Role of Su(Hw) zinc finger 10 and interaction with CP190 and Mod(mdg4) proteins in recruiting the Su(Hw) complex to chromatin sites in Drosophila

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Abstract

Su(Hw) belongs to the class of proteins that organize chromosome architecture and boundaries/insulators between regulatory domains. This protein contains a cluster of 12 zinc finger domains most of which are responsible for binding to three different modules in the consensus site. Su(Hw) forms a complex with CP190 and Mod(mdg4)-67.2 proteins that binds to well-known Drosophila insulators. To understand how Su(Hw) performs its activities and binds to specific sites in chromatin, we have examined the previously described su(Hw)¹ mutation that disrupts the 10th zinc finger (ZF10) responsible for Su(Hw) binding to the upstream module. The results have shown that Su(Hw)¹ loses the ability to interact with CP190 in the absence of DNA. In contrast, complete deletion of ZF10 does not prevent the interaction between Su(Hw)Δ¹⁰ and CP190. Having studied insulator complex formation in different mutant backgrounds, we conclude that both association with CP190 and Mod (mdg4)-67.2 partners and proper organization of DNA binding site are essential for the efficient recruitment of the Su(Hw) complex to chromatin insulators.

Introduction

Eukaryotic gene expression is regulated by a complex interplay of different regulatory elements. Recently, it has been found that chromosomes in the genomes of higher eukaryotes are organized into a series of discrete topologically associating domains (TADs). [1–5]. In addition, specific interactions between enhancers and promoters, between several promoters, or more complex interactions between multiple enhancers and promoters have been described [6–9]. Mechanisms involved in the formation of chromosome architecture are not yet completely elucidated. However, a model has arisen that a special class of architectural proteins is involved in the organization of specific long-distance interactions between different types of regulatory elements [10–12].
Transcription factors involved in the activity of insulators have been attributed to the category of architectural proteins. Insulators, or chromatin boundaries, are genomic regulatory elements (nucleoprotein complexes) that can block the action of an enhancer on a promoter when interposed between them [12–16]. It was also shown in transgenic lines, that the majority of insulators can support specific distant interactions between enhancers or silencers and promoters [17–22]. Some insulators are able to interpose a barrier between the transcriptionally active chromatin and heterochromatin [21,23–26].

The best studied DNA binding architectural/insulator proteins belong to the large and poorly characterized class of transcription factors with clusters of zinc fingers (ZFs) of C2H2 type [10,27]. The CTCF protein, evolutionarily conserved among bilateral metazoans, contains a cluster of 11 ZFs, while the Su(Hw) protein specific to insects contains 12 ZFs also organized into one cluster [28,29]. The C2H2 ZF clusters recognize long DNA binding motif in which each ZF recognizes three nucleotides [30,31]. As follows from limited experimental data, only part of C2H2 domains are usually involved in DNA binding, with the rest being involved in interactions with proteins or RNAs [32,33]. Most of genomic Su(Hw) binding regions have one or two consensus site(s) [34,35], while studies on transgenic lines have shown that only four reiterated binding sites for Su(Hw) can function affectively [36].

The Su(Hw) protein directly interacts with three proteins characterized in detail previously: Mod(mdg4)-67.2, CP190, and E(y)2/Sus1 [37–43]. In addition, several new interactions of the Su(Hw) insulators have recently been described [44–46].

Mod(mdg4)-67.2 and CP190 are BTB/POZ domain proteins that are recruited to chromatin by the Su(Hw) protein [37,40,43]. Mod(mdg4)-67.2 is one of protein isoforms produced by the mod(mdg4) gene, also known as Evar3-93D, which encodes a large set of proteins containing the BTB domain at the N-terminus and different C-terminal domains [37,40,41]. As shown previously, Mod(mdg4)-67.2 specifically interacts with Su(Hw) through an isoform-specific C-terminal acidic domain. As most of the described BTB domains in higher eukaryotes, the BTB/POZ domain of CP190 forms stable homodimers, while the BTB domain of Mod(mdg4)-67.2 belongs to a specific class of BTB domains that form multimeric complexes [47–51]. Su(Hw) and many other insulator proteins are involved in recruiting of CP190 to the chromatin [43,51–55].

The genome-wide studies have shown that binding sites for the Su(Hw), Mod(mdg4)-67.2, and CP190 proteins could be grouped into two main classes which are characterized by the binding of either Su(Hw) alone (SBS-O) or of all the three proteins (SBS-CM) [35,56,57]. In the interphase nucleus the Su(Hw), CP190, and Mod(mdg4)-67.2 proteins colocalize in speckles, named insulator bodies [43,58–62]. It was speculated [60] that protein complexes are formed in the insulator bodies before binding to the chromatin.

The recent data on the ‘Su(Hw) code’ [56,57] show that Su(Hw) binds to a compound consensus sequence (approximately 26 nucleotides) comprised of three modules. The ZF6–ZF9 cluster binds to the main, central module; the ZF2–ZF4 cluster, to the downstream CG-rich module; and the ZF10–ZF12 cluster, to the upstream AT-rich module (Fig 1). It was shown that the Su(Hw)F protein failed to bind to the gypsy insulator consisting from 12 binding sites organized mainly by up and central modules [56,57]. The su(Hw)F mutation involves the point substitution of the first cysteine in ZF10 with tyrosine [63], which affects the critical Zn-coordinating residue and thereby radically alters the structure of the C2H2 domain. As a result, the Su(Hw)F has inactive the ZF10-ZF12 cluster that is essential for binding to the up AT-rich module in the site. Thus, the Su(Hw)F protein can’t effectively bind to the gypsy insulator by using only interaction between the ZF6-ZF9 cluster and the central module in the 12 reiterated sites.
Here we have found that Su(Hw)\textsuperscript{f} loses the ability to interact with CP190 in the absence of DNA. However, deletion of ZF10 (Su(Hw)\textsuperscript{Δ10}) restores the CP190–Su(Hw) interaction, suggesting that the point mutation in ZF10 affect conformation of Su(Hw)\textsuperscript{f} leading to loss of interaction with CP190. The Su(Hw)\textsuperscript{Δ10} mutant binds to the gypsy insulator better than does Su(Hw)\textsuperscript{f} and partially restores its enhancer-blocking activity but, in contrast to the wild-type Su(Hw) protein, fails to interact with the gypsy insulator in the mod(mdg4)\textsuperscript{u1} mutant background. These results suggest that CP190 and Mod(mdg4)-67.2 are critical for binding of the mutant Su(Hw)\textsuperscript{Δ10} (lacking ZF10) to the gypsy insulator consisting mainly of the upstream and central modules. Like the wild-type Su(Hw), the mutant Su(Hw)\textsuperscript{f} protein can efficiently bind to four reiterated binding sites that each consist of three modules. Thus, stable binding of Su(Hw) depends on association with CP190 and Mod(mdg4)-67.2 partners and proper organization of DNA binding site.

Materials and methods

The constructs for yeast two-hybrid assay, transgenic constructs, and details of experimental and analytical procedures are described in the Supplementary Data (S1 Appendix).

Germ-line transformation, genetic crosses, and phenotypic analysis

All flies were maintained at 25°C on the standard yeast medium. To obtained transgenic lines carrying a random $P$ transposon insertion, the construct together with P25.7wc—a $P$ element with defective inverted repeats used as a transposase source [64]—were injected into $y\ ac\ w^{1118}$ preblastoderm embryos. The resulting flies were crossed with $y\ ac\ w^{1118}$ flies, and the transgenic progeny were identified by their eye color. Chromosome localization of various transgene insertions was determined by crossing the transformants with the $y\ ac\ w^{1118}$ balancer stock containing dominant markers, $In(2RL)\text{CyO}$ for chromosome 2 and $In(3LR)\text{TM3,Sb}$ for chromosome 3. The transformed lines were examined by Southern blot hybridization to check for transposon integrity and copy number.

The lines with excisions of Su(Hw) binding sites were obtained by crossing flies carrying the transposons with the Cre recombinase-expressing line $y^{6}, w^{1118};\text{CyO, P[w^{+},cre]/Sco; +}$. All excisions were confirmed by PCR analysis.

To test the effects of Su(Hw) protein on yellow gene expression, lines containing the yellow transposons were crossed into $su(Hw)^{v}/su(Hw)^{v}$ or $su(Hw)^{v}/su(Hw)^{e04061}$ mutant background as described previously [38]. $Su(Hw)^{v}$ is a deletion of the $su(Hw)$ gene; $su(Hw)^{v}$ encodes a...
protein with a point substitution in ZF10 that retains some ability to bind to DNA; and su (Hw)^{04061} is a mutation generated by inserting the PiggyBac element near the start codon [65].

The yellow (y) phenotype was determined from the level of pigmentation of the abdominal cuticle and wings in 3- to 5-day-old males developing at 25˚C. The level of pigmentation (i.e., of y expression) was estimated on an arbitrary five-grade scale, with the wild-type expression and the absence of expression assigned scores 5 and 1, respectively. The pigmentation of bristles on the thorax and head was also assessed in 3- to 5-day-old males developing at 25˚C. Its variegation was scored on a five-grade scale, with score 1 denoting the loss of pigmentation in all bristles on the thorax and head, score 5 denoting the wild-type pigmentation of all bristles, and intermediate scores being as follows: e-v, extreme variegation (only 1–3 bristles on the thorax and head are pigmented); m-v, moderate variegation (about half of bristles are yellow); and w-v, weak variegation (only 1–3 bristles on thorax and head are yellow). No less than 50 flies from each y line were independently scored by two observers.

The white (w) phenotype was determined from eye pigmentation in adult flies. Wild-type white expression determined the bright red eye color (R); in the absence of white expression, the eyes were white (W). Intermediate levels of white expression (in increasing order) were reflected in the eye color ranging from yellow (Y), through orange (Or), to brown (Br).

To obtain transgenic flies with insertion in 38D, the DNA of reporter constructs was injected into preblastoderm embryos of y1 M[vas-int-Dm]ZH-2A w^+; M[3xP3-RFP.attP']ZH-38D genotype [66]. The resulting flies were crossed with y^{2}scD1 w^{1118}ct^{6} flies, and the progeny carrying the transgene in the 38D region were identified by their eye color. The generation of transgenic lines and construct introduction into the mod(mdg4)^{u1} or Su(Hw)^{v}/ Su(Hw)^{e04061} background were performed as described [38].

**Cells lysate preparation and immunoprecipitation experiments**

The S2 cells culture was obtained from the stock center of Vavilov Institute of General Genetics, Russian Academy of Sciences (http://vigg.ru/index.php?id=337) [67,68]. The S2 cells cultured as described [69] were transformed using the Effectene Transfection Reagent (Qiagen) as recommended by the manufacturer. To prepare cell lysate, approximately 10^7 cells were scraped from the plate and washed with two portions of PBS at 4˚C, with pelleting at 1000 g for 5 min after each wash. The washed pellet was resuspended in 300 μL of lysis buffer (10 mM HEPES, pH 7.9; 450 mM NaCl, 5 mM MgCl$_2$, 0.5% NP-40, 1 mM DTT, Protease Inhibitor Cocktail (Roche) and 1U/mL DNase I); incubated on ice for 45 min, with pipetting up and down three to four times to disrupt cell clumps; centrifuged at 15 000 g, 4˚C, for 15 min; and the supernatant was transferred to a new tube. An aliquot of the supernatant (10% of total volume) was stored to be used as input control #1, and the rest was diluted with four volumes of dilution buffer (10 mM HEPES, pH 7.9; 5 mM MgCl$_2$, 1 mM DTT, Protease Inhibitor Cocktail). The diluted lysate was again centrifuged at 15 000 g, 4˚C, for 15 min, and the supernatant was transferred to a new tube, with an aliquot (10% of total volume) of it being stored as input control #2. The lysate was then supplemented with 40 μL of FLAG Sepharose beads (Sigma) and incubated overnight at 4˚C on a rotary shaker. The beads were gently pelleted by centrifugation (700–1000 rpm at 4˚C, ~1 min), an aliquot of the supernatant was stored as output control, and the beads were washed with three portions of IP150 buffer (10 mM Tris-HCl, pH 7.5; 150 mM NaCl, 10mM MgCl$_2$, 1 mM EDTA, 1 mM EGTA, 0.3 mM DTT, 0.1% NP-40, 10% glycerol), 10 min each, with pelleting between washes. The resulting immunoprecipitate was boiled with 1× Laemmli buffer (25 μL per sample) for 10 min, resolved by SDS-PAGE (20 μL per lane), and immunoblotted with appropriate antibodies (see below).
**Yeast two-hybrid assay**

Experiments were performed using plasmids and protocols from Clontech. For growth assays, plasmids were transformed into yeast pJ69-4A cells, which were plated onto media without tryptophan and leucine and grown at 30˚C for 3 days. Thereafter, the cells were plated onto selective media without tryptophan, leucine, histidine, and adenine (high stringency conditions) or without tryptophan, leucine, histidine and 5 mM 3-aminotriazole (medium stringency conditions), and their growth was compared after 2–3 days.

**Chromatin preparation and ChIP analysis in pupae**

The material (about 150–200 mg of adult male flies, sufficient for four to five independent immunoprecipitations) was homogenized in 5 ml of buffer IP-S+ (10 mM Tris-HCl, pH 7.5; 10 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 250 mM sucrose with PMSF, leupeptin, and pepstatin A) at 4˚C using a Dounce homogenizer with type A pestle. The homogenate was filtered through a BD Falcon filter into a 50-mL tube and pelleted by centrifugation at 4000 g, 4˚C for 5 min. The supernatant was discarded, and the pellet was washed with three 3-mL portions of buffer IP-S+, with pelleting at 4000 g for 5 min after each wash. The final pellet was resuspended in 0.5 mL of IP-10+ buffer (10 mM Tris-HCl, pH 7.5; 10 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1mM DTT, 0,1% NP-40, 10% glycerol, and Roche Complete Protease Inhibitor Cocktail) and homogenized at 4˚C using a Dounce homogenizer with type B pestle. The homogenate was supplemented with an equal volume of IP-850 buffer (10 mM Tris-HCl, pH 7.5; 850 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0,1% NP-40, 10% glycerol, and Roche Complete Protease Inhibitor Cocktail), mixed gently, and left on ice for 30–60 min. This was followed by 4 rounds of centrifugation at 14 000 g for 15 min, with the supernatant being each time transferred to a new tube without disturbing the pellet. Immediately before experiments, the lysate was diluted with three volumes of buffer IP-0 buffer (10 mM Tris-HCl, pH 7.5; 10 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1% NP-40, 10% glycerol, and Roche Complete Protease Inhibitor Cocktail), centrifuged as above, and transferred to a new tube. To reduce nonspecific background, supernatant was pre-cleared by incubation with protein A or protein G agarose beads at 4˚C for 30 min, with constant stirring. Agarose was pelleted by brief centrifugation, and the supernatant was collected for chromatin immunoprecipitation with appropriate antibodies (see below). After overnight incubation at 4˚C on a rotary shaker, protein A or protein G agarose beads were added to collect the precipitated complexes, and incubation was continued for 2 h under the same conditions. Agarose was pelleted by centrifugation (700–1000 rpm at 4˚C, ~1 min), the supernatant was carefully removed, and the pellet was washed with the following buffers (1 mL each, for 3–5 min on a rotary shaker); Low Salt Wash Buffer (20 mM Tris-HCl, pH 8.0, with 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 150 mM NaCl), High Salt Wash Buffer (20 mM Tris-HCl, pH 8.0, with 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 500 mM NaCl), LiCl Wash Buffer (10 mM Tris-HCl, pH 8.0, with 0.25 M LiCl, 1% NP40, 1% deoxycholate, and 1 mM EDTA), and two portions of TE Buffer. The complex was removed from the agarose by elution with two 250-μL portions of elution buffer (0.1 M NaHCO3 with 1% SDS), 15 min each, at room temperature with rotation. The eluates were pooled, supplemented with 20 μL of 5 M NaCl, and heated at 65˚C for 4 h to reverse the complex–DNA crosslinks. Then 20 μL of 1 M Tris-HCl (pH 6.5), 10 μL of 0.5 M EDTA, and 2 μL of Proteinase K solution (10 mg/mL) were added, and the mixture was incubated for 1 h at 45˚C. DNA was recovered by phenol/chloroform extraction and ethanol precipitation and solubilized in water for PCR. PCR products were amplified from at least two separate immunoprecipitates from three
different chromatin preparations. Primer sequences used in PCR for ChIP analysis are shown in the S1 Table.

**Antibodies**

Specific antibodies and working dilutions were as follows: monoclonal mouse anti-FLAG (1:300) from Sigma (# F 1804), and polyclonal rat anti-CP190 (1:500), polyclonal rabbit anti-Mod(mdg4)-67.2 (1:500), rabbit anti-Su(Hw) N-terminal domain (1:200), rabbit anti-Chromator (1:300), and rat anti-Skeletor (1:200) raised in our lab and described previously [59,60].

**Results**

**The Su(Hw)$^f$ protein interacts with Mod(mdg4)-67.2 but not with CP190 in vitro**

The Su(Hw)$^f$ protein in the ovaries fails to bind to 60% of genomic Su(Hw) sites but can still bind to a consensus site in vitro, although with a weaker efficiency than the wild-type protein [56]. Since Mod(mdg4)-67.2 and CP190 may be involved in Su(Hw) recruitment to chromatin [35,41,60], we tested the possibility that Su(Hw)$^f$ loses the ability to interact with any of these proteins.

The Su(Hw) protein (Fig 2A) contains an N-terminal acidic domain (152 aa–208 aa), 12 ZF domains (219 aa–632 aa), and a C-terminal acidic domain (892 aa–945 aa) [37,63]. It has been shown that Mod(mdg4)-67.2 interacts with the N-terminal domain [70] and the C-terminal domain, including the aa 716 to 880 region [40] (Fig 2A). The BTB domain of CP190 interacts with two adjacent regions in the N-terminal part of Su(Hw) [71].

To test for the interaction between the insulator proteins, we used the yeast two-hybrid assay (Y2H), which is effective for studying complete proteins that are difficult to express in a bacterial in vitro system (S1 Fig). The method is based on fusing the GAL4 activation and DNA binding domains to the N- or C-end of the test protein or its part. We compared the interactions of the wild-type and Su(Hw)$^f$ mutant with CP190 and Mod(mdg4)-67.2 and found that Su(Hw)$^f$ did not interact with Cp190 (Fig 2A), but still interacted with Mod(mdg4)-67.2.

The loss of association between CP190 and Su(Hw) might be explained by a change in Su(Hw) conformation due to a defect in the structure of ZF10. To test this possibility, we used the mutant Su(Hw)$^{Δ10}$ protein in which ZF10 was completely deleted and found that Su(Hw)$^{Δ10}$ interacted with both CP190 and Mod(mdg4)-67.2 like the wild-type protein (Fig 2A). These results suggest that ZF10 is not directly required for the Su(Hw)–CP190 interaction, but structurally defective ZF10 affects the ability of mutant Su(Hw) to interact with CP190.

To confirm the results of Y2H assay, we tested the above interactions in co-IP experiments on S2 cells in which Su(Hw), Su(Hw)$^f$, and Su(Hw)$^{Δ10}$ tagged with a triple FLAG epitope were expressed (Fig 2B and S2 Fig). We observed co-immunoprecipitation between FLAG-Su(Hw)$^+$ or FLAG-Su(Hw)$^{Δ10}$ and endogenous Mod(mdg4)-67.2 and CP190 proteins. At the same time, FLAG-Su(Hw)$^f$ effectively co-precipitated with Mod(mdg4)-67.2 but only weakly co-precipitated with endogenous CP190. Taken together, these results show that structural alteration of ZF10 by point mutation in the Su(Hw)$^f$ protein interferes with the ability of the mutant to interact with CP190.

**Normal ZF10 and interaction with CP190 and Mod(mdg4) are essential for binding of Su(Hw) to chromatin**

To further compare the role of ZF10 and CP190/Mod(mdg4)-67.2 partners in the binding of Su(Hw) to chromatin sites, we produced transgenic lines expressing either wild-type FLAG-
tagged protein (Su(Hw)$^+$) or its FLAG-tagged mutant variants (Su(Hw)$^f$ and Su(Hw)$^{Δ10}$) under control of the Actin5C (Act5C) promoter. As shown previously, Su(Hw)$^f$ in the ovaries and larvae binds only to the "f-retained" sites (such as 62D, 50A, 87E), but not to "f-lost" sites (such as 1A2, 66E, and gypsy) [56]. To test the affinity of Su(Hw)$^f$ mutant for the "f-lost" sites we also expressed this protein under control of the ubiquitin-63E (Ubi) promoter. A phiC31-based integration system was used to insert the transgenes at the 38D site. After that, the transgenes were introduced into the su(Hw)$^−$ background (su(Hw)$^v$/su(Hw) e04061). The expression of the Su(Hw) transgenes was verified by the Western blot analysis with the anti-FLAG

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antibodies (S3 Fig) The Su(Hw) variants under control of Act5C were expressed at similar levels, while the expression of Su(Hw)Δ10 under control of the Ubi promoter was at least two to three times stronger than in that lines.

The interactions between the Su(Hw) variants and Mod(mdg4)-67.2 or CP190 were also assayed by co-IPs from the extract prepared from the 2-day old males of the corresponding transgenic lines (S4 Fig). This experiment confirmed the results obtained in S2 cells: Su(Hw)Δ10 showed only weak co-precipitation with endogenous CP190 but normal co-precipitation with Mod(mdg4)-67.2, while Su(Hw)Δ10 effectively co-precipitated with both proteins.

Next, we studied binding of the insulator proteins by ChIP at the previously characterized Su(Hw) sites [73] in pupae of the transgenic lines (Fig 3). As expected, we observed a positive Su(Hw) binding signal at all test sites in the Su(Hw)+ transgenie line. In the su(Hw)− pupae, we still observed residual Su(Hw) binding to insulator site 62D, which was correlated with Mod(mdg4)-67.2 binding to the same site. Residual binding of CP190 was observed at 62D, 50A and 87E sites. These results are in agreement with data on the critical role of Su(Hw) in recruiting of the Mod(mdg4)-67.2 isoform [40], while CP190 is known to be recruited to chromatin by many DNA-binding proteins [35,54].

In the transgenic line expressing Su(Hw)Δ10 under control of the Act5C promoter, this protein was found to bind to the f-lost 62D, 50A, and 87E sites, similar to wild-type Su(Hw), but not to the f-retained sites 1A2, 66E, and gypsy (Fig 3). We also observed that the Mod(mdg4)-67.2 and CP190 proteins were recruited to the 62D, 50A, 87E sites but not to the 1A2, 66E and gypsy sites, suggesting that Su(Hw)Δ10 was able to recruit the CP190 and Mod(mdg4)-67.2 proteins to chromatin. Thus, Su(Hw)Δ10 failed to bind the gypsy and 1A2 sites in female gonads [56], larvae [73] and pupae (this study).

In contrast, Su(Hw)Δ10 bound not only to the 62D, 50A, 87E sites but also (at a medium level) to the 1A2, 66E, and gypsy insulators. The C190 and Mod(mdg4)-67.2 proteins were also recruited to the 1A2, 66E, and gypsy sites. These results showed that Su(Hw)Δ10, compared to Su(Hw)Δ10, can better bind to the f-lost sites. However, when Su(Hw)Δ10 was expressed at a higher level (under control of the Ubi promoter), we observed not only better binding of Su(Hw)Δ10 to the 62D, 50A, 87E sites, but also moderate binding of the mutant protein to the 1A2, 66E and gypsy sites Thus, the increasing expression of Su(Hw)Δ10 provides for its binding to the f-lost sites (Fig 3).

These results correlated with phenotypes of the y2 allele in different Su(Hw) backgrounds (Fig 4A). In the y2 mutation (Fig 4A), gypsy is inserted between the yellow promoter and the enhancers controlling expression in the wings and body cuticle [74]. The Su(Hw) insulator blocks activity of the enhancers that results in a weak yellow expression in wing blades and body cuticle (y2 phenotype) [74,75]. In flies bearing a combination of Su(Hw)Δ10 and y2 yellow expression was completely restored, like in y2; su(Hw)Δ10 flies. In contrast, the expression of Su(Hw)Δ10 supported the enhancer-blocking activity of the gypsy insulator in the y2 allele, as in the case of Su(Hw)+. Thus, Su(Hw)Δ10 binds to the gypsy sequences and almost completely restores its enhancer-blocking activity. On the other hand, the overexpression of Su(Hw)Δ10 failed to restore even partial blocking of the yellow enhancers by the gypsy insulator in the y2 allele.

We also tested the effect of different Su(Hw) variants on the activity of the gypsy insulator in cut and scute loci (S5 Fig). In the ct6 allele, gypsy is located between the cut promoter and the wing margin enhancer separated by 85 kb [37]. The mutant phenotype in the ct6 allele is a consequence of complete blocking of the margin enhancer by the insulator. In scD1N allele, downstream insertion of gypsy inhibits scute expression in several specific areas of the epithelium and, in particular, blocks the formation of several types of bristles [76]. All Su(Hw) mutant variants expressed in the su(Hw)+ background (su(Hw)y2/su(Hw)Δ10Δ10) failed to support the ability
Fig 3. Evaluation of binding of the insulator protein in the mod(mdg4) transgenic lines. Variants of the Su(Hw) protein were expressed in the y<sup>2</sup>sc<sup>D</sup>1<sup>ct</sup>6; P(Su(Hw))<sup>-38D/P(Su(Hw))<sup>-38D; su(Hw)<sup>+/su(Hw)10061</sup> lines, where P(Su(Hw)) are Su(Hw)<sup>−</sup>-Act5C – P[w<sup>+</sup>]; WAB-Su(Hw)1-945-FLAG]; P[w<sup>+</sup>]; WAB-Su(Hw)1-945-FLAG; Su(Hw)<sup>1-945-Act5C</sup> – P[w<sup>+</sup>]; WAB-Su(Hw)1-945-FLAG]; P[w<sup>+</sup>]; WAB-Su(Hw)1-945-FLAG]; Su(Hw)<sup>-Act5C</sup> – P[w<sup>+</sup>]; WAB-Su(Hw)1-945-FLAG]; P[w<sup>+</sup>]; WAB-Su(Hw)1-945-FLAG]; Su(Hw)<sup>-Ubi–P[w<sup>+</sup>]; UbqW-Su(Hw)<sup>-FLAG</sup>; P[w<sup>+</sup>]; UbqW-Su(Hw)<sup>-FLAG</sup>]. The y<sup>2</sup>sc<sup>D</sup>1<sup>ct</sup>6; su(Hw)<sup>−</sup>/su(Hw)10061 line is designated as Su(Hw)<sup>−</sup>. Quantitative PCR (qPCR) was performed at the selected Su(Hw) regions. The ras64B coding region (Ras) was used as a negative control. The percent recovery of immunoprecipitated DNA (Y axis) was calculated relative to the amount of input DNA. Error bars indicate standard deviation of three independent biological replicates.

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Fig 4. Effects of the su(Hw) mutations on the activity of gypsy insulator in the mod(mdg 4)u1 background. (A) Effects of the mod(mdg4)u1 mutation on yellow expression in transgenic lines. A schematic of the y^2 allele (drawn not to scale): the yellow wing (Ew) and body (Eb) enhancers are shown as partially overlapping gray ovals; the bristle enhancer (Ebr), as a gray oval in the yellow intron; the transcription start site is indicated by an arrowhead. The gypsy insertion is shown as a triangle with the black circle (Gy) marking Su(Hw) binding sites. Analysis of the transgenic lines was performed in the y^2scD1ct6; mod(mdg4)+/mod(mdg4)− background (mod−) or in the y^2scD1ct6; mod(mdg4)u1/ mod(mdg4)u1 background (mod+). Numbers in the "mod−" and "mod+" lines indicate a relative level of yellow expression in the body cuticle/wing blades/bristles, which ranged from 2 (pigmentation as in the y^2 allele) to 5.
of the gypsy insulator to block cut or scute enhancer in the cf<sup>f</sup> and sc<sup>D1</sup> alleles, showing that Su(Hw)<sup>Δ10</sup>, like Su(Hw)<sup>f</sup>, does not support the enhancer-blocking activity of the gypsy insulator in tissues where the cut or scute genes are required.

Taken together, these results indicated that the CP190–Su(Hw) interaction has an effect on binding of the Su(Hw) complex to the chromatin. The Su(Hw)–Mod(mdg4)-67.2 interaction was also suggested to have a role in this process [60].

As shown previously, the mod(<sup>mdg4</sup>)<sup>u1</sup> mutation leads to a direct repression of yellow in <i>y<sup>2</sup></i> flies that results in the nonpigmented bristles and a variegated pigmentation in the abdominal segments. We found that mod(<sup>mdg4</sup>)<sup>u1</sup> in combination with Su(Hw)<sup>Δ10</sup> transgene almost completely suppressed the <i>y<sup>2</sup></i> mutant phenotype, indicating that Su(Hw)<sup>Δ10</sup> failed to bind the gypsy sequence in the absence of the Mod(<sup>mdg4</sup>)-67.2 protein (Fig 4B).

### Comparing the functional activity of the gypsy insulator and four Su(Hw) binding sites

The Su(Hw) protein binds to the sites integrated in the regulatory regions that are also bound by unknown transcription factors, which may facilitate the binding of Su(Hw)<sup>f</sup> to DNA. To find out whether the presence of an appropriate DNA binding motif is sufficient for recruiting the Su(Hw)<sup>f</sup> protein, we used a DNA fragment with four reiterated Su(Hw) motifs (S<sup>4k</sup>) corresponding to site 3 in the gypsy insulator (S6 Fig). This fragment contained all three modules corresponding to the consensus site.

We compared the Su(Hw)<sup>+</sup> and Su(Hw)<sup>f</sup> proteins for binding to the S<sup>4</sup> element and the ability to mediate the enhancer-blocking and barrier activity of the Su(Hw) sites. As reporters, we used the yellow gene and the white gene that was inserted on the 3' side of yellow in tail-to-head orientation. The white gene accounts for eye pigmentation and is regulated by the eyespecific enhancer [77]. To test the enhancer-blocking activity, constructs were made (Fig 5A), in which the eye enhancer was inserted between the yellow wing and body enhancers, and the S<sup>4</sup> fragment flanked by <i>loxP</i> sites was inserted between the enhancers and promoters at ~893 relative to the yellow transcription start site. As a control, we used the previously characterized transgenic line carrying the construct with the same configuration of the regulatory elements and the gypsy insulator flanked by the <i>loxP</i> sites (Fig 5A). We obtained 12 transgenic lines, each carrying one copy of the construct with the S<sup>4</sup> sites. The S<sup>4</sup> fragment effectively blocked the yellow and white enhancers, with deletion of S<sup>4</sup> restoring the expression of the reporter genes. These results showed that the S<sup>4</sup> sites blocked the enhancers at a comparable level with the gypsy insulator (Fig 5A and S7 Fig). In the su(Hw)<sup>y</sup> su(Hw)<sup>y</sup> background, the Su(Hw)<sup>f</sup> protein completely blocked the enhancers in transgenic lines carrying the S<sup>4</sup> but not in transgenic...
Fig 5. Comparison of the enhancer-blocking activity of the gypsy (Gy) and S*4 insulators. (A) Effects of the su(Hw) and mod(mdg4) mutations on yellow and white expression in transgenic lines. In the scheme of the construct (drawn not to scale), the yellow wing (Ew) and body (Eb) enhancers are shown as shaded ovals; the eye enhancer (Ee) inserted between them, as a white oval; the yellow (Y) and white (W) genes, as arrows indicating the direction of transcription; and the gypsy and S*4 insulators, as shaded triangles. Downward arrows indicate loxP target sites for the Cre recombinase; the same sites in the construct names are denoted by parentheses. The “yellow” column shows the numbers of transgenic lines with the yellow pigment level in the abdominal cuticle (reflecting the activity of the body enhancer); in most of the lines, the pigment level in wing blades (reflecting the activity of the wing enhancer) closely correlated with these scores. The level of pigmentation (i.e., of y expression) was estimated on an arbitrary five-grade scale, with wild-type expression and the absence of expression assigned scores of 5 and 1, respectively. Wild-type white expression determined the bright red eye color (R); in the absence of white expression, the eyes were white (W).
Intermediate levels of pigmentation, with the eye color ranging from pale yellow to yellow (Y) from dark yellow to orange (Or), from dark orange to brownish red (Br) reflect the increasing levels of expression. In the N/T ratio, N is the number of lines in which flies acquired a new phenotype upon deletion (Δ) of the specified DNA fragment, or on the mutant background, and T is the total number of lines examined for each particular construct. (B) ChIP-qPCR analysis of insulator proteins binding to the gypsy or S
trans insulator in transgenic lines. The Eye(S
eye)YW lines included in the analysis are designated # 1 and # 2. The ras64B coding region (Ras) was used as a control devoid of Su(Hw) binding sites. The percent recovery of immunoprecipitated DNA (Y axis) was calculated relative to the amount of input DNA. Error bars indicate standard deviation of three independent biological replicates. The abbreviations of mutant backgrounds: su(Hw)
tf/TM6,Tb, su(Hw) —su(Hw)/su(Hw), mod —mod(md4)4/md(modmd4)
white or S
su(Hw)
expression. In the N/T ratio, N is the number of lines in which flies acquired a new
background (Fig 5A). The inactivation of Mod(md4)−67.2 in the lines carrying the gypsy insulator (Fig 5A). As expected, both test elements lost their enhancer-blocking activity in the su(Hw)+/ su(Hw)+/su(Hw)+041064 background. Taken together, these results showed that S4sites were fully active in the transgenic lines expressing the mutant Su (Hw)f protein.

In two independent S4× transgenic lines, binding of the Su(Hw), Mod(md4)-67.2, and CP190 proteins was tested by means of ChIP, with the 62D and 1A2 sites being used as an internal control. The binding of these proteins was also tested in the control transgenic line carrying the gypsy insulator. Chromatin was isolated from pupae of transgenic lines with either su(Hw)+/TM6,Tb, su(Hw)+/su(Hw)+041064 or su(Hw)+/su(Hw)+ background (Fig 5B). The results showed that all three proteins—Su(Hw), CP190 and Mod(md4)-67.2—bound to the S4sites in the two selected transgenic lines and to the gypsy insulator in the su(Hw)+/TM6,Tb background, while the Su(Hw)f protein effectively bound to the S4 sites but not to the gypsy insulator. Thus, Su(Hw)f proved to bind to the reiterated four sites containing all three modules (S5 Fig). Importantly, CP190 bound to the S4 sites with the same efficiency in both su(Hw)+ and su(Hw)f backgrounds. These results confirm that CP190 is able to interact with the Su(Hw) sites after its binding to DNA.

According to the ‘Su(Hw) code’ model, the DNA binding site determines which of the ZF domains are not involved in DNA binding and may therefore be available for association with additional proteins such as E(y)2/Sus1 [42,56,57]. To test whether gypsy and S4 display different properties, we compared their activity in transgenic lines crossed into the mod(md4)4× background (Fig 5A). The inactivation of Mod(md4)-67.2 in the mod(md4)+ site had little effect on the activity of the gypsy insulator, which was manifested in the partial release of the eye enhancer, while the yellow enhancers remained inactive. In contrast, the mod(md4)+ mutation led to almost complete inactivation of the enhancer-blocking activity of the S4 insulator. Interestingly, inactivation of mod(md4)+ only moderately affected the binding of Su (Hw) and CP190 to both gypsy and S4 insulators (Fig 5B). Thus, the Su(Hw) and CP190 proteins still bound to the sites, but their ability to block enhancers was lost only in the case of the S4 sites.

In addition to blocking the enhancers, one copy of the gypsy insulator can also counteract the repressive effect of the Polycomb Response Element (PRE) [17,78,79]. PREs are bound by proteins of the Polycomb group (PcG), inducing the silencing of both endogenous target genes and reporter genes [80]. As shown previously, E(y)2/Sus1 is recruited to the Su(Hw) insulators and is required for blocking of PRE-mediated repression by the gypsy insulator [42]. Since ZFs 7–12 were mapped to interact with E(y)2/Sus1, we ask whether S4 functions differently from the gypsy insulator in protecting reporter expression from PRE-mediated repression. In the constructs (Fig 6), the 660-bp core PRE from the bxd region of homeotic gene Ultrabithorax [79] was inserted between the wing and body enhancers. The loxP-flanked S4 fragment was inserted at −893 between the yellow promoter and PRE. Only those transgenic lines were included in this experiment in which the PRE repressed the yellow and white genes after deletion of the insulators. As a result, we found that S4 effectively blocked PRE-mediated silencing of the yellow and white expression (Fig 6). Crossing into the su(Hw)+ background resulted in
the silencing of yellow and white expression. Once again, $S^{x4}$ could protect yellow and white expression from PRE-mediated silencing in the $su(Hw)^f$ background. Thus, inactivation of ZF10 in the Su(Hw)$^f$ protein does not affect the ability to block PRE-mediated silencing.

**Discussion**

Here we have found that the Su(Hw)$^f$ protein cannot interact with CP190 in the absence of DNA. It is likely that structural alteration of ZF10 leads to a change in the conformation of the Su(Hw) protein that prevents the interaction of its N-terminal domain with CP190. The proper conformation of Su(Hw) is restored after binding to DNA, which allows the interaction
between its N-terminal domain and the BTB domain of CP190. This finding makes it possible to estimate the role of the Su(Hw)–CP190 interaction in recruiting the insulator complex to chromatin.

In the *Drosophila* interphase cell nucleus, the Su(Hw), Mod(mdg4)-67.2, and CP190 proteins colocalize in speckles (insulator bodies) that might function as a depot of proteins involved in the regulation of transcription and insulation [60,61,81,82]. The insulator bodies could facilitate a formation of complexes between Su(Hw)–Mod(mdg4)-67.2–CP190 and other transcription factors. It appears that Mod(mdg4)-67.2 and CP190 interact with neighboring DNA binding (architectural) proteins and in this way facilitate the recruitment of ‘mature’ Su(Hw) insulator complexes to the corresponding chromatin sites. The efficiency of Su(Hw) complex recruiting depends on the presence of essential modules in the consensus site, the number of such sites, and the interaction with neighboring partners (Fig 7A). According to the ‘Su(Hw) code’ [56,57], ZF10 and ZF4 of the Su(Hw) protein are required for interaction with the upstream AT-reach module and the downstream CG-reach module of the consensus binding site (Fig 1). The Su(Hw)f protein looses the ability to recognize the upstream module at DNA and at the same cannot interact with CP190 before recruitment to chromatin (Fig 7B). For this reason, only overexpressed Su(Hw)f can weakly bind to the 12 sites in the *gypsy* insulator. It seems likely (although not confirmed) that Su(Hw)f binds only to the third site in the *gypsy* insulator that contains all three modules. This may explain the inability of overexpressed Su(Hw)f to block the yellow enhancers, in contrast to Su(Hw)Δ10 that interacts with CP190. The relatively weak binding of Su(Hw)Δ10 to the *gypsy* insulator is indicative of the main contribution of ZF10 to the Su(Hw) complex recruitment to the *gypsy* sites. Moreover, inactivation of Mod(mdg4)-67.2 also completely blocks the recruitment of Su(Hw)Δ10 to the *gypsy* insulator and other “f-lost” sites. Thus, the recruitment of the mutant Su(Hw) protein incapable of recognizing the upstream module in the consensus site is completely dependent on Mod(mdg4)-67.2 and CP190 partners.

The Su(Hw) code model [57] predicts that the consensus site determine which of Su(Hw) ZF domains are not involved in DNA binding and, hence, are free to interact with other proteins and/or complexes (Fig 7C). For example, the ZF domains of the Su(Hw) and dCTCF proteins are required for recruitment of E(y)2/Sus1, which is involved in blocking of PRE-mediated silencing [11,42]. The interaction of E(y)2/Sus1 with Su(Hw) was previously mapped to the region of ZFs 7–12 [42]. Here we found that ZF10 is not required for blocking of PRE by the Su(Hw) sites. Thus, it appears that E(y)2/Sus1 interacts with ZF11 and/or ZF12, which are not highly required for DNA binding [57].

Interestingly, the *gypsy* insulator (12 sites with different modules structure) and four Su(Hw) sites (S<sup>−4</sup>) with three modules show the same efficiency in blocking the yellow enhancers in transgenic lines. Inactivation of Mod(mdg4)-67.2 did not significantly change the enhancer-blocking activity of the *gypsy* insulator. At the same time, Mod(mdg4)-67.2 is critical for the insulator activity of the S<sup>−4</sup> sites. Since Mod(mdg4)-67.2 inactivation only weakly affected binding of the Su(Hw) and CP190 proteins to both *gypsy* and S<sup>−4</sup> sites, this difference in insulation may be explained by the existence of an unknown protein/complex involved in enhancer blocking that is recruited to the *gypsy* but not to the S<sup>−4</sup> site. For example, this unknown protein/complex can supposedly interact with ZF4, which is essential for binding to the S<sup>−4</sup> sites but not to *gypsy*. Further study is required for testing the relevance of the code model.

**Conclusions**

The mechanisms regulating the chromatin binding of architectural proteins, such as Su(Hw), are not yet well understood. Our results support the model that the recruitment of the Su(Hw)
Fig 7. A model explaining differences in the Su(Hw) complex recruitment to the consensus sites and to sites in the gypsy insulator. (A) The Su(Hw) complex is recruited to consensus site(s) consisting of three modules. Most of ZFs are involved in stable interaction with the consensus site. The interactions of C190 and Mod(mdg4) with neighboring proteins (dotted arrows) are not critical for recruiting the insulator complex. (B) The insulator complex carrying Su (Hw) with mutated ZF10 is recruited to the sites in the gypsy insulator. The binding of the Su(Hw) complex to DNA is unstable and strongly depends on CP190 and Mod(mdg4)-67.2 (thick arrows) that may stabilize interaction with surrounding proteins. (C) The insulator complex with the Su(Hw)+ protein is recruited to the gypsy sites. ZFs 1–4 are not involved in binding to these sites and may recruit unknown proteins/complex (un-p) that improve the enhancer-blocking activity of the Su(Hw) complex in the absence of the Mod(mdg4)-67.2 protein.

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complex depends on many protein–protein and DNA–protein interactions at the target chromatin sites.

Supporting information

S1 Appendix. Materials and methods.

S1 Table. Primer sequences used in PCR for ChIP analysis.

S1 Fig. Test for two-hybrid interaction strength. (1)—no interaction « - », (2)—« + », (3)—« ++ ».

S2 Fig. Co-immunoprecipitation between Su(Hw) variants fused to the FLAG epitope and the Skeletor protein. The FLAG-Su(Hw), FLAG-Su(Hw), and FLAG-Su(Hw) were expressed in the S2 cells. The immunoprecipitated complexes were washed with buffers containing 150 mM NaCl before loading onto the SDS-PAGE for Western blot analysis. The PVDF membrane was probed with antibodies against the Skeletor protein. Each panel represents a single FLAG immunoprecipitation experiment with the particular Su(Hw) variant. Su(Hw) variants do not interact with the Skeletor protein. The bottom panel shows the result of immunoprecipitation between Chromator and Skeletor proteins that interact with each other [83]. All results were reproduced in two independent experiments.

S3 Fig. Western blot analysis for the expression of Su(Hw) protein and its derivatives in vivo. Su(Hw) isolated from adult flies as described (Gdula et al., 1997) [84] was resolved by electrophoresis in 7.5% SDS-PAGE, electroblotted onto a PVDF membrane, and probed with antibodies against Su(Hw) or FLAG epitope (designated αSu(Hw) and αFLAG, respectively). Anti-tubulin staining (αTub) was used as loading control. The name of alleles included in analysis are indicated above the figure. Expression analysis of transgenic lines was performed on the y; su(Hw); su(Hw) background. Analysis of transgenic lines was performed on the y; su(Hw); su(Hw) background. All results were reproduced in three independent experiments.

S4 Fig. Results of co-immunoprecipitation between the Su(Hw) protein and its derivatives fused to FLAG epitope and the Mod(mdg4)-67.2 or CP190 protein. The immunoprecipitated complexes were washed with 150 mM NaCl-containing buffers before loading onto SDS-PAGE for Western blot analysis. The PVDF membrane was consecutively probed with antibodies against the indicated proteins (CP190 or Mod-67.2) or FLAG epitope. "Input" is the input fraction (10% of lysate used for immunoprecipitation); "Output IP," the supernatant after immunoprecipitation; "IP," the immunoprecipitate. The names of alleles included in analysis are indicated above the figure. Analysis of transgenic lines was performed on the y; su(Hw); su(Hw) background. All results were reproduced in three independent experiments.

S5 Fig. Effect of Su(Hw) and its derivatives on the activity of the gypsy insulator in the ct and sc alleles. (A) Schemes (not to scale) of the ct allele. The gray oval (Wme) indicates the wing margin enhancer controlling cut expression in the wings. The transcription start site indicated by arrowheads. The gypsy insertion is shown as a triangle with the black circle (Gy) marking Su(Hw) binding sites. The names of alleles included in analysis are under the photos.
showing the cut wing phenotype. (B) Scheme of the yellow/ac/sc region in scD allele. The coordinates of the AS-C region are as defined by Campuzano et al. (1985) [76]. The gypsy (scD1) insertion is shown as a triangle with an arrow. Thick horizontal gray arrows show the positions and direction of yellow and AS-C gene transcripts. Filled oval indicate the endogenous Su(Hw) insulator. The standard nomenclature for each bristle is as follows: HU, humeral; AOR, anterior orbital; PS, presutural; ASA, anterior supra-alar; OC, ocellar; PV, postvertical; ANP, anterior notopleural; SC, scutellar. Only the bristles affected in the ac and sc mutations are shown. Empty boxes indicate that the corresponding bristles are present (wild-type phenotype). Filled boxes indicate the absence of the corresponding bristle(s) in more 90% of the flies.

S6 Fig. The sequence of the oligonucleotide used to produce the synthetic region consisting of the Su(Hw) sites (S×4). This oligonucleotide contains gypsy insulator sequences corresponding to binding site 3 and includes the consensus sequence with three modules that represents the highest-affinity Su(Hw) binding site [57].

S7 Fig. Examples of abdominals and eyes pigmentation. Photos represent the abdominal and eyes pigmentation in the 3-day-old males. Numbers indicate the scores of yellow expression in the abdominal segments, which ranged from 1 (pigmentation as in y1 allele) to 5 (pigmentation as in wild-type flies). Wild-type white expression determined the bright red eye color (R); in the absence of white expression, the eyes were white (W). Intermediate levels of pigmentation are yellow (Y), orange (Or), and brownish red (Br).

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