoters without being trapped by one of them.

The “Looping model” is an alternative to the “tracking model”. In this model, distal enhancers physically interact with the appropriate promoters through formation of chromatin loops (Bondarenko, Liu, Jiang, & Studitsky, 2003; Kyrchanova, Toshchakov, Parshikov, & Georgiev, 2007). Using RNA Fluorescence In Situ Hybridization (FISH), Ronshaugen and Levine visualized that the distal enhancers often loop to the Abd-B promoter region. Many insulators contain Matrix/Scaffold attachment regions (MARs/SARs), which are known to associate with the nuclear matrix and have been proposed to provide attachment sites for chromatin loops. Studies of insulator interactions indicate that insulators may interfere with chromatin looping formation through interactions with similar elements or sub-nuclear structures (Cai & Shen, 2001; Nabirochkin, Ossokina, & Heidmann, 1998). For example, two tandem arranged su(Hw) insulators abolish enhancer-blocking activity instead of strengthening it,

allowing the enhancer to by-pass. However, when enhancers or promoters are flanked by insulators, enhancer-promoter interaction is blocked more thoroughly (Cai & Shen, 2001). This evidence suggests that the closely positioned su(Hw) insulators interact with each other exclusively and cannot interact with other elements to form the proper chromatin structure, which is necessary for the correct enhancer activity. Another similar example is the *scs/scs'* insulator pair that flanks the 87A7 heat shock puff in *Drosophila* polytene chromosomes. The *scs* insulator is bound by the protein Zw5, whereas the *scs'* insulator is bound by BEAF. Chromosome conformation capture (3C) experiments showed that the two sites colocalize, and coimmunoprecipitation assays demonstrated that the two proteins interact, thus providing a mechanism for anchoring the loop (Blanton, Gaszner et al. 2003). This idea is straightforward, and well supported. However, the question of precisely how loop formation interferes with enhancer function remains an active area of study.

1.4.2. Models of the barrier function

The propagation of the silencing complex along the chromatin is illustrated by the study on the *Drosophila* Heterochromatin Protein 1 (HP1). HP1 binds to histone H3 that is methylated at lysine 9 (H3K9me), a hallmark of heterochromatin. A histone methyltransferase (HMT) is then recruited by the H3K9me-bound HP1 to methylate the next H3K9, forming the silencing cascade. Barriers are thought to stop the propagation of silencing effect by breaking such chain reactions. The barrier function of insulators was thought to be either disabling or counteracting to the silencing chromatin propagation.

For the disabling model, insulators could recruit nucleosome remodeling complexes to deplete or substitute the substrate of the methyltransferase. For example, yeast nucleosome

remodeling complex Swi/Snf, which mobilizes nucleosomes, is recruited to the barriers (Oki, Valenzuela, Chiba, Ito, & Kamakaka, 2004). A barrier downstream to the *Drosophila white* locus was found to replace histone H3 with an H3 variant, H3.3, which is a poor substrate for the HMTs of the silencing complex (Nakayama, Nishioka, Dong, Shimojima, & Hirose, 2007).

For the counteracting model, insulators may actively recruit activating complexes such as acetyltransferases to add active marks on chromatin. In the yeast native HMR locus, the histone acetylation occurs at the barrier (Oki et al., 2004). Additional evidence comes from the chicken *β -globin* locus. The 5'HS4 insulator coincides with a histone hyper-acetylation site, which is critical for protection against long-term silencing in mammalian cells (Mutskov, Farrell, Wade, Wolffe, & Felsenfeld, 2002). The histone hyper-acetylation is maintained by histone-modifying enzymes that are recruited by the barrier protein USF1. Knockdown of USF1 activity compromises recruitment of histone modifications and causes encroachment of H3K9 methylation (West, Huang, Gaszner, Litt, & Felsenfeld, 2004).

1.5. Chromatin boundaries in *Drosophila* Hox gene clusters

Homeobox-containing genes are found in many organisms, including worms, fish, frogs, birds, mammals, and plants (McGinnis, Levine, Hafen, Kuroiwa, & Gehring, 1984). Hox genes encode a series of transcription factors which typically switch on cascades of other genes and function in patterning the anterior-posterior body axis. Thus, by providing the identity of particular body regions, Hox genes determine the specific properties of the body segments. Interestingly, Hox genes are found in clusters, and the relative gene order within these clusters is conserved between organisms. That is, the order of related HOX genes in *Drosophila* and in

mice is the same. In addition, the order of Hox genes on the chromosome is related to where they are expressed along the anterior-posterior body axis.

Hox gene expression is initiated by the actions of many transcription factors during embryogenesis (Shimell, Simon et al. 1994). Curiously, these expression patterns are maintained after disappearance of the transcription factors. The active or inactive state is memorized through chromatin structure and transmitted to daughter cells through cell division. Although the mechanism of this epigenetic gene expression is not clear, genetic studies have identified many genes involved in the process (Kennison 1995). These genes are classified into two groups. *Polycomb* group (PcG) genes mainly govern the maintenance of the inactive state, and *Trithorax* group (*trx*) genes are mainly responsible for the maintenance of the active state.

In *Drosophila* Hox gene complexes, there are extensive cis-regulatory regions containing tissue specific regulatory elements that can direct the Hox gene expression over a long distance. For instance, *infra abdominal* (*iab*) enhancers are found upstream to the *Abd-B* gene of the *BX-C*. Each of those *iab* enhancers is responsible for *Abd-B* expression in a specific parasegment (PS). Previous studies have shown that the *iab* enhancers are divided into independent domains, and there are boundary elements defining each domain. Fab7 insulator, for example, is located between *iab*-6 and *iab*-7. Knock out of Fab7 leads to an ectopic activation of *iab*-7 in PS11, which is controlled by *iab*6 in wild-type. Two other insulators, Mcp and Fab-8 are found to separate the *iab*-4/5 and *iab*-7/8 respectively.

The SF1 chromatin boundary is found in *Antennapedia* Complex (ANT-C), located between the Hox gene *Scr* and the pair-rule gene *ftz* (Belozerov et al., 2003). The *ftz* gene has a group of regulatory elements that are located upstream or downstream to its promoter driving a seven-stripe expression pattern (Harding, Rushlow, Doyle, Hoey, & Levine, 1986). The *Scr*

expression is driven by *Scr* cis-regulatory elements and then maintained by Polycomb/Trithorax responding elements (PRE/TRE). The regulatory elements of the two genes are interspersed in common intergenic regions. Since some *ftz* enhancers are promiscuous and could interact with *Scr* promoter (Gorman & Kaufman, 1995), there must be a regulatory mechanism to ensure specific enhancer-promoter interaction. The presence of SF1 facilitates the proper enhancer-promoter interactions and protects *ftz* gene from PRE silencing effect. According to the looping model, we hypothesize that SF-1 may function by interacting with additional boundaries downstream of *ftz* to form an independent *ftz* functional loop domain. Additionally, based on our lab's most recent finding, SF-1 interacts with multiple genomic regions within the ANT-C (Mo Li and Haini Cai, unpublished).

1.6. Summary of hypothesis and purpose of study

Put all the information together, there are still many questions remaining unanswered about insulator functions: Do insulators always interact with the same partners? Are there dynamic changes in insulator interactions during animal development? Since Hox gene expression is known to be tissue specific, and we believe that SF1 and other potential boundary elements function to regulate Hox gene expression, are insulator interactions also tissue specific? In the field of insulator function study, so far no tissue specific experiments have been performed to look at the chromatin structure changes between different tissues. To answer the raised questions and to further understand the function and mechanism of SF1 and other insulators, I will use SF1 as an example to test the insulator interactions in different tissue regions, which have different *ftz/Scr* expression patterns. We propose that chromatin boundaries form different structural conformations in different tissues or at different developmental stages, partitioning

DNA regulatory regions into different functional domains, to alter gene expression patterns at different locations or stages.

CHAPTER 2

MATERIALS AND METHODS

2.1. Drosophila stocks

Fly stock used in this study is W¹¹¹⁸ line. Stock was fed standard cornmeal, molasses, yeast, and agar medium and was maintained at 25°C.

2.2. Reporter plasmid constructs

The P-element constructs used for tissue specific labeling were based on the pCaSPeR vector. All of the early embryonic enhancer elements were generated by PCR and cloned into pCRII/TOPO vector (Invitrogen). The primers used: acaaatcgcatagata and tatcgagtcacatcagcattac for Hairy 1; acaaatcgcatagata and tatcgagtcacatcagcattac for IAB5; acaaatcgcatagata and tatcgagtcacatcagcattac for SF2-2. The enhancer sequences were cut out with EcoRI and inserted into a unique RI site in the CAebGFP and CAebRFP vectors (Cai lab) (Figure 4). Orientation of enhancers was determined by restriction digestion and PCR. Qiagen plasmid Midi kit was used to extract and purify the constructs that are going to be used for fly embryo microinjection. Purified constructs were injected into W¹¹¹⁸ preblastoderm embryos. Embryo microinjection was contracted to a fly injection company (Rainbow transgenic flies, Inc.).

2.3. Generation of transgenic lines and genetic crosses.

Injected larvae were fed in standard cornmeal, molasses, yeast, and agar medium and were maintained at 25°C allowing them to develop to adulthood. Each of those flies was then crossed to 3-5 W^{1118} flies. Their progenies were screened for red eye color, which indicates the positive transgenic lines. Positive lines were checked by genomic PCR as well. The heterozygous flies were self-crossed in order to obtain homozygous flies (Figure 5).

2.4. Embryo cell dissociation

This procedure was modified based on existing cell disruption protocol (Lawrence S. B. Goldstein 1994). Approximately 5000-6000 homozygous H1, SF2-2 GFP and IAB5 RFP flies were fed on apple juice agar plates and yeast paste plates (50% apple juice, 2% agar, 1% ethyl alcohol and 1% acetic acid). If crosses were needed, homozygous virgin female from one transgenic line were fed for two days before being crossed with homozygous male flies from a different line to generate double positive heterozygous line. Flies were then fed three to four days at 25 °C incubator with frequent feeding (2-3 times /day) before egg collection. Following pre-collection for 4 hours, all the embryos were collected and allowed to develop for another 4 or 10 hours at 25 °C incubator to obtain either 4-8 or 10-14 hour old embryos, respectively. All the collected embryos were washed by ddH₂O to rinse off the yeast paste. Embryos were dechorionated in 6% solution of sodium hypochlorite (regular bleach) for 3 minutes on a 125 µm medal mesh. The dechorionated embryos were rinsed by ddH₂O. Paper tissue was used to absorb excess water from the bottoms of the mesh. 150-200 µl of block dried embryos were transferred into a 5ml glass Dounce homogenizer filled with 2 mL of 1XPBS buffer (0.0162 M Na₂HPO₄,

0.15 M NaCl, 0.0027 M KCl, 0.0014 M KH₂PO₄, pH=7.2) and were homogenized by 30-50 up-and-down strokes of the loose pestle (Pestle A) on ice. Foaming or air bubbles were avoided by handling pestle movement carefully and slowly. The 2 ml homogenate was filtered through a 125 µm medal mesh into a 15ml plastic centrifuge tube and kept on ice. The cell homogenizing step was repeated until all collected embryos were used up. The homogenate was then pelleted by centrifugation at 860 g for 1 min at 4°C. The pellet was re-suspended in 2 ml ice-cold HyQ SFX-Insect serum-free medium (HyClone), followed by centrifugation at 860 g for 1 min at 4°C. The pellet was suspended in 1mL of PBS buffer (pH=7.2) containing 0.05-0.1% trypsin and was incubated at 37°C for 10 min on a nutator (Shigenobu, Arita, Kitadate, Noda, & Kobayashi, 2006). The concentration of trypsin used depends on the developmental stage of embryos. For samples at early developmental stages, 0.05% trypsin was used. For samples at late developmental stages, 0.1% trypsin was used. After filtration through a 40 µm mesh, 1 ml of ice-cold HyQ SFX Insect serum-free medium (HyClone) was added to stop the reaction. The dissociated cells were pelleted by centrifugation at 860 g for 1 min and re-suspended in 3 ml ice-cold HyQ SFX Insect serum-free medium. 8 ul of cell suspension were transferred onto a hemocytometer. Cell morphology was verified under microscope. Cells were also counted and the concentration was adjusted to about 10^8 cells/ mL and stored on ice. Samples were used for cell sorting within 20 minutes after dissociation. The suspension was filtered through a 40 µm filter (CTEGD Flow Cytometry Facility provided) immediately before cell sorting. To slow down the aging process of the cells, samples were kept on ice all the time.

2.5. FACS analysis and cell sorting

MoFlo cell sorter (CTEGD Flow Cytometry Facility) was used to sort GFP/RFP positive cells. Fluorescent cells were sorted by gating for GFP/RFP positive and auto-fluorescence negative events. Light reflecting parameters, such as doublet profile, forward light scattering profile, side light scattering profile were used to exclude cell clusters, doublets, and none cell debris. For GFP/RFP and auto-fluorescence detection, I used the wavelength at 525 nm and 575 nm, respectively. Cells were sorted through a flow chamber with a 100 μ m flow cell tip under 9 psi sheath fluid pressure. The sorting was carried out at a rate of 10000-20000 counts/second. The sorted cells were collected into 5 ml tubes filled with 1ml of S2 medium. To slow down the aging process of the cells, the sample chamber of the cell sorter was cooled down to 4°C throughout the sorting process.

Isolated cells were spun down at 860 g for 1 min at 4°C and then re-suspended in 200 μ l S2 medium on ice till use. 8 μ l of isolated cells were transferred onto a hemocytometer. Cell morphology and fluorescence was verified under Zeiss laser-scanning con-focal microscope (LSM 510 META). Total cell population and cells with fluorescence were counted. The percentage of the successfully dissociated and enriched single fluorescence cells among the total cell population was calculated using the following formula: $p = s/t$, where p represents the percentage of single cells with only correct fluorescence, s represents the cell number of single cells with only correct fluorescence, and t represents total cell population including single fluorescent cells, cells in the fluorescent aggregates and non-fluorescent single cell and clusters. The dissociated samples were also verified and counted before cell sorting. The percentage of each fluorescent cell population before sorting (p') was generated using the same formula described above. The fold of enrichment (FE) of each fluorescent cell population after sorting

was calculated by comparing the percentage of single fluorescent cells after sorting (p) with the percentage of single fluorescent cells before sorting (p'): $FE = p / p'$.

2.6. 3C analysis using enriched cells

Sorted cells were pelleted by spin for 5 minutes at 800 g at 4 °C. Cells were re-suspended and washed in 5 ml cell lysis buffer (0.34 M Sucrose, 10 mM Tris pH 8.0, 10 mM NaCl, 0.25 mM PMSF, 0.1% BME, without Triton X-100) in 15 ml centrifuge tubes. Cells were then spun down at 800g for 5 minutes and re-suspended in 5 ml cell lysis buffer devoid of Triton X-100 but containing 1% formaldehyde. After being spun down at 800g for 5 minutes, cells were fixed for 10 minutes at room temperature on a nutator. Fixation was quenched with addition of glycine to final 0.125 M. Cells were spun down at 800 g for 5 minutes at 4 °C and washed twice with 5 ml cell lysis buffer (with Triton). Cells were spun down at 800 g for 5 minutes at 4 °C and then washed once with 5 ml loop assay buffer (10 mM Tris-HCl pH 7.9, 5 mM MgCl₂, 20 mM NaCl, 1 mM DTT). Pelleted cells were re-suspended with 1X NEB EcoR I restriction buffer. SDS was added to a final 0.1%. The reaction was incubated at 37 °C for 10 minutes to remove any non-crosslinked proteins from DNA. Triton X-100 was added to 1 % concentration to sequester SDS and to allow subsequent restriction digestion. The DNA was digested with EcoR I (1000 unit/reaction) at 37 °C over night. The restriction enzyme was inactivated by incubating at 65 °C for 25 minutes. The reaction was diluted 40 X in 1X ligase buffer. DNA was ligated at room temperature for 20 minutes followed by an over night incubation at 16 °C with T4 DNA ligase (10 ul/reaction, 400 units/ul). Cross-link was reversed by over night incubation at 50 °C in the presence of 10ug/ml of proteinase K. DNA was purified by phenol-Chloroform extraction twice and ethanol precipitation. Purified DNA was re-suspended in 50 ul Gibco H₂O. DNA was quantified using Fluorometer. To generate the control template, purified genomic DNA was

digested with the same restriction enzyme and ligated in a concentration of ~500ng/μl. The appropriate amount of template DNA for PCR was determined by serial dilution. The specificity of the PCR products was ensured by a two-step nested PCR strategy. Primers were designed to make the final PCR products 200-300bp long. PCR products were analyzed on 2 % agarose gel and imaged by a Biorad UV Gel Doc imager. Quantitation of PCR products was done using the Gel Analyzer function of ImageJ (Rasband, W.S. <http://rsb.info.nih.gov/ij/>).

To obtain quantitative and more consistent PCR results, a two step nested Real-Time PCR was performed with Applied Biosystems 7500 Real-Time PCR system. Data was collected and processed with 7500 Real-Time PCR sequence detection software v1.3.1.

Primers used for both conventional PCR and Real-time PCR were the same:

SF1o: ACGAATGACCTATGCCATTTATCT	SF1i: CAAAATCGATTTTGGGTGTGTCT
R1o: TCGAGGGTTCCTTACCTCT	R1i: CGACGCAAGCACACAGA
R2o: CAGGAGCTAAAAATGCGTTCA	R2i: TTCGCAACATTTTGGCCTG
R3o: GAAAGGTTGCTCAGAACAGGG	R3i: CCGCACAAATTACATAATCACG
R4o: CGACAAAAGACGCCAAGACA	R4i: ATGCGAATGGGAGAAATGCG
R5o: TGAAAGGAGTTACTCGCAACT	R5i: TAATTTAGCAGGTCGGGCG
R6o: CACGATCGATCGCCAATCAT	R6i: GCCAAACAGGTGTGTCTGG
R7o: TGG CGC TCA AAA GCG TT	R7i: GGCCTGGCTTCGACTTTT
R8o: AATGTCGTATCACACAGGCG	R8i: GGCAATCCGTCCTCACATT
R9o: GCGACAAGTGTGTGCGTAG	R9i: GCGCTTTGCCTTTGGACAT
R10o: GCCTTCTATTCGGGTATCTCT	R10i: ATAACCACTGCAGTCGGTTAG
R11o: TGTCAGAAATCCACTCGAGC	R11i: TCCATCTCTCGATCCATCAA

CHAPTER 3

RESULTS

3.1 SF1 interacts with chromatin boundaries within the the *Scr-ftz* region

In order to test the looping hypothesis and search for SF1 long range interacting element, Chromosome Conformation Capture (3C) technique was used to probe the entire *Drosophila* ANT-C. 3C is a powerful new tool for studying the spatial organization of the genome (Dekker et al., 2002). Formaldehyde-mediated crosslinking is utilized in 3C to capture spatial juxtaposition of DNA. The likelihood of two DNA fragments being crosslinked is an indication of spatial proximity. The crosslinking frequency is quantitated using a ligation-based PCR strategy, and expressed as the ratio (ie. relative crosslinking frequency) of signal obtained by PCR on crosslinked template to that obtained on a control template. The control template includes all possible ligation products present in equal quantity. It also corrects for variations in PCR amplification efficiency of different primer sets, and is universally adopted as a necessary control for 3C (Dekker, 2006). Several sets of control experiments are averaged to generate a consensus control profile. Other control experiments that omit the crosslinking or ligation steps are used to demonstrate the specificity of a positive signal. Recently, the use of 3C has revealed long-rang regulatory interactions in the β -globin, H19/Igf2 and olfactory receptor loci (Ling et al., 2006; Lomvardas et al., 2006; Tolhuis et al., 2002). 3C is therefore an ideal tool for studying the organization of complex genetic loci, for example, the Hox gene complexes. However, 3C with conventional PCR reactions can only generate semi-quantitative results, and variations among independent trials are higher than desired. of several reactions is not ideal. To improve

consistency and obtain accurate and quantitative data, we chose Real-Time PCR for most of our tests.

DNA extracted from whole embryos was used as template. The SF1 region was used as the anchor of boundary interactions. Primers were designed to cover every EcoR I restriction digestion fragment within ANT-C and to give 200-300 base pair products. The chromatin region tested is depicted in figure 2. Nested PCR was performed to ensure the specificity of PCR products. The whole-embryo 3C profile showed that SF1 interacts with three chromatin regions, R2, R6/7 and R9/10, within the ANT-C (figure 2). R2 is situated 3' to the *ftz*-DE enhancer of *ftz* (Calhoun and Levine 2003) and 5' to the *Scr* distal enhancer PS2 (Gorman and Kaufman 1995); R6/7 is located 3' to the PS2 and 5' to an *Scr* distal silencer, which has PRE characteristics; and R9/10 is sitting 3' to the *Scr* distal silencer and 5' to the homeotic gene *Antp* (figure 2). These three elements were then cloned and tested for insulator activity both in *Drosophila* S2 cells and in embryos. R9/10 showed strong insulator activity, while R2 and R6/7 didn't show insulator activity (data not shown). It is interesting to note that all three SF1 interacting fragments were located at intergenic regions between different *ftz* and *Scr* regulatory elements. Because of their special genomic locations, insulator activities and prominent SF1 interacting abilities, we propose that these SF1 interacting elements cooperate with SF1 to separate the regulatory elements of *ftz* and *Scr* into different functional domains by forming chromatin loops.

3.2 Chromatin structure of *Scr-ftz* region is distinct at different developmental stages.

The expression of Hox genes has two well known characteristics. First, Hox genes are activated by enhancers at early embryogenesis stage (Boncinelli, Simeone et al. 1991). The chromatin structure is then maintained by PRE/TRE mediated silencing/activating

mechanisms(Busturia and Morata 1988; Kennison 1995). Second, the expression of Hox genes is collinear with body AP axis, which means in different body segments, there are different Hox gene expression patterns (Prince, Price et al. 1998). If nuclear organization contains epigenetic information, then it can be inferred that it is cell-lineage specific. Additionally, nuclear organization and transcriptional regulation are known to be intimately related. The fact that gene expression patterns define cell identity also implies that nuclear organization is cell-type specific. Therefore, elements, such as insulators, that are involved in the establishment of nuclear organization and transcriptional regulation must be regulated between cell types. Since chromatin structure plays an important role in Hox gene regulation, we were interested in the following two questions: 1) Are the boundary-boundary interactions within Hox gene complexes the same at early and late developmental stages?, and 2) Are the boundary-boundary interactions within Hox gene complexes the same in different body segments?

To investigate the boundary-boundary interactions in Hox gene complexes during early and late developmental stages, we collected *Drosophila* embryos after allowing egg-lay for 4 hours. The eggs were then divided into two halves and aged at 25 °C for 4 or 10 hours, respectively, resulting in 4-8-hour or 10-14-hour-old embryos. The 4-8-hour-old embryos represent embryos at the early developmental stages. In those embryos, germband extension has just started, gap genes expression has stopped, and the transcription of Hox genes has started. The 10-14-hour-old embryos represent embryos at the late developmental stages. In those embryos, body axis patterning is completed, the activation of Hox genes has passed, and the transcription state of Hox genes is maintained by PRE/TRE. After DNA extraction, similar 3C procedure was performed using early and late embryos. As expected, the 3C profile obtained indicated different SF1 interactions in the early and late embryos. In the early embryos, SF1

interacted with R1, R5-7, and R10 more frequently than with other regions tested. In the late embryos, the interaction frequency of SF1-R1 and R5-7 dropped significantly, whereas SF1-R10 interaction remained the same (figure 3). There were no significant differences in the SF1 interaction frequency of R2-4 and R8-10 between the early and late samples.

3.3 A novel cell isolation technique for tissue specific 3C analysis.

To answer whether there are different SF1 interactions in distinct body segments, we decided to use the Fluorescence Activated Cell Sorting (FACS) technique to enrich cells from specific body segments for 3C. To utilize the FACS technique, cells needed to be labeled with fluorescence reporters. Constructs as shown in figure 4 were generated. Green fluorescence protein (GFP) and red fluorescence protein (RFP) reporters were introduced into a pCaSPeR vector based plasmid, which contained a mini-white eye color reporter. Early embryonic enhancers Hairy 1(H1), SF2-2 and infra-abdominal-5 (iab-5) were inserted between the divergently transcribed mini-white and GFP or RFP genes, driving their expressions in specific body segments. H1 is one of the enhancers of the pair rule gene *Hairy* (Eckert, Aranda et al. 2004), governing the first stripe of Hairy expression. It drives RFP expression within parasegment 0 to 1 (figure 6A). SF2-2 is discovered by our lab (unpublished), which directs *ftz* stripe 1 and stripe 5 expression. SF2-2 drives GFP expression at parasegment 2 and 10 (figure 6B). iab-5 is from the Abd-B locus (Kuziora and McGinnis 1988). It regulates Abd-B expression in the abdominal region and drives RFP expression at parasegment 10, 11 and 12 (figure 6C).

W¹¹¹⁸ flies were used for embryo microinjection. Embryos were collected and injected after 30 minutes of egg-lay. Injection was done within another 30 minutes. Survived larvae were picked and maintained in standard food at 25 °C to allow them to develop to adulthood. Adults

were crossed to the W¹¹¹⁸ line. The F1 flies were screened for red-eye marker, which indicated germline transformation. Positive lines were checked by genomic PCR as well. Flies from same lines were self-crossed to generate homozygous lines. The expression patterns of the respective fluorescent markers in the embryos laid by different homozygous lines were verified under confocal microscope. All fluorescent lines emitted fluorescent lights in correct patterns, indicating that embryonic enhancers were driving the expression of fluorescent proteins in the designed tissues specific pattern.

As shown in figure 7, to dissociate cells, embryos were collected, washed and dechorinated after being aged for 4 and 10 hours at 25 °C. 200 ul embryos were then transferred into a glass Dounce homogenizer. Cells were dissociated but not broken by 30-50 slow passes with type A (loose) pestle. Homogenate was treated with 0.1% trypsin at 37 °C for 10 minutes to further dissociate cell aggregates. Dissociated cells were filtered, rinsed, and checked under microscope to ensure that cells were intact and and separate from each other (figure 8A). Dissociated cells were subject to cell sorting using a a MoFlo cell sorter. Cells of interest were collected based on their forward and side light scatter characteristics and the fluorescent signals they emitted after excited by laser with 525 nm or 575 nm wavelength. Throughout this entire procedure, cells were kept at 4 °C to prevent the developmental and cell fate changes.

Collected cells were concentrated. The morphology of cells was verified. Cells were counted on a hemocytometer under confocal microscope (figure 8B-D). For quality assurance purpose, total cell population and cells with fluorescence were counted. The percentage of the successfully dissociated and enriched single fluorescence cells among the total cell population was calculated using the formula described in the materials and methods section. The dissociated samples were also verified and counted before cell sorting. The percentage of each fluorescent

cell population before sorting (p') was generated using the same formula. The fold of enrichment (FE) of each fluorescent cell population after sorting was calculated by comparing the percentage of single fluorescent cells after sorting (p) with the percentage of single fluorescent cells before sorting (p'): $FE = p / p'$. Prior to cell sorting, cells with either green or red fluorescence made up about 10% of the total cell population. After cell sorting, the percentage of the fluorescent cells increased to about 50%. The fold of enrichment was more than 4.0 (figure 8). Because both SF2-2 and *iab-5* drive fluorescence protein expression in parasegment 10, we crossed SF2-2 GFP line with *iab-5* RFP line to generate double positive embryos, which have green stripe in parasegment 2, red stripes in parasegment 11 and 12, and yellow stripe in parasegment 10 (figure 6D). Therefore, we could separate cells from parasegment 2, 11 and 12, and 10 from whole embryos using a single sorting experiment. Meanwhile, since embryos were aged to different developmental stages, we could also obtain cells not only from different body segments, but also at different developmental stages.

3.4 Chromatin structure within the *Scr-ftz* region is distinct within different body segments.

As described in the introduction, *Scr* expression is active in the body regions posterior to parasegment 2 during early embryogenesis. At late developmental stages, *Scr* transcription is no longer active; however, its transcriptional state is maintained by PRE/TRE. In body regions anterior to parasegment 2, *Scr* expression is always off. In order to compare the chromatin structure between *Scr* on and off tissues, cells from H1 (anterior) and *iab-5* (posterior) were used to represent *Scr* off and *Scr* on tissue, respectively.

3C profiles of different samples showed dramatic differences. To compare SF1 interactions in different tissues, 3C profiles of anterior and posterior tissues with the same

developmental stages were compared together. At early stage, anterior tissues had no SF1 interaction through out the entire *Scr-ftz* region, while posterior tissues had multiple SF1 interacting points, including R1, R4, R6, and R10 (figure 9A). At late stages, anterior tissues showed SF1 interactions only in R3 and R10 regions, while posterior tissues showed similar SF1-R3 and SF1-R10 interactions except that the interaction frequency was lower in R3 and higher in R10 when they are compared to the anterior tissues. There was another interacting point for posterior tissues in R6, where SF1 interaction frequency was significantly higher than that in the anterior tissues (figure 9B).

To compare SF1 interactions at different developmental stages, samples from the same body region but at different developmental stages were compared. In anterior tissue, at early stages, there was no SF1 interaction through out the entire *Scr-ftz* region. However, at late stages, the anterior tissue showed SF1 interaction at R3 and R10 (figure 9C). In posterior tissue, at early stages, there were multiple SF1 interactions at R1, R4, R6, and R10. While the posterior tissue became older, only R10 still maintained SF1 interaction (figure 9D).

3.5 Conclusion

In conclusion, based on the previous findings of SF1 interacting elements within *Scr-ftz* region, the work described in this thesis extended the study of boundary-boundary interactions by comparing the chromatin structure of the *Scr-ftz* region at different developmental stages and in distinct body regions. A novel cell isolation technique was created based on FACS cell sorting. This technique utilized early embryonic enhancers to drive fluorescent reporter protein expressions at specific body regions, labeling the body regions for the following fluorescence activated cell sorting. This method allowed us to enrich cells from any specific body segments as

long as the proper tissue specific enhancers are available. Because cells were enriched by more than 4 folds, the outcomes of our experiments were much stronger and cleaner without the interference of the background or contamination. Our 3C data of SF1 interactions showed that there were significant differences of the 3D chromatin structure in samples with different developmental stages and gene expression patterns. The results supported our hypothesis that chromatin structure plays a crucial role in gene regulation, and boundaries can form dynamic interactions with other boundaries during different developmental stages and in different body regions. These dynamic interactions between boundaries may suggest a regulatory significance of changing chromatin structure. The chromatin structure differences between distinct developmental stages and body tissues showed dynamic boundary-boundary interactions in different cells types, providing snap shots of the changing process of the chromatin structures and its related gene regulation.

CHAPTER 4

DISCUSSION

So far, little is known about the mechanistic role of chromatin boundaries in gene regulation. There are several models put forth by different groups trying to explain the mechanism of insulator function. Although previous work mainly focused on deciphering the mechanisms by which insulators block the communication between an enhancer and a promoter, the field has recently shifted to a more global view of insulators. We have shown a new way of probing into the mechanisms and biological functions of chromatin boundaries, specifically SF1. Because the cells used for the experiments were enriched from specific tissue regions with different gene expressions, the results were more specific and straightforward, reflecting the real fly biology. Additionally, cells at different developmental stages were enriched and the boundary interactions in those cells were compared, allowing us to see the dynamic interactions between insulators during fly development. This project provided us more insight about the mechanism of chromatin boundaries and helped us to understand the precision of gene regulation.

In this study, we found that in whole embryos with a wide developmental range, SF1 interacted with three chromatin elements, R2, R6/7 and R9/10. We wondered what the biological significance of these interactions was and what their regulatory mechanisms were. Since Hox gene expression has developmental stage difference and tissue specificity, we asked whether the chromatin structure of Hox gene region changed when their expression pattern was changing. We used *Scr-ftz* region as an example, since it contained multiple cis-regulatory elements for both homeotic gene *Scr* and non-homeotic gene *ftz*.

To test the chromatin structure difference between different developmental stages, 4-8-hour and 10-14-hour-old embryos were collected as samples. In 4-8-hour-old embryos, Hox gene expression has just started, and body segmentation is occurring (Arbeitman, Furlong et al. 2002). In 10-14-hour-old embryos, Hox gene expression has stopped, and body segments are formed. The reason to skip 8-10hr stage is because during this stage, Hox gene expression is decreasing and PRE/TRE mediated silencing/activating maintenance has not fully established yet. In order to obtain true early and late samples, we didn't collect 8-10hr embryos. As expected, 3C profiles on early and late samples showed different SF1 interactions. Though both early and late samples showed strong SF1-R10 interactions, early sample contained higher SF1-R1 and SF1-R5-7 interaction frequency. Since whole embryo represented all the cells from different body segments, it was likely that in early stages, there were stronger SF1-R1 and SF1-R5-7 interactions, or there were more cells that contained these SF1 interactions. This observation was consistent with our looping model. Either SF1-R1 loop or SF1-R5-7 loop could facilitate the *Scr* promoter-enhancer interactions depending on which model to use (figure 10). Interestingly, there was no difference of SF1 interactions at R10. Both early and late samples showed strong SF1-R10 interactions, indicating R10 was a constitutive SF1 interacting point. According to its location, SF1-R10 loop could isolate the entire *ftz* transcription unit from Hox gene region. It could also facilitate the *Scr* distal PRE to interact with its promoter to maintain the transcription state (figure 10).

To test the chromatin structure difference between distinct body segments, a novel FACS based cell isolation technique was created. Transgenic fly lines were generated. Cells from specific body segments in those transgenic lines were labeled by different fluorescence. An efficient cell dissociation procedure was created based on existing protocols (Lawrence S. B.

Goldstein 1994). This procedure can be used on cell dissociation for samples at both early and late developmental stages, resulting in intact and well separated cells. After cell dissociation, single cells were run through cell sorter. Fluorescent cells were collected as samples for 3C analysis. This technique enabled us to do experiments on cells from any specific body regions. Enriched samples can provide stronger and purer signals without the interference of the background and contaminations.

3C analysis on the sorted samples showed huge dynamics of SF1 interactions at different developmental stages or in different body regions. At the early stage, anterior tissue showed no SF1 interactions throughout the entire *Scr-ftz* region. It was consistent with the transcription state of this region, because neither *Scr* nor *ftz* was active in this tissue. However, posterior tissue showed multiple SF1 interactions, including R1, R4, R6 and R10. Except SF1-R4 interaction, the rest was very similar to the 3C profile on whole embryo at the early stage. The reason could be that all the regions posterior to parasegment 2 express *Scr*, and therefore, “*Scr* on” region made up of most of the embryo tissue. Since posterior tissue is part of the “*Scr* on” tissue, it is not surprising to see similar 3C profile between them. Because R4 also sits at the dividing point of *Scr* enhancer and distal PRE element, its interaction with SF1 could have the same biological function as SF1-R6 interaction, but is used in other posterior tissues. At the late stage, anterior tissue showed two SF1 interactions at R3 and R10. It is easier to explain SF1-R10 interaction, for R10 could facilitate *Scr* promoter-PRE interaction, allowing *Scr* transcription state to be maintained at the late stages. The reason of SF1-R3 interaction is not clear. It could be an unknown mechanism that allows SF1 to interact with *Scr* enhancers to ensure they are not contacting the promoter during late stages. Posterior tissue showed similar 3C profile except that

there was another SF1-R6 interaction. R6 could interact with R3 and SF1 together to loop out the *Scr* enhancers, so it ensures no enhancer-promoter communication.

Based on the 3C analysis on mixed un-aged whole embryo samples, aged whole embryos samples, and sorted cells at different developmental stages and from different body regions, we saw significant differences in terms of SF1 interactions. These different SF1 interactions within distinct body regions or developmental stages indicate multiple SF1 interactions of the same *Scr-ftz* region are alternating depends on the cells' Hox gene expression patterns. As specific boundary-boundary interactions correlate with specific gene expression patterns, our results suggested the potential regulatory roles of SF1 interactions. The chromatin structure differences between distinct developmental stages and body tissues showed the dynamic boundary-boundary interactions in different cells types, providing snap shots of the changing process of the chromatin structure with its correlated gene expression.

There are some limitations of our work. First, tissue specific samples were limited by the availability of embryonic enhancers. For the posterior “*Scr* on” tissues, since we don't have enhancers which can drive single stripe at posterior regions, we utilized *iab-5* enhancer. The expression pattern driven by *iab-5* enhancer is three posterior stripes at *ftz* stripe 5, 6, and 7. Hence, the posterior tissue we enriched was a mixture of cells from these three posterior stripes. Because cells from each stripe could have their own chromatin structures, the mixed samples may introduce an interruption on the 3C outcomes. It might be the reason that we saw multiple SF1 interactions in the sorted posterior tissues (figure 9). Second, due to the availability of fluorescent markers, only several colors could be used for cell sorting. To solve the above two problems, we used a combination of different enhancers with different colors to further divide the tissue regions. Various fluorescent markers with stronger signals also could be used

(Giepmans, Adams et al. 2006). This allows us to label cells with multiple colors and isolate cells with different identities by one round of cell sorting.

Several questions remain open regarding the mechanisms of the regulation of different boundary-boundary interactions. For example, what protein factors are required for proper interactions? How do chromatin boundaries find their interacting partners? How to control the frequency and timing of different interactions? In the future, researchers can use RNAi assay and deficiency genetic screen to search for the insulator protein factors. More 3C assays will be performed on samples with much narrower developmental windows and body regions. A dynamic boundary-boundary interaction graph can be generated according to these assays. It will provide us a more detailed picture of chromatin structure changes in different tissues and developmental stages.

Figure 1. Functions and characteristics of Insulator.

Insulators can block improper enhancer-promoter interactions and protect transgenes from chromosomal position effect. As shown in the figure below, insulator 2 blocks green enhancer from interacting with yellow gene promoter. Insulator 1 protects green gene from chromosomal position effect caused by the nearby silencing effect. Insulator 1 and 2 together organize the genomic region into independent functional domains. The domain on the left is inactive. Chromatin in this domain is compacted. It has silencing effect to the nearby region. The chromatin in the middle and right domains is relaxed. Therefore, the transcription of green and yellow genes is active in the middle and right domains, respectively. Insulators are indicated by brown ovals; histones are indicated by orange cycles; DNA is indicated by black line; enhancers are indicated by colored boxes.

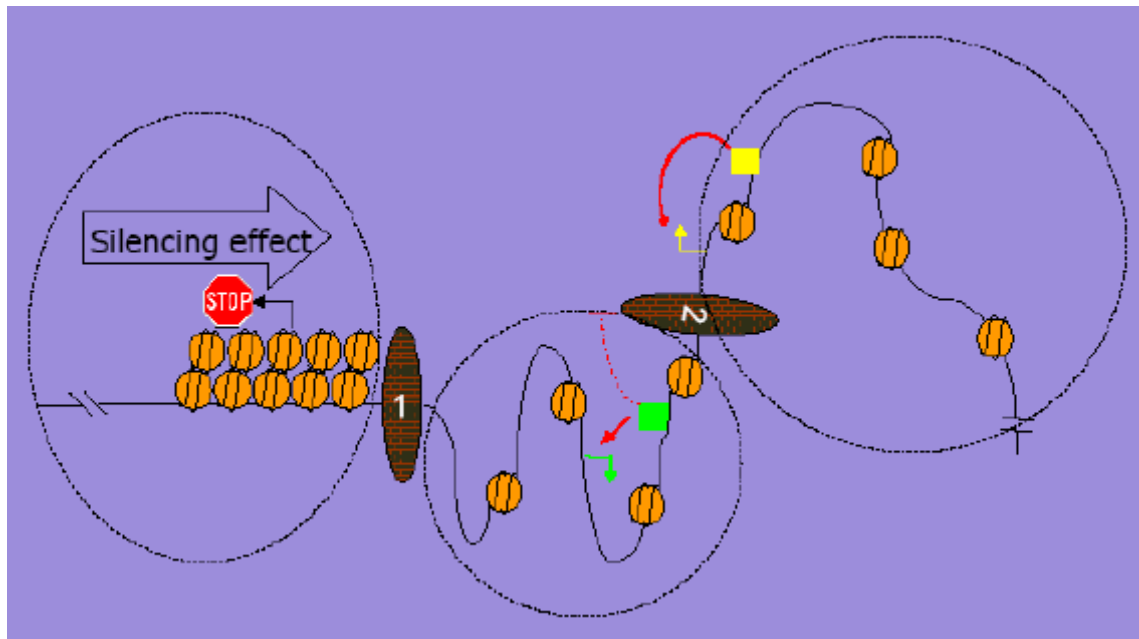


Figure 2. Whole embryo 3C reveals SF1 interaction points within *Scr-ftz* region.

3C analysis performed on samples extracted from whole embryos with a wide range of developmental stages showed three SF1 interacting elements, R2, R6-7 and R9-10. The map on top indicates the *Scr-ftz* region. *Scr* and *ftz* transcription start sites are marked by two divergently pointed orange and green arrows, respectively. The positions of those primers for the nested PCR are marked by arrowheads. SF1 primers are indicated by red arrowheads. The primers for the testing regions are indicated by black arrowheads. EcoR I restriction sites within the *ftz* downstream region are marked by black vertical lines. The shaded green boxes represent *ftz* regulatory elements and the shaded orange boxes represent *Scr* regulatory elements. SF1 is indicated by a brown oval. The three SF1 interacting points are indicated by grey circles. Downstream Antp gene is indicated by a blue box. Map is not drawn in scale.

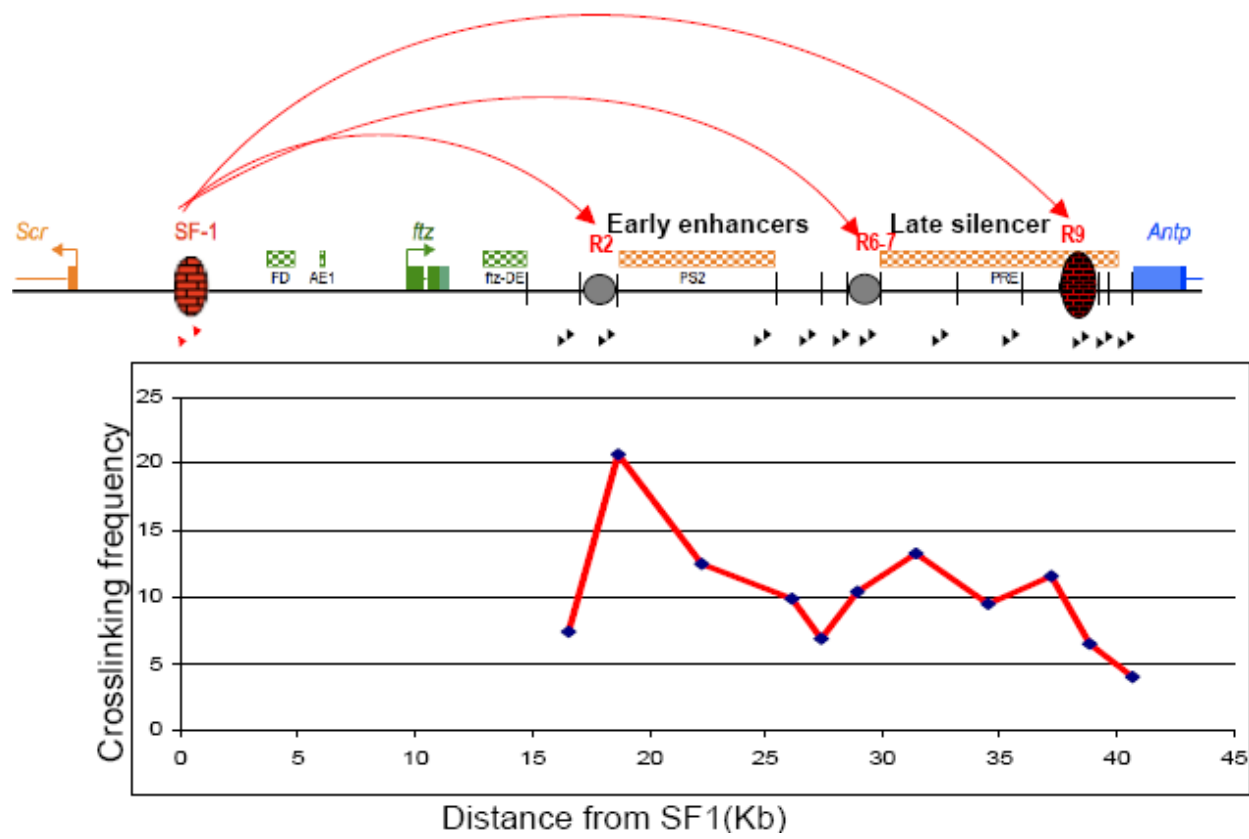


Figure 3. 3C analysis showed different SF1 interaction profiles on whole embryo samples at early and late developmental stages.

DNA samples were extracted from whole embryos at early and late developmental stages. 3C profiles for both samples are plotted based on their SF1 interaction frequency. Early sample is indicated by green dashed line, and late sample is indicated by red solid line. SF1 interacting points were marked using grey circles on the *Scr-ftz* region map.

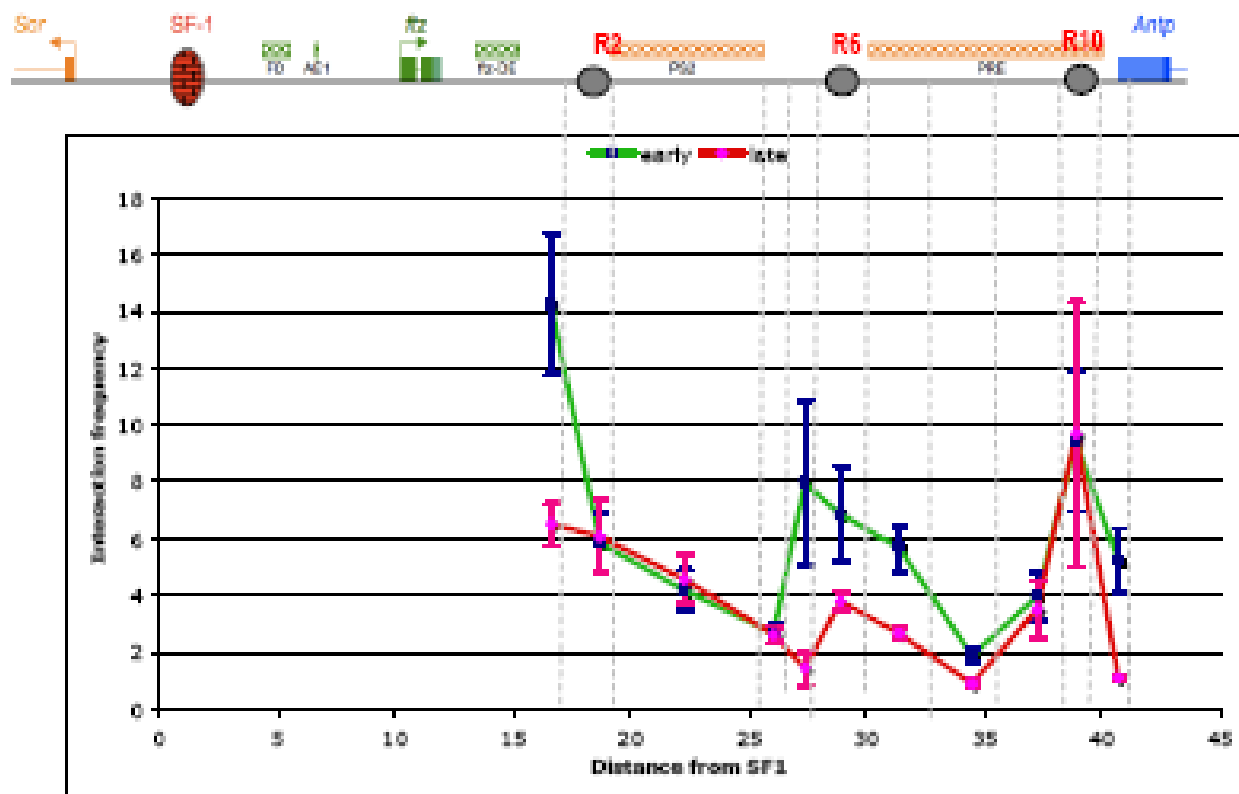


Figure 4. Constructs used for generating transgenic fly lines.

Mini-white and fluorescent reporter genes are divergently transcribed. Embryonic enhancers (H1, SF2-2 and IAB5) were inserted between the two genes, driving the GFP/RFP expression in specific tissue regions.

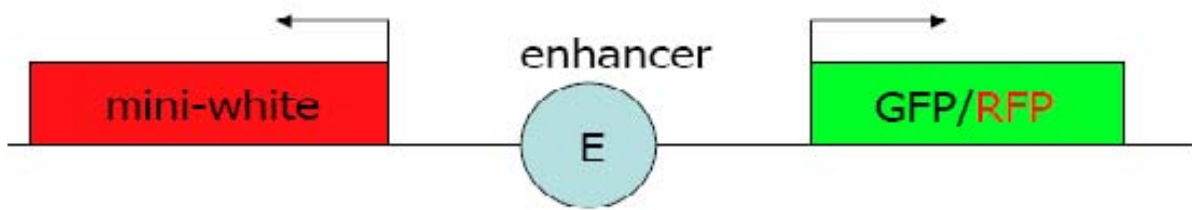


Figure 5. Cross scheme to obtain homozygous transgenic fly lines.

After embryo microinjection, survived larvae were maintained in standard food media at 25 degree allowing them to develop to adulthood. Those flies were then crossed to W^{1118} flies. The progenies were *Screened* for red eye color, which indicates the positive transgenic lines. The heterozygous flies were self-crossed to generate homozygous flies.

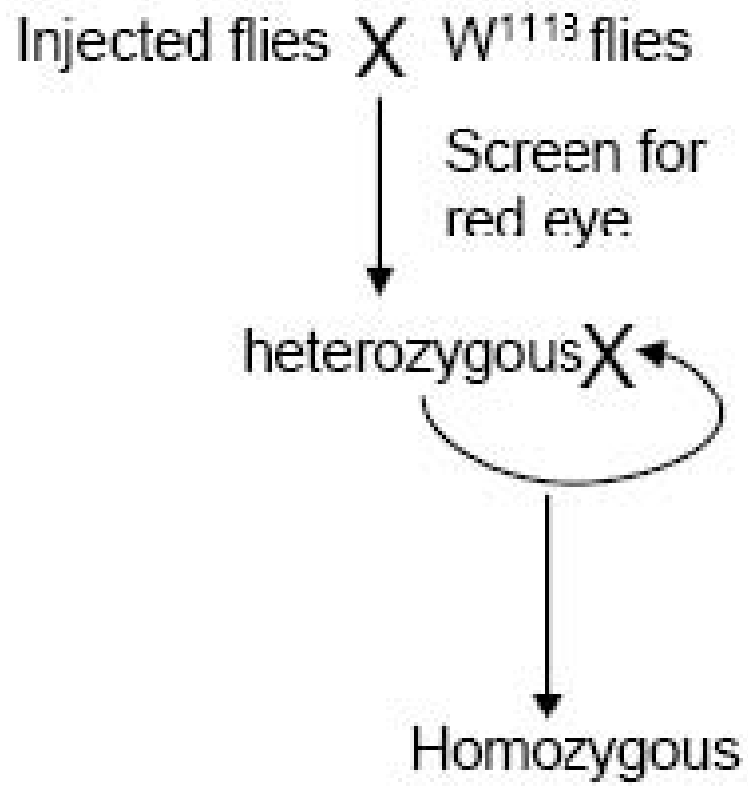


Figure 6. Fluorescent marker labeled transgenic embryos.

Transgenic embryos were generated with fluorescence protein markers driven by early embryonic enhancers.

A) CAH1RFP line expressing RFP at parasegment 0-1. B) CASF2-2GFP line expressing GFP at parasegment 2 and 10. C) CAIAB5RFP line expressing RFP at parasegment 10, 11, and 12. D) CASF2-2GFP & CAIAB5RFP double positive line expressing GFP at parasegment 2, both GFP and RFP resulting in a yellow stripe at parasegment 10, and RFP at parasegment 11 and 12.

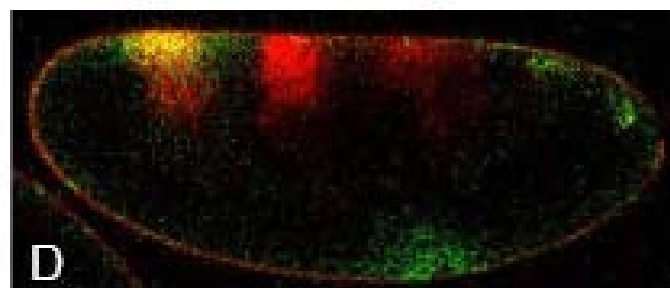
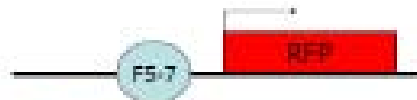
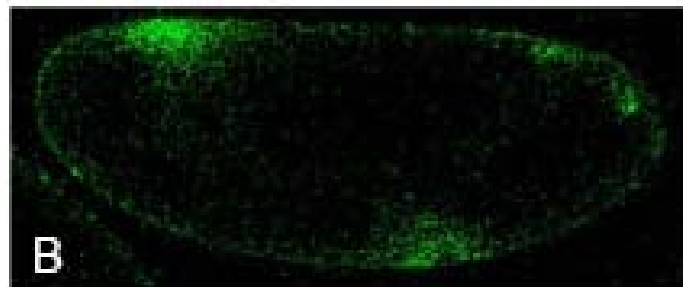
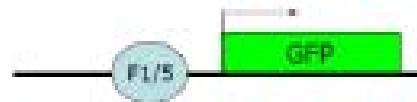
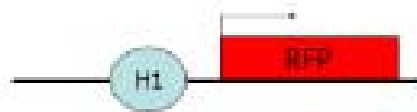
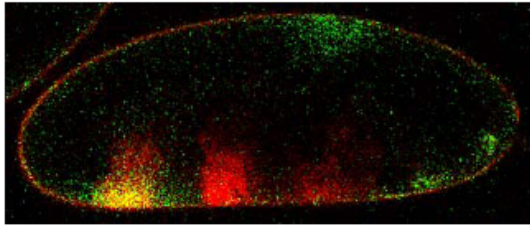


Figure 7. Flow chart of cell dissociation procedure and FACS cell sorting.

Steps of embryo cell dissociation and FACS based cell sorting are shown in the following flow chart.

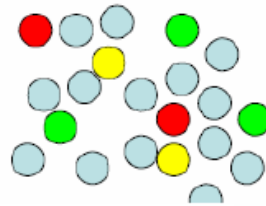
Fluorescence labeled embryos were collected, washed and dechorinated. Embryos were disrupted in glass dounce homogenizer. Dissociated cells were checked to see if they were separated from each other. Trypsin treatment was followed to further dissociate cell clusters. Homogenate was then run through FACS cell sorter. Based on their fluorescent colors, cells were collected into different tubes. After sorting, sorted cells were checked under microscope to see whether they were intact and their fluorescent colors were correct.



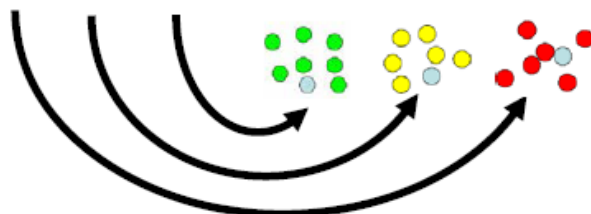
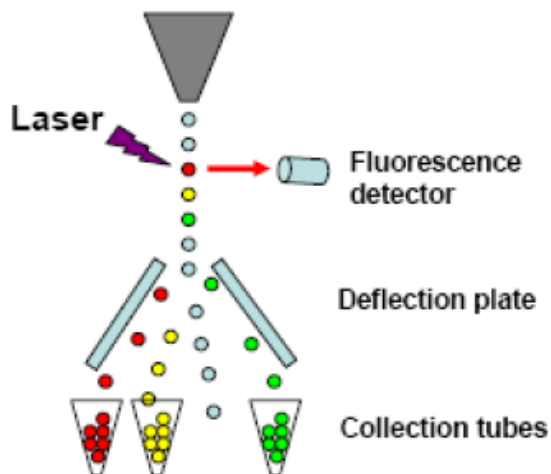
Generation of fluorescence labeled embryos.



Cell dissociation using Dounce homogenizer, followed by trypsin treatment



Quality check of dissociated cells

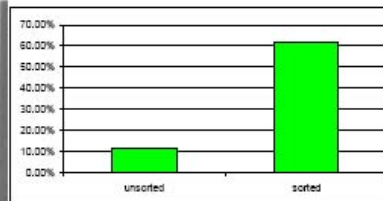
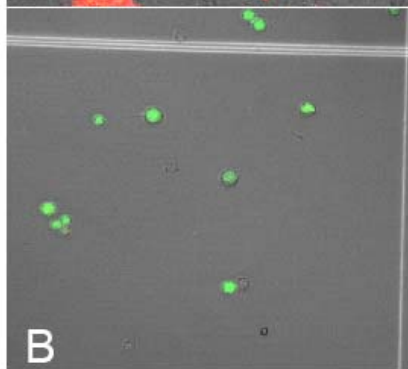
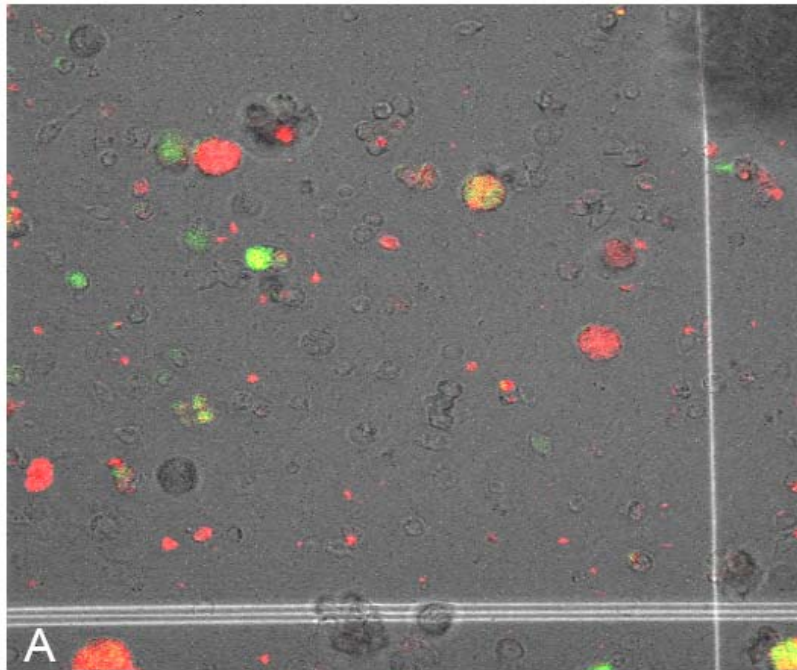


Quality check of sorted cells

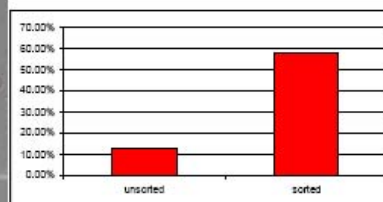
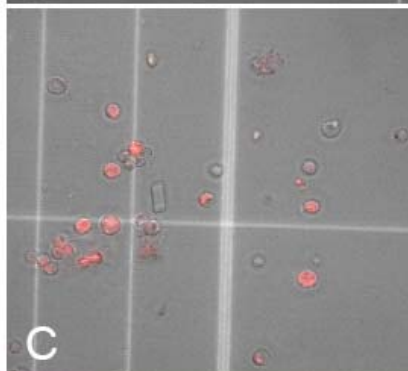
Figure 8. Sorted fluorescent cells were checked under con-focal microscope.

Sorted cells were collected into different tubes, concentrated, checked under microscope.. A small amount of cells were transferred onto a hemocytometer for a cell count. Unsorted cells were counted as well as control.

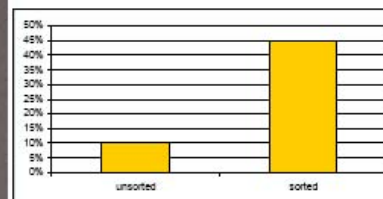
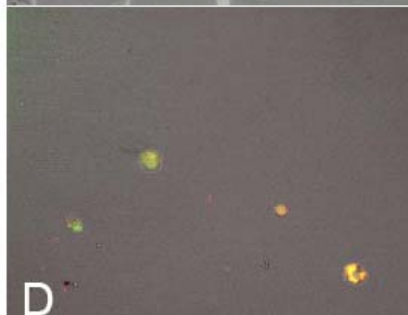
A) Unsorted cells contain dark, red, green and yellow cells. B) In sorted green cells tube, 62% of total cells are green. The fold of enrichment (FE) is 5.24. C) In sorted red cells tube, 58% of total cells are red. The fold of enrichment is 4.59. D) In sorted yellow cells, 45% of total cells are yellow. The fold of enrichment is 4.48.



FE=5.24



FE=4.59



FE=4.48

Figure 9. 3C profiles of samples from different body regions and at different developmental stages.

Quantitative Real-Time PCR was performed on samples from different body regions and at distinct developmental stages. 3C profiles of anterior tissue at early stages (HE), anterior tissue at late stages (HL), posterior tissue at early stages (IE), and posterior tissue at late stages (IL) were compared. Anterior tissue is indicated by green color; posterior tissue is indicated by red color; early tissue is indicated by dashed line; and late tissue is indicated by solid line. The result is combined from two independent experiments. The *Scr-ftz* expression in every isolated tissue is summarized in the table.

A) Comparison of SF1 interaction in anterior tissue at early and late stages. B) Comparison of SF1 interaction in posterior tissue at early and late stages. C) Comparison of SF1 interaction in anterior and posterior tissue at early stages. D) Comparison of SF1 interaction in anterior and posterior tissue at late stages.

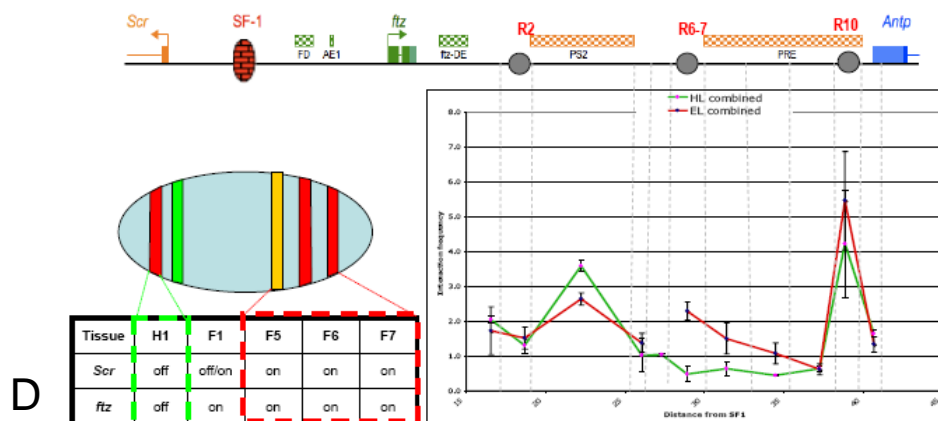
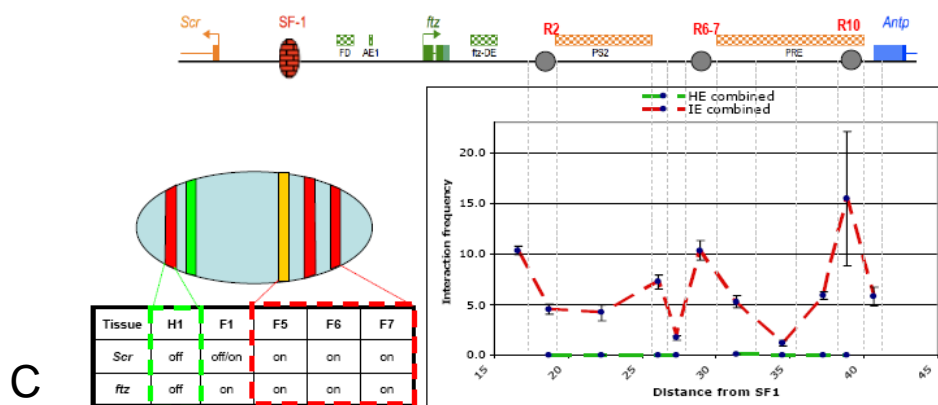
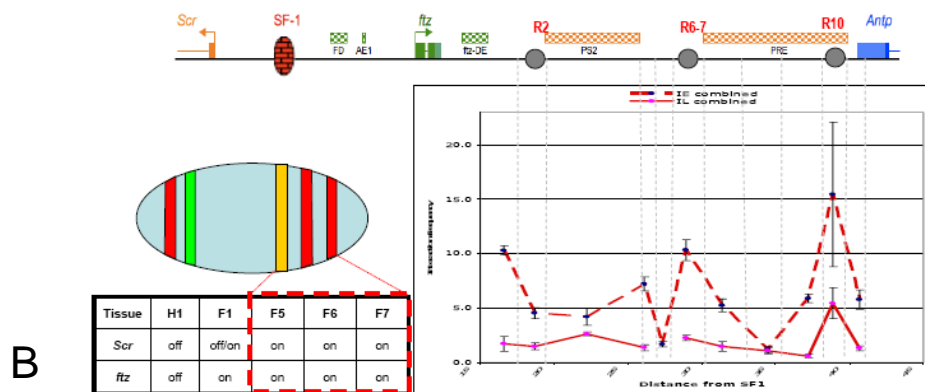
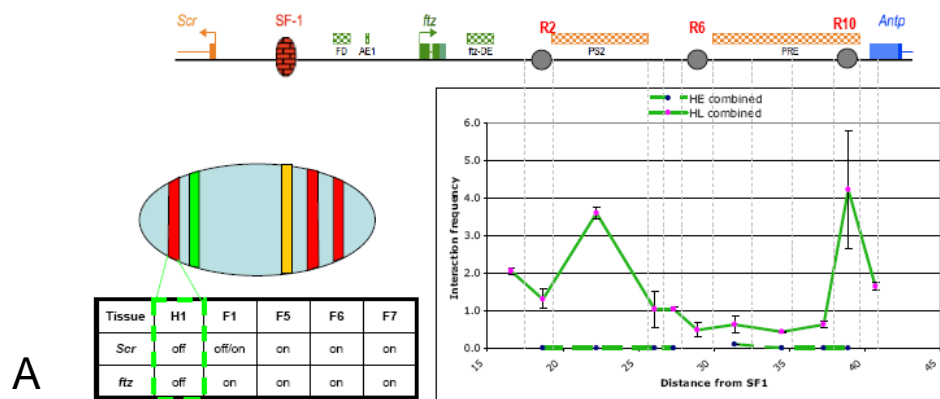
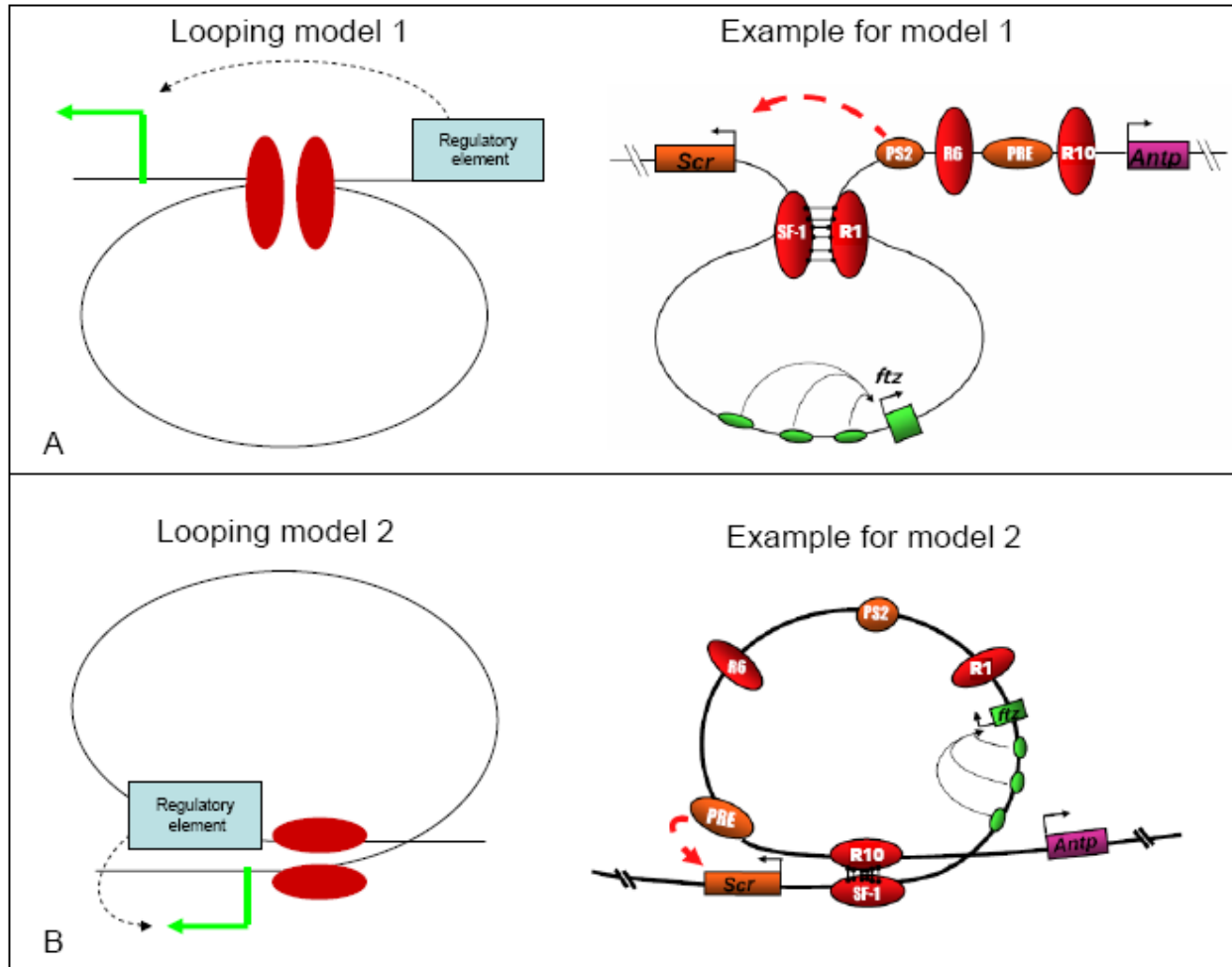


Figure 10. Models of different SF1 interactions.

Two possible models of different SF1 interactions are provided to explain their biological functions. Examples for each model are also shown in this figure.

A) In model 1, when two boundaries interact, the downstream boundary pulls its 3' regulatory element closer to the green promoter, facilitating their communication. SF1 and R1 interaction seen in this study is shown here as an example for this model. B) In model 2, when two boundaries interact, the downstream boundary pulls its 5' regulatory element closer to the green promoter, facilitating their communication. SF1 and R10 interaction seen in this study is shown here as an example for this model. Boundaries are indicated as red ovals, promoters are indicated by green arrows, regulatory elements are indicated by blue boxes, and DNA is indicated by black lines.



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