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Characterization of **BEAF** Mutations Isolated by Homologous Recombination in Drosophila

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**ABSTRACT**

The Drosophila BEAF-32A and BEAF-32B proteins bind to the scs’ insulator and to hundreds of other sites on Drosophila chromosomes. These two proteins are encoded by the same gene. We used ends-in homologous recombination to generate the null BEAF\(^{32A-KO}\) allele and also isolated the BEAF\(^{32B-KO}\) allele that eliminates production of only the BEAF-32A protein. We find that the BEAF proteins together are essential, but BEAF-32B alone is sufficient to obtain viable flies. Our results show that BEAF is important for both oogenesis and development. Maternal or zygotic BEAF is sufficient to obtain adults, although having only maternal BEAF impairs female fertility. In the absence of all BEAF, a few fertile but sickly males are obtained.

Using both a chromosomal position-effect assay and an enhancer-blocking assay, we find that BEAF is necessary for scs’ insulator function. Lack of BEAF causes a disruption of male X polytene chromosome morphology. However, we did not find evidence that dosage compensation was affected. Position-effect variegation of the \(v^{wm4h}\) allele and different variegating transgenes was enhanced by the knockout mutation. Combined with the effects on male X polytene chromosomes, we conclude that BEAF function affects chromatin structure or dynamics.

**ENHANCERS** can act over large distances and are capable of activating transcription from diverse promoters (Kermekchiev 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 \(Ig\overline{f}2\) gene (Bell and Felsenfeld 2000; Harr et al. 2000). This insulator is not methylated on the maternal chromosome, allowing binding of the CTCF protein, which blocks activation of \(Ig\overline{f}2\) by a downstream enhancer. The insulator is methylated on the paternal chromosome, which prevents binding by CTCF and allows activation of \(Ig\overline{f}2\) 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 (Reik et al. 1995; Frevel et al. 1999). 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 Parnell and Geyer 2000; Hogg et al. 2001). 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 (Labbador and Corces 2002; Kuhn and Geyer 2003; Gaszner and Felsenfeld 2006). 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 article 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 (Zhao et al. 1995; Hart et al. 1997). A few other genomic BEAF-binding sites have been identified, and they function as insulators in transgenic fly assays (Cuvier et al. 1998, 2002). 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 \(~\)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 knock-out mutation in the BEAF gene (BEAF<sup>32A<sub>Δatg</sub></sup>). In the process, we also isolated an allele that eliminates the ability to produce the 32A protein (BEAF<sup>32A<sub>Δatg</sub></sup>). We found that the 32B protein is sufficient to obtain healthy, viable flies. In contrast, eliminating both BEAF proteins reveals that BEAF is essential. Both oogenesis and development are affected by a lack of BEAF. We demonstrate that BEAF is required for the insulator activity of scs', but not for 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 that 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 construction and germline transformation:** Cloning of the BEAF gene as a 5-kb BglI fragment generated from genomic DNA by PCR has been described (Figure 1A), as has generation of transgenic flies containing this gBPF rescue transgene (Gilbert et al. 2006). Site-directed mutagenesis was used to introduce mutations into this gene (Figure 1B; Quikchange, Stratagene, La Jolla, CA). One mutation eliminated the ATG start codon of BEAF-32A and destroyed an Nsi site. A second mutation eliminated the ATG start codon of BEAF-32B and created an ApaI 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 BamHI site. A fourth mutation introduced an I-SceI site into the intron between the unique 32B exon and the shared exon. The I-SceI 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 ~280 bp downstream of the 32B mutation and ~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 NsiI site of pTV2 (Rong et al. 2002). This plasmid (0.4 μg/μl) was co-injected with the helper plasmid pRT25.7wc (0.1 μg/μl) into preblastoderm y<sup>+/w;2>y<sup>0.25</sup> embryos to generate P[w<sup>+</sup>/mBF] transgenic flies (Spradling 1986).

A 2-element plasmid encoding a BEAF:EGFP fusion gene was also constructed (referred to as GGBP for green fluorescent BEAF; Figure 1D). The stop codon of the BEAF gene was mutated to a KpnI site, pEGFP-N3 (CLONTECH) was modified by deleting a 600-bp Asdl-BglII fragment encoding the CMV-E 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 ~265 bp upstream from the 5' end of this fragment. An EcoRI-J/Hind fragment, from the BEAF promoter through the SV40 polyadenylation site, was cloned into pM2 (Cuvelier 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<sup>+</sup>/GGBP] 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°. The yellow (y) enhancer blocking lines (2scs<sup>y</sup> inserted at 19D; scs inserted at 80E; scs 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<sup>26H</sup>), 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 (http://flystocks.bio.indiana.edu).

**Isolation of BEAF mutations by ends-in homologous recombination:** Flies with P[w<sup>+</sup>/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<sup>+</sup>/mBF] females were crossed to 70FLP/70FLP/YM6 males. Larvae were given one heat shock at 38° for 1 hr in a water bath. For crosses with P[w<sup>+</sup>/mBF] CyO, white-eyed female progeny with CyO were crossed to y<sup>+/w;2>y<sup>0.25</sup> males and progeny with red eyes but lacking CyO were crossed to CyO/Sp<sup>i</sup> flies to screen for potential homologous recombination events. For crosses with P[w<sup>+</sup>/mBF] on the X chromosome, white-eyed female progeny from the first cross were crossed to 70FLP/70FLP/YM6 males and the larvae were given a 1-hr 38° heat shock. This eliminated background in the next generation caused by progeny with the original P[w<sup>+</sup>/mBF] transposon. Males with red eyes were then crossed to CyO/Sp<sup>i</sup> females to screen for potential homologous recombination events. For the P[w<sup>+</sup>/mBF] CyO strategy, ~82,500 chromosomes were screened ([1100 vials × 150 flies/vial]/2) because of the CyO chromosome. Eight mobilizations were recovered, only one of which was due to homologous recombination. For the strategy using P[w<sup>+</sup>/mBF] on the X chromosome, ~100,500 chromosomes were screened (670 vials × 150 flies/vial). Three mobilizations were recovered, all of which were due to homologous recombination.

Homologous recombination was confirmed by genomic PCR. Ends-in homologous recombination results in a gene duplication with the mini-white marker gene between the two copies. Primer pairs that would specifically amplify the upstream gene copy, the downstream gene copy, or the original single-copy BEAF gene, all as 5-kb fragments, were used. Amplified DNA was sequenced and analyzed by restriction digestions. We found that one recombination event resulted in both gene copies having a mutated 32A ATG, but the 32B and shared sequences were intact. This is the BEAF<sup>32A<sub>Δatg</sub></sup> allele, and the chromosome is w<sup>+</sup>. The other three recombination events had at least one wild-type gene copy. One was determined to have the 32A and 32B ATG mutations as well as the tandem stop codons in the downstream gene copy. This gene duplication was reduced to a single copy by crossing flies to a 70FL-Crel Sb/TM6 line and giving the larvae a 1-hr 38° heat shock (Rong et al. 2002). w<sup>+</sup> mosaic males were selected and crossed to CyO/Sp<sup>i</sup> females. In the following generation, flies with CyO but lacking the 70FL-Crel Sb chromosome were selected and individually crossed to CyO/Sp<sup>i</sup> flies again. Flies that eclosed and lacked Sp<sup>i</sup> were then self-crossed. Flies were
screened by PCR and restriction digestion to identify the BEAF\textsuperscript{\textalpha-KO} chromosome, which is \textit{w}. Primer sequences used for mutagenesis, PCR, and sequencing are available upon request.

**Viability assays:** To examine the effect of the lack of maternal BEAF on female fertility and egg viability, flies of the genotypes indicated in Table 1 were crossed in fly cages sealed with grape juice agar plates smeared with yeast paste. The agar plates were changed every 24 hr 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\textsuperscript{\textalpha-KO} female virgins, the BEAF\textsuperscript{\textalpha-KO} chromosome was placed over a Cy\textit{O} GFP \textit{w}\textsuperscript{\textalpha} balancer chromosome, and third instar larvae were placed in PBS and sorted by fluorescence microscopy. Homozygous BEAF\textsuperscript{\textalpha-KO} larvae were placed in a new vial to pupate and eclose. Surprisingly, this treatment improved the fecundity of the BEAF\textsuperscript{\textalpha-KO} flies and vigor of their progeny.

To determine the viability of BEAF\textsuperscript{\textalpha-KO} flies provided with maternal BEAF, six males and six females of the genotype BEAF\textsuperscript{\textalpha-KO}/Cy\textit{O} were placed in a vial for 3 days and then transferred to a new vial for an additional 3 days. The number of BEAF\textsuperscript{\textalpha-KO} and BEAF\textsuperscript{\textalpha-KO}/Cy\textit{O} adults that eclosed was recorded.

**Ovary dissection and DAPI staining:** Wild-type or BEAF\textsuperscript{\textalpha-KO} females were mated with wild-type males for 4 days before dissection. Ovaries were dissected in PBS (0.9% NaCl, 14 mM Na\textsubscript{2}HPO\textsubscript{4}, 6 mM NaH\textsubscript{2}PO\textsubscript{4}, pH 7.3). The dissected ovaries were fixed with 4% formaldehyde in PBS for 15–30 min and then stained with DAPI (250 ng/ml DAPI in PBS/0.1% Triton-X100) or propidium iodide (100 ng/ml) plus RNase A (200 \textmu g/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 Axioplan microscope equipped with a Spot RT Slider CCD camera. For viewing GFP fluorescence, salivary glands were fixed for 1 min with 3.7% paraformaldehyde in PBS plus 5% Triton X-100, stained 20 min with 100 ng/ml DAPI in PBS plus 2% Triton X-100, and washed 2 min in 50% glycerol. The chromosomes were then gently spread in a fresh drop of 50% glycerol and viewed immediately.

**Scanning electron microscopy:** Flies were prepared and SEM was performed as previously described (Gilbert et al. 2006).

**RESULTS**

**Generation of mutant BEAF alleles by homologous recombination:** The BEAF gene encodes two related 32-kDa proteins, BEAF-32A and BEAF-32B (Figure 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 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 (Figure 1B). Each mutation either created or destroyed a restriction site. Flies containing this \textit{mBF} transgene were used to generate flies with mutant alleles of BEAF by ends-in homologous recombination (Rong and Golli 2000, 2001; Rong et al. 2002). This commonly results in a gene duplication bracketing the mini-\textit{white} marker gene (Figure 1C). Using primer pairs anchored in genomic sequences outside of the transgene sequences and in the mini-\textit{white} sequences (indicated in Figure 1C), we confirmed four such gene duplication events by individual PCR amplification of the BEAF gene upstream and downstream of the mini-\textit{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{\textalpha-KO} allele, and the chromosome is \textit{w}\. 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 \textit{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{\textalpha-KO} allele, and the chromosome is \textit{w}\. 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
(Figure 2). For this PCR analysis, gene-specific primers were not used. Instead, primer pairs that generated 500-bp fragments from all BEAF genes present were used (indicated in Figure 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<sup>Δ KO</sup> flies used antibodies specific for either the 32A or the 32B protein, while the Western analysis of BEAF<sup>Δ<sub>AR</sub> KO</sup> flies used an antibody that recognizes both BEAF proteins. These analyses demonstrated that BEAF<sup>Δ KO</sup> flies only have the 32A ATG mutation and make 32B protein, but no detectable 32A protein (Figure 2, B and C). Similarly, the BEAF<sup>Δ<sub>AR</sub> KO</sup> flies have all three of the BEAF mutations and do not produce any detectable BEAF protein (Figure 2, E and F).

**Effects of the BEAF mutations on Drosophila viability:** Flies homozygous for the BEAF<sup>Δ KO</sup> 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, and the 32A protein is not necessary.

In contrast, flies homozygous for the BEAF<sup>Δ<sub>AR</sub> KO</sup> 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 2 weeks if pampered. Females, on the other hand, have reduced fertility and appear to be very sensitive to environmental conditions. When BEAF<sup>Δ<sub>AR</sub> KO</sup> female virgins were collected from their parental vial and crossed to BEAF<sup>Δ<sub>AR</sub> KO</sup> 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<sup>Δ<sub>AR</sub> KO</sup> females laid <5% <sup>of</sup> the number of eggs laid by wild-type females, the number of larvae obtained per female was <1% <sup>of</sup> the number from wild-type females, and the number of pupae and adults was <0.2% <sup>of</sup> 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 that maternal BEAF suffices to obtain adults.

To facilitate the collection of BEAF<sup>Δ<sub>AR</sub> KO</sup> 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 w<sup>1</sup> balancer. Third instar larvae were placed in PBS and sorted by fluorescence microscopy. BEAF<sup>Δ<sub>AR</sub> KO</sup> larvae lacked GFP and were placed into new vials to pupate and eclose. The resulting BEAF<sup>Δ<sub>AR</sub> KO</sup> females (confirmed by white eyes and lack of curly wings) were used for crosses to BEAF<sup>Δ<sub>AR</sub> KO</sup> 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<sup>Δ<sub>AR</sub> KO</sup> females, confirming that BEAF is important for oogenesis (Table 1). BEAF<sup>Δ<sub>AR</sub> KO</sup> females laid similar numbers of eggs and had similar larval hatch rates of ~40% whether they were mated with BEAF<sup>Δ<sub>AR</sub> KO</sup> or with 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 1). The adults with zygotic BEAF appeared normal, a 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 vials to which the larvae were transferred.

### Table 1

<table>
<thead>
<tr>
<th>Stage</th>
<th>$\varphi$ BEAF\textsuperscript{AB-KO} × $\delta$ BEAF\textsuperscript{AB-KO}</th>
<th>$\varphi$ BEAF\textsuperscript{AB-KO} × $\delta$ BEAF</th>
<th>$\varphi$ BEAF × $\delta$ BEAF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No./($\xi$)(day)</td>
<td>Viability*</td>
<td>Total</td>
</tr>
<tr>
<td>Embryo</td>
<td>5.07</td>
<td>1669</td>
<td>4.65</td>
</tr>
<tr>
<td>Larva</td>
<td>1.97</td>
<td>0.39</td>
<td>647</td>
</tr>
<tr>
<td>Pupa</td>
<td>0.81</td>
<td>0.41</td>
<td>265</td>
</tr>
<tr>
<td>Adult</td>
<td>0.17</td>
<td>0.21</td>
<td>56</td>
</tr>
</tbody>
</table>

A total of 46 BEAF\textsuperscript{AB-KO} females were used in crosses to BEAF\textsuperscript{AB-KO} males, 27 BEAF\textsuperscript{AB-KO} females were used in crosses to BEAF males, and 18 BEAF females were used in crosses to BEAF males.

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

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.

The pupal and adult viability from the BEAF females is an underestimate because of mortality caused by overcrowding in the vials to which the larvae were transferred.
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 postembryonic 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 BEAFAB-KO genotype was confirmed for six adults by PCR and restriction digestion analysis (data not shown).

To determine if development of homozygous BEAFAB-KO flies that had maternal BEAF is impaired, we recorded the number of BEAFAB-KO and BEAFAB-KO/CyO flies as they eclosed from six lightly populated vials derived from BEAFAB-KO/CyO parents (Table 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 1–2 days later than their heterozygous siblings, indicating a slight developmental delay.

Multiple transgenic fly lines with either the gBF or the 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 BEAFAB-KO flies also have a rough-eye phenotype, and this is also rescued by the BEAF transgenes (Figure 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 BEAFAB-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 and Western and Northern results that indicated that the BEAF proteins are ubiquitous.

BEAF is required for normal oogenesis: Because BEAFAB-KO females had low fertility that was rescued by gBF and GFBF transgenes, we decided to examine their ovaries. BEAFAB-KO and wild-type females were mated with wild-type males for 4–7 days prior to dissection. The number of ovarioles per ovary did not appear to differ between BEAFAB-KO and wild-type females, but mutant ovaries were smaller than those from wild type (Figure 4, A–C). This was because most ovarioles from mutant females lacked mature oocytes, whereas most ovarioles from wild-type females ended with a mature oocyte (Figure 4, D and E).

In normal egg-chamber development, there are 16 interconnected germine cells enveloped by a layer of somatic follicle cells. Of these, 15 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 Kambsellis 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, which could be due to the fusion of two egg chambers or to an extra round of cell division (Figure 4, G and 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 (Figure 4H), and sometimes the chamber was nearly the size of a mature oocyte (Figure 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 Figure 4, J–K). In yet other cases, an egg chamber the size of a mature oocyte had large, oddly shaped nurse-cell nuclei distributed throughout (Figure 4M). This presumably represents egg-chamber degeneration by a pathway different from 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

### Table 2

<table>
<thead>
<tr>
<th>Day:</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>BEAFAB-KO/ CyO</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>BEAFAB-KO/ BEAFAB-KO</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>% of total</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>

*Percentage of all eclosed flies that have the genotype BEAFAB-KO/BEAFAB-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.*

*Running total, not daily totals.*
absence of BEAF. If fertilized, some of these oocytes are capable of developing into adults, especially if provided with zygotic BEAF (Table 1).

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

In the position-independent expression assay, mini-white was bracketed by the M2 derivative of **scs**' on the 5'<sup>′</sup> side and by the **scs** insulator on the 3'<sup>′</sup> side (Cuvier et al. 1998). The M2 insulator has two copies of the high-affinity BEAF-binding site of **scs**', with one copy replacing the low-affinity binding site normally present. Because the mini-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'<sup>′</sup> insulator, chromosomal position effects should lead to the activation of mini-white in some fly lines and result in darker eye pigmentation. This was observed: ~90% of insulated fly lines have yellow or light-orange eyes, while <50% of fly lines insulated only at the 3'<sup>′</sup>-end have such light eye pigmentation (our unpublished results and Cuvier et al. 1998). We had three fly lines with the M2 transposon inserted in the **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 **BEAF<sup>AB-KO</sup>** background (Figure 5A), indicating a loss of protection from chromosomal position effects in the absence of 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).

Three different insulators were tested in the enhancer-blocking assay. An **scs**' dimer (2**scs**'), **scs**, or gypsy insulator was located between the **y** wing and body enhancers and the **y** gene. “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. 2006).
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 (Figure 5B). Removal of each of the three insulators resulted in darker pigmentation. In the BEAFAB-KO background, the 2scs’ flies had a level of pigmentation similar to that of 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 noted 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 BEAFAB-KO background, this diffuse spreading extends further to encompass one-half or more of each segment (Figure 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 gBF and GFBF transgenes (data not shown). Therefore this spreading of the pigmentation is not related to the 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 y that is involved in determining the pigmentation pattern.

**BEAF mutations perturb male polytene 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 X chromosome of BEAFAB-KO males from heterozygous mothers exhibited obvious structural defects (Figure 6A). The banding pattern was lost, and the chromosome appeared shorter and puffier. This is less extreme than the results that 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 (Gilbert et al. 2006). Normal X chromosome morphology is restored in the presence of a GFBF transgene, demonstrating that the phenotype is due to a lack of BEAF protein (Figure 6B).

Polytene chromosomes were also prepared from larvae from BEAFAB-KO inter se crosses. As for males that
had maternal BEAF, the 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 (Stuckenholtz et al. 1999). The DCC associates only 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 twofold higher rates than in females (Bone et al. 1994; Hamada et al. 2005). Figure 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, and MLE and the dosage-compensation-associated histone modification, acetylation of H4 lysine 16 (data not shown). Therefore, 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+ mutation normally shows dosage compensation (males and females have similar eye pigment levels) and the w 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 (Figure 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.

**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<sup>AB-KO</sup> allele on PEV. The <i>w<sup>en1</sup></i> gene and three different insertions of a transposon carrying a <i>y</i> 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 <i>w<sup>en1</sup></i> gene is caused by a chromosomal inversion on the X chromosome (Tartof <i>et al</i>. 1989). The KV732 and KV600 fly lines have the <i>y</i> transgene inserted near the pericentromeric heterochromatin of the X chromosome, while KV123 is near the pericentromeric heterochromatin of chromosome arm 3L (Yan <i>et al</i>. 2002). PEV effects on <i>w<sup>en1</sup></i> are determined by comparing the number of pigmented ommatidia in flies of different genotypes (Figure 7A) or by extracting and quantitating the pigment (Figure 7B). PEV effects on <i>y</i> are determined by comparing the number of darkly pigmented spots on abdomens of flies of different genotypes (Figure 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<sup>AB-KO/BEAF</sup> males and a stronger enhancement in BEAF<sup>AB-KO</sup> males (Figure 7 and data not shown). Thus the lack of BEAF allows heterochromatin to spread and to 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 <i>et al</i>. 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.

**DISCUSSION**

As a tool for studying BEAF function, we generated the BEAF<sup>AB-KO</sup> knockout allele by homologous recombination. In the process, we also isolated the BEAF<sup>A-KO</sup> allele that cannot produce the 32A protein. Flies homozygous for the BEAF<sup>A-KO</sup> allele are healthy and viable, indicating that the 32B protein is sufficient for normal development. In contrast, flies homozygous for the BEAF<sup>AB-KO</sup> allele cannot be maintained as a stable line. Maternal BEAF is sufficient to obtain fertile adults, although the resulting BEAF<sup>AB-KO</sup> flies eclose 1–2 days later than their BEAF<sup>AB-KO/CyO</sup> siblings and are sickly. Also, although equal numbers of males and females are obtained, the fertility of the BEAF<sup>AB-KO</sup> 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 BEAFAB-KO flies. A number of different phenotypes were observed, although ovaries from a given BEAFAB-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 that we found between BEAF and spindle-E (spn-E) (Rox et al. 2007). 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 (Kellium and Schedl 1991, 1992). This led to the identification of the BEAF proteins as scs'-binding proteins (Zhao et al. 1995; Hart et al. 1997). 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 BEAFAB-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 to 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 the nucleosome remodeling factor (NURF) (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 affect mainly the male X chromosome and stronger mutations to affect all chromosomes of both males and females (Wang et al. 2001). Also, BEAFAB-KO animals are healthier than BEAFAB-KO animals and we observed a weaker effect on the male X chromosome in BEAFAB-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 BEAFAB-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 BEAFAB-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. 1998, 2002). 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 blot of adults in Figure 2F). Using several of our GFB transgenic fly lines in the BEAFGFP 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 (Sarge and Park-Sarge 2005; Bantignies and Cavalli 2006). 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 in 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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