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Genetic Analysis of the Boundary Element Associated Factors-BEAF32A and BEAF-32B

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GENETIC ANALYSIS OF THE BOUNDARY ELEMENT ASSOCIATED FACTORS-
BEAF-32A AND BEAF-32B

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
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by
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ABSTRACT

Insulators are DNA elements that have been shown to restrict the communication between enhancers and promoters in transgenic assays. In my PhD work I have attempted to highlight the function and importance of insulators by using the Boundary Element Associated Factors- BEAF-32A and BEAF-32B as model insulator binding proteins. These proteins bind to scs’ insulator sequence. We generated a null $BEAF^{AB-KO}$ allele and $BEAF^{a-KO}$ allele by using homologous recombination method. BEAF-32B was found to be sufficient to obtain viable flies. Our results show that BEAF is required for both oogenesis and development. Position independent expression and enhancer-blocking assays showed that BEAF confers insulator function to scs’ sequence. The role of BEAF in chromatin structure and dynamics was shown by the fact that absence of BEAF caused the male X-chromosome morphology to be disrupted and also through position-effect variegation assay. The above process of gene targeting by homologous recombination to obtain $BEAF$ knock-out resulted in the generation of second-site mutations. We showed through several experimental and control crosses that second-site mutations are generated to a significant extent during the process of homologous recombination. Next we showed that BEAF has a role in maintaining patterns of gene expression. We expressed a dominant negative form of BEAF (BID) under GAL4 UAS control by an eye driver which resulted in a rough eye phenotype and this effect could be rescued by introducing an extra copy of a $BEAF$ transgene. Using this assay as a tool we screened for dominant mutations that modified this eye phenotype. The genes identified in this assay mostly belonged to transcription factors involved in head development, or general transcription factors and insulator binding proteins. We then validated the results obtained in the above genetic screen by showing how several genes which positively interacted with BEAF in the above assay show aberrant levels and
patterns of gene expression in BEAF knock-out flies. We also looked at accessibility of certain proteins to DNA in a BEAF knock-out background and found that DNA binding is only subtly affected in the absence of BEAF. The work done here established BEAF as an essential protein. It also showed how BEAF might be involved in the regulation of several important genes. Knowledge gained from these studies would certainly help us to understand the importance of insulators in Drosophila and also in other higher forms of organisms.
CHAPTER ONE
BACKGROUND
Genetic material inside the eukaryotic nucleus is organized into chromatin which is composed of DNA packed with proteins (Laemmli 1978). By electron microscopy two types of chromatin can be visualized, heterochromatin and euchromatin (Friedlander and Hauschteck-Jungen 1986; Lehmann et al. 1987; Stack 1984; Weith 1985). Heterochromatin is transcriptionally inactive and is the condensed form of chromatin. It is seen as dense patches of chromatin. Some lines the nuclear membrane; however, it is broken by clear areas at the pores so that transport is allowed (Fremster 1974). Euchromatin on the other hand is less dense and mainly located away from the nuclear membrane in the nucleoplasm. Euchromatin participates in the active transcription of DNA to mRNA products. The unfolded structure allows gene regulatory proteins and RNA polymerase complexes to bind to the DNA sequence, which can subsequently initiate the transcription process (Bender 2004; Elgin and Weintraub 1975; Gilbert 2002; Pfeifer and Riggs 1991; Shirley and Anderson 1977).

In humans the nuclear diameter is $10^{-5}$ m and the length of DNA is $\sim2$m. To pack this long DNA into the nucleus, DNA needs to undergo several levels of organization (Fuentes-Mascorro et al. 2000; Takeyasu et al. 2004). The first level is the 2 nm DNA filament. One hundred and forty six base pairs of 2nm DNA is wrapped around histone octamers that consist of two molecules each of the core histones H2A, H2B, H3 and H4 to form an 11 nm “beads-on-a-string” structure. This DNA-wrapped histone complex is known as a “nucleosome”. Histone H1 and a spacer region of DNA separate these nucleosomes. This typical beads on a string structure can be visualized by electron micrographs (Allen et al. 1993; Fritzschke et al. 1994; Gusse and Chevaillier 1980). The next level of coiling produces the 30 nm nucleoprotein fibers (Felsenfeld et al. 1996; Gasser 1995; Manuelidis and Chen 1990; Van Holde and Zlatanova 1996). The 30nm fibers are further packed onto a scaffold (Newport 1987;
Moments before cell division, after the DNA replication or S phase of the cell cycle has taken place, the chromatin gets condensed into separate metaphase chromosomes (Benbow 1992). In these metaphase chromosomes the extended scaffold is folded into a helical structure and further packed into a highly compacted structure.

The genome contains regulatory elements known as enhancers which are able to activate expression of their target promoters from distal locations which can be tens of kilobases distant from target promoters (West and Fraser 2005). Many studies have illustrated the fact that enhancer-promoter communication is potentially promiscuous (Kermekchiev et al. 1991). Understanding how enhancers interact with their cognate promoters from such distances is an important area of research. The principal models proposed to explain distal enhancer function invoke some kind of enhancer-promoter communication, either through protein-protein interactions resulting in the formation of DNA loops (looping model), the free sliding of proteins recruited by the enhancer along the DNA (scanning model), or the establishment of modified chromatin domains between the enhancer and the promoter by facilitator proteins which generate a progressive chain of higher order complexes along the chromatin fiber (linking model) (Blackwood and Kadonaga 1998; Bulger and Groudine 1999; Dillon and Sabbattini 2000; Dorsett 1999; Engel and Tanimoto 2000; Martin et al. 1996; Ptashne 1986).

Opposite to the function of enhancers, silencers are DNA sequences that nucleate heterochromatin by the recruitment of repressors (Dhillon and Kamakaka 2002; Rusche et al. 2003). Histones in the heterochromatic region of yeast have been found to be hypoacetylated at histone H3 and H4 compared to transcriptionally active regions, which show hyperacetylation at these histones (Braunstein et al. 1996). Methylation on histone H3 is also used as a signature to distinguish silenced regions from transcriptionally active regions (Dhillon and Kamakaka 2002; Rusche et al. 2003).
Heterochromatic regions are also found to be enriched with various chromodomain containing proteins and these are regions where transcription factor accessibility is very low (Ahmed et al. 2001; Andersen and Horvitz 2007; Bjerling et al. 2004; Hinkins et al. 2005; Jae Yoo et al. 2002; Lechner et al. 2005). Various proteins like Clr4/Su(var)3–9, HP1, Swi6 and Sir proteins influence the formation of silencing in Drosophila, mammals and yeast (Bultman and Magnuson 2000; Eskeland et al. 2007). Models for the spreading of these proteins, and so the spreading of the condensed chromatin state have been proposed (Grewal and Jia 2007).

Enhancer-promoter fidelity is thought to be maintained by subdivision of chromosomes into functional domains. Communication is allowed to occur only within a domain. Models propose the existence of chromatin domain insulators (also known as boundary elements) which create domain boundaries by insulating the interaction of regulatory elements occurring in different domains (Geyer and Clark 2002). Insulators have been found to block communication between enhancers and promoters when placed between them in transgenic assays. But when they are placed upstream or downstream of them they have no effect. This quality of insulators distinguishes them from silencers (Cai and Levine 1995; Scott and Geyer 1995). Transgenic constructs have been used to bracket transgenes with insulators to protect against chromosomal position effects. This function of insulator is thus thought to be responsible in formation of autonomous gene expressing domains (Cuvier et al. 1998; Kellum and Schedl 1991b). Similar to this function insulators are also known to act as barriers by protecting genes from spreading silent heterochromatic regions located in adjacent domains. Thus an open chromatin domain with potentially active genes and an adjacent closed chromatin domain with
inactive genes would be kept separate (Litt et al. 2001; Noma et al. 2001; Prioleau et al. 1999).

Characterization of insulator activity has been done extensively in *Drosophila melanogaster*. For instance, boundary elements have been reported in the Drosophila Bithorax complex. Identity of parasegments 5-14 of Drosophila is specified by the bithorax complex (Karch et al. 1994). Proteins Ubx, Abd-A and Abd-B are three major classes of proteins encoded in the bithorax complex (Gyurkovics et al. 1990). Parasegment identity (PS10-PS13) at the posterior end of the fly is specified by the Abd-B (Barges et al. 2000). The Abd-B regulatory region is subdivided into separate iab domains. The iab domains are flanked by boundaries like Mcp, Fab-7, and Fab-8. These elements restrict the activity of the iab enhancers (Gruzdeva et al. 2005). For instance the iab-6 enhancer is insulated from iab-7 by Fab-7 element. Studies have been performed in which removal of Fab-7 element has resulted in homeotic transformation of parasegment 11 into parasegment 12 (Gyurkovics et al. 1990; Hagstrom et al. 1997). Also the identity of PS12 is specified by initiators located on the proximal side of Fab-8 in the iab-7 region, while initiators that specify PS13 identity are located on the distal side of Fab-8, in iab-8. Transgenic assays have been used to demonstrate that Fab-8 has enhancer blocking activity and that it can insulate reporter constructs from the regulatory action of the iab-7 and iab-8 initiators (Barges et al. 2000).

In vertebrates, the first example of an insulator element was provided by a hypersensitive site of the chicken beta-globin locus, cHS4 (Wai et al. 2003). This insulator at the chicken β-globin locus contains a binding site for the 11 zinc-finger protein CTCF. Binding of CTCF is required for insulator function (Recillas-Targa et al. 1999). Insulator activity has also been found in the mouse. Here the two imprinted genes, *insulin-like growth factor 2 (Igf2)*, and *H19*
are ~80 kb apart and are expressed only from the paternal and maternal alleles respectively (Banerjee et al. 2001; Kurukuti et al. 2006). Both these genes share a set of enhancers which are located downstream of the H19 gene. Expression of these two genes are regulated by an imprinting control region (ICR) located upstream of the H19 locus. CTCF binds to the ICR in the maternal allele, blocking communication between the enhancers and the Igf2 gene. Hence only the H19 gene is maternally expressed. On the paternal chromosome the ICR is methylated and this inhibits CTCF binding. The Igf2 promoter interacts with the downstream enhancers which drives expression of Igf2. The hypermethylated ICR at the same time causes silencing of the H19 promoter (Holmgren et al. 2001; Kanduri et al. 2002; Kurukuti et al. 2006; Ling et al. 2006; Pant et al. 2004; Szabo et al. 2004; Yang et al. 2003).

Scs and scs’ are Drosophila sequences located at the proximal and distal boundaries of the 87A7 heat-shock puff of polytene chromosomes (Farkas and Udvardy 1992; Udvardy et al. 1985). They are two of the first sequences demonstrated to have insulator activity in the enhancer blocking and position independent expression assays. In the enhancer blocking assay an insulator sequence is placed between an enhancer and a transgene. Insulators have been found to block this enhancer-transgene communication when placed between them. But when they are placed upstream or downstream they have no effect (Kellum and Schedl 1991). In the position-independent expression assay, a transgene lacking an enhancer is bracketed by insulator sequences on either side. It has been shown that since the transgene lacks an enhancer, bracketing it with insulators leads to low levels of expression of the transgene. In the absence of insulators, chromosomal position effects result in activation of the transgene (Geyer 1997; Kellum and Schedl 1991; Roy et al. 2007; Zhang and Liu 2004). Another well-studied insulator in Drosophila is the 340 bp gypsy element from the gypsy retrotransposon. This element also has enhancer activity like the scs and scs’ insulators. The zinc finger protein su(Hw) binds to the gypsy element, which has
12 su(Hw) binding sites, and confers insulator function (Geyer and Corces 1992; Roseman et al. 1993).

The Boundary Element Associated Factor (BEAF) was purified based on binding to scs’ (Zha et al. 1995). This led to cloning cDNAs for the two BEAF proteins BEAF-32A and BEAF-32B (Hart et al. 1997). Both these proteins are derived from the same BEAF gene presumably by alternative transcription initiation. The proteins only differ in their amino termini where BEAF-32A has 81 amino acids and 32B has 80 amino acids. The amino terminus is the DNA binding domain which is an atypical Zn finger (BED finger) (Aravind 2000). The rest of the 202 amino acids are identical, being derived from the same exon for both proteins. These 202 amino acids can be further divided into two regions, the middle M domain (amino acids 81 to 203) and the C-terminal domain (amino acids 203-282). No function has been associated with the middle domain. The C-terminal domain is required for interactions between BEAF subunits, resulting in homo or heterocomplex formation by 32A and 32B (Hart et al. 1997). This region has a potential leucine zipper and a BESS domain (Bhaskar and Courey 2002; Delattre et al. 2002; Ratnaparkhi et al. 2008). One or both of these presumably mediate interactions between BEAF subunits. From immunostaining of polytene chromosomes isolated from salivary glands of third instar larvae, BEAF localizes to hundreds of sites which are predominantly interbands and puff borders (Hart et al. 1997). Very little is known about functional differences between 32A and 32B.

Genetic tools have been developed to study BEAF function and mechanism. First a dominant negative form of BEAF was designed. This gene encode the BEAF self-interaction domain (BID) but lacks the N-terminal DNA binding domains. BID binds to BEAF proteins by its self-interaction domain and it acts as a dominant negative form of BEAF by interfering with
DNA binding. Immunoprecipitation data shows that BID and BEAF physically interact in vivo (Gilbert et al. 2006). The BID protein is expressed using the GAL4-UAS system. This system has proved to be a powerful tool for studying gene expression in *Drosophila melanogaster* (Brand and Perrimon 1993; Duffy 2002). The system has two parts: first an expression cassette containing the *GAL4* gene encoding the yeast transcription factor protein GAL4, and a cassette containing the UAS (Upstream Activation Sequence) to which GAL4 specifically binds to activate gene transcription. The *GAL4* driver gene is placed under the influence of a promoter showing a regulatory pattern of interest, while the UAS controls expression of target gene (in this case *BID*). When *BID* is ubiquitously expressed by a daughterless driver using this GAL4-UAS system the embryo is unable to survive past embryogenesis. This indicates that *BEAF* is an essential gene required during embryo development stages. Expression of *BID* in salivary glands by using a GAL4 salivary gland driver system leads to a global disruption of polytene chromosome structure and to a loss of the BEAF immunostaining pattern. The effect of BID on the polytene chromosome indicates that BEAF has a role in chromatin structure or dynamics. This was further verified by testing the effect of BID protein on position-effect variegation (PEV) of the *w^m4h* gene. A chromosomal inversion on the X chromosome brought this gene near pericentric heterochromatin. This rearrangement results in variegated expression in eyes due to silencing of *w^m4h* by variable spreading of pericentric heterochromatin, which is detected as varying numbers of pigmented ommatidia (Tartof et al. 1989). The level of variegation is very sensitive to mutations that directly or indirectly affect chromatin organization. BID expression resulted in enhancement of PEV by lowering the *w^m4h* gene expression in eyes (Gilbert et al. 2006). BID was also expressed in the adult fly eye by the help of a GAL4 eye driver. This gave rise to a rough eye phenotype in which the eye ommatidia are disorganized. These chromosomal
and eye effects can be reversed and rescued by adding a third copy of the \textit{BEAF} gene demonstrating BID specifically interferes with BEAF function (\textit{Gilbert et al. 2006}). BID interferes with scs’ insulator function in both a position independent expression and enhancer-blocking assay confirming that BEAF has insulator activity.

As a second tool to study BEAF function, knock-out alleles of \textit{BEAF} were generated by homologous recombination. This is the focus of chapter two. Using ends-in homologous recombination (\textit{Rong and Golic 2000; Rong et al. 2002}), a knock-out of 32A (\textit{BEAF}^{a-KO}) and a full knock out (\textit{BEAF}^{AB-KO}) flies were generated. It was observed that the 32B protein alone can maintain healthy, viable flies. But elimination of both BEAF proteins demonstrated that \textit{BEAF} is an essential gene. Although essential, adult flies lacking BEAF are obtained. Oogenesis and development are both affected by a lack of BEAF. As shown for the BID protein, we found that BEAF is essential for the insulator activity of scs’ but not of the scs or gypsy insulators (which use the Zw5 and su(Hw) proteins, respectively). Like results obtained with BID, results with \textit{BEAF}^{AB-KO} also indicate that BEAF plays a role in chromatin structure or dynamics.

While generating the knock-out mutations of BEAF by the above gene targeting technique (\textit{Roy et al. 2007a; Zhao et al. 1995}), we obtained three types of unusual results. First we obtained unexpected recombination products, and the other two involved the apparent generation of second-site mutations during homologous recombination or during reduction of the resulting gene duplication to a single copy. The \textit{BEAF} mutations were separated from the non-targeted mutations by meiotic recombination (\textit{McKim et al. 2002}). We have since found two other reports that mention the presence of second-site mutations after generating mutations in Drosophila by homologous recombination (\textit{Lankenau et al. 2003; O’Keeffe et al. 2007}), suggesting the occurrence of these events is not uncommon. In chapter five we used the
procedure that can result in ends-in homologous recombination to look for second-site lethal mutations generated on the third chromosome. Our results clearly show that the method used to promote gene targeting by homologous recombination causes a modest but significant increase in the rate of spontaneous, non-targeted lethal mutations.

In the third chapter we describe a screen for proteins that genetically interact with BEAF. For this, we used the rough eye phenotype caused by \textit{BID} expression in eye imaginal discs. We reasoned that mutations in genes that are important for BEAF function would modify this phenotype. Identification of these genetic interactions should provide insight into BEAF function. Based on models of function and our evidence that BEAF affects chromatin, we thought that BEAF might interact with proteins involved in chromatin structure or dynamics. Of 30 such genes tested, \textit{Nipped A} and \textit{spindle-E} were the only genes that showed positive interactions (enhanced the rough eye phenotype). Instead, most interactions were with insulator binding proteins and transcription factors involved in head development. All these interactions can be interpreted as downstream effects of interfering with BEAF function, supporting the hypothesis that BEAF plays an important role in maintaining global patterns of gene regulation during processes such as eye development. The existence of cross-talk between different classes of insulators is also apparent from this work, which could be an indirect interaction based on their common roles in maintaining gene expression patterns. The interaction with \textit{spn-E} might indicate that BEAF function involves RNAi pathways (PAL-BHADRA \textit{et al.} 2004). If so, following up on this could provide insight into how BEAF functions.

According to our working model, BEAF has a global role in maintaining patterns of gene expression. We explored this hypothesis in the fourth chapter, extending the work described in chapter three. We show how the absence of BEAF leads to altered expression levels and patterns
of several genes that genetically interacted with BEAF. We also look at accessibility of proteins to DNA in a $BEAF^{AB-KO}$ background. To observe expression levels and patterns, the genes *fushi tarazu, suppressor of Hairy wing, Deformed, bicoid* and *proboscipedia* were selected based on their strong interaction with BEAF in the rough eye assay. To observe binding to polytene chromosomes, the genes *Dref* and *maleless (mle)* were chosen. *Dref* was selected because previous evidence showed that BEAF and DREF compete for overlapping binding sites in DNA, and that this competition occurs in vivo (HART et al. 1999). Although male Drosophila have one X chromosome rather than the two present in females, they require the same amount of gene products encoded by the X. This difference in chromosome number is taken care in flies by the dosage compensation complex which binds to the male X chromosome to double the final amount of X-encoded gene products (KELLEY and KURODA 1995; KELLEY et al. 1995). MLE binds to only the male X chromosome since it is a component of the Drosophila dosage compensation complex (COPPS et al. 1998). Our initial reason for choosing MLE was to mark the X chromosome. But we found MLE binding was subtly affected in the absence of BEAF.

We extracted RNA from wild-type and $BEAF^{AB-KO}$ embryos, collected at different time points. q-RT-PCR was conducted on these RNA samples with gene specific primers to look at levels of gene expression. $su(Hw)$ was over-expressed in $BEAF^{AB-KO}$ embryos to a significant extent. The levels of *ftz, Dfd, pb* and *bcd* dropped especially at later time points in the absence of BEAF. The *Dref* level was unaffected in $BEAF^{AB-KO}$ embryos. Next we looked at patterns of gene expression in embryos by in situ hybridization. *ftz, Dfd* and *bcd* showed altered expression patterns in $BEAF^{AB-KO}$ embryos while *su(Hw)* and *Dref* expression patterns remained unaltered. Polytene chromosomes were immunostained with antibodies for DREF and MLE to look for any alteration of binding of these proteins in the absence of BEAF. No obvious difference in DREF...
binding was observed in a $BEAF^{AB\text{-}KO}$ background. There was however subtle differences in MLE binding to male X polytene chromosomes in the absence of BEAF.

Current models propose that chromatin insulators define domain boundaries by insulating elements within a domain from interactions with elements located in other domains, and in this way they maintain enhancer-promoter fidelity and long distance gene regulation (Geyer and Clark 2002; Sasaki et al. 2000; West and Fraser 2005). Here in this work BEAF has been used as a model system to study insulator function and mechanism. Studies have shown that BEAF binds to the scs’ insulator sequence and confers insulator activity (Gilbert et al. 2006). Here we studied $BEAF$ function by developing genetic tools. We demonstrated that BEAF is required for scs’ insulator function through various assays. The role of BEAF in maintaining chromatin structure and dynamics was implicated in this work. BEAF was also found to play an important role in embryogenesis and oogenesis, presumably through effects on gene regulation. Finally these studies support the proposed role of insulators in maintaining gene regulation by demonstrating that expression levels and patterns of some genes are altered in the absence of BEAF. A future challenge will be to elucidate mechanisms by which BEAF interacts with chromatin structure and affects gene regulation.

**Literature Cited**


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CHAPTER TWO

CHARACTERIZATION OF BEAF MUTATIONS ISOLATED BY HOMOLOGOUS RECOMBINATION IN DROSOPHILA*

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Introduction

Enhancers can act over large distances and are capable of activating transcription from diverse promoters (Kermechiev et al. 1991). Chromatin domain insulators are thought to help prevent promiscuous interactions between enhancers and promoters by dividing chromosomes into domains such that interactions can occur within domains but cannot occur between elements located in different domains. Perhaps the best known example that illustrates the importance of insulators is the imprinted mammalian insulator downstream of the Igf2 gene (Bell and Felsenfeld 2000; Hark et al. 2000). This insulator is not methylated on the maternal chromosome, allowing binding of the CTCF protein which blocks activation of Igf2 by a downstream enhancer. The insulator is methylated on the paternal chromosome, which prevents binding by CTCF and allows activation of Igf2 by the downstream enhancer. Inactivation of the insulator on both chromosomes can lead to Beckwith-Wiedemann fetal overgrowth syndrome and the development of Wilms’ tumor (Frelve et al. 1999; Reik et al. 1995). In Drosophila, deletion of the Fab-7 insulator in the bithorax complex leads to homeotic transformation of adult abdominal segment 6 (AS6) into another copy of the more posterior AS7 (Mihaly et al. 1997).

There are differences between insulators in certain assays, indicating that different molecular mechanisms can result in insulator activity (for examples, see (Hogga et al. 2001; Parnell and Geyer 2000). In addition, some insulators are composite elements with separate components responsible for blocking enhancer-promoter communication and for acting as a barrier against chromosomal position effects (Recillas-Targa et al. 2002). It is not clear how any insulator functions at the molecular level. The various models that have been proposed include acting as promoter decoys, influencing chromatin structure or dynamics, and nuclear organization (Gaszner and Felsenfeld 2006; Kuhn and Geyer 2003; Labrador and Corces...
These models are not mutually exclusive. To understand how insulators function, it is necessary to study the proteins involved in insulator activity.

We are interested in the two 32 kDa Drosophila boundary element-associated factors, BEAF-32A and BEAF-32B. Throughout this paper we refer to these proteins together as “BEAF” and individually as “32A” or “32B”. BEAF binds to the scs’ insulator as well as to hundreds of other sites on chromosomes (Hart et al. 1997; Zhao et al. 1995). A few other genomic BEAF binding sites have been identified, and they function as insulators in transgenic fly assays (Cuvier et al. 2002; Cuvier et al. 1998). This suggests that BEAF-dependent insulators are a common class of insulator in Drosophila. 32A and 32B are derived from the same gene. They have unique amino-terminal DNA binding domains of about 80 amino acids, but the remaining 200 amino acids are encoded by a shared exon. BEAF forms complexes with itself, and this is mediated by a region near the carboxy-terminus (Hart et al. 1997). Because there were no mutations available in the BEAF gene, we previously designed a transgene under GAL4 UAS control that encodes a dominant negative BEAF protein (Gilbert et al. 2006). Here we expand on that work by generating and characterizing mutations in the BEAF gene.

We used ends-in homologous recombination (Rong and Golic 2000; Rong et al. 2002), to generate a knockout mutation in the BEAF gene (BEAF<sup>ΔB-KO</sup>). In the process, we also isolated an allele that eliminates the ability to produce the 32A protein (BEAF<sup>ΔA-KO</sup>). We find that the 32B protein is sufficient to obtain healthy, viable flies. In contrast, eliminating both BEAF proteins reveals that BEAF is essential. Oogenesis and development are both affected by a lack of BEAF. We demonstrate that BEAF is required for the insulator activity of scs’, but not of the scs insulator (which binds the Zw5 protein; (Gaszner et al. 1999) or the gypsy insulator (which binds the su[Hw] protein; (Harrison et al. 1989). We also provide evidence that BEAF function
affects chromatin. This confirms and extends results we obtained with the dominant negative BEAF protein, and supports the hypothesis that BEAF functions by affecting chromatin structure or dynamics.

**Materials and Methods**

**DNA Constructions and Germline Transformation**

Cloning of the *BEAF* gene as a 5 kb *Bgl*II fragment generated from genomic DNA by PCR has been described (Fig. 2.1A), as has generation of transgenic flies containing this *gBF* rescue transgene (GILBERT *et al.* 2006). Site-directed mutagenesis was used to introduce mutations into this gene (Fig. 2.1B; Quikchange, Stratagene). One mutation eliminated the ATG start codon of *BEAF-32A* and destroyed an *Nsi*I site. A second mutation eliminated the ATG start codon of *BEAF-32B* and created an *Apa*I site. Alternative ATG codons for both 32A and 32B are in the wrong reading frames. A third mutation introduced two tandem stop codons into the exon shared by both 32A and 32B and destroyed a *Bam*HI site. A fourth mutation introduced an *I-Sce*I site into the intron between the unique 32B exon and the shared exon. The *I-Sce*I site is 3.7 kb downstream of the 5' end of the cloned sequences and 1.2 kb upstream of the 3' end. It is also about 280 bp downstream of the 32B mutation and about 300 bp upstream of the introduced stop codons. All mutations were confirmed by restriction digestions and sequencing. The resulting mutant *BEAF* (*mBF*) gene was cloned into the *Not*I site of pTV2 (RONG *et al.* 2002). This plasmid (0.4 μg/μl) was co-injected with the helper plasmid pπ25.7wc (0.1 μg/μl) into preblastoderm *y¹ w⁶⁷c²³* embryos to generate P[w⁺ mBF] transgenic flies (SPRADLING 1986).

A *P*-element plasmid encoding a *BEAF-EGFP* fusion gene was also constructed (referred to as *GFBF* for *Green Fluorescent BEAF*; Fig. 2.1D). The stop codon of the *BEAF* gene was mutated to a *Kpn*I site. pEGFP-N3 (CLONTECH) was modified by deleting a 600 bp *AseI-Bgl*II
fragment encoding the CMV-IE promoter. A 2.7 kb EcoRI-KpnI BEAF gene fragment was ligated into the modified pEGFP-N3 plasmid to fuse EGFP sequences in frame at the carboxy end of the BEAF sequences. About 900 bp of sequences upstream of the BEAF-32A ATG are present. This likely contains all regulatory elements of the BEAF promoter since a divergent gene, CG10155, is reported to initiate transcription about 265 bp upstream from the 5’ end of this fragment. An EcoRI-AflII fragment, from the BEAF promoter through the SV40 polyadenylation site, was cloned into pM2 (Cuvier et al. 1998). pM2 is a derivative of pCaSpeR4 with the scs’-derived M2 and scs insulators, so the fusion gene is insulated. This P[w+ GFBF] construct was injected into embryos as described above to generate transgenic fly lines.

**Drosophila Stocks**

Flies were maintained on standard cornmeal, yeast and sugar medium with Tegosept. Crosses were performed at 25°C. The yellow (y) enhancer blocking lines (2scs’ inserted at 19D; scs inserted at 60A; gypsy inserted at 25C) have been previously described (Kuhn et al. 2004). Generation of the M2 mini-white position-independent expression lines is described in (Gilbert et al. 2006). The y variegating lines KV732 (X heterochromatin band 29H), KV600 (X 26H) and KV123 (3L 48H) were kindly provided by G. H. Karpen (University of California at Berkeley). All other fly lines used were from the Bloomington Drosophila Stock Center (flystocks.bio.indiana.edu).

**Isolation of BEAF Mutations by Ends-in Homologous Recombination**

Flies with P[w+ mBF] on the X or the CyO balancer chromosome were used to generate mutations in the BEAF gene by homologous recombination (Rong and Golic 2000). BEAF is on the second chromosome. Briefly, P[w+ mBF] females were crossed to 70I-SceI 70FLP/TM6 males. Larvae were given one heat shock at 38°C for one hour in a water bath. For crosses with
$\text{P[w}^+ \text{ mBF]} \text{CyO, white eyed female progeny with CyO were crossed to } y^1 \text{ w}^{67c23} \text{ males and}

\text{progeny with red eyes but lacking CyO were crossed to CyO/Sp}^I \text{ flies to screen for potential}

\text{homologous recombination events. For crosses with P[w}^+ \text{ mBF] on the X chromosome, white}

\text{eyed female progeny from the first cross were crossed to 70FLP/70FLP males and the larvae}

\text{were given a one hour 38°C heat shock. This eliminated background in the next generation}

\text{caused by progeny with the original P[w}^+ \text{ mBF] transposon. Males with red eyes were then}

\text{crossed to CyO/Sp}^I \text{ females to screen for potential homologous recombination events. For the}

\text{P[w}^+ \text{ mBF] CyO strategy approximately 82,500 chromosomes were screened ([1100 vials x 150}

\text{flies/vial]/2 because of the CyO chromosome). Eight mobilizations were recovered, only one of}

\text{which was due to homologous recombination. For the strategy using P[w}^+ \text{ mBF] on the X}

\text{chromosome approximately 100,500 chromosomes were screened (670 vials x 150 flies/vial).}

\text{Three mobilizations were recovered, all of which were due to homologous recombination.}

\text{Homologous recombination was confirmed by genomic PCR. Ends-in homologous}

\text{recombination results in a gene duplication with the mini-white marker gene between the two}

\text{copies. Primer pairs were used that would specifically amplify the upstream gene copy, the}

\text{downstream gene copy or the original single copy BEAF gene, all as 5 kb fragments. Amplified}

\text{DNA was sequenced and analyzed by restriction digestions. We found that one recombination}

\text{event resulted in both gene copies having a mutated 32A ATG, but the 32B and shared sequences}

\text{were intact. This is the BEAF}^{d-KO} \text{ allele, and the chromosome is w}^+. \text{The other three}

\text{recombination events had at least one wild-type gene copy. One was determined to have the 32A}

\text{and 32B ATG mutations as well as the tandem stop codons in the downstream gene copy. This}

\text{gene duplication was reduced to a single copy by crossing flies to a 70I-CreI Sb/TM6 line and}

\text{giving the larvae a one hour 38°C heat shock (Rong et al. 2002). w}^+ \text{ mosaic males were selected}
and crossed to CyO/Sp\(^{1}\) females. In the following generation flies with CyO but lacking the 70I-CreI Sb chromosome were selected and individually crossed to CyO/Sp\(^{1}\) flies again. Flies that eclosed and lacked Sp\(^{1}\) were then self-crossed. Flies were screened by PCR and restriction digestion to identify the BEAF\(^{AB-KO}\) chromosome, which is w'. Primer sequences used for mutagenesis, PCR and sequencing are available upon request.

**Viability Assays**

To examine the effect of lack of maternal BEAF on female fertility and egg viability, flies of the genotypes indicated in Table 2.1 were crossed in fly cages sealed with grape juice agar plates smeared with yeast paste. The agar plates were changed every 24 hours and embryos were counted. Hatched larvae were counted and transferred with a brush to vials, and pupae and adults were counted as they appeared. To facilitate collection of BEAF\(^{AB-KO}\) female virgins, the BEAF\(^{AB-KO}\) chromosome was placed over a CyO GFP w\(^{+}\) balancer chromosome and third instar larvae were placed in PBS and sorted by fluorescence microscopy. Homozygous BEAF\(^{AB-KO}\) larvae were placed in a new vial to pupate and eclose. Surprisingly, this treatment improved the fecundity of the BEAF\(^{AB-KO}\) flies and vigor of their progeny.

To determine the viability of BEAF\(^{AB-KO}\) flies provided with maternal BEAF, six males and six females of the genotype BEAF\(^{AB-KO}/CyO\) were placed in a vial for three days, then transferred to a new vial for an additional three days. The number of BEAF\(^{AB-KO}\) and BEAF\(^{AB-KO}/CyO\) adults that eclosed was recorded.

**Ovary Dissection and DAPI Staining**

Wild-type or BEAF\(^{AB-KO}\) females were mated with wild-type males for 4 days before dissection. Ovaries were dissected in PBS (0.9% NaCl, 14 mM Na\(_2\)HPO\(_4\), 6 mM NaH\(_2\)PO\(_4\), pH 7.3). The dissected ovaries were fixed with 4% formaldehyde in PBS for 15-30 min., then
stained with DAPI (250 ng/ml DAPI in PBS/0.1% Triton-X100) or propidium iodide (100 ng/ml) plus RNase A (200 μg/ml) for 30 min. The stained ovaries were transferred to a glass slide with a drop of 60% glycerol in PBS and observed with a Zeiss Axioskop microscope equipped with a SPOT RT Slider CCD camera (Diagnostic Instruments, Inc.) or a Leica TCS-SP2 confocal microscope.

**Insulator and Position Effect Variegation (PEV) Assays**

All test genes were on the X or third chromosome, and the presence of these chromosomes could be followed by eye pigmentation. The cross strategy took advantage of the normal fertility of BEAF4AB-KO males. Males from the test lines were crossed to CyO/Sp1 females. In the next generation females with the test gene and CyO were selected and crossed to BEAF4AB-KO males. Females with the test gene and BEAF4AB-KO/CyO were selected and crossed again to BEAF4AB-KO males. BEAF4AB-KO flies with one copy of the test gene were compared with wild-type flies with one copy of the test gene (generated by crossing test flies to w y flies). For PEV assays, phenotypes of BEAF4AB-KO/CyO flies with one copy of the test gene were also recorded. Eyes were photographed using darkfield illumination with a x4 objective on a Zeiss Axioskop microscope equipped with a Spot RT Slider CCD camera (Diagnostic Instruments, Inc.). Abdomens were photographed at 50x magnification using fiberoptic illumination on a Zeiss Stemi 2000 stereomicroscope equipped with a Spot RT Slider CCD camera. Eye pigment was quantitated by homogenizing the heads of 20 males in 200 μl 0.1% ammonium hydroxide, extracting once with chloroform, and determining the OD480 of the solution (ASHBURNER 1989).

**Immunostaining Polytene Chromosomes**

Polytene chromosomes were prepared from salivary glands of healthy, wandering third instar larvae and immunostained as previously described (GILBERT et al. 2006). For this purpose,
a fly line with the \textit{BEAF}_{AB-KO} allele over a \textit{w}^{+} \textit{CyO GFP} balancer. Homozygous \textit{BEAF}_{AB-KO} larvae derived from this line were identified by the lack of green fluorescent protein. Affinity-purified rabbit anti-BEAF antibody was used at a 1:50 dilution. Rabbit antibodies against the X-chromosome dosage compensation complex components MOF, MLE, MSL-1, MSL-2 and MSL-3 were kindly provided by M.I. Kuroda (HHMI and Harvard Medical School) and J.C. Lucchesi (Emory University), and were used at 1:500 dilutions (except MSL-2: 1:250). Rabbit anti-histone H4-acetyl-lysine 16 was purchased from Upstate Biotech (07-329) and used at a 1:400 dilution. Texas Red or FITC-conjugated goat anti-rabbit secondary antibodies were used at 1:400 dilutions (Jackson, West Grove, PA). Chromosomes were stained with 100 ng/ml DAPI. Slides were viewed with a Zeiss Axioskop microscope equipped with a Spot RT Slider CCD camera. For viewing GFP fluorescence, salivary glands were fixed for 1 minute with 3.7% paraformaldehyde in PBS plus 5% Triton X-100, stained 20 minutes with 100 ng/ml DAPI in PBS plus 2% Triton X-100, and washed 2 minutes in 50% glycerol. The chromosomes were then gently spread in a fresh drop of 50% glycerol and viewed immediately.

\textbf{Scanning Electron Microscopy}

Flies were prepared and SEM was performed as previously described (\textit{Gilbert et al.} 2006).

\textbf{Results}

\textbf{Generation of Mutant \textit{BEAF} Alleles by Homologous Recombination}

The \textit{BEAF} gene encodes two related 32 kDa proteins, BEAF-32A and BEAF-32B (Fig. 2.1A). These proteins have different amino-terminal DNA binding domains encoded by unique exons, while the remainder of the proteins are identical and are encoded by a shared exon. We constructed a mutant \textit{BEAF} transgene (\textit{mBF}) by introducing point mutations to eliminate the 32A
and 32B ATG start codons and insert two tandem stop codons into the shared exon (Fig. 2.1B). Each mutation either created or destroyed a restriction site. Flies containing this mBF transgene were used to generate flies with mutant alleles of BEAF by ends-in homologous recombination (Rong and Golic 2000; Rong and Golic 2001; Rong et al. 2002). This commonly results in a gene duplication bracketing the mini-white marker gene (Fig. 2.1C). Using primer pairs anchored in genomic sequences outside of the transgene sequences and in the mini-white sequences (indicated in Fig. 2.1C), we confirmed four such gene duplication events by individual PCR amplification of the BEAF gene upstream and downstream of the mini-white gene. Sequence and restriction digestion analyses found that one gene duplication had the 32A ATG mutation in both gene copies, but lacked the other mutations. We refer to this as the BEAF\textsuperscript{32A-KO} allele, and the chromosome is w\textsuperscript{+}. The other three gene duplications had at least one wild-type BEAF allele. One had all three BEAF mutations in the downstream copy. This was reduced to a single copy using I-CreI endonuclease (Rong et al. 2002), and flies retaining all three mutations were identified by PCR analysis. We refer to this as the BEAF\textsuperscript{32A\textendash}32B-KO allele, and the chromosome is w\textsuperscript{−}.

Flies homozygous for both mutant alleles were able to eclose. We analyzed these flies by PCR and Western blotting to confirm that they had the BEAF mutations (Fig. 2.2). For this PCR analysis, gene-specific primers were not used. Instead, primer pairs were used that generated 500 bp fragments from all BEAF genes present (indicated in Fig. 2.1B). Each fragment encompassed a site that was mutated in the mBF transgene to allow detection of the mutations by restriction analysis. The Western analysis of BEAF\textsuperscript{32A-KO} flies used antibodies specific for either the 32A or the 32B protein, while the Western analysis of BEAF\textsuperscript{32A\textendash}32B-KO flies used an antibody that recognizes both of the BEAF proteins. These analyses demonstrated that BEAF\textsuperscript{32A-KO} flies only have the 32A ATG mutation and make 32B protein, but no detectable 32A protein (Fig. 2.2B, C). Similarly,
the $BEAF^{AB-KO}$ flies have all three of the BEAF mutations and do not produce any detectable BEAF protein (Fig. 2.2E, F).

**Figure 2.1 Strategy for targeted mutagenesis by homologous recombination.** (A) Map of the BEAF gene, showing part of the upstream divergent CG10155 gene and downstream convergent knot (kn) gene. Arrows indicate the direction of transcription, and thin lines represent introns. Note the unique 5’ exons and shared 3’ exon for BEAF-32A and BEAF-32B. (B) The BEAF gene was cloned as a 4.9 kb BglII fragment (gBF). Mutations were introduced at four locations to make the mutant mBF clone. The mBF gene was used for targeted mutagenesis by ends-in homologous recombination (Rong et al. 2002). Bg: BglII sites; N: destroyed NsiI site; A*: created ApaI site; S*: created I-SceI site; Bm: destroyed BamHI site. A: coding sequences unique to 32A. B: coding sequences unique to 32B. C: coding sequences common to both 32A and 32B. Arrows indicate primer pairs used for PCR. See Materials and Methods for details. (C) Schematic of the gene duplication expected from ends-in homologous recombination, with the mini-white marker gene between the duplicated BEAF gene. Arrows indicate primer pairs used for gene-specific PCR amplification of the 5’ or 3’ gene copy. (D) Schematic of the GFBF gene. The stop codon of the BEAF gene was converted to a KpnI site. Genomic BEAF sequences on an EcoRI-KpnI fragment were inserted upstream of EGFP sequences in the correct reading frame, with an SV40 polyadenylation sequence downstream of the EGFP sequences. E: EcoRI; K: KpnI; Af: AflII. See Materials and Methods for details.
Figure 2.2 Molecular characterization of \textit{BEAF}^{A-KO} and \textit{BEAF}^{AB-KO} flies. (A) Schematic of the duplicated \textit{BEAF}^{A-KO} gene and the mini-white marker gene (grey box). Black boxes: mutated \textit{BEAF} sequences; open boxes: wild-type \textit{BEAF} sequences. See Figure 2.1B for details. (B) Restriction analysis of 500 bp PCR products generated from homozygous \textit{BEAF}^{A-KO} flies (A-KO lanes), the \textit{mBF} plasmid (Mut lanes) or wild-type flies (Wt lanes). In \textit{BEAF}^{A-KO} flies, only the 32A ATG mutation is present (\textit{NsiI} lanes); the 32B ATG is not mutated (\textit{ApaI} lanes) and the two tandem stop codons are not present (\textit{BamHI} lanes). C. Western analysis of \textit{BEAF}^{A-KO} (A-KO lanes) and wild-type (wt lanes) embryo nuclear extracts with antibodies specific for 32A (anti-32A lanes) or 32B (anti-32B lanes). Antibody specificity is demonstrated by loading 32A protein (Ec A lanes) or 32B protein (Ec B lanes) expressed in \textit{E. coli}. Note that the anti-32A antibody cross-reacts with another protein, presumably yolk protein. This demonstrates that more total protein was loaded in the A-KO anti-32A lane, yet 32A protein was not detected. Similar amounts of total protein were loaded in the \textit{BEAF}^{AB-KO} and wild-type lanes for the anti-32B blot. D. Schematic of the single-copy \textit{BEAF}^{AB-KO} gene. Black boxes: mutated \textit{BEAF} sequences. See Figure 2.1B for details. E. Restriction analysis of 500 bp PCR products generated from homozygous \textit{BEAF}^{AB-KO} flies (AB-KO lanes), the \textit{mBF} plasmid (Mut lanes) or wild-type flies (Wt lanes). The 32A ATG mutation, 32B ATG mutation and the mutation introducing two tandem stop codons are all present. See Panel B for details. F. Western analysis of \textit{BEAF}^{AB-KO} (AB-KO lane) and \textit{BEAF}^{A-KO} (A-KO lane) adult flies with an antibody that recognizes both forms of \textit{BEAF}. The cross-reactive band just above \textit{BEAF} demonstrates that similar amounts of total protein were loaded in both lanes, but no \textit{BEAF} is detected in the AB-KO lane. See Panel C for details.
Effects of the BEAF Mutations on Drosophila Viability

Flies homozygous for the BEAF\textsuperscript{A-KO} allele are viable. They appear healthy, have normal fertility, and can be maintained as a homozygous stock. Thus the 32B protein is sufficient for survival, the 32A protein is not necessary.

In contrast, flies homozygous for the BEAF\textsuperscript{AB-KO} allele cannot be maintained as a stock. They are weaker than their heterozygous siblings with the CyO balancer chromosome, and die within a few days if they are not transferred to a new vial containing a limited number of flies. However, the males have normal fertility when crossed to wild-type females and can live at least two weeks if pampered. Females, on the other hand, have reduced fertility and appear to be very sensitive to environmental conditions. When BEAF\textsuperscript{AB-KO} female virgins were collected from their parental vial and crossed to BEAF\textsuperscript{AB-KO} males, they laid few eggs and no larvae were obtained. When crossed to wild-type males, they still laid few eggs but some larvae were obtained. For crosses to wild-type males, counting indicated that BEAF\textsuperscript{AB-KO} females laid less than 5% the number of eggs laid by wild-type females, the number of larvae obtained per female was less than 1% of the number from wild-type females, and the number of pupae and adults was less than 0.2% of the number obtained from wild-type females. As described next, different results were obtained when the experimental protocol was modified. Nevertheless, this result demonstrates that BEAF is important for oogenesis and/or development, and maternal BEAF suffices to obtain adults.

To facilitate the collection of BEAF\textsuperscript{AB-KO} females that had not had an opportunity to mate with males with a wild-type BEAF gene, the mutant chromosome was placed over a CyO GFP \textsuperscript{w\textsuperscript{+}} balancer. Third instar larvae were placed in PBS and sorted by fluorescence microscopy. BEAF\textsuperscript{AB-KO} larvae lacked GFP, and were placed into new vials to pupate and eclose. The
resulting $BEAF^{AB-KO}$ females (confirmed by white eyes and lack of curly wings) were used for crosses to $BEAF^{AB-KO}$ or wild-type males. To our surprise, these females laid approximately five times more eggs than their isogenic siblings collected from parental vials. In addition, larvae, pupae and adults were obtained from inter se crosses. Wild-type females still laid over six times more eggs than these $BEAF^{AB-KO}$ females, confirming that BEAF is important for oogenesis (Table 2.1). $BEAF^{AB-KO}$ females laid similar numbers of eggs and had similar larval hatch rates of about 40% whether they were mated with $BEAF^{AB-KO}$ or wild-type males. This hatch rate was less than half that obtained for wild-type flies, indicating that BEAF is also important for embryonic development. Consistent with this, we have previously shown that expression of a dominant negative form of BEAF leads to embryonic lethality (Gilbert et al. 2006).

Zygotic BEAF rescued some animals that lacked maternal BEAF, as indicated by the higher proportion of animals from $BEAF^{AB-KO}$ mothers that survived to pupal and adult stages if they had $BEAF$ fathers rather than $BEAF^{AB-KO}$ fathers (Table 2.1). The adults with zygotic BEAF appeared normal, roughly equal number of females and males were obtained, and they were fertile. Survival rates to pupal and adult stages remained lower than that obtained for wild-type animals, and the observed wild-type viability was lowered by mortality caused by overcrowding in the wild-type vials. The lowest survival rate for $BEAF^{AB-KO}$ animals was obtained at the pupa to adult transition (about 20%). Only about 5% of these adults were females and these females died shortly after eclosing. At least one-third of the males also died shortly after eclosing, but those that survived were fertile. Thus BEAF is important for post-embryonic development, especially of females. Most of the adults, whether or not they survived long, appeared normal. However, a few individuals had various obvious defects in eye, wing, leg, thorax or abdomen.
morphology. The $BEAF^{AB-KO}$ genotype was confirmed for six adults by PCR and restriction digestion analysis (data not shown).

Table 2.1 Fertility of $BEAF^{AB-KO}$ females and effect of zygotic BEAF

<table>
<thead>
<tr>
<th>Stage</th>
<th>No./Viability</th>
<th>Total</th>
<th>No./ Viability</th>
<th>Total</th>
<th>Viability</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(♀)(day)</td>
<td></td>
<td>(♀)(day)</td>
<td></td>
<td>(♀)(day)</td>
<td></td>
</tr>
<tr>
<td>Embryo</td>
<td>5.07 1669 4.65</td>
<td>897 32.0</td>
<td>4067</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larva</td>
<td>1.97 0.39 647 1.86</td>
<td>0.40 359 32.0</td>
<td>1.0b 4067</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pupa</td>
<td>0.81 0.41 265 1.15</td>
<td>0.62 222 26.4</td>
<td>0.82c 3350</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>0.17 0.21 56 0.79</td>
<td>0.68 152 21.8</td>
<td>0.83c 2767</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A total of 46 $BEAF^{AB-KO}$ females were used in crosses to $BEAF^{AB-KO}$ males; 27 $BEAF^{AB-KO}$ females were used in crosses to $BEAF$ males; 18 $BEAF$ females were used in crosses to $BEAF$ males.

a Viability is the fraction of animals that progress from the previous developmental stage to the indicated stage.

b The $BEAF$ females laid a high density of eggs on the collection plates, making it difficult to count embryos. Therefore the number of embryos was estimated to be the same as the number of larvae collected.

c The pupal and adult viability from the $BEAF$ females is an underestimate because of mortality caused by crowding in the vials the larvae were transferred to.
## Table 2.2 Viability of $BEAF^{AB-KO}/BEAF^{AB-KO}$ flies

<table>
<thead>
<tr>
<th>Day:</th>
<th>Number of flies eclosing of the indicated genotype</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>$BEAF^{AB-KO}/CyO$</td>
<td></td>
<td>43</td>
<td>68</td>
<td>74</td>
<td>46</td>
<td>30</td>
<td>34</td>
<td>18</td>
<td>25</td>
<td>9</td>
<td>8</td>
<td>355</td>
</tr>
<tr>
<td>$BEAF^{AB-KO}/BEAF^{AB-KO}$</td>
<td></td>
<td>2</td>
<td>25</td>
<td>28</td>
<td>19</td>
<td>16</td>
<td>24</td>
<td>18</td>
<td>13</td>
<td>7</td>
<td>8</td>
<td>160</td>
</tr>
<tr>
<td>Percent of total $^a$</td>
<td></td>
<td>4.4%</td>
<td>19.6%</td>
<td>22.9%</td>
<td>24.3%</td>
<td>25.6%</td>
<td>27.9%</td>
<td>29.7%</td>
<td>30.0%</td>
<td>30.5%</td>
<td>31.1%</td>
<td>31.1%</td>
</tr>
</tbody>
</table>

$^a$ Percentage of all eclosed flies that have the genotype $BEAF^{AB-KO}/BEAF^{AB-KO}$ (running total, not daily totals). One-third of the eclosed flies should have this genotype if it does not affect viability, because $CyO/CyO$ is embryonic lethal.
To determine if development of homozygous $BEAF^{AB-KO}$ flies that had maternal BEAF is impaired, we recorded the number of $BEAF^{AB-KO}$ and $BEAF^{AB-KO}/CyO$ flies as they eclosed from six lightly populated vials derived from $BEAF^{AB-KO}/CyO$ parents (Table 2.2). Nearly one-third of the flies were homozygous, with roughly equal numbers of males and females. This indicates that survival to adulthood is not affected when the only BEAF present is maternally provided. However, homozygous flies eclosed one to two days later than their heterozygous siblings, indicating a slight developmental delay.

To determine if development of homozygous $BEAF^{AB-KO}$ flies that had maternal BEAF is impaired, we recorded the number of $BEAF^{AB-KO}$ and $BEAF^{AB-KO}/CyO$ flies as they eclosed from six lightly populated vials derived from $BEAF^{AB-KO}/CyO$ parents (Table 2.2). Nearly one-third of the flies were homozygous, with roughly equal numbers of males and females. This indicates that survival to adulthood is not affected when the only BEAF present is maternally provided. However, homozygous flies eclosed one to two days later than their heterozygous siblings, indicating a slight developmental delay.

Multiple transgenic fly lines with either the $gBF$ or $GFBF$ transgene were able to rescue the defects in fertility and vigor. We previously reported that producing a dominant negative form of BEAF in eye imaginal discs leads to a rough eye phenotype (Gilbert et al. 2006). Homozygous $BEAF^{AB-KO}$ flies also have a rough eye phenotype, and this is also rescued by the $BEAF$ transgenes (Fig. 2.3). Hence these defects are due to the lack of BEAF protein and not to an unrelated mutation on the chromosome. The $GFBF$ transgene is driven by a 900 bp $BEAF$ promoter fragment and is insulated from chromosomal position effects. Therefore we expect production of the GFBF proteins to reflect that of the endogenous BEAF proteins. Fluorescence microscopy of homozygous $BEAF^{AB-KO}$ animals rescued by four different $GFBF$ transgenes all
gave the same result. Green fluorescent BEAF was observed in every nucleus at every life stage for all tissues examined (data not shown). This is consistent with previous immunolocalization, Western and Northern results that indicated the BEAF proteins are ubiquitous.

**Figure 2.3** *BEAF^{AB-KO}* flies have a rough eye phenotype that is rescued by *BEAF* transgenes. Scanning electron micrograph of a *BEAF^{AB-KO}* fly (left panel) shows that it has a rough eye phenotype. Introducing a single copy of a *gBF* (not shown) or *GFBF* transgene (right panel) rescues this phenotype, resulting in wild-type eye morphology. *BEAF^{A-KO}* flies do not have a rough eye phenotype (not shown).

**BEAF Is Required for Normal Oogenesis**

Because *BEAF^{AB-KO}* females had low fertility that was rescued by *gBF* and *GFBF* transgenes, we decided to examine their ovaries. *BEAF^{AB-KO}* and wild-type females were mated with wild-type males for 4 to 7 days prior to dissection. The number of ovarioles per ovary did not appear to differ between *BEAF^{AB-KO}* and wild-type females, but mutant ovaries were smaller than those from wild-type (Fig. 2.4A, B, C). This was because most ovarioles from mutant females lacked mature oocytes, whereas most ovarioles from wild-type females ended with a mature oocyte (Fig. 2.4D, E).
Figure 2.4 Effect of the $BEAF^{AB-KO}$ mutation on oogenesis. Females were mated with wild-type males for four days before dissecting out their ovaries and staining DNA with DAPI or propidium iodide plus RNase A. KO: $BEAF^{AB-KO}$ ovaries; Wt: wild-type ovaries. Panels F-M were obtained by confocal microscopy. See text for details.
In normal egg chamber development there are 16 interconnected germline cells enveloped by a layer of somatic follicle cells. Fifteen become nurse cells with large polyploid nuclei, and the other cell becomes the oocyte. Yolk and the contents of the nurse cells begin to accumulate in the oocyte at stage 8, leading to gradual enlargement of the oocyte (Mahowald and Kambysellis 1980). We did not note any difference between mutant and wild-type germaria, which is where oogenesis initiates. However, we observed a variety of mutant phenotypes that generally became apparent around stage 8 or later. Some egg chambers had too many nurse cell nuclei that could be due to the fusion of two egg chambers or an extra round of cell division (Fig. 2.4G, I). Others had small, brightly staining nuclei that presumably represented an intermediate step in egg chamber degeneration. Sometimes such an egg chamber was small like a stage 7 chamber (Fig. 2.4H), and sometimes the chamber was nearly the size of a mature oocyte (Fig. 2.4L). In other cases the ovariole had a stage 7 or 8 egg chamber adjacent to what appeared to be a mature oocyte, with intermediate stages missing (compare Fig. 2.4J to K). In yet other cases, an egg chamber the size of a mature oocyte had large, oddly shaped nurse cell nuclei distributed throughout (Fig. 2.4M). This presumably represents egg chamber degeneration by a different pathway than that being used in egg chambers with small, brightly staining nuclei. Typically an ovary pair from a female exhibited multiple examples of only one of the phenotypes shown. Thus BEAF is important for oogenesis, particularly at the stages when oocyte size dramatically increases by vitellogenesis and transport of material from the nurse cells. Occasionally a mature oocyte is formed in the absence of BEAF. If fertilized, some of these oocytes are capable of developing into adults especially if provided with zygotic BEAF (Table 2.1).
The \textit{BEAF^{AB-KO}} Mutation Affects \textit{scs’} Insulator Function

Mutating the \textit{BEAF} binding sites in \textit{scs’} eliminates insulator activity (Cuvier \textit{et al.} 1998), and expression of a dominant negative form of \textit{BEAF} interferes with \textit{scs’} insulator activity (Gilbert \textit{et al.} 2006). To extend these results to the \textit{BEAF^{AB-KO}} allele, we used two transgene assays. One tested the ability of insulators bracketing the mini-\textit{white} gene to protect against chromosomal position effects, leading to position-independent expression of mini-\textit{white}. The other assay tested the ability of insulators to block communication between the wing and body enhancers and the promoter of the \textit{y} gene.

In the position-independent expression assay, mini-\textit{white} was bracketed by the M2 derivative of \textit{scs’} on the 5’ side and by the \textit{scs} insulator on the 3’ side (Cuvier \textit{et al.} 1998). The M2 insulator has two copies of the high affinity \textit{BEAF} binding site of \textit{scs’}, with one copy replacing the low affinity binding site normally present. Because the mini-\textit{white} gene lacks an enhancer, bracketing it with insulators should lead to low levels of expression resulting in flies with yellow or light orange eyes. In the absence of the 5’ insulator, chromosomal position effects should lead to the activation of mini-\textit{white} in some fly lines and result in darker eye pigmentation. This is observed: ~90% of insulated fly lines have yellow or light orange eyes, while <50% of fly lines insulated only at the 3’ end have such light eye pigmentation (unpublished results and (Cuvier \textit{et al.} 1998). We had three fly lines with the M2 transposon inserted in the \textit{X} chromosome, and one line with an insertion in chromosome 3. Females heterozygous for these transposons had yellow or light orange eyes. Three of these lines had darker pigmentation in a \textit{BEAF^{AB-KO}} background (Fig. 2.5A), indicating a loss of protection from chromosomal position effects in the absence of \textit{BEAF}. The insertion in the fourth line is apparently not subject to position effects. Two of these fly lines were also tested in the presence
of a dominant negative BEAF protein (M2-9 and M2-10) and showed similar activation of mini-
white (Gilbert et al. 2006).

Figure 2.5 scs’ does not function as an insulator in BEAF<sup>AB-KO</sup> flies, but the scs and gypsy
insulators are still functional. (A) The M2 insulator, an scs’ derivative, does not protect against
chromosomal position effects in the absence of BEAF protein. Eyes of 3- to 4-day old females
heterozygous for different M2 transposons and homozygous for BEAF or BEAF<sup>AB-KO</sup> (KO) are
shown. See text for details. (B) A dimer of the scs’ insulator does not block communication
between the y wing and body enhancers and the y promoter in the absence of BEAF protein.
Lack of BEAF protein does not affect the ability of the scs and gypsy insulators to block this
enhancer-promoter communication. Shown are abdomens of 3- to 4-day old females
homozygous for BEAF or BEAF<sup>AB-KO</sup> (KO) and heterozygous for the indicated transposons, with
(Ins) or without [del(Ins)] the indicated insulator between the enhancer and promoter. See text
for details.
Three different insulators were tested in the enhancer-blocking assay. An scs’ dimer (2scs’), scs or gypsy insulator was located between the $y$ wing and body enhancers and the $y$ gene. The scs and gypsy insulators do not have BEAF binding sites. “Sibling” lines in which the insulators had been removed by the Cre recombinase were also used. This allowed us to compare the level of $y$-dependent body pigmentation due to the same transposon integration site in the presence and absence of the insulators. Previous studies with these fly lines found that these insulators do not form the boundaries of heat shock puffs in polytene chromosomes (Kuhn et al. 2004). The level of pigmentation in the dorsal abdomen of 3- to 4-day old females was recorded for flies heterozygous for the enhancer-blocking transposons with and without the insulators in the presence of BEAF, and with the insulators in the absence of BEAF (Fig. 2.5B). Removal of each of the three insulators resulted in darker pigmentation. In the $BEAF^{AB-KO}$ background the 2scs’ flies had a similar level of pigmentation as their “siblings” lacking the insulator. Enhancer-blocking by the scs and gypsy insulators was not affected. We conclude that BEAF is required for the function of 2scs’.

While performing this experiment we noticed that the abdominal pigmentation pattern is altered in all flies lacking BEAF. Pigmentation is concentrated in a thin stripe at the posterior edge of the dorsal side of the abdominal segments, except for the two most posterior segments in males which are fully pigmented. The pigment spreads in a diffuse manner to encompass around one third of each segment. In the $BEAF^{AB-KO}$ background this diffuse spreading extends further to encompass one half or more of each segment (Fig. 2.5B). We subsequently found that this spreading occurs in male and female flies. The abdominal pigmentation pattern is visible even in the absence of a functional $y$ gene, although the color is yellow-brown instead of gray-black. The diffuse spreading of the pigmentation also occurs in the absence of a functional $y$ gene, and is
rescued by \textit{gBF} and \textit{GFBF} transgenes (data not shown). Therefore this spreading of the pigmentation is not related to the \textit{y} transgene used in the enhancer-blocking assay, but is related to the lack of BEAF protein. Perhaps it is due to deregulation of some gene upstream of \textit{y} that is involved in determining the pigmentation pattern.

\textbf{BEAF Mutations Perturb Male Polytene \textit{X}-Chromosome Morphology}

If insulators function by affecting chromatin structure or dynamics, the lack of BEAF could affect chromatin structure. To observe interphase chromatin, polytene chromosome squashes were prepared from salivary glands of third instar larvae. The \textit{X} chromosome of \textit{BEAF}^4\textit{AB-KO} males from heterozygous mothers exhibited obvious structural defects (Fig. 2.6A). The banding pattern was lost, and the chromosome appeared shorter and puffier. This is less extreme than results we previously obtained by producing a dominant negative form of BEAF in salivary glands, in which the structure of all polytene chromosomes was disrupted in both males and females (\textit{Gilbert et al.} 2006). Normal \textit{X} chromosome morphology is restored in the presence of a \textit{GFBF} transgene, demonstrating that the phenotype is due to a lack of BEAF protein (Fig. 2.6B).

Polytene chromosomes were also prepared from larvae from \textit{BEAF}^{4\textit{AB-KO}} inter se crosses. As for males that had maternal BEAF, the \textit{X} polytene chromosome from these males showed obvious structural defects but the somatic chromosomes usually appeared normal. There was large variation in polytene chromosome structure from female larvae, ranging from severe disruption of all chromosomes to normal appearance (data not shown). No female larvae survived to become healthy adults, raising the possibility that the variable morphology of their polytene chromosomes represents the variable health of these larvae. Thus the lack of maternal
BEAF did not lead to a more severe disruption of polytene chromosome structure except in cases where we believe the health of the larvae was poor.

To positively identify the $X$ chromosome and to determine if the dosage compensation complex (DCC) was affected, we immunostained polytene chromosomes for DCC proteins (Stuckenholz et al. 1999). The DCC only associates with the male $X$ chromosome, where it mediates acetylation of histone H4 on lysine 16. This causes $X$-linked genes in males to be transcribed at 2-fold higher rates than in females (Bone et al. 1994; Hamada et al. 2005). Figure 2.6 shows that association of the DCC protein MOF with the male $X$ chromosome is not affected in the absence of BEAF. The same is true of the DCC proteins MSL-1, MSL-2, MSL-3, MLE and the dosage compensation associated histone modification, acetylation of H4 lysine 16 (data not shown). So the DCC localizes and functions normally despite the altered morphology of the $X$ chromosome.

We used alleles of the $X$-linked $w$ gene to further test effects on dosage compensation by examining eye pigmentation. The $w^{a}$ mutation normally shows dosage compensation (males and females have similar eye pigment levels) and the $w^{c}$ mutation does not (males have less eye pigment than females) (Lerach et al. 2005). Eye pigment levels were not affected by the BEAF$^{AB-KO}$ allele, indicating no effect on dosage compensation in this assay (data not shown).

Polytene $X$ chromosomes from BEAF$^{AB-KO}$ males also had perturbed morphology (Fig. 2.6C). However, the phenotype was less severe and more variable. The $X$ chromosome morphology ranged from normal or near normal to moderately perturbed. Thus flies lacking 32A protein are not completely normal even though adults have no obvious phenotypes, are healthy and have normal fertility.
Figure 2.6 BEAF mutations cause a disruption of male X polytene chromosome structure. (A) Salivary gland polytene chromosomes prepared from a wild-type male third instar larvae exhibit a normal banding pattern when the DNA is stained with DAPI. One chromosome arm of polytene chromosomes prepared from a BEAF<sup>AB-KO</sup> male has lost the banding pattern and appears shorter and broader. Indirect immunostaining with an antibody against MOF shows that it is the X chromosome that appears abnormal. (B) The GFBF transgene rescues the abnormal phenotype of the BEAF<sup>AB-KO</sup> male polytene X chromosome. Upper panels show chromosomes stained with DAPI and gently spread in 50% glycerol without acid treatment to allow direct visualization of green fluorescent BEAF fusion proteins. Lower panels show chromosomes that have undergone normal fixation, with the X chromosome identified by indirect immunostaining with an antibody against MOF. (C) Polytenes from BEAF<sup>A-KO</sup> males show a similar X chromosome phenotype, but it is less extreme and more variable. Note that 32B protein can be detected on these chromosomes by indirect immunofluorescence with an antibody against BEAF. The X chromosome is identified by indirect immunostaining with an antibody against MOF.
Position-Effect Variegation (PEV) Is Enhanced in the Absence of BEAF

As a second test of the ability of BEAF to affect chromatin organization, we examined the effect of the $BEAF^{AB-KO}$ allele on PEV. The $w^{m4h}$ gene and three different insertions of a transposon carrying a $y$ gene were used. In all four cases, PEV is due to variable spreading of pericentromeric heterochromatin that silences the reporter gene in some cells. The $w^{m4h}$ gene is caused by a chromosomal inversion on the $X$ chromosome (TARTOF et al. 1989). The KV732 and KV600 fly lines have the $y$ transgene inserted near the pericentromeric heterochromatin of the $X$ chromosome, while KV123 is near the pericentromeric heterochromatin of chromosome arm 3L (YAN et al. 2002). PEV effects on $w^{m4h}$ are determined by comparing the number of pigmented ommatidia in flies of different genotypes (Fig. 2.7A), or by extracting and quantitating the pigment (Fig. 2.7B). PEV effects on $y$ are determined by comparing the number of darkly pigmented spots on abdomens of flies of different genotypes (Fig. 2.7C). The level of variegation is very sensitive to mutations that directly or indirectly affect chromatin organization.

The phenotypes of males with one copy of the PEV reporter gene were recorded. In all cases we found a slight enhancement of PEV in $BEAF^{AB-KO}/BEAF$ males and a stronger enhancement in $BEAF^{AB-KO}$ males (Fig. 2.7 and data not shown). Thus the lack of BEAF allows heterochromatin to spread and silence the reporter genes in a larger number of cells. This is consistent with our previous results, in which we found that BEAF is a triplo-suppressor of PEV while a dominant negative form of BEAF is an enhancer of PEV (GILBERT et al. 2006). These PEV assays are consistent with the model that BEAF forms barrier elements which maintain genes in transcriptionally active states by isolating them from surrounding silent heterochromatin.
Figure 2.7 The $BEAF^{AB-KO}$ mutation enhances variegation of $w^{m4h}$ and variegating $y$ transgenes. (A) Males heterozygous for $BEAF^{AB-KO}$ and hemizygous for $w^{m4h}$ show mildly enhanced variegation of $w^{m4h}$. Males homozygous for $BEAF^{AB-KO}$ and hemizygous for $w^{m4h}$ show a stronger enhancement of $w^{m4h}$ variegation. Eyes of 4- to 5-day old males are shown. (B) Enhancement of $w^{m4h}$ variegation was quantitated by extracting pigment from male heads of the indicated genotypes and measuring the OD$_{480}$. (C) Variegation of a $y$ transgene located in the pericentromeric heterochromatin of chromosome arm 3L is enhanced in males heterozygous for $BEAF^{AB-KO}$, and more strongly enhanced in males homozygous for $BEAF^{AB-KO}$. The $y$ transgene is in the $KV123$ transposon, and is heterozygous. Abdomens of 2- to 3-day old males are shown. Similar results were obtained with two other variegating $y$ transgenes located on the $X$ chromosome ($KV732$ and $KV600$).
Discussion

As a tool for studying BEAF function, we generated the $BEAF^{AB-KO}$ knockout allele by homologous recombination. In the process, we also isolated the $BEAF^{A-KO}$ allele that cannot produce the 32A protein. Flies homozygous for the $BEAF^{A-KO}$ allele are healthy and viable, indicating that the 32B protein is sufficient for normal development. In contrast, flies homozygous for the $BEAF^{AB-KO}$ allele cannot be maintained as a stable line. Maternal BEAF is sufficient to obtain fertile adults, although the resulting $BEAF^{AB-KO}$ flies eclose one to two days later than their $BEAF^{AB-KO}/CyO$ siblings and are sickly. Also, although equal numbers of males and females are obtained, the fertility of the $BEAF^{AB-KO}$ females is compromised. Crosses with these females demonstrated that zygotic BEAF is also sufficient to obtain equal numbers of fertile males and females, and fertile males can be obtained even in the absence of BEAF. However, in the absence of maternal BEAF less than half of the embryos hatch and there is a drastic reduction in the number of adults obtained. The absence of all BEAF results in female lethality by the pharate adult stage or shortly after eclosing. In addition, driving expression of a transgene encoding a dominant negative form of BEAF by daughterless-GAL4 leads to embryonic lethality (GILBERT et al. 2006). Thus BEAF plays an important role during development, particularly in females, although sickly adults can be obtained that lack BEAF.

The lowered female fertility led us to inspect ovaries from $BEAF^{AB-KO}$ flies. A number of different phenotypes were observed, although ovaries from a given $BEAF^{AB-KO}$ female normally exhibited only one phenotype. We conclude that BEAF plays an important role during oogenesis as well as during development. While the defects in oogenesis could be due to deregulation of genes in the absence of BEAF, it could also be at least partly related to the genetic interaction we found between BEAF and spindle-E ($spn-E$) (ROY et al. 2007b). The protein encoded by $spn-E$ is
a helicase subunit of an RNA interference complex that plays a role in oogenesis (KENNERDELL et al. 2002) and heterochromatin formation (PAL-BHADRA et al. 2004). It is of interest to note that a genetic interaction between the RNAi machinery and gypsy insulator function has been reported (LEI and CORCES 2006), and that the su(Hw) insulator protein also plays a role in oogenesis (HARRISON et al. 1993). In addition, the JIL-1 histone H3 kinase plays a role in modulating chromatin structure and is essential at all stages of development as well as for oogenesis (ZHANG et al. 2003).

The scs’ insulator was originally identified because it forms a special chromatin structure that appeared to localize to one end of the heat shock puff at 87A of polytene chromosomes (UDVARDY et al. 1985). It was subsequently shown to function as an insulator in the first transgenic enhancer blocking and position independent expression assays to be done (KELLUM and SCHEDL 1991b; KELLUM and SCHEDL 1992). This led to the identification of the BEAF proteins as scs’ binding proteins (HART et al. 1997; ZHAO et al. 1995). The importance of the BEAF binding sites in scs’ for insulator activity has been shown using both cultured cells (ZHAO et al. 1995) and transgenic flies (CUVIER et al. 1998), and additional genomic BEAF binding sites were shown to have insulator activity (CUVIER et al. 1998). However, it is possible that some other protein binds to these sites in vivo to confer insulator activity. It was also shown that a dominant negative form of BEAF interferes with scs’ insulator activity (GILBERT et al. 2006), although this protein might affect proteins in addition to BEAF. Here we show that BEAF is required for the insulator activity of scs’. Using both a position independent expression assay and an enhancer blocking assay, we found that scs’ loses insulator activity in the absence of BEAF protein. In the enhancer blocking assay we also tested the scs and gypsy insulators, which lack BEAF binding sites, and found that these insulators work in the absence of BEAF.
The altered appearance of the X polytene chromosome in BEAF^{AB-KO} male mutant larvae provides dramatic evidence for a role for BEAF in chromatin organization. This is further supported by the PEV assays which indicate that BEAF helps limit heterochromatin spreading. Mutations in genes encoding other chromatin proteins have a similar effect on the male X chromosome. This includes ISWI, which is the catalytic subunit of multiple chromatin remodeling complexes including NURF (nucleosome remodeling factor) (DEURING et al. 2000); the NURF301 subunit of NURF (BADENHORST et al. 2002); and the heterochromatin proteins Su(var)3-7 and HP1 (SPIERER et al. 2005). This supports models in which insulators function by affecting chromatin structure or dynamics.

It is curious that only the male X chromosome is affected, whereas global structural alterations are observed in all chromosomes of males and females when a dominant negative form of BEAF is produced in larval salivary glands (GILBERT et al. 2006). It is likely that the chromatin organization of the male X chromosome is especially susceptible to disruption due to some feature associated with dosage compensation. A candidate for such a feature is the hyperacetylation of lysine 16 of histone H4 (BONE et al. 1994), which interferes with formation of 30 nm chromatin fibers (SHOGREN-KNAAK et al. 2006). Evidence that the male X chromosome is more sensitive to disruption is derived from mutations in the histone H3 kinase, JIL-1. When polytene chromosomes were observed using an allelic series of JIL-1 mutations, weak mutations were found to mainly affect the male X chromosome and stronger mutations affected all chromosomes of both males and females (WANG et al. 2001). Also, BEAF^{A-KO} animals are healthier than BEAF^{AB-KO} animals and we observed a weaker effect on the male X chromosome in BEAF^{A-KO} animals. This suggests that the dominant negative has a stronger effect than the lack of BEAF. This is consistent with the lethal effect of producing the dominant negative protein in
embryos, whereas homozygous $BEAF^{AB-KO}$ adults are obtained. We assume that the dominant negative has a stronger effect because it actively interferes with BEAF activity, while the gradual disappearance of maternal BEAF mitigates the effect of the knockout. Perhaps the dominant negative protein also interferes with the function of proteins in addition to BEAF. If so, it is likely that these proteins normally interact with BEAF since the phenotypes caused by the dominant negative and by $BEAF^{AB-KO}$ are similar and can be rescued by BEAF transgenes. The future identification of any such proteins should provide insight into how BEAF functions.

We have shown here that the BEAF proteins have insulator activity. BEAF binds to hundreds of sites on polytene chromosomes (ZHao et al. 1995), and other genomic binding sites have insulator activity (Cuvier et al. 2002; Cuvier et al. 1998). Yet 32A is not essential, adults can be obtained with only maternal BEAF, some embryos hatch with only zygotic BEAF, and a small number of fertile males are obtained in the absence of all BEAF. This is somewhat reminiscent of mutations in the su(Hw) insulator protein, which lead to female sterility but otherwise are not lethal (Harrison et al. 1993). BEAF is normally present at all life stages (for example, see the Western of adults in Fig. 2.2F). Using several of our GFBF transgenic fly lines in the $BEAF^{AB-KO}$ background, in which the transgene is insulated and driven by 900 bp of BEAF promoter sequences, we observe green fluorescent BEAF in all nuclei of all tissues at all life stages that we have looked at (data not shown). If BEAF is normally ubiquitous and contributes to gene regulation by forming boundaries between hundreds of domains, why are the effects of a lack of BEAF so limited? The answer is not known at present. One possibility is that the misregulation of genes caused by malfunctioning insulators is minor enough that fitness is reduced without being immediately lethal. Another possibility that we find particularly intriguing is that there could be some type of epigenetic memory mechanism, similar to what has been
proposed for Polycomb group proteins (Bantignies and Cavalli 2006; Sarge and Park-Sarge 2005). This epigenetic memory has been shown to be meiotically inheritable (Cavalli and Paro 1998). Loss of this “epigenetic memory” could be stochastic, resulting in deregulation of different genes in different individuals or clonal populations of cells. This could result in the variable timing of death in the absence of BEAF, and the single phenotype observed per ovary but different phenotypes in different ovaries. The knockout mutations described here will be useful tools in future studies aimed at discovering proteins that interact with BEAF and for investigating the role of BEAF in gene regulation and chromatin organization. This will ultimately lead to an understanding of the molecular mechanisms used in insulator function.

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CHAPTER THREE

A GENETIC SCREEN SUPPORTS A BROAD ROLE FOR THE DROSOPHILA INSULATOR PROTEINS BEAF-32A AND BEAF-32B IN MAINTAINING PATTERNS OF GENE EXPRESSION*

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Introduction

Proper regulation of gene expression is essential for developmental programs and signal transduction-mediated responses to environmental signals. Yet as illustrated by many studies of regulatory regions, enhancer-promoter communication is potentially promiscuous (Kermekchiev et al. 1991). One means of maintaining enhancer-promoter fidelity is thought to involve the subdivision of chromosomes into functional domains such that communication can only occur within a domain. In this model, chromatin domain insulators (also known as boundary elements) define domain boundaries by insulating elements within a domain from interactions with elements located in other domains (Geyer and Clark 2002). In transgene assays, insulators must be located between an enhancer and promoter to block communication. They have no effect if located upstream or downstream, indicating insulators do not act as silencers (Cai and Levine 1995; Scott and Geyer 1995). Bracketing transgenes with insulators protects against chromosomal position effects, creating a domain in which expression is driven solely by regulatory elements in the transgenic construct (Cuvier et al. 1998; Kellum and Schedl 1991b). Perhaps related to this protection, insulators can act as barriers that prevent different chromatin states in adjacent domains from spreading into each other and consequently influencing gene expression. Thus an open chromatin domain with potentially active genes and an adjacent closed chromatin domain with inactive genes would be kept separate (Litt et al. 2001; Noma et al. 2001; Prioleau et al. 1999). Enhancer blocking and protection from chromosomal position effects are separable activities at least in some insulators (Recillas-Targa et al. 2002). Despite the appeal of this model, to our knowledge only one study of the vertebrate insulator protein CTCF has provided evidence that insulators play an extensive role in maintaining patterns of gene expression (Mukhopadhyay et al. 2004). Here we provide
evidence for cross-talk between different classes of insulators in Drosophila, and for a broad role for BEAF-dependent insulators in maintaining patterns of gene expression.

The boundary element-associated factors BEAF-32A and BEAF-32B are 32 kDa proteins derived from the same gene (HART et al. 1997). They bind to the ses’ insulator, and these binding sites are essential for insulator activity. Immunostaining of polytene chromosomes indicates that there are several hundred BEAF binding sites in the Drosophila genome, and other binding sites that have been tested have insulator activity (CUVIER et al. 1998). This indicates that BEAF-dependent insulators are common in Drosophila. To gain insight into BEAF function, we designed a GAL4 UAS-controlled transgene encoding a dominant negative form of BEAF (GILBERT et al. 2006). This protein, BID, has the carboxy-terminal BEAF self-interaction domain but lacks the amino-terminal DNA binding domains found in BEAF-32A and 32B. Therefore it should form complexes with the BEAF proteins and interfere with DNA binding. We have previously shown that BEAF is the major target of BID, and BID interferes with the binding of BEAF to polytene chromosomes. In support of a link between BEAF-dependent insulator activity and chromatin structure or dynamics, BID expression causes a global disruption of polytene chromosome morphology and also enhances position effect variegation.

Expression of the BID transgene in eye tissue via an ey-GAL4 driver leads to a rough eye phenotype that is rescued by a third copy of the BEAF gene (GILBERT et al. 2006). We reasoned that mutations in genes that are important for BEAF function would modify this phenotype. Identification of these genetic interactions should provide insight into BEAF function. This assay did not provide evidence for general interactions between BEAF and proteins involved in chromatin structure or dynamics. Of over 30 such genes tested, only Nipped-A and spindle-E showed an interaction. Instead, most interactions were with insulator binding proteins and
transcription factors involved in head development. This supports the hypothesis that BEAF plays an important role in maintaining global patterns of gene regulation during processes such as eye development. It also indicates there is cross-talk between different classes of insulators, which could be an indirect interaction based on their common roles in maintaining gene expression patterns. Finally, the interaction with *spn-E* suggests that BEAF function might involve RNAi pathways (Pal-Bhadra et al. 2004).

**Materials and Methods**

**Drosophila Stocks**

Flies were raised at 25°C on standard cornmeal, yeast and sugar medium with Tegosept. Construction of the *BID* transgene, generation of transgenic fly lines, and construction of the *ey-GAL4/CyO; BID.3A/BID.3A* line was previously described (Gilbert et al. 2006). All experiments reported here used a third chromosome insertion called *BID.3A*, hereafter referred to simply as *BID*. *MRTF*KO and *UAS-MRTF* flies were kindly provided by Z. Han and E.N. Olson (Han et al. 2004). *P[ftz]* and *ftz11* flies were kindly provided by H.M. Krause (Schwartz et al. 2001). All other flies were obtained from the Bloomington Drosophila Stock Center (flystocks.bio.indiana.edu), and information concerning the deficiencies and mutations used in this study can be found at Flybase (www.flybase.org).

**Rough Eye-Based Modifier Screen**

Male *ey-GAL4/CyO; BID/BID* flies were crossed to virgin female flies containing chromosomal deficiencies or mutations of interest. All crosses were done at 25°C as described above. The resulting progeny were scored under a dissecting microscope and preserved for scanning electron microscopy. At least ten animals of the respective genotypes were scored to determine the reproducibility of the phenotype. No differences were observed between male and
female progeny of the same genotype. In addition, progeny with CyO were compared to progeny with ey-GAL4 to confirm that the phenotype was due to BID expression. There was no disruption of eye development in the absence of ey-GAL4 for any of the deficiencies or mutations tested. However, certain balancers enhanced the rough eye phenotype. In particular, certain TM3 balancers enhanced. Care was taken to account for balancers.

To perform the rescue crosses, fly lines with the rescue construct homozygous or balanced together with the relevant mutation over a balancer chromosome were constructed. These flies were crossed to the ey-GAL4/CyO; BID/BID line and appropriate progeny were scored.

**Scanning Electron Microscopy**

Flies were fixed in FAA (16% formaldehyde, 5% acetic acid, 45% ethanol) for at least 24 hours, then put through a dehydration series of ethanol (10 min each 75%, 87%, 94%, 97%, 4x 100%) followed by 2x 30 min in 100% hexamethyldisilazane. Flies were dried overnight in a hood and stored in a dessicator. Flies were sputter coated and photographed in a Cambridge Stereoscan 260 SEM at 15 kV.

**Results**

**Expression of a Dominant-Negative BEAF Transgene in Eye Imaginal Discs Leads to a Rough Eye Phenotype that Is Rescued by a Third Copy of the BEAF Gene**

The BEAF gene encodes two 32 kDa proteins, BEAF-32A and 32B. We previously reported the design and characterization of a transgene under GAL4 UAS control that encodes the BEAF self-interaction domain (BID) but lacks a DNA binding domain (Gilbert et al. 2006) (Fig. 3.1A, B). We found that the BID protein acts as a dominant-negative antagonist of BEAF function in transgenic flies by several criteria. Of relevance for the present study is the finding that using an ey-GAL4 driver to express BID in eye discs interferes with eye development,
resulting in a mild rough eye phenotype. The effect is mainly seen in the posterior half of the eye, particularly along the posterior margin, in the center of the eye, and in the posterior-dorsal quadrant. The phenotype is more extreme when the \textit{BID} transgene is homozygous. Evidence that the effect is specifically due to interference with BEAF activity derives from rescue of the rough eye phenotype when a third copy of \textit{BEAF} is provided as a transgene (Fig. 3.1C). Here we take advantage of this phenotype to screen for genetic interactions that modify the \textit{BID}-dependent rough eye phenotype. A wide variety of biological processes have been studied using similar eye-based screens (THOMAS and WASSARMAN 1999).

\textbf{Chromosomal Deficiency Screen}

As a first step in screening for genetic interactions, we crossed \textit{ey-GAL4}/\textit{CyO; BID/BID} flies to 57 second chromosome and 96 third chromosome deficiency lines from the Bloomington Stock Center deficiency kits (Fig. 3.2 and Table 3.4). Haplo-insufficiency of one or more genes deleted by a deficiency could enhance or suppress the \textit{BID}-dependent rough eye phenotype. Approximately one-third of the deficiencies appeared to suppress the mild rough eye phenotype observed in flies heterozygous for \textit{ey-GAL4} and \textit{BID}. Because the eye phenotype was weak to begin with, we suspected there were a high number of “false positives”. Therefore we ignored this data and focused on deficiencies that enhanced the phenotype. We found that 19 of 153 deficiencies, or 12%, enhanced the phenotype (Fig. 3.3 and Table 3.1). Due to overlaps between deficiencies, these results identify at least 16 chromosomal regions that harbor genes that genetically interact with \textit{BEAF}.
Figure 3.1 The BEAF-interaction-domain (BID) protein causes a rough eye phenotype that is rescued by an extra copy of the BEAF gene. (A) The carboxy-terminal half of the BEAF coding sequences were joined in frame to sequences encoding an HA epitope tag and SV40 NLS (black box). This is in pUAST (BRAND and PERRIMON 1993), and so is under GAL4 UAS control (ovals) and has an SV40 polyadenylation site (not shown). (B) BEAF-32A and 32B have unique amino-terminal DNA binding domains of 80 amino acids (hatched boxes). The rest of the proteins are identical, being derived from the same exon. This includes a 120 amino acid central portion of unknown function (open box) and an 80 amino acid carboxy-terminal domain that mediates interactions between BEAF proteins (gray box) (HART et al. 1997). The BID protein has an amino-terminal HA epitope tag and SV40 NLS joined to the carboxy terminal half of BEAF. Thus BID should form complexes with 32A and 32B, inhibiting DNA binding by BEAF complexes in a manner analogous to the Drosophila Emc and vertebrate Id proteins (CAMPUZANO 2001; NORTON et al. 1998). (C) Scanning electron micrographs of flies of the indicated genotypes demonstrate that driving BID expression with ey-GAL4 leads to a rough eye phenotype. This phenotype is more extreme when BID is homozygous, and is rescued to near wild-type by a third copy of the BEAF gene provided by a transgene inserted at different third chromosome locations. Like BID/BID flies, ey-GAL4/CyO flies have normal eyes (not shown).
Figure. 3.2 Chromosomal deficiencies screened for enhancement of the *ey-GAL4/+; BID/+* rough eye phenotype. The numbered and lettered subdivisions of salivary gland polytene chromosomes are indicated, with centromeres shown as filled circles. Thick red lines indicate deficiencies that enhance, and thin black lines indicate deficiencies that do not. Four of 57 second chromosome deficiencies and fifteen of 96 third chromosome deficiencies enhanced the phenotype. This includes three overlapping deficiencies from chromosome arm 3L region 62 and two overlapping deficiencies from 3R region 89. Positions of tested genes that enhance the phenotype are also indicated (blue vertical bars). See Tables 3.1, 3.2 and Table 3.4 for more information.
Table 3.1 Chromosomal deficiencies that enhance the rough eye phenotype

<table>
<thead>
<tr>
<th>Deficiency</th>
<th>Cytological Position</th>
</tr>
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<tbody>
<tr>
<td>Df(2R)ST1</td>
<td>42B3-5;43E15-18</td>
</tr>
<tr>
<td>Df(2R)BSC11</td>
<td>50E6-F1;51E2-4</td>
</tr>
<tr>
<td>Df(2R)Jp8</td>
<td>52F5-9;52F10-53A1</td>
</tr>
<tr>
<td>Df(2R)Dll-MP</td>
<td>60E1-2;60E6</td>
</tr>
<tr>
<td>Df(3L)R-G7</td>
<td>62B8-9;62F2-5</td>
</tr>
<tr>
<td>Df(3L)BSC23</td>
<td>62E8;63B5-6</td>
</tr>
<tr>
<td>Df(3L)Exel6091</td>
<td>62E8;62F5</td>
</tr>
<tr>
<td>Df(3L)ZP1</td>
<td>66A17-20;66C1-5</td>
</tr>
<tr>
<td>Df(3L)vin7</td>
<td>68C8-11;69B4-5</td>
</tr>
<tr>
<td>Df(3L)fz-M21</td>
<td>70D2-3;71E4-5</td>
</tr>
<tr>
<td>Df(3L)BSC20</td>
<td>76A7-B1;76B4-5</td>
</tr>
<tr>
<td>Df(3L)Pc-2q</td>
<td>78C5-6;78E3-79A1</td>
</tr>
<tr>
<td>Df(3L)BSC21</td>
<td>79E5-F1;80A2-3</td>
</tr>
<tr>
<td>Df(3R)Scr</td>
<td>84A1-2;84B1-2</td>
</tr>
<tr>
<td>Df(3R)BSC24</td>
<td>85C4-9;85D12-14</td>
</tr>
<tr>
<td>Df(3R)ry506-85C</td>
<td>87D1-2;88E5-6</td>
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<tr>
<td>Df(3R)sbd105</td>
<td>88F9-89A1;89B9-10</td>
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<tr>
<td>Df(3R)P115</td>
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<tr>
<td>Df(3R)3450</td>
<td>98E3;99A6-8</td>
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</table>

\(^a\)Flies with the indicated deficiencies were crossed to *ey-GAL4*/CyO; *BID/BID* flies. Eyes of progeny heterozygous for the deficiency, *ey-GAL4* and *BID* were more disordered than reference heterozygous *ey-GAL4*; *BID* flies.
Figure. 3.3 Representative scanning electron micrographs showing the enhancement of the ey-GAL4/+; BID/+ rough eye phenotype by deficiencies. The first panel shows a reference eye. The other panels show eyes of the same genotype combined with deficiencies that uncover the indicated regions. All deficiencies that clearly enhanced the phenotype are shown, and are listed in Table 3.1.
**Mutant Allele Screen**

Our goal was to use the BID-dependent loss of BEAF function to identify interactions with specific genes. To this end, we used the rough eye phenotype to screen a variety of mutant alleles. A total of 91 mutant alleles were tested, encoding 80 proteins. Mutations in 17 genes enhanced the rough eye phenotype (Fig. 3.4 and Table 3.2), while the others did not (Table 3.3). In addition to listing the alleles that did or did not enhance the rough eye phenotype, Tables 3.2 and 3.3 correlate these results to those obtained with deficiencies that uncover the tested alleles.

Three criteria were used to select mutant alleles for testing. One criterion was to identify available mutations in genes deleted by deficiencies that enhanced the rough eye phenotype. We focused on genes encoding proteins known or predicted to function in the nucleus. Thirty-seven genes were selected, including a few from deficiencies that appeared to weakly interact but were excluded from Table 3.1. Other genes of interest did not have available mutations. Fourteen of the interacting genes we identified fall into this category, such as Distal-less (Dll), Myocardin-related transcription factor (MRTF) and fushi tarazu (ftz) (see below).

The second criterion was based on the hypothesis that insulators function by affecting chromatin structure or dynamics. In support of this model, we previously found that the presence of the BID protein results in a global disruption of salivary gland polytene chromosome morphology and removal of BEAF from these chromosomes. We also found that BEAF is a triplo-suppressor and haplo-enhancer of position-effect variegation (GILBERT *et al.* 2006). Based on this hypothesis we selected mutations in genes encoding proteins involved in insulator activity, covalent histone modifications, chromatin remodeling, chromatin structure, or whose activity is thought to involve chromatin. While this category overlapped with the first, 35 genes were selected solely based on this reasoning. This criterion resulted in the identification of a
couple genetic interactions, such as *zeste-white 5* (*Zw5*, also called *deformed wings, dwg*) and *Nipped-A* (see below).

**Figure 3.4** Representative scanning electron micrographs showing the enhancement of the *ey-GAL4/+; BID/+ rough eye phenotype by mutant alleles.* The first panel shows a reference eye. The other panels show eyes of the same genotype combined with the indicated mutant alleles. All alleles that clearly enhanced the phenotype are shown, and are listed in Table 3.2.
Table 3.2 Mutant alleles that enhance the rough eye phenotype

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele</th>
<th>Function&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Cytology</th>
<th>Tested Df&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>deformed wings (zeste-white 5)</td>
<td>dwg&lt;sup&gt;11-32&lt;/sup&gt;</td>
<td>IBP</td>
<td>3B3</td>
<td></td>
</tr>
<tr>
<td>Nipped-A</td>
<td>Nipped-A&lt;sup&gt;NC116&lt;/sup&gt;</td>
<td>HAT</td>
<td>41E1</td>
<td></td>
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<tr>
<td>Distal-less</td>
<td>Dll&lt;sup&gt;f&lt;/sup&gt;</td>
<td>TF</td>
<td>60E2</td>
<td>Df(2R)Dll-MP&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>Myocardin-related</td>
<td>MRTF&lt;sup&gt;KO&lt;/sup&gt;</td>
<td>TF</td>
<td>62F2-3</td>
<td>Df(3L)BSC23&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>transcription factor</td>
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<td></td>
<td></td>
<td>Df(3L)R-G7&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>TBP-associated factor 6</td>
<td>Taf6&lt;sup&gt;f&lt;/sup&gt;</td>
<td>GTF</td>
<td>76B9</td>
<td>Df(3L)kto2</td>
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<td></td>
<td></td>
<td></td>
<td>Df(3L)XS533</td>
</tr>
<tr>
<td>TBP-associated factor 1</td>
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<td>GTF</td>
<td>84A1</td>
<td>Df(3R)Scr&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>labial</td>
<td>lab&lt;sup&gt;14&lt;/sup&gt;</td>
<td>TF</td>
<td>84A1</td>
<td>Df(3R)Scr&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>proboscipedia</td>
<td>pb&lt;sup&gt;s&lt;/sup&gt;</td>
<td>TF</td>
<td>84A5</td>
<td>Df(3R)Scr&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>zen&lt;sup&gt;3&lt;/sup&gt;</td>
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<td>Df(3R)Scr&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>Df(3R)Scr&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>84A5</td>
<td>Df(3R)Scr&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>fushi tarazu</td>
<td>ftz&lt;sup&gt;2&lt;/sup&gt;</td>
<td>TF</td>
<td>84A6</td>
<td>Df(3R)Scr&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>su(Hw)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>IBP</td>
<td>88B3</td>
<td>Df(3R)ry506-85C&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>su(Hw)&lt;sup&gt;8&lt;/sup&gt;</td>
<td></td>
<td>88B3</td>
<td></td>
</tr>
<tr>
<td>spindle E</td>
<td>spn-&lt;sup&gt;E1&lt;/sup&gt;</td>
<td>RNAi</td>
<td>89A5</td>
<td>Df(3R)sbd105&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>abdominal A,</td>
<td>abd-A&lt;sup&gt;D24&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Abdominal B</td>
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<td>TF, TF</td>
<td>89E2-5</td>
<td>Df(3R)P115&lt;sup&gt;e&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>a</sup>Flies with the indicated mutations were crossed to ey-GAL4/CyO; BID/BID flies. Eyes of progeny heterozygous for the mutation, ey-GAL4 and BID were more disordered than reference heterozygous ey-GAL4; BID flies.

<sup>b</sup>: GTF: general transcription factor; HAT: histone acetyltransferase complex; IBP: insulator binding protein; RNAi: RNA interference; TF: transcription factor.

<sup>c</sup>: Deficiency that enhances the rough eye phenotype.
Table 3.3 Mutant alleles that do not enhance the rough eye phenotype

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele</th>
<th>Function</th>
<th>Cytology</th>
<th>Tested Df</th>
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<td>IBP</td>
<td>3B3</td>
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<tr>
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<td>HDAC</td>
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<td>ChRem</td>
<td>21B4-5</td>
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<td>DC</td>
<td>23F3</td>
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<td>TcoR/HDAC</td>
<td>49B5-7</td>
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<td>PcG/PRC1</td>
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<td>$Psc^{c22}$</td>
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<td>$lat^6$</td>
<td>C</td>
<td>49F7-8</td>
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<td>short stop</td>
<td>$shot^{103010}$</td>
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<td>Additional sex combs</td>
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<td>Distal-less</td>
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<tr>
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<td>$bab1^{Agal4-5}$</td>
<td>TF</td>
<td>62E2-F1</td>
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(Table con’d.)

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<th>Gene</th>
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<th>Genotype</th>
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<td>Rpd3</td>
<td>Df(3L)BSC23, Df(3L)Exel6091, Df(3L)GN24, Df(3L)JD198, Df(3L)ZN47</td>
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<sup>a</sup>Flyes with the indicated mutations were crossed to ey-GAL4/CyO; BID/BID flies. Eyes of progeny heterozygous for the mutation, ey-GAL4 and BID were not more disordered than reference heterozygous ey-GAL4; BID flies.

<sup>b</sup>: ABP: actin binding protein; ChRem: chromatin remodeling complex; ChStr: chromatin structure; DC: dosage compensation; DNA-BP: DNA binding protein; DNARep: DNA replication; HDAC: histone deacetylase complex; HetCh: heterochromatin protein; HK: histone kinase; HMT: histone methyltransferase; IPIP: insulator protein-interacting protein; MBP: microtubule binding protein; MED: mediator complex; NA: nucleosome assembly; ORC: origin replication complex; PcG: polycomb group protein; PM: protein metabolism; PRC1: polycomb repressor complex 1; PRC2: polycomb repressor complex 2; SP: secretory pathway; ST: signal transduction; TcoF: transcription co-factor; TcoR: transcriptional co-repressor; TrxG: trithorax group protein.

<sup>c</sup>: Deficiency that enhances the rough eye phenotype.
Table 3.4

Chromosomal deficiencies tested for genetic interactions

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<tr>
<th>Deficiency(^a)</th>
<th>Cytological Position</th>
<th>Enhancement</th>
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<td>(Df(2L))net-PMF</td>
<td>21A1;21B7-8</td>
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<td>(Df(2L))BSC4</td>
<td>21B7-C1;21C2-3</td>
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<td>22D2-3;22F1-2</td>
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(Table con’d.)

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\begin{align*}
Df(3L)66C-G28 & \quad 66B8-9;66C9-10 \\
Df(3L)BSC13 & \quad 66B12-C1;66D2-4 \\
Df(3L)h-i22 & \quad 66D10-11;66E1-2 \\
Df(3L)Scf-R6 & \quad 66E1-6;66F1-6 \\
Df(3L)BSC35 & \quad 66F1-2;67B2-3 \\
Df(3L)AC1 & \quad 67A2;67D11-13 \\
Df(3L)BSC14 & \quad 67E3-7;68A2-6 \\
Df(3L)vin5 & \quad 68A2-3;69A1-3 \\
Df(3L)vin7 & \quad 68C8-11;69B4-5 \quad \text{enhances} \\
Df(3L)eyg^{C1} & \quad 69A4-5;69D4-6 \\
Df(3L)BSC10 & \quad 69D4-5;69F5-7 \\
Df(3L)BSC12 & \quad 69F6-70A1;70A1-2 \\
In(3LR)C190^{i} Ubx^{42TR} & \quad 70A1-2;70C3-4 \\
Df(3L)fz-GF3b & \quad 70C1-2;70D4-5 \\
Df(3L)fz-M21 & \quad 70D2-3;71E4-5 \quad \text{enhances} \\
Df(3L)XG5 & \quad 71C2-3;72B1-C1 \\
Df(3L)brm11 & \quad 71F1-4;72D1-10 \\
Df(3L)st-f13 & \quad 72C1-D1;73A3-4 \\
Df(3L)81k19 & \quad 73A3-74F \\
Df(3L)BSC8 & \quad 74D3-75A1;75B2-5 \\
Df(3L)W10 & \quad 75A6-7;75C1-2 \\
Df(3L)Cat & \quad 75B8;75F1 \\
Df(3L)ED4782 & \quad 75F2;76A1 \\
Df(3L)fz2 & \quad 75F10-11;76A1-5 \\
Df(3L)BSC20 & \quad 76A7-B1;76B4-5 \quad \text{enhances} \\
Df(3L)kto2 & \quad 76B1-2;76D5 \\
Df(3L)XS533 & \quad 76B4;77B \\
Df(3L)rdgC-co2 & \quad 77A1;77D1 \\
Df(3L)ri-79c & \quad 77B-C;77F-78A \\
Df(3L)ri-XT1 & \quad 77E2-4;78A2-4 \\
Df(3L)ME107 & \quad 77F3;78C8-9 \\
Df(3L)Pc-2q & \quad 78C5-6;78E3-79A1 \quad \text{enhances} \\
Df(3L)Pc-kni & \quad 78C4--5;78C9--D1;79E2--3 \\
Df(3L)Pc-Mk & \quad 78A2;78C9 \\
Df(3L)ED4978 & \quad 78D5;79A2 \\
Df(3L)Ten-m-AL29 & \quad 79C1-3;79E3-8 \\
Df(3L)HD1 & \quad 79D3-E1;79F3-6 \\
Df(3L)BSC21 & \quad 79E5-F1;80A2-3 \quad \text{enhances} \\
Df(3R)ME15 & \quad 81F3-6;82F5-7 \\
Df(3R)3-4 & \quad 82F3-4;82F10-11 \\
\end{align*}
\]
(Table con’d.)

| Df(3R)e1025-14 | 82F8-10;83A1-3 |
| Df(3R)ED5177 | 83B4;83B6 |
| Df(3R)BSC47 | 83B7-C1;83C6-D1 |
| **Df(3R)Scr** | **84A1-2;84B1-2** enhances |
| **Df(3R)p712** | **84B1-2;84D11-12 or** |
| **Df(3R)ED5177** | **83B4;83B6** |
| **Df(3R)p-XT103** | **85A2;85C1-2** |
| **Df(3R)BSC24** | **85C4-9;85D12-14** enhances |
| **Df(3R)p-XT103** | **85B4-6;85B6** |
| **Df(3R)BSC24** | **85C4-9;85D12-14** enhances |
| **Df(3R)p-XT103** | **85B4-6;85B6** |

| **a**flies with the indicated deficiencies were crossed to ey-GAL4/CyO; BID/BID flies. Eyes of progeny heterozygous for the deficiency, ey-GAL4 and BID were not more disordered than reference heterozygous ey-GAL4; BID flies. |
The third criterion was based on a high-throughput two hybrid screen for protein interactions between Drosophila proteins (Giot et al. 2003). Five proteins were identified as interacting with BEAF. Four were encoded by conceptual genes with no available mutations. The fifth was katanin-60, a microtubule severing protein with an available mutation (katanin-60\textsuperscript{UY1645}). Because so few proteins directly connected to BEAF, we followed the interaction chains out to look for other candidates to test. We found a mutant allele of a transcriptional co-repressor (CtBP\textsuperscript{B7De-10}), four transcription factors (esg\textsuperscript{k6606}, Sna\textsuperscript{18}, gt\textsuperscript{O292}, kni\textsuperscript{9}), an actin binding protein (shot\textsuperscript{A03010}) and a protein involved in signal transduction (RhoBTB\textsuperscript{EP03099}). The encoded proteins had at most two proteins between them and BEAF in the interaction chain. None of the eight mutant alleles from this line of inquiry showed a genetic interaction with BEAF in the rough eye screen (Table 3.3).

A more limited screen was previously done using a UAS-BEAF-32A transgene with a GMR-GAL4 driver, which also leads to a rough eye phenotype (Yamaguchi et al. 2001). Overexpression of UAS-BEAF-32A should affect the composition of BEAF complexes, impairing the function of 32B-dependent insulators but not 32A-dependent insulators. Expression of BID should impair the function of all BEAF-utilizing insulators by reducing the number of DNA binding domains in BEAF complexes. It is difficult to compare deficiency results because only those that affected the rough eye phenotype were identified, and of those identified most were different than those we used. Fourteen genes were screened, and three interactions with UAS-BEAF-32A overexpression were found (DII, kohtalo and suppressor of hairy wing) (Yamaguchi et al. 2001). Of the genes seven tested in both screens, there was only one difference. Kohtalo encodes a transcription factor that is a subunit of the Mediator complex.
(JANODY et al. 2003). The \( \text{kto}^l \) mutation enhanced the rough eye phenotype caused by \( UAS-BEAF-32A \) expression, but not by \( BID \) expression.

**Interactions with Insulator-Related Proteins**

Mutant alleles encoding several proteins implicated in insulator activity were tested. The \( BEAF^{KG06094} \) allele has a transposon inserted into the intron separating the unique 32A and 32B exons. Ubiquitous expression of \( BID \) is lethal, suggesting BEAF-32A, 32B or both are essential proteins (GILBERT et al. 2006). Yet \( BEAF^{KG06094} \) is viable, and it had no effect on the BID-dependent rough eye phenotype. However, a deficiency that deletes the \( BEAF \) gene enhanced the phenotype (Table 3.3). Some BEAF binding sites overlap with binding sites for the transcription factor DREF (DNA replication-related factor), and there is evidence that BEAF and DREF compete for binding to these sites (HART et al. 1999). Neither the \( Dref^{KG09294} \) allele nor the deficiency that deletes \( Dref \) enhanced the rough eye phenotype. The chromosomal protein D1 and BEAF can cooperatively bind to some DNA sequences (CUVIER et al. 2002). It is not known how common this interaction is since these proteins show different localizations on chromosomes. D1 mainly binds to heterochromatic regions containing certain repetitive DNA sequences (AULNER et al. 2002; RODRIGUEZ ALFAGEME et al. 1980) while BEAF binds to euchromatin (ZHAO et al. 1995). The lethal mutant allele \( D1^{EYO5004} \) did not enhance the rough eye phenotype, although a deficiency that deletes \( D1 \) did (Table 3.3).

Mutant alleles of two other insulator binding proteins showed an interaction in the eye assay. The Zw5 protein binds to the scs insulator and is encoded by the \( dwg \) gene (GASZNER et al. 1999). Physical interactions between Zw5 and BEAF have been reported (BLANTON et al. 2003). Two \( dwg \) alleles were tested; \( dwg^{17-32} \) enhanced the rough eye phenotype and \( dwg^8 \) did not. However, it was previously reported that \( dwg^8 \) (called \( zw5^{62j1} \)) enhanced the rough eye
phenotype caused by UAS-BEAF-32A overexpression (BLANTON et al. 2003). The suppressor of hairy wing [su(Hw)] protein binds to an insulator found in the gypsy retrotransposon, and is essential for its insulator activity (GEYER and CORCES 1992). Like the deficiency that removes \( su(Hw) \), the \( su(Hw)^2 \) and \( su(Hw)^8 \) alleles enhanced the rough eye phenotype. This was also found to be the case with UAS-BEAF-32A overexpression (YAMAGUCHI et al. 2001). We found no enhancement by the \( su(Hw)^3 \) allele.

The mod(mdg4) and dTopors proteins interact with su(Hw) (CAPELSON and CORCES 2005; GERASIMOVA et al. 1995). The \( mod(mdg4)^{L3101} \), \( mod(mdg4)^{03852} \) and \( Topors^{05515} \) alleles did not enhance the phenotype, nor did the deficiencies that deleted these genes. The GAGA factor (GAF) is encoded by the \( trl \) gene, and has been implicated in insulator activity (OHTSUKI and LEVINE 1998). GAF has also been shown to play roles in transcriptional activation and repression, presumably related to its role in chromatin remodeling (TSUKIYAMA et al. 1994) and interactions with Polycomb group repressor proteins (MULHOLLAND et al. 2003), and in male X-chromosome dosage compensation (GREENBERG et al. 2004). GAF and mod(mdg4) each have a BTB domain, and these domains appear to be functionally interchangeable (READ et al. 2000). The \( trl^{P2325} \) allele did not enhance the eye phenotype although a deficiency that uncovers this gene did.

**Interactions with Transcription Factors**

Most of the other mutant alleles that enhanced the rough eye phenotype were in genes encoding transcription factors or general transcription factors (Table 3.2, Fig. 3.4). However, this is not a general effect. Of the 29 transcription factors tested, 18 did not enhance the phenotype (Table 3.3). Three others had alleles that enhanced and other alleles that did not, as described below. Seven of the eleven transcription factors that showed an effect were in the
Antennapedia complex (ANTC). The alleles were $lab^{14}$, $pb^{5}$, $zen^{3}$, $bcd^{12}$, $Dfd^{6}$, $Scr^{2}$, $ftz^{3}$ and $ftz^{11}$. The only tested allele from the ANTC that did not show an interaction was $Antp^{10}$. A major role of the ANTC is to specify fates in the developing head (DeNeell 1994; Diedrich et al. 1989).

The bithorax complex (BXC) encodes transcription factors involved in specifying fates in the developing thorax and abdomen (Lewis 1998; Maeda and Karch 2006). Genes of the ANTC and BXC play critical roles in specifying development of the Drosophila body plan, just as their vertebrate homologs play similar critical roles in vertebrate development. Individual mutations in the BXC did not enhance the rough eye phenotype, although the double mutation $abd-A^{D24}$ and $Abd-B^{D18}$ did. These particular mutations were not tested individually, so it is not known if either alone would enhance.

The other two transcription factor genes that showed an interaction were Distal-less and Myocardin-related transcription factor. We found that $Dll^{5}$ enhanced the rough eye phenotype while the weaker $Dll^{9}$ allele did not; both alleles were previously found to enhance the rough eye phenotype caused by $UAS-BEAF-32A$ overexpression (Yamaguchi et al. 2001). $MRTF^{KO1}$ is a null allele generated by homologous recombination (Han et al. 2004). $Dll$ is involved in developmental processes including limb and antennae development (Dong et al. 2001), while $MRTF$ is involved in development of the tracheal system (Han et al. 2004).

Alleles of two general transcription factor genes, $Taf1^{R14}$ and $Taf6^{I}$, were tested and found to enhance the phenotype. Both proteins are part of the TFIID complex that includes the TATA-binding protein. With the exception of $Taf6$, all of these transcription factor genes are uncovered by deficiencies that enhance the rough eye phenotype (Table 3.2).
Interactions with Nipped-A and spindle-E (spn-E)

The other two genetic interactions identified were with Nipped-A$^{NC116}$ and spn-E$^{1}$. Nipped-A is a subunit of the SAGA histone acetyltransferase complex, although it appears to be a multifunctional protein that also functions independently of SAGA (Gause et al. 2006). Some subunits are shared between the SAGA and TFIID complexes (Lee et al. 2000), indicating that these complexes interact. The two TFIID subunit genes tested in our assay enhanced the rough eye phenotype, so the interaction with Nipped-A could reflect the relationship between SAGA and TFIID. Alternatively, it could reflect an interaction between BEAF and some other aspect of Nipped-A function.

The spn-E gene encodes a helicase that is part of a protein complex involved in RNA interference. This complex plays a role in oocyte maturation (Kennerdell et al. 2002) and heterochromatin formation (Pal-Bhadra et al. 2004). Mutations in spn-E affect heterochromatin by affecting the localization of Heterochromatin Protein 1 and dramatically reducing the level of histone H3 lysine 9 methylation, presumably by affecting the activity of the methyltransferase SU(VAR)3-9. We tested mutant alleles for the genes encoding these two proteins [Su(var)205$^{5}$, Su(var)3-9$^{1}$] and found that they did not enhance the BID-dependent rough eye phenotype.

Most Tested Genes Encoding Proteins Involved in Chromatin Function Do Not Enhance the BID-Dependent Rough Eye Phenotype

As indicated in Table 3.3, many mutant alleles that we tested did not enhance the rough eye phenotype. As already mentioned, this includes a number of transcription factors. Other tested alleles encoded various proteins involved in chromatin structure or function. This includes genes involved in nucleosome assembly, chromatin remodeling, covalent histone modifications (histone acetyltransferases, deacetylases, methyltransferases, a kinase), male X chromosome...
dosage compensation, transcriptional co-repression, polycomb group and trithorax group genes. Genes involved in DNA repair, mitosis and signal transduction were also tested and found to have no effect. These results suggest that there is not a general interaction in this assay between BEAF and proteins involved in various aspects of chromatin structure or dynamics.

**Specificity of the Genetic Interactions**

Eighteen chromosomes with mutant alleles of interest enhanced the $BID$-dependent rough eye phenotype (Table 2). None of these chromosomes resulted in a rough eye phenotype in the absence of $BID$ expression (data not shown). This indicates an interaction between the proteins encoded by the mutant alleles and $BEAF$. If this is the case, then supplying an extra functional copy of the genes should eliminate the enhanced phenotype. We tested this for two genes. A $P[ftz]$ transgene (SCHWARTZ et al. 2001) was used together with the $ftz^{3}$ and $ftz^{11}$ alleles, and a $UAS-MRTF$ transgene (HAN et al. 2004) was used with the $MRTF^{K01}$ allele. The rescue transgenes reversed the effects of the mutant alleles (Fig. 3.5). Extrapolating from these results, it is likely that there are genetic interactions between all genes identified in this screen and $BEAF$.

**Discussion**

Insulators are thought to divide chromosomes into functionally independent domains, preventing communication between enhancers and promoters located in different domains. As such, they play an important role in gene regulation and perhaps in nuclear organization. Yet very little is known about molecular mechanisms employed by insulators. Models propose that insulators function by influencing chromatin structure or dynamics, nuclear organization, or by acting as promoter decoys (CAPELSON and CORCES 2004; KUHN and GEYER 2003; LABRADOR and CORCES 2002; WEST et al. 2002). Using a GAL4 UAS-controlled transgene encoding a
dominant negative form of the BEAF proteins (*BID*), we found evidence linking BEAF function to chromatin structure or dynamics (Gilbert et al. 2006). Here we extend those results by using this system to perform a screen aimed at identifying factors that genetically interact with BEAF. Mutant alleles of 17 genes were found to enhance the *BID*-dependent rough eye phenotype. Most of these genes map to chromosomal deficiencies that also enhanced the phenotype.

![Figure 3.5: Transgenes providing an extra wild-type copy of mutant alleles rescue the enhanced rough eye phenotype.](image)

**Figure. 3.5 Transgenes providing an extra wild-type copy of mutant alleles rescue the enhanced rough eye phenotype.** The rough eye phenotype observed in flies heterozygous for *ey-GAL4* and *BID* is enhanced in the presence of heterozygous *ftz*³ or *ftz*¹¹ mutations. This enhanced phenotype is reversed in the presence of a heterozygous *ftz* transgene driven by the *ftz* promoter. Similarly, the enhanced rough eye observed in the presence of the heterozygous *MRTF KO1* allele is reversed in the presence of a heterozygous *UAS-MRTF* transgene. Genotypes are as indicated.
Most of the identified genes encode proteins that fall into two classes: insulator binding proteins or transcription factors. Two of three *su(Hw)* alleles and one of two *dwg* alleles enhanced the rough eye phenotype. Similar results were previously obtained by overproduction of BEAF-32A protein (Blanton *et al.* 2003; Yamaguchi *et al.* 2001). Both *su(Hw)* and *dwg* encode insulator binding proteins, indicating an interaction between different classes of insulators. However, mutant alleles encoding other proteins that interact with BEAF or *su(Hw)* did not enhance the phenotype. This includes genes that encode DREF, D1, mod(mdg4) and Topors. While it is possible that this indicates that these proteins do not influence the activity of insulators dependent on BEAF or *su(Hw)*, there are other possible explanations for this. The mutations tested for these four genes are P-element insertions in or near the transcription units, and their effects on these genes have not been well characterized. Perhaps they are not null mutations or affect neighboring genes. Perhaps if other mutations in these genes were tested an effect would be observed, particularly for cases where deficiencies showed an enhancement. Functional redundancy with other proteins or sufficient production of protein from the single wild-type gene are also possible explanations. Another possibility is that interactions would be detected with an assay that could confidently detect suppression of the phenotype. More studies are needed to determine the relationship of these other proteins to insulator activity and BEAF function.

Genetic interactions were detected with only one third of the tested transcription factors. Most of these transcription factors were from the ANTC, which is involved in head development, or were general transcription factors. A double mutation from the BXC, which is involved in thorax and abdomen development, also showed an interaction. The other two identified transcription factors were Dll and MRTF. Like transcription factors encoded by the ANTC, Dll
regulates genes involved in head development. MRTF regulates genes involved in development of the tracheal system. The tracheal system encompasses the entire body, including the head. Why did these transcription factors show an interaction, while others did not? Consideration of the assay leads to a possible explanation. The assay is based on eye development, rather than any particular insulator. If certain genes involved in eye development are insulated by BEAF-dependent insulators, then impairing BEAF function could lead to faulty regulation of those genes and cause a disruption of eye development. A decrease in the level of transcription factors involved in the regulation of these genes could exacerbate the faulty regulation. This could also explain the genetic interactions with TFIID subunits. A similar result could be obtained with transcription factors that regulate genes adjacent to key genes involved in eye development, if a BEAF-dependent insulator separates the adjacent genes. For instance, this could account for the interaction with the *abd-A, Abd-B* double mutant. However, this would be a much less common occurrence. It should be emphasized that it is not known how BEAF functions, and these genetic interactions could be indirect. A high-throughput 2-hybrid assay did not find evidence for direct interactions between BEAF and transcription factors (Giot et al. 2003). Nevertheless, according to this explanation our results support an important role for BEAF in assuring proper gene regulation during eye development. Because it is unlikely that the role of BEAF is limited to eye development, our results suggest that BEAF plays an important role in maintaining global patterns of gene expression.

We previously provided evidence that *BID* expression specifically targets BEAF function, leads to a global disruption of salivary gland polytene chromosome structure, and enhances pericentromeric heterochromatin-dependent position effect variegation (PEV). In addition, a third copy of BEAF suppresses PEV (Gilbert et al. 2006). Yet we tested mutant
alleles of over 40 proteins involved in chromatin structure or dynamics, and only two showed an interaction in our eye-based assay. A similar eye-based screen for genetic interactions with the chromatin remodeling protein brahma (brm) also failed to find interactions with chromatin proteins other than members of the brm protein complex (ARMSTRONG et al. 2005). Interactions with proteins involved in transcription and signaling were also found. This supports our explanation that the interactions we observed might have been limited by the design of the assay. We did not observe an enhancement of the rough eye phenotype with the brm$^2$ mutation or a mutation in the brm subunit moira (mor$^1$). A screen involving the activity of a specific BEAF-dependent insulator rather than a developmental process might provide different results, and help determine the relationship between BEAF function and chromatin.

Of the mutant alleles we tested that encode chromatin proteins, only Nipped-A and spn-E enhanced the rough eye phenotype. As mentioned, the interaction with Nipped-A might be related to its role as a subunit of the SAGA histone acetyltransferase complex, and the close association between SAGA and TFIID. The interaction with spn-E could indicate an interplay between BEAF function and heterochromatin. However, spn-E mutations cause a reduction in histone H3 lysine 9 methylation, a heterochromatin mark mediated by the SU(VAR)3-9 methyltransferase, and redistribution of HP1 from heterochromatin to euchromatin (PAL-BHADRA et al. 2004). Mutations in the genes encoding these proteins did not enhance the rough eye phenotype. As mentioned above, it is possible that there are redundant activities that mask the effects of these mutations, or that these proteins are not produced in limiting amounts, or that other mutations in these genes would show an effect (especially for genes where we used poorly characterized mutations, as discussed earlier). Another intriguing possibility for future study is that the RNA interference machinery is involved in BEAF-dependent insulator activity. It has
recently been reported that the RNAi machinery affects Gypsy insulator function (LEI and CORCES 2006). In particular, the helicase Rm62 antagonizes and the Argonaute proteins piwi and aubergine enhance the activity of the Gypsy insulator.

This work provides strong evidence for a role for BEAF in maintaining global patterns of gene regulation. It also provides evidence for cross-talk between different classes of insulators, perhaps by affecting the regulation of different sets of genes. Together with a previous study of the vertebrate CTCF insulator protein (MUKHOPADHYAY et al. 2004), our results help validate the hypothesis that insulators play an extensive role in gene regulation. The other interesting result to come out of this screen was the interaction with \textit{spn-E}. Using this assay to test mutations in other genes involved in RNAi will help determine if BEAF-dependent insulators are affected by RNAi pathways, as is the su(Hw)-dependent Gypsy insulator. A screen involving the activity of a specific BEAF-dependent insulator rather than a developmental process would help explore and extend the results presented here and in our previous study using the BID protein (GILBERT et al. 2006).

**Literature Cited**


MUKHOPADHYAY, R., W. YU, J. WHITEHEAD, J. XU, M. LEZCANO et al., 2004 The binding sites for the chromatin insulator protein CTCF map to DNA methylation-free domains genome-wide. Genome Res 14: 1594-1602.


CHAPTER FOUR

LACK OF THE DROSOPHILA BEAF INSULATOR PROTEINS AFFECTS EXPRESSION OF GENES ENCODING PROTEINS INVOLVED IN GENE REGULATION
Introduction

For optimum growth and development gene expression needs to be tightly co-ordinated. However, enhancers are capable of interacting with any promoter in transgenic constructs; there is little specificity (Kermekchiev et al. 1991). Evidence indicates that this promiscuity of enhancers can be restricted to a certain promoter by insulator sequences. In doing so, insulators are thought to divide chromosome into domains such that interactions may be allowed within domains but not between different domains (Capelison and Corces 2004; Geyer 1997). Evidence for this model comes from experiments showing that insulators can block communication when placed between an enhancer and its promoter not when placed upstream or downstream of them (Gohl et al. 2008; Nabirochkin et al. 1998; Scott and Geyer 1995; Zhao and Dean 2004). Insulators can also protect from position effects caused by condensed chromatin (Cuvier et al. 1998; Recillas-Targa et al. 2002). This function of insulators makes it possible for transcriptionally active genes to be separate from adjacent silent condensed regions (Bickmore et al. 2004; Litt et al. 2001). This is known as the barrier activity of insulators (Bell et al. 1999). Examples of insulators have been found in different species (e.g. yeast, Drosophila, mammals). The Drosophila scs and scs’ sequences located at the proximal and distal boundaries of the 87A7 heat-shock puff of polytene chromosomes are two of the first insulators to be characterized (Farkas and Udvardy 1992; Udvardy et al. 1985). Well studied examples of a vertebrate insulator are the insulator sequence located between the insulin-like growth factor 2 (Igf2) and H19 genes (Bell and Felsenfeld 2000) and the HS4 DNaseI hypersensitive site from the chicken β globin locus (Abruzzo and Reitman 1994; Inoue et al. 1999; Stamatoyannopoulos et al. 1995).
Here we provide evidence to show that the Boundary Element Associated Factor (BEAF) plays a role in gene expression. The two 32kDa proteins BEAF-32A and BEAF-32B arise from a single BEAF gene probably by alternative transcription initiation. The two proteins only differ in their N-terminal DNA binding domain, with the rest of the proteins being derived from the same exon and so are identical (HART et al. 1997). This includes a C-terminal interaction domain that mediates interactions between BEAF subunits. Both these proteins bind to the scs’ insulator, and confer insulator activity. Many BEAF binding sites are found on polytene chromosomes when immunostained (HART et al. 1997; ZHAO et al. 1995). This shows that BEAF is an insulator binding protein which is spread throughout the genome.

To study BEAF we developed two genetic tools. First a transgene encoding a dominant negative form of BEAF was designed which includes the BEAF self-interaction domain (BID) but lacks an N-terminal DNA binding domain (GILBERT et al. 2006). BID expression is controlled by a GAL4-inducible promoter. BID protein was shown to physically interact with BEAF in vivo by co-immunoprecipitation. BID expression in the eyes driven by an ey-GAL4 driver resulted in rough eye phenotype that could be rescued by an extra copy of BEAF provided by a transgene. This showed that BEAF plays a role in eye development. BID expression also resulted in disruption of salivary gland polytene chromosome morphology of third instar larvae. An extra copy of BEAF rescued this phenotype. Furthermore, BID interfered with scs’ insulator function in both position independent expression and enhancer-blocking assays. In the enhancer blocking assay an insulator sequence is placed between an enhancer and a transgene. Insulators have been found to block enhancer-transgene communication when placed between them. But insulators placed upstream or downstream have no effect (KELLUM and SCHEDL 1991). In the position-independent expression assay, a transgene lacking any enhancer is bracketed by insulator sequences on either side. It has been shown that since the transgene lacks an enhancer, bracketing it with insulators
leads to low levels of expression of the transgene. In the absence of the insulator, chromosomal position effects result in activation of the transgene (Geyer 1997; Kellum and Schedl 1991a; Zhang and Liu 2004). When BID was ubiquitously expressed by a daughterless driver the embryo was unable to survive past embryogenesis. This indicates that BEAF is an essential gene required during embryo development stages. Secondly we generated a knock-out of BEAF using homologous recombination (Roy et al. 2007a). We showed that flies with this BEAF knock-out allele (BEAF\textsuperscript{AB-KO}) lacked scs’ insulator function as shown by enhancer blocking and position independent expression assays. Absence of BEAF also affected oogenesis as BEAF\textsuperscript{AB-KO} females had almost half the size of ovary compared to wild-type and were almost sterile. Both genetic tools show that the BEAF proteins are essential and are required for scs’ insulator function.

We used the rough eye phenotype caused by BID in a genetic screen and found evidence for cross-talk between different classes of insulator proteins and for a broad role for BEAF in maintaining patterns of gene expression (Roy et al. 2007b). Most mutant alleles that enhanced the rough eye phenotype encoded general transcription factors, transcription factors involved in head development, and insulator binding proteins. In this paper we test the hypothesis that genes encoding the identified transcription factors should be mis-regulated in the absence of BEAF.

We looked at expression levels and patterns of ftz, bcd, pb, su(Hw), Dfd and Dref. Using q-RT-PCR, we show that expression levels of most of genes were affected in the absence of BEAF. Using in situ hybridization, we show that patterns of expression of several of the above genes were altered in BEAF\textsuperscript{AB-KO} embryos. To look at binding site accessibility, we immunostained third instar salivary gland polytene chromosomes. For this purpose we were limited to proteins which are present in salivary glands for which we had antibodies. We
immunostained for DREF and MLE and found DREF binding remained unaffected. However subtle differences in MLE binding to DNA was observed in the absence of the BEAF.

Materials and Methods

Scanning Electron Microscopy

Flies were prepared and SEM was performed as previously described (ROY et al. 2007b).

Embryo Collection

Embryos were collected from flies homozygous for wild-type or the BEAF<sup>AB-KO</sup> allele. BEAF<sup>AB-KO</sup> flies cannot be maintained as a stock. To avoid maternal BEAF we used the BEAF<sup>AB-KO</sup> /CyO P[w<sup>+</sup> GFP] line producing the green fluorescent protein (GFP). Homozygous BEAF<sup>AB-KO</sup> larvae were isolated based on lack of GFP under a fluorescent microscope, placed in vials to eclose, and used to set up fly cages. Embryos were collected on grape-juice agar plates. For q-RT-PCR assays, staged embryos were aged to give 0-2 hrs, 2-4 hrs and 4-8 hrs collections. These embryos were then dechorionated and flash-frozen with liquid nitrogen and stored at -80° C prior to RNA extraction. For in-situ hybridization assays embryos were collected twice a day. These embryos were fixed and stored using a published protocol (LECUYER et al. 2008).

q-RT-PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen<sup>TM</sup> Life Technologies). Embryos with the null BEAF<sup>AB-KO</sup> mutation (ROY et al. 2007a) or wild-type for BEAF were used. Primers were purchased from Integrative DNA Technologies. The RT primers shown in Table 4.1 were used to prime gene-specific reverse transcription with SuperScript<sup>TM</sup> III reverse transcriptase in a 20-µl total volume reaction that included RT primers (2µM), 1x RT buffer (Invitrogen<sup>TM</sup> Life Technologies), 5 mM MgCl<sub>2</sub>, 10mM DTT, RNAase OUT<sup>TM</sup> (40U/µl) and 0.5 mM deoxynucleotide triphosphate mix (dNTP). The reaction was incubated at 50°C for 50 min
and then terminated by incubation at 85°C for 5 min. After reverse transcription, the cDNA generated was used to carry out quantitative PCR. 1x Taq buffer, 1.5 mM MgCl₂, 200 µM deoxynucleotide triphosphates (dNTP), 100 nM forward and reverse primer (Table 4.2), a 20,000 fold stock diluted SYBR Green, and Taq polymerase (1U/reaction) were added to make a final volume of 20 µl. PCR was performed in an ABI 7000 Prism Sequence Detector (Perkin-Elmer) with preheating at 94°C for 2 min, followed by 40 cycles of melting (94°C for 30 s), annealing (55°C for 30 s) and extension (72°C for 30 s), ending with a holding temperature of 72°C for 4 mins. The level of *Trf* mRNA, which encodes a general transcription factor, was used as an internal control for the RNA samples. Our results indicate that the level of *Trf* RNA is unaffected by the lack of BEAF. Three independent RNA preparations of both genotypes were used, and triplicate q-PCR reactions for each RNA preparation were done. The relative BEAF<sup>AB-KO</sup> level is calculated by first subtracting the wild-type gene C<sub>t</sub> value from the wild-type *Trf* C<sub>t</sub> value. This gave us a ΔC<sub>t</sub><sup>WT</sup> (Wt *Trf*- WT Gene) value. A similar ΔC<sub>t</sub><sup>BEAF<sub>AB-KO</sub></sup> for the BEAF<sup>AB-KO</sup> sample was also calculated. The relative value is obtained by dividing the ΔC<sub>t</sub><sup>BEAF<sub>AB-KO</sub></sup> / ΔC<sub>t</sub><sup>WT</sup>. The relative values of three independent RNA extractions were calculated and an average final relative value was determined. This was done for all three staged embryo collections.

**Table 4.1: Gene specific RT primers**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicoid</td>
<td>5'-CCCTGGGAACCATTTACACGGATCTT-3’</td>
</tr>
<tr>
<td>Dref</td>
<td>5’-TGAACAGATCCTTGTTGCGGTGCT-3’</td>
</tr>
<tr>
<td>Deformed</td>
<td>5’-TTCGTCGGTGGTTCCGAGGAG-3’</td>
</tr>
<tr>
<td>ftz</td>
<td>5’-TTCTTCACGCCCAGATTGGTGAGCAGA-3’</td>
</tr>
<tr>
<td>Proboscipedia</td>
<td>5’-TTCAGTGTGTCAAGGCGCTTATCTGAG-3’</td>
</tr>
<tr>
<td>su(Hw)</td>
<td>5’-TTACGCCCAGCCTCATCGGTGCA-3’</td>
</tr>
<tr>
<td>Trf</td>
<td>5’-GCAAGCTTGTTGCCATGAACCTTTACGG-3’</td>
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</tbody>
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### Table 4.2: Quantitative PCR primers

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicoid</td>
<td>5’-AGAGGCTTAGCAGGAACCGAAGAT-3’</td>
<td>5’-AACACGCCTCCTCATCCAGGCTAAT</td>
</tr>
<tr>
<td>Dref</td>
<td>5’-ATACACGCAATTGGGCACAGCATC-3’</td>
<td>5’-TCCAAAGTGAGCGCCAATGTATACGCT-3’</td>
</tr>
<tr>
<td>Deformed</td>
<td>5’-TCCAATTATGCCAATGCCACTCCCG-3’</td>
<td>5’-TATCCATTTGCGGATCCACCCACT-3’</td>
</tr>
<tr>
<td>ftz</td>
<td>5’-AACAGCCAGGAGCCACTACAGCTA-3’</td>
<td>5’-TGGTCACCTGCTCTCTGATTGTGT-3’</td>
</tr>
<tr>
<td>Proboscipedia</td>
<td>5’-ACGACTGAGTGTGTGTGT-3’</td>
<td>5’-GTTTGCTAACCCTGCAAATGGTG-3’</td>
</tr>
<tr>
<td>su(Hw)</td>
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<td>5’-AATTCACGCACCACACACGCCATT-3’</td>
</tr>
<tr>
<td>Trf</td>
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<td>5’-CTTGGCGCCGTTGAAAGCTAGC-3’</td>
</tr>
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</table>

### In-Situ Hybridization

Wild-type and **BEAF**<sub>4B</sub>-**KO** embryos were collected, fixed and hybridized with gene specific biotinylated RNA probes based on the protocols of Lécuyer *et al.* ([Lecuyer et al. 2008](#)) and Kosman *et al.* ([Kosman et al. 2004](#)). Gene specific primer pairs with a T7 promoter on one primer and a T3 promoter on the other were used along with genomic DNA to PCR amplify ~1 kb regions of the corresponding genes. Transcription reactions were then conducted using Biotin RNA labeling mix with T3 or T7 RNA polymerase to synthesize strand-specific biotinylated RNA probes (Roche Applied Science). The gene-specific primers used for the PCR amplification are listed in Table 4.3. The post-hybridization probe detection was carried out using the TSA<sup>TM</sup> BIOTIN SYSTEM Kit (Perkin Elmer Life and Analytical Sciences, Inc.) with DAB (diaminobenzidine) and CoCl<sub>2</sub>.

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Table 4.3 Primers used for PCR amplification of in situ hybridization templates

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward Primer (including clamp and T3 RNA polymerase core promoter sequence)</th>
<th>Reverse primer (including clamp and T7 RNA polymerase core promoter sequence)</th>
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<tr>
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<td>5’-CCAAGCCTTCATTAACCCCTCA CTAAAG GGAGAACATGAGCGACCG GGAATAAGACCT-3’</td>
<td>5’-CAGAGATGCATAATACGACTCTACTATAGGGAGCTTAAAGAGACAACAGCCTCAGGTC-3’</td>
</tr>
<tr>
<td>Dref</td>
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<td>5’-CAGAGATGCATAATACGACTCTACTATAGGGAGCTTAAAGAGACAACAGCCTCAGGTC-3’</td>
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<tr>
<td>Deformed</td>
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<td>5’-CAGAGATGCATAATACGACTCTACTATAGGGAGCTTAAAGAGACAACAGCCTCAGGTC-3’</td>
</tr>
<tr>
<td>ftz</td>
<td>5’-CCAAGCCTTCATTAACCCCTCA ACTAAAG GGAGAACATGAGCGACCG GGAATAAGACCT-3’</td>
<td>5’-CAGAGATGCATAATACGACTCTACTATAGGGAGCTTAAAGAGACAACAGCCTCAGGTC-3’</td>
</tr>
<tr>
<td>Proboscipedia</td>
<td>5’-CCAAGCCTTCATTAACCCCTCA ACTAAAG GGAGAACATGAGCGACCG GGAATAAGACCT-3’</td>
<td>5’-CAGAGATGCATAATACGACTCTACTATAGGGAGCTTAAAGAGACAACAGCCTCAGGTC-3’</td>
</tr>
<tr>
<td>su(Hw)</td>
<td>5’-CCAAGCCTTCATTAACCCCTCA ACTAAAG GGAGAACATGAGCGACCG GGAATAAGACCT-3’</td>
<td>5’-CAGAGATGCATAATACGACTCTACTATAGGGAGCTTAAAGAGACAACAGCCTCAGGTC-3’</td>
</tr>
</tbody>
</table>

**Immunostaining Polytene Chromosomes**

Polytene chromosome squashes were prepared from salivary glands of healthy third instar larvae and immunostained as previously described (Gilbert et al. 2006). We used the BEAF^{AB-KO} / CyO P[w^{+}GFP] line producing the green fluorescent protein (GFP). Homozygous BEAF^{AB-KO} larvae were isolated based on lack of GFP using a fluorescent microscope. Mouse anti-DREF antibody was used at a 1:400 dilution (Hart et al. 1999). Rabbit anti-MLE antibody was used at a 1:400 dilution. FITC or Texas Red-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies were used at 1:400 dilutions (Jackson, West Grove, PA) respectively for DREF and MLE. 100 ng/ml DAPI was used to stain the polytene chromosomes. A Zeiss Axioskop microscope equipped with a Spot RT Slider CCD camera was used to view and photograph the slides.
Results

Genes Show Altered Levels of Expression in $BEAF^{4B\cdot KO}$ Embryos

Expression of the $BID$ transgene, which encodes a dominant negative form of BEAF, in eye tissue via an ey-GAL4 driver results in a rough eye phenotype that can be rescued by introducing an extra copy of $BEAF$ via a transgene (GILBERT et al. 2006). We used this tool to look for genetic interactions with BEAF (ROY et al. 2007b). We hypothesized that genes that are important for BEAF function would modify this phenotype when mutations for such genes are introduced in a $BID$ background. This led to the discovery of 17 genes that enhanced the rough eye phenotype. Rescue transgenes of some of those genes which were also introduced reversed the effect thus confirming the interaction. Results obtained with $ftz$ are shown in Fig. 4.1. The interactions we found were with transcription factors, general transcription factors and insulator binding proteins, which we interpreted as downstream effects on gene regulation caused by a lack of BEAF function rather than proteins involved in BEAF function.

If the genetic interactions represent downstream effects, then expression of the above genes might be altered in the absence of BEAF. To test this, levels of expression of five genes [$su(Hw)$, $Dfd$, $ftz$, $pb$ and $bcd$] which showed positive interaction with BEAF in the rough eye assay (ROY et al. 2007b) were compared between wild-type and $BEAF^{4B\cdot KO}$ embryos. We also included the gene for the transcription factor $Dref$ (Fig. 4.2A-F). Although the allele of $Dref$ ($Dref^{kg09294}$) tested did not enhance the rough eye phenotype, there is evidence that BEAF and DREF can compete for binding to overlapping binding sites (HART et al. 1999). Wild-type and $BEAF^{4B\cdot KO}$ fly cages were set up and embryos were collected on grape juice agar plates and aged to 0-2 hrs, 2-4 hrs and 4-8 hrs. RNA was extracted from staged embryos followed by q-RT-PCR, using gene-specific primers for the initial RT step, to compare the levels of mRNA present in
Figure 4.1 Rough eye phenotype caused by interfering with BEAF function, and enhancement of the phenotype by the ftz[3] allele. (A) Flies homozygous for the BID transgene without a GAL4 driver have normal eyes. (B) Flies heterozygous for BID and an ey-GAL4 driver have moderately rough eyes (C) Flies homozygous for BID and heterozygous for ey-GAL4 driver show extreme rough eye phenotype (D) The rough eye phenotype in flies heterozygous for BID and ey-GAL4 is rescued to near wild-type by a third copy of the BEAF gene provided by a transgene (E) The rough eye phenotype in flies heterozygous for BID and ey-GAL4 is enhanced by introduction of the ftz[3] mutant allele. (F) The enhanced rough eye phenotype caused by the ftz[3] allele is rescued by providing an extra wild-type copy of ftz allele.
Figure 4.2 Genes show altered levels of expression in $BEAF^{AB-KO}$ embryos. RNA isolated from 0-2 hr, 2-4 hr and 4-8 hr embryos was used in q-RT-PCR to determine expression levels in $BEAF^{AB-KO}$ embryos relative to wild-type embryos. Shown are results from triplicate reactions on three independent RNA samples of each genotype, normalized to wild-type. Bars represent standard deviations. Results are shown for (A) $su(Hw)$, (B) $ftz$, (C) $Dfd$, (D) $pb$, (E) $bcd$ and (F) $Dref$. 
wild-type and \textit{BEAF}^{AB-KO} embryos in each collection (Fig. 4.2A-F). \textit{su}(Hw) showed an overexpression in \textit{BEAF}^{AB-KO} embryos at all three stages. Although variable in the three RNA collections, the level of \textit{su}(Hw) over-expression was a dramatic 1000–fold at the 4-8 hr stage of embryo development. For \textit{ftz}, \textit{Dfd}, \textit{pb} and \textit{bcd} the mRNA level dropped in \textit{BEAF}^{AB-KO} embryos at the 4-8 hour stage. Results were more variable earlier. This variability, together with the large standard deviations between experiments, could indicate that the effects of a lack of BEAF on gene regulation is somewhat stochastic. This is similar to the variable ovary phenotypes we have observed (Roy et al. 2007a). \textit{Dref} expression levels, on the other hand, remained unaffected in \textit{BEAF}^{AB-KO} embryos.

**Genes Show Altered Patterns of Expression in \textit{BEAF}^{AB-KO} Embryos**

Having established that expression levels of transcription factors can be affected by a lack of BEAF, we next looked for effects on expression patterns. This was done by in situ hybridization to wild-type and \textit{BEAF}^{AB-KO} embryos using strand-specific RNA probes (Fig.4.3). Sense-strand probes gave no signals, indicating hybridization specificity (not shown). First we looked at the Drosophila segmentation gene \textit{ftz}. At the cellular blastoderm stage, \textit{ftz} is expressed in a pattern of seven transverse stripes (Fig.4.3A) (Doe et al. 1988). This pattern was clearly altered in \textit{BEAF}^{AB-KO} embryos (Fig. 4.3B). The \textit{ftz} stripes were more diffuse in many \textit{BEAF}^{AB-KO} embryos, often with altered spacing between stripes.

The pattern of \textit{Dfd} expression also was affected to a significant extent in \textit{BEAF}^{AB-KO} embryos. At the cellular blastoderm stage in wild-type embryos \textit{Dfd} mRNA accumulates in a stripe approximately 6 cells wide (Fig. 4.3C) (Jack and McGinnis 1990). After the germ band is fully extended \textit{Dfd} is restricted to the maxillary (Mx) and the mandibular (Mn) segments (6 hrs after egg laying; AEL) (Fig. 4.3C middle panel). During the germ band retraction stage (10 hr
there is strong expression of \textit{Dfd} in the cells of the maxillary segment which border the labial lobe, while the anterior-lateral cells of the maxillary segment no longer express \textit{Dfd} (Fig. 4.3C right panel). In addition, at this time, \textit{Dfd} is also expressed in one or two rows of cells in the anterior portion of the dorsal ridge bordering on the optic lobe (Jack and McGinnis 1990). These patterns are altered in \textit{BEAF}^{AB-KO} embryos. \textit{Dfd} mRNA in \textit{BEAF}^{AB-KO} embryos fails to form a defined stripe at the cellular blastoderm stage (Fig. 4.3D left panel). During the germ band extension stage \textit{Dfd} is not specifically expressed in the Mx and Mn segments. Rather it is expressed all along the anterior-posterior axis on the ventral side of the embryo (Fig. 4.3D middle panel). In the germ-band retraction phase, \textit{Dfd} mRNA expression is shifted slightly away from the wild-type location.

Examination of the localization of \textit{bcd} mRNA revealed that it is only subtly affected in the absence of BEAF. \textit{bcd} mRNA is localized to the anterior pole in a freshly laid wild-type egg (Fig. 4.3E) (Irion and St JohnSTON 2007). In \textit{BEAF}^{AB-KO} embryos \textit{bcd} mRNA is less tightly confined to the anterior pole of the embryo. A slight gradient of distribution away from the anterior pole towards the center of the embryo body axis is observed (Fig. 4.3F).

We found that \textit{su(Hw)} gave the same ubiquitous expression pattern in wild-type (Fig. 4.3G) and \textit{BEAF}^{AB-KO} embryos (Fig. 4.3H). However, the \textit{BEAF}^{AB-KO} embryos always gave darker staining. This is in line with our q-RT-PCR results that indicated that \textit{su(Hw)} is overexpressed in the absence of BEAF.

As expected, \textit{Dref} was also ubiquitously expressed at all stages in wild-type embryos (Fig. 4.3I). Both the \textit{Dref} expression pattern and level appears unaffected in the \textit{BEAF}^{AB-KO} embryos (Fig. 4.3J). Unlike our results for \textit{su(Hw)}, \textit{BEAF}^{AB-KO} embryos did not exhibit darker
Figure 4.3 Genes show altered patterns of expression in BEAF^{AB-KO} embryos. (A) *ftz* expression pattern in wild-type embryos as determined by in situ hybridization using a strand specific biotinylated probe. (B) *ftz* expression in BEAF^{AB-KO} embryos. (C) Deformed mRNA expression pattern in wild-type embryos at early cellular blastoderm stage (left panel), germ-band extension stage (middle panel) and at germ-band retraction stage (right panel). (D) Deformed expression pattern in BEAF^{AB-KO} embryos at the above mentioned stages. (E) Bicoid expression in wild-type embryos (F) Bicoid expression pattern in BEAF^{AB-KO} embryos. (G) *su(Hw)* mRNA expression pattern in wild-type. (H) *su(Hw)* expression in BEAF^{AB-KO} embryos (I) Wild-type Dref expression (J) BEAF^{AB-KO} Dref expression.
staining. This is again consistent with our q-RT-PCR result which found no change in \textit{Dref} mRNA levels in the absence of BEAF.

Finally, we did not observe any effect on \textit{pb} expression patterns in \textit{BEAF}^{\text{AB-KO}} embryos (data not shown). However, expression is limited to very small patches of cells in the labial and maxillary lobes at the germ band retraction stage of development (Pultz \textit{et al.} 1988). We found \textit{pb} difficult to detect, so pattern differences could be present in \textit{BEAF}^{\text{AB-KO}} embryos. Any such differences must be subtle.

\textbf{Accessibility of Proteins to DNA Was Subtly Affected in the Absence of BEAF}

To determine if accessibility of DNA binding proteins to their binding sites is altered in the absence of BEAF, we performed immunostaining on polytene chromosomes from salivary glands of third instar larvae. For this purpose, we chose to use an antibody against DREF, because it is known to be expressed in salivary glands. We also used an antibody against MLE to assist in identifying male X chromosomes (Copps \textit{et al.} 1998). MLE is a component of the dosage compensation complex that specifically binds to the X chromosome in males. We reasoned that any DNA binding protein might show altered binding if chromatin structure is perturbed in the absence of BEAF.

DREF binds to many locations on Drosophila chromosomes. We compared the DREF binding pattern on male X polytene chromosomes (Fig. 4.4A) and autosomal chromosome arm 3L (Fig. 4.4B). Polytene chromosomes from at least six larvae of each genotype were compared to document the reproducibility of immunostaining patterns. Comparison of wild-type and \textit{BEAF}^{\text{AB-KO}} chromosomes to each other indicated that the DREF binding patterns were very similar, if not identical. Although subtle differences could be found, there were also subtle differences between larvae of the same genotype.
Figure 4.4 Accessibility of proteins to binding sites on chromosomes is largely unaffected in the absence of BEAF. (A) Wild-type and BEAF<sup>AB-KO</sup> male polytene X-chromosomes from salivary glands of third instar larva immunostained with DREF antibody (green). Various sites have been numbered to facilitate comparison between chromosomes from wild-type and BEAF<sup>AB-KO</sup> animals. No clear, reproducible differences in DREF binding were detected (B) Comparison of DREF binding to Chromosome arm 3L of wild-type and BEAF<sup>AB-KO</sup> animals, as in (A). Again, no clear, reproducible differences in DREF binding were found (C) Binding of MLE to the male polytene X-chromosome is compared between wild-type and BEAF<sup>AB-KO</sup> animals. Two reproducible differences between binding of MLE to WT and BEAF<sup>AB-KO</sup> X chromosomes were found, indicated by arrow.
We also compared the binding pattern of MLE on male X chromosomes (Fig. 4.4C). Once again, the patterns were very similar on wild-type and $BEAF^{AB-KO}$ chromosomes. There were however two locations where MLE binding reproducibly differed between the two genotypes (indicated by the arrows in Fig. 4.4C). We conclude that the effects of a lack of BEAF on the accessibility of DNA binding proteins to their binding sites is subtle.

**Discussion**

Our rough eye screen led to the identification of mutant genes that enhanced the phenotype caused by the BID dominant negative BEAF protein. Most of these belonged to the category of transcription factors, general transcription factors or insulator binding proteins (Roy *et al.* 2007b). We reasoned that these interactions were due to downstream effects of interfering with BEAF function. This lead us to hypothesize that lack of BEAF might cause mis-regulation of these genes. Alternatively, lack of BEAF could alter regulation by altering accessibility of transcription factors to their binding site targets. To test this we looked at gene expression levels and patterns, and protein binding site accessibility in $BEAF^{AB-KO}$ animals. Our results support the breakdown of the regulation of levels and patterns of gene expression, but not changes in the accessibility to DNA binding sites.

For the most part these effects were fairly subtle, which is consistent with the fact that maternal BEAF is sufficient to get adults, and about 40% of embryos lacking maternal and zygotic BEAF can still hatch (Roy *et al.* 2007). On the other hand, the tested allele of Dref did not show an interaction in the rough eye assay, and no effect on expression levels or patterns was detected. This is also true for Trf expression levels, which were used to normalize the q-RT-PCR results. On the one hand, there is no reason to think that lack of BEAF only affects the tested genes. On the other hand, it is clear that not all genes are affected by a lack of BEAF. The
absence of BEAF in BEAf_{AB-KO} adult flies gives rise to a rough eye phenotype and also results in several ovary phenotypes (Roy et al. 2007) suggesting that genes in these pathways are likely most susceptible to a lack of BEAF. Also most of the transcription factors which interacted with BEAF in the rough eye assay are from the Antennapedia complex which is involved in various vital processes such as Drosophila head development, brain development, specification of segmental identity, embryonic pattern specification, and anterior/posterior axis specification. BEAF might be involved in these multiple pathways and all these genes are probably functioning downstream of BEAF.

It is difficult to predict at this point whether the detected effects on gene expression are due to direct or indirect effects of a lack of BEAF. The absence of BEAF might be disrupting the expression of transcription factor genes which gives rise to a chain reaction causing the mis-regulation of several genes including those we tested.

Insight into the number of genes whose regulation is affected by a lack of BEAF, and the pathways they participate in, could be gained by performing a genome wide microarray in BEAf_{AB-KO} individuals to look at all the genes affected by BEAF. This knowledge when combined with a genomic map of BEAF binding sites produced in other work in the lab, can eventually lead to a better understanding of the role of insulator proteins ranging from Drosophila to mammals.

Literature Cited


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CHAPTER FIVE

TARGETED GENE REPLACEMENT IN DROSOPHILA CAN BE ACCOMPANIED BY SECOND-SITE MUTATIONS
Introduction

Since the introduction of gene targeting by homologous recombination in Drosophila in 2000, it has been readily adopted by Drosophila researchers as an important part of their toolkit for generating mutations (Rong and Golic 2000). To carry out gene targeting by the ends-in method in Drosophila, a donor construct carrying mutated DNA from the gene to be targeted is randomly inserted into the genome by P-element-mediated transformation. Then, a site-specific recombinase (FLP) and a site-specific endonuclease (I-SceI) are used to generate, in vivo, an extra-chromosomal DNA molecule that carries a double-stranded break (DSB) within the gene of interest (Fig. 5.1).

Figure 5.1 Gene targeting by homologous recombination (ends-in method).

The presence of the DSB stimulates homologous recombination between the excised donor and the homologous chromosomal target locus (Rong and Golic 2000; Rong and Golic 2001). One possible fate of an ends-in targeting molecule would be integration at the locus of homology, producing a tandem duplication of the targeted gene. The target locus duplication can be reduced to a single copy by homologous recombination between the repeated sequence elements. This event is stimulated by an I-CreI-generated DSB between the repeats (Rong et al. 2002).
While using this technique to generate mutations in the \textit{BEAF} gene (Roy et al. 2007a; Zhao et al. 1995), we obtained three types of unusual results. One was the generation of unexpected recombination products, and the other two involved the apparent generation of second-site mutations during homologous recombination or during reduction of the resulting gene duplication to a single copy. The \textit{BEAF} mutations were separated from the non-targeted mutations by meiotic recombination (McKim et al. 2002). This was omitted from the paper describing the effects of the \textit{BEAF} mutations because it would have detracted from the main points of that paper (Roy et al. 2007a). We have since found two other reports that mention the presence of second-site mutations after generating mutations in Drosophila by homologous recombination (Lankenau et al. 2003; O'Keeffe et al. 2007), suggesting the occurrence of these events is not uncommon.

Although second-site mutations can occur with any mutagenesis technique, it is a serious issue that might easily be overlooked with gene-targeting since this method is not expected to generate non-targeted mutations. We conducted a survey of eight publications describing phenotypes associated with alleles generated by homologous recombination in Drosophila, and found that three did not mention experiments that would have determined whether non-targeted mutations affected observed phenotypes (Beall et al. 2004; Donaldson et al. 2004; Egli et al. 2003; Han et al. 2004; Hittinger et al. 2005; Lankenau et al. 2003; Seum et al. 2002; Sogame et al. 2003). Four papers did not mention testing for rescue of mutant phenotypes by wild-type transgenes, and three of these four only reported experiments done with the mutant chromosome in a homozygous state. They did not combine mutant alleles with each other or with an appropriate chromosomal deficiency (which would keep second-site mutations heterozygous). Because of our experience with this method, combined with published work that appears to
overlook the possibility of second-site mutations, we feel it is important to raise awareness of the necessity to use care when analyzing mutations generated by gene targeting.

Here in this paper we used the ends-in gene targeting protocol to calculate the frequency of second-site lethal mutations introduced on the third chromosome. An isogenized third chromosome without any lethal mutations was used for this purpose. Our results show that the gene targeting by homologous recombination method does increase the frequency at which lethal second-site mutations occur.

**Materials and Methods**

**Isogenizing the Third Chromosome**

The third chromosome used in these crosses must be free of any lethal mutations. For this purpose it was isogenized. The third chromosome we selected is marked by a P[\textit{mus301,w}^+] transgene (McCaffrey \textit{et al.} 2006). The \textit{w}^+ confers orange eye color. Homozygous males of the P[\textit{mus301,w}^+]/P[\textit{mus301,w}^+] genotype were crossed with TM3/ET50 females. Male progeny of the genotype P[\textit{mus301,w}^+]/TM3 emerging from this cross were individually crossed to TM3/ET50 females again. Progeny flies of the genotype P[\textit{mus301,w}^+]/TM3 were then self-crossed, and flies homozygous for *P[\textit{mus301,w}^+]/ *P[\textit{mus301,w}^+] (*= non-lethal isogenized chromosome) from these vials were then self-crossed and maintained as a stable line.

**Fly Crosses**

The following types of crosses were conducted. The first three crosses were controls and remaining two were experimental crosses. 1) **Negative control**: The control line lacks the mutant \textit{BEAF} transgene (P[\textit{w}^+ \textit{mBF}]) on the X chromosome (Roy \textit{et al.} 2007a) and transgenes for any of the recombinases (I-\textit{Cre} or I-\textit{Sce} or FLP) (Rong and Golic 2000; Rong \textit{et al.} 2002). The P[\textit{w}^+ \textit{mBF}] is flanked on either side by FRT sites which are recognized by the FLP recombinase.
It also has sequences for the site-specific endonucleases I-SceI and I-CreI (Rong and Golic 2000; Rong et al. 2002; Roy et al. 2007a). The following crosses were conducted to develop the control line. Homozygous *P[\text{mus301}, \text{w}^+]*/*P[\text{mus301}, \text{w}^+]* males were crossed with TM3/ET50 virgin females. The vials were emptied after 3 days and progeny larvae were heat shocked for 1 hr at 37°C in a water-bath. Adult *P[\text{mus301}, \text{w}^+]*/TM3 males eclosing from these vials were then crossed individually with TM3/ET50 females. At this stage ~250 vials were set up. *P[\text{mus301}, \text{w}^+]*/TM3 progeny emerging from these vials were self-crossed. In the next generation, vials giving rise to ~1/3rd of flies homozygous for *P[\text{mus301}, \text{w}^+]*/ *P[\text{mus301}, \text{w}^+]* are considered non-lethal events while vials with either none or very few (≤ 5% of total population) homozygotes are considered lethal or semi-lethal events respectively. 2) I-CreI control and I-SceI FLP control: These control lines lack the P[w^+\text{mBF}]. To establish these two lines, homozygote males of the genotype *P[\text{mus301}, \text{w}^+]*/*P[\text{mus301}, \text{w}^+]* were crossed to I-CreI Sb/TM6 or I-SceI FLP/TM3 females separately. Vials were emptied after 3 days and the progeny larvae were heat shocked as mentioned above to produce the FLP recombinase and site-specific endonucleases (Rong and Golic 2000; Rong and Golic 2001; Rong et al. 2002). *P[\text{mus301}, \text{w}^+]*/ I-CreI Sb or *P[\text{mus301}, \text{w}^+]*/I-SceI FLP males emerging from this cross were then individually mated with TM3/ET50 females. At this point ~150 vials for each control line were set up. *P[\text{mus301}, \text{w}^+]*/TM3 adults eclosing (eliminating I-CreI Sb and I-SceI FLP chromosomes) were then self-crossed and in the next generation the number of lethal vs. non-lethal events were calculated. 3) Experimental lines for I-CreI or I-SceI FLP: First P[w^+\text{mf}] / P[w^+\text{mf}]; I-CreI Sb/TM6 and P[w^+\text{mf}] / P[w^+\text{mf}]; I-SceI FLP/TM3 stable lines were established. Males homozygous for *P[\text{mus301}, \text{w}^+]*/ *P[\text{mus301}, \text{w}^+]* were crossed with females from each of the above two lines separately. Vials were emptied of flies after 3 days and they
were heat shocked as mentioned above. Males of the P[w^mBF]; *P[mus301,w^+]/ I-CreI Sb or P[w^mBF]; *P[mus301,w^+]/ I-SceI FLP were isolated and crossed to TM3/ET50 females. Around ~150 vials were set up at this point. In the next step males of the genotype *P[mus301,w^+]/TM3 (getting rid of P[w^mBF], I-SceI FLP and I-CreI Sb) were crossed with TM3/ET50 females. Progeny males and females of *P[mus301,w^+]/TM3 genotype were then self-crossed. Progeny from these crosses were scored to determine the number of lethal vs. non-lethal events.

**Results**

We followed the protocol for gene targeting by homologous recombination (Rong and Golic 2000; Rong and Golic 2001; Rong et al. 2002) to determine rates of occurrence of lethal second-site mutations. The three control crosses lacked a donor transgene containing recognition sites for FLP, I-SceI and I-CreI. One control cross also lacked heat-shock inducible transgenes for producing FLP, I-SceI and I-CreI, while the other two controls had transgene encoding either I-CreI or both FLP and I-SceI. The mutant BEAF transgenic P[w^mBF] donor construct was on the X chromosome (Roy et al. 2007a) and the target BEAF gene is located on the second chromosome. Therefore we selected the third chromosome to record the rate of introduction of non-targeted lethal mutations. As explained in the materials and methods, five types of crosses were conducted and the number of lethal vs. non-lethal events occurring on the third chromosome was calculated for each type of cross.

Table 5.1 shows the results obtained from these experiments. For the negative control cross we observed 5 lethal events on the third chromosome out of 237 crosses (2.1%). For the I-CreI and I-SceI FLP controls we observed a lethal mutation rate of 2.8 % (3/105) and 3.8% (4/108), respectively. For the experimental lines for I-CreI and I-SceI FLP which included the
Table 5.1

Number of lethal events introduced in the third chromosome due to second site mutations during homologous recombination

<table>
<thead>
<tr>
<th>Genotypes*¹</th>
<th>Number of crosses*²</th>
<th>Non-lethal events</th>
<th>Lethal events</th>
<th>% Lethal events</th>
<th>Chi^2</th>
<th>alpha</th>
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<tr>
<td>Control</td>
<td>237</td>
<td>232</td>
<td>5</td>
<td>2.1%</td>
<td></td>
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</tr>
<tr>
<td>Control (I-CreI Sb)</td>
<td>105</td>
<td>102</td>
<td>3</td>
<td>2.8%</td>
<td>0.17794</td>
<td>0.67</td>
</tr>
<tr>
<td>Control (I-SceI FLP)</td>
<td>108</td>
<td>104</td>
<td>4</td>
<td>3.8%</td>
<td>0.74197</td>
<td>0.39</td>
</tr>
<tr>
<td>I-CreI Sb with P[w^+ mBF]</td>
<td>118</td>
<td>109</td>
<td>9</td>
<td>7.6%</td>
<td>6.33034</td>
<td>0.012</td>
</tr>
<tr>
<td>I-SceI FLP with P[w^+ mBF]</td>
<td>114</td>
<td>107</td>
<td>7</td>
<td>6.1%</td>
<td>3.78729</td>
<td>0.052</td>
</tr>
</tbody>
</table>

*¹ Genotypes here mean the control and the experimental lines explained in the paper.

*² The number of crosses also represents the number of times the gene targeting protocol was adopted for each genotype.
P[w+mBF] transgene, we found an increase in the frequency of second-site mutations. The line expressing I-CreI recombinase in the presence of the P[w+mBF] transgene gave 9 lethal events out of 118 crosses (7.6%) on the third chromosome. Finally, for the experimental line expressing the recombinase I-SceI FLP along with the P[w+mBF] transgene we observed 7 lethal events out of 114 crosses set up (6.1%). A chi-square analysis comparing the percentage of lethal events for each condition to the negative control indicated there is no significant difference in the number of lethal events occurring in the I-CreI or I-SceI FLP controls compared to the negative control. On the other hand the alpha value for the experimental I-CreI crosses was about 0.01 and for the I-SceI FLP crosses it was about 0.05. Therefore the rates at which lethal mutations occur using the experimental lines were significantly higher than the control lines.

The results obtained here indicate that inducing expression of I-CreI or I-SceI FLP in the presence of a transgene with recognition sites for these enzymes stimulates the rate of second site mutations.

**Discussion**

Gene targeting by homologous recombination in Drosophila is a valuable tool. However, there is a distinct possibility that second-site mutations are often introduced into chromosomes when using this technique despite the expectation that only targeted mutagenesis will occur. Although this possibility is not addressed in many publications reporting use of this technique, detecting and eliminating second-site mutations was essential for the accurate analysis our BEAF mutations (ROY *et al*. 2007). While the mechanism responsible for introducing the second-site mutations remains uncharacterized, our results and those of others highlight the need for care in working with mutations generated using homologous recombination.
Here in this paper we carried out the gene targeting method in experimental lines using a mutant BEAF transgene. Our goal was to measure the rate of production of lethal mutations on a nontargeted chromosome in the presence and absence of the I-CreI or I-SceI FLP transgenes, with and without a transgene containing recognition sites for these enzymes. Our results clearly indicate a significant increase in the rate of formation of non-targeted or second-site lethal mutations in the experimental lines.

As with other mutagenesis methods, well-established techniques such as backcrossing, mapping, complementation with a wild-type transgene, and the use of multiple independently derived alleles must be used to verify that observed phenotypes are attributable to the mutation of interest. Bearing this potential complication in mind, gene targeting is a powerful technique that allows the generation of mutant alleles that would otherwise be difficult to obtain.

**Literature Cited**


CHAPTER SIX
SUMMARY
Insulators are thought to organize genomes into independent domains of gene expression, thus contributing to gene regulation. Evidence indicates that insulators operate among different species from insects to mammals. The exact mechanism by which insulators function is unknown. During my PhD years I have tried to decipher the mechanism of insulator function by genetically analyzing BEAF (Boundary Element Associated Factor) which was discovered because it binds to the Drosophila scs’ insulator. There are two forms of BEAF proteins generated from one gene, BEAF-32A and BEAF-32B.

As genetic tools for studying BEAF, chapter two describes the generation of BEAF knock-out alleles by end-in homologous recombination method. Two forms of BEAF knock-out were created by this method: BEAF^{AB-KO} which prevents production of both 32A and 32B, and BEAF^{A-KO} which prevents production of 32A. Flies expressing only 32B were found to be healthy and fertile which indicated that 32A is not an essential protein. However the BEAF^{AB-KO} allele is lethal and affects both oogenesis and development. Using the BEAF^{AB-KO} allele in enhancer blocking and position independent expression assays we found that the insulator function of scs’ is conferred by BEAF, but it is not required for scs or gypsy insulator function. BEAF has a role in maintaining chromatin structure or dynamics. This function of BEAF was confirmed by the observation that the male X polytene chromosome is perturbed in BEAF^{AB-KO} larvae and by position-effect variegation assays.

The chromosomes with the BEAF knock-out alleles generated with the homologous recombination technique also had lethal second-site mutations. In chapter five we carried out several experimental and control crosses to determine if the method stimulates the non-targeted lethal mutation rate, using the third chromosome as the reporter. Our results indicated the gene targeting method does indeed stimulate the spontaneous non-target mutation rate.
The third chapter describes a screen for genetic interactions with \textit{BEAF}. Using an eye driver GAL4-UAS system to express a truncated form of BEAF lacking the N-terminal DNA binding domain but possessing the BEAF Interaction Domain (BID), results in a rough eye phenotype that can be rescued by expression of an extra copy of a \textit{BEAF} gene. Mutations in several genes were identified that enhanced this rough eye phenotype. Most of these interacting genes belonged to the class of transcription factors and insulator binding proteins. This work supported the hypothesis that \textit{BEAF} maintains global patterns of gene expression.

The genetic interactions uncovered in the third chapter are probably due to mis-regulation of these transcription factors caused by the absence of BEAF. To confirm this in the fourth chapter, we looked at levels and patterns of expression of genes which strongly interacted with BEAF in the eye assay. We also looked at accessibility of proteins to DNA. A comparison of BEAF and \textit{BEAF}^{AB-KO} embryos found that most of the genes tested showed altered levels and patterns of expression in the absence of BEAF. However, by immunostaining polytene chromosomes we found that the DNA binding of proteins was only subtly affected by the lack of BEAF.

The work presented highlights the overall importance of BEAF in flies. These studies established \textit{BEAF} as an essential gene which has roles in processes in Drosophila such as embryogenesis, eye development and oogenesis. They also show that BEAF affects chromatin structure and/or dynamics. BEAF was found to interact with transcription factors taking part in important processes like development of the anterior part of the Drosophila body and embryonic pattern specification. We showed that expression levels and patterns of these genes are greatly dependent on the presence of BEAF. The genes whose expression is altered in the absence of BEAF are probably functioning downstream to BEAF in these pathways. Future extension of this
work should be a genome-wide microarray conducted in $BEAF^{AB-KO}$ flies to identify genes whose expression is altered in the absence of BEAF. When combined with a genomic map of BEAF binding sites produced in other work in the lab, information obtained from such microarray analysis would be instrumental in mapping BEAF to pathways where it plays an integral role.
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