doi:10.1016/j.jmb.2011.07.052 J. Mol. Biol. (2011) 412, 423–436

Contents lists available at www.sciencedirect.com

Journal of Molecular Biology

journal homepage: http://ees.elsevier.com.jmb

# *Drosophila* BTB/POZ Domains of "ttk Group" Can Form Multimers and Selectively Interact with Each Other

**Artem Bonchuk[1,2], Stepan Denisov[1], Pavel Georgiev[1]* and Oksana Maksimenko[1]***

[1]*Department of the Control of Genetic Processes, Institute of Gene Biology, Russian Academy of Sciences, 34/5 Vavilov Street, Moscow 119334, Russia*
[2]*Faculty of Biology, Moscow State University, Leninskie Gory, 1/73, Moscow 119992, Russia*

*Received 8 June 2011; accepted 21 July 2011*
Available online 29 July 2011

***Edited by M. Gottesman***

***Keywords:***
GAGA;
BTB domain;
Mod(mdg4);
CP190;
chromatin insulator

The BTB (*b*ric-a-brac, *t*ramtrack and *b*road complex)/POZ (*po*xvirus and *z*inc finger) domain is a conserved protein–protein interaction motif contained in a variety of transcription factors involved in development, chromatin remodeling, insulator activity, and carcinogenesis. All well-studied mammalian BTB domains form obligate homodimers and, rarely, tetramers. Only the BTB domain of the *Drosophila* GAGA factor (GAF) has been shown to exist as higher-order multimers. The BTB domain of GAF belongs to the "ttk group" that contains several highly conserved sequences not found in other BTB domains. Here, we have shown by size-exclusion chromatography, chemical cross-linking, and nondenaturing PAGE that four additional BTB domains of the ttk group—Batman, Mod(mdg4), Pipsqueak, and Tramtrack—can form multimers, like GAF. Interestingly, the BTB domains of GAF and Batman have formed a wide range of complexes and interacted in the yeast two-hybrid assay with other BTB domains tested. In contrast, the BTB domains of Mod(mdg4), Pipsqueak, and Tramtrack have formed stable high-order multimer complexes and failed to interact with each other. The BTB domain of *Drosophila* CP190 protein does not belong to the ttk group. This BTB domain has formed stable dimers and has not interacted with domains of the ttk group. Previously, it was suggested that GAF oligomerization into higher-order complexes facilitates long-range activation by providing a protein bridge between an enhancer and a promoter. Unexpectedly, experiments in the *Drosophila* model system have not supported the role of GAF in organization of long-distance interaction between the yeast GAL4 activator and the *white* promoter.



## Introduction

The BTB (*b*ric-a-brac, *t*ramtrack and *b*road complex) domain [also known as the POZ (*po*xvirus and *z*inc finger) domain] is a versatile protein–protein interaction motif that participates in a wide range of cellular functions, including transcription regulation (for review, see Ref. [1]). Transcription factors with a BTB domain at the N-terminus comprise a large important class of molecules involved in development and carcinogenesis. As shown in crystallographic studies,

**Corresponding authors.* E-mail addresses: georgiev_p@mail.ru; mog@genebiology.ru.

Abbreviations used: DBD, DNA-binding domain; 3-AT, 3-aminotriazole; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether) *N*,*N*′-tetraacetic acid.

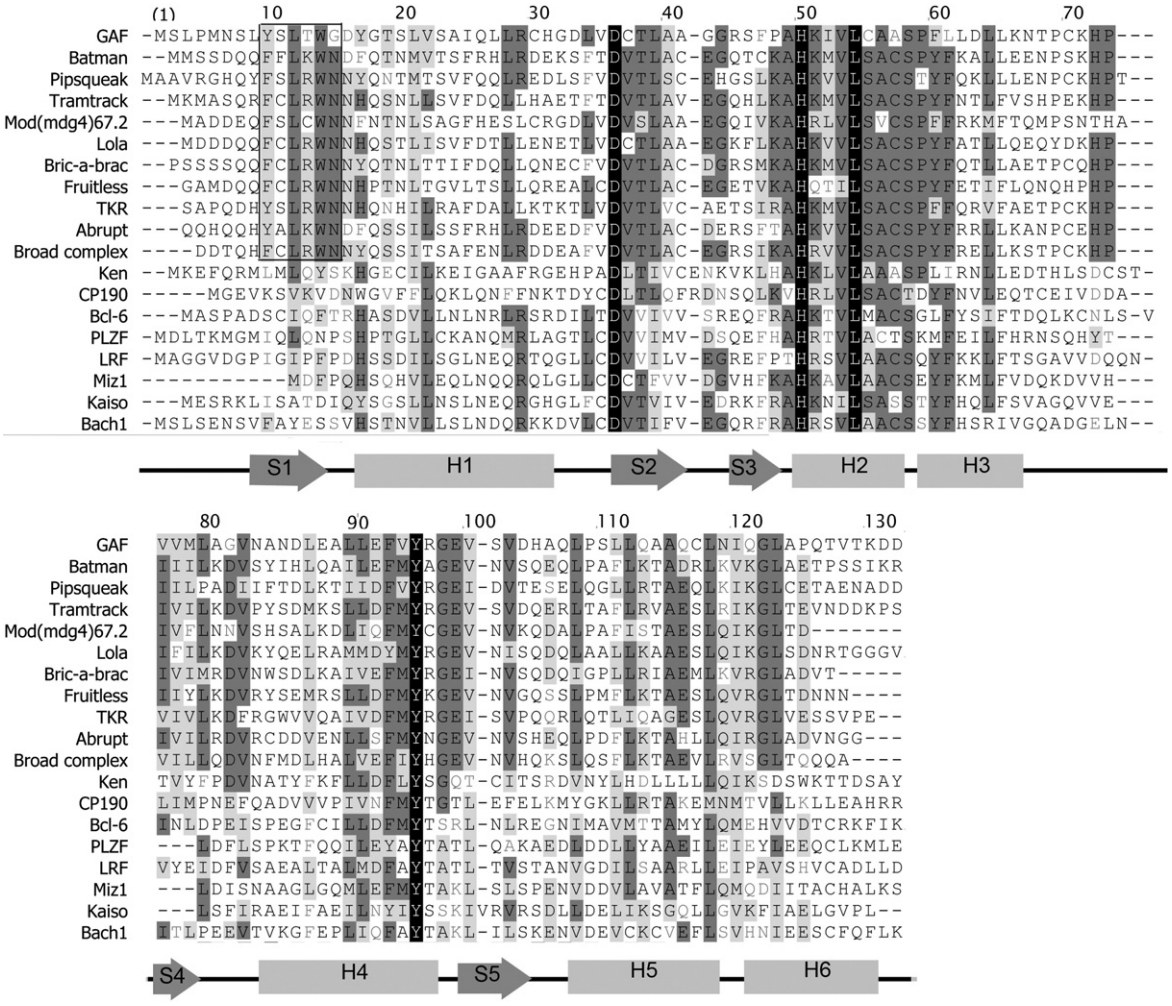


**Fig. 1.** Alignment of BTB domain sequences. Conservative N-terminal regions of BTBs of the ttk group are shown in the frame. Positions of α-helices (H) and β-strands (S) are indicated according to data on crystal structures of several BTB domains.

the BTB domains from PLZF (*p*romyelocytic *l*eukemia *z*inc *f*inger),[2,3] BCL6,[4,5] LRF/ZBTB7,[6,7] Bach1,[8] and FAZF[9] are tightly interwound homodimers. In the case of Miz1, there are contradicting crystallographic data that BTB exists as either homodimer[9] or homotetramer.[10]

A significant amount of data indicates that BTB domains are involved in the recruitment of non-BTB-containing regulator proteins.[11–13] Another set of experiments have shown that BTB domains of different proteins in some cases can specifically interact with each other, but it is unknown how such interactions proceed. Rarely, heterodimerization of two different BTBs has been observed. For example, there is indirect experimental evidence that PLZF and FAZF,[14] Bach2 and MAZR,[15] and Miz1 and Bcl6[16] participate in gene regulation through BTB–BTB interactions.

Experimental data indicate that BTB domains from some BTB-Zf proteins can mediate higher-order self-association.[17–19] In *Drosophila,* oligomerization of the BTB domain of GAGA binding factor (GAF) is considered to be important for regulating the transcriptional activity of chromatin and supporting long-distance interactions between enhancers and promoters.[20,21]

The BTB domain of GAF belongs to the "ttk group" that includes BR-C, Batman, Pipsqueak, Bab, Mod(mdg4), and several other important transcription factors.[22] This group contains several highly conserved sequences that are not found in other BTB domains (Fig. 1). The average sequence homology within the ttk group is 49%, whereas that between this group and other BTB domains is only 24%. Importantly, BTBs of several proteins from the ttk group can interact with the BTB of GAF.[22–26]

One member of the ttk group, Mod(mdg4)-67.2 [Mod(mdg4)], interacts with the Su(Hw) protein and is involved in the enhancer-blocking activity of Su (Hw)-dependent insulators. The current model

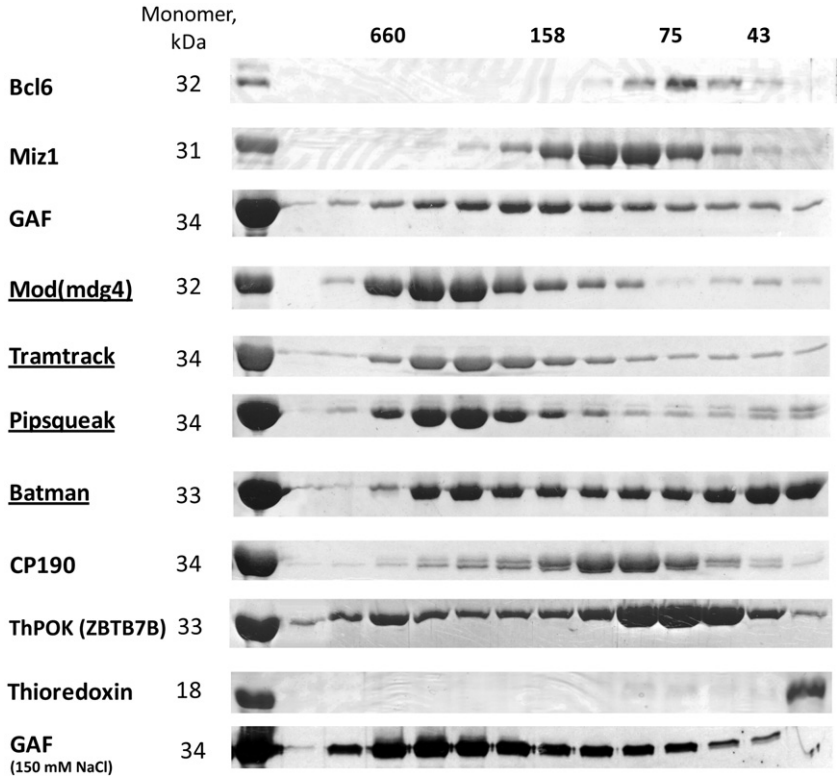


**Fig. 2.** Sephacryl S200 size-exclusion chromatography of thioredoxin-fused BTBs. Elution volumes of protein molecular weight standards are indicated. GAF-interacting BTB domains are underlined. Bcl6 and Miz1 were used as controls for dimeric and tetrameric domains, respectively. Th-POK is a recently identified vertebrate homolog of GAF.

suggests an important role for Mod(mdg4) in the interaction between Su(Hw) insulators.[27] It is hypothesized that the BTB domains form multimers that bring together distantly located insulator elements.[20] Interestingly, Su(Hw) also interacts with the BTB-containing CP190 protein.[28] This protein is essential for enhancer blocking of the Su (Hw) insulator and also interacts with other *Drosophila* insulator proteins, dCTCF[29,30] and BEAF.[31] Although the BTB domain of CP190 does not belong to the ttk group, it is suggested to play a significant role in supporting long-distance interactions between insulators.

Here, we compared for the first time oligomerization properties of several BTB domains from the ttk group and CP190 with those of well-studied BTBs of Miz1 and Bcl6. We also examined a possible role of GAF in supporting long-distance interactions between regulatory elements in the *Drosophila* model.

## Results

### BTB domains of GAF and several other transcriptional factors can form multimers in solution

The BTB of *Drosophila* GAF belongs to the group of BTB domains containing several conserved regions that distinguish them from other BTB domains found in *Drosophila* and vertebrates (Fig. 1). Homodimerization appears to be common to BTB domains, while the BTB domain of GAF displays the unique property to form multimeric complexes.[18,19] It has been shown that GAF, intact or with the deleted C-terminal Q domain, forms multimeric complexes only in the presence of the BTB domain.[18,19] Thus, the ability of the GAF BTB domain alone to form multimeric complexes has never been demonstrated directly,[18,19] and the size of such multimers also remains uncertain. Hence, the question has arisen as to the possibility of multimerization of the GAF BTB domain and other BTB domains that resemble this domain of the GAF protein.

We included in analysis well-studied BTB domains of Batman, Pipsqueak, Tramtrack, and Mod (mdg4) belonging to the ttk group, which were shown to interact selectively with GAF through the BTB domain.[23–26] As controls, we used well-characterized BTB domains of human proteins Bcl6 and Miz1, which can form dimers[4] and tetramers,[10] respectively. We also examined the BTB domain of CP190, which has no significant homology to BTBs from the ttk group, and BTB from a vertebrate homolog of GAF identified recently.[32]

Initially, pure recombinant BTB domains obtained in bacterial cells were studied by means of size-exclusion chromatography and electrophoresis under nondenaturing conditions. To improve BTB

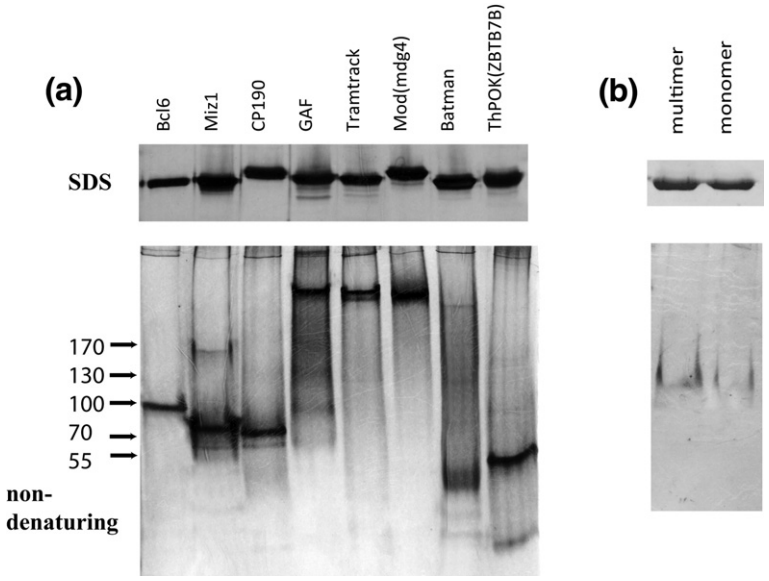


**Fig. 3.** Denaturing and nondenaturing polyacrylamide gel electrophoresis (a) of different BTB domains and (b) of the Batman BTB domain recovered from multimer and monomer fractions obtained by size-exclusion chromatography.

domains folding and solubility, we fused them with a thioredoxin tag.

In agreement with previous data, size-exclusion chromatography showed that Bcl6 formed dimers, while Miz1 formed tetramers (Fig. 2). The elution profile of the GAF BTB domain was surprisingly broad. GAF migrated at a rate indicative of apparent molecular mass between 40 kDa (monomer) and 600 kDa (at least, octamer). It is noteworthy that the BTB of Batman showed a similar elution profile, suggesting that it can exist in different forms, from a monomer to multimers.

In contrast, the BTB domains of Mod(mdg4), Pipsqueak, and Tramtrack proteins formed mainly high-order oligomers. The BTB domain of CP190 was eluted as a tetramer, as was the BTB domain of Miz1. Thus, the above BTB domains can be divided in three groups with respect to their capacity for oligomerization or multimerization: (1) stable multimers [Mod(mdg4), Pipsqueak, and Tramtrack], (2) unstable multimers (GAF and Batman), and (3) stable tetramers (CP190).

In gel-filtration experiments, we used 400 mM NaCl buffer because high salt usually inhibits unspecific interactions. To avoid the possibility that high-salt buffer can induce multimerization, we repeated the experiment with the BTB domain of GAF at 150 mM NaCl, using the same salt concentration in the protein purification procedure. We did not observe any appreciable differences between gel-filtration profiles of the GAF BTB domain in 400 or 150 mM NaCl buffer (Fig. 2).

The results of electrophoresis under nondenaturing conditions (with a 10× lower concentration of BTB domains in 150 mM NaCl buffer) confirmed the distribution of these BTBs into three groups (Fig. 3a). The BTB domains of Mod(mdg4) and Tramtrack in nondenaturing PAGE experiments migrated as stable multimers. The BTB domain of GAF formed several different complexes, from dimers to high-order multimers. Interestingly, the BTB domain of Batman migrated as a low-molecular-weight substance, and this was the only BTB that produced an abundant monomer fraction in size-exclusion chromatography. As a further test, we performed nondenaturing PAGE of this protein recovered from high- and low-molecular-weight fractions of size-exclusion chromatography at a concentration of about 5 μM and obtained similar results (Fig. 3b). Therefore, multimers of Batman proved to be the least stable compared to other domains tested. Interestingly, dimer formation by Batman, in contrast to other examined BTB domains, also appeared to be unstable, as followed from the presence of a major monomer fraction. The BTB of Bcl6 had the highest p*I* (calculated at 6.43 for thioredoxin fusion, compared to p*I*<6.0 in all other BTB–thioredoxin fusions) and migrated even more slowly than Miz1; thus, it was difficult to correctly determine the oligomerization status of Miz1 and CP190 using nondenaturing PAGE (Fig. 3a). However, they were most probably represented by dimers, and only a minor fraction of Miz1 existed as tetramers.

To further characterize the properties of BTB multimers, we performed chemical cross-linking by glutaraldehyde (Fig. 4). Similar results were obtained using other cross-linking agents such as EGS or DSP quenched with ethanolamine or Tris (data not shown). We used the same conditions as in nondenaturing electrophoresis experiments. Cross-linked BTB domains of Bcl6, Miz1, and CP190 migrated as dimers. Once again, the BTB domain of Miz1 was found only as dimers, suggesting that such a form is characteristic of this BTB at lower concentrations. After chemical cross-linking of GAF, Mod(mdg4), and Tramtrack, we revealed multimers

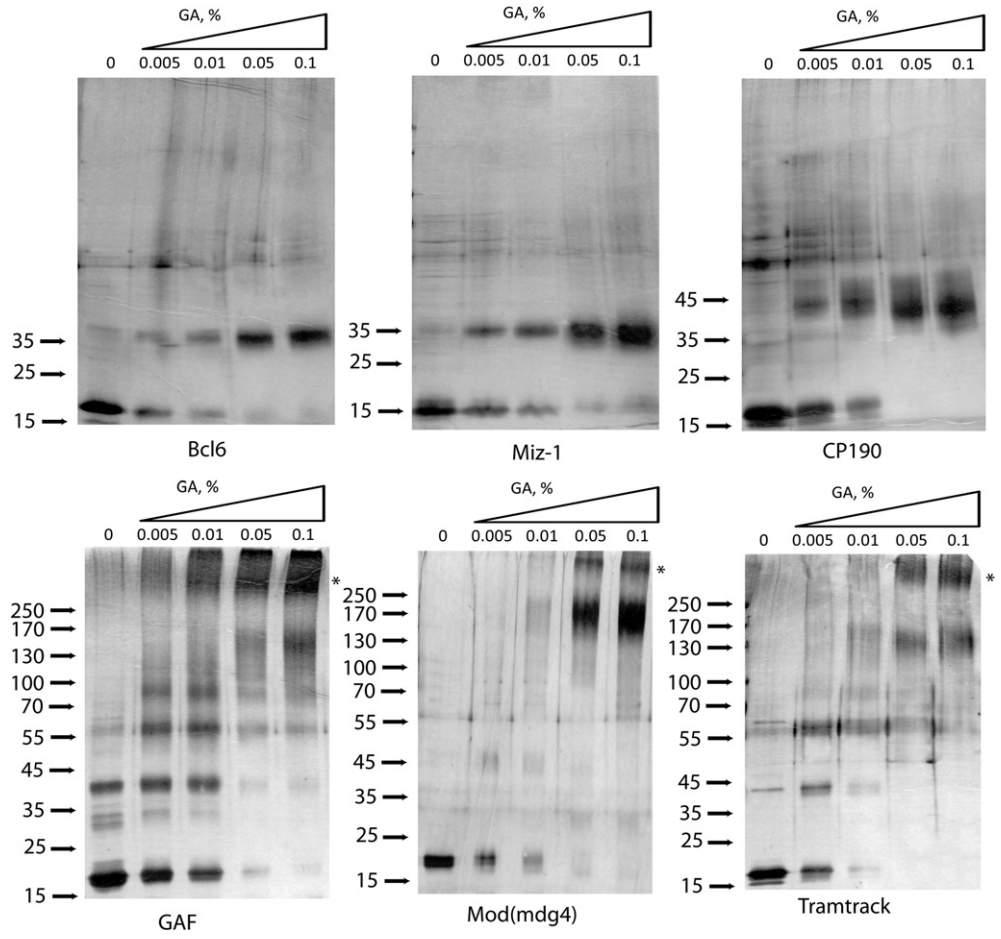


**Fig. 4.** Cross-linking of BTB domains by incubation with increasing concentration of glutaraldehyde (GA). Proteins were separated in 5–12% gradient polyacrylamide gels and visualized by silver staining (for experimental details, see Materials and Methods). The additional heavy band at the top of the gels in the lower row (asterisk) appears to be a result of unspecific reactions. However, since no such artifacts were observed in cross-linked samples of dimerizing BTBs at the same concentration (the upper row), this band could consist of domains that failed to assemble into ordered octamers but formed large multimers, as well as result from inter-octamer cross-linking. Additional bands near 70 kDa correspond to traces of the DnaK chaperone in protein preparations.

with molecular masses between 130 and 170 kDa, which expectedly contained an even number of subunits and, hence, were probably octamers consisting of 18-kDa monomers (in this case, without thioredoxin and 6× His tag) represented by the corresponding band (Fig. 4). The BTB domains of Mod(mdg4) and Tramtrack formed stable multimers, while the BTB of GAF existed in several forms, including dimers. Cross-linking of Pipsqueak, Batman, and Abrupt BTB domains also revealed presence of major multimers with mass near 150 kDa (data not shown).

Notably, dimers in small amounts were visible on SDS-PAGE gels even in samples without the cross-linking agent, indicating the high stability of dimer formation by the BTB domains tested. Therefore, it can be assumed that a dimer is a stable unit of an octamer.

To study possible concentration dependence of GAF multimer formation, we performed nondenaturing PAGE of GAF BTB at protein concentrations ranging from 5 to 100 μM but revealed no obvious shift in the multimer-to-monomer ratio (Fig. 5a). The concentration of proteins loaded onto the gel-filtration column was about 60 μM (Fig. 2); during chromatography, proteins were diluted almost 10-fold, but in all cases (except for Batman and GAF), we observed sharp peaks indicating that multimers are stable at the concentrations tested. Nondenaturing PAGE and cross-linking experiments were performed with 5 μM proteins (Figs. 3 and 4). Recently, a $K_d$ of less than 2 μM for dimer formation was determined for three BTB domains;[9] previously, a $K_d$ of 110 μM was reported for dimer–tetramer association of Miz1.[10] Therefore, our experiments show that multimers of the GAF BTB domain are

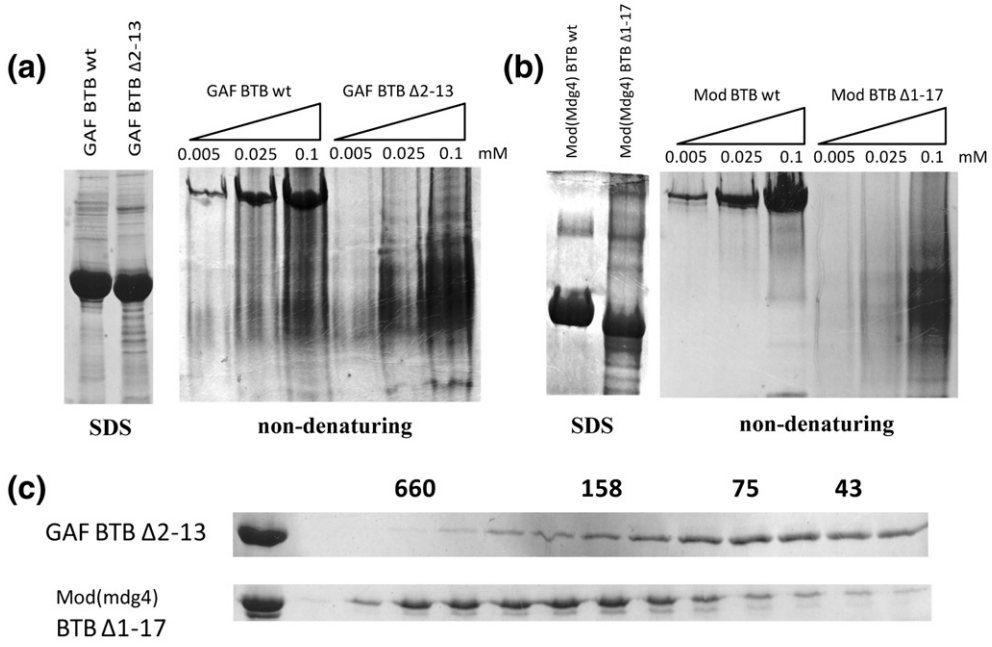


**Fig. 5.** Denaturing and nondenaturing electrophoresis of wild-type and N-terminal-mutated BTB domains of (a) GAF and (b) Mod(mdg4) and (c) Sephacryl S200 size-exclusion chromatography of N-terminal-mutated BTB domains of GAF and Mod(mdg4).

stable in solution with a $K_d$ of at least 5 μM; multimers of Mod(mdg4) and Tramtrack BTB domains appear to be even more stable; Batman forms less stable multimers, with $K_d$ estimated to be between 10 and 60 μM.

Recently, Th-POK (Zbtb7b) was identified as a vertebrate homolog of GAF.[32] Our experiments (Figs. 2 and 3a) showed that the Th-POK BTB domain can form only dimers in solution, like the BTB domains of many other human transcription factors investigated to date.

It seems likely that tetramer formation by the BTB domains of Miz1 and CP190 requires high protein concentrations. At physiologically relevant concentrations, these BTB domains preferentially form dimers.

Taken together, these results show that multimerization is a property common to a large group of *Drosophila* BTB domains with similar structure.

## Conserved N-terminal sequence of GAF-related BTB domains is involved in oligomerization

All *Drosophila* BTB domains that form multimers in solution have the characteristic hydrophobic sequence FxLRWN in the β1 region that is absent in BTB domains of other species. This conserved segment has high β-strand propensity, consistent with the presence of interchain β1 contacts across dimers. It has been proposed that exposed hydrophobic residues in this sheet region may participate in organization of the β-strand and drive strong dimer–dimer associations.[1,33] The dimerizing BTB domain of Th-POK, a vertebrate homolog of GAF, also lacks this conserved N-terminal sequence.

To test the role of this sequence, we generated a deletion of 2–13 aa in the GAF BTB domain ($BTB^{GAF\Delta}$) with simultaneous substitution of the highly conserved tryptophan residue by methionine (Fig. 1). Nondenaturing electrophoresis and size-exclusion chromatography showed that the oligomerization ability of the mutant BTB domain was dramatically reduced, indicating the importance of the amino-terminal conserved region for stabilization of BTB domain oligomers (Fig. 5a and c). This result also proves that the observed oligomerization is not due to unspecific aggregation or disulfide bond formation.

A deletion of the first 17 aa from the BTB domain of Mod(mdg4) ($BTB^{Mod\Delta}$) also markedly reduced its ability to form oligomers (Fig. 5b and c). Unfortunately, a major fraction of the mutated BTB domain expressed in bacteria was insoluble (obviously because of misfolding), which also resulted in the slower mobility in size-exclusion chromatography similar to the profile of natively unfolded proteins.

## Testing interactions between BTB domains in the yeast two-hybrid assay

As shown previously, the BTB domain of GAF can interact with BTB domains of Batman,[25,34] Pipsqueak,[26] Tramtrack,[23,33] and Mod(mdg4).[24] The question arises as to whether these BTB

**Table 1.** Interactions between BTB domains fused with the activation domain (AD) or DBD of GAL4 in the yeast two-hybrid assay

| | GAL4 AD BTB | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| GAL4 DBD BTB | GAF | Tramtrack | Mod(mdg4) | Pipsqueak | Batman | CP190 | Bcl6 | Mod(mdg4) Δ1–18 | GAF Δ2–13 | GAF BTB Δ2–13 AD | pGAD |
| GAF | +++ | +++ | ++ | +++ | +++ | +/− | +/− | +/− | **+++** | +++ | − |
| Tramtrack | +++ | +++ | − | − | +++ | − | − | − | + | +++ | − |
| Mod(mdg4) | +++ | − | +++ | − | +++ | − | − | + | +/− | + | − |
| Pipsqueak | +++ | − | − | +++ | +++ | − | − | − | + | +++ | − |
| Batman | +++ | +++ | +++ | +++ | +++ | − | − | − | +++ | +++ | − |
| CP190 | − | − | − | − | − | +++ | − | − | − | − | − |
| Bcl6 | − | − | − | − | − | − | +++ | − | − | − | − |
| Mod(mdg4) Δ1–18 | − | − | +++ | − | − | − | − | − | − | − | − |
| GAF Δ2–13 | **+++** | +++ | − | +++ | +++ | − | − | − | **+/−** | **+/−** | − |

The activation domain was placed either in front of the test protein or behind it (GAF BTB Δ2–13 AD). The plus symbols indicate the relative strength of two-hybrid interaction, with +++ referring to the vigorous growth of yeast in the presence of 5 mM 3-AT (selective inhibitor of the HIS3 gene product) and + or +/− referring to its inability to grow on 3-AT containing medium; − indicates the absence of interaction, which could be easily distinguished from +/− visually.

domains can interact with each other in different combinations. To test these protein–protein interactions, we used the yeast two-hybrid assay. The results confirmed that the BTB of GAF can interact with other BTB domains capable of forming multimeric complexes (Table 1).

The BTB of GAF and all other BTB domains forming multimers failed to interact with BTB domains of human Bcl6 (obligate homodimer, used as negative control) and CP190. The BTB domain of Batman interacted with the same BTB domains as did GAF BTB. In contrast, the BTB domains of Mod (mdg4), Pipsqueak, and Tramtrack failed to interact with each other. These results suggest that the stability of multimeric forms of BTB domains correlates with their ability to interact with heterologous BTB domains.

Unexpectedly, we found that $BTB^{GAF\Delta}$ failed to effectively interact with itself in the yeast two-hybrid assay. To confirm this result, we fused the activation domain of GAL4 (GAL4AD) to the C-terminus of the $BTB^{GAF\Delta}$ domain. Again, only a weak interaction was observed, suggesting that the $BTB^{GAF\Delta}$ domains formed unstable oligomers. In contrast, the $BTB^{GAF\Delta}$ domain effectively interacted with the wild-type BTB of GAF. Thus, the N-terminal domain of wild-type BTB can stabilize its complex with $BTB^{GAF\Delta}$, which lacks this domain. $BTB^{GAF\Delta}$ can also interact with BTBs of Batman, Pipsqueak, and Tramtrack, but not of Mod(mdg4).

Taken together, these results suggest that the β1 region is important for dimer stabilization rather than for multimerization of BTB domains.

## GAF fails to support distant activation of the promoter by GAL4 activator in *Drosophila*

As shown previously, GAF can stimulate transcription by linking the GAL4-dependent enhancer to its cognate promoter in human cells[20] and yeast.[21] In both studies, heterologous model systems were used to examine long-distance interactions between GAF binding sites. Hence, we decided to test the ability of GAF to support the enhancer–promoter communication in *Drosophila* using the GAL4/*white* assay described previously.[35,36] This assay is based on the finding that the yeast GAL4 activator bound to sites located upstream of the *yellow* gene fails to stimulate the *white* promoter placed downstream of the *yellow* 3′ end. The DNA fragment containing five binding sites for GAF was constructed as described previously.[20]

In the test construct (Fig. 6a), 10 GAL4 binding sites (G4) were inserted at −893 relative to the *yellow* transcription start site. As a result, the distance between the *white* gene and G4 was almost 5 kb. To examine the functional interaction between two GAF binding fragments, one element flanked by frt sites[37] was inserted near G4, and the other, flanked by lox sites,[38] was inserted near the *white* promoter. The presence of the frt and lox sites made it possible to delete the GAF binding sites and to compare stimulation of transcription by GAL4 in transgenic lines before the deletion of the regulatory elements and after it (control). The GAF binding regions were inserted in opposite orientations. As a control, we used the previously described transgenic lines carrying Mcp elements instead of GAF binding sites in the same positions.[36]

In 12 resultant transgenic lines, flies had eye color ranging from pale yellow (pY) to dark yellow (dY) indicative of basic activation of the *white* promoter. Eye pigmentation remained unchanged after deletion of GAF binding sites, which indicated that GAF had no effect on the activity of the *white* promoter. Using cross-linking chromatin immunoprecipitation of chromatin isolated from pupae of one transgenic line and by gel retardation assay *in vitro*, we found

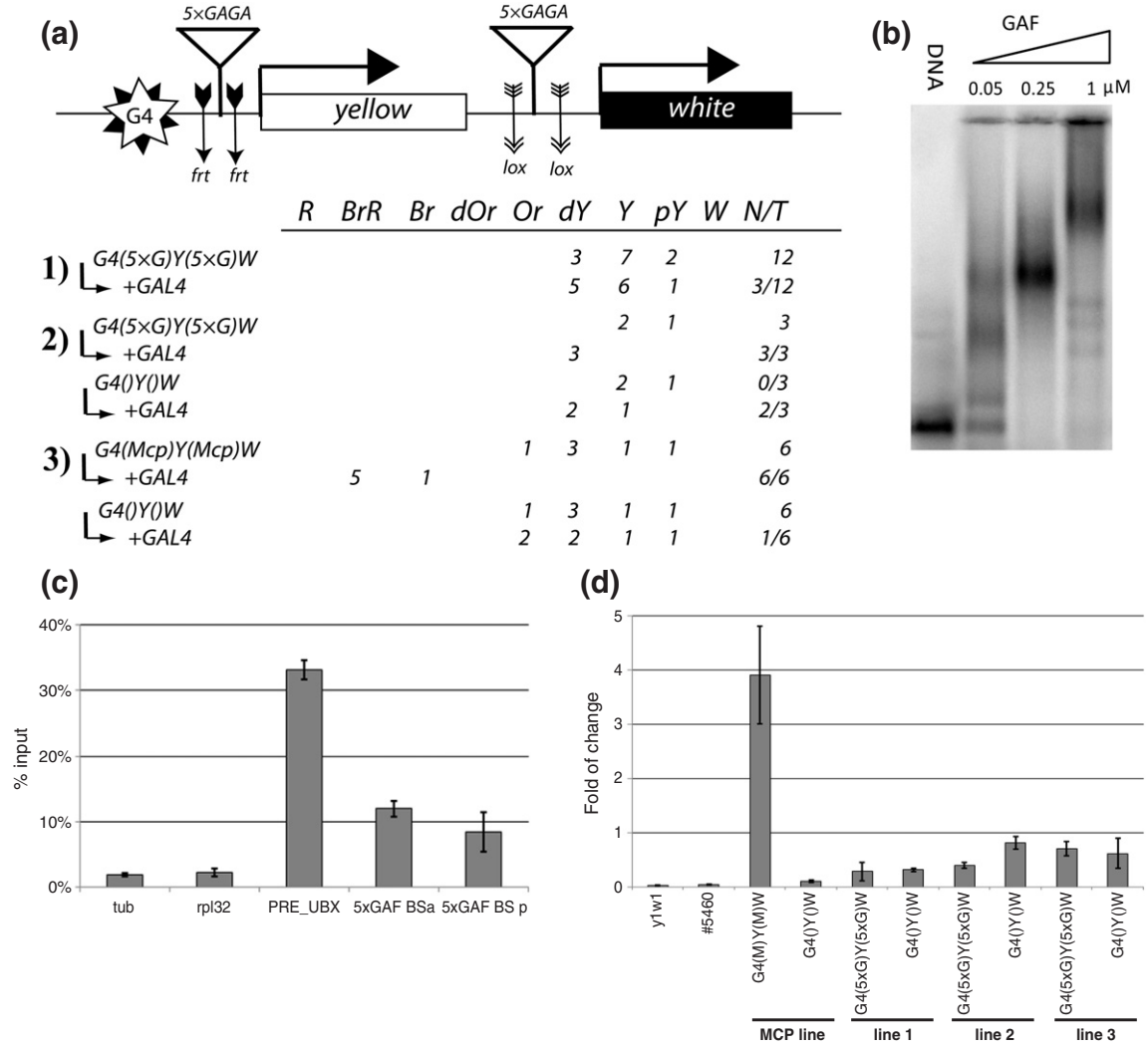


**Fig. 6.** Testing the ability of GAF to support long-distance interactions in *Drosophila*. (a) Testing the functional interaction between GAF binding sites in the GAL4/*white* model system. GAL4 binding sites (G4) are at a distance of 5 kb from the *white* promoter. Five GAF binding sites (5× GAGA) are flanked by sites for site-specific recombinases Cre and FLP. (1) GAL4 induced a slight increase of eye pigmentation in 3 out of 12 transgenic lines tested. (2) After deletion of the GAF binding sites, GAL4 stimulated *white* expression to the same level in 2 out of 3 transgenic lines. (3) Mcp elements instead of 5× GAGA strongly induced *white* expression upon GAL4 activation. Results with G4(Mcp)Y(Mcp)W were obtained in the previous study.[36] The wild-type *white* expression determined the bright-red eye color (R); in the absence of *white* expression, the eyes were white (W). Intermediate levels of pigmentation, with the eye color ranging from pale yellow (pY) [through yellow (Y), dark yellow (dY), orange (Or), dark orange (dOr) and brown (Br)] to brownish red (BrR), reflect the increasing levels of *white* expression. Figures in columns show the numbers of lines in which flies acquired a new w phenotype upon induction of GAL4. (b) Interaction of recombinant GAF with the DNA fragment containing five GAF binding sites used in transgenic construct analyzed in the electrophoretic mobility shift assay. (c) Results of immunoprecipitation experiments with chromatin isolated from flies carrying the above transgenic construct and treated with anti-GAF antibodies. Real-time PCR was performed with primers for *tubulin*, *rpl32*, *Ultrabithorax* polycomb response element (PRE), and GAF binding sites located upstream of the *yellow* gene (5× GAF BSa) and near the promoter of the *white* gene (5× GAF BSp) in transgenic construct. (d) Results of quantitative real-time PCR with embryonic RNAs for determining the levels of *white* mRNA accumulation. Embryos of the $yacw^{1118}$ line (designated y1w1) and GAL4 driver line (designated no. 5460) were used as a negative control, and embryos of the G4(M)Y(M)W line[36] were used as a positive control for induction of *white* expression. After excision of Mcp elements (designated M), *white* stimulation by GAL4 was strongly reduced. To test the role of GAF in *white* stimulation by GAL4, we measured *white* transcription in three transgenic lines G4(5× G)Y(5× G)W and their derivative lines with excised GAF binding sites. Individual transcript levels determined by quantitative PCR were normalized relative to *rp47* and *rpl32* for the amount of input cDNA. Error bars represent standard deviation of values obtained by analyzing three independently isolated RNA samples.

that GAF effectively bound to both DNA fragments (Fig. 6b and c). To express GAL4 protein, we used the transgenic line carrying the GAL4 gene under the control of the ubiquitous tubulin promoter. GAL4 only weakly stimulated transcription in 3 out of 12 transgenic lines. Deletion of both GAF binding sites had almost no effect on the ability of GAL4 to stimulate *white*. In contrast, two Mcp elements facilitated activation of *white* by GAL4, which was indicative of interaction between the Mcp elements[36] (Fig. 6a).

It appeared that GAF could support enhancer–promoter interactions in embryos, in which this protein is expressed at the highest level. To test this possibility, we used the GAL4/*white* assay to compare the levels of *white* mRNA accumulation in embryos before and after the deletion of GAF elements. If GAF were able to support long-distance interaction between the binding sites located near the GAL4 activator and the *white* promoter, GAL4 would effectively stimulate *white* expression only in the presence of GAF binding sites. Unexpectedly, we observed no significant effect of GAF elements on the level of *white* expression in any of the three transgenic lines tested (Fig. 6d), whereas two Mcp elements in the control transgenic line effectively supported *white* stimulation by GAL4.

Thus, GAF failed to support long-distance communication in the GAL4/*white* assay.

## Discussion

The results of this study show that BTB domains of the ttk group can multimerize. They contain conserved regions that are absent in other BTBs, which is an evidence that the ability to form large multimers is unique to this group of *Drosophila* BTB domains. Preliminary data suggest that the vertebrate homolog of GAF has a similar role in the regulation of HOX gene expression, but its BTB domain exists mainly as a homodimer.

It appears that the presence of several conserved regions in BTB domains of the ttk group is the factor accounting for their ability to form large multimers (see Fig. 1). An interaction between dimers was previously observed in crystals of the PLZF BTB domain.[39] This interaction involved contacts between intermolecular β-sheets β1–β5 of neighboring dimers, but its strength proved insufficient for supporting multimer formation by PLZF BTB in unsaturated solution. The appearance of additional hydrophobic residues in these regions might have resulted in stabilization of cross-dimer interactions. For this reason, we have examined the role of the N-terminal FxLRWN region characterized by regular arrangement of hydrophobic residues, which indicates its involvement in organization of the β-strand. We have found that the conserved FxLRWN sequence of Mod(mdg4) and GAF BTB domains is required for their ability to form large multimers. In the yeast two-hybrid assay, BTBs of mutant GAF and Mod(mdg4) have failed to self-associate effectively, suggesting that even their dimers are unstable. In contrast, they have proved to interact with wild-type BTB domains, which is evidence that mutant BTB domains are properly folded and can form stable dimers with the wild-type counterpart.

According to crystallographic studies, an important component of the hydrophobic dimerization interface in PLZF and Bcl6 is the association of the long form elements β1 and α1 of one monomer with the core structure of the other monomer. One component of the dimerization interface is an intermolecular antiparallel β-sheet formed between β1 of the first monomer and β5 of the second monomer.[2] However, although nine human BTB transcription factors lack β1, they still can form dimers. For example, the BTB of Miz1 lacks the residues necessary for forming the N-terminal β1 but forms stable dimers and even tetramers.[10] On the other hand, the deletion of the N-hook from Bach1 BTB resulted in the conversion of the homodimer into a stable monomer in solution, indicating that the N-region promotes homodimerization.[8]

The BTB domains of the ttk group have the unique ability to specifically interact with each other. Such interactions can proceed either via formation of heterodimers between two BTB domains or via association between two different homodimers or larger oligomers. The available data on the ability of mammalian BTB domains to form heterodimers are contradictory. There are many examples of interaction between BTB-containing transcription factors. Thus, Miz1 recruits Bcl6 to several promoters,[16] and the PLZF and FAZF proteins co-localize in nuclear speckles and can form heterodimers.[14] In both cases, however, it has not been directly shown that the heterodimerization of BTB domains is the factor accounting for the interaction between Miz1 and Bcl6 or between FAZF and PLZF. Since BTB domains interact with many different non-BTB proteins, it is possible that the BTB domain of one protein interacts with an unidentified region of another BTB protein. For example, Mod(mdg4) and CP190 interact *in vivo* and *in vitro* through non-BTB domains[40] (unpublished data).

In this study, we have observed that $BTB^{GAF\Delta}$ can still interact with other BTB domains with almost the same specificity as the wild-type BTB. Thus, it is likely that such an interaction proceeds through heterodimer formation by BTB proteins. Alternatively, $BTB^{GAF\Delta}$ can form unstable homodimers capable of interacting with homodimers of another BTB. This interaction stabilizes unstable $BTB^{GAF\Delta}$ homodimers or protects them from degradation. It is noteworthy that although $BTB^{GAF\Delta}$ is similar to the wild-type BTB in the specificity of heteromeric

interactions, it fails to effectively homodimerize. Therefore, heteromeric interactions in this case are likely to proceed mainly through heterodimer formation by BTB proteins or take place at the interface that is not involved in oligomerization. However, this apparently does not apply to the GAF–Mod(mdg4) interaction.

As CP190 and Mod(mdg4) are involved in the activity of Su(Hw) and dCTCF insulators,[30] it has been suggested that the BTB domains of these proteins are involved in specific interaction between insulators. Here, we have found that the BTB domains of these proteins are completely different. In contrast to Mod(mdg4), the BTB domain of CP190 forms stable dimers and fails to interact with BTB domains of the ttk group. Thus, the Mod(mdg4) and CP190 proteins appear to play different roles in the activity of *Drosophila* insulators.

The role of BTB domains of the ttk group in organization of long-distance interactions either between insulators or between an enhancer and a promoter is still debatable. All experimental data supporting this model have been obtained with GAF. It has been shown that the BTB domain mediates GAF multimerization into higher-order oligomers that cooperatively bind to multiple sites present in its natural target promoters.[18,19] Electron microscopic[19] and DNA pull-down experiments[20] confirmed that GAF complexes can form a protein link between separate DNA elements *in vitro*. There is experimental evidence that GAF can facilitate gene activation in human 911 cells[20] and yeast[21] by acting as an anchor that links the remote GAL4 binding sites to the promoters.

In our experiments, we have used the same GAF binding region as in the previous studies,[20,21] and GAF has proved to effectively bind to these sites *in vitro* and *in vivo*. This protein is strongly expressed in embryos, in the eye imaginal discs of larvae, and in adult flies.[41] However, we have observed no functional interaction between GAF binding sites resulting in stimulation of *white* by the remote GAL4 activator. A probable explanation to these unexpected results is that long-distance interactions in all previous studies were analyzed in heterologous model systems that lacked many of BTB transcription factors interacting with GAF. These interactions could interfere with the ability of GAF to support long-distance interactions in *Drosophila*.

There are many pieces of indirect experimental evidence for the role of GAF in supporting the enhancer–promoter communication or insulation. It has been shown that the promoter-proximal GAF sites in the *Ubx* gene are implicated in regulation by the ABX and BXD distal regulatory elements.[42] The GAF sites in the *engrailed* promoter are essential for enhancer-dependent activation.[43] The stage-specific activity of GAF is supported by the observation that the *eve* promoter in embryos possesses intrinsic insulator properties that are critically dependent on GAF function.[44] In all these cases, however, GAF may well play only an auxiliary role by facilitating the binding of other transcription factors involved in the above activities. For example, such a role has been suggested for GAF in the case of Fab-7 and SF1 insulators, which have many GAF binding sites.[45,46]

Thus, further studies are necessary for elucidating the role of BTB domains of the ttk group in long-distance interactions and their other possible activities in transcriptional regulation.

## Materials and Methods

### Plasmid construction

DNA fragments coding for Bcl6 (residues 1–129), Miz1 (1–117), GAF (1–131), Tramtrack (1–117), Pipsqueak (1–122), Batman (1–128), Mod(mdg4) (1–119), Abrupt (53–198), CP190 (1–126), and Th-POK (1–141) were PCR amplified from HEK293 cells cDNA (Bcl6, Miz1, and Th-POK) or cDNA from Schneider line 2 cells (*Drosophila* proteins) and subcloned into pET32a(+) vector (Novagen) with a modified multiple cloning site in-frame with thioredoxin and N-terminal 6× His. GAF with a deletion in the BTB domain was amplified using corresponding primers; the BTB domain of Mod(mdg4) bearing a 17-aa deletion was digested with EagI from pET32 wild-type construct and subcloned into NotI site of modified pET32a vector. To make fusions with the GAL4 DNA-binding domain (DBD) and activating domain for yeast two-hybrid screen, we digested the BTB domain coding sequences from pET32 constructs with BglII and XhoI or SalI and subcloned them into BamHI and SalI sites of pGAD24 and pGBT9 (Clontech) with filled-in EcoRI site. Details of cloning are available on request.

### Expression and purification of BTB domains

*Escherichia coli* BL21(DE3) bacteria transformed with the pET32–BTB fusion construct were grown to an $A_{600}$ of 1.0 at 37 °C and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 25 °C for 4–5 h. The cells were pelleted at 5000*g* for 15 min, resuspended in buffer A [30 mM Tris–HCl (pH 8.0), 400 mM NaCl, 5 mM β-mercaptoethanol, and 20 mM imidazole] containing 1 mM PMSF and Calbiochem Complete Protease Inhibitor Cocktail VII (1 μl/ml), and lysed by sonication. The homogenate was centrifuged at 20,000*g* for 30 min, and the supernatant fluid was applied onto a Ni-NTA column equilibrated in buffer A. After extensive washing with buffer A, proteins were eluted with elution buffer (buffer A containing 200 mM imidazole) and analyzed immediately or stored after adding glycerol (50% w/w).

### Gel-filtration chromatography and nondenaturing electrophoresis

Proteins equilibrated in 20 mM Na phosphate buffer (pH 7.4) with 400 mM NaCl and 5 mM β-mercaptoethanol

were adjusted to a concentration of about 2 mg/ml (60 μM) and applied onto a Sephacryl S200 16/60 column (GE Healthcare) running in the same buffer at a flow rate of 1 ml/min at room temperature. The column was pre-equilibrated with protein molecular weight standards (GE Healthcare), the void volume measured to be 50 ml. Eluted fractions (2.5 ml) were collected, precipitated with trichloroacetic acid, and analyzed by SDS-PAGE followed by Coomassie staining. For nondenaturing electrophoresis, protein samples in the same buffer were adjusted to an appropriate concentration, mixed with loading buffer [62.5 mM Tris–HCl (pH 6.8) with 1% β-mercaptoethanol and 10% glycerol], and incubated for 1 h. Electrophoresis was performed in 7% PAGE (pH 8.8) with 5% upper gel (pH 6.8) in Tris-glycine electrode buffer, pH 8.3. Gels were Coomassie or silver stained.

### Cross-linking experiments

Proteins were expressed and extracted, as previously described, using buffer A with 40 mM Hepes–KOH (pH 7.7) instead of Tris and were immobilized on Co-IDA agarose (Biontex) from a cleared bacterial lysate. The resin was washed two times with buffer A–Hepes and two times with thrombin cleavage and cross-linking buffer [20 mM Hepes—KOH (pH 7.7), 150 mM NaCl, 20 mM imidazole, 2.5 mM $CaCl_2$, and 1 mM β-mercaptoethanol]. BTB domains were eluted overnight with 1 U/ml biotinylated human thrombin (Novagen) at 20 °C. Thrombin was removed by incubating the resulting supernatant fluid with Streptavidin agarose (Pierce) for 10 min. Protein concentration was adjusted to 5 μM for 1 h. Cross-linking was performed with indicated concentrations of glutaraldehyde at room temperature for 10 min and quenched with 50 mM glycine. Samples were resolved in 5–12% gradient acrylamide gels and visualized by silver staining.

### Two-hybrid screen

Two-hybrid assays were carried out using yeast strain pJ69-4A, plasmids, and protocols obtained from Clontech. For growth assays, plasmids were transformed into yeast strain pJ69-4A by the lithium acetate method as described by the manufacturer and were plated on media without tryptophan and leucine. After 3 days of growth at 30 °C, the cells were plated on selective media without tryptophan, leucine, and histidine, and their growth was compared after 2–3 days in the presence or absence of 5 mM 3-aminotriazole (3-AT). Each assay was repeated twice.

### Electrophoretic mobility shift assay

The full-length 6× His-tagged GAF expression plasmid pKH-GAF was described previously.[47] The protein was produced using the same procedure as described above, except that 100 μM $ZnCl_2$ was added to all buffers. The $^{32}$ATP-end-labeled DNA fragment was mixed with indicated amounts of recombinant GAF in 25 mM Hepes buffer (pH 7.6) containing 100 mM NaCl, 0.1 mM $ZnCl_2$, 0.05% NP40, 1 mM DTT, 5% glycerol, and 50 ng/μl poly (dI-dC). The mixture was incubated at 25 °C for 30 min and analyzed in 1% agarose in 0.5× Tris–borate–ethylenediaminetetraacetic acid (EDTA).

### Generation and analysis of transgenic flies

The construct and the P25.7wc plasmid were injected into *yacw*$^{1118}$ preblastoderm embryos.[48] The resultant flies were crossed with *yacw*$^{1118}$ flies, and the transgenic progenies were identified by their eye color. Lines with DNA fragment excisions were obtained by crossing the flies bearing the transposons with the FLP (*w*$^{1118}$; *S2CyO*, *hsFLP*, *ISA/Sco*; +) or Cre (*yw*; *Cyo*, *P*[*w*$^{+}$,*cre*]/*Sco*; +) recombinase-expressing lines.[37,38] Cre recombinase induces 100% excisions in the next generation. A high level of FLP recombinase (almost 90% efficiency) was produced by daily heat-shock treatment for 2 h during the first 3 days after hatching. Details of the crosses used for genetic analysis and excision of functional elements are available upon request. To induce GAL4 expression, we used the modified *yw1118*; *P*[*w*$^{+}$, *tubGAL4*]*117*/*TM3*,*Sb* line (Bloomington Center no. 5138), in which the marker *mini-white* gene was deleted as described previously.[36] To induce GAL4 expression in embryos, we also used the *w*∗; *P*{*w*[+*mW.hs*]=*GAL4-da.G32*}*UH1* line (Bloomington Center no. 5460). To determine the levels of *white* expression, we visually estimated the degree of pigmentation in the eyes of 3- to 5-day-old males developing at 25 °C. Wild-type *white* expression determined the bright-red eye color (R); in the absence of *white* expression, the eyes were white (W). Intermediate levels of eye pigmentation (in increasing order) were reflected in the eye color ranging from pale yellow (pY) to yellow (Y), dark yellow (dY), orange (Or), dark orange (dOr), and, finally, brown (Br) or brownish red (BrR).

### Chromatin immunoprecipitation

Chromatin was prepared from mid-late pupae. Samples (500 mg) was ground in liquid nitrogen in a mortar and resuspended in 10 ml of buffer A [15 mM Hepes—KOH (pH 7.6), 60 mM KCl, 15 mM NaCl, 13 mM EDTA, 0.1 mM ethylene glycol bis(β-aminoethyl ether) *N*,*N*′-tetraacetic acid (EGTA), 0.15 mM spermine, 0.5 mM spermidine, 0.5% NP40, and 0.5 mM DTT] containing 0.5 mM PMSF and Complete (EDTA-free) Protease Inhibitor Cocktail V (Calbiochem). The suspension was homogenized in a Dounce homogenizer with pestle B and filtered through a nylon cell strainer (BD Biosciences). The homogenate was transferred onto 3 ml of buffer A with 10% sucrose (AS), and the nuclei were pelleted by centrifugation at 4000*g* at 4 °C for 5 min. The pellet was resuspended in 5 ml of buffer A, homogenized again in a Dounce homogenizer, and transferred onto 1.5 ml of buffer AS, and the nuclei were collected by centrifugation. The nuclear pellet was resuspended in wash buffer [15 mM Hepes—KOH (pH 7.6), 60 mM KCl, 15 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 0.1% NP40, and protease inhibitors] and cross-linked with 1% formaldehyde at room temperature for 15 min. Cross-linking was stopped by adding glycine to a final concentration of 125 mM. The nuclei were washed with three 10-ml portions of wash buffer, resuspended in 1.5 ml of nuclei lysis buffer [15 mM Hepes (pH 7.6), 140 mM NaCl, 1 mM EDTA, 0.1 mM

EGTA, 1% Triton X-100, 0.5 mM DTT, 0.1% sodium deoxycholate, 0.1% SDS, and protease inhibitors], and sonicated on ice with a Branson Sonifier 150 for 5×20 s at 1-min intervals. Debris was removed by centrifugation at 14,000*g*, 4 °C, for 10 min. Chromatin was pre-cleared in protein G agarose (Pierce) blocked with bovine serum albumin and salmon sperm DNA. An aliquot of such pre-cleared chromatin was used as an input sample. Pre-cleared chromatin samples were incubated with rat antibodies against GAF (1:200) and with nonspecific IgG purified from preimmune rat serum at 4 °C overnight, and chromatin–antibody complexes were then collected using blocked protein G agarose at 4 °C for 5 h. After several rounds of washing with lysis buffer as such or with 500 mM NaCl, LiCl buffer [20 mM Tris–HCl (pH 8), 250 mM LiCl, 1 mM EDTA, 0.5% NP40, 0.5% sodium deoxycholate, and protease inhibitors], and Tris–EDTA buffer, the DNA was eluted with elution buffer [50 mM Tris–HCl (pH 8), 1 mM EDTA, and 1% SDS], the cross-links were reversed, and the precipitated DNA was extracted with phenol/chloroform. The enrichment of specific DNA fragments was analyzed by real-time PCR, using a CFX96 Thermal Cycler (Bio-Rad).

#### RNA isolation and real-time PCR

Total RNA was isolated using TRI reagent (MRC) according to the manufacturer's instructions. RNA was treated with two units of Turbo DNase I (Ambion) for 30 min at 37 °C to eliminate genomic DNA. The synthesis of cDNA was performed using 2 μg of RNA and ArrayScript reverse transcriptase (Ambion). The amounts of specific cDNA fragments were analyzed by real-time PCR, using a CFX96 Thermal Cycler (Bio-Rad). At least three independent experiments with each primer set were performed for three independent RNA samples. Individual expression values were normalized with reference to *rp47*, *rpl32* mRNA. Relative levels of mRNA expression were calculated using the cycle threshold method.

The primers used for real-time PCR experiments were as follows: tub (5′-gctttcccaagaagctcataca-3′ and 5′-ggttcagtgcggtattatccag-3′), rpl32 (5′-gttcgatccgtaaccgatgt-3′ and 5′-ccagtcggatcgatatgctaa-3′), rp47 (5′-tgtccttccagcttcaagatgaccatc-3′ and 5′-cttgggcttgcgccatttgtg-3′), PRE_UBX (5′-gccacgccccttcac-3′ and 5′-gccctctctcttttgagttatcg-3′), 5× GAF BSa (5′-gcctcctggccttacaatttact-3′ and 5′-cctctcgttcattgctctctcg-3′), 5× GAF BSp (5′-actgcactggatatcattgaacttatc-3′ and 5′-tggacagagaaggaggcaaaca-3′), and *white* (5′-gcaaatgtcagcacacgatcat-3′ and 5′-gtgggctcatcgcagatca-3′).

## Acknowledgements

We are grateful to A. Parshikov for his help in producing transgenic flies and to N. A. Gorgolyuk for his help in preparing the manuscript. Dr. P. Verrijzer kindly provided us with the pKH-GAF vector. This study was supported by the Ministry of Science and Education of the Russian Federation, project no. P1165 (to O.M.); the Molecular and Cell Biology Program of the Russian Academy of Sciences (to P.G.); Russian Foundation for Basic Research grant no. 09.04.01232-a (to O.M.); and a stipend from the Dynasty Foundation (to O.M.). Experiments were performed using the equipment of the Institute of Gene Biology, Russian Academy of Sciences facilities supported by the Ministry of Science and Education of the Russian Federation.

## References

1. Stogios, P. J., Downs, G. S., Jauhal, J. J., Nandra, S. K. & Prive, G. G. (2005). Sequence and structural analysis of BTB domain proteins. *Genome Biol.* **6**, R82.
2. Ahmad, K. F., Engel, C. K. & Prive, G. G. (1998). Crystal structure of the BTB domain from PLZF. *Proc. Natl Acad. Sci. USA*, **95**, 12123–12128.
3. Li, X., Lopez-Guisa, J. M., Ninan, N., Weiner, E. J., Rauscher, F. J., III & Marmorstein, R. (1997). Overexpression, purification, characterization, and crystallization of the BTB/POZ domain from the PLZF oncoprotein. *J. Biol. Chem.* **272**, 27324–27329.
4. Ahmad, K. F., Melnick, A., Lax, S., Bouchard, D., Liu, J., Kiang, C. L. *et al.* (2003). Mechanism of SMRT corepressor recruitment by the BCL6 BTB domain. *Mol. Cell*, **12**, 1551–1564.
5. Ghetu, A. F., Corcoran, C. M., Cerchietti, L., Bardwell, V. J., Melnick, A. & Prive, G. G. (2008). Structure of a BCOR corepressor peptide in complex with the BCL6 BTB domain dimer. *Mol. Cell*, **29**, 384–391.
6. Schubot, F. D., Tropea, J. E. & Waugh, D. S. (2006). Structure of the POZ domain of human LRF, a master regulator of oncogenesis. *Biochem. Biophys. Res. Commun.* **351**, 1–6.
7. Stogios, P. J., Chen, L. & Prive, G. G. (2007). Crystal structure of the BTB domain from the LRF/ZBTB7 transcriptional regulator. *Protein Sci.* **16**, 336–342.
8. Ito, N., Watanabe-Matsui, M., Igarashi, K. & Murayama, K. (2009). Crystal structure of the Bach1 BTB domain and its regulation of homodimerization. *Genes Cells*, **14**, 167–178.
9. Stogios, P. J., Cuesta-Seijo, J. A., Chen, L., Pomroy, N. C. & Prive, G. G. (2010). Insights into strand exchange in BTB domain dimers from the crystal structures of FAZF and Miz1. *J. Mol. Biol.* **400**, 983–997.
10. Stead, M. A., Trinh, C. H., Garnett, J. A., Carr, S. B., Baron, A. J., Edwards, T. A. & Wright, S. C. (2007). A beta-sheet interaction interface directs the tetramerisation of the Miz-1 POZ domain. *J. Mol. Biol.* **373**, 820–826.
11. Pointud, J. C., Larsson, J., Dastugue, B. & Couderc, J. L. (2001). The BTB/POZ domain of the regulatory proteins bric à brac 1 (BAB1) and bric à brac 2 (BAB2) interacts with the novel *Drosophila* $TAF_{II}$ factor $BIP2/dTAF_{II}155$. *Dev. Biol.* **237**, 368–380.
12. Defossez, P. A., Kelly, K. F., Filion, G. J., Perez-Torrado, R., Magdinier, F., Menoni, H. *et al.* (2005). The human enhancer blocker CTC-binding factor interacts with the transcription factor Kaiso. *J. Biol. Chem.* **280**, 43017–43023.
13. Chopra, V. S., Srinivasan, A., Kumar, R. P., Mishra, K., Basquin, D., Docquier, M. *et al.* (2008). Transcriptional activation by GAGA factor is through its direct interaction with dmTAF3. *Dev. Biol.* **317**, 660–670.

14. Hoatlin, M. E., Zhi, Y., Ball, H., Silvey, K., Melnick, A., Stone, S. *et al.* (1999). A novel BTB/POZ transcriptional repressor protein interacts with the Fanconi anemia group C protein and PLZF. *Blood*, **94**, 3737–3747.
15. Kobayashi, A., Yamagiwa, H., Hoshino, H., Muto, A., Sato, K., Morita, M. *et al.* (2000). A combinatorial code for gene expression generated by transcription factor Bach2 and MAZR (MAZ-related factor) through the BTB/POZ domain. *Mol. Cell. Biol.* **20**, 1733–1746.
16. Phan, R. T., Saito, M., Basso, K., Niu, H. & Dalla-Favera, R. (2005). BCL6 interacts with the transcription factor Miz-1 to suppress the cyclin-dependent kinase inhibitor p21 and cell cycle arrest in germinal center B cells. *Nat. Immunol.* **6**, 1054–1060.
17. Yoshida, C., Tokumasu, F., Hohmura, K. I., Bungert, J., Hayashi, N., Nagasawa, T. *et al.* (1999). Long range interaction of *cis*-DNA elements mediated by architectural transcription factor Bach1. *Genes Cells*, **4**, 643–655.
18. Espinas, M. L., Jimenez-Garcia, E., Vaquero, A., Canudas, S., Bernues, J. & Azorin, F. (1999). The N-terminal POZ domain of GAGA mediates the formation of oligomers that bind DNA with high affinity and specificity. *J. Biol. Chem.* **274**, 16461–16469.
19. Katsani, K. R., Hajibagheri, M. A. & Verrijzer, C. P. (1999). Co-operative DNA binding by GAGA transcription factor requires the conserved BTB/POZ domain and reorganizes promoter topology. *EMBO J.* **18**, 698–708.
20. Mahmoudi, T., Katsani, K. R. & Verrijzer, C. P. (2002). GAGA can mediate enhancer function in trans by linking two separate DNA molecules. *EMBO J.* **21**, 1775–1781.
21. Petrascheck, M., Escher, D., Mahmoudi, T., Verrijzer, C. P., Schaffner, W. & Barberis, A. (2005). DNA looping induced by a transcriptional enhancer *in vivo*. *Nucleic Acids Res.* **33**, 3743–3750.
22. Zollman, S., Godt, D., Prive, G. G., Couderc, J. L. & Laski, F. A. (1994). The BTB domain, found primarily in zinc finger proteins, defines an evolutionarily conserved family that includes several developmentally regulated genes in *Drosophila*. *Proc. Natl Acad. Sci. USA*, **91**, 10717–10721.
23. Bardwell, V. J. & Treisman, R. (1994). The POZ domain: a conserved protein–protein interaction motif. *Genes Dev.* **8**, 1664–1677.
24. Melnikova, L., Juge, F., Gruzdeva, N., Mazur, A., Cavalli, G. & Georgiev, P. (2004). Interaction between the GAGA factor and Mod(mdg4) proteins promotes insulator bypass in *Drosophila*. *Proc. Natl Acad. Sci. USA*, **101**, 14806–14811.
25. Mishra, K., Chopra, V. S., Srinivasan, A. & Mishra, R. K. (2003). *Trl*-GAGA directly interacts with *lola like* and both are part of the repressive complex of *Polycomb* group of genes. *Mech. Dev.* **120**, 681–689.
26. Schwendemann, A. & Lehmann, M. (2002). Pipsqueak and GAGA factor act in concert as partners at homeotic and many other loci. *Proc. Natl Acad. Sci. USA*, **99**, 12883–12888.
27. Ghosh, D., Gerasimova, T. I. & Corces, V. G. (2001). Interactions between the Su(Hw) and Mod(mdg4) proteins required for gypsy insulator function. *EMBO J.* **20**, 2518–2527.
28. Pai, C. Y., Lei, E. P., Ghosh, D. & Corces, V. G. (2004). The centrosomal protein CP190 is a component of the *gypsy* chromatin insulator. *Mol. Cell*, **16**, 737–748.
29. Mohan, M., Bartkuhn, M., Herold, M., Philippen, A., Heinl, N., Bardenhagen, I. *et al.* (2007). The *Drosophila* insulator proteins CTCF and CP190 link enhancer blocking to body patterning. *EMBO J.* **26**, 4203–4214.
30. Gerasimova, T. I., Lei, E. P., Bushey, A. M. & Corces, V. G. (2007). Coordinated control of dCTCF and *gypsy* chromatin insulators in *Drosophila*. *Mol. Cell*, **28**, 761–772.
31. Bushey, A. M., Ramos, E. & Corces, V. G. (2009). Three subclasses of a *Drosophila* insulator show distinct and cell type-specific genomic distributions. *Genes Dev.* **23**, 1338–1350.
32. Matharu, N. K., Hussain, T., Sankaranarayanan, R. & Mishra, R. K. (2010). Vertebrate homologue of *Drosophila* GAGA factor. *J. Mol. Biol.* **400**, 434–447.
33. Pagans, S., Ortiz-Lombardia, M., Espinas, M. L., Bernues, J. & Azorin, F. (2002). The *Drosophila* transcription factor tramtrack (TTK) interacts with trithorax-like (GAGA) and represses GAGA-mediated activation. *Nucleic Acids Res.* **30**, 4406–4413.
34. Faucheux, M., Roignant, J. Y., Netter, S., Charollais, J., Antoniewski, C. & Theodore, L. (2003). *batman* interacts with *Polycomb* and *trithorax* group genes and encodes a BTB/POZ protein that is included in a complex containing GAGA factor. *Mol. Cell. Biol.* **23**, 1181–1195.
35. Kyrchanova, O., Chetverina, D., Maksimenko, O., Kullyev, A. & Georgiev, P. (2008). Orientation-dependent interaction between *Drosophila* insulators is a property of this class of regulatory elements. *Nucleic Acids Res.* **36**, 7019–7028.
36. Kyrchanova, O., Toshchakov, S., Parshikov, A. & Georgiev, P. (2007). Study of the functional interaction between Mcp insulators from the *Drosophila bithorax* complex: effects of insulator pairing on enhancer–promoter communication. *Mol. Cell. Biol.* **27**, 3035–3043.
37. Golic, K. G. & Lindquist, S. (1989). The FLP recombinase of yeast catalyzes site-specific recombination in the *Drosophila* genome. *Cell*, **59**, 499–509.
38. Siegal, M. L. & Hartl, D. L. (2000). Application of Cre/loxP in *Drosophila*. Site-specific recombination and transgene coplacement. *Methods Mol. Biol.* **136**, 487–495.
39. Li, X., Peng, H., Schultz, D. C., Lopez-Guisa, J. M., Rauscher, F. J., III & Marmorstein, R. (1999). Structure–function studies of the BTB/POZ transcriptional repression domain from the promyelocytic leukemia zinc finger oncoprotein. *Cancer Res.* **59**, 5275–5282.
40. Golovnin, A., Biryukova, I., Romanova, O., Silicheva, M., Parshikov, A., Savitskaya, E. *et al.* (2003). An endogenous Su(Hw) insulator separates the *yellow* gene from the *Achaete-scute* gene complex in *Drosophila*. *Development (Camb.)*, **130**, 3249–3258.
41. Chintapalli, V. R., Wang, J. & Dow, J. A. (2007). Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. *Nat. Genet.* **39**, 715–720.
42. Laney, J. D. & Biggin, M. D. (1992). *zeste*, a nonessential gene, potently activates *Ultrabithorax* transcription in the *Drosophila* embryo. *Genes Dev.* **6**, 1531–1541.

43. Orihara, M., Hosono, C., Kojima, T. & Saigo, K. (1999). Identification of *engrailed* promoter elements essential for interactions with a stripe enhancer in *Drosophila* embryos. *Genes Cells*, **4**, 205–218.
44. Ohtsuki, S. & Levine, M. (1998). GAGA mediates the enhancer blocking activity of the *eve* promoter in the *Drosophila* embryo. *Genes Dev.* **12**, 3325–3330.
45. Schweinsberg, S., Hagstrom, K., Gohl, D., Schedl, P., Kumar, R. P., Mishra, R. & Karch, F. (2004). The enhancer-blocking activity of the Fab-7 boundary from the *Drosophila bithorax* complex requires GAGA-factor-binding sites. *Genetics*, **168**, 1371–1384.
46. Belozerov, V. E., Majumder, P., Shen, P. & Cai, H. N. (2003). A novel boundary element may facilitate independent gene regulation in the Antennapedia complex of *Drosophila*. *EMBO J.* **22**, 3113–3121.
47. Agianian, B., Leonard, K., Bonte, E., Van der Zandt, H., Becker, P. B. & Tucker, P. A. (1999). The glutamine-rich domain of the *Drosophila* GAGA factor is necessary for amyloid fibre formation *in vitro*, but not for chromatin remodelling. *J. Mol. Biol.* **285**, 527–544.
48. Karess, R. E. & Rubin, G. M. (1984). Analysis of P transposable element functions in *Drosophila*. *Cell*, **38**, 135–146.