Chapter 3. Interactors of GAGA factor involved in the regulation of bithorax complex
Abstract

Epigenetic inheritance to maintain the expression state of the genome is essential during development. In *Drosophila*, the cis regulatory elements, called the Polycomb Response Elements (PREs) function to mark the epigenetic cellular memory of the corresponding genomic region with the help of PcG and trxG proteins. While the PcG genes code for the repressor proteins, the trxG genes encode activator proteins. The observations that some proteins may function both as PcG and trxG member and that both these group of proteins act upon common cis elements indicate at least a partial functional overlap among these proteins. Trithoraxlike (GAGA factor/GAF) was initially identified as a trxG member but later was shown to be essential for PcG function on several PREs including the *iab-7PRE*. Although first isolated as transcriptional activator of the *Drosophila* homeotic gene Ultrabithorax (*Ubx*), the mechanism of this activation is not known. In order to understand how GAF functions in PcG context (repressive pathway) and also as a trxG (activation pathway), we have looked for the interactors of this protein. We identified *lola like/batman, lola* and *bip2/dmTAF3* as strong interactors of GAF factor in a yeast two-hybrid screen using GAF as bait. *lola! also interacts with polyhomeotic* and, like *Trl*, both *lola, ph* and *lola* are needed for *iab-7PRE* mediated pairing dependent silencing of mini-white transgene. These observations suggest a possible mechanism of how GAF plays a role in maintaining the repressed state of target genes involving *lola!, which may function as a mediator to recruit PcG complexes. On the other hand *bip2/dmTAF3* (also known as dTAF1155) is a component of the TFIID complex in yeast and hence was tested for its role in the activation pathway, we found it to be a part of the activation pathway of GAF. We acquired mutations in *dmTAF3* and showed that, in *Trl* mutant background, they affect transcription of *Ubx* leading to enhancement of *Ubx* phenotype. These results reveal that the gene activation pathway involving GAF is through its direct interaction with dmTAF3.
Chapter 3: Interactors of GAGA factor

3.1 Introduction

During development, differential expression patterns of genes that define cell types once established need to be epigenetically maintained. Specific DNA sequences and the protein complexes working in combination confer this cellular memory. In *Drosophila*, early regulatory network of genes, referred to as segmentation genes, setup the expression pattern of homeotic genes during the first 2–3 h of development. After this initial period, segmentation proteins disappear but the expression pattern of homeotic genes set up early on is maintained by a mechanism that involves the protein product of a number of genes grouped into Polycomb group (PcG) and trithorax group (trxG) of genes. Generally, these two groups of genes function in opposite directions. Mutations in the PcG genes lead to ectopic activation of homeotic genes, which suggests that their normal function is to repress genes. The trxG genes, on the other hand, are the positive regulators of the hox genes (Kennison, 1993; Pirrotta, 1998; Simon, 1995).

Protein products of PcG and trxG genes are recruited to the target loci through common DNA sequences referred to as Polycomb Response Elements, PRE (Chan et al., 1994; Muller and Bienz, 1991; Simon et al., 1993). Although there are no significant sequence similarities among different PREs that have been functionally characterized so far, there is at least one sequence motif that is conserved (Mihaly et al., 1998) which turned out to be the binding site for a PcG member, *pleiohomeotic, pho* (Brown et al., 1998). Among the other sequence motifs that are present in most PREs is the GAGA binding site. We have shown recently that GAGA motifs are necessary for *iab-7PRE* (Mishra et al., 2001). The significance of GAF binding sites in PRE function has been demonstrated by us and others in case of different PRE by showing that GAF protein is a part of the protein complex that is assembled at these cis elements (Busturia et al., 2001; Hagstrom et al., 1997; Hodgson et al., 2001; Horard et al., 2000; Mishra et al., 2001; Strutt et al., 1997). The association with PcG complexes appears to be due to direct interaction of GAF with LOLAL (Faucheux et al., 2003). These observations suggest that multiple sequence motifs, in certain combinations, contribute to PRE function, probably, by recruiting different subsets of proteins that form the chromatin complex at different loci or same locus in different cell type. In addition, GAF also interacts with dSAP18, a member of the dSin3 complex that has histone deacetylase activity (Espinas et al., 2000).
Involvement of GAF in repressive function of PcG proteins, however, raises a paradox as GAF has been identified as an activator protein, an enhancer of PEV and a member of trxG of genes (Biggin and Tjian, 1988; Farkas et al., 1994; Soeller et al., 1993). The GAF factor was first identified as an in vitro transcription activator of Ubx (Biggin and Tjian, 1988). Later, GAF was found to be a positive transcription factor for the engrailed gene and was cloned based on its binding to the sequence GAGAG (Soeller et al., 1993). Independently, the phenotype of mutations of the Trithorax like (Trl) gene suggested it to be a positive regulator of Abd-B, as well as Ubx. Molecular characterization of Trl led to the discovery that it encodes the GAF (Farkas et al., 1994). However, the function of GAF is not restricted to gene-specific transcriptional activation since Trl mutations are dominant enhancers of PEV, indicating that GAF counteracts heterochromatic silencing (Farkas et al., 1994). An anti-trithorax (TRX) antibody immunoprecipitates GAF from nuclear extracts, suggesting that GAF can physically associate with at least one trxG complex (Poux et al., 2002). Interestingly, binding of TRX to a subregion of the bithoraxoid (bxd) regulatory element of Ubx, which has properties of both a maintenance element (Tillib et al., 1999) and a silencer element, is dependent on GAGA sites (Poux et al., 2002). Since TRX does not have a DNA-binding activity on its own, it is likely that GAF, probably together with PSQ, another GAGA binding factor that is a PeG member (Lehmann et al., 1998) (Huang et al., 2002), is required to mediate TRX binding to certain sites within the bxd element, whereas binding to other sites seems to be mediated by, as yet, unidentified proteins (Tillib et al., 1999).

GAF has been implicated in chromatin remodeling and was shown to modify the accessibility of promoters by altering nucleosome positioning (Lehmann, 2004; Tsukiyama et al., 1994). GAF causes nucleosome disruption in an energy-dependent reaction that requires other proteins as well (Tsukiyama et al., 1994; Tsukiyama et al., 1995). The upstream regulatory region of the D. melanogaster hsp26 gene includes two DNase I-hypersensitive sites that encompass the critical heat shock elements. This chromatin structure is required for heat shock-inducible expression and depends on two (CT)n(GA) n elements bound by GAF (Farkas et al., 2000; Lu et al., 1993; Wilkins and Lis, 1997). In transgenic assays, removal of these GAGA binding sites leads to the loss of heat shock-inducible hsp26 expression and drastic reduction of nuclease accessibility in the chromatin of the regulatory region. Chromatin immunoprecipitation experiments
showed that the decrease in TFIID binding did not reduce GAF binding (Leibovitch et al., 2002). In contrast, the loss of GAGA binding resulting from (CT)n mutations decreased TFIID binding. These data suggested that both GAF and TFIID are necessary for formation of the appropriate chromatin structure at the hsp26 promoter and predicted a regulatory mechanism in which GAF binding precedes and contributes to the recruitment of TFIID (Leibovitch et al., 2002). However, the question of how GAF leads to the recruitment of TFIID remains elusive.

In addition to these functions, association of GAF with the GA-rich centric heterochromatin in early embryos (Platero et al., 1998; Raff et al., 1994) indicated why variety of nuclear cleavage cycle defects are displayed by Trl mutants (Bhat et al., 1996). These data and the observation that GAF is required for enhancer blocking activity of chromatin domain boundary elements (Belozerov et al., 2003; Schweinsberg et al., 2004), suggests that GAF is a multifunctional protein that mediates gene-specific regulation but also plays a global role in higher-order chromatin-mediated regulatory mechanisms as well as chromosome function.

How does GAF carryout these apparently opposing functions? It is now emerging that PcG and trxG proteins function on the same cis elements and therefore PRE and TRE may be closely linked or overlapping (Orlando et al., 1998; Strutt et al., 1997; Tillib et al., 1999). The mechanism of this assembly of chromatin structure that is capable of maintaining expression of linked genes is not clear yet. The GAF has been shown to be involved in ATP dependent nucleosome remodeling (Tsukiyama et al., 1994). Recent studies provide a direct link between chromatin remodeling and PcG mediated maintenance of the repressive chromatin (Czermin et al., 2002; Muller et al., 2002). Any direct link between this remodeling and GAF remains to be established. On the contrary, GAF protein was not detected in the ESC-E(Z) protein complex capable of histone H3 lysine 27 methylation which may be one of the steps in remodeling process that ensures nucleosomes stay in repressive chromatin conformation (Muller et al., 2002). There still may, however, be a more dynamic role of GAF in the assembly of the PcG protein complex (Poux et al., 2001a).
3.2 Objectives

Our lab is interested in identifying the factors, which bring about the PRE function. GAF was initially identified as a trxG member but later was shown to be essential for PcG function on several PREs. It also binds to iab-7PRE and is required for its repressive function and has been shown to be required for the function of the Fab-7 boundary.

We decided to investigate the mechanism behind the function of GAF as a component of the repressive PcG by identifying the direct interacting partners of this protein. We used the yeast two-hybrid system to screen a cDNA library made from Drosophila embryos. During this screen we identified lola like (lola) aka batman, lola as one of the strong interactors of GAF. Since lola1 has previously been shown to interact with polyhomeotic, ph-p, a known PcG gene, it is likely that PRE function of GAF is mediated by lola. During this screen we identified dmTAF3 as one of the strong interactors of GAF. Since TAF3 (TBP-associated factor 3) is a component of the TFIID general transcription factor, we tested if the interaction observed in two-hybrid assays has functional significance in the context of the transcriptional activation role of GAF in Drosophila.
lolalike (lolal) / batman (ban)
Figure 3.1 Molecular map showing the domains of GAGA factor, LOLAL and LOLA proteins
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3.3 Results

3.3.1 Characterization of new members of repressive pathway of GAF

3.3.1.1 lolalike (lolal)/batman (ban)

3.3.1.1.1 Results

3.3.1.1.1.1 Identification of lolal as a potential partner for GAF

GAF contains three distinct domains, the BTB/POZ domain, Zn finger domain and a polyQ domain (Figure 3.1) (Farkas et al., 1994). The BTB/POZ domains of several proteins have been reported to mediate homo- and heterodimerizations (Bardwell and Treisman, 1994) and can be functionally swapped between two proteins (Read et al., 2000). We reasoned that GAF may carry out its diverse roles by recruiting different proteins to the target DNA sequences and the BTB domain may mediate this. A yeast two-hybrid screen was carried out using the BTB domain of GAF, amino acids 1-245 (Figure 3.1) as the bait (performed by Krishnaveni Mishra and Arumugam Srinivasan) (Mishra et al., 2003). The 0–16h embryo cDNA library was screened to identify potential partners for GAF protein. Out of the 46 strong interacting partners 14 turned out to be on single gene lolalike/batman (lolal). All the clones had complete coding region of the gene and LOLAL protein also showed interaction with the full length GAF when used as a bait.

To further verify the GAGA–LOLAL interaction a GST pull-down assay was performed by expressing full length LOLAL-GST fusion protein in Escherichia coli. Nuclear extracts were prepared from 0–16h wild-type Drosophila embryos and incubated with either GST or GST–LOLAL. The complexes were pulled down using glutathione Sepharose and tested for the presence of GAF protein by Western blotting (Krishnaveni Mishra and Arumugam Srinivasan) (Mishra et al., 2003). It was found that GAF protein comes down specifically with GST–LOLAL. In the light of yeast two-hybrid interaction studies this assay suggests that GAF and LOLAL interact directly. It has been shown that GAF factor associates with PC, PH and PSC (Poux et al., 2001a; Poux et al., 2001b). Since our results show a direct interaction between LOLAL and GAF, we tested if PC is
Figure 3.2 Interaction of *lolal* with PcG and trxG genes

Panel (A) shows the extra sex comb phenotype, indicated by arrows, in *lolal*<sup>x02512</sup>/Pc<sup>f1</sup> and ph<sup>104</sup>/y; *lolal*<sup>x02512</sup>/+ males. I, II, III indicate anterior, middle and posterior legs. Similar phenotype is seen in Pc<sup>1</sup> mutation background. Panel (B) shows the wing phenotype of *lolal*<sup>x02512</sup>/+ ;Pc<sup>1</sup>/+ flies. All flies of this genotype with two different alleles of Pc showed this defect. Panel (C) shows the rough eye phenotype with irregular margin as found in *lolal*<sup>x02512</sup>/+ ;mor<sup>1</sup>/+ flies. This is a variable phenotype with flies of this genotype showing normal to rough eye defect to varying degree.
also pulled down by GST–LOLAL. It was found that GST–LOLAL does enrich PC (Mishra et al., 2003). The two pull-down experiments suggest that LOLAL is a part of the PcG protein complex. LOLAL could be the adaptor between GAF and PC as the latter two coimmunoprecipitate (Horard et al., 2000). From these experiments it could be concluded that LOLAL interacts with GAF in vitro and exists in a complex that also contains PC.

Earlier reports suggested that lolal enhances the sex comb phenotype of ph (Faucheux et al., 2001). Our results mentioned above suggest that, at least in vitro, LOLAL can be associated with the GAF containing complex that includes PC and other PcG proteins. Hence GAF and PH interaction was tested using the nuclear extracts of 0–16h old embryo and larvae of transgenic flies carrying FLAG-tagged PH (Shao et al., 1999). These were incubated with bacterially produced GST–LOLAL, M2 coupled agarose (Sigma). FLAG-PH bound complex isolated in this manner was analyzed by western blot for the presence of GAF. It was observed that the association of GAF with PH in embryos was dependent on externally provided GST–LOLAL, while in the larval extracts, PH was found to be associated with GAF factor, irrespective of whether or not external GST–LOLAL was added (Mishra et al., 2003).

3.3.1.1.1.2 Genetic interactions of lolal

3.3.1.1.1.2.1 Interaction of lolal with other PcG and trxG genes

The sex comb phenotype of polyhomeotic (ph^{410}) is enhanced by lolal^{802512} and, by this criterion; lolal can be classified as a PcG member. Often members of the PcG interact with one another and also with the trxG members. We tested lolal in different PcG and trxG mutant backgrounds, (Table 3.1). Since most of these mutations are recessive lethal, only the heterozygote combinations, except for ph which is homozygous viable, could be tested. As expected, we found sex combs on the middle leg and often on the third leg in ph^{410}/y; lolal^{802512}/+ males, (Figure 3.2A). We found similar interaction of lolal^{802512} with Polycomb (PcJ) and Polycomblike (Pcl^{71}) (Table 3.1). Interestingly, ph^{410} in combination with lolal^{802512} showed partial homeotic transformation of A4 to A5, A5 to A6 and A6 to A7, (Figure 3.3B). In contrast, Additional sex combs (Asx^{XPF23}) showed
### Table 3.1 Interaction of *lolal* with PcG and trxG genes

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Phenotype</th>
<th>Comments</th>
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<tbody>
<tr>
<td><em>PcI</em>(^{T1})</td>
<td>Sex comb on the 2nd leg</td>
<td>All male flies, sex comb teeth per leg 4 ± 1</td>
</tr>
<tr>
<td></td>
<td>Sex comb on the 3rd leg</td>
<td>All male flies, sex comb teeth per leg 3 ± 1</td>
</tr>
<tr>
<td><em>PcI</em></td>
<td>Sex comb on the 2nd leg</td>
<td>All male flies, sex comb teeth per leg 5 ± 1</td>
</tr>
<tr>
<td></td>
<td>Sex comb on the 3rd leg</td>
<td>All male flies, sex comb teeth per leg 2 ± 1</td>
</tr>
<tr>
<td></td>
<td>Curved wings with posterior margin defect</td>
<td>All flies show the phenotype</td>
</tr>
<tr>
<td><em>ph</em>(^{T10})</td>
<td>Sex comb on the 2nd leg</td>
<td>All male flies, sex comb teeth per leg 3 ± 2</td>
</tr>
<tr>
<td></td>
<td>Sex comb on the 3rd leg</td>
<td>12.5% male flies, sex comb teeth per leg 3 ± 1</td>
</tr>
<tr>
<td></td>
<td>Segmental transformation</td>
<td>All male flies show reduction in 6th segment</td>
</tr>
<tr>
<td><em>Asx</em>(^{NF23})</td>
<td>Bristle on the 6th male sternite</td>
<td>26.3% male flies</td>
</tr>
<tr>
<td><em>mor</em>(^I)</td>
<td>Rough eye (In females)</td>
<td>This phenotype is quite variable with flies showing very mild to strong defects</td>
</tr>
<tr>
<td><em>trg</em></td>
<td>Segmental transformation</td>
<td>69.2% males show partial A7 to A6 transformation (47% males show this phenotype when <em>trg</em> is brought paternally)</td>
</tr>
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\(^{a}\) *Psc*\(^I\), *Scm*\(^{B5.13B}\), *ashl*\(^{B1}\), *trx*\(^{ed}\), *osa*\(^{60099}\), *pho*\(^{CV}\) and *Trl*\(^{13C}\) did not show any interaction in double heterozygous conditions based on such clear visible phenotypic criteria.

\(^{b}\) Phenotype shown is the most prominent one seen with the corresponding allele in *lolal*\(^+/\) background. Phenotype reported for *Asx*\(^{NF23}\), *PcI*\(^{T1}\) and *ph* were seen when the PcG mutation was brought maternally. In case of *PcI*, the *PcI*\(^+/\) + */CyO* siblings also showed 5 ± 1 sex comb teeth per 2nd leg, while this genotype did not show any sex comb teeth on the 3rd leg.
Figure 3.3  Genetic interactions of *lolal* with PeG and trxG mutations

Cuticular structures of the adult male abdomen. Anterior is top and posterior is bottom. The abdomen was split mid-dorsally and flattened. Cuticle preparation of differential mutant combinations with *lolal* is shown. (A) is wild-type fly; (B) is *ph*^410/*ph*^410; *lolal*^B02512/+* showing strong reduction in the size of 6th tergite (indicated by black arrowhead) and additional pigmentation of the 4th tergite (indicated by white arrow). (C) is *lolal*^B02512/+; *Asx*^XP23Jj/+ abdomen with few additional bristles on the 6th sternite (indicated by arrow), wild-type male cuticle is devoid of bristles in this region. We almost always found two bristles in these flies. The rest of the cuticle appears normal. (D) is *lolal*^B02512/+;*trg/* cuticle preparation. These flies show development of a small tergite (indicated by arrowheads). These preparations have several bristles in the 6th sternite, which is not due to *lolal* as these bristles are present in similar numbers in *trg/* + flies.
partial A6 to A5 transformation in the abdomen, (Figure 3.3C). \(Pc^I\) in combination with \(lolal^{k02512}\) also gave a wing phenotype (Figure 3.3B). All \(lolal^{k02512}/+;\; Pc^I/+\) flies had crumbled wings with defective margin. Among the trxG mutations tested, \(moira\; (mor^I)\) had phenotype of rough eye (Figure 3.2C) and \(trithoraxgleich\; (trg)\) showed homeotic transformation in the abdomen (Figure 3.3D). While \(trg/+\) flies have several bristles on the 6th sternite in males, in the combination of \(lolal^{k02512}\), additional segment was formed (shown by arrow mark in Figure 3.3D) indicating partial anteriorization, transformation of A7 to A6. Contrary to the expectations based on the biochemical studies discussed above, any apparent interaction of \(lolal^{k02512}\) with \(Trl\; 13c\) in heterozygous condition was not observed. Similarly, among the mutations that we tested, \(osa^{00090}\; ,\; trithorax\; (trx^red)\), \(absent\; small\; homeotic\; 1\; (ash1)\), \(Posterior\; sex\; combs\; (Psc^I)\), \(Sex\; combs\; multiple\; (Scm^{85.13b})\) did not show any interaction with \(lolal^{k02512}\). Interestingly, it has been shown recently that \(Trl\) also enhances \(ph\) phenotype in a fashion similar to what we see in case of \(lolal\) and \(ph\) (Hodgson et al., 2001). This raises the possibility that there may be a networking of \(Trl-lolal-ph\) and, perhaps, other PcG proteins in the PRE mediated repression mechanisms. These interactions of \(lolal\) with \(ph\) and \(Pc\) suggest that association of \(lolal\) may be the key step of PcG function of GAF.

3.3.1.1.1.2.2 Pairing dependent silencing of iab-7PRE is dependent on lolal and ph

The pairing-dependent silencing assay (PDSA) is widely used for checking the PRE nature of a given sequence. Previous studies have shown that mutations in PcG genes relieve this pairing-dependent silencing (Chan et al., 1994; Gindhart, Jr. and Kaufman, 1995; Hagstrom et al., 1997; Kassis, 1994). Earlier studies from our lab had already shown that transgenic lines carrying \(iab-7\; PRE\) next to \(mini-white\) reporter, \(PRE-mw\) (\(iab-7PRE-mw\)), show pairing dependent silencing of the \(mini-white\) and that like several PcG mutations, this silencing was affected by \(Trl\) mutations (Hagstrom et al., 1997; Mishra et al., 2001). The PRE-mw lines were used to test if \(lolal\) is required for the PS function. Since \(lolal\) was shown to genetically interact with \(ph\) (Faucheux et al., 2001) as well as in GST pull down in our lab, \(ph\) was also checked in this PS assay. In several transgenic lines, \(iab-7PRE-mw\; /\; iab-7PRE-mw\) flies show lighter eye color as compared to the \(iab-7PRE-mw\; /+\) flies (Mishra et al., 2001). All the lines tested showed clear requirement of both \(lolal\) and \(ph^{410}\) for the PS function, as the eye color of \(iab-7PRE-mw\)
Figure 3.4 Effect of lolal and ph on the pairing dependent silencing of iab-7PRE-mini white (PRE-mw) transgenic lines

Eye color of female flies of similar age is compared. The eye color of PRE-mw homozygous flies in wild-type background is always lighter than those in lolal^{K0212}/CyO (A) or ph^{410}/ph^{410} (B) background.
/iab-7PRE-mw flies showed clear enhancement when brought in lolal (ban^{Df(2R)311a}) or ph^{410} (ph-p) background (Figure 3.4).

3.3.1.1.2 Discussion

Chromatin organization is a vital component of the mechanism regulating gene expression in eukaryotes. The molecular mechanisms of how these regulatory processes take place, how active and inactive regions of chromatin are established during development and maintained through a number of cell divisions, is still far from understood. In Drosophila, extensive genetic and biochemical studies on the homeotic gene regulation have implicated PcG and trxG group of genes in the maintenance of expressed state at the level of higher order chromatin organization (Pirrotta, 1997; Simon, 1995). It is emerging that high molecular weight complexes consisting of few 'core' PcG proteins and other variable components exist (Chopra and Mishra, 2005; Hodgson et al., 2001; Horard et al., 2000; Poux et al., 2001a; Saurin et al., 2001; Simon et al., 1993). The variable members may determine stage specific or locus specific function. For example, pho has been shown to be more crucial for late larval and pupal stages as compared to the early embryonic development (Poux et al., 2001a).

One of the key steps in the PcG/trxG mediated maintenance is the recruitment of the multi-protein complex of correct composition onto the PRE. Recent studies have shown that more than one or perhaps several recruiting processes take place in concert. It is likely that different recruitment possibilities provide the necessary variation that is needed for the establishment and maintenance of varying transcriptional states at hundreds of different loci. Our studies identify a new member in this process. GAF bound to specific sites on the PREs recruits LOLAL, which in turn, through direct or indirect means, incorporates PH into the complex. This raises a question whether the other recruiting agents like pho, zeste, etc. (Americo et al., 2002; Brown et al., 1998) function in cooperation or competition with each other. Also, it is not clear if the complex is assembled de novo on the PREs, a pre-assembled complex is recruited or partly assembled sub-complexes are recruited. Since large complexes of PcG proteins can be isolated, we can conclude that such structures, once assembled are stable. It is not
clear though if these complexes are stable during cell division or they assemble each time a cell divides.

loal was originally identified in the Drosophila gene disruption project and named so because of similarity to lola (longitudinals lacking) (Spradling et al., 1999). Later on this mutation was also found to enhance the homeotic phenotype of polyhomeotic, ph, and renamed as batman (Faucheux et al., 2001). loal encodes a protein of 127 amino acids that contains a BTB domain of about 90 amino acids, leaving only few residues at both ends of the protein for any other functional motif/domain. (Simon et al., 1995) Unlike multi-domain proteins, the ones made of a single domain alone may function as adaptor modules to bring together two different molecules/complexes. GAF is known to activate transcription of several genes. In this context, loal may function to inhibit this activation role of GAF; further studies will be required to differentiate between these mechanisms.

As most of the PREs contain GAF binding sites, it is likely that at least some PcG complexes are recruited by GAF through its direct interaction with LOLAL. These findings establish a molecular link between GAF and the PcG complex. Since GAF functions in several other processes that do not seem to be directly linked to the PRE function, it is likely that there are several interactors of GAF. We are studying such interactors and initial observations suggest that a large number of proteins can interact with GAF with a potential to target a repressive or activator function to different loci. It is not clear though, how the selection of appropriate partner is made. Is it in the context in which GAF is bound or different heterodimers pre-exist in the nucleus and these are then recruited to appropriate loci? New assays will have to be designed to appropriately answer these questions. The genetic interaction studies (Table 3.1) show that loal interacts with a variety of PcG and trxG mutations (Figure 3.3 and 3.4). This underscores the important role of this protein in the regulation of developmental genes. Interestingly, we find that loal interactions with ph mutation leads to transformation of 2nd (and some times 3rd) leg to 1st leg, an apparent anteriorization type of homeotic transformation in thoracic but in the abdominal region same combination leads to posteriorization type of homeotic transformation, pigmentation of A4 (A4 to A5) reduction in the size of A6 (A6 to A7). However, appearance of sex comb in 2nd and 3rd legs is also known to be due to derepression of Scr in posterior segments thereby explaining this phenotype as due to
loss of the repression function of the PcG proteins. Furthermore, trxG and PcG mutations upon interaction with lolal can give a similar phenotype. In lolal context, Asx and trg both show partial A6 to A5 transformation in abdominal region. Pc is involved in pairing dependent silencing complex recruited by iab-7 PRE. In this study we show that ph is also involved in the PS function of iab-7 PRE. While it was known that lolal enhances the homeotic phenotype of ph, we demonstrate that both ph and lolal are involved in establishing the repressive complex at the iab-7 PRE. This indicates that lolal and ph function in coordination to set up a repressive complex.

Taken together, these results suggest that lolal may be acting along with GAF or with other partners in different complexes in a locus or stage specific manner. Depending on the context it could be an activator or repressor function. Since not only ‘ON’ or ‘OFF’ but also several ‘levels of expression states’ for a given hox gene or indeed other regulated loci are maintained, it is likely that a unique combination of trxG and PcG proteins may be needed for each varying levels of expression state of a given locus. Affinity pull-down experiments (Mishra et al., 2003), show that GAF, LOLAL, PH and PC proteins coexist in a complex. This is also in agreement with the genetic interaction studies, where we found interaction of lolal with ph and Pc. Genetic and biochemical studies also suggest that, like Trl, lolal could also be not specifying the kind of complex to be assembled. It is likely, therefore, that specificity of the GAF partner, PcG or trxG member, does not come from the lolal. It is even possible that lolal could also be a multifunctional adapter of GAF in assembling multi-protein complexes. The specificity may come from yet another factor or even from the transcriptional activity around the locus. Recent observations that transcription process itself may contribute to the cellular memory may support this view (Bender and Fitzgerald, 2002; Hogga and Karch, 2002; Rank et al., 2002). This might bring together the ability of GAF to support transcription and recruitment of multi-protein complexes and nucleosome remodeling activity in one mechanistic context. GAF has been suggested to be involved in creating a nucleosome free region (Hagstrom et al., 1997). The first step in establishing a PcG complex may be through GAF mediated nucleosome remodeling of the chromatin on the PRE region to create more accessibility for other proteins. The recruitment of a protein complex to the accessible region may take place through GAF or by other factors that can anchor the complex onto DNA. As the next step LOLAL could mediate recruitment of initial
complex, for example, ESC-E(Z) protein complex, which modifies nearby histone tails to covalently mark the region for the recruitment of another complex, like PRC1. Consistent histone modifications and remodeling may be needed to maintain the chromatin conformation. Our studies would place LOLAL as the factor binding to the DNA bound GAF even when the rest of the complex is not recruited and therefore help in subsequent recruitment steps. In this context, the exact function of proteins like LOLAL becomes very important. Further studies will be required to clarify these issues.

3.3.1.1.3 Summary

- LOLAL directly interacts with GAF
- lolal is a new PcG member as it enhances PcG phenotypes
- lolal disrupts pairing dependent silencing and hence is a part of the repressive complex at iab-7PRE
- Ph is also a part of the iab-7PRE repressive complex as it disrupts pairing dependent silencing
- LOLAL may function as an adaptor protein and help recruit other PcG members to the PRE bound GAF
longitudinals lacking (lola)
3.3.1.2 longitudinals lacking (lola)

3.3.1.2.1 Results

3.3.1.2.1.1 Identification of LOLA as an interacting partner for GAF

LOLA was obtained in a yeast two-hybrid screen using GAF as bait as described in section 4.3.1.1.1.1. The direct interaction was further confirmed by GST pull down assays as described in section 4.3.1.1.1.2. lola encodes a transcription factor required for axon growth and guidance in the embryo (Giniger et al., 1994). LOLA contains a BTB/POZ domain and a Zn finger domain. Through its Zn finger domain it can bind to DNA and via its BTB/POZ domain it can interact with other proteins. lola mutant lack longitudinal connectives of embryonic ventral nerve cord (Seeger et al., 1993). In such mutant embryos growth cones, that normally pioneer longitudinal pathways, initially extend but then stall, thereby, lacking most longitudinal axon pathways (Giniger et al., 1994). lola encodes twenty five mRNAs that generate nineteen different proteins. All the isoforms share four exons that encode a common amino terminus, which contains the BTB domain. In addition, all but one of these transcription factors are spliced to unique exons encoding one or a pair of zinc-finger motifs (Giniger et al., 1994; Ohsako et al., 2003).

Since we observed LOLA and GAF interaction, we explored the possibility of this mutation behaving as PcG member. There are many genes that have been shown to encode BTB domain containing proteins and most of them behave as transcriptional repressors. This BTB subfamily of transcriptional repressors includes the human oncogenes BCL6 and PLZF. In these oncogenes, the BTB domain is crucial for oncogenesis through the recruitment of PcG and HDAC complexes (Barna et al., 2002; Melnick et al., 2002).
Figure 3.5  Interaction of *lola* with PcG genes

The figure shows the extra sex comb phenotype, indicated by arrows, in *Pc^{T1}/lola^{00642}* and *lola^{00642}/ + ; Pc^{+}/+* males. T1, T2 and T3 legs indicate anterior, middle and posterior legs. In the wild type flies the T2 and T3 legs lack presence of sex combs but in *Pc* and *PcI* sex combs are seen on T2 legs (arrowheads). This phenotype is enhanced in *lola^{00642}* background as seen by the appearance of more sex combs on T2 and T3 legs.
3.3.1.2.1.2 Genetic interactions of lola

3.3.1.2.1.2.1 Interaction of lola with other PcG and trxG genes

Often members of PcG interact with one another and also with the trxG members (Ali and Bender, 2004). lola was tested in different PcG and trxG mutant backgrounds, Table 3.2. Since most of these mutations are homozygous lethal, only the heterozygote combinations could be tested. As expected, sex combs on the 2nd and 3rd pair of legs in lola00642/+; Pc1l+/ males was found (Figure 3.5B, Table 3.2). Similar presence of sex combs on 2nd and 3rd legs in lola00642/+; Pc2l/+ and lola00642/+; Pc1l/+ male fly was found (Figure 3.5C, Table 3.2). In addition to the sex comb phenotype an uneven wing margin phenotype in lola00642/+; Pc12058l/+ was observed. The enhancement of sex comb phenotype clearly demonstrates that lola is indeed a new PcG member. Contrary to the expectations based on the biochemical studies discussed above, any apparent interaction of lola00642 with Trl13c and TrlR85 in heterozygous conditions was not seen. Among the mutations that we tested Psc1, ScmR5.13B, E(z)S1, lola, Su(Z)2, brm, ese2, ash1 B1, trx red, osa00090, pho CV did not show any genetic interaction with lola00642.

3.3.1.2.1.2.2 Pairing dependent silencing caused by iab-7PRE is dependent on lola

As described earlier PDSA is widely used for checking the PRE nature of a given sequence and previous studies have shown that mutations in PcG genes relieve this pairing-dependent silencing (Chan et al., 1994; Gindhart, Jr. and Kaufman, 1995; Hagstrom et al., 1997; Kassis, 1994). Earlier studies from our lab had already shown that transgenic lines carrying iab-7 PRE next to mini-white reporter, PRE-mw, show pairing dependent silencing of the mini-white and that like several PcG mutations, this silencing was affected by Trl mutations (Hagstrom et al., 1997; Mishra et al., 2001). From the above genetic interaction screen it was clear that lola is a new PcG member and hence iab-7PRE-mw lines was used to test if lola is required for the PS function. In several transgenic lines, iab-7PRE-mw / iab-7PRE-mw flies show lighter eye color as compared to the iab-7PRE-mw / + flies (Mishra et al., 2001). Two different lines containing iab-7PRE-mw (WT44 and WT45) were tested. Both the lines showed clear requirement of
Table 3.2 Interaction of \textit{lola} with PcG and trxG genes$^a$

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Phenotype$^b$</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>$PcI^T$</td>
<td>Sex comb on the 2nd leg</td>
<td>All male flies, sex comb teeth per leg 4 ± 1</td>
</tr>
<tr>
<td></td>
<td>Sex comb on the 3rd leg</td>
<td>All male flies, sex comb teeth per leg 3 ± 1</td>
</tr>
<tr>
<td>$Pc^I$</td>
<td>Sex comb on the 2nd leg</td>
<td>All male flies, sex comb teeth per leg 5 ± 1</td>
</tr>
<tr>
<td></td>
<td>Sex comb on the 3rd leg</td>
<td>38% of male flies, sex comb teeth per leg 4 ± 2</td>
</tr>
<tr>
<td>$Pc^2$</td>
<td>Sex comb on the 2nd leg</td>
<td>25% of male flies, sex comb teeth per leg 5 ± 1</td>
</tr>
<tr>
<td></td>
<td>Sex comb on the 3rd leg</td>
<td>12.12% of male flies, sex comb teeth per leg 3 ± 1</td>
</tr>
<tr>
<td>$PcI^{2038}$</td>
<td>wing margin not even</td>
<td>All flies show this phenotype</td>
</tr>
</tbody>
</table>

$^a$ $Psc^I$, $Scm^{R5;13B}$, $E(z)S^2$, \textit{lola}, $Su(Z)2^*$, $brm$, $esc^2$, $ashI^{Bl}$, $trx^{red}$, $osa^{00090}$ $pho^{CV}$ and $Trl^{R85}$ did not show any interaction in double heterozygous conditions based on such clear visible phenotypic criteria.

$^b$ Phenotype shown is the most prominent one seen with the corresponding allele in \textit{lola}^{00642}/+ background. Phenotype reported for $PcI$ and $Pc$ were seen when the PcG mutation was brought maternally. In case of $Pc^I$ and $Pc^2$, the +/\textit{CyO}; $Pc^I$/+ siblings also showed 5 ± 1 sex comb teeth per 2nd leg, while this genotype did not show any sex comb teeth on the 3rd leg.
Figure 3.6  *lola* disrupts pairing dependent silencing of *iab-7PRE*

Effect of *lola* on the pairing dependent silencing of two different *iab-7PRE* -mini white (*PRE-mw*) transgenic lines 1) WT 44, 2) WT 45. Eye color of female flies of similar age is compared. The eye color of *PRE-mw* homozygous flies in wild-type background is always lighter than those in *lola<sup>00642</sup>/CyO* background.
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*lola* for the PS function, as the eye color of *iab-7PRE-mw / iab-7PRE-mw* flies showed enhancement when brought in *lola*<sup>00642</sup> background, (Figure 3.6). This shows that *lola* disrupts pairing dependent silencing and is required for the repressive property of the *iab-7PRE*.

### 3.3.1.2.2 Discussion

Chromatin organization is mandatory for gene expression in eukaryotes as the DNA is bound to histones and they have to be modified to “open” or “close” the DNA for gene expression or repression, respectively. The molecular mechanisms of how these regulatory processes take place – how active and inactive regions of chromatin are established during development and maintained through a number of cell divisions – is still far from understood. In *Drosophila*, extensive genetic and biochemical studies on the homeotic gene regulation have implicated PcG and trxG group of genes in the maintenance of expressed state at the level of higher order chromatin organization (Pirrotta, 1997; Simon, 1995). It is emerging that high molecular weight complexes consisting of few ‘core’ PcG proteins and other variable components exist (Hodgson et al., 2001; Horard et al., 2000; Poux et al., 2001a; Saurin et al., 2001; Simon et al., 1993). The variable members may determine stage specific or locus specific function. For example, *pho* has been shown to be more crucial for late larval and pupal stages as compared to the early embryonic development (Poux et al., 2001a). One of the key steps in the PcG/trxG mediated maintenance is the process of recruitment of the multi-protein complex of correct composition onto the PRE. Recent studies have shown that more than one or perhaps several recruiting processes take place simultaneously. It is probable that different recruitment possibilities or modes provide the necessary variation that is needed for the establishment and maintenance of varying transcriptional states at numerous loci.

These studies have identified a new member, LOLA, involved in this repressive process mediated by PcG members. LOLA has a DNA binding Zn finger domain apart from a BTB/POZ domain. The BTB domain containing proteins are generally known to behave as transcriptional repressors (Melnick et al., 2002). GAF can bind to *iab-7PRE* and recruit LOLA, which in turn, through direct or indirect means, incorporates other PcG members of the complex. An alternate mechanism to explain this interaction is that
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LOLA binds to the *iab-7PRE*, via its Zn finger domain, independent of GAF, and then interacts with GAF and other PcG members to form repressive complex. Hence it is a new addition to the repertoire of DNA binding PcG proteins that include GAF, PHO, ZESTE, PHOL, PSQ. This raises a question whether the other recruiting agents like PHO, ZESTE, etc., (Americo et al., 2002; Brown et al., 1998) function in cooperation or in competition with each other. Also, it is not clear if the complex is assembled *de novo* on the PREs, a pre-assembled complex is recruited or partly assembled sub-complexes are recruited. Since large complexes of PcG proteins can be isolated, we can conclude that such structures, once assembled are stable. It is not clear though if these complexes are stable during cell division or they assemble each time a cell divides. Recently, *lola* and *pipsqueak* (*psq*), another BTB domain containing protein that binds to GAGA sequences, have been implicated to have role in tumorigenesis (Ferres-Marco et al., 2006). It has been shown that when both *lola* and *psq* genes are over expressed in a sensitized background (i.e., in over expressed *Delta (Dl)* gene belonging to the Notch pathway) induced the formation of metastatic tumors in *Drosophila*. In the same study it was observed that the aberrant silencing of tumor suppressor genes, like *Retinoblastoma family (Rbf)*, occur as a result of over expression of *lola* and *psq* leading to increase in H3K27 and H3K9 methylation which is the hallmark of epigenetic gene silencing. PSQ-LOLA proteins could bind to the silenced genes and enforce silencing by recruitment of HDAC or PcG repressors. The study classified *lola and psq* to be "epigenetic silencers" as they behaved as PcG members and could enhance the sex comb phenotype of Pc mutants (Ferres-Marco et al., 2006). It is also to be noted that the BTB/POZ domain containing proteins are conserved in humans too and many of them have been implicated to bring about many developmental roles (Bardwell and Treisman, 1994).

It is known that most of the PREs contain GAGA binding sites (Mishra et al., 2001) and it is likely that some PcG complexes could be recruited by GAF through its direct interaction with LOLA, or LOLA could bind and interact with GAF and then recruit other PcG members. This finding establishes a molecular link between GAF and the PcG complex and elucidates new interacting partners of GAF in its repressive role. Since GAF functions in several other processes that do not seem to be directly linked to the PRE function, it is likely that there are several interactors of GAF. A large number of proteins can interact with GAF with a potential to target a repressive or activator role.
function to different loci. It is not clear though how the selection of appropriate partner is made. New assays will have to be designed to appropriately answer these questions.

The genetic interaction studies (Table 3.2) show that lola interacts with a variety of PcG and trxG mutations (Figure 3.5). This highlights the important role of this protein in the regulation of developmental genes. We find that lola interacts with Pc and Pcl mutations giving rise to transformation of 2nd and 3rd leg to 1st leg, likely due to derepression of Scr in posterior segments. Furthermore, trxG and PcG mutations upon interaction with lola can give a similar phenotype. It is known that GAF is involved in pairing dependent silencing complex recruited by iab-7 PRE (Mishra et al., 2001). In this study we show that lola is also involved in the PS function of iab-7 PRE (Figure 3.6). This indicates that lola and Trl together function to set up a repressive complex at the iab-7PRE. Taken together, these results suggest that lola may be acting along with GAF or with other partners in different complexes in a locus or a stage specific manner. GAF can function as an activator or repressor depending on the context of its interacting partners. Not only ‘ON’ or ‘OFF’ but also several ‘levels of expression states’ for a given hox gene or indeed other regulated loci are maintained, it is likely that a unique combination of trxG and PcG proteins may be needed for each varying levels of expression state of a given locus.

We know that GAF has been suggested to be involved in creating a nucleosome free region (Hagstrom et al., 1997) so it could bind to the PREs making it accessible to other PcG proteins for repression. The recruitment of a protein complex to the accessible region may take place through GAF or by LOLA itself via its BTB/POZ domain. As the next step LOLA could mediate recruitment of initial complex, for example, ESC-E(Z) protein complex, which modifies nearby histone tails to covalently mark the region for the recruitment of another complex, like PRC1. Consistent histone modifications and remodeling may be needed to maintain the chromatin conformation. This study would place LOLA as the factor binding to the DNA bound GAF or even binding to DNA and then interacting with GAF, even when the rest of the complex is not recruited and therefore help in subsequent recruitment steps. In this context, the exact function of proteins like LOLA becomes very important.
a) Repressive role of GAF: GAF can bind to \textit{iab-7PRE} (6) and interact with LOLAL (6) as well as LOLA. LOLA can also bind to \textit{iab-7PRE} (9) and interact with GAF via its BTB domain. LOLA can also first bind to PRE and then interact with GAF. LOLA functions as an adaptor protein to recruit other proteins like PH (6).

b) Model of PRC1 targeting to \textit{iab-7PRE}:

For simplicity, similar DNA binding proteins are circled (dotted lines) and treated as single targeting domains. Cooperative interactions between DNA binding proteins and subunits of PRC1 are indicated by arrows. These interactions are described in the text and in references 1-5. Based on the available data, the interaction of GAF with PRC1 subunits requires the adaptor proteins LOLAL and CORTO (10, 4). This repressive complex is now termed as "Polycombiosome" (7,8).

References:
9) Pirrotta V. (unpublished observations)
3.3.1.2.3 Summary

- LOLA directly interacts with GAF
- \textit{lola} is a new PcG member as it enhances PcG phenotypes
- \textit{lola} disrupts pairing dependent silencing and hence is a part of the repressive complex at \textit{iab-7PRE}

3.3.1.3 Conclusions

The study provides new insights on the interacting partners of GAF required for its repressive role at the \textit{iab-7PRE} locus. LOLAL and LOLA both posses BTB domain that is also present in GAF and is involved in protein-protein interactions. LOLAL can function as an adaptor protein in recruiting other PcG complexes, as it possesses only the BTB domain. LOLA, on the other hand, can bind to DNA directly (unpublished results V. Pirrotta), and then interact with GAF and recruit other PcG complex proteins and bring about silencing of the target gene at the \textit{iab-7PRE} locus (Figure 3.7 a). There might be another possibility that LOLA can interact with GAF and then bind to the PRE. Essentially, the outcome of binding is to increase co-operativity among PcG members and bring about silencing of target gene. The PcG repressive complexes have been coined a new term called as “Polycombiosome” (Blastyak et al., 2006; Mohd-Sarip et al., 2005) (Figure 3.7b) as there are distinct kind of complexes which modify histones (PRC2) and creates binding sites for the other set of PcG complex (PRC1) to bind and lead to formation of inaccessible chromatin structure (Ringrose and Paro, 2004). In this complex LOLA as well as LOLAL are important components along with GAF. The PcG members binding to \textit{iab-7PRE} are PHO, DSP1, GAF, PSQ, LOLA and GRH and all these DNA binding PcG members recruit repressive complex and bring about silencing of the target gene. Members like LOLAL and CORTO mediate this recruitment as they have been shown to interact with PRC1 components and DNA binding components.
Brìc à brac Interacting Protein 2
(bip2/dmTAF3)
Figure 3.8  Structure of GAGA factor and dmTAF3 proteins
3.3.2 Characterization of a new member of activation pathway of GAF

3.3.2.2 Bric à brac Interacting Protein 2 (bip2/\textit{dmTAF3})

3.3.2.2.1 Results

3.3.2.2.1.1 Identification of \textit{dmTAF3} as an interacting partner of GAF

GAF can act both as an activator and repressor of transcription. In order to understand the mechanism of these diverse functions, we identified the proteins that interact with GAF. The N-terminal 245 amino acids of GAF, which contain the BTB domain was used in the Y2H screen and interacting proteins were isolated (as described in section 3.3.1.1.1.1) (Mishra et al., 2003). Out of the 45 clones that passed the stringent tests for true interactions, two identical clones containing the middle region of a gene known as \textit{dmTAF3} or \textit{bip2} was recovered (Figure 3.7). \textit{bip2} was first identified as \textit{Bric à brac Interacting Protein 2}, in a two-hybrid screen for interacting partners of \textit{Bric à brac 1} and \textit{2} (Pointud et al., 2001) and demonstrated to be dTAF_{II}155, the \textit{Drosophila} homologue of yeast TAF_{II}47 (Gangloff et al., 2001). TAF_{II}S are TATA binding protein (TBP) associated proteins that comprise TFIID, one of the general factors required for initiation of transcription by RNA polymerase II. TAFIIIs are thought to contribute to TFIID function through contacts with other transcription factors, histones and or DNA (Chen and Hampsey, 2002). From here on, \textit{dmTAF3} will be used instead of \textit{bip2}, according to the unified nomenclature proposed by (Tora, 2002). The \textit{dmTAF3} polypeptide contains a histone fold domain (HFD) at the N-terminus (aa 1 – 75) and a plant homeo domain (PHD) (aa 1342 – 1392) at the C terminus (Pointud et al., 2001). The 1.8-kb insert of our clones interacting with GAF contained amino acids 480 to 1073 of \textit{dmTAF3} (Figure 3.8).

To confirm that the interaction of GAF with \textit{dmTAF3} is specific, a GST pull down experiment was carried out in our lab (Krishnaveni Mishra and Arumugam Srinivasan, unpublished data) and indeed it was found that GAF and \textit{dmTAF3} directly interact. A part of \textit{dmTAF3} protein was expressed in \textit{E. coli} as a GST-fusion protein and incubated with \textit{Drosophila} nuclear extracts. We found that GAF was specifically pulled down by GST-\textit{dmTAF3} but not by GST alone. Furthermore, an abundant nuclear protein, HP-1,
**Figure 3.9** Haltere to wing transformation is enhanced in *dmTAF3* mutant background

The *Trl^{R85/TM2}, Ubx* flies show haltere to wing transformation (a), which is enhanced in *dmTAF* background (*Trl^{R85/TM2}, Ubx; dmTAF3^{F0a+}/dmTAF3^{F0a-}* (b).

**Figure 3.10** UBX levels in third instar larvae imaginal discs in different mutant backgrounds

The normal levels of UBX as seen in the peripodial cells of the WT wing (a). The levels of UBX decrease in *Trl^{R85/TM2}, Ubx* (b) and *dmTAF3^{F0a+}/dmTAF3^{F0a-}* (c) wing imaginal discs. Further decreased staining of UBX is seen in the case of *Trl^{R85/TM2}, Ubx; dmTAF3^{F0a+}/dmTAF3^{F0a-}* disc (c). The images were taken at constant exposure and magnification.
did not interact with GST-dmTAF3, indicating that the interaction with GAF was specific. The GST-dmTAF3 fusion protein contained amino acids 612 to 1073, which overlaps with the region that was found to interact with BAB1 and 2 (aa 757 to 1091) (Pointud et al., 2001). This region of dmTAF3 may therefore interact with specific BTB-containing polypeptides and may link transcriptional activators to RNA Pol II (K. Mishra and A. Srinivasan, unpublished data). It was subsequently found that in immunoprecipitation experiments GAF and dmTAF3 interact in vivo (A. Srinivasan and R.K. Mishra, unpublished data).

3.3.2.1.1.2 dmTAF3 and Trl enhance the Ubx phenotype

We used the homozygous viable dmTAF3Fa allele, which was known to have reduced levels of dmTAF3 transcripts, for its interaction with GAF. GAF is known to be an activator of Ubx transcription and mutations in Trl gene dominantly enhance the phenotype of Ubx heterozygous flies - haltere to wing transformation (i.e., T3 to T2) (Farkas et al., 1994). If dmTAF3 is required for GAF-mediated transcriptional activation of Ubx, then in the sensitized genetic background of doubly heterozygous Ubx and Trl flies, mutations in dmTAF3 should enhance the haltere to wing transformation phenotype. Haltere to wing transformation in the case of TrlR85/TM2, Ubx background was observed in only ~3% of the flies (Figure 3.9a, upper panel). This frequency of transformation remained, but the severity of the transformation was remarkably increased in mutant dmTAF3 context (TrlR85/TM2, Ubx; dmTAF3Fa4/dmTAF3Fa4) (Figure 3.8b, lower panel). In particular, we have never observed the three strongest transformations shown in Figure 3.9b, lower panel in flies bearing wild-type dmTAF3 alleles. This transformation phenotype being an indication of insufficiency of Ubx activity, our observation firmly establishes a role of dmTAF3, together with GAF, in the maintenance of Ubx gene expression in vivo.

3.3.2.1.1.3 UBX levels are reduced in Trl and dmTAF3 double mutant context

We also directly checked UBX levels in different mutant combinations. The amount of UBX protein was detected by anti-UBX antibody staining of the wild type and mutant imaginal discs. UBX staining is seen in the peripodial membrane cells of the wing discs
Figure 3.11  *Ubx-lacZ* transgene is repressed in *Trl* and *dmTAF3* mutation backgrounds

The activity staining of the *TM3, Ubx-lacZ (Blue Balancer)* line acts as a read out of the dependence of transcription factors binding to the UCR elements of *Ubx* Gene. The X-Gal staining reveals the expression of the transgene in wing (a) haltere (b) and leg (c) imaginal discs in the transgenic line [*TM3, Ubx lacZ / +*]. The X-Gal staining of the *TM3, Ubx lacZ* is abolished completely from the wing disc in *dmTAF3^{fla401/}
dmTAF3^{fla401}* (d) and *Trl^{R85}* (g) mutant backgrounds. While X-Gal staining is lost from the anterior compartments of the haltere and leg discs in *dmTAF3^{fla401/}
dmTAF3^{fla401}* (e,f) and *Trl^{R85}* (h, i) backgrounds respectively. The double mutant imaginal disc [*Trl^{R85}/TM3 Ubx lacZ, Ubx: dmTAF3^{fla401/}
dmTAF3^{fla401}*] show severely reduced staining of *lacZ* in posterior compartment of haltere (k) and leg (l) imaginal discs.
and everywhere in the haltere disc (Brower, 1987). The endogenous level of UBX was estimated in different mutant combinations using anti-UBX antibody staining of the wild type and mutant imaginal discs from third instar larvae. In wing imaginal discs, UBX staining is seen in the peripodial membrane cells (Figure 3.10a). A decrease in UBX staining in TrlR85/TM2, Ubx (Figure 3.10b) background was observed. In dmTAF3Fa4a/dmTAF3Fa4a homozygous mutants (Figure 3.10c), a decrease in UBX staining was seen in spite of the presence of two copies of wild type Ubx genes. In the TrlR85/TM2, Ubx; dmTAF3Fa4a/dmTAF3Fa4a background, expression of UBX was further decreased (Figure 3.10d) suggesting that GAF and dmTAF3 are both required for normal levels of expression of Ubx gene.

3.3.2.1.1.4 Trl and dmTAF3 are involved in transcriptional activation of Ubx-lacZ

In order to confirm that the enhanced Ubx phenotype in Trl, dmTAF3 double mutant background is due to reduction in Ubx transcription and not due to an indirect effect, the TM3, Sb, Ubx-lacZ “Blue balancer” (Irvine et al., 1991; Moore et al., 1998) was used as a detector of Ubx transcription. This TM3Sb, Ubx-lacZ line contains the Upstream Control Region (UCR), i.e., the bxd-pbx regulatory element and the promoter of the Ubx gene cloned upstream of the lacZ reporter. This line is one of the transgenic lines, which was generated to dissect out the functions of the large UCR of the Ubx gene (Irvine et al., 1991). In our hands, the Blue balancer gave a pattern of LacZ staining shown in Figures 3.11 & 3.12i a, b & c. The change in the expression of Ubx-lacZ, tested by β-galactosidase activity and antibody staining, in different genetic backgrounds was used as a direct readout of the effect of Trl and dmTAF3 mutations on the Ubx promoter. Earlier work has shown that dmTAF3 is expressed in all the imaginal structures of the third instar larvae (Pointud et al., 2001), hence 3rd instar imaginal discs were stained for the lac Z activity (Figure 3.11). We found that the Ubx-lacZ transgene is expressed everywhere in wing, haltere and leg discs (Figure 3.11 a,b,c). In particular, the wing staining of Ubx-lacZ is in all cells, which is in contrast to endogenous Ubx expression that is limited to the peripodial membrane of wing disc (Brower, 1987). This might be due to the absence of wing-specific repressive elements in UCR cloned adjacent to the lacZ (Irvine et al., 1991). The expression of TM3, Ubx-lacZ was observed throughout the wing disc, which was confirmed by taking sections through the wing disc after anti β-
Figure 3.12 Antibody staining of *Ubx-lacZ* transgene

i) *Ubx-lacZ* transgene is repressed in *Trl* and *dmTAF3* mutation backgrounds. The anti beta-gal (green) and anti engrailed (red) antibody staining in the imaginal discs. The expression of the *TM3, Ubx-lacZ* line is in the wing (a) haltere (b) and leg (c) discs. The *lacZ* expression is lost from the wing disc of *TM3, Ubx-lacZ/+; dmTAF3*F advertise/ *dmTAF3*F advertise (d) and *TrlR/ TM3, Ubx-lacZ* (f) completely. However the *lacZ* staining is retained in the posterior compartments of *TM3, Ubx-lacZ/+; dmTAF3*F advertise/ *dmTAF3*F advertise haltere and leg discs (e) and similar loss of staining is seen in the anterior compartment of *TrlR/ TM3, Ubx-lacZ* haltere (g) and leg discs (h), as seen by the overlay images of *lacZ* and engrailed expression.

ii) *Ubx-lacZ* transgene is expressed all throughout the wing disc. The anti beta-gal (green) and anti engrailed (red) antibody staining in the wing imaginal disc of *TM3, Ubx-lacZ* line. (a) 2 micro metre sections have been taken of the wing discs and the expression of *lacZ* reporter is seen all throughout the wing disc contrary to the endogenous UBX expression which is seen only in the peripodial membrane of the wing. (b) the anti-engrailed expression is seen in the posterior compartment of the wing disc and (c) is the overlay.
galactosidase antibody staining by using confocal microscopy [Figure 3.12 (ii)]. In \textit{dmTAF3}^{F4a}\textbackslash dmTAF3^{F4a} mutant background, \textit{Ubx-lacZ} activity staining was lost in wing discs (Figure 3.11 d) and became restricted to the posterior compartment in haltere (Figure 3.11e) and leg discs (Figure 3.11f). Similar effect was observed in the \textit{Trl}^{R85/+} background: wing disc staining was abolished (Figure 3.11g) and haltere and leg discs retained the lacZ staining only in the posterior compartments (Figure 3.11h,i). The compartment specificity was confirmed by double labeling with anti-\textit{ENGRAILED} and anti-\textit{β}-galactosidase antibodies (Figure 3.12). The activity staining in the posterior compartments was also severely reduced in \textit{Trl}^{R85}/\textit{Ubx-lacZ}; \textit{dmTAF3}^{F4a}/\textit{dmTAF3}^{F4a} (double mutant) mutant haltere (Fig. 3.11k) and leg (Fig. 3.11l) imaginal discs. The severe reduction in the \textit{Ubx-lacZ} staining in posterior compartments of leg and haltere discs of double mutant imaginal discs further suggests that \textit{Trl} and \textit{dmTAF3} both are needed for \textit{Ubx} activation in the posterior compartments as no effect was seen in single mutant combinations.

The anti-\textit{β}-galactosidase antibody staining revealed a pattern identical to the one found by \textit{lacZ} activity staining. Wild-type discs showed the presence of \textit{β}-galactosidase throughout the wing, haltere and leg discs [Figure 3.12(i)a,b,c]. Antibody staining was not observed in the wing discs of \textit{TM3}, \textit{Ubx-lacZ}/+; \textit{dmTAF3}^{F4a}/\textit{dmTAF3}^{F4a} larvae [Figure 3.12(i)d] whereas the \textit{β}-galactosidase staining was observed only in the posterior compartment of haltere and leg discs [Figure 3.12(i)e]. In \textit{Trl}^{R85}/\textit{TM3}, \textit{Ubx-lacZ} background, the wing discs showed no anti-\textit{β}-galactosidase staining [Figure 3.12(i)f] by contrast to the posterior compartments of leg and haltere discs [Figure 3.12(i)g, h].

All these experiments show that both \textit{dmTAF3} and \textit{GAF} are required for the transcriptional activation of \textit{Ubx}. We also tried to look for a change in the expression of a \textit{lacZ} reporter inserted within the endogenous \textit{Ubx} gene (Casares et al., 1997), but found no effect of \textit{Trl} or \textit{dmTAF3} heterozygous backgrounds (data not shown). This might be due to the presence of the Downstream Regulatory Region (DCR) of \textit{Ubx} gene that could bring redundant transcription factors on the endogenous \textit{Ubx} promoter. The endogenous regulation of \textit{Ubx} is a highly complex phenomenon and many factors other than \textit{GAF} and \textit{dmTAF3} are likely to be required for precise tissue-specific activation or repression.
Figure 3.13 Proposed model of events leading to formation of proper chromatin structure at the *hsp26* promoter

Nucleosomes are represented by large orange ovals, with a dashed outline indicating their increased instability or movement. The GAGA factor (GAGA) (blue), chromatin remodeling complex (NURF) (green), TATA binding protein (TBP) (pink), TFIIID complex (purple), RNA polymerase II (RPol II) (shaded black), unknown protein or protein complex (X) (red), GAGA factor binding site (yellow box) and TATA box (white box). For simplicity, only one GAGA factor site is indicated. While X is suggested here to serve a bridging function. According to our data this unknown factor X which helps in recruitment of TFIIID complex is dmTAF3.

Adapted from Leibovitch et.al. MCB, Sept. 2002, p. 6148–6157
3.3.2.1.2 Discussion

Our study shows that dmTAF3 interacts with the BTB/POZ domain of GAF. The BTB/POZ domain is an evolutionarily conserved protein-protein interaction domain found in numerous developmentally regulated transcription factors (Albagli et al., 1995; Bardwell and Treisman, 1994). The BTB/POZ domain, first identified in Drosophila and poxviruses (Albagli et al., 1995), was shown to be involved in homophilic and heterophilic protein-protein interactions (Bardwell and Treisman, 1994; Hoatlin et al., 1999). Our finding that GAF interacts with dmTAF3 reveals a possible mechanism on how GAF could recruit the TFIID complex to carry out transcriptional activation.

The fact that a dmTAF3 mutation enhances the phenotype of Ubx mutation in a sensitized background, i.e., heterozygous for the gene encoding GAF, gives a strong evidence that TAF3 is a direct partner of GAF in the activation pathway. The colocalization of GAF and dmTAF3-Myc over expressed protein on polytene chromosomes further confirmed this in vivo interaction (R.K. Mishra, unpublished data). The effect is likely to be at the level of transcription as shown by the modified expression of Ubx-lacZ transgene in dmTAF3 and Trl mutant background. A simple model proposes that GAP contributes to Ubx transcription by its binding to specific sites near the promoter (via its Zinc fingers) and then recruits the transcriptional machinery by interaction of its BTB/POZ domain with dmTAF3 (Figure 3.13). Our study clearly demonstrates the role of dmTAF3 in the activation pathway mediated by GAF and provides a missing link between trxG proteins and the transcriptional machinery, but we found no effect of the dmTAF3 knock-down mutation in the expression of Ubx-lacZ in the posterior compartments of leg and haltere discs. This could be due to the fact that the mutation is not a complete loss-of-function, but it is equally possible that additional factors are also involved, especially when Ubx is strongly expressed. Further studies will be required to identify these components.

Recently, the requirement of GAF was shown for the transcriptional activation of hsp26 gene. The upstream regulatory region of the D. melanogaster hsp26 gene includes two DNase I-hypersensitive sites that encompass the critical heat shock elements. This chromatin structure is required for heat shock-inducible expression and depends on two (CT)n•(GA)n elements bound by GAF (Farkas et al., 2000; Lu et al., 1993; Wilkins and
Lis, 1997). In transgenic assays, removal of these GAF binding sites leads to the loss of heat shock-inducible hsp26 expression and drastic reduction of nuclease accessibility in the chromatin of the regulatory region. Chromatin immunoprecipitation experiments showed that the decrease in TFIID binding did not reduce GAF binding (Leibovitch et al., 2002). In contrast, the loss of GAF binding resulting from (CT)n mutations decreased TFIID binding. These data suggested that both GAF and TFIID are necessary for formation of the appropriate chromatin structure at the hsp26 promoter and predicted a regulatory mechanism in which GAF binding precedes and contributes to the recruitment of TFIID (Leibovitch et al., 2002). However, the question of how GAF leads to the recruitment of TFIID remains elusive (Figure 3.12). These studies have shown that GAF can interact with dmTAF3, which is a TFIID associated factor and is required for activation of Ubx. It provides the missing link between GAF and TFIID complex recruitment and places dmTAF3 involvement in the activation role of GAF (Figure 3.13).

3.3.2.1.3 Summary

- dmTAF3 directly interacts with GAF
- dmTAF3 and Trl enhance the Ubx phenotype i.e., the haltere to wing pathway
- dmTAF3 and Trl double mutant lead to reduction in UBX levels
- Trl and dmTAF3 are involved in transcriptional activation of Ubx-lacZ transgene

3.4 Conclusions

The long standing question that how GAF can bring about so many roles is of interest and our studies have tried to answer some of these questions as to how GAF can perform two opposite roles viz. activation and repression. We have been able to characterize two new direct interactors of GAF i.e lola and lolal to be important for the repressive role of GAF and dmTAF3/bip2 a TAF to be involved in bringing about the activation functions of GAF. These findings also prompt to speculate GAF to behave like a “molecular switch” for repressed and activated states of target genes. It has been known that GAF binding sites are found in promoters as well as in PREs of many genes. The interaction of GAF either with a transcriptional activator like dmTAF3 or with PeG proteins such as
GAF can bind to the GAGAG sequences present at promoter region of target genes and in turn recruit dmTAF3 protein by interaction via its BTB domain. dmTAF3 can in turn help dock the TFIID transcriptional machinery and bring about the activation of the target genes. In the cell where target gene needs to be repressed, GAF can interact with PcG proteins bound at the corresponding PREs and bring about repression by recruiting PcG complex by a looping mechanism.
LOLAL (Faucheux et al., 2003; Mishra et al., 2003) or LOLA, raises the possibility that GAF functions like a switch that can recruit either activator or repressor complexes at a target promoter and then maintains the transcriptional state (Figure 3.14). In cell types in which a gene needs to be active, GAF bound at the promoter sites would interact with activators, like dmTAF3, and maintain the active state. By contrast, in cell types where a promoter needs to be silenced, GAF would interact with PcG proteins like LOLAL and LOLA associated with even distant PREs, by a looping mechanism (Schwartz et al., 2004), and bring about a repressive chromatin context, which probably involves histone tail modifications.

3.5 Summary

- LOLAL and LOLA directly interact with GAF
- LOLAL and LOLA are new PcG members as they enhance PcG phenotypes
- LOLAL, LOLA and PH are required for pairing dependent silencing at the \( iab-7PRE \)
- LOLAL and LOLA are two new interacting partners for repressive pathway of GAF
- dmTAF3 directly interacts with GAF
- \( dmTAF3 \) enhances \( Ubx \) phenotype i.e., haltere to wing transformation
- dmTAF3 is required for \( Ubx \) transcription along with GAF
- \( dmTAF3 \) and \( Trl \) both are required for the transcriptional activation of \( Ubx-lacZ \) transgene
- dmTAF3 is a new interacting partner of GAF for its activation pathway function