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Cell

Poly(ADP-ribosyl)ation Regulates Insulator Function and Intrachromosomal Interactions in *Drosophila*

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SUMMARY

Insulators mediate inter- and intrachromosomal contacts to regulate enhancer-promoter interactions and establish chromosome domains. The mechanisms by which insulator activity can be regulated to orchestrate changes in the function and three-dimensional arrangement of the genome remain elusive. Here, we demonstrate that *Drosophila* insulator proteins are poly(ADP-ribosyl)ated and that mutation of the poly(ADP-ribose) polymerase (*Parp*) gene impairs their function. This modification is not essential for DNA occupancy of insulator DNA-binding proteins dCTCF and Su(Hw). However, poly(ADP-ribosyl)ation of K566 in CP190 promotes protein-protein interactions with other insulator proteins, association with the nuclear lamina, and insulator activity in vivo. Consistent with these findings, the nuclear clustering of CP190 complexes is disrupted in *Parp* mutant cells. Importantly, poly(ADP-ribosyl)-ation facilitates intrachromosomal interactions between insulator sites measured by 4C. These data suggest that the role of insulators in organizing the three-dimensional architecture of the genome may be modulated by poly(ADP-ribosyl)ation.

INTRODUCTION

Insulator proteins mediate inter- and intrachromosomal interactions that bring together distant regulatory elements in the genome (Merkenschlager and Odom, 2013; Phillips-Cremins and Corces, 2013; Van Bortle and Corces, 2013). The functional consequences of insulator-mediated chromosomal interactions depend on the location of their binding sites relative to different regulatory elements, epigenetic features, and the presence of other nuclear factors. For example, looping between two insulator sites that separate an enhancer from the promoter of a gene will abolish enhancer-promoter communication and block transcription (Guo et al., 2011; Wood et al., 2011), whereas interactions between two insulator sites that bring an enhancer in close proximity to a promoter facilitate activation of transcription (Guo et al., 2012; Liu et al., 2011; Monahan et al., 2012; Xu et al., 2011).

In addition to, or as a consequence of, their role in regulating specific transcription processes, insulators may also have a more general role in the three-dimensional organization of the genome. Results from high-resolution mapping of intrachromosomal interactions using chromosome conformation capture (3C)-related techniques suggest that the genome may be spatially organized into large topologically associating domains (TADs) (Dixon et al., 2012; Hou et al., 2012; Lieberman-Aiden et al., 2009; Nora et al., 2012; Sexton et al., 2012). Each TAD is demarcated by relatively sharp domain borders and is defined by high frequency of intradomain interactions with limited contacts to other domains across the genome. Insulator proteins are enriched at TAD borders, suggesting that they may play a role in their establishment and thus the physical organization of chromosomes during interphase. In *Drosophila*, TAD borders contain clusters of different insulator proteins named "aligned insulators," whereas discrete insulator sites are enriched inside TADs (Hou et al., 2012; Van Bortle et al., 2012). In vertebrates, in addition to CTCF, TAD borders are enriched in SINE elements and tRNA genes (Dixon et al., 2012), which contain binding sites for the insulator protein TFIIIC (Van Bortle and Corces, 2012). It is then possible that aligned insulators play a role in organizing the genome into domains, whereas single insulator sites inside TADs regulate interactions between regulatory sequences to control the expression of individual genes.

CTCF is the main insulator protein characterized in vertebrates, although recent results suggest that tRNA genes may also have insulator function in human cells (Raab et al., 2012). CTCF binds to specific sequences in the genome and mediates interactions among CTCF insulator sites in a process that is stabilized by cohesin (Parelho et al., 2008; Rubio et al., 2008; Stedman et al., 2008; Wendt et al., 2008). In *Drosophila*, there are several sequence-specific DNA-binding insulator proteins that bind distinct genomic sites, including the *Drosophila* homolog of CTCF (dCTCF), Suppressor of Hairy-wing [Su(Hw)], and boundary element-associated factor (BEAF-32). These DNA-binding proteins recruit two common factors, centrosomal protein 190 (CP190) and Modifier of mdg4 [Mod(mdg4)], which are necessary for insulator activity (Gerasimova et al., 2007; Mohan et al., 2007; Pai et al., 2004). CP190 and Mod(mdg4) contain BTB domains that mediate protein-protein interactions, thereby serving as a bridge to bring together distant insulator sites.

Contacts between distant insulator sites via distinct chromatin loops result in clustering of these sites; a subset of clusters containing a large number of insulator sites can be visualized as ''insulator bodies'' in the nucleus (Gerasimova et al., 2000). Many insulator bodies are present close to the nuclear periphery, and the attachment of insulator complexes to the nuclear lamina has been shown to be important for insulator activity in *Drosophila* (Capelson and Corces, 2005). These observations suggest that insulator function can be controlled, in principle, by regulating the binding of Su(Hw), dCTCF, or BEAF-32 to DNA, by modulating the interactions between these proteins and CP190 and/or Mod(mdg4), or by controlling their interaction with the nuclear matrix, but the mechanisms by which these interactions are regulated have not been explored in detail.

It is plausible that posttranslational modification of insulator proteins may allow them to control interactions between specific sites in the genome in order to regulate different patterns of gene expression. Indeed, the activity of insulator proteins in *Drosophila* has been shown to be modulated by SUMO conjugation and ubiquitination (Capelson and Corces, 2005, 2006). Similarly, vertebrate CTCF undergoes phosphorylation (El-Kady and Klenova, 2005), SUMOylation (MacPherson et al., 2009), and poly(ADP-ribosyl)ation (PARylation) (Yu et al., 2004). PARylation is the catalysis of a negatively charged polymer, poly(ADP-ribose) (PAR), from the donor nicotinamide adenine dinucleotide (NAD^+) onto a target protein by poly(ADP-ribose) polymerase (PARP). Although the activity of the CTCF insulator has been shown to be regulated by PARylation in mammalian cells, the underlying mechanism of this effect and the extent of its consequences on the establishment of inter- and intrachromosomal interactions remain unclear. For example, at the *H19* imprinting control region (ICR), inhibition of PARylation impairs CTCF-mediated maternal imprinting without affecting its DNA binding (Yu et al., 2004). On the other hand, epigenetic silencing of the $p16^{INK4a}$ tumor suppressor gene is associated with defective PARylation of CTCF and the loss of CTCF binding (Witcher and Emerson, 2009).

In this study, we analyze the role of PARylation in the function of *Drosophila* insulator proteins. We find that CP190, dCTCF, Mod(mdg4)2.2, and Su(Hw) are PARylated in vitro and in vivo. Inhibition of PARylation leads to weaker interactions between CP190 and dCTCF, as well as their association with the nuclear lamina. Furthermore, intrachromosomal interactions and nuclear clustering of CP190 are disrupted in *Parp* mutant cells, suggesting that PARylation stabilizes chromatin looping between distant insulator sites. Taken together, the results suggest that PARylation regulates the ability of insulators to organize the *Drosophila* genome by facilitating interactions among insulator sites.

RESULTS

Drosophila Insulator Proteins Undergo Poly(ADP-ribosyl)ation

To explore the possibility that *Drosophila* insulator proteins are modified by PARylation, we immunoprecipitated CP190, Su(Hw), Mod(mdg4)2.2, and dCTCF proteins from *Drosophila* S2 cell extracts. Precipitated fractions were then subjected to western blot analysis, probing first with antibodies that recognize the PAR modification, followed by antibodies against each of the insulator proteins. We detected 190 kDa and 130 kDa PARylated products that correspond to the CP190 and Su(Hw) proteins, respectively (Figures 1A and 1B). Similarly, we observed a 120 kDa PARylated product that corresponds to dCTCF (Figure S1A available online). These results suggest that CP190, dCTCF, and Su(Hw) insulator proteins are PARylated in vivo. Consistent with this finding, dCTCF could be immunoprecipitated with 10H antibody, which specifically recognizes PAR moieties on modified proteins (Figure S1A). Western blot analysis of immunoprecipitated Mod(mdg4)2.2 protein with PAR antibody produced a smear with several distinct bands. One of these bands migrates at about 120 kDa and corresponds to a minor product observed in the western blot probed with antibody to the Mod(mdg4)2.2 isoform, also known as Mod(mdg4)67.2 (Figure 1C, top arrowhead). This result suggests that, unlike CP190 and dCTCF proteins, Mod(mdg4)2.2 may undergo multiple rounds of PARylation. Next, we sought to find whether these insulator proteins can be PARylated in vitro. Glutathione S-transferase (GST)-tagged insulator proteins purified from *E. coli* were subjected to in vitro PARylation using biotinylated NAD+ as a substrate. As a control, GST tag could not be PARylated in vitro (Figure S1B). Both GST-CP190 and GST-dCTCF proteins were PARylated only in the absence of the PARP inhibitor 3-aminobenzamide (3AB) (Figure 1D). The presence of PARylated products that migrate closely to the unmodified proteins (middle and lower panels) suggests that, unlike mammalian CTCF, both CP190 and dCTCF proteins only undergo a single round of PARylation (Figure 1D). Consistent with in vivo data, GST-Mod(mdg4)2.2 and GST-Su(Hw) proteins can also be PARylated in vitro (Figure S1B).

PARylation was previously shown to occur at a novel poly(ADP-ribose)-binding zinc finger (PBZ) motif in a number of eukaryotic proteins that are involved in the DNA damage response and checkpoint regulation (Ahel et al., 2008). Analyses of insulator protein sequences suggest that CP190, and to a lesser extent dCTCF, may contain similar PBZ motifs (Figure S1C). To test whether this putative PBZ domain plays an active role in PARylation of CP190 and dCTCF, we substituted lysine 566 (K566) of CP190 and lysine 434 (K434) of dCTCF with alanine residues by site-directed mutagenesis. In vitro PARylation of CP190 is severely impaired when K566 is mutated, indicating that CP190 contains a similar PBZ motif crucial for this posttranslational modification (Figure 1E). On the other hand, mutation of K434 of dCTCF has no consequence on PARylation (data not shown), consistent with the report that PARylation of CTCF occurs specifically in the N-terminal region (Farrar et al., 2010). The in vivo role of the K566 residue was then examined by studying the biochemical properties of transiently expressed myc-tagged wild-type (WT) and K566A mutant CP190 in S2 cells (Figure S1D). Comparable expression of WT and K566A myc-tagged CP190 protein was observed in S2 cells 48 hr after calcium phosphate transfection (Figure S1D). Consistent with in vitro assays (Figure 1E), the PAR moiety was detected on WT, but not on K566A CP190, protein after immunoprecipitation (IP) with myc antibody (Figure 1F), confirming that lysine 566 is required for PARylation in vivo.

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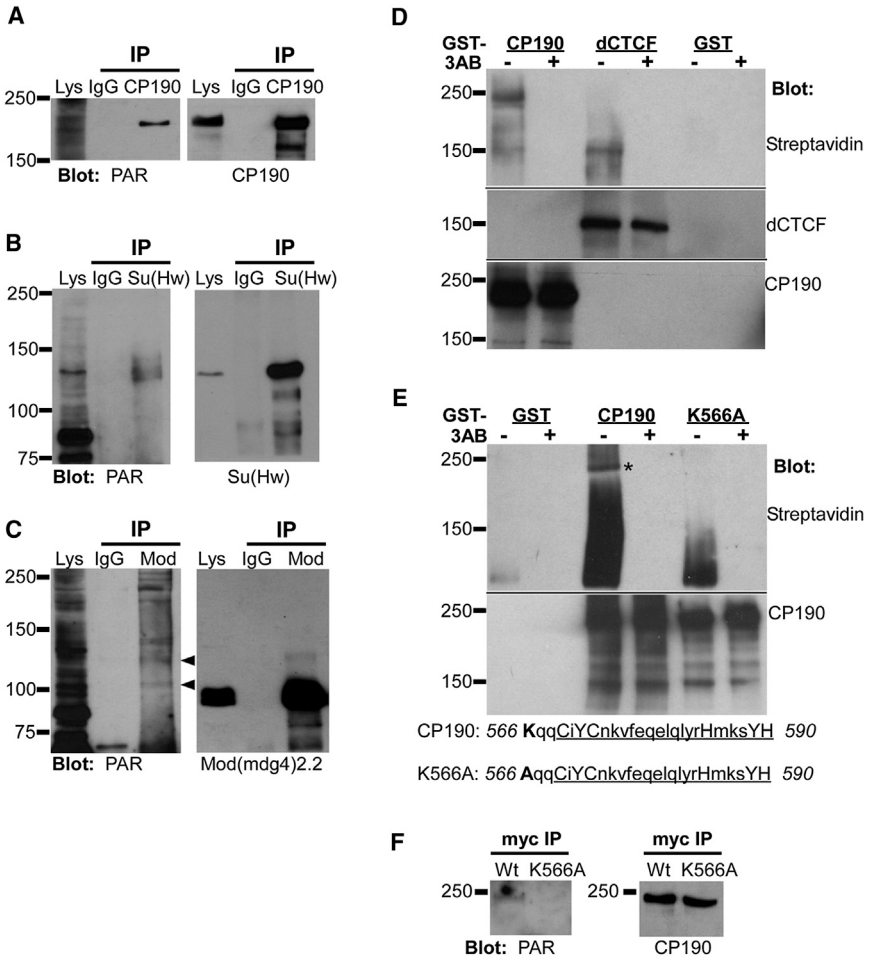

Figure 1. *Drosophila* Insulators Undergo PARylation In Vivo and In Vitro

(A–C) CP190, Su(Hw), and Mod(mdg4)2.2 are PARylated in S2 cells. Cell lysates (Lys) were immunoprecipitated with either preimmune serum (IgG) or antibodies that recognize different insulator proteins. The various fractions were subjected to western analysis with PAR antibody followed by antibodies to different insulator proteins. Black arrowheads point to the possible PARylated form of Mod(mdg4)2.2 protein.

(D) CP190 and dCTCF can be PARylated in vitro. GST, GST-tagged CP190, and GST-tagged dCTCF were PARylated in vitro using biotin-NAD+ as a substrate in the presence or absence of 3AB. In vitro products were western blotted with Streptavidin-HRP, followed by dCTCF and CP190 antibodies.

(E) The K566 residue within the putative PBZ domain is essential for PARylation of CP190. GST, GST-tagged CP190, and GST-tagged CP190:K566A proteins were PARylated in vitro and western blotted with streptavidin-HRP and CP190 antibody. Asterisk indicates the location of CP190 in the gel. The sequence of the PBZ domain and the location of the K566A mutation are indicated at the bottom of the panel.

(F) CP190:K566A protein is not PARylated in vivo. Lysates from S2 cells transfected with WT or K566A mutant (KA) CP190-myc constructs were immunoprecipitated with myc antibody and probed with PAR and CP190 antibodies.

See also Figure S1.

Mutation of *Parp* Affects the Function of the *gypsy* and *Fab-8* Insulators

We next tested whether a mutation of the *Parp* gene in $Parp^{CH1}$/+ flies alters insulator function using two reporter mutant strains, *yellow-2* (y^2) and *cut-6* (ct^6). These strains were generated by the insertion of the *gypsy* retrotransposon, which contains multiple Su(Hw) binding sites, between the enhancer and promoter sequences of the *yellow* and *cut* genes. Binding of Su(Hw), Mod(mdg4)2.2 and CP190 proteins to the *gypsy* transposon blocks the communication between these regulatory elements. As a result, adult y^2ct^6 flies have light abdominal pigmentation and cut wing margins (Figures 2A and 2B). Normal pigmentation is partially restored in hypomorphic $CP190^{4-1}/CP190^{H312}$ mutant flies (Pai et al., 2004), which show darker pigmentation owing to intermediate y^2 expression (Figure 2A). In y^2 flies that are heterozygous $Parp^{CH1}$/+, the majority of the animals also exhibit darker abdominal pigmentation. Combination of either the $CP190^{4-1}/CP190^{H312}$ or $mod(mdg4)^{T6}$ mutations with $Parp^{CH1}$/+ further reduces insulator activity, with more flies exhibiting the darkest pigmentation (Figures 2A and S1E). Similarly, expression of the *cut* gene is partially rescued in $mod(mdg4)^{T6}$ mutants, which show one or multiple notches in the wing margin (Figure 2B). Although $Parp^{CH1}$/+ did not suppress the ct^6 phenotype, the majority of $mod(mdg4)^{T6}/mod(mdg4)^{T6}$; $Parp^{CH1}$/+ double mutants have round wing margins, indicative of impaired insulator activity (Figure 2B). Taken together, these results show that PARylation is required for proper Su(Hw) insulator function in vivo.

We next asked whether mutation of the *Parp* gene would also affect the activity of the *Fab-8* insulator, which requires the dCTCF and CP190 proteins for function (Gerasimova et al., 2007; Mohan et al., 2007). The $Fab\text{-}8^{60.39.2}$ reporter line carries a transgene that includes the *Fab-8* insulator positioned between the eye enhancer and the coding region of the *white* gene (Barges et al., 2000). The presence of the *Fab-8* insulator blocks enhancer-promoter communication, resulting in lower *white* expression and flies with light orange eyes. Male $CTCF^{y+6}$/+ mutant flies have red eyes, suggesting that loss of a copy of the *dCTCF* gene is sufficient to abolish *Fab-8* insulator activity at the *white* locus (Figure 2C). Similarly, $Parp^{CH1}$/+ flies exhibit red eye coloration in all *Fab-8* reporter lines (Figures 2C, 2D, and S1F). This result suggests that PARylation is also required for the activity of the dCTCF insulator at the *Fab-8* site.

Because PARylation regulates the function of many nuclear proteins, it remains possible that the effects of mutating and inhibiting Parp activity on CP190 function may be an indirect consequence of impairing other nuclear processes. To rule out this possibility, we examined insulator activity in strains

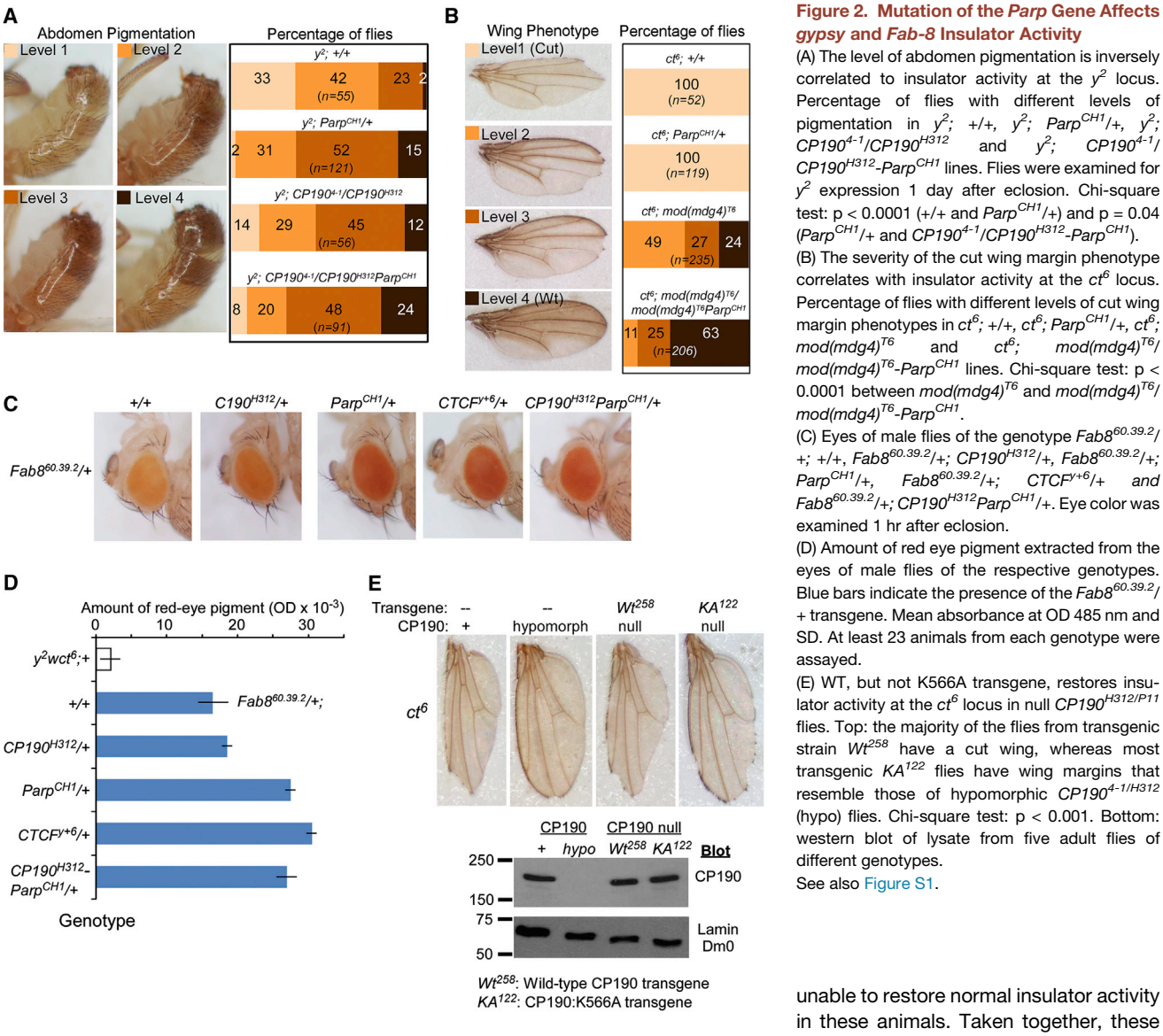

Figure 2. Mutation of the *Parp* Gene Affects *gypsy* and *Fab-8* Insulator Activity

(A) The level of abdomen pigmentation is inversely correlated to insulator activity at the y^2 locus. Percentage of flies with different levels of pigmentation in y^2; +/+, y^2; $Parp^{CH1}$/+, y^2; $CP190^{4-1}/CP190^{H312}$ and y^2; $CP190^{4-1}/CP190^{H312}$-$Parp^{CH1}$ lines. Flies were examined for y^2 expression 1 day after eclosion. Chi-square test: $p < 0.0001$ (+/+ and $Parp^{CH1}$/+) and $p = 0.04$ ($Parp^{CH1}$/+ and $CP190^{4-1}/CP190^{H312}$-$Parp^{CH1}$).

(B) The severity of the cut wing margin phenotype correlates with insulator activity at the ct^6 locus. Percentage of flies with different levels of cut wing margin phenotypes in ct^6; +/+, ct^6; $Parp^{CH1}$/+, ct^6; $mod(mdg4)^{T6}$ and ct^6; $mod(mdg4)^{T6}/mod(mdg4)^{T6}$-$Parp^{CH1}$ lines. Chi-square test: $p < 0.0001$ between $mod(mdg4)^{T6}$ and $mod(mdg4)^{T6}/mod(mdg4)^{T6}$-$Parp^{CH1}$.

(C) Eyes of male flies of the genotype $Fab8^{60.39.2}$/+; +/+, $Fab8^{60.39.2}$/+; $CP190^{H312}$/+, $Fab8^{60.39.2}$/+; $Parp^{CH1}$/+, $Fab8^{60.39.2}$/+; $CTCF^{y+6}$/+ and $Fab8^{60.39.2}$/+; $CP190^{H312}Parp^{CH1}$/+. Eye color was examined 1 hr after eclosion.

(D) Amount of red eye pigment extracted from the eyes of male flies of the respective genotypes. Blue bars indicate the presence of the $Fab8^{60.39.2}$/+ transgene. Mean absorbance at OD 485 nm and SD. At least 23 animals from each genotype were assayed.

(E) WT, but not K566A transgene, restores insulator activity at the ct^6 locus in null $CP190^{H312/P11}$ flies. Top: the majority of the flies from transgenic strain Wt^{258} have a cut wing, whereas most transgenic KA^{122} flies have wing margins that resemble those of hypomorphic $CP190^{4-1/H312}$ (hypo) flies. Chi-square test: $p < 0.001$. Bottom: western blot of lysate from five adult flies of different genotypes.

See also Figure S1.

carrying a transgene expressing the CP190 K566A mutation. We used P-element-mediated transformation to obtain four independent strains expressing either a WT or K566A CP190 mutant transgene and assayed their effects on the ct^6 phenotype as described above (Figure S1G). Decrease of insulator activity in hypomorphic $CP190^{4-1}/CP190^{H312}$ mutant flies allows the expression of the *cut* gene, resulting in the formation of a round wing margin (Figures 2E and S1H). The majority (65% or more) of the flies from two WT CP190 transgenic lines exhibit severe notches in their wing margin (Figures 2E and S1H), implying that the WT CP190 transgene successfully restores the insulator activity in *CP190* mutant flies and blocks the expression of the *cut* gene. On the other hand, despite comparable levels of CP190 expression, the majority of the flies from two independent CP190:K566A transgenic lines have a more rounded wing margin resembling that of *CP190* mutant flies (Figures 2E and S1H). This indicates that CP190:K566A is unable to restore normal insulator activity in these animals. Taken together, these results demonstrated that PARylation of the lysine 566 residue of CP190 is required for proper in vivo insulator function.

PARylation Facilitates Interactions between Insulator Proteins

The results described above suggest that the activity of *Drosophila* insulators is regulated by PARylation of the K566 residue of CP190. Consistent with this hypothesis, CP190 and Parp proteins colocalize at many genomic sites on polytene chromosomes (Figure S2A). To further understand the underlying mechanism of this regulation, we examined the effect of blocking PARylation on insulator proteins in *Drosophila* S2 cells. Cells treated with 3AB exhibit morphological changes and turn from a semiadherent spherical shape to fully adherent cuboidal or fibroblast-like cells (Figure S2B). To confirm the effectiveness of drug inhibition by 3AB, we carried out immunoprecipitation of cellular lysates with 10H antibody, which specifically

recognizes PAR moieties on modified proteins. Western blot analysis of immunoprecipitated products with PAR antibody indicates that proteins from cells treated with 3AB are significantly less PARylated when compared to control cells (Figure S2C). Moreover, western blot analysis of CP190 protein immunoprecipitated from cellular lysates with PAR antibody indicates that CP190 is also less PARylated in cells treated with 3AB (Figure S2D). Because PARylation has been reported to regulate transcription (Krishnakumar et al., 2008), we compared the level of insulator proteins between control and 3AB-treated S2 cells. Western analyses indicate that the levels of CP190, Mod(mdg4)2.2, Su(Hw) and dCTCF are unaffected in cells treated with 3AB (Figure S2E). Consistent with this result, there is no significant reduction in the level of CP190 and Mod(mdg4)2.2 on polytene chromosome isolated from *Parp* mutant larvae (Figure S2F).

The in vivo function of the *gypsy* and *Fab*-8 insulators in *Drosophila* requires interactions between individual insulator sites. It is possible that PARylation may affect insulator activity by modulating protein-protein interactions among insulator proteins. To test this, we immunoprecipitated CP190 from cell lysates of control and 3AB-treated S2 cells and examined whether its association with other insulator proteins is regulated by PARylation. CP190 and dCTCF are specifically pulled down by CP190 antibody and not by preimmune serum, and there is a considerable reduction (~40%–60%) of dCTCF protein pulled down with CP190 upon 3AB treatment (Figure 3A, n = 6). To ensure that PARylation is directly responsible for this outcome, we examined the effect of the K566A mutation on the interaction between CP190 and dCTCF in S2 cells expressing myc-tagged WT and K566A mutant CP190. Results indicate that CP190:K566A myc-tagged protein showed a significant reduction in its interaction with endogenous dCTCF protein (Figure 3B), suggesting that PARylation of CP190 at K566 promotes interaction between dCTCF and CP190 proteins in vivo.

PARylation Facilitates Interactions of Insulator Proteins with the Nuclear Matrix

PARP-1 has been reported to associate with the nuclear lamina (Vidaković et al., 2004). Binding of the *gypsy* insulator to the nuclear lamina via dTopors (Capelson and Corces, 2005) and the association of vertebrate CTCF with nucleophosmin and the nuclear matrix (Yusufzai et al., 2004) suggest that tethering of insulator elements to subnuclear sites may be a common strategy used by insulator proteins for nuclear organization. To test whether PARylation of *Drosophila* insulator proteins affects their association with the nuclear matrix, we examined the effect of 3AB treatment on this process. We detect enrichment of different insulator proteins in the nuclear matrix, which is characterized by the presence of Lamin Dm0 and the absence of histones (Figure 3B) (Kallappagoudar et al., 2010). When PARylation is inhibited, the nuclear matrix localization of CP190, Su(Hw), dCTCF, and Mod(mdg4)2.2 is significantly reduced (Figures 3C and S2G), whereas the level of these proteins remains unaffected in the soluble nuclear fraction (Figures 3C and S2G). To rule out possible pleiotropic effects caused by 3AB inhibition, we knocked down *Parp* using double-stranded RNA (dsRNA). There is a significant reduction in the level of Parp and PARylated proteins in S2 cells after dsRNA treatment (Figure S2H). Consistent with other results, protein-protein interactions between CP190 and dCTCF (Figure S2I), as well as the association of insulator proteins with the nuclear lamina (Figure S2J), are significantly impaired in *Parp* knockdown cells. Taken together, these data indicate that the enzymatic activity of Parp is required for optimal complex formation of insulator proteins at the nuclear lamina, a process that is necessary for insulator activity (Capelson and Corces, 2005).

DNA Occupancy of Insulator Proteins at a Subset of Genomic Sites Is Reduced upon Inhibition of PARylation

PARylation has different effects on the ability of proteins to bind DNA. Therefore, comparison of the genome-wide distribution of insulator proteins in control and 3AB-treated S2 cells may provide insights into the actual mechanism by which PARylation regulates insulator function in *Drosophila*. To examine this question, we mapped the genome-wide occupancy of *Drosophila* insulator proteins CP190, Su(Hw), dCTCF, and Mod(mdg4)2.2 in control and 3AB-treated S2 cells by chromatin IP followed by deep sequencing (ChIP-seq). Interestingly, inhibition of PARylation does not have a general effect on the genome-wide localization of these proteins (Figures 4A, S3A, and S3B). Instead, only a subset of insulator sites is affected by inhibition of PARylation (Figure S3B). To more accurately determine the effect of PARylation on the DNA binding patterns of insulator proteins, we examined the fold differences of the normalized raw signals between control and 3AB-treated samples. Inhibition of PARylation results in a loss or greater than 2-fold decrease in DNA binding at 650 CP190 sites, 311 CTCF sites, 227 Mod(mdg4)2.2 sites, and 56 Su(Hw) sites. On the other hand, only 12 CP190 sites, 9 CTCF sites, and 84 Mod(mdg4)2.2 sites exhibit new or increased binding after 3AB treatment (Figure 4B). These 3AB-responsive sites contain the same consensus sequence as other insulator sites in the genome (Figure S3C). Validation of ChIP-seq data by quantitative PCR (qPCR) confirmed reduced binding of CP190 at specific genomic sites upon drug inhibition (Figure S3D). Of the 650 3AB-downregulated CP190 binding sites, 64 overlap with 3AB-responsive Mod(mdg4)2.2 sites, and 21 overlap with affected Su(Hw) and Mod(mdg4)2.2 sites (Figure 4B), suggesting that insulator components may be coregulated by PARylation at selected genomic sites, although modification of just one insulator protein may be sufficient to disrupt its function. The small overlap between the 3AB-downregulated CP190 and dCTCF sites also suggests that PARylation may regulate binding of these two proteins independently at distinct genomic locations. Only one site (asterisk) shows reduced binding of all four insulator proteins upon 3AB inhibition (described as bait A below). To ensure that these effects are directly caused by alteration in PARylation of insulator proteins, we asked whether binding of the myc-tagged CP190:K566A mutant protein at 3AB-responsive CP190 sites may also be impaired by performing ChIP with myc antibody in S2 cells expressing this mutant protein. We observed significant reduction in the occupancy of CP190:K566A protein at several 3AB-responsive CP190 sites (Figure S3E). As a control, we did not see significant differences between the occupancy of WT and K566A mycCP190 proteins at CP190 sites unaffected by 3AB treatment (Figure S3E).

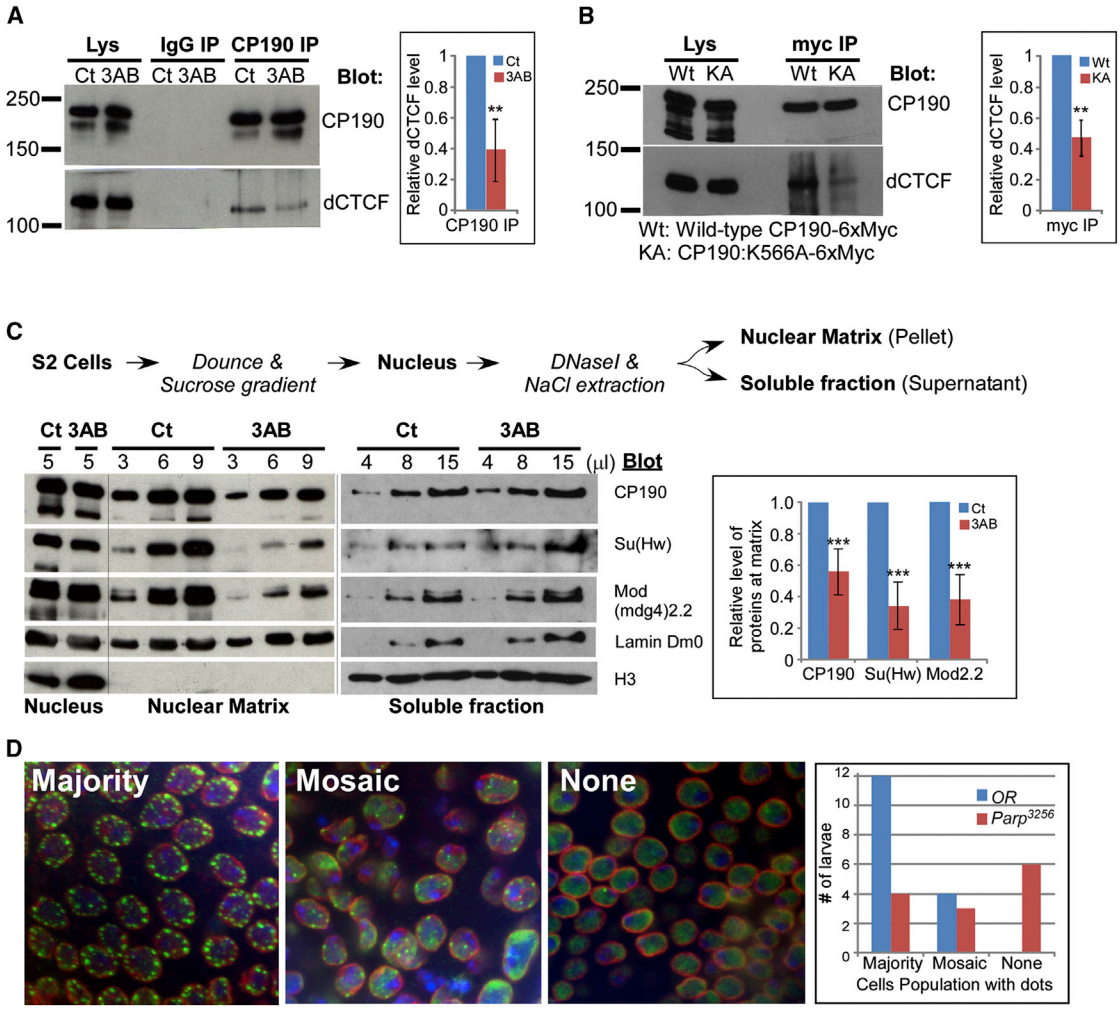

Figure 3. Interaction between Insulator Proteins and Their Association with the Nuclear Lamina Are Stabilized by PARylation

(A) PARylation stabilizes interactions between CP190 and dCTCF proteins. Lysate (Lys) from control (Ct) and 3AB-treated cells was immunoprecipitated with preimmune serum (IgG) or CP190 antibody (CP190). These fractions were subjected to western blot analysis with CP190 and dCTCF antibodies. Quantification of the relative level of dCTCF protein that was pulled down by CP190 antibody and SD from six independent experiments (**$p < 0.005$).

(B) CP190:K566A protein interacts weakly with dCTCF in S2 cells. Lysates from cells transfected with WT or KA construct were immunoprecipitated with myc antibody and probed with CP190 and dCTCF antibodies. Quantification of the relative level of dCTCF protein that was pulled down by myc antibody and SD from three independent experiments (**$p < 0.008$).

(C) PARylation promotes the association of insulator proteins with the nuclear lamina. Nucleus, nuclear matrix, and soluble fractions isolated from control and 3AB-treated cells were western blotted with CP190, Su(Hw), Mod(mdg4)2.2, Lamin Dm0, and histone H3 antibodies. Mean band intensities quantified by ImageJ software and SD from at least five independent experiments (***CP190, $p = 0.0004$, $n = 6$; Su(Hw), $p = 0.00001$, $n = 7$; Mod(mdg4)2.2, $p = 0.0006$, $n = 5$).

(D) Formation of CP190 insulator bodies is impaired in *Parp*^{03256} mutant larvae. Immunolocalization of CP190 (green) and Lamin Dm0 (red) in diploid nuclei from imaginal wing disc cells with DNA stained by DAPI (blue). Histogram depicting the distribution of the nuclear CP190 staining pattern in *OR* (WT) and *Parp*^{03256} mutant larvae.

See also Figure S2.

PARylation Facilitates the Binding of CP190, dCTCF, and Mod(mdg4)2.2 at Independent Insulator Sites

In order to obtain further insights into the nature of the insulator sites affected by PARylation, we examined their location with respect to various genomic features. Although dCTCF, CP190, and Mod(mdg4)2.2 binding sites are enriched at transcription start sites (TSSs), 3AB-responsive insulator sites are preferentially located 0.4 to 1.4 kb upstream of TSSs (Figures 4C and S3F). Although most genomic CP190 sites are within 8 kb of one another, the distance between adjacent affected CP190 sites is >13 kb (Figure 4D). Similarly, the distance between 3AB-downregulated dCTCF and CP190 sites is greater than the average distance between genomic dCTCF and CP190 sites (Figure S3G). These results suggest that 3AB-responsive insulator binding sites do not cluster with one another and may not correspond to aligned insulators located at TAD borders.

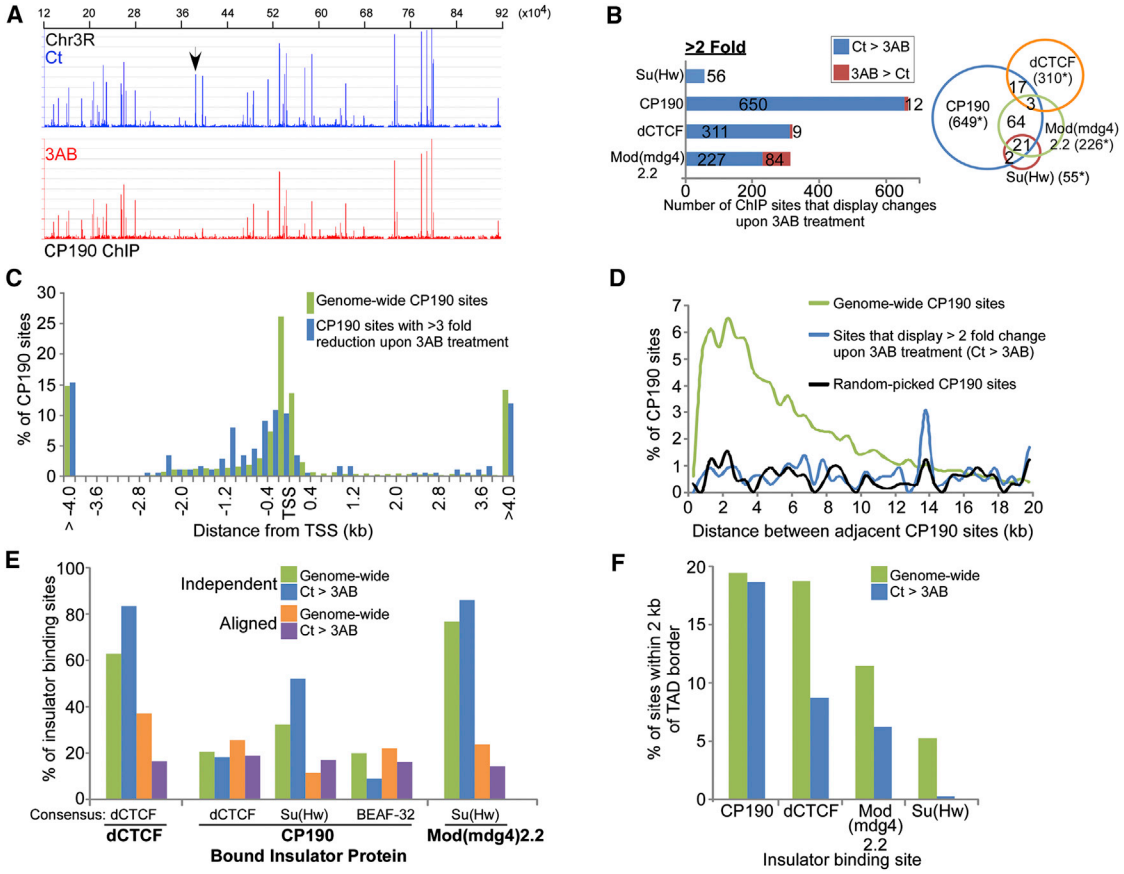

Figure 4. A Subset of Insulator Binding Sites Is Regulated by PARylation

(A) Comparison of CP190 ChIP-seq peaks across an 840 kb region of *Drosophila* chromosome 3R in control (Ct) and 3AB-treated S2 cells. Arrow indicates site at which CP190 binding is disrupted by 3AB treatment.

(B) Graphs representing the number of Su(Hw), CP190, dCTCF, and Mod(mdg4)2.2 binding sites that exhibit greater than 2-fold changes between Ct and 3AB samples (left). Right: Venn diagram of the overlap between different 3AB-downregulated insulator sites. The asterisk represents a site where binding of four insulator proteins was reduced by 3AB treatment.

(C) Distribution of genomic CP190 binding sites (green) and sites with more than 3-fold reduction in the 3AB treated cells (blue) with respect to TSS. Each interval on the x axis represents a 200 bp window.

(D) Graph representing the distance between adjacent genomic (green), 3AB-downregulated (blue), and randomly picked (black) CP190 sites.

(E) Percentage of independent and aligned insulator binding sites affected by 3AB treatment. Genome-wide refers to insulator binding sites that contain consensus motifs and are bound by different insulator proteins. Ct > 3AB refers to 3AB-downregulated insulator binding sites.

(F) Percentage of genome-wide and 3AB-downregulated insulator sites that are within 2 kb of TAD borders.

See also Figure S3.

Drosophila TADs are demarcated by clusters of dCTCF, Su(Hw), BEAF-32, Mod(mdg4), and/or CP190 insulator proteins, suggesting that aligned sites, defined by the presence of at least two sequence-specific DNA-binding insulator proteins and CP190, may play a more critical role in maintaining domain boundaries than independent sites, which are bound by only one DNA-binding insulator protein (Van Bortle et al., 2012). We therefore sought to find out whether 3AB-responsive insulator protein binding occurs at aligned or independent sites. Inhibition of PARylation preferentially reduces the binding of dCTCF, CP190, and to a lesser extent, Mod(mdg4)2.2 at independent insulator sites (Figure 4E). In accordance, most 3AB-downregulated dCTCF, Mod(mdg4)2.2, and Su(Hw) sites are located away (>2 kb) from TAD borders when compared to their genomic counterparts (Figure 4F). In addition, results from *k*-means clustering analysis suggest that CP190 sites susceptible to PARylation tend to reside within H3K27me3-enriched genomic regions (Figure S3H). Therefore, these results suggest that PARylation preferentially promotes the binding of insulator proteins at specific independent sites within TADs.

Mutation of *Parp* Affects the Formation of Insulator Bodies

PARylation may regulate insulator activity at specific sites in the genome by promoting long-range interactions between distant insulator sites. In diploid cells, interactions between distant CP190 sites lead to chromatin looping and the formation of insulator bodies, which correlates with normal in vivo insulator

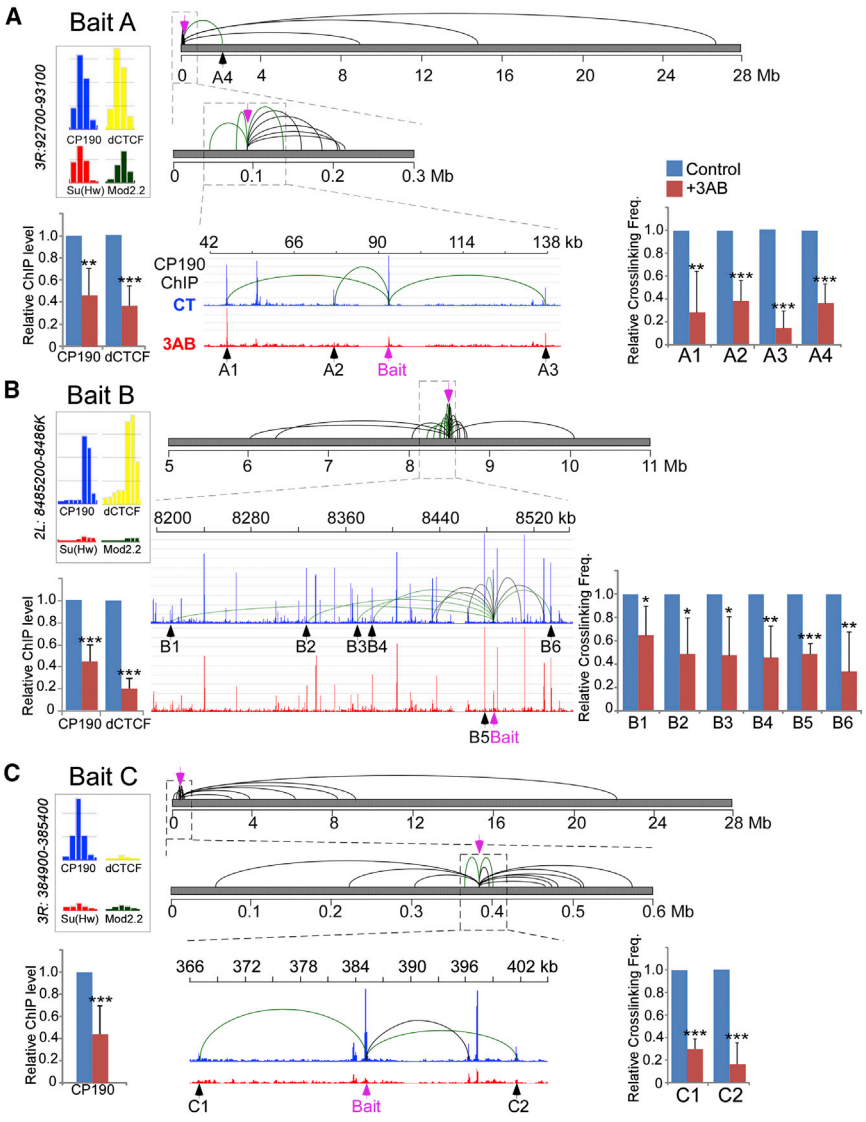

Figure 5. Intrachromosomal Interactions between Specific Distant CP190 Binding Sites Are Regulated by PARylation

(A) Bait A represents an aligned insulator site with dCTCF and Su(Hw) consensus motifs. Left: ChIP signal of four insulator proteins in control cells (top). Relative ChIP-qPCR of CP190 and dCTCF at the bait and the SD from four independent experiments (bottom). Right: graphical depiction of the intrachromosomal interactions between bait A and 12 CP190 binding sites in control cells. 3AB-responsive interactions are represented by a green line. Middle: ChIP signal of CP190 surrounding bait A in control (CT) and 3AB-treated cells. Graph of relative crosslinking frequency between bait A and the four affected sites in control and 3AB samples and SD from four independent experiments.

(B) Bait B represents an independent dCTCF site that is bound by CP190 protein, ChIP-qPCR validation, and SD from four independent experiments (left). 19 intrachromosomal interactions between the bait B fragment and other CP190 binding sites were validated in control cells (middle); 6 of these interactions were reduced by 3AB treatment with error bars indicating the SD from 4 independent experiments (right).

(C) Bait C represents a class of CP190 binding sites devoid of DNA consensus motifs for BEAF-32, Su(Hw), and dCTCF proteins. Left: validation of CP190 binding at bait C by ChIP-qPCR with SD from seven independent experiments. 18 intrachromosomal interactions between bait C and other CP190 binding sites were validated in control cells (middle) of which two interactions were downregulated by 3AB. Error bars indicate SD from four independent experiments (right).

$^{*}p < 0.05$, $^{**}p < 0.02$, and $^{***}p < 0.005$.

See also Figures S4 and S5.

function (Capelson and Corces, 2005; Lei and Corces, 2006). If PARylation is necessary for mediating long-range interactions, we would expect either reduction or disruption in the formation of CP190 insulator bodies in *Parp* mutant animals. To explore this possibility, we examined the presence of CP190 insulator bodies in wing imaginal discs isolated from WT *Oregon R* and $Parp^{C03256}$ mutant flies (Kotova et al., 2010). $Parp^{C03256}$ is a hypomorphic allele that expresses a short isoform of the Parp protein lacking the first zinc finger. In imaginal wing discs dissected from WT larvae, the majority of the cells contain multiple insulator bodies detected with CP190 antibodies (Figure 3D, left), whereas a small fraction of imaginal discs show a mosaic pattern: ~50% of the cells contain insulator bodies, and the rest do not. On the other hand, imaginal discs from 6 out of 13 *Parp* mutant larvae examined were completely devoid of insulator bodies, whereas 3 exhibited a mosaic pattern in the wing imaginal discs. Four of the mutant wing discs analyzed contained insulator bodies in the majority of their cells. Because 27% of $Parp^{C03256}$ mutant animals survive to pupae (Kotova et al., 2010), it is possible that these escapers have residual levels of PARylation during the larval stage, explaining the lack of complete penetrance in the insulator body phenotype. The results suggest that PARylation may play an important role in mediating clustering between distant CP190 sites to form insulator bodies.

PARylation Is Required for Intrachromosomal Interactions Mediated by Insulator Proteins

To further explore the role of PARylation in mediating interactions between distant insulator sites, we used circular chromosome conformation capture (4C) experiments (Göndör et al., 2008). Bait fragments were selected at sites at which binding of CP190 is significantly disrupted by 3AB treatment. Baits containing different combinations of insulator DNA-binding proteins were then chosen, and the ChIP-seq signal was confirmed by qPCR (Figures S4 and 5). Bait A, located on chromosome arm 3R between positions 91,997 and 92,920, is an example of an

aligned insulator site that contains Su(Hw) and dCTCF proteins, which in turn recruit CP190 and Mod(mdg4)2.2. Binding of the four insulator proteins was reduced by 3AB inhibition (Figures 5A and S4, left). Bait B, located on chromosome arm 2L between positions 8,485,115 and 8,485,894, is an independent insulator site that is bound by dCTCF and CP190. Binding of both dCTCF and CP190 was significantly reduced by 3AB treatment (Figures 5B and S4, middle). Finally, bait C, located on chromosome 3R between positions 384,720 and 385,398, resembles a recently characterized class of insulator site that contains only CP190 protein (Schwartz et al., 2012). Consistent with their weak ChIP signal intensity, there is no DNA consensus sequences for either Su(Hw) or dCTCF in this bait fragment (Figure S4, right). Binding of CP190 protein was also significantly reduced upon inhibition of PARylation (Figures 5C and S4, right).

A modified 4C protocol (Göndör et al., 2008) was used as a strategy to identify new CP190 interacting sites in S2 cells (Figures S5A–S5C). Analysis of the results from the 4C experiments indicates the existence of extensive interactions between these three bait sequences and other loci across the genome. We focused on the interacting fragments that contain CP190 binding sites and validated several of these interactions with site-specific primers on multiple 3C and 4C samples (Figure S5D), most of which occur in *cis* (Figure 5). We reasoned that the reduction in the binding of insulator proteins on the bait fragments upon 3AB treatment could result in the loss of their long-range interactions with multiple distant CP190 sites. Furthermore, because PARylation stabilizes interactions between CP190 and dCTCF (Figures 3A, 3B, and S2I), we speculated that long-range DNA interactions mediated by baits A and B may be more drastically affected by 3AB treatment compared to bait C. To address this possibility, we prepared multiple 3C libraries from control and 3AB-treated S2 cells (n = 4 for each condition) and tested the ligation efficiency of individual interacting sites with site-specific primers using qPCR (Figure S5E).

Inhibition of PARylation led to distinct outcomes with each of the selected baits. Of the 12 long-range DNA interactions that are mediated by bait A, four interactions are significantly reduced by 3AB treatment. Three of these affected interacting sites lie within 45 kb of the bait (Figure 5A, A1–A3), whereas one is located ~2 Mb away (Figure 5A, A4). Of the 19 long-range DNA interactions mediated by bait B, 6 are reduced upon 3AB inhibition (Figure 5B). The distance between the bait and these 3AB-affected interacting sites ranges between 6.5 kb (B5) and 274 kb (B1). Finally, of the 19 long-range DNA interactions mediated by bait C, only two neighboring sites (C1 and C2), located within 20 kb of the bait, are affected by 3AB treatment (Figure 5C). The weak signals of dCTCF and Su(Hw) at bait fragment C suggest that these interactions may be mediated primarily by CP190 (Figure S4, right). The reduced crosslinking frequency between site C1 and bait C in the absence of CP190 supports the notion that PARylation of CP190 is sufficient to stabilize the interaction between these two CP190 sites (Figure S5F). As a control, we examined the interaction of bait 28 with neighboring distant insulator sites in the well-characterized bithorax complex locus (Lanzuolo et al., 2007). In agreement with the observation that CP190 and dCTCF at bait 28 are not significantly perturbed upon 3AB inhibition, none of the 16 intrachromosomal interactions between bait 28 and distant CP190 sites are downregulated by 3AB treatment (Figure S5G). Taken together, the results suggest that PARylation of insulator proteins may affect their ability to organize the 3D architecture of the genome through stabilization of interactions between different distant insulator sites.

DISCUSSION

Insulator proteins play an important role in chromatin organization, but the mechanisms by which insulator activity can be regulated to orchestrate the establishment of distinct patterns of intra- and interchromosomal interactions during cell differentiation are poorly understood. Here, we present evidence suggesting that *Drosophila* insulator proteins CP190, dCTCF, Mod(mdg4)2.2, and Su(Hw) are PARylated and that mutations in the *Parp* gene impair the activity of the *gypsy* and *Fab-8* insulators in vivo. Consistent with reports indicating that binding of vertebrate CTCF to DNA is independent of PARylation (Farrar et al., 2010), we find that inhibition of PARylation only causes a moderate change in the genome-wide occupancy of insulator DNA-binding proteins dCTCF and Su(Hw). Instead, interaction of CP190 with insulator DNA-binding proteins is decreased in the absence of PARylation. Because CP190 is involved in mediating interactions among insulator sites, it is likely that PARylation regulates the ability of insulators to mediate contacts between distant sites in the genome. This conclusion is strongly supported by the fact that PARylation of CP190 protein at lysine 566 is required for its in vivo function.

Eukaryotic genomes are organized into physical domains that are remarkably stable between cell types and even species (Dixon et al., 2012). Although borders between TADs are enriched in aligned insulators in *Drosophila* and contain CTCF, SINE elements, and tRNA genes in mice and humans, the majority of insulator binding sites lie within TADs (Dixon et al., 2012; Hou et al., 2012; Sexton et al., 2012). This differential distribution points to the possible existence of two functional classes of insulator sites in the genome. One class is composed of sites that are relatively constant during cell differentiation and are present at TAD borders. The second one may be composed of independent insulator sites within TADs that may have a role in regulating intradomain interactions to affect specific transcriptional outcomes. Consistent with this hypothesis, CTCF is primarily involved in mediating intradomain interactions in pre-pro B cells (Lin et al., 2012). Moreover, recent studies indicating that the large, invariant TADs can be hierarchically organized by CTCF, cohesin, and/or Mediator complexes into constitutive and cell-type-specific subtopologies support the idea that interactions within TADs can be regulated during cell differentiation (Phillips-Cremins et al., 2013). Our results suggest that PARylation of insulator proteins may represent a mechanism used by cells to regulate intrachromosomal contacts during their response to stimuli and cell lineage commitment. Significant disruption in the formation of insulator bodies in *Parp* mutants suggests that nuclear clustering of distant insulator sites may require PARylation. This clustering is mediated by CP190 and Mod(mdg4), which in turn interact with the insulator DNA-binding proteins dCTCF and Su(Hw). By modulating the interactions

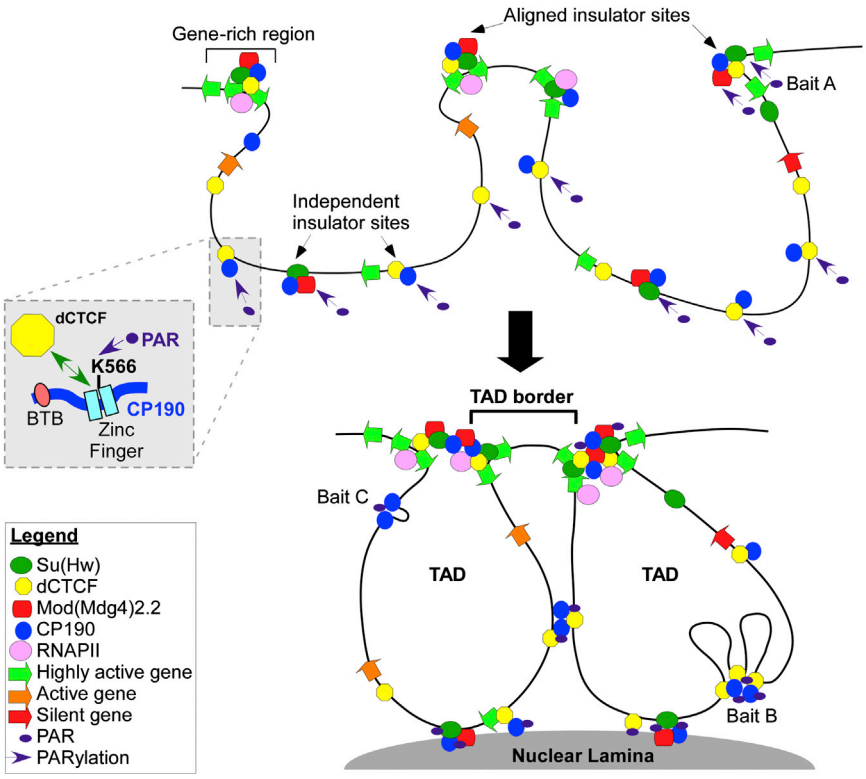

Figure 6. Model of How PARylation Regulates Insulator-Mediated Chromosome Organization

PARylation at CP190 K566 promotes its interaction with dCTCF. PARylation modulates the binding of insulator proteins within TADs, which in turn affects the intrachromosomal interactions between distant insulator sites and their association with the nuclear lamina.

between these two sets of proteins, PARylation may influence insulator-mediated chromatin looping both within topological domains and between TAD borders to elicit either a local transcriptional response or global architectural reorganization of the genome (Figure 6). One observation from our studies is that PARylation-sensitive CP190 binding sites are enriched within H3K27me3-marked chromatin domains. Although Polycomb group proteins are recruited by PARP-1 to DNA lesions during the UV damage response (Chou et al., 2010), it remains to be seen whether PARylation of CP190 can be targeted by the Polycomb complex at specific genomic sites. A recent report found that Tip60-mediated H2AK5 acetylation at the 5′ end of the *Hsp70* genes is critical for the activation and spread of Parp prior to nucleosome eviction (Petesch and Lis, 2012), suggesting that additional mechanism may be present to target PARylation to specific insulator binding sites.

EXPERIMENTAL PROCEDURES

Fly Strains and Cell Culture

Parp^{CH1}/Tm3 was obtained from Dr. Allan Spradling, *Parp^{C03256}/Tm6b* was obtained from Dr. Alexei Tulin, and *Fab-8* reporter strains were obtained from Dr. Paul Schedl. P-element-mediated transformation was carried out by injecting either WT or K566A mutant CP190 transgenes directly into *y^{2}wct^6; CP190^{H312}/Tm6b* embryos together with *Δ2-3* plasmid (O'Connor and Chia, 1993).

To inhibit PARylation, 10^7 S2 cells were first seeded with 4.5 ml of medium in a T25 flask. Cells were then added with either 0.5 ml of sterilized water (control) or 120 mM of 3AB (Sigma) to obtain a final concentration of 12 mM and harvested after 16 hr of incubation at 25°C.

Poly(ADP-ribosyl)ation Assays, IP, and Nuclear Matrix Preparation

Cloning and expression of recombinant insulator proteins are described in the Extended Experimental Procedures. In vitro poly(ADP-ribosyl)ation was carried out in a 30 μl reaction buffer containing 0.1 to 0.5 μg of GST proteins, 50 mM Tris buffer (pH8.0), 10 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, and 25 μM 6-Biotin-17 NAD (Trevigen) or 0.5 mM NAD (Sigma), 80 ng of bovine, or 30 ng human PAR polymerase (Alexis Biochemicals) in the presence or absence of 12 mM 3AB (Sigma). After 1 hr incubation at 25°C, GST protein was washed twice with 1 ml of wash buffer (50 mM Tris, pH8.0, 1 mM DTT, 10 mM $MgCl_2$, 0.2 mM PMSF). The product was boiled in Laemmli SDS buffer and subjected to western blotting.

IP of insulator proteins was carried out as previously published (Pai et al., 2004). For nuclear matrix preparation, the nuclear fraction was isolated from S2 cells with 1.8 M sucrose nuclear isolation buffer, digested with DNase I, and extracted with two rounds of incubation with high-salt buffer (Kallappagoudar et al., 2010). Detailed methods are described in the Extended Experimental Procedures.

ChIP-Seq and 3C on 4C Analysis

ChIP and generation of sequencing libraries were performed as previously described (Bushey et al., 2009; Wood et al., 2011). Sequences were mapped to the dm3 genome with Bowtie 0.12.3 (Langmead et al., 2009) using default settings. Peaks were then called with MACS 1.4.0alpha2 (Zhang et al., 2008) using equal numbers of unique reads for input and ChIP samples and a p value cutoff of 1×10^{-10}. Up- and downregulated CP190, dCTCF, and Mod(mdg4) 2.2 sites between control and 3AB treatment were determined as previously described (Wood et al., 2011) and explained in the Extended Experimental Procedures. Histone modification states in S2 cells were obtained from modENCODE (Kharchenko et al., 2011). 3C and 4C were performed as previously described (Göndör et al., 2008; Hagège et al., 2007) with S2 cells but using a four base cutter, DpnII (NEBs). The 4C method is summarized in Figure S5A and is fully described in the Extended Experimental Procedures. At least four biological replicates, with 5×10^6 cells per experiment, were used to compare the effect of 3AB treatment on crosslinking frequency between the bait and the distant insulator sites.

ACCESSION NUMBERS

ChIP-seq data are available from NCBI's Gene Expression Omnibus (GEO) under accession number GSE41354.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.08.052.

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