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# Role of Mod(mdg4)-67.2 Protein in Interactions between Su(Hw)-Dependent Complexes and Their Recruitment to Chromatin

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**Abstract**—Su(Hw) belongs to the class of proteins that organize chromosome architecture, determine promoter activity, and participate in formation of the boundaries/insulators between the regulatory domains. This protein contains a cluster of 12 zinc fingers of the C2H2 type, some of which are responsible for binding to the consensus site. The Su(Hw) protein forms complex with the Mod(mdg4)-67.2 and the CP190 proteins, where the last one binds to all known *Drosophila* insulators. To further study functioning of the Su(Hw)-dependent complexes, we used the previously described *su(Hw)$^{E8}$* mutation with inactive seventh zinc finger, which produces mutant protein that cannot bind to the consensus site. The present work shows that the Su(Hw)$^{E8}$ protein continues to directly interact with the CP190 and Mod(mdg4)-67.2 proteins. Through interaction with Mod(mdg4)-67.2, the Su(Hw)$^{E8}$ protein can be recruited into the Su(Hw)-dependent complexes formed on chromatin and enhance their insulator activity. Our results demonstrate that the Su(Hw) dependent complexes without bound DNA can be recruited to the Su(Hw) binding sites through the specific protein–protein interactions that are stabilized by Mod(mdg4)-67.2.

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*Keywords*: transcription regulation, Su(Hw), Mod(mdg4), protein–protein interactions, chromatin insulators

## INTRODUCTION

In higher eukaryotes, regulation of gene expression becomes more complex due to the cell differentiation during embryonic development. Cell specialization is determined by the expression of various combinations of transcription factors, which are encoded by a large group of developmental genes that control differentiation [1]. Another large group of genes encodes housekeeping proteins that are essential for functioning of all cells. In *Drosophila*, housekeeping genes are grouped in clusters, and all their regulatory elements are usually found in close proximity to the promoters they regulate. Unlike housekeeping genes, developmental genes usually have complex, extensive regulatory systems consisting of a large number of enhancers, each of which determines gene expression in a specific group of cells and over a certain time period [2-4]. Enhancers can stimulate promoters while being located at distances from them, in some cases more than hundreds of kb. Regulation of interactions between the enhancers and promoters is controlled by a special group of regulatory elements called insulators [5-7]. Certain combinations of insulators can interact with each other to form chromatin domains that enhance/block long-distance interactions between the enhancers and promoters [5, 8, 9].

In *Drosophila*, architectural proteins bind to the gene promoters and insulators. Characteristic feature of these proteins are clusters consisting of five or more

*Abbreviations*: BTB, bric-a-brac, tramtrack, and broad complex; CP190, centrosomal protein 190 kD; Mod(mdg4), modifier of mdg4; SBS, Su(Hw) binding sites; Su(Hw), suppressor of Hairy-wing; TTK, Tramtrack group; Y2H, yeast two hybrid; ZF, zinc fingers.

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zinc fingers (ZF) of the C2H2 type [10, 11]. It has been shown that the architectural proteins specifically bind to the extended motifs of 12-15 bp by its 4-5 C2H2 domains. In *Drosophila*, the best described architectural protein is Su(Hw) (suppressor of Hairy-wing), which has about 2000 binding sites in the genome [12-15]. Su(Hw) was originally discovered as an insulator protein that blocks interactions between the enhancers and gene promoters by binding to 12 sites within the *gypsy* retrotransposon [12-14]. Subsequently, it was shown that Su(Hw) is involved in formation of promoters and is capable of repressing transcription from some of them [15, 16]. In the center of the Su(Hw) protein there is a cluster consisting of 12 C2H2 ZFs, which bind to the consensus sequence [17]. At the N-terminus of the Su(Hw) protein there are two conserved regions that interact with the BTB (bric-a-brac, tramtrack, and broad complex) domain of the CP190 protein (centrosomal protein 190 kD), which is involved in formation of active insulators and promoters of housekeeping genes [18, 19]. At the C-terminus of the protein, a domain is mapped that interacts with one of 30 isoforms of the Mod(mdg4) protein (modifier of mdg4) [20-22]. All Mod(mdg4) isoforms at the N-terminus have a TTK (Tramtrack group)-like BTB domain, which is responsible for formation of hexamers [23, 24]. The Mod(mdg4)-67.2 isoform has a unique C-terminus that interacts exclusively with the C-terminal domain of the Su(Hw) protein. In addition, it was found that the Su(Hw)-dependent complexes could also include other proteins: HIPP1 (HP1 and insulator partner protein 1), which interacts with the same C-terminal region of Su(Hw) as Mod(mdg4)-67.2 [25-27], ENY2 (enhancer of yellow 2), which interacts with the zinc fingers 11 and 12 of the Su(Hw) protein [28], and RNA-binding proteins [29, 30]. All isoforms of the Mod(mdg4) protein, like the CP190 protein, are SUMOlated and, as a result of multiple protein–protein interactions, form speckles in the nucleus [31-33]. It is assumed that multimerization of the BTB domains and interaction between the SUMO (small ubiquitin-like modifier) proteins form the speckle core, to which "passenger" proteins such as Su(Hw) and other architectural proteins are attached. According to the proposed model, speckles function as reservoirs of architectural proteins that bind to new DNA during its replication [32, 33].

In the present work, we investigated the ability of the mutant protein Su(Hw)$^{E8}$, which does not by itself bind DNA, to be recruited to the Su(Hw) dependent chromatin sites. A point replacement of histidine at position 459 with tyrosine in the Su(Hw)$^{E8}$ mutant [34] leads to the destruction of the seventh C2H2 domain, which is necessary for binding of the protein to chromatin [17]. As a result, Su(Hw)$^{E8}$ cannot bind to Su(Hw)-binding sites *in vitro* and is not detected on the polytene chromosomes [17]. However, we have demonstrated that in the presence of the chromatin-binding mutant of Su(Hw) with N-terminus deletion (Su(Hw) ΔN), the Su(Hw)$^{E8}$ the protein is recruited to the Su(Hw)-dependent insulator sites. Efficient binding of Su(Hw)$^{E8}$ is mediated by the Mod(mdg4)-67.2 protein.

## MATERIALS AND METHODS

**Generation of recombinant genetic constructs.** All constructs for Y2H (yeast two hybrid) assay were created on the basis of the pGBT9 vector containing the DNA-binding domain of the yeast GAL4 protein (Clontech, USA).

To create the pGBTSu(Hw)$^{E8}$ construct, the PCR product 5′-ggaacagcacaagtcacgtg 3′/5′ caccaatgcagaaaa cttcttgtc-3′ was treated with BglII endonuclease, and the PCR product 5′-gcccttaaaaagTatcgacgct-3′/5′-aatccgt gcgttccataat-3′ with endonuclease EagI. Su(Hw) cDNA was used as a template for PCR. The resulting DNA fragments were co-cloned into the plasmid pGBTSu(Hw) digested with BglII and EagI.

To create the pGBTSu(Hw)$^{E8}$Δ114 construct, the XhoI-AflII fragment containing deletion of 114 a.a. from pGBTSu(Hw)Δ114, was cloned into the plasmid pGBTSu(Hw)$^{E8}$ digested with XhoI and AflII.

To create the pGBTSu(Hw)$^{E8}$Δ283 construct, the EagI-SalI fragment containing a 17-aa deletion from pGBTSu(Hw)Δ283 was cloned into the pGBTSu(Hw)E8 plasmid treated with EagI and SalI.

Plasmids pGBTSu(Hw), pGBTSu(Hw)Δ283, pGADMod(mdg4)-67.2, and pGADCP190 were obtained and described previously [18, 20].

**Yeast two-hybrid system.** Analysis of protein interactions in Y2H was performed using plasmids and protocols from Clontech. Plasmids were transformed into a yeast strain pJ69-4A by lithium acetate method as described by the manufacturer and plated on a medium without tryptophan and leucine. After 3 days of growth at 30°C, the cells were subcultured onto a selective medium without tryptophan, leucine, histidine, and adenine, and growth of yeast colonies was compared after 2-3 days. As a negative control, interaction of Su(Hw) protein derivatives expressed in the pGBT9 vector with the pGAD24 vector was tested. Interactions of the full-length Su(Hw) protein with the Mod(mdg4)-67.2 or CP190 proteins, described previously, served as a positive control [18, 20]. Each experiment was repeated three times.

**Analysis of *Drosophila* transgenic lines phenotype.** All flies were kept at 25°C on a standard yeast medium (Bloomington Drosophila Stock Center). Effects of different combinations of mutations were assessed independently by two investigators. Level of expression of the *yellow* and *cut* phenotypes was assessed in males aged 3-5 days, developing at 25°C. Changes in

*yellow* gene expression (in the body and wings) were assessed on a five-point scale, where 5 corresponds to wild-type pigmentation; 2 corresponds to the level of pigmentation associated with the $y^2$ mutation; 3 and 4 – partial activation of basal transcription; 1 – no expression. Flies in which expression of the *yellow* allele was characterized previously were used as a standard. Changes in the *cut* gene expression were assessed by counting the number of gaps occurring along the edge of the wing plate. Wild-type flies and flies carrying the $ct^6$ allele were used as a reference. Representative wing shapes shown in Fig. 2b were selected as "average" from the series of wings arranged in increasing order of severity of their mutant phenotype. In each transgenic line, the phenotype of at least 50 flies was assessed.

Transgenic lines Su(Hw)+, Su(Hw)Δ62, Su(Hw)Δ52, Su(Hw)Δ114, and Su(Hw)J, targeted with the 3xFLAG epitope, were obtained and described previously [18, 35]. Combination of the $mod(mdg4)^{u1}$ mutation or combinations of the $su(Hw)^v/su(Hw)^2$ and $su(Hw)^v/su(Hw)^{E8}$ mutations with transgenic lines was carried out in accordance with the scheme described previously [36]. All details of genetic crosses can be provided upon request.

**Immunostaining of polytene chromosomes.** *Drosophila* 3rd instar larvae were grown at 18°C under standard conditions. Staining of polytene chromosomes was performed according to the previously described method [37]. The following primary antibodies were used: anti-Su(Hw) (rabbit) 1 : 300 and anti-FLAG 1 : 50 (mouse). The secondary antibodies used were FITC-AffiniPure Donkey Anti-Rabbit 1 : 200 and Cy5-AffiniPure Donkey Anti-Mouse 1 : 200 (Jackson Immuno Research, USA). Analysis was performed using a Zeiss fluorescence microscope (Axio Observer.Z1, Germany) equipped with an OptiGrid structured illumination microscopy system (Qioptiq, Luxembourg). The Fiji program was used for image processing.

**Chromatin immunoprecipitation.** Isolation of chromatin from the pupal stage of *Drosophila* development and subsequent immunoprecipitation procedure were carried out in accordance with the method described previously [35]. The following antibodies were used for immunoprecipitation: anti-Su(Hw) (rabbit) 1 : 200 and anti-FLAG 1 : 300 (mouse). Amount of immunoprecipitated DNA was determined by quantitative PCR using SYBR green (Bio-Rad, USA, Cat# 170-8882). Sequences of primers used in PCR:

62D fw – 5′ TTTGGGCTTGGTGAGAACAG 3′
62D rev – 5′ TGATACCAGGCGAACAGAAATC 3′
50A fw – 5′ ATACAAAGTGGTTTCAGCCAAGAAG 3′
50A rev – 5′ TTGATAAATAGTCCAGCACGCATAC 3′
87E fw – 5′ GGATGTTACA TTGAGAGTGCTTAGG 3′
87E rev – 5′ TTTGCGTTTCGGCTGCTGTC 3′
1A2 fw – 5′ ACCACACATCAGTCATCGTGT 3′
1A2 rev – 5′ CTTCGTCTACCGTTGTGC 3′
gypsy fw – 5′ TTCTCTAAAAAGTATGCAGCACTT 3′
gypsy rev – 5′ CACGTAATAAGTGTGCGTTGA 3′
ras fw – 5′ GAGGGATTCCTGCTCGTCTTCG 3′
ras rev – 5′ GTCGCACTTGTTACCCACCATC 3′

Each experiment was performed in triplicate biological replicates.

**Antibodies.** We used polyclonal antibodies against the N-terminal domain of the Su(Hw) protein described previously [32, 33], and monoclonal antibodies against the FLAG tag (Sigma, USA, Cat# F 1804).

**Statistical analysis** was performed using Student's *t*-test.

## RESULTS

**The $Su(Hw)^{E8}$ protein is able to directly interact with the Mod(mdg4)-67.2 and CP190 proteins.** We have previously shown that the mutant $Su(Hw)^f$ protein, in which the tenth ZF is inactivated, loses its ability to interact with the CP190 protein *in vitro* [35]. To test how mutation in the seventh ZF of the $Su(Hw)^{E8}$ protein affects interaction with two other components of the Su(Hw)-dependent complex, CP190 and Mod(mdg4)-67.2 proteins, we used a yeast two-hybrid system. Based on the pGBT9 vector, three constructs were created. The first construct expressed in yeast the full-length protein $Su(Hw)^{E8}$, the second expressed its derivative $Su(Hw)^{E8}$Δ114 with deletion of the region 88-202 aa responsible for interaction with the CP190 protein, and the third expressed the derivative $Su(Hw)^{E8}$Δ283, in which the region of interaction with the Mod(mdg4)-67.2 protein (760 to 778 aa) was deleted (Fig. 1a). We then tested direct interactions of the $Su(Hw)^{E8}$ variants with the full length CP190 and Mod(mdg4)-67.2, which were expressed in the pGAD24 vector. The $Su(Hw)^{E8}$ protein interacted with all insulator proteins in the same way as the wild type protein. The $Su(Hw)^{E8}$Δ114 protein lost its ability to interact with CP190, and the $Su(Hw)^{E8}$Δ283 protein lost its ability to interact with Mod(mdg4)-67.2 (Fig. 1a). Thus, the mutation in the seventh ZF does not affect interaction of the $Su(Hw)^{E8}$ protein with other components of the insulator complex. Therefore, the $Su(Hw)^{E8}$ protein can be recruited to chromatin through interaction with the CP190 and Mod(mdg4)-67.2 proteins.

**$Su(Hw)^{E8}$ protein restores insulator function of the mutant Su(Hw) proteins, interaction of which with CP190 is impaired.** To study the Su(Hw)-dependent insulation, two model systems are usually used, which are generated by integrating the transposable element *gypsy* into the *yellow* ($y^2$) and *cut* ($ct^6$) loci. The *yellow* gene is responsible for pigmentation of the *Drosophila* cuticle [38]. In the wild type, the body, wings, and bristles of flies are dark colored.

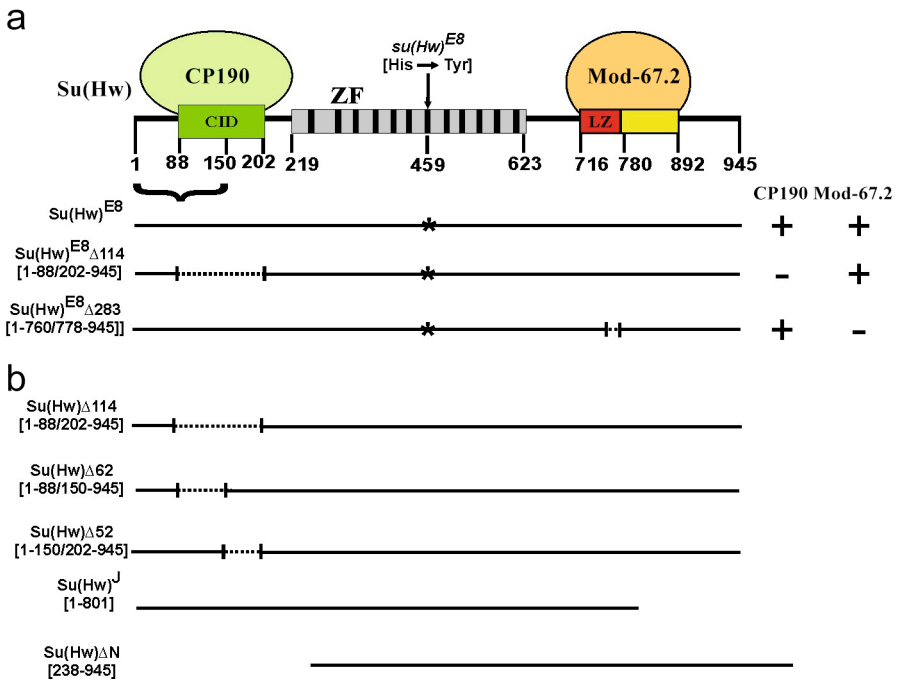


**Fig. 1.** Su(Hw) protein derivatives. a) Full length Su(Hw) protein is shown schematically. Domain designations: CID, domain interacting with CP190; ZF, zinc fingers; LZ, leucine zipper. The diagram shows the regions of interaction with the Mod(mdg4)-67.2 (Mod-67.2) and CP190 proteins, which are depicted as ovals. Vertical arrow indicates the *su(Hw)*$^{E8}$ mutation. The bracket below the diagram indicates the region used for generation of antibodies. The numbers indicate amino acid residues that limit domains and derived forms. The names of the derivatives are indicated on the left, size of the derivatives is indicated by the segments, the dotted lines indicate internal deletions, and the asterisk indicates the *su(Hw)*$^{E8}$ mutation. To the right of the diagrams are the results obtained in the Y2H. "+", presence of interaction and "–", absence of interaction. b) Deletion derivatives used in genetic experiments and immunostaining of polytene chromosomes.

In the $y^2$ allele, the *gypsy* retrotransposon was inserted between the body and wing enhancers and the *yellow* gene promoter (Fig. 2a). In this case, the Su(Hw) insulator completely blocks activation of the *yellow* expression in the body and wings, that results in yellow pigmentation of the body and wing blades of the mutant flies (Fig. 2b). However, bristles of the flies remain pigmented, because the bristle enhancer is located in the intron of the gene [39].

In the $ct^6$ allele (Fig. 2a), *gypsy* is located between the wing margin enhancer and the *cut* promoter, separated from each other by a distance of more than 70 kb. Wing margin enhancer is responsible for the development of the wing edge. In this case, the insulator completely blocks the wing margin enhancer, resulting in almost completely cut off the wing edge and the wing bristles are absent (Fig. 2b) [22, 40].

The CP190 protein binds to two regions, 88-150 aa and 150-202 aa, at the N-terminus of the Su(Hw) protein (Fig. 1a) [18]. Using genetic crosses different transgenes were integrated into the second chromosome of the $y^2ct^6$; $su(Hw)^v/su(Hw)^2$ line: expressing either the full length Su(Hw)+ protein or it derivatives – Su(Hw) Δ62 (deletion of the region 88-150 aa), Su(Hw)Δ52 ( deletion of region 150-202 aa), and Su(Hw)Δ114 (deletion of both regions interacting with CP190 (Fig. 1b). Combination of the mutations $su(Hw)^v/su(Hw)^2$ inactivates the native Su(Hw) protein, which allows us to analyze the effect of mutant proteins on the $y^2ct^6$ phenotype (Fig. 2b) [18].

Inactivation of the Su(Hw) protein in the $y^2ct^6$; $su(Hw)^v/su(Hw)^2$ line restored *yellow* expression in the $y^2$ allele and *cut* expression in the $ct^6$ allele, demonstrating that the Su(Hw) protein binding is critical for insulation. Introduction of the Su(Hw)+ transgene leads to complete restoration of insulation (Fig. 2b). We previously showed that the CP190 protein is also required for the Su(Hw) dependent insulation [18]. In the lines with expression of the Su(Hw)Δ62 and Su(Hw)Δ52 proteins exhibiting weakened binding of the CP190 protein to the Su(Hw) dependent complex, the insulator completely blocked the body and wing enhancers of the *yellow* gene ($y^2$ phenotype). However, the CP190 deficiency produced much stronger effect on the *cut* gene phenotype. In the case of Su(Hw)Δ52 protein expression, insulation in the $ct^6$ allele weakened: numerous, but separate, gaps were present along the edge of the wing and some of the bristles developed. The Su(Hw) Δ62 protein demonstrated only weak insulator activity: 1-2 gaps appeared along the edge of the wing. In the Su(Hw)Δ114 line, the CP190 protein did not bind to the insulator complex. As a result, there was no insulator

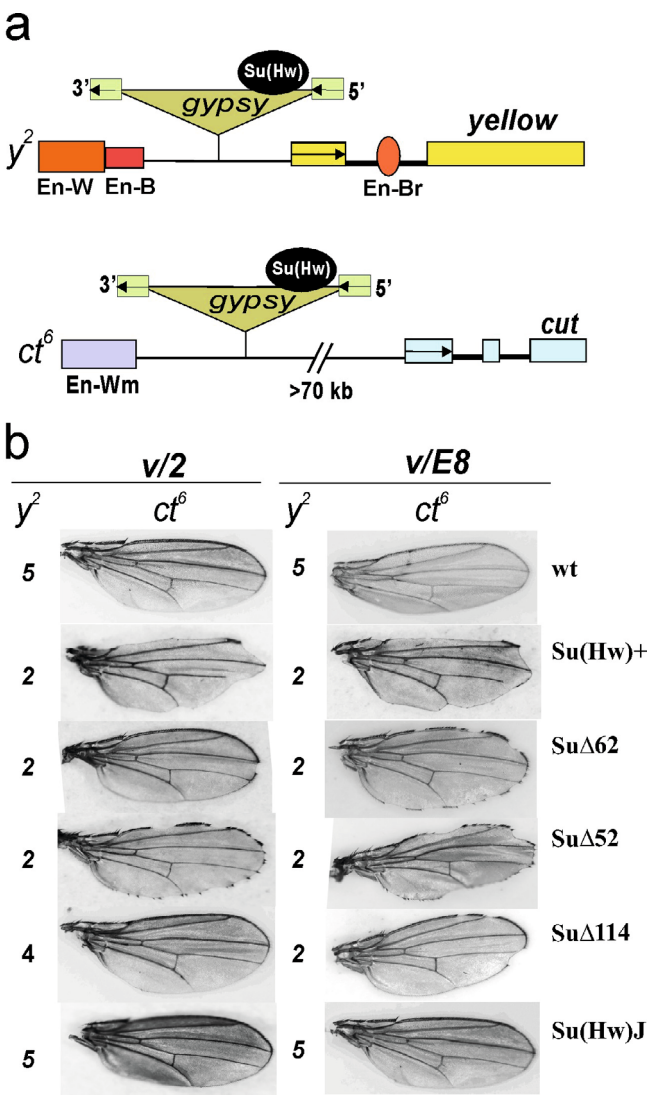


**Fig. 2.** Effect of Su(Hw)$^{E8}$ protein on *gypsy*-dependent insulation. a) Schematic representation of the $y^2$ and $ct^6$ alleles. Exons of the *yellow* and *cut* genes are shown as rectangles. Gene transcription initiation sites are indicated by arrows. The *gypsy* retrotransposon is depicted as a triangle. Rectangles at its ends represent LTRs (long terminal repeats), orientation of which is indicated by arrows. Designations: Su(Hw), insulator Su(Hw); En-W, wing enhancer, En-B, body enhancer; En-Br, enhancer of bristles; En-Wm is a wing margin enhancer. b) Effect of Su(Hw) derivatives on activity of the *gypsy* insulator in the $y^2$ and $ct^6$ alleles analyzed in the $su(Hw)^v/su(Hw)^2$ (v/2) and $su(Hw)^v/su(Hw)^{E8}$ (v/E8) mutant background. The lines used for phenotypic analysis are indicated in the right column: wt, $y^2ct^6$, Su(Hw)+, the transgene expressed as the full length protein. Schemes and names of other derivatives are shown in Fig. 1. The numbers in column $y^2$ show the level of expression of the *yellow* gene in the cuticle of the body and wings. The photographs show changes in the wing phenotype of the *cut* gene in different mutant backgrounds.

activity: the flies had the wild-type wings, and color of the cuticle was also close to normal (Fig. 2b).

Next we studied insulation in the $y^2ct^6;su(Hw)^v/su(Hw)^{E8}$ line (Fig. 2b). In this case, expression of the Su(Hw)$^{E8}$ protein did not restore insulation. Unexpectedly, insulation has been partially restored when the transgenes expressing Su(Hw) derivatives were introduced to the $y^2ct^6;su(Hw)^v/su(Hw)^{E8}$ line. In the Su(Hw)Δ62 line, the number of wing gaps increased significantly, and in the Su(Hw)Δ52 line, flies had a wing phenotype close to $ct^6$. Even in the Su(Hw)Δ114 line, the $ct^6$ allele showed weak insulation: individual wing gaps appeared. At the same time, insulation in the $y^2$ allele was restored almost completely (Fig. 2b). Analysis of the results suggests that the derivatives of Su(Hw) recruit the Su(Hw)$^{E8}$ protein capable of binding with the CP190 protein, to the sites of the *gypsy* insulator, that leads to restoration of insulation.

Since the Su(Hw)Δ114 protein does not interact with CP190, recruitment of the Su(Hw)$^{E8}$ to the Su(Hw)-dependent chromatin sites could be carried out through the Mod(mdg4)-67.2 protein, which binds to the C-terminal region of Su(Hw) (716-892 aa) responsible for insulation and transcriptional repression [20, 21]. To test the role of Mod(mdg4)-67.2 in the recruitment of the Su(Hw)$^{E8}$ protein, we used the Su(Hw)J transgene expressing a mutant protein with deletion of 144 aa at the C-terminal [16]. In the mutant protein Su(Hw)J (aa 1-801), the region of interaction with Mod(mdg4)-67.2 is deleted, so Su(Hw)J binds only to CP190 (Fig. 1b). In the $y^2ct^6;su(Hw)^v/su(Hw)^2$ mutant background, phenotypes of the flies expressing the Su(Hw)J and Su(Hw)Δ114 proteins are similar: expression of *yellow* and *cut* is restored (Fig. 2b). However, in the line expressing Su(Hw)J in the $su(Hw)^v/su(Hw)^{E8}$ background, restoration of insulation did not occur. The obtained data are consistent with the putative role of the Mod(mdg4)-67.2 protein in recruitment of the Su(Hw)$^{E8}$ protein to the Su(Hw)-dependent chromatin sites.

**Mod(mdg4)-67.2 mediates recruitment of the Su(Hw)$^{E8}$ to the binding sites of the Su(Hw)ΔN protein with polytene chromosomes.** To confirm recruitment of the Su(Hw)$^{E8}$ protein to the chromatin sites through the Mod(mdg4)-67.2 protein, we used transgenic lines expressing a Su(Hw)ΔN derivative with deletion of the N-terminal domain of the Su(Hw) (from 1 to 238 aa) tagged with the 3xFLAG (Fig. 1b) [20]. The Su(Hw)ΔN derivative, similar to the Su(Hw)Δ114, interacts only with the Mod(mdg4)-67.2 protein. On the polytene chromosomes of *Drosophila* larvae from the $y^2ct^6;Su(Hw)\Delta N\text{-}FLAG/\ Su(Hw)\Delta N\text{-}FLAG;su(Hw)^v/su(Hw)^2$ line, the Su(Hw)ΔN protein can be identified using antibodies against FLAG, but not with antibodies against the N terminal domain (1-150 aa) of the Su(Hw) protein (Figs. 1 and 3). In the $y^2ct^6$ line, the insertion sites of the *gypsy* retrotransposon are located at the end of the X chromosome distal to the chromocenter. Immunostaining with antibodies against FLAG showed that the Su(Hw)ΔN protein binds to chromatin less ef-

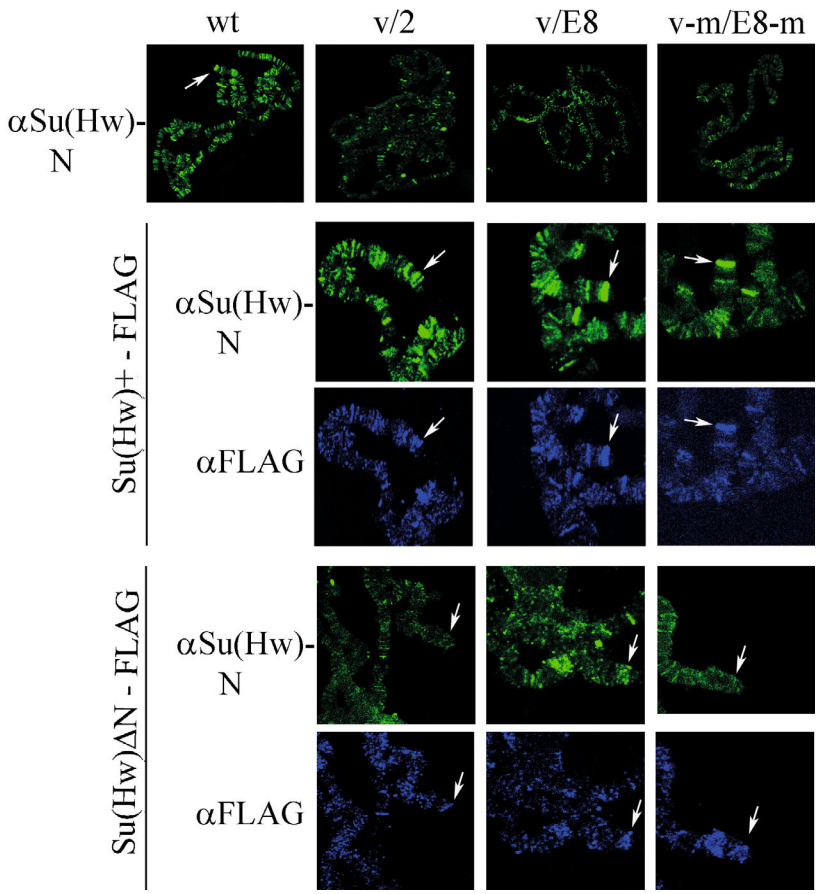


**Fig. 3.** Binding of the Su(Hw)$^{E8}$ protein to polytene chromosomes. Immunostaining of polytene chromosomes of the salivary glands of third instar larvae from the *y*$^2$*ct*$^6$ (wt) lines, *y*$^2$*ct*$^6$*;su(Hw)*$^v$*/su(Hw)*$^2$ (v/2), *y*$^2$*ct*$^6$*;su(Hw)*$^v$*/su(Hw)*$^{E8}$ (v/E8), *y*$^2$*ct*$^6$*;su(Hw)*$^v$*mod(mdg4)*$^{u1}$*/su(Hw)*$^{E8}$*mod(mdg4)*$^{u1}$ (v-m/E8-m), and from the same lines expressing the Su(Hw)ΔN-FLAG or Su(Hw)+-FLAG proteins. Antibodies against the FLAG epitope (αFLAG) and against the N-terminal domain of the Su(Hw) protein (αSu(Hw)-N) were used in the experiments. Arrows indicate *gypsy* insertion at the end of X chromosome.

ficiently in comparison with the full length Su(Hw)+ protein, since stability of the Su(Hw) binding is mediated by the CP190 protein (Fig. 3) [18].

In the *y*$^2$*ct*$^6$*;su(Hw)*$^v$*/su(Hw)*$^{E8}$ line, as in the *y*$^2$*ct*$^6$*;su(Hw)*$^v$*/su(Hw)*$^2$ line, antibodies against the N-terminal domain of Su(Hw) did not stain the Su(Hw)-binding sites. However, in the *su(Hw)*$^v$*/su(Hw)*$^{E8}$ mutant background, when the Su(Hw)ΔN derivative was expressed, these antibodies effectively stained numerous Su(Hw) binding sites, including the *gypsy* sites at the end of the chromosome X (Fig. 3). Consequently, antibodies against the N-terminal domain identified the full-length Su(Hw)$^{E8}$ protein, which was recruited to chromatin through interaction with the Su(Hw)ΔN protein.

Introduction of the *mod(mdg4)*$^{u1}$ mutation, which completely inactivates the Mod(mdg4)-67.2 protein, did not change anti-FLAG staining in the lines expressing Su(Hw)ΔN. However, in the *y*$^2$*ct*$^6$;Su(Hw)ΔN-FLAG/Su(Hw)ΔN-FLAG;*su(Hw)*$^v$*mod(mdg4)*$^{u1}$*/su(Hw)*$^{E8}$ *mod(mdg4)*$^{u1}$ line, staining with antibodies against the N terminal domain of Su(Hw) completely disappeared (Fig. 3). Thus, in the absence of the Mod(mdg4)-67.2 protein, the Su(Hw)ΔN protein lost its ability to interact with Su(Hw)$^{E8}$ and recruit it to its binding sites.

**Mod(mdg4)-67.2 mediates association of the Su(Hw)$^{E8}$ with the binding sites of the Su(Hw)ΔN protein.** To further confirm our results using chromatin immunoprecipitation, we tested binding level of the Su(Hw)$^{E8}$ protein in the *y*$^2$*ct*$^6$*;su(Hw)*$^v$*/su(Hw)*$^{E8}$ line with five most studied Su(Hw)-dependent insulators [41, 42]. To detect the Su(Hw)$^{E8}$ protein, antibodies against the N-terminal domain of Su(Hw) were used. As expected, the Su(Hw) protein was not detected at the tested sites in either the *su(Hw)*$^v$*/su(Hw)*$^2$ or *su(Hw)*$^v$*/su(Hw)*$^{E8}$ mutant background (Fig. 4a).

We next demonstrated, using anti-FLAG antibodies, that in the lines expressing the Su(Hw)ΔN-FLAG derivative, the Su(Hw)ΔN protein binds to all SBSs (Su(Hw) binding sites) both in the presence and absence of the Mod(mdg4)-67.2 protein (Fig. 4b). Introduction of the *mod(mdg4)*$^{u1}$ mutation slightly reduced the level of Su(Hw) binding, since Mod(mdg4)-67.2, similarly to CP190, stabilizes association of the insulator complex with SBS [20].

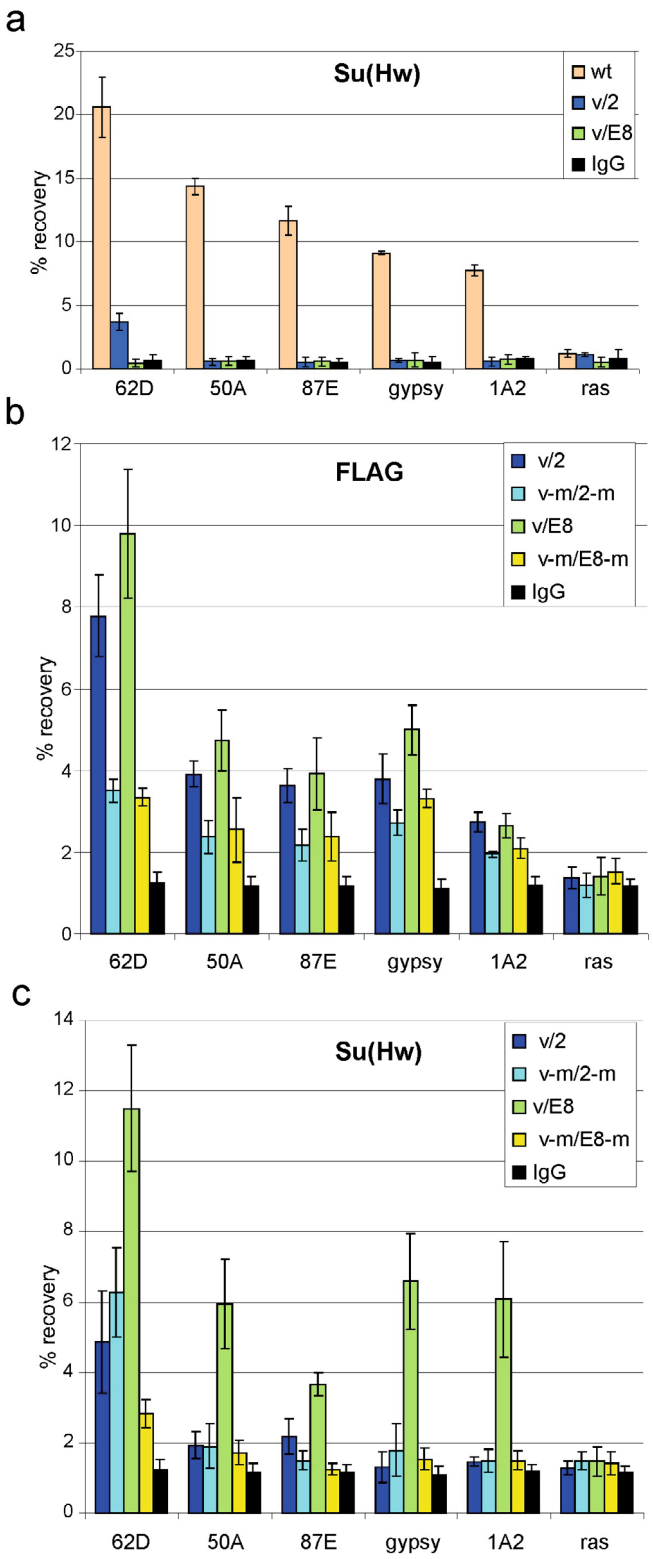


**Fig. 4.** Binding of Su(Hw)$^{E8}$ protein to SBS. a) Testing of Su(Hw) protein binding in the $y^2ct^6$ lines using antibodies against the N-terminal domain of Su(Hw). b) Su(Hw)ΔN protein binding testing in the $y^2ct^6$; Su(Hw)ΔN-FLAG/ Su(Hw)ΔN-FLAG lines using antibodies against FLAG epitope. c) Su(Hw)$^{E8}$ protein binding testing in the $y^2ct^6$; Su(Hw)ΔN-FLAG/Su(Hw)ΔN-FLAG lines using antibodies against the N-terminal domain of Su(Hw). Coding region of the *ras64B* gene (ras) was used as a control that did not contain binding sites for the Su(Hw) protein. Percent of the recovery of immunoprecipitated DNA (Y axis) was calculated relative to the input amount of DNA. The names of selected Su(Hw) dependent sites are indicated at the bottom (X axis). Standard deviation of three independent biological replicates is shown. Significance levels (Student's *t*-test) $p < 0.05$. Designations: wt, wild type; v-m/2-m, combination of mutations *su(Hw)$^v$mod(mdg4)$^{u1}$/su(Hw)$^2$ mod(mdg4)$^{u1}$*; IgG, immunoglobulins. Other designations are as in Figs. 2 and 3.

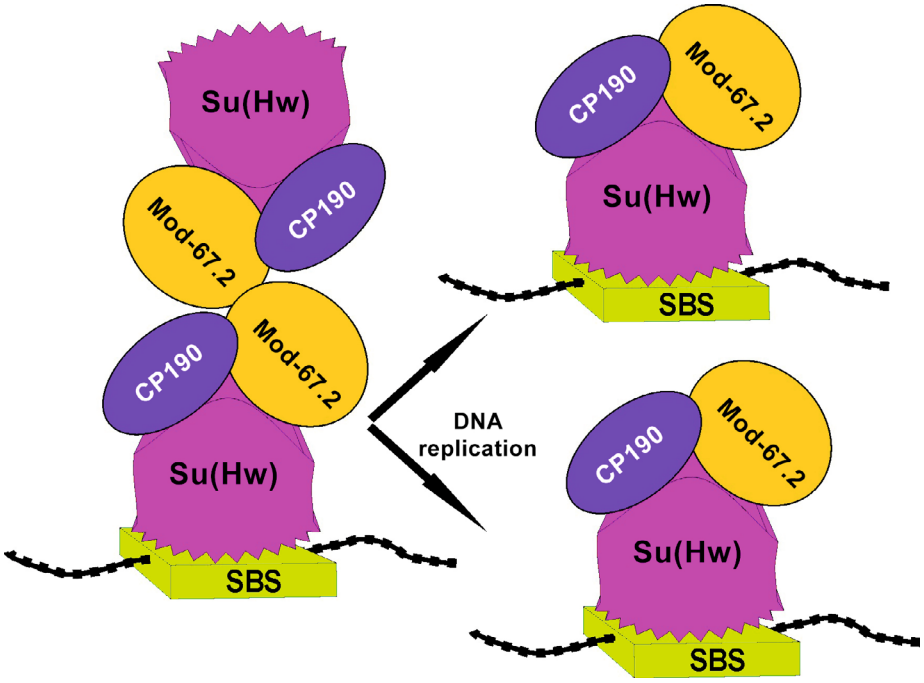


**Fig. 5.** Model of recruitment of the Su(Hw)-dependent complexes to SBS during DNA replication.

Analysis of the level of Su(Hw) binding in the $y^2ct^6$; Su(Hw)ΔN-FLAG/Su(Hw)ΔN-FLAG; $su(Hw)^v/su(Hw)^2$ line showed that the protein was absent at four of the five sites (Fig. 4c). Introduction of the $mod(mdg4)^{u1}$ mutation into this line had no effect on Su(Hw) binding. Presence of some Su(Hw) at the most efficient binding site 62D is explained by the residual expression in the $su(Hw)^2$ allele [34]. This once again confirms that antibodies against the N-terminal domain of Su(Hw) are able to recognize the native protein, but not the mutant derivative Su(Hw)ΔN.

In the $y^2ct^6$; Su(Hw)ΔN-FLAG/Su(Hw)ΔN-FLAG; $su(Hw)^v/su(Hw)^{E8}$ line the level of binding of the Su(Hw) protein at all tested sites increased 1.5-3-fold in comparison with the mutant background $su(Hw)^v/su(Hw)^2$ (Fig. 4c). When the $mod(mdg4)^{u1}$ mutation was introduced, $Su(Hw)^{E8}$ did not bind to four tested sites, and at the site 62D its binding decreased to the level of the $su(Hw)^v/su(Hw)^2$ mutant background. The obtained data completely confirm that the Mod(mdg4)-67.2 protein plays a decisive role in the interactions between the complex formed at the Su(Hw)-dependent sites and the $Su(Hw)^{E8}$ protein.

## DISCUSSION

The obtained results demonstrate that through interaction with the Mod(mdg4)-67.2 protein, the mutant $Su(Hw)^{E8}$ protein, which is unable to bind to the consensus DNA sequence, not only can be recruited to SBS, but also enhance insulator activity of the Su(Hw)-dependent complexes. According to the previously proposed model [33], speckles are the site of formation of the protein complexes, which subsequently bind to DNA. The Mod(mdg4) isoforms and the CP190 protein take part in the formation of speckles and recruit there other architectural proteins, including the Su(Hw) protein. It can be assumed that formation of the Su(Hw)ΔN-$Su(Hw)^{E8}$/CP190 complex occurs in the speckles and is stabilized by multimerization of the Mod(mdg4)-67.2 protein, which interacts with all components of this complex. As a result of the subsequent binding of the complex to SBS, the $Su(Hw)^{E8}$/CP190 proteins are also recruited to them, which leads to partial restoration of the activity of the *gypsy* insulator dependent on the CP190 protein.

The obtained results suggest that the Su(Hw) protein is capable of being recruited to the chromatin Su(Hw) binding sites without directly interacting with the DNA consensus sequence. It should be noted that in almost all regulatory elements SBS are present as a single copy [41]. Recruitment of the chromatin-unbound Su(Hw) protein to the single SBSs can increase efficiency of recruitment of the partner proteins, thereby increasing functional activity of the Su(Hw) dependent insulator. Also, Su(Hw) proteins, not associated with DNA, can bind to the newly synthesized DNA during the replication process, competing with nucleosomes, and thus effectively reproduce insulators during the cell division (Fig. 5). To experimentally confirm the proposed model, further studies of the mechanisms of insulator complexes formation in the speckles and on chromatin are required.

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