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Ibf1 and Ibf2 are novel CP190-interacting proteins required for insulator function

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Abstract

Insulators are DNA-protein complexes that play a central role in chromatin organization and regulation of gene expression. In *Drosophila* different proteins, dCTCF, Su(Hw), and BEAF bind to specific subsets of insulators most of them having in common CP190. It has been shown that there are a number of CP190-binding sites that are not shared with any other known insulator protein, suggesting that other proteins could cooperate with CP190 to regulate insulator activity. Here we report on the identification of two previously uncharacterized proteins as CP190-interacting proteins, that we have named Ibf1 and Ibf2. These proteins localize at insulator bodies and associate with chromatin at CP190-binding sites throughout the genome. We also show that Ibf1 and Ibf2 are DNA-binding proteins that form hetero-oligomers that mediate CP190 binding to chromatin. Moreover, Ibf1 and Ibf2 are necessary for insulator activity in enhancer-blocking assays and Ibf2 null mutation cause a homeotic phenotype. Taken together our data reveal a novel pathway of CP190 recruitment to chromatin that is required for insulator activity.

Keywords CP190; CTCF; Fab-8; insulator

Subject Categories Chromatin, Epigenetics, Genomics & Functional Genomics; Transcription

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Introduction

Insulators are DNA-protein complexes that are thought to play a role in chromatin organization and the regulation of gene expression by mediating intra- and interchromosomal interactions (Maeda & Karch, 2007; Wallace & Felsenfeld, 2007; Van Bortle & Corces, 2013). Insulators have been characterized in all organisms, from yeast to humans (West *et al*, 2002; Valenzuela & Kamakaka, 2006). In *Saccharomyces cerevisiae* and *S. pombe* insulators are primarily promoters of highly active RNA pol II and RNA pol III transcribed genes or TFIIIC-bound sequences that block the spread of silencing (Oki & Kamakaka, 2005; Noma *et al*, 2006). In metazoans, although there are some examples of gene promoters functioning as insulators (Lunyak *et al*, 2007; Chopra *et al*, 2009) and it has recently been shown that tRNA genes have insulator activity (Ebersole *et al*, 2011; Raab *et al*, 2012), most of the insulators are autonomous DNA-protein complexes. Vertebrates possess only one known insulator protein, CTCF, while in *Drosophila* different proteins, dCTCF, Su(Hw), BEAF and GAGA, bind to the different insulators characterized. In addition, CP190 protein seems to be common to all of them (Gurudatta & Corces, 2009; Ahanger *et al*, 2013). Several studies indicate that insulator elements and the proteins they bind play a fundamental role in development and disease. In *Drosophila*, the bithorax and antennapedia complexes contain several transcriptional regulatory sequences that orchestrate the complex spatio-temporal expression of the homeotic genes present in these regions. Several studies have shown that the proper interplay between these regulatory sequences requires the function of different insulators (Karch *et al*, 1994; Barges *et al*, 2000; Belozerov *et al*, 2003; Perez-Lluch *et al*, 2008). Recently, CTCF occupancy was mapped in different cell types and showed much more widespread differential occupancy than previously suggested (Shen *et al*, 2012). Cell type-specific CTCF sites significantly overlapped with enhancers suggesting a role of these insulator sites in regulation of gene expression. On the other hand, it has been reported that CTCF-binding sites conserved between cell types represent an organizational pattern present in all cells, regardless of the developmental stage and tissue (Kim *et al*, 2007; Cuddapah *et al*, 2009) and it has been proposed that they delineate chromatin structures required for conserved genome functions that can be related to human diseases (Martin *et al*, 2011).

Several genome-scale mapping analysis of insulator protein-binding sites have been performed in flies and mammals (Kim *et al*, 2007; Bushey *et al*, 2009; Negre *et al*, 2010; Schwartz *et al*, 2012). Insulator proteins localize to thousands of sites characterized by conserved target sequences (Schwartz *et al*, 2012; Van Bortle *et al*, 2012). These observations and data obtained in the analysis of insulator protein recruitment in *Drosophila* in response to different stimuli, such as heat-shock response or ecdysone signaling (Wood *et al*, 2011) suggest that insulator activity must be controlled at the level of insulator protein binding to DNA, wherein differences in DNA motifs can influence protein occupancy levels and features of insulator function. It has been reported that distinct rules govern the binding of an

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insulator protein to different classes of sites, which sometimes involve cooperation between several insulator proteins. It has been also shown that there are a number of CP190-binding sites that are not shared with any other known insulator protein, suggesting that there should exist more proteins that cooperate with CP190 to regulate insulator activity (Schwartz *et al*, 2012).

Here, we report on the identification of two novel DNA-binding proteins that mediate CP190 recruitment to chromatin and on the characterization of their role in insulator activity.

Results

Identification of two novel CP190-interacting proteins

Multiprotein complexes containing CP190 were purified to identify previously undescribed interacting proteins. To this end, a S2 *Drosophila* embryonic cell line expressing a CP190-TAP fusion protein was obtained. Nuclear extracts from that stable cell line were used to purify proteins that are associated with the CP190-TAP following a modified TAP procedure (see Materials and Methods). Eluates were analysed by SDS-polyacrylamide gradient gel electrophoresis which revealed multiple bands (Fig 1A). LC/MS analyses without prior electrophoretic separation were carried out and several different polypeptides that copurified with CP190-TAP in three independent purifications were identified (Table 1 and Supplementary Table S1). As expected, some of them corresponded to previously characterized CP190-associated proteins such as CP60 and the insulator factors CTCF, mod(mdg4)67.2 and Su(Hw) (Table 1). In addition to them, the products of two genes, CG8436 and CG9740 that consistently copurified with CP190-TAP were identified (Mascot scores of 500–250 and several identified peptides corresponding 30–50% sequence coverage). Since the molecular function of these proteins was not known we propose to name CG8436 Insulator binding factor 1 (Ibf1) and CG9740 Insulator binding factor 2 (Ibf2). A few other polypeptides were also identified but with lower scores.

We performed coimmunoprecipitation experiments to confirm the interaction between CP190, Ibf1 and Ibf2 and to this end we raised antibodies against CP190, Ibf1 or Ibf2 proteins (see Materials and Methods and Supplementary Fig S1). Epitope-tagged proteins, Ibf1-HA and Ibf2-V5, were transiently expressed in S2 cells and immunoprecipitation with αCP190 antibodies resulted in coprecipitation of both Ibf1-HA and Ibf2-V5 (Fig 1B, upper and middle panels). No coimmunoprecipitation was observed when no antibodies were added. Moreover, immunoprecipitation assays of endogenous proteins showed that Ibf2 was significantly enriched in the immunoprecipitated samples with the different antibodies (Fig 1B, lower panel). This assay also showed that immunoprecipitation with αIbf1 results in quantitative coprecipitation of Ibf2 suggesting that both proteins form a stable complex in the cell.

Having identified Ibf1 and Ibf2 as CP190-interacting proteins we asked whether these proteins colocalize in the cell. Immunolocalization assays in third instar larvae imaginal discs showed that both proteins are distributed in a non-uniform pattern in nuclei of diploid cells where they overlap with CP190 at insulator body sites (Fig 1C).

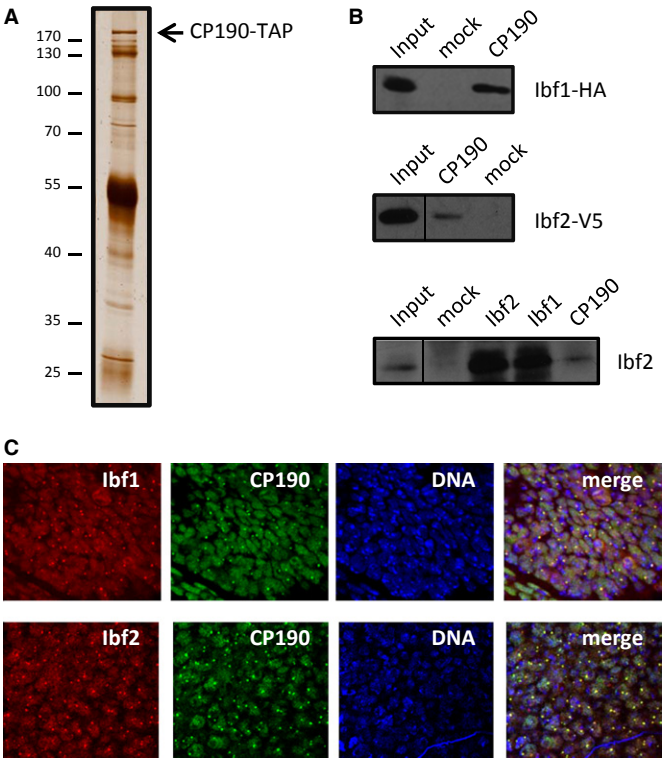

Figure 1. Ibf1 and Ibf2 associate with CP190.

A Silver staining of 10% of the eluted proteins from CP190-TAP purification.

B Western blot using αHA (upper panel) or αV5 (middle panel), and αIbf2 (lower panel) of nuclear extracts of either transiently transfected S2 cells with tagged-proteins (upper and middle panel) or S2 cells (lower panel) that were subjected to immunoprecipitation with the indicated antibodies (αCP190, αIbf1, αIbf2) or no antibodies (mock). Input corresponds to 10% of the immunoprecipitated material.

C Immunolocalization of CP190 (green) and Ibf1 (red, upper panel) or Ibf2 (red, lower panel) in imaginal disc cells. DNA is stained with DAPI (blue).

Source data are available online for this figure.

Ibf1 and Ibf2 colocalize with CP190-binding sites throughout the genome

In order to obtain high-resolution information about the genome-wide chromatin association of Ibf1 and Ibf2 and their relationship with CP190 distribution pattern, we performed ChIP-seq analyses in S2 cells using αCP190, αIbf1 and αIbf2 antibodies. We found 2,070 CP190 peaks, 613 Ibf1 peaks and 788 Ibf2 peaks (Fig 2A). Comparison of Ibf1 and Ibf2 ChIP-seq profiles indicates extensive overlap between them (Fig 2B, total number of binding sites and Fig 2C, binding profiles over a large region of chromosome 2L as an example). Indeed, more than 99% of Ibf1 overlap with Ibf2-binding sites and the higher signals observed in the Ibf2 ChIP-seq analysis are due, most likely, to technical differences in the immnunoprecipitation efficiency (Fig 2C). These data along with the coimmunoprecipitation results reported above indicate that Ibf1 and Ibf2 form hetero-dimers that bind chromatin altogether. These analyses also showed that CP190 binds to most of the Ibf1 and Ibf2 sites (85% and 80% respectively) whereas only 25% of CP190 binding sites are also sites for Ibf1 and Ibf2 (Fig 2B and C). Therefore, these

Table 1. CP190-interacting partners identified. List of identified CP190-interacting proteins ranked by the total number of peptides found by mass spectrometry in one representative experiment.

Name	Peptides	Score	Coverage (%)	Function
CP190	39	2676	47.4	Insulator protein
CP60	17	1135	48.0	Microtubule-associated protein
Su(Hw)	10	547	13.0	Insulator protein
CG8436	9	619	49.2	Unknown
Mod(mdg4)67.2	7	502	17.7	Insulator protein
CG9740	5	341	51.8	Unknown
Pita	4	193	10.0	DNA binding protein
CTCF	2	199	6.0	Insulator protein

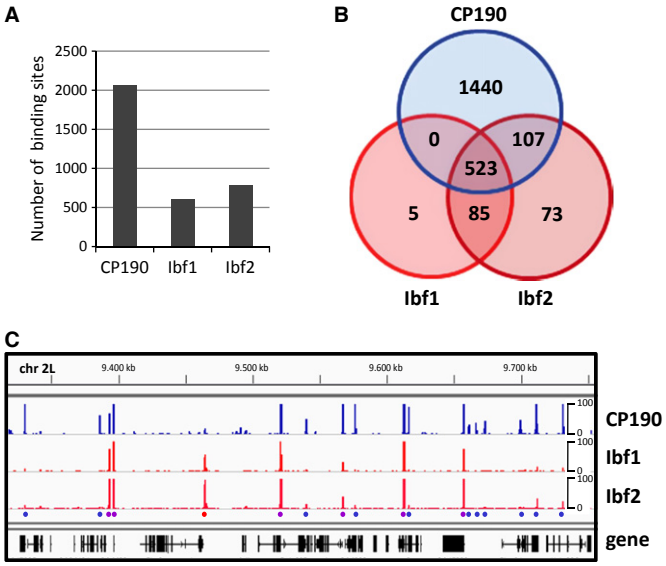

Figure 2. Genome-wide Ibf1, Ibf2 and CP190 colocalization.

A A summary of the whole-genome peak analysis for CP190, Ibf1 and Ibf2. We defined peaks as locations within the enriched regions with a coverage difference between IP and input above 100 reads.

B A Venn diagram indicating the number of binding sites that overlap between CP190, Ibf1 and Ibf2.

C Representative ChIP-seq data for CP190, Ibf1 and Ibf2 over a 400-kb region of chromosome 2L. CP190 peaks are indicated by a blue circle, Ibf1/Ibf2 peaks are indicated by a red circle and overlapping peaks are indicated by purple circles.

results suggest that Ibf1 and Ibf2 participate in only a subclass of CP190 insulators.

We have also examined the location of Ibf1 and Ibf2 binding sites in relation to genetic features and we have analyzed the distribution based on whether sites are located either in intergenic regions, inside genes or overlapping the start or the end of the most proximal gene. As reported for other insulator proteins, Ibf1 and Ibf2 are preferentially located in intergenic regions (intergenic + promoter proximal in Supplementary Fig S2). Ibf1 and Ibf2 distribution patterns are essentially identical between them and similar to CP190 pattern although CP190 is more enriched at transcriptional start sites than the others (Supplementary Fig S2).

We next compared Ibf1 and Ibf2 genomic locations with that of the other insulator proteins taking advantage of the ChIP-chip/ChIP-seq results of the modENCODE database. More than 90% of CP190-binding sites from this study correspond to CP190 sites in the ChIP-chip assay of the modENCODE project being the much greater number of CP190-binding sites found by the modENCODE project likely the consequence of a difference in the levels of stringency applied (Supplementary Fig S3, compare upper and lower panels). Moreover, similar to the comparison reported above, most of Ibf1 and Ibf2 binding sites overlap with modENCODE CP190 sites (Supplementary Fig S4).

Comparison of Ibf1 and Ibf2 with the other insulator-binding proteins showed a stronger overlap between Ibf1/Ibf2 and CTCF peaks than either Su(Hw) or BEAF (Supplementary Fig S4). Recently it has been shown that CP190-binding sites can be found either alone or overlapping with different combinations of the insulator proteins Su(Hw), CTCF and BEAF (Schwartz *et al*, 2012). We found that Ibf1 and Ibf2 are present in only some of the co-binding groups reported by Schwartz *et al* (Supplementary Fig S5). Indeed, about 70% of co-bound CP190-CTCF sites, either containing Su(Hw) and mod(mdg4) or not, are also bound by Ibf1 and Ibf2 while these proteins can rarely be found in CP190-BEAF or CP190-Su(Hw)-mod(mdg4) groups. On the other hand, Ibf1 and Ibf2 can be found in some of the CP190 standalone sites but not in CTCF, Su(Hw) or BEAF standalone sites. Altogether these results indicate that Ibf1 and Ibf2 always associate with CP190 insulators which can also contain other insulator proteins, preferentially CTCF.

Ibf1 and Ibf2 cooperate with CP190 to regulate gene expression

To characterize a role of Ibf1 and Ibf2 on gene expression and their relationship with CP190 we analyzed genomic changes in gene expression after depletion of these proteins in S2 cells. Since we could not obtain a significant knock-down after Ibf1 RNAi treatment we performed the analyses for CP190 and Ibf2 (see later in the text results indicating that Ibf2 mutation is likely to correspond to Ibf1/Ibf2 mutation regarding chromatin regulation). RNAi treatments decrease both CP190 and Ibf2 mRNA levels 4–5-fold and protein levels 2–3-fold (Supplementary Fig S6). As previously reported for CP190 and other insulator proteins (Bartkuhn *et al*, 2009; Schwartz *et al*, 2012), there is only a small number of genes that showed clear changes in their levels of expression after CP190 or Ibf2 knock-downs. We found both repressive and activating effects on gene

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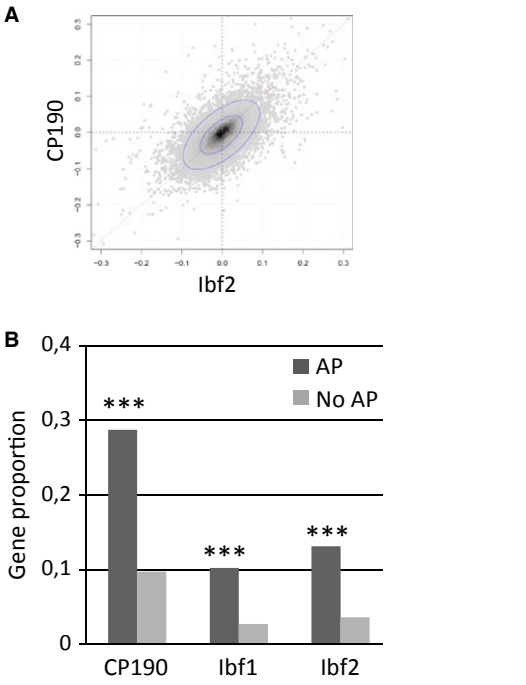

Figure 3. Ibf1 and Ibf2 cooperate with CP190 to regulate gene expression.

A Log2 fold-changes in knockdown Ibf2 or CP190 conditions versus mock RNAi are plotted for visualization of the correlation between both conditions. Each dot represents a gene. Blue circles indicate 50% and 90% of total number of genes, respectively. The diagonal line corresponds to a perfect correlation of +1.

B The graph shows proportion of genes containing CP190, Ibf1 or Ibf2 peaks. AP indicates genes with alternative promoters and No AP genes without alternative promoters. *** indicates that differences are statistically significant with $P < 0.0001$ in Fisher exact tests.

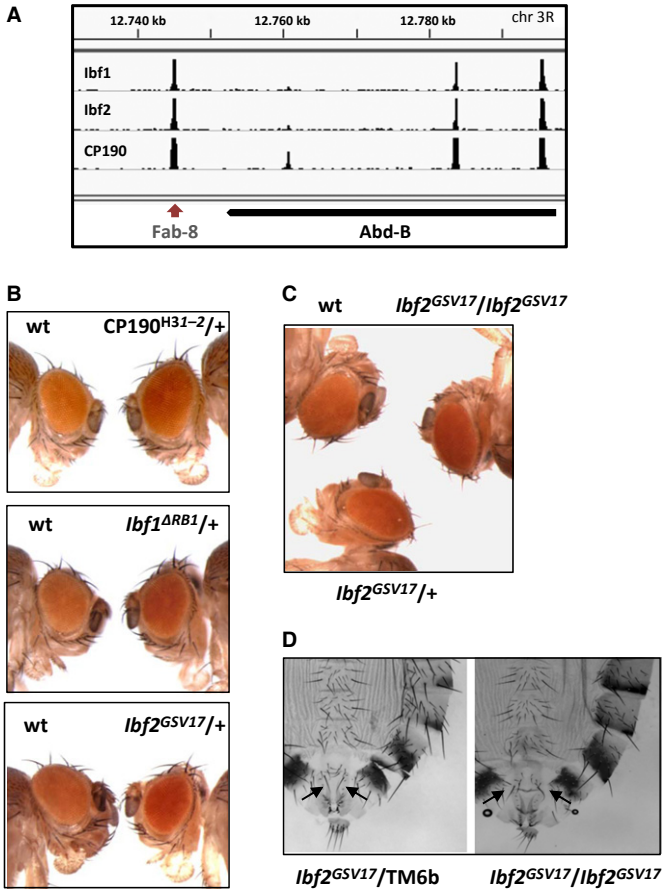

Figure 4. Insulator activity of Ibf1 and Ibf2.

A ChIP-seq data for Ibf1, Ibf2 and CP190 on the *Abd-B* gene. Location of the Fab-8 boundary element is indicated.

B Eye color of representative flies in enhancer-blocking assays using the transgenic line F8134.1 in wild-type and heterozygous $CP190^{H31-2}$, $Ibf1^{\Delta RB1}$ and $Ibf2^{GSV17}$ mutant backgrounds.

C Eye color of representative flies in enhancer-blocking assays using the transgenic line F8109.6 in wild-type, heterozygous and homozygous $Ibf2^{GSV17}$ mutant backgrounds.

D Abdomen cuticles of $Ibf2^{GSV17}$ heterozygous and homozygous females. Arrows indicate bristles in the A7 sternite showing different orientations, either toward the mid axis in heterozygous females (left panel) or towards the lateral sides in homozygous females (right panel).

expression. As shown in Fig 3A genes that change significantly their expression levels (those outside the blue circles) are mainly located in the upper right and lower left quadrants, meaning that they are genes that change in the same direction in both depletions. However, it must be noted that several of those differentially expressed genes do not fall close to the diagonal meaning that they are highly affected in one knockdown but not in the other. In order to evaluate the degree of co-regulation between Ibf2 and CP190 we plotted changes in expression for all the genes in the two knockdown conditions (Fig 3A) and we computed the Spearman's rank correlation between log2 fold changes. The Spearman's correlation value obtained was 0.588 (P value < 0.0001), suggesting that these proteins cooperate in the regulation of gene expression.

Moreover, as it has been previously reported for vertebrate CTCF-binding sites (Kim *et al*, 2007), we have found that there is a significant concentration of Ibf1/Ibf2/CP190-binding sites at genes that display alternative promoter usage (Fig 3B). Indeed, the proportion of genes with different TSSs that contain peaks of these proteins is threefold compared with genes that do not contain alternative promoters suggesting that Ibf1, Ibf2 and CP190 can be functionally linked in the regulation of levels of alternative transcripts or their tissue specificity.

Ibf1 and Ibf2 are necessary for insulator activity

We next asked whether Ibf1 and Ibf2 play a role in insulator function and to answer this question we performed enhancer-blocking assays. The ChIP-seq assays reported above showed strong Ibf1 and Ibf2 binding to the Fab-8 boundary element of the BX-C (Fig 4A). Thus, to perform enhancer-blocking assays we obtained transgenic lines containing the Fab-8 boundary element (Barges *et al*, 2000) located between the white enhancer and the miniwhite reporter gene (see Materials and Methods). We obtained several lines showing enhancer-blocking activity of Fab-8 (see Materials and Methods, Fig 4 and Supplementary Fig S7).

On the other hand we obtained mutant alleles of Ibf1 and Ibf2 in a white background by mobilizing P elements present in either

CG8436/Ibf1 or *CG9740/Ibf2* genomic regions (Supplementary Fig S8). The PBac[RB]CG8436^{e03576} strain, which express normal levels of Ibf1 (data not shown), contains a P element inserted in the first intron of *Ibf1* close to the second exon of the gene. After mobilization of the transposon one of the lines obtained (*Ibf1*$^{\Delta RB1}$) harbors a deletion of DNA sequences from the second exon until the end of the *Ibf1* gene and the promoter and part of the coding sequence of the contiguous *VhaM8.9* gene. Therefore, this strain is expressing only the first exon of *Ibf1*, likely rendering the gene nonfunctional. However, it also corresponds to a *VhaM8.9* mutant allele, since the deletion includes the transcription start site and part of the coding sequences of this gene. Homozygotes *Ibf1*$^{\Delta RB1}$ did not reach third instar larval stage due to, at least in part, VhaM8.9 mutation. The p[GSV6]GS16482 strain contains a P element inserted in the coding sequence of the *Ibf2* gene which interrupts the Ibf2 protein at amino acid 22. After mobilization of this element we selected for lines (*Ibf2*^{GSV}) that no longer express the *white* gene from the transposon sequences but still contain part of the transposon that renders the *Ibf2* gene nonfunctional (see Fig 6C, upper panel, Western blot assay showing absence of Ibf2 protein in *Ibf2*^{GSV17} mutant background). Both, *Ibf2*^{GSV17} homozygotes and *Ibf2*^{GSV17}/Df(3R)ED5339 transheterozygotes showed reduced viability (60–70%), male sterility and many of the eclosed flies were short-lived.

We found a significant relief in enhancer-blocking using transgenic lines containing the Fab-8 boundary element in both Ibf1 and Ibf2 mutant backgrounds, since heterozygote *Ibf1*$^{\Delta RB1}$ or *Ibf2*^{GSV17} mutant flies showed a clear increase in the eye pigmentation similar to the one observed in the *CP190*$^{H31\text{-}2}$ mutant background (Fig 4B). Indeed, 66 out of 74 *Ibf1*$^{\Delta RB1}$ heterozygote individuals and 149 of 152 *Ibf2*^{GSV17} heterozygote individuals have darker eyes compared with wild-type flies. Similar effects were obtained using other independent Fab-8 transgenic lines and another *Ibf2* mutant background, *Ibf2*^{GSV20} (Supplementary Fig S7). Moreover, a small but significant increase in eye color was seen in *Ibf2*^{GSV17} homozygous condition (Fig 4C). Thus, we can conclude that Fab-8-mediated enhancer-blocking requires both Ibf1 and Ibf2.

Furthermore, *Ibf2*^{GSV17} homozygous females exhibit a loss-of-function homeotic transformation similar to the one observed in *Fab-8*^{416} flies, which carry a deletion of the Fab-8 boundary element (Barges *et al*, 2000). In adult mutant females the bristles in the A7 sternite have lost their orientation, which in wild-type is toward the mid axis, and resemble that normally found in more anterior sternites. Indeed, bristles in 70% of *Ibf2*^{GSV17} homozygous females exhibit a vertical outward orientation indicating A7 to A6 transformation while only 14% of *Ibf2*^{GSV17} heterozygous females show this homeotic transformation (Fig 4D). This homeotic transformation is also present at similar frequency in homozygotes for the P-element insertion in the original p[GSV6]GS16482 strain and in *Ibf2*^{GSV17}/Df(3R)ED5339 transheterozygotes (75% and 72% respectively).

Ibf1 and Ibf2 are related proteins that contain a BED finger DNA-binding domain

Ibf1 and Ibf2 are encoded by two genes, CG8436 and CG9740, located one next to the other in chromosome 3R at position 85D6 (Supplementary Fig S8). These two genes are transcribed in divergent orientations with TSS separated by 470 bp, giving rise to two polypeptides of 242 and 195 amino acids respectively. A BLAST search of similar proteins in the *Drosophila melanogaster* databases performed with either Ibf1 or Ibf2 retrieved in both cases just one hit, which was either Ibf2 or Ibf1 respectively. Sequence alignment by ClustalW showed 18.7% of identity between both proteins with a highly conserved region in the N-terminal part of them (Supplementary Fig S9). A visual inspection of these regions showed a pattern of cysteines and histidines which could form a metal-chelating structure. Further analysis revealed that these proteins contain a BED finger, a DNA-binding domain that is present in the chromatin-boundary element binding proteins BEAF and DREF (Aravind, 2000). They share a signature of $Cx_2Cx_nHx_{3\text{-}5}$[H/C], with x_n a variable spacer that is predicted to form a zinc finger (Fig 5A). BED proteins also contain a motif of two highly conserved aromatic residues separated by a polar residue which is partially conserved in Ibf1 and Ibf2 proteins. Besides, these proteins also possess a region enriched in basic residues located N-terminal to the BED domain, which in other proteins has been suggested to be involved in DNA contacts (Aravind, 2000).

The presence of DNA-binding ability in diverse BED domains suggests that Ibf1 and Ibf2 could bind directly to DNA. Therefore, we searched for conserved motifs in the ChIP-seq data using the rGADEM package. In Ibf1, Ibf2 and CP190 binding sequences we found not only the canonical CTCF and Su(Hw) motifs and a motif that has been reported to be related to CP190 binding, either directly or by means of an unknown protein (Schwartz *et al*, 2012), but also a new motif that we have named Ibf (Fig 5B). While CTCF, Su(Hw) and CP190 consensus motifs are present in similar percentages in either Ibf1, Ibf2 or CP190 binding sequences (Fig 5C), the Ibf motif was preferentially enriched in Ibf1 or Ibf2 binding sites (58–57%) compared to CP190-binding sites (21%). Moreover, this motif is present in only 7% of the CP190 peaks that do not contain Ibf1/Ibf2 whilst it is present in 50% of the CP190-binding sites that also bind Ibf1/Ibf2 (data not shown). Therefore, we hypothesized that this motif corresponds to the Ibf motif and we analyzed DNA binding *in vitro* by electrophoretic mobility shift assays (EMSA). We used a radioactively labeled oligonucleotide containing the Ibf motif and bacterially expressed purified Ibf1 and Ibf2 proteins (Fig 5D). These assays showed that both proteins bind specifically to the Ibf sequence since the retarded complex was eliminated when non labeled Ibf oligonucleotide was used as competitor but not when the competitor contains a mutated Ibf motif (see Materials and Methods for sequences). Thus, Ibf1 and Ibf2 bind directly to DNA suggesting that they could be involved in CP190 recruitment to chromatin at sites where they colocalize.

Ibf1/Ibf2 complex mediate CP190 binding to chromatin

To further characterize the mechanisms involved in Ibf1 and Ibf2 insulator function we analyzed whether Ibf1, Ibf2 and CP190 binding to chromatin depend on each other. To this end we performed immunolocalization assays in mutant alleles using either the *Ibf2*^{GSV17} mutant allele that corresponds to a null *Ibf2* mutation (see above in the text and Fig 6C) or the combination of *CP190*^{P1}/*CP190*^{P11} alleles that behaves as a null *CP190* mutation (Pai *et al*, 2004). These assays showed that the levels of Ibf1 on polytene chromosomes in the *Ibf2*^{GSV17} mutant allele are dramatically reduced (Fig 6A). Since lack of Ibf1 binding in the Ibf2 mutant background could also reflect a contribution to synthesis and/or stability of the

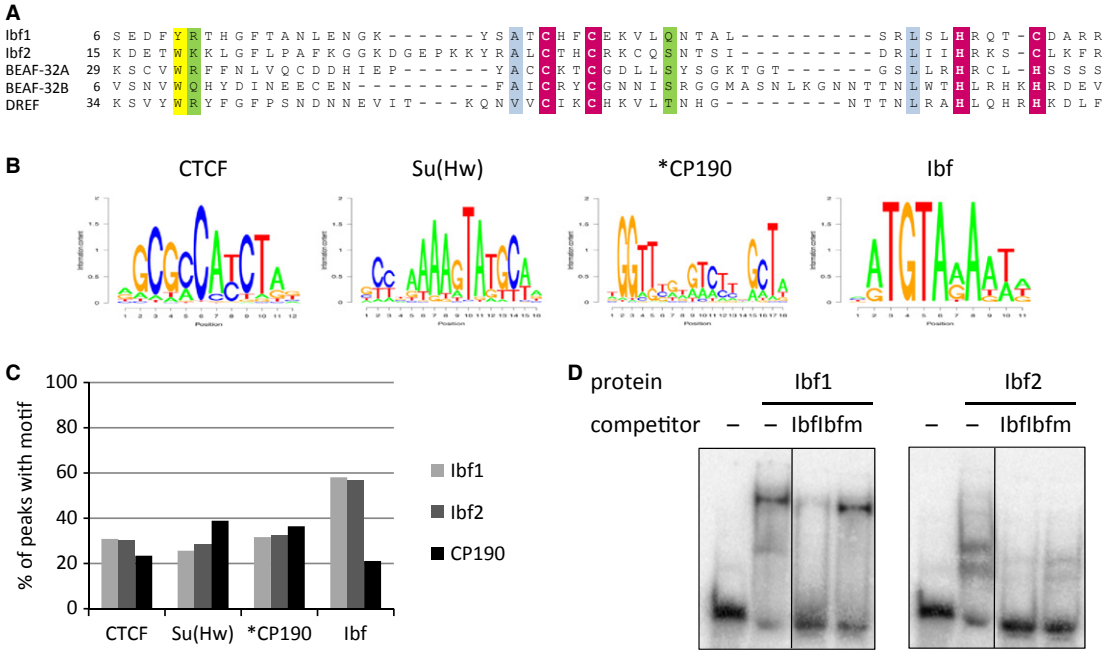

Figure 5. Characterization of Ibf1 and Ibf2 DNA-binding activities.

A Alignment of Ibf1, Ibf2, BEAF and DREF BED finger domains. Numbers on the left side indicate the position of the amino acid in the corresponding protein. The shading is according to the BED finger domain consensus: the metal-chelating residues are shown in red shading whereas yellow, green and blue shading indicate aromatic, polar and hydrophobic residues respectively.

B The logo representation of sequence motifs characteristic of CTCF, Su(Hw), CP190 and Ibf binding sites defined by the rGADEM algorithm. * in CP190-binding site indicates that the motif has been reported to be related to CP190 binding either directly or by means of an unknown protein.

C The presence of CTCF, Su(Hw), CP190 and Ibf motifs within Ibf1, Ibf2 and CP190 genomic binding sites.

D EMSA analyses of Ibf1 and Ibf2 DNA-binding activity. The Ibf motif probe was incubated with bacterially expressed proteins. Where indicated, a competitor oligonucleotide containing either the Ibf motif (Ibf) or a mutated Ibf motif (Ibfm) was added.

Source data are available online for this figure.

protein we have analyzed Ibf1 mRNA and protein levels in Ibf2 mutant lines. While Ibf1 mRNA levels are slightly increased in Ibf2 mutant larvae (Fig 6B), protein levels are reduced by a factor between two and four (Fig 6C). These results strongly suggest destabilization of Ibf1 protein due, most likely, to its inability to associate with Ibf2 in the complex that binds chromatin. Similar results were reported for several components of the polycomb PRC2 complex when their binding to chromatin was impaired (Tan *et al*, 2007). On the contrary, both Ibf1 and Ibf2 appear normal in CP190 mutant polytene chromosomes (Fig 6D and Fig 6E respectively). Altogether these results indicate that Ibf1 needs Ibf2 to associate with chromatin whereas these proteins do not require CP190 to bind to chromatin.

On the other hand, CP190 appears to be present at normal levels in polytene chromosomes in the Ibf2 mutant background (Supplementary Fig S10A). However, the results reported above show that the majority of CP190-binding sites do not correspond to Ibf1 and Ibf2 sites (Fig 2) and therefore shouldn't be affected in Ibf1/Ibf2 mutant conditions rendering the analyses in polytene chromosomes not conclusive. To unambiguously determine whether CP190 binding depends on Ibf1/Ibf2 we performed ChIP assays in wild-type and $Ibf2^{GSV17}$ mutant larvae that behave as an Ibf1/Ibf2 null mutation regarding chromatin regulation, since we have found that Ibf1 is not able to bind chromatin in the absence of Ibf2 (Fig 6A). Our analyses showed a reduction in CP190 binding in the Ibf2 mutant background at the Fab-8 boundary element of the BX-C, where Ibf1 and Ibf2 colocalize with CP190 (Fig 6F). Moreover, our results also show a clear reduction in CP190 binding at other sites where the three proteins colocalize (CP1 and CP2 in Fig 6F) while CP190 remained associated to regions that do not correspond to Ibf1/Ibf2 binding sites (CP3 and CP4 in Fig 6F). In order to obtain genome-wide information about the effect of Ibf depletion on CP190 binding to chromatin we performed ChIP-seq analyses in wild-type and $Ibf2^{GSV17}$ mutant larvae using αCP190 antibodies. These assays show that in wild-type larvae CP190 is bound to a fraction of the Ibf1/Ibf2 binding sites reported above (Fig 6G). This is in agreement with a previous report showing cell type-specific binding for another insulator protein (Shen *et al*, 2012). These assays also show that depletion of Ibf2 causes a reduction in the presence of CP190 on Ibf-binding sites (Fig 6G and see as an example Supplementary Fig S10, peak highlight with a red asterisk). Moreover, there are many CP190 peaks which, although still present in the mutant background, show a clear reduction in the levels of the protein (see for example Supplementary Fig S10, peak highlight with a blue asterisk). Altogether, these results indicate that Ibf proteins participate in CP190 recruitment to chromatin at many sites where they colocal-

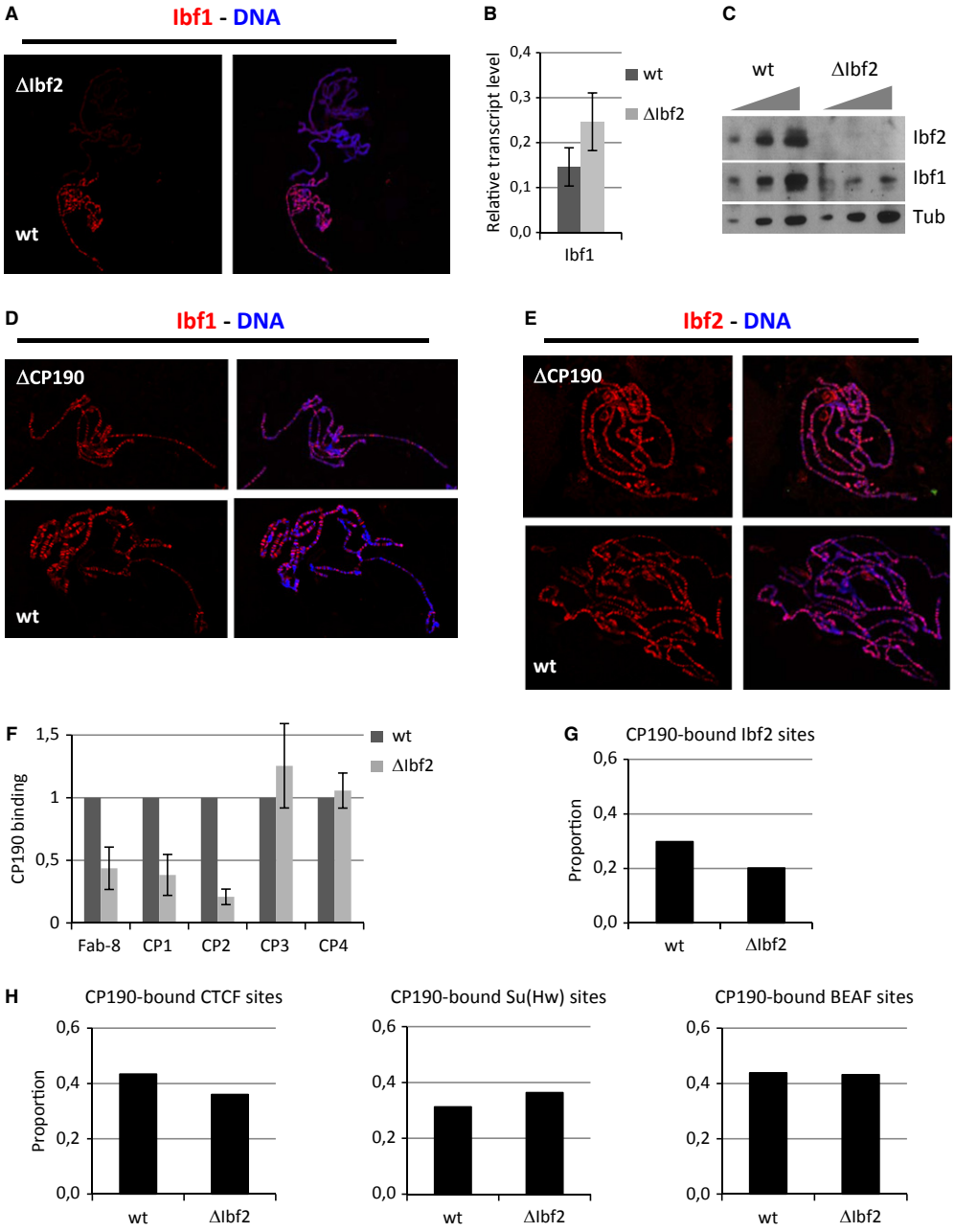

Figure 6. Ibf1/Ibf2 complex mediates CP190 binding to chromatin.

A Patterns of immunolocalization of Ibf1 (red) in polytene chromosomes obtained from mutant *Ibf2*^{GSV17} larvae that were mixed and squashed together with control wild-type larvae. DNA is stained with DAPI (blue).

B Quantitative RT-PCR analysis of the levels of Ibf1 in control wild-type and mutant *Ibf2*^{GSV17} larvae. Relative Ibf1 mRNA expression was determined in relation to Rp49 expression. Data are presented as mean ± s.d.

C Western blot analyses with αIbf1 or αIbf2 antibodies of protein extracts (twofold increasing amounts) from control wild-type and mutant *Ibf2*^{GSV17} larvae. The signal obtained with αTubulin antibodies was used as a loading control.

D Patterns of immunolocalization of Ibf1 (red) in polytene chromosomes obtained from mutant *CP190*$^{P1/P11}$ and wild-type larvae. DNA is stained with DAPI (blue).

E Patterns of immunolocalization of Ibf2 (red) in polytene chromosomes obtained from mutant *CP190*$^{P1/P11}$ and wild-type larvae. DNA is stained with DAPI (blue).

F ChIP-qPCR using antibodies against CP190 and primers for the Fab-8 boundary element and a set of randomly chosen CP190-binding sites that are either Ibf1/Ibf2 binding sites (CP1 and CP2) or not (CP3 and CP4). Average enrichments (normalized to the input sample) are plotted as the ratio of precipitated DNA in wild-type or *Ibf2*^{GSV17} mutant relative to wild-type larvae. Error bars show s.e.m. of three independent biological replicates.

G Proportion of CP190 peaks in either wild-type or mutant *Ibf2*^{GSV17} larvae that overlap Ibf2 sites identified above (Fig 2).

H Proportion of CP190 peaks in either wild-type or mutant *Ibf2*^{GSV17} larvae that overlap CTCF, Su(Hw) or BEAF sites (modENCODE _283_331 and _274 files respectively).

Source data are available online for this figure.

ize. Nevertheless, these assays also show that there are some CP190-Ibf2 overlapping sites that are unaffected by Ibf2 depletion (see for example Supplementary Fig S10, peak highlight with a black asterisk) suggesting that CP190 binding to these sites depends on other insulator DNA-binding proteins. As reported above, our results point to a special participation of Ibf1/Ibf2 complex in CP190 insulators that also contain CTCF. In order to characterize this further we analyzed the proportion of CP190-binding sites in either wild-type or mutant $Ibf2^{GSV17}$ larvae that overlap CTCF, Su(Hw) or BEAF sites. These analyses reveal that CP190 sites that are lost in the Ibf2 mutant condition overlap significantly with CTCF sites while CP190 sites that are maintained in the absence of Ibf2 more often correspond to Su(Hw) sites (Fig 6H).

Overall, we can conclude that the Ibf1/Ibf2 complex mediates CP190 recruitment to chromatin at sites where they colocalize and that these proteins are required for insulator function.

Discussion

In this study we have identified Ibf1 and Ibf2 as two novel CP190-interacting proteins and we have shown that these proteins are able to bind DNA with affinity and specificity. The mechanistic interdependencies between CP190 and sequence-specific DNA-binding proteins such as Su(Hw), CTCF and BEAF, although extensively analyzed in immunolocalization and ChIP assays, are still not fully understood. It has been shown that CP190 depends on Su(Hw) to associate with the gypsy insulator but this dependence is not clear for endogenous Su(Hw)-dependent insulators (Pai *et al*, 2004). CP190 and CTCF cooperate with each other to bind chromatin but different studies showed different degrees of interdependence (Gerasimova *et al*, 2007; Mohan *et al*, 2007; Wood *et al*, 2011). Recently, a study taking into account quantitative aspects of binding revealed several classes of binding sites occupied by specific combinations of insulator proteins and proposed the existence of still unknown players (Schwartz *et al*, 2012). Our results demonstrate a novel pathway of CP190 recruitment to chromatin which involves binding to a complex formed by two previously uncharacterized proteins, Ibf1 and Ibf2. These findings add more complexity to insulators and could explain previously controversial observations on the mechanistic interdependencies between CP190 and the previously characterized DNA-binding proteins CTCF, Su(Hw) and BEAF. Nevertheless, there are still several CP190-binding sites that neither colocalize with previously known insulator proteins nor with Ibf1 and Ibf2 suggesting that there may be additional unknown DNA-binding proteins involved in insulator function in *Drosophila*.

In this study we have shown that Ibf1 and Ibf2 contain a BED finger which is a DNA-binding domain that is also present in the insulator protein BEAF and several transposases distributed over the eukaryotic crown group. This suggests that in *Drosophila* the BED finger has been recruited for cellular functions from transposases to regulate his own chromatin structure. It has been shown that BEAF insulator protein separates close head-to-head genes with different patterns of transcriptional regulation (Yang *et al*, 2012). On the other hand, previously reported studies showed concentration of CTCF-binding sites at genes that contain alternative promoters in vertebrates (Kim *et al*, 2007) and the correlation of CTCF binding to the protocadherinα gene with alternative isoform expression (Monahan *et al*, 2012). Our results show that Ibf1, Ibf2 and CP190-binding sites are clearly enriched at genes with alternative promoters suggesting a role for these proteins in the selection of promoters in distinct cell types along development.

Recent data indicate that chromatin insulators outgrow the classical barrier and enhancer-blocking roles that defined these elements; they appear to be involved in mediating long-range inter- and intra-chromosomal arrangements that can direct the nuclear co-localization of specific sequences playing a crucial role in the regulation of gene expression. The insulator proteins CP190 in *Drosophila* and CTCF-cohesin in mammals have been shown to mediate protein-protein interactions resulting in the organization of the chromatin into loops. It is tempting to speculate that Ibf1 and Ibf2, besides recruiting CP190 to chromatin, can help this protein in the establishment or maintaining of the interactions between different insulator elements; we have shown that both, Ibf1 and Ibf2, are able to bind specifically to the same DNA motif but the results of genome-wide analyses presented here strongly suggest that they do not compete for binding to DNA. Ibf1 and Ibf2 are similar proteins encoded by two genes that are located one next to the other in the genome suggesting that they arose from a gene duplication event. Our analyses show that both proteins are required for insulator activity since mutation of any of them has an effect in enhancer-blocking assays and our results indicate that Ibf1 and Ibf2 form a stable complex in the cell. It has been shown that duplication of genes coding for homodimers is frequently accompanied by conservation of protein interactions permitting functional diversification (Pereira-Leal *et al*, 2007). Ibf1 and Ibf2 contain, besides the N-terminal DNA-binding domain, a conserved region at the C-terminal part that could account for Ibf1-Ibf2 protein interactions. Although further investigation will be needed to characterize whether both, Ibf1 and Ibf2, or which one of them, support chromatin contacts and/or protein-protein interactions with CP190, our results clearly show that Ibf1/Ibf2 complex binds to chromatin and to CP190 and they suggest that this complex might be able to interact with two different insulator elements contributing to the loop organization of the chromatin.

Materials and Methods

Fly stocks

p[GSV]GS16483 was obtained from DGRC (Indiana University), pBac[RB]CG8436^{e03576} from the Exelixis Collection (Harvard Medical School) and Df[3R]ED5339 was obtained from Bloomington Drosophila Stock Center (Indiana University). CP190^{P1} and CP190^{P11} were obtained from V. Corces (Pai *et al*, 2004).

Ibf2 mutant lines were obtained by mobilization of the P element from p[GSV]GS16483 followed by selection of lines that either kept part of the transposon or delete enough DNA sequences to also affect Ibf1 gene. We obtained 40 white minus lines, among them, none with a lesion affecting both genes and 13 lines that kept parts of the transposon. The integrity of the genomic sequences on both sides of the insert in lines $Ibf2^{GSV17}$ and $Ibf2^{GSV20}$ was checked by PCR (data not shown).

The Fab-8 sequence (Barges *et al*, 2000) was obtained by PCR amplification of a DNA fragment from 3R:12744472 to 3R:12745518 using the following primers: 5′-CTTTGACGAGTTTTCCAAGC-3′ and

5′-GGGCTACCAGTGCGTGGCC-3′. The PCR product was inserted in a pCasper between the enhancer of the *white* gene and a mini-white reporter gene, as described in (Perez-Lluch *et al*, 2008). When this construct was introduced into flies 60% of the transgenic lines obtained (five out of eight) showed reduced eye pigmentation ranging from yellow to dark orange. Lines with light eyes were used in enhancer-blocking assays and the eye color was determined 24 h after eclosion for control and mutant backgrounds.

Antibodies

Rat α-Ibf-1 and α-Ibf-2 polyclonal antibodies were raised against bacterially expressed recombinant proteins containing the full-length Ibf1 and Ibf2 and were used at a 1:2,500 dilution for Western blot. Rabbit α-CP190 polyclonal antibodies were raised against a bacterially expressed recombinant protein containing amino acids 125–1096 of CP190 and was used at 1:4,000 dilution for Western blot. Rat α-HA was purchased from Roche (12CA5), mouse αV5 was purchased from Invitrogen (R960-25), rabbit α-GFP was purchased from Invitrogen (A-6455), and mouse α-βtubulin was purchased from Sigma (A2547).

Purification and characterization of multiprotein complexes containing CP190

Purification of multiprotein complexes was performed from stable S2 cell lines expressing CP190-TAP. Full-length CP190 was TAP-tagged at the C-terminus using plasmid pMK33-CTAP where expression of the TAP-tagged protein is driven by a metallothionein inducible promoter, and stable S2 lines obtained according to (Veraksa *et al,* 2005). Expression of CP190-TAP was induced by overnight treatment with 0.07 mM $CuSO_4$. Crude nuclear extracts were prepared according to (Dignam *et al*, 1983) and precleared by incubation with BSA-Dynabeads for 2 h at 4°C. Extracts were, then, applied to IgG-Dynabeads (Invitrogen) and incubated overnight at 4°C. After incubation, beads were extensively washed and eluted with 25 mM Tris-HCl (pH 6.8), 5% glycerol, 1% SDS. Eluted material was analysed by SDS-PAGE. A single band from the stacking gel was cut and analyzed by standard LC/MS, performed in the Proteomics Unit of the "Institut de Recerca de la Vall d'Hebron" (Barcelona).

Coimmunoprecipitation experiments

Assays were performed with extracts prepared from S2 cells or transiently transfected S2 cells with Ibf1-HA (LD32555, obtained from DGRC) or Ibf2-V5 (full-length Ibf2 was cloned into a pAc5.1). Cells were lysed with 1% Triton X-100, 300 mM NaCl, 50 mM Tris pH 8, 1 mM EDTA, 1 mM DTT. Incubation with α-CP190, α-Ibf1, α-Ibf2 or no antibodies was performed at 4°C for 2 h. After incubation with Protein A/G Agarose (Santa Cruz Biotech), beads were pelleted by centrifugation, washed and analysed by Western blot.

Immunostaining experiments

Immunostaining experiments were performed in polytene chromosomes and imaginal discs of third instar larvae with α-Ibf1 (1:400), α-Ibf2 (1:500) and α-CP190 (1:1,000) according to (Font-Burgada *et al*, 2008). For visualisation, slides were mounted in Mowiol (Calbiochem-Novabiochem) containing 0.2 ng/ml DAPI (Sigma) and visualised in a Nikon Eclipse E-800 inverted microscope or in a confocal Leica TCS SP2-AOBS microscope.

ChIP experiments

For ChIP, chromatin was prepared according to (Lloret-Llinares *et al*, 2012) from cultured S2 cells and from third instar larvae according to (Carbonell *et al*, 2013). Immunoprecipitations were basically performed as described in (Orlando *et al*, 1997) using rat α-Ibf1, α-Ibf2, and rabbit α-CP190 polyclonal antibodies. For ChIP-qPCR, triplicates from three independent biological replicates were analyzed (see Supplementary Table S2 for primers used in these experiments).

For ChIP-seq, 10 ng of DNA, quantified by Qubit dsDNA HS Assay Kit (Invitrogen) were used for library preparation. End-repair, adenylation, ligation of adapters and PCR enrichment for 18 cycles was performed using TruSeqRNA Sample Prep Kit (Illumina) according to manufacturer's recommendations. Purified libraries were quantified by Qubit dsDNA HS Assay Kit (Invitrogen) and size distribution was evaluated using Bioanalyzer DNA 1000 assay (Agilent). Single-end sequencing of 50 nucleotides was performed on Solexa/Illumina. See Supplemental experimental procedures for details on data processing and quality control, sequence alignment, peak calling and downstream analyses.

Expression profiling

S2 cells (2.5×10^6) were transfected with 15 μg dsRNA. As a mock RNAi treatment cells were transfected with LacZ dsRNA (see Supplementary Table S2 for primers used to produce Ibf1, Ibf2, CP190 and LacZ dsRNAs). The transfection was repeated after 48 h and 72 h and cells collected for RNA extraction at 96 h, using Rneasy mini kit (Qiagen). Duplicates were processed for each RNAi condition.

cDNA library preparation and amplification were performed from 25 ng total RNA using WTA2 (Sigma-Aldrich) with 19 cycles of amplification. 8 μg cDNA was subsequently fragmented by DNAseI and biotinylated by terminal transferase obtained from GeneChip Mapping 10Kv2 Assay Kit (Affymetrix). Hybridization mixture was prepared according to Affymetrix protocol. Each sample target was hybridized to a GeneChip Drosophila Genome 2.0 Array (Affymetrix). The arrays were scanned in a GeneChip Scanner 3000 (Affymetrix). CEL files were generated from DAT files using GCOS software (Affymetrix). To generate the log2 expression estimates, overall array intensity was normalized between arrays and the probe intensity of all probes in a probeset summarized to a single value using RMA (Robust Multichip Average) algorithm (Irizarry *et al*, 2003).

To measure the degree of co-regulation between the mutants, we computed the pair-wise Spearman's rank correlation between samples.

Electrophoretic mobility shift assays

DNA probes were obtained by annealing oligonucleotides for the Ibf motif: 5′-TCTGTGTTTTGACTATTTTACATTTGACCATGGAT-3′ and 5′-TCTGATCCATGGTCAAATGTAAAATAGTCAAAACA-3′ and for a mutated Ibf motif: 5′-TCTGTGTTTTGACTGGCCTTCTGTTGAC

CATGGAT-3′ and 5′-TCTGATCCATGGTCAACAGAAGGCCAGTCAAA ACA-3′ in Tris pH 7.5 10 mM and $MgCl_2$ 10 mM. Annealed oligonucleotides were end-labeled using [α-^{32}P]dATP and Klenow fragment (Fermentas). Recombinant full-length Ibf1 or Ibf2 was added to the purified probe in a 10 μl final volume of binding reaction containing 20% Glycerol, 50 mM Hepes pH 7.8, 120 mM KCl, 1 μg/μl BSA, 0.01 μg/μl ssDNA and 2 mM DTT. For DNA competition analysis, binding was competed using 60-fold excess of either Ibf probe or mutated Ibf probe. Binding reactions were incubated on ice for 15 min and then resolved on 8% non-denaturing polyacrylamide gels. Gels were dried and subjected to autoradiography.

Accession numbers

ChIP-seq and expression profiling data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE47559.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions
Conceived and designed the experiments: SC, MLE. Performed the experiments: SC, UF, MLE. Analyzed the data: SC, UF, OR, EP, MLE. Wrote the paper: SC, MLE.

Conflict of interest
The authors declare that they have no conflict of interest.

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