The chromosomal association/dissociation of the chromatin insulator protein Cp190 of *Drosophila melanogaster* is mediated by the BTB/POZ domain and two acidic regions

Daniel Oliver, Brian Sheehan, Heather South, Omar Akbari, Chi-Yun Pai*

Abstract

**Background:** Chromatin insulators or boundary elements are a class of functional elements in the eukaryotic genome. They regulate gene transcription by interfering with promoter-enhancer communication. The Cp190 protein of *Drosophila melanogaster* is essential to the function of at least three-types of chromatin insulator complexes organized by Su(Hw), CTCF and BEAF32.

**Results:** We mapped functional regions of Cp190 in vivo and identified three domains that are essential for the insulator function and for the viability of flies: the BTB/POZ domain, an aspartic acid-rich (D-rich) region and a C-terminal glutamic acid-rich (E-rich) region. Other domains including the centrosomal targeting domain and the zinc fingers are dispensable. The N-terminal CP190BTB-D fragment containing the BTB/POZ domain and the D-rich region is sufficient to mediate association with all three types of insulator complexes. The fragment however is not sufficient for insulator activity or viability. The Cp190 and CP190BTB-D are regulated differently in cells treated with heat-shock. The Cp190 dissociated from chromosomes during heat-shock, indicating that dissociation of Cp190 with chromosomes can be regulated. In contrast, the CP190BTB-D fragment didn’t dissociate from chromosomes in the same heat-shocked condition, suggesting that the deleted C-terminal regions have a role in regulating the dissociation of Cp190 with chromosomes.

**Conclusions:** The N-terminal fragment of Cp190 containing the BTB/POZ domain and the D-rich region mediates association of Cp190 with all three types of insulator complexes and that the E-rich region of Cp190 is required for dissociation of Cp190 from chromosomes during heat-shock. The heat-shock-induced dissociation is strong evidence indicating that dissociation of the essential insulator protein Cp190 from chromosomes is regulated. Our results provide a mechanism through which activities of an insulator can be modulated by internal and external cues.

Background

Chromatin in the eukaryotic cell nucleus is organized into sub-regions of various transcriptional activities. Chromatin insulators, also known as boundary elements, are a unique class of functional elements in eukaryotic genomes. They are thought to separate differently regulated sub-regions along chromatin fibers. Deletion of an insulator can cause abnormal expression of local genes resulting in developmental defects. For example, deletion of the Fab-7 insulator in Bithorax complex of *Drosophila melanogaster* results in body segment transformation [1].

Chromatin insulators interfere with promoter-enhancer interactions only when they are positioned between a promoter and the enhancer. The *gypsy* insulator of *Drosophila melanogaster* is one of the best characterized insulators. Insertion of a copy of the *gypsy* insulator sequence in a gene or its regulatory region interferes with interactions between local enhancers and the promoter thus causing mutant phenotypes in many genes [2,3]. The *gypsy* insulator is a 340 to 430 base pair sequence containing 8 or 12 copies of a consensus repeat sequence, some of which bind the Suppressor of...
Hairy-wing [Su(Hw)] zinc finger protein, which is required for insulator activity [2,4-6]. Su(Hw) organizes a protein complex on the gypsy insulator. Identified proteins in the complex include Su(Hw), the Centrosomal Protein 190 (Cp190), Modifier of mdg 4 67.2 [Mod(mdg 4)67.2], and several other proteins [7-13]. The Cp190 protein is essential for gypsy insulator function too [11] and is present in other types of chromatin insulator complexes such as the CTCF complex which mediates the insulator activity at the Fab-8 insulator in the Bithorax complex [14-16], and the BEAF32 complex [16,17].

Cp190 has three conserved protein motifs: (1) The Broad-complex, Tramtrack and Bric-Abrac (BTB) homologous domain, also known as the Poxvirus and Zinc Finger (POZ) domain; (2) three copies of C2H2 zinc fingers; and (3) the C-terminal E-rich domain. In addition to these three domains, previous studies identified a centrosomal targeting domain (CENT) for localizing the Cp190 protein to centrosomes during mitosis [18]. To understand the roles of these domains in insulator function, we used genetic complementation using P-element transgenes expressing domain-truncated Cp190 mutants. We identified an additional acidic D-rich region which is involved in the association of Cp190 with insulator complexes. We found that the BTB domain, the D-rich region and an acidic C-terminal E-rich region are essential to the function of Cp190 in the gypsy insulator. The zinc fingers and the centrosomal targeting domain are dispensable. Our results indicate that the three essential domains have distinct roles in insulator binding and function.

Results
Cp190 domain-truncated mutants
To determine functional domains essential for the function of Cp190 in the gypsy chromatin insulator, we performed genetic complementation with P-element transgenes carrying Cp190 mutants, each lacking a predicted functional domain (Figure 1A). Since Cp190 is expressed ubiquitously in cells of all examined tissues in all developmental stages and that CP190 mutations were rescued by a CP190 cDNA driven by the Ubiquitin Ubi63e promoter [19], we expressed Cp190 proteins using the P-element vectors containing the Ubiquitin Ubi63e promoter [20]. Each P-element transgene contains a full-length or a mutated CP190 cDNA fragment fused to either the green fluorescent protein (GFP), the red fluorescent protein (RFP) or a 6x-Myc tag (Figure 1A). The molecular tags allow detection of the transgenic fusion proteins by anti-tag antibodies or by GFP or RFP fluorescence. At least two independent insertions of each P-element were crossed into homozygous CP190 mutant backgrounds. These include CP190\textsuperscript{ΔM}, GFP-CP190dZnF and mRFP-CP190 transgenic lines.

We next determined if the transgenes rescue the lethality of homozygous CP190\textsuperscript{ΔM}. Expression of mRFP-CP190 encoded by P[Ubi63e::mRFP-CP190, w\textsuperscript{+}], or GFP-CP190dZnF lacking all three zinc fingers encoded by P[Ubi63e::GFP-CP190dZnF, mini-w\textsuperscript{+}], fully rescued the lethality of homozygous CP190\textsuperscript{ΔM}. The rescued adults were healthy and fertile, showing that the GFP-CP190dZnF and the mRFP-CP190 proteins support all essential Cp190 functions. We confirmed the published result that the CP190ΔM transgene which lacks the centrosomal-targeting CENT region (Figure 1A) rescues lethality of the homozygous CP190\textsuperscript{ΔM} mutant [19].

The zinc finger and centrosomal targeting domains are also not required for gypsy insulator activity. The insulator function was evaluated using two gypsy insertion mutations that cause adult phenotypes: the cut wing phenotype of the ct\textsuperscript{c} mutation (Figure 2A) and the body cuticle pigmentation phenotype of the y\textsuperscript{2} mutation. ct\textsuperscript{c} wing margins lack bristle cells (Figure 2A top left). The ct\textsuperscript{c} margin phenotype is suppressed in a CP190-deficient background. For example, in the homozygous viable CP190\textsuperscript{144-1} flies, some margin bristles appear between veins L3 and L5 and wings are
rounder in shape (Figure 2A, middle left), indicating that the gypsy insulator activity is partially reduced. Most CP190\textsuperscript{3}/CP190\textsuperscript{P1} flies die as pupae, but a few adult escapers (2\%) have wings with wild-type appearance (Figure 2A, bottom left), indicating greatly reduced insulator activity. A copy of mRFP-CP190, GFP-CP190dZnF or the CP190\textsuperscript{ΔM} transgenes restored the gypsy insulator function in the homozygous CP190\textsuperscript{3}, which cause substantial wing margin loss (Figure 2A, right column).

In contrast, the BTB domain of Cp190 is required for viability and insulator activity. Neither the myc-tagged myc-CP190dBTB encoded by P[Ubi63e::myc-CP190dBTB, mini-w\textsuperscript{+}], nor the GFP-tagged GFP-CP190dBTB encoded by P[Ubi63e::GFP-CP190dBTB, mini-w\textsuperscript{+}], rescue the lethality of homozygous CP190\textsuperscript{3} although they were expressed at substantial levels (Figure 1). To evaluate if the myc- or GFP-CP190dBTB transgenes rescue the defective gypsy insulator function, the transgenes were crossed into the homozygous viable

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& & & \textbf{H4-1} & & & \textbf{Leathality} & \textbf{Rescued} & \textbf{Gypsy Rescued} & \textbf{Association} \\
\hline
\textbf{CP190} & RFP & BTB & CENT & ZnF & E-rich & 755\textsuperscript{+} & + & + & + \\
\textbf{CP190dBTB} & Myc & BTB & CENT & ZnF & E-rich & 1066 & - & - & - \\
\textbf{CP190dZnF} & GFP & BTB & CENT & & & 1066 & + & + & + \\
\textbf{CP190ΔM} & & & & & & 1066 & + & + & + \\
\textbf{CP190dCT(En15)} & & & & & & 1578 & - & - & + \\
\textbf{CP190BTB-D} & GFP & BTB-D & CENT & ZnF & & & - & - & - \\
\textbf{CP190BTB} & GFP & BTB-D & CENT & & & & & - & - \\
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\caption{Protein structure and expression levels of CP190 deletion mutants. (A) Schematic diagram of CP190 deletion mutants and their genetic complementation phenotypes. The full-length Cp190 is tagged with mRFP (mRFP-CP190). Each CP190 mutant contains a deletion of one of the functional domains. CP190dBTB lacks the BTB domain and is tagged with myc (myc-CP190dBTB) or with GFP (GFP-CP190dBTB, not shown); CP190dZnF lacks all three zinc fingers and is tagged with GFP (GFP-CP190dZnF); CP190\textsuperscript{ΔM} lacks the centrosomal targeting domain (CENT); CP190BTB-D lacks CENT, zinc fingers and the E-rich domain and is tagged with GFP (GFP-CP190BTB-D); CP190BTB has only the BTB domain and is tagged with GFP (GFP-CP190BTB). The CP190dCT(En15) is the predicted protein from the EMS-induced CP190\textsuperscript{En15} mutant, which lacks two zinc fingers and the E-rich region. The amino acids at the junction of each deletion are indicated. (B-C) Expression of the mutated Cp190 proteins revealed by the anti-Cp190 immunoblot (B) or by anti-GFP immunoblots (C). All transgenic lines were crossed into the homozygous CP190\textsuperscript{3} background. Proteins extracted from about two 3\textsuperscript{rd} instar larvae containing the indicated transgene were loaded per each lane. Similar results were also obtained from pupae 24-48 hours after pupation. Underneath the blots were stripped filters re-probed with the anti-actin antibody as a loading control.}
\end{figure}
The BTB, but not the zinc finger or CENT domains, is essential for association of Cp190 with the Su(Hw)-Mod(mdg4)67.2 insulator complex

The y locus at the tip of the X chromosome contains a gypsy insertion in y2 flies. Proteins in the Su(Hw) insulator complex including Su(Hw), Mod(mdg4)67.2 and Cp190 can be detected at the y locus in y2 flies by immunostaining of salivary gland polytene chromosomes [11]. We used immunostaining of y2 polytene chromosomes to assay association between the mutated Cp190 proteins and the Su(Hw) complex. We found that both the GFP-Cp190dZnF and the Cp190ΔM proteins bind to the y locus (Figure 2B), indicating that the CENT domain and the zinc fingers are not required for association of Cp190 with the gypsy insulator, consistent with the genetic complementation results which show that these domains are not essential for gypsy chromatin insulator activity. In contrast the myc-Cp190dBTB protein was no longer present at the gypsy site in the y locus (Figure 3A, white arrows), indicating that the association of the myc-Cp190dBTB protein with the Su(Hw) complex at the gypsy insulator is weak or non-existent.

In addition, we noticed that the myc-Cp190dBTB protein still associated with many sites on chromosomes although it was absent from the Su(Hw) complex at gypsy, suggesting that other regions in Cp190 may mediate binding to other types of chromosome-associated complexes. We compared the distribution of the GFP-Cp190dZnF and the mRFP-Cp190 proteins in living cells of salivary glands dissected from 3rd instar larvae. The fully functional mRFP-Cp190 is associated with polytene chromosomes as multiple bands in the cell nucleus, but is not detectable in extra-chromosomal spaces (Figure 3E, yellow arrows). Although significant amounts of the GFP-Cp190dBTB protein were detected on polytene chromosomes and colocalized with the mRFP-Cp190 (Figure 3E, bottom row), it had a more diffuse pattern and could be detected extra-chromosomally (Figure 3E, yellow arrows). This result is consistent with the immunostaining result of polytene chromosomes which shows that Cp190dBTB still associates with polytene chromosomes at many sites.

The polytene staining results described above indicate that the Cp190dBTB protein does not associate with the Su(Hw)-Mod(Mdg4)67.2 complex at gypsy, which is supported by immunoprecipitation assays. We showed previously that proteins in the Su(Hw) complex, such as Su
Figure 3 The BTB domain is necessary but not sufficient for association with the Su(Hw) complex. (A) Distribution of myc-CP190dBTB and Cp190 on polytene chromosomes. The polytene chromosomes of the y' (upper panel) and of the y', P[Ubi63e::myc-CP190dBTB], CP190O/CP190- (lower panel) flies were stained with anti-CP190 (left column) and anti-Mod(mdg4)67.2 (middle column). Shown are the tips of X chromosomes with the y locus (white arrows) and a band near y (yellow arrows). (B) Co-immunoprecipitation of Cp190 and Mod(mdg4)67.2. ImmunobLOTS of anti-Cp190 (top panel) and anti-Mod(mdg4)67.2 (bottom panel). Proteins were immunoprecipitated with anti-Myc from y' ct6, P[Ubi63e::myc-CP190dBTB]/+; CP190O/CP190- pupae (lanes 1 and 2), with anti-Cp190 from y' ct6 pupae (lanes 3 and 4), and with anti-Myc (lane 5) or pre-immune (lane 6) from y' ct6 pupae. Input controls from myc-CP190dBTB (lane 7) and from y' ct6 pupae. (C-D) Anti-Cp190 ChIP of known Su(Hw), CTCF, and BEAF32 loci assayed by Real-Time PCR (percentage of input DNA, n=3), from y' ct6 flies (C) and from ct6, P[Ubi63e::myc-CP190dBTB]; CP190O/CP190- flies (D). The 1A6 region is the negative control [12]. All results were normalized to Fab-8. (E) Distribution of the mRFP-CP190 (red, left column), GFP-CP190dBTB (green, middle column) in a living salivary gland cell nucleus from a 3rd instar larva. An extra-chromosomal space containing GFP-CP190dBTB signals but not mRFP-CP190 signals (yellow arrows). The closer views (bottom row) are crops indicated by the white squares in the upper row. The white arrows point to two bands containing both mRFP-CP190 and GFP-CP190dBTB. (F) Distribution of GFP-CP190BTB-nls (green, left) and mRFP-CP190 (red) in the cell nucleus of a living salivary gland from a 3rd instar larva. The white arrows point an extra-chromosomal space containing GFP-CP190BTB-nls but not mRFP-CP190. The yellow arrows point to two mRFP-CP190 bands on polytene chromosomes that did not have detectable GFP-CP190BTB-nls signals.
(Hw) and Mod(mdg4)67.2, co-precipitated with Cp190 [11]. We precipitated the myc-CP190dBTB protein with anti-MYC from extracts of the y^2 w ct^6, P[Ubi63e::myc-CP190dBTB, mini-w^+/+; CP190^3/TM6B, Tb pupae (Figure 3B, lanes 1 and 2) and detected very weak signals of co-precipitated Mod(mdg4)67.2, in contrast to precipitation of wildtype Cp190 (Figure 3B, lanes 3 and 4). The anti-Myc and anti-Cp190 immunoprecipitation reactions were specific since neither Cp190 nor Mod(mdg4)67.2, in contrast to the CTCF and BEAF32 sites, signals of the three tested Su(Hw) sites (gypsy, IA2, and 62D) are significantly weaker than the signal at Fab-8 (Figure 3D, black bars) and are indistinguishable with the negative control region 1A6, suggesting that the BTB domain is critical for association of Cp190 with the Su(Hw) complexes at these loci, consistent with the results of co-IP experiments and the polytene chromosome staining experiments.

The CP190dBTB protein lacking the BTB domain does not associate with the Su(Hw) complex. We thus tested if the BTB domain is sufficient to associate with insulators. We generated flies carrying the P[Ubi63e::GFP-CP190dBTB-ns, w^+] which encodes the fusion protein containing the GFP and the BTB domain of Cp190 fused to the nuclear localization signal of the Drosophila Transformer protein (GFP-CP190BTB-ns) (Figure 1A and 1C). Distribution of this GFP-tagged Cp190 mutant protein in the cell nucleus is significantly different from that of the mRFP-CP190. First, the GFP-CP190BTB-ns protein localizes to extra-chromosomal spaces but the mRFP-CP190 does not (Figure 3F, white arrows). Second, the GFP-CP190BTB-ns is not present at most of the strong mRFP-CP190 bands on polytene chromosomes in the cell nucleus (Figure 3F, yellow arrows). Third, we could not detect signals of the GFP-CP190BTB-ns protein, stained by the anti-GFP antibody, on the polytene chromosomes spreads (data not shown). These results suggest that the BTB domain alone is not sufficient to associate with the Su(Hw) insulator complexes.

The BTB domain and an Aspartic acid-rich (D-rich) region of Cp190 are sufficient for association with gypsy, CTCF and BEAF32 sites. The predicted protein of CP190^{En15} labeled as CP190dCT(En15) (Figure 1A), contains the BTB and CENT (centrosomal-association) domains, but lacks two of the three zinc fingers and the C-terminal E-rich domain. Genetic tests indicate that the CP190dCT (En15) protein cannot support insulator activity. Loss-of-function CP190 mutations dominantly enhance the effects of the homozygous mod(mdg4)^{76} mutation on gypsy-dependent phenotypes [11]. The CP190^{En15} allele was obtained in a newly conducted genetic screen of EMS-mutagenized flies for dominant enhancers of mod (mdg4)^{76}. The CP190^{En15} mutation dominantly enhances y^2, om^P^1-D11, and ct^5 all three gypsy-dependent

Role of BTB domain in the association of Cp190 with multiple types of Cp190-containing insulator complexes. Cp190 associates with diverse insulators including Su(Hw), CTCF and BEAF32 [14-17]. To more closely investigate the role of the BTB domain in association between Cp190 and the three types of Cp190-containing insulator complexes, we performed chromatin immunoprecipitation (ChIP) assays (Figure 3C, D, and Supplemental Table S2 in Additional file 1). We tested Su(Hw)-associated gypsy loci, IA2 and 62D [12,22,23], CTCF-associated Fab-8, CTCF2, CTCF12, CTCF13, BXC100 and BXC114 loci [17,24], and BEAF32A- or BEAF32B-associated ssc', BEAF-A2, BEAF-A3, BEAF-A3, BEAF-B12, BEAF-B13 and BEAF-B16 loci [25]. We included a site in chromosome locus 1A6 as a negative control [12]. Signals from all loci were normalized to the signal of Fab-8 to reveal the relative strength of association of Cp190 with tested sites in comparison with the association of Cp190 with the Fab-8 region. The results indicate that Cp190 associates with Su(Hw) complexes at gypsy, IA2 and 62D, but not with the 1A6 negative control region (Figure 3C, black bars). Cp190 also associates with CTCF sites at Fab-8, CTCF12, BXC100, BXC114, but not at CTCF2 and CTCF13 (Figure 3C, grey bars). Cp190 binds to BEAF32 sites at ssc', A2, and B16, but not at A3, AB3, B12, and B13 (Figure 3C, white bars). Association with the tested regions is specific and we did not detect these sites in ChIP samples precipitated with pre-immune serum (Supplemental Table S2 in Additional file 1).

We next determined the binding of the myc-CP190dBTB at the Cp190-positive sites and the negative control 1A6 site. The signal of myc-CP190dBTB at Fab-8 is significantly higher than the 1A6 negative control region, suggesting that substantial amounts of the myc-CP190dBTB protein lacking the BTB domain still associates with the Fab-8 region (Figure 3D). The signal of Fab-8 is weaker than those of BXC114, SSC', BEAF-A2 and BEAF-B16. Since the signal of the wild-type Cp190 at Fab-8 is stronger than the signals at BXC114, SS'S, BEAF-A2 and BEAF-B16, the results indicate that the BTB domain contributes partially to the association of Cp190 with Fab-8, although the domain is not critical for the association.
phenotypes in CP190<sup>En15</sup>/+, mod(mdg4)<sup>T6</sup> flies, indicating that the gypsy insulator function is reduced (Figure 4A). Homozygous CP190<sup>En15</sup> is pupal lethal, but we found four halfway eclosed CP190<sup>En15</sup>/CP190<sup>P11</sup> adults that survived for some 18 hours without significant locomotion after removal from the pupal case. The cuticle color of these y<sup>2</sup> w ct<sup>6</sup>; CP190<sup>En15</sup>/CP190<sup>P11</sup> adults was darker than the y<sup>2</sup> w ct<sup>6</sup> flies (Figure 4B, upper panel) and the wings had fully developed margins (Figure 4B lower panel), indicating that the gypsy insulator was non-functional.

Although the gypsy insulator is non-functional in CP190<sup>En15</sup> flies, the CP190dC(En15) protein is still present at gypsy insulators. CP190dC(En15) binds polytene chromosomes (Figure 5A, top) and colocalizes with the Su(Hw) protein at the y locus in y<sup>2</sup> mutants (Figure 5A, white arrows). CP190dC(En15) also co-localizes with Su (Hw) and Mod(mdg4)67.2 proteins in diploid cells (Figure 5B). In contrast, the CP190dC(En15) protein is no longer present at the y locus of the y<sup>2</sup> polytene chromosome in the mod(mdg4) mutant (Figure 5A, bottom panel). This result supports the idea that the interaction between the BTB domains of Cp190 and Mod(mdg4) 67.2 contributes to the binding of Cp190 with the Su (Hw) insulator complex. BTB domains often mediate dimers with other BTB-containing proteins, and thus we posit that the Cp190 BTB domain interacts with the Mod(mdg4)67.2 BTB domain and that Mod(mdg4)67.2 recruits Cp190 lacking the C-terminal E-rich domain.

ChIP assays with homozygous CP190<sup>En15</sup> pupae indicate that CP190dC(En15) associates with all sites that bind wild-type Cp190 (Figure 5C and 5D, and Supplemental Table S4 in additional file 1), because the signals of all tested sites were significantly higher than the 1A6 negative control region. The signals at 1A2 and 62D were stronger than Fab-8, whereas in the wild-type Cp190 ChiP results the signals at 1A2 and 62D were weaker than Fab-8 (Figure 3C and 5D). The result suggests that the C-terminal E-rich domain contributes partially to the association of Cp190 with the CTCF complexes at Fab-8.

The CP190dC(En15) protein associates with all Cp190-containing insulator complexes but the GFP-Cp190BTB-nls does not. We thus reasoned that another part of the Cp190 protein in addition to the BTB domain must also be essential for the association. We noticed that there is a D-Rich acidic region between the zinc fingers and the BTB domain. This D-rich region is in the CP190dC(En15) protein, but not in the GFP-Cp190BTB-nls protein (Figure 1A). We generated flies carrying the PuBi::GFP-Cp190BTB-D, w+ which encodes a Cp190 fragment containing both the BTB and the D-rich domain (Figure 1A). GFP-Cp190BTB-D protein localizes to polytene chromosomes as distinct bands and not to extra-chromosomal spaces in living salivary glands (Figure 6). In addition, this GFP-fusion protein co-localized completely with the mRFP-Cp190 on polytene chromosomes (Figure 6j-R). In diploid larval cells, e.g. brain cells and imaginal disc cells, the GFP-Cp190BTB-D protein exists as speckles and co-localizes with mRFP-Cp190 (data not shown). These results indicate that this N-terminal Cp190 fragment is sufficient to associate with most of the Cp190-containing insulator complexes in living cells.

*Figure 4* The C-terminal E-rich domain is essential to Cp190’s insulator function. (A) Genetic assays to test functionality of the gypsy insulator in CP190<sup>En15</sup>. Shown are morphological phenotypes of the y<sup>2</sup> w om<sup>6</sup>b<sup>D11</sup> ct<sup>6</sup>; mod(mdg4)<sup>T6</sup> e female (left, control) and the y<sup>2</sup> w om<sup>6</sup>b<sup>D11</sup> ct<sup>6</sup>; CP190<sup>En15</sup> mod(mdg4)<sup>T6</sup> e mod(mdg4)<sup>T6</sup> e female (right, En15). The En15 fly has a darker abdomen cuticle color (enhanced y<sup>2</sup> phenotype) compared to the control fly. The arrowhead points to the partially suppressed ct<sup>6</sup> wing shape phenotype which lacks some wing margin bristle cells. The arrow points to the fully suppressed ct<sup>6</sup> wing shape (enhanced ct<sup>6</sup> phenotype). The om<sup>6</sup>b<sup>D11</sup><sup>En15</sup> pigmentation pattern of the eye of the control female fly is shown on the upper left and the om<sup>6</sup>b<sup>D11</sup><sup>En15</sup> pigmentation pattern of the En15 female which has expanded white region in the equatorial part of the eye (enhanced om<sup>b</sup>b<sup>D11</sup> phenotype) is shown on the lower right. (B) The body cuticle pigmentation (upper panel) and the wing shape (lower panel) of the CP190<sup>P11</sup>/CP190<sup>P11</sup> mutant (left column) and CP190<sup>P11</sup> flies (right column). Arrows point to different pigmentation of the abdomens.
Although it associates with all Cp190 sites, GFP-Cp190BTB-D, like the CP190dC(En15), is not functional in the insulator complexes and lacks essential Cp190 functions. y2; w+; PcP190 H4-1 flies have the same y2 body cuticle pigmentation and ct6 wing shape phenotypes as the y2 w ct6; P(EN15);GFP-Cp190BTB-D flies (data not shown). The GFP-Cp190-BTB-D transgene also does not rescue the lethality of homozygous CP1903. From at least 500 F1 offspring flies of the y2 w ct6; P(Ubi63e::GFP-Cp190BTB-D, mini-w+); CP1903/TM6B, Tb parents, we obtained no CP1903 homozygous adults.

The mRFP-Cp190 redistributed to extra-chromosomal spaces during heat-shock whereas the CP190BTB-D fragment remained associated.

The heat shock response in the Drosophila melanogaster has been intensively-studied. When fruit flies are stressed with heat, the transcription of most of the normal genes in cells is shut off and newly synthesized...
Figure 6 The N-terminal Cp190 fragment containing the BTB domain and D-rich region colocalizes with the full-length Cp190 protein.

(A-I) The distribution of the GFP-CP190BTB-D (green, B, E, H, M, Q) and the mRFP-CP190 (red, C, F, I, N, R) proteins in the cell nucleus of a living salivary gland. (D-F) and (G-I) are two of the optical sections from the same cell shown in (A-C) and were analyzed by deconvolution algorithm. The same deconvolution processed optical section as (D-F) is marked with the ROI1 (green) and ROI2 (purple) (J). (K-R) The intensity profile chart of the ROI1 (K) and the closer views of ROI1 (L-N). The ROI1 is indicated as a green line in (J) and in (L). The intensity profile chart of the ROI2(O) and the closer views of ROI2 (P-R). The ROI2 is indicated as a purple line in (P) and in (J).
RNA species correspond to a small number of heat-induced genes [26,27]. The phenomenon of global changes in transcription is correlated with increased phosphorylation of the histone H3 Serine 10 (H3S10) at the heat-induced loci and with a sharp decrease of the global level of H3S10 phosphorylation at other loci [28]. We hypothesized that the global changes of transcription may involve changes in chromatin insulators at a global level. We thus monitored the distribution of mRFP-CP190 and GFP-CP190BTB-D proteins in cells of the salivary gland after heat-shock. We found that after 30 minutes of heat shock at 37°C, significant amounts of mRFP-CP190 localized to the extra-chromosomal space (Figure 7A-C, white arrows), although association of the protein with chromosomes was still obvious. After 50 minutes of heat shock, the mRFP-CP190 signals were mostly diffused and the protein was clearly present at extra-chromosomal spaces (Figure 7D-I, arrows). The result indicates that the heat treatment induced dissociation of the Cp190 protein from the originally bound insulator sites on chromosomes. On the other hand, we did not detect significant changes of the distribution of the GFP-CP190BTB-D protein which remained bound to polytene chromosomes as sharp bands and was not detectable in the extra-chromosomal spaces (Figure 7E and 7H, arrow heads).

To determine if Cp190 tightly associates with chromosome without heat-shock treatment, we analyzed the exchange rates of GFP-CP190BTB-D and mRFP-CP190mRFP using the Fluorescence Recovery After Photobleaching (FRAP) technique. We did not detect significant recovery of both GFP-CP190BTB-D and mRFP-CP190 signals in the bleached area two minutes after photobleaching, indicating that no significant exchanges of the two Cp190 proteins within two minutes on chromosomes (Figure 7), K, N, O, R, S, white arrows, and Figure 7V left chart, BTBD-NHS and FL-NHS).

In the cells heat-shocked for 30 minutes, we detected signals of extra-chromosomal mRFP-CP190 (Figure 7M). The signals were significantly weaker in the bleached area right after photobleaching (Figure 7Q, and V, right chart), indicating that the extra-chromosomal signals were not background and were real signals representing the mRFP-CP190 molecules which were not associated with chromosomes. The result is consistent with the conclusion above that Cp190 may dissociate from chromosomes in response to a heat-shock treatment.

In contrast with the non-heat-shocked cells, we detected significant recovery of mRFP-CP190 signals in the bleached area within 2 minutes (Figure 7U and 7V, FL-HS), indicating that a fraction of the mRFP-CP190 rapidly moved into the bleached area. The result indicates that the heat-shocked cells contained a fraction of fast-moving mRFP-CP190 which was not present in cells before the heat treatment.

The redistributed mRFP-CP190 molecules in the bleached area were either in extra-chromosomal space where Cp190 may move more freely or were associated with chromosomes during the recovering period. It is noticeable that the distribution pattern of the recovered signals in the bleached area was different from the pattern before photobleaching (Figure 7M and 7U). In most of the bleached area, the signals that reappeared lacked distinct bands. These signals might represent mRFP-CP190 in extra-chromosomal space. However, a few bands reappeared at locations overlapping with bands that existed before photobleaching (Figure 7M and 7U, yellow arrows), implying that the mRFP-CP190 may be exchanged at a higher rate at these locations on chromosomes. All evidence from the heat-shock treatment indicates that a mechanism exists for regulating the association/dissociation of Cp190 with chromosomes. In contrast with the mRFP-CP190, the GFP-CP190BTB-D protein in the heat-shocked gland cells remained bound to chromosomes tightly. We didn’t detect significant recovery of the GFP-CP190BTB-D signal in the bleach area 2 minutes after photobleaching. This result is similar to the non-heat-shocked cells (Figure 7R, T, and 7V left chart BTBD-HS), suggesting that the CP190BTB-D lacking the C-terminal E-rich domain of Cp190 is incapable of responding to the heat-shock treatment and thus remained associating with chromosomes.

**Discussion**

**The BTB domain of Cp190 has multiple essential roles for fly development in addition to the association of Cp190 with the Su(Hw) complex**

Multiple lines of evidence indicate that the BTB domain is required for association of Cp190 with the Su(Hw) insulator complex: (1) the CP190dBTB protein which lacks the BTB domain does not associate with the gypsy insulator sequence in ChIP assays and does not localize to the gypsy site on polytene chromosomes; (2) proteins in the Su(Hw) complex are not co-precipitated with myc-CP190dBTB, but are co-precipitated with wild-type Cp190. Lack of association between the CP190dBTB protein and the Su(Hw) complex at the gypsy insulators in a CP190 mutant may result in defective functionality of the insulator which is also supported by the genetic complementation result that expression of the protein does not rescue the defective gypsy insulator activity in homozygous CP190 mutants. It is likely that the BTB domain interacts with the BTB domain of Mod(Mdg4) 67.2 because Mod(mdg4)67.2 lacking the BTB domain fails to interact with Cp190 in two-hybrid assays and is not functional in vivo [29].
Figure 7 The full sized Cp190 dissociated from polytene chromosomes during heat-shock while the CP190BTB-D fragment remained bound. (A-I) Salivary glands dissected from 3rd instar larvae expressing both mRFP-CP190 and GFP-CP190BTB-D proteins were treated with heat-shock for 30 minutes (A-C), for 50 minutes (D-I). (J-V) Salivary glands without heat-shock (J, K, N, O, R, S) or heat-shock for 30 mins (L, M, P, Q, T, U) were analyzed by the Fluorescence Recovery After Photobleach (FRAP) technique. The distribution of GFP-CP190BTB-D (green, B, E, and H) and the distribution of mRFP-CP190 (red, C, F, I) indicate that mRFP-CP190 dissociated from the chromosomes and was present in extra-chromosomal spaces (arrows). The squares in D, E, and F mark the region that is enlarged in G, H, and I. The arrow heads in G, H, and I points to two randomly sampled bands of GFP-CP190BTB-D on polytene chromosomes. (J-U) Salivary glands without the pretreatment of heat-shock (J, K, N, O, R, S) or heat-shocked for 30 minutes (L, M, P, Q, T, U) were analyzed by the FRAP technique. Images were taken before photobleaching (J, K, L, M), right after photobleaching (N, O, P, Q) and 2 minutes after photobleaching (R, S, T, U). White arrows point to areas that were photobleached. Yellow arrows point to bands reappeared after photobleaching. (V) Quantitative analysis of the fluorescence of GFP-CP190BTB-D (BTBD) and mRFP-CP190 (FL) before photobleaching (white bars), 0 minute (grey bars) and 2 minutes (black bars) after photobleaching from multiple nuclei (n >= 3) in heat-shocked treated (HS) or non-heat-shocked treated (NHS) salivary glands. The Relative Fluorescence Intensity (RFI) of chromosomal regions (left chart) and the RFI of the mRFP-CP190 in HS nuclei in extra-chromosomal regions (right chart).
In addition to the critical role in the association of Cp190 with the Su(Hw) complex, the BTB domain of Cp190 must have other essential roles for viability of flies. This is because the homozygous su(Hw) null is female sterile, however CP190
 flies expressing the GFP-CP190dBTB or myc-CP190dBTB proteins are still inviable, indicating that the CP190dBTB proteins are unable to support at least one function for viability in other Cp190-containing complexes. Both the polytene staining results and ChIP assays from myc-CP190dBTB indicate that the BTB domain is not essential for association with the CTCF or BEAF32 complexes, but quantitatively contributes to the association with these complexes. Thus either the CTCF or BEAF32 complexes containing the myc-CP190dBTB are defective in function or the BTB domain is involved in an activity essential for fly survival but unrelated to the three types of insulators.

The E-rich domain contributes quantitatively to the association of Cp190 with all three types of insulator complexes and is essential for Cp190’s functions

The C-terminal E-rich region is not necessary for the association of Cp190 with all three types of insulator complexes, because the CP190dCT(En15) fragment that lacks the whole E-rich region localizes to all the tested Cp190 wild-type containing Su(Hw), CTCF and BEAF sites in ChIP assays. This conclusion is well supported by the complete co-localization of the GFP-CP190BTB-D fragment with the mRFP-CP190 full-length protein on polytene chromosomes in the living salivary gland cell nucleus. The E-rich domain however may still contribute to the association of Cp190 with the Su(Hw) complex since the Cp190 wild-type protein still associates with the Su(Hw) complex in the mod(mdg4)domain mutant [11], but the CP190dCT(En15) fragment lacking the E-rich region does not. The interaction between the E-rich region and the Su(Hw) protein may stabilize Cp190 in the Su(Hw) insulator complex, although the interaction is not essential for association. More importantly, the E-rich domain is required for the essential function of Cp190 because the homozygous CP190
 fly is lethal and the P[Ub:i::GFP-CP190BTB-D, w+] transgene does not rescue the lethality of the homozygous CP190
 mutant. It is likely that the E-rich domain is required by all the Cp190-containing insulator complexes.

The dissociation of Cp190 with chromosomes is a regulated process and requires the function of the E-rich domain

ChIP-chip results from several groups published recently showed that not all Su(Hw) complexes, CTCF complexes or BEAF32 complexes contain Cp190 [16,17]. We also found that some tested chromatic regions containing CTCF complexes or BEAF32 complexes which were not associated with significant amounts of Cp190. This phenomenon argues that the recruitment of Cp190 to each individual insulator site may be regulated. This view is supported by the dynamic distribution of Cp190 during heat-shock. Significant amounts of mRFP-CP190 may dissociate from bound sites and localize to the extra-chromosomal space, implying that a mechanism exists for regulating the association/dissociation of Cp190 with chromosomes.

Cp190 binds tightly to chromosomes when flies were cultured in normal temperature. We didn’t detect significant exchange of either the full-size Cp190 protein or the CP190BTB-D fragment on chromosomes. In cells treated with heat-shock, the full-size Cp190 protein dissociated from chromosomes and redistributed into the extra-chromosomal space. This indicates that dissociation of Cp190 is a regulated process. In the same heat-shocked cells, CP190BTB-D which lacks the C-terminal part of Cp190 was still tightly bound to chromosomes while the full-size Cp190 dissociated. This phenomenon strongly suggests that the C-terminal part of Cp190 must be essential for the dissociation. A possible mechanism for this phenomenon is that modifications to the C-terminal part of Cp190, for example phosphorylation, would weaken the interaction between Cp190 and other proteins in insulator complexes. Genetic evidence indicates that insulator complexes without Cp190 are not functional. Dissociation of Cp190 therefore may down-regulate activities of insulators thus affecting the expression of local genes. Further characterization of the interactions will be necessary to understand the molecular mechanism through which Cp190 is recruited differently to the insulator complexes at different genetic locations. However since relatively less information about the composition of the CTCF and the BEAF32 complexes is known, more detailed analysis of the molecular interactions will require identification of more components in the two types of chromatin insulator complexes.

Conclusions

We have determined sub-regions of the Cp190 protein required for fly survival, for association with Cp190-containing insulators and for the gypsy insulator activity. The N-terminal CP190BTB-D fragment of Cp190 containing the BTB domain and the D-rich acidic region is sufficient for association with chromosomes. The fragment however is insufficient for insulator activity and for fly survival during development. The middle portion of the Cp190 protein, including the CENT domain which mediates centrosomal localization and the zinc finger domain, is dispensable for critical insulator functions. The C-terminal E-rich acidic region strengthens
association of Cp190 with most insulator sites and is essential for Cp190’s insulator function.

We have shown evidence that dissociation of Cp190 from its bound sites on chromosomes is a regulated process. Cp190 dissociated from chromosomes when cells were treated with heat-shock. In contrast, the CP190BTB-D lacking the E-rich domain did not dissociate from chromosomes during heat-shock, indicating that the E-rich region is required for this dissociation process. Previous findings have demonstrated that the E-rich region is required for this dissociation process. Cp190 full-length cDNA in the pBluescript SK+ vector was mutagenized with the Quickchange XL Mutagenesis Kit (Stratagene) using 5′-gcacaagagacatcagagccaggctttggaggatggc-3′ and 5′-gccccctccaaagctctctcattgagccacgcgcgc-3′ primers. The obtained clone (pSK-.CP190dZnF) with anticipated deletion was confirmed by sequencing. To create the entry clone pENTR. CP190dZnF, the CP190dZnF fragment in pSK-.CP190dZnF was amplified using 5′-ccacacggctacgggcaagccag-3′ and 5′-tagctcctctctccgcgccgc-3′ primers.

Methods
Antibodies
Rabbit and rat anti-Cp190 antibodies were reported previously [11]. A rat anti-CP190BTB-D antibody was used for the immunoblot in Figure 1B. The antibody was generated by immunizing rats (Pocono Rabbit Farm and Laboratory Inc.) with the 6X-His-CP190BTB-D fusion protein purified from the BL21 E. coli transformed with pET15B.CP190BamH1 in which a BamH1 digested CP190 cDNA was inserted in frame into pET15B vector. One of the rabbit anti-Cp190 antibodies was successfully used in immunoprecipitation experiments [11] and in ChIP assays [17]. The rabbit anti-Cp190 antibody was used in the ChIP assays, immunofluorescence stainings of polytene chromosomes, and immunoprecipitation experiments in this study. The rat anti-Mod(mdg4)67.2 polyclonal antibody was reported earlier [30]. The rat anti-actin antibody in immunoblots was purchased from Abcam Co. (ab50591-100). The rabbit-anti-GFP antiserum was raised by immunizing rabbits with purified bacteria-expressed His-GFP protein (Pocono Rabbit Farm and Laboratory Inc.).

The CP190 mutants
P-elements containing CP190 truncations were generated by inserting the full-length or truncated CP190 cDNA fragments into pENTR/D-Topo (Invitrogen) which were subsequently recombined with pUGW or pURW destination vectors [20]. All P-elements obtained were introduced into flies with the traditional germ line transformation procedures and were crossed into CP190 deficient background by classical genetic manipulation. Flies were cultured in 23°C or 26°C environmental chambers.

To generate the P-element encoding the GFP-CP190dBTB, we performed PCR using the full-length CP190 cDNA (LD02352, Research Genetics) as the template and the 5′-caccaggaacatcagagccag-3′ and 5′-tagctcctctctgcgcgc-3′ as the primers. The amplified CP190dBTB fragment was inserted into pENTR/D-Topo vector (Invitrogen) to obtain the entry clone pENTR. CP190dBTB. The pENTR.CP190dBTB was recombined with destination vectors pUMW or pUGW vectors [20] using Clonase II (Invitrogen) to become pUMW. CP190dBTB for generating flies carrying P[Ubi63e::myc-CP190dBTB, w+]/ or pUGW.CP190dBTB for generating flies carrying P[Ubi63e::GFP-CP190dBTB, w+]. To generate the deletion of zinc fingers in the CP190 protein, the CP190 full-length cDNA in the pBluescript SK+ vector was mutagenized with the Quickchange XL Mutagenesis Kit (Stratagene) using 5′-gcaaaaagagcttgagagcagggctttggaggatggc-3′ and 5′-gctctctccaaagctctctcattgagccacgcgcgc-3′ primers. The obtained clone (pSK-.CP190dZnF) with anticipated deletion was confirmed by sequencing. To create the entry clone pENTR. CP190dZnF, the CP190dZnF fragment in pSK-.CP190dZnF was amplified using 5′-ccacacggctacgggcaagccag-3′ and 5′-tagctcctctctccgcgccgc-3′ primers. The resulting fragment was inserted into the pENTR/D-Topo vector (Invitrogen) to generate the entry clone pENTR. CP190dZnF. The insert was subsequently recombined into pUGW [20] using Clonase II to obtain the pUGW. CP190dZnF for generating flies carrying P[Ubi63e::GFP-CP190dZnF, w+]. For flies expressing GFP-CP190BTB-nls fusion protein we performed fusion-PCR to fuse the CP190 cDNA fragment amplified by 5′-ccacacggctacgggcaagccag-3′ and 5′-ctctctctctctccgcgccgc-3′ primers and the cDNA fragment encoding the nuclear localization sequence (NLS) of the Drosophila melanogaster Transformer protein amplified by 5′-gcaaaaagagcttgagagcagggctttggaggatggc-3′ and 5′-gctctctccaaagctctctcattgagccacgcgcgc-3′. The resulting fragment was inserted into the pENTR/D-Topo to obtain the entry clone pENTR.CP190BTB-nls which was subsequently recombined with the destination vector pUGW using Clonase II to obtain the pUGW.CP190BTB-nls which was injected into flies for generating flies carrying the P[Ubi63e::CP190BTB-nls, w+]. For flies expressing the GFP-CP190BTB-D fusion protein, the CP190 cDNA fragment amplified by 5′-ccacacggctacgggcaagccag-3′ and 5′-gcgcaccgtcgcgcgc-3′ was inserted into the pENTR/D-Topo to obtain the entry clone pENTR.CP190BTB-D which was subsequently recombined with the destination vector pUGW using Clonase II to obtain the pUGW.CP190BTB-D which was injected into flies to generate flies carrying P[Ubi63e::GFP-CP190BTB-D, w+]. The fly stocks carrying the CP190ΔM and the CP190ΔM were obtained from Dr. J. W. Raff [19]. All the transgenic lines evaluated are on the second chromosome, except the P[Ubi63e::mRFP-CP190, w+] which were all inserted on the 3rd chromosome. We recombined two independent 3rd chromosome P[Ubi63e::mRFP-CP190, w+] transgenic insertions onto the chromosome containing the CP190ΔM.
mutation. Both transgenic lines express similar amounts of the encoded mRFP-CP190 fusion protein and behaved the same in the genetic complementation assays. The CP190mut mutation on the recombined chromosomes was confirmed by sequencing reactions using endogenous CP190 specific primers (data not shown) and is evident by lack of the wild-type Cp190 protein in the protein lysates prepared from the \( y^2 \) w ct; \[Ubi63e::mRFP-CP190, w+\] CP190+/TM6B, Tb flies and evaluated at least 500 offspring flies and we could not find homozygous CP190mut adults. In contrast, from the control cross containing \( y^2 \) w ct; CP190mut/TM6B, Tb parents, we evaluated 52 adult offspring adult flies and observed 16 homozygous CP190transgene adults. All 13 CP190transgene adults, which is close to the expected Mendelian ratio if the transgene rescues (1/6). All 13 CP190transgene adults, indicating that \( y^2 \) w ct; CP190+/TM6B, Tb parents, we evaluated at least 500 offspring flies and we could not find a single homozygous CP190mut adult.

For the genetic complementation analysis of P[Ubi63e::mRFP-CP190, w+], from the genetic cross of the \( y^2 \) w ct; P[Ubi63e::mRFP-CP190, w+] CP190+/TM6B, Tb and \( y^2 \) w ct; CP190+/TM6B, Tb parents, we evaluated 52 adult offspring adult flies and observed 16 Tb+ homozygous CP190transgene adults. The ratio (16/52) is close to the expected Mendelian ratio (1/3) if the transgene rescues. In contrast, from the control cross containing \( y^2 \) w ct; CP190+/TM6B, Tb parents, we evaluated at least 500 offspring flies and we could not find a single homozygous CP190mutd.

For the genetic complementation analysis of P[Ubi63e::GFP-CP190dZnF, mini-w+], from the genetic cross of the \( y^2 \) w ct; P[Ubi63e::GFP-CP190dZnF, mini-w+/+; CP190+/TM6B, Tb and \( y^2 \) w ct; CP190+/TM6B, Tb parents, we evaluated 112 adult offspring adult flies and observed 16 Tb+ homozygous CP190transgene adults, which is close to the expected Mendelian ratio if the transgene rescues (1/6). All 13 CP190transgene adults were \( w^+ \), indicating that they contain the GFP-CP190dZnF transgene \(( y^2 \) w ct; P[Ubi63e::GFP-CP190dZnF, mini-w+/+; CP190dZnF). For the genetic complementation analysis of P[Ubi63e::myc-CP190dBTB, mini-w+] and P[Ubi63e::GFP-CP190dBTB, mini-w+], three transgenic P[Ubi63e::myc-CP190dBTB, mini-w+] lines and two transgenic P[Ubi63e::GFP-CP190dBTB, mini-w+] lines on the second chromosome were introduced into the CP190+/TM6B, Tb genetic background. We evaluated at least 500 progeny from the P[Ubi63e::myc-CP190dBTB, mini-w+)//+; CP190+/TM6B, Tb parents or the P[Ubi63e::GFP-CP190dBTB, mini-w+)/+; CP190+/TM6B, Tb parents of each transgenic line. We observed at least 10 homozygous CP190transgene larvae and pupae in each line but could not find homozygous CP190transgene adults, indicating that P[Ubi63e::myc-CP190dBTB, mini-w+] and P[Ubi63e::GFP-CP190dBTB, mini-w+] transgenes do not rescue lethality of the homozygous CP190mus.

Chromatin Immunoprecipitation
ChiP was performed from pupae (0.1 g) by dounce homogenization in 1 ml of ice-cold PBSMT (2.5 mM MgCl2, 3 mM KCl, and 0.3% Triton X-100 in PBS) plus protease inhibitors (Complete protease inhibitor tablet cocktail, Roche). Homogenized cells were cross-linked by 1% formaldehyde solution and were sonicated to obtain 200-1000 bps DNA fragments. ChiP was performed using the rabbit anti-Cp190 [11,17], mouse anti-MYC 500 μl (9E, Hybridoma Bank at the University of Iowa), or preimmune serum. For regular PCR analysis, DNA was serial diluted and amplified with gypsy-specific primers 5'-GGCCGCCGAATTCGTGTGCGTTGAATTATTGCACC-AAA-3' 5'-GGTGATGCAATATAAATCTTTTATTG-3' and the Fab-8 specific primers 5'-GGCA CAAATCGTTATTGG-3' and 5'- GCAAGGAGAGTTCCATCC-3'. For Real-Time PCR analysis, the DNA samples were mixed with primers (see Supplement Table S1 in additional file 1) and Fast SYBR Green master mix (Applied Biosystems). The PCR reactions were performed in a Fast 7500 Real-Time PCR system (Applied Biosystems) using a standard program with 60°C annealing temperature and 45 seconds of elongation time.

Microscopy
Polytene chromosome spreads were prepared as described previously [11]. For live salivary gland cell imaging, freshly dissected glands were cultured in the serum-free insect medium (Invitrogen) and were examined immediately. For the heat-shock experiments, 3rd instar larvae containing the transgenes were heated in a 37°C water bath. Salivary glands from heat-treated larvae were dissected in the pre-warmed serum-free insect medium and viewed immediately under microscope. All fluorescent images were taken by the Leica DMI5000 scope and the Leica FX350 camera. The deconvolution analysis was performed with the AF6000 software (Leica) using the standard preset condition. The analysis of FRAP was performed with a confocal microscope (Olympus) and processed by the FluoView software (Olympus). For FRAP experiments, untreated or heat-shock treated (37°C water bath for the desired time) third instar larvae were dissected in a cold serum-free insect medium (Invitrogen). The dissected salivary glands were transferred into a culture chamber and were investigated immediately under the confocal microscope. For quantitative analysis of fluorescence of GFP-CP190BTB-D and mRFP-CP190 before and after photobleaching, the “Relative Fluorescence Intensities” of randomly sampled chromosomal regions or extra-
Characterization of the dominant enhancer phenotypes of CP190<sup>En15</sup>

The CP190<sup>En15</sup> mutation dominantly enhances the effects of the mod(mdg4)<sup>T6</sup> mutation on the y<sup>2</sup>, omb<sup>P1-D11</sup>, and ct<sup>6</sup> all three gypsy-dependent phenotypes (Figure 4A). The altered phenotypes consistently indicate that the gypsy insulator has reduced functionality in the heterozygous CP190<sup>En15</sup> flies: (1) wings of the y<sup>2</sup> w omb<sup>P1-D11</sup> ct<sup>6</sup>; CP190<sup>En15</sup> mod(mdg4)<sup>T6</sup>/mod(mdg4)<sup>T6</sup> flies have a wild-type shape and margins, suggesting a complete loss-of-function of the gypsy insulator at the ct<sup>6</sup> locus (Figure 4A, the fly on the right, arrow), whereas the wings of the y<sup>2</sup> w omb<sup>P1-D11</sup> ct<sup>6</sup>; mod(mdg4)<sup>T6</sup>/mod(mdg4)<sup>T6</sup> flies have underdeveloped margins, indicating that the gypsy insulator at the ct<sup>6</sup> locus in the fly strain is weak but is still functional (Figure 4A, the fly on the left, arrowhead); (2) y<sup>2</sup> w omb<sup>P1-D11</sup> ct<sup>6</sup>; CP190<sup>En15</sup> mod(mdg4)<sup>T6</sup>/mod(mdg4)<sup>T6</sup> flies have more darkly pigmented body cuticle and wings than y<sup>2</sup> w omb<sup>P1-D11</sup> ct<sup>6</sup>; mod(mdg4)<sup>T6</sup>/mod(mdg4)<sup>T6</sup> flies, indicating a weaker gypsy insulator activity in the CP190<sup>En15</sup> mod(mdg4)<sup>T6</sup>/mod(mdg4)<sup>T6</sup> flies; (3) in y<sup>2</sup> w omb<sup>P1-D11</sup> ct<sup>6</sup>; CP190<sup>En15</sup> mod(mdg4)<sup>T6</sup>/mod(mdg4)<sup>T6</sup> flies, the eyes have a wider un-pigmented region in the equatorial part of the eye comparing to that of the y<sup>2</sup> w omb<sup>P1-D11</sup> ct<sup>6</sup>; mod(mdg4)<sup>T6</sup>/mod(mdg4)<sup>T6</sup> flies (Figure 4A, lower right and upper left closer views). The omb<sup>P1-D11</sup> marker is a gypsy-dependent pigmentation pattern in the eyes [31]. The w omb<sup>P1-D11</sup> female flies have evenly pigmented eyes due to a P[lacW] and a gypsy inserted in the optomotor blind (omb) locus. The partially degraded gypsy insulator function in the y<sup>2</sup> w omb<sup>P1-D11</sup> ct<sup>6</sup>; mod(mdg4)<sup>T6</sup>/mod(mdg4)<sup>T6</sup> female flies results in a slightly un-pigmented area in the equatorial part of the eyes (Figure 4A, top left closer view) [11]. The phenomenon of a wider un-pigmented area in the eyes of the y<sup>2</sup> w omb<sup>P1-D11</sup> ct<sup>6</sup>; CP190<sup>En15</sup> mod(mdg4)<sup>T6</sup>/mod(mdg4)<sup>T6</sup> female flies than the y<sup>2</sup> w omb<sup>P1-D11</sup> ct<sup>6</sup>; mod(mdg4)<sup>T6</sup>/mod(mdg4)<sup>T6</sup> female flies suggests that the insulator function in the CP190<sup>En15</sup> mod(mdg4)<sup>T6</sup>/mod(mdg4)<sup>T6</sup> flies is even weaker than in the mod(mdg4)<sup>T6</sup>/mod(mdg4)<sup>T6</sup> flies.

Additional material

Additional file 1: Real-Time PCR analysis of ChIP assays. Primers for the Real-Time PCR analysis of ChIP assays (Table S1). Raw data for the y<sup>2</sup> ct<sup>6</sup> anti-Cp190 ChIP (Table S2), for the myc-CP190dBTB anti-Cp190 ChIP (Table S3), for the CP190D(En15) anti-Cp190 ChIP (S4).

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Authors’ contributions

DO generated four of the constructs for tagged CP190 mutant proteins, including GFP-CP190BTB, myc-CP190dBTB, GFP-CP190dBTB, and CP190dZnF, performed the original Western blot of the Figure 1B, determined the localization of CP190dZnF, CP190d(En15) on polytene chromosomes with immunofluorescence staining in Figure 2B, 3A, and 3A; determined the localization of CP190d(En15) protein in diploid cells in Figure 5B; performed immunoprecipitation experiment and revealed that CP190dBTB does not associated with the Su(Hw)-mod(mdg4)67.2 complex; precipitated the chromatin associated with CP190 in CP190<sup>D</sup>, CP190dBTB, and CP190dZnF flies, and performed the initial analysis of the association of the CP190 with the gypsy insulator complexes in these flies. BS performed the Real-Time PCR analysis of all the tested sites with the ChIP samples, reproduced the Western blot result of Figure 1B; performed the Western blots of Figure 1C, characterized the localization of mRFP-CP190, GFP-CP190dBTB, and GFP-CP190dZnF in Figure 3E and 3F; determined the localization of mRFP-CP190 and GFP-CP190dBTB-D proteins in the cell nucleus. HS generated the GFP-CP190BTB-D expression flies, characterized the localization of GFP-CP190BTB-D in polytene cells. OA determined the localization of CP190DM protein at the y locus on polytene chromosomes. OP generated the CP190<sup>En15</sup> mutant and characterized the gypsy-related phenotypes of this mutant; supervised the progress of the project. All authors read and approved the final manuscript.

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