SUMO conjugation is required for the assembly of *Drosophila* Su(Hw) and Mod(mdg4) into insulator bodies that facilitate insulator complex formation

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Summary

Chromatin insulators are special regulatory elements involved in modulation of enhancer–promoter interactions. The best studied insulators in *Drosophila* require Suppressor of Hairy Wing [Su(Hw)], Modifier of mdg4 [Mod(mdg4)] and centrosomal 190 kDa (CP190) proteins to be functional. These insulator proteins are colocalized in nuclear speckles named insulator bodies. Here, we demonstrate that post-translational modification of insulator proteins by small ubiquitin-like modifier (SUMO) and intact CP190 protein is crucial for insulator body formation. Inactivation of SUMO binding sites in Mod(mdg4)-67.2 leads to the inability of the mutant protein and Su(Hw) to be assembled into insulator bodies. In vivo functional tests show that a smaller amount of intact Mod(mdg4)-67.2, compared with the mutant protein, is required to restore the normal activity of the Su(Hw) insulator. However, high expression of mutant Mod(mdg4)-67.2 completely rescues the insulator activity, indicating that sumoylation is not necessary for enhancer blocking. These results suggest that insulator bodies function as a depot of sumoylated proteins that are involved in insulation and can facilitate insulator complex formation, but are nonessential for insulator action.

Key words: Chromatin insulator, CP190, Heat shock, Nuclear speckles, Sumoylation

Introduction

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 (for reviews, see Brasset and Vaury, 2005; Gaszner and Felsenfeld, 2006; Kuhn and Geyer, 2003; Ohlsson et al., 2010; Valenzuela and Kamakaka, 2006). In addition, most of insulators can support long-distance interactions of distantly located enhancers/silencers and promoters, thereby regulating communication between them. Some insulators can also function as boundaries between transcriptionally active chromatin and heterochromatin.

To date, chromatin insulators have been characterized in a variety of species, which is indicative of their universal importance in the regulation of gene expression. The best-characterized *Drosophila* insulator was found in the regulatory region of the *gypsy* retrovirus (Geyer et al., 1986; Geyer and Corces, 1992; Kim et al., 1996). It contains 12 degenerated repeats of the binding motif for the zinc-finger protein Suppressor of Hairy wing [Su(Hw)], which is essential for its function (Holdridge and Dorsett, 1991; Geyer and Corces, 1992). At the same time, most endogenous Su(Hw) insulators contain fewer than four Su(Hw) binding sites (Kuhn-Parnell et al., 2008; Nègre et al., 2010; Parnell et al., 2006).

Three proteins, Mod(mdg4)-67.2, CP190 and E(y)2/Sus1, interact directly with Su(Hw) and are required for the activity of Su(Hw)-dependent insulators (Büchner et al., 2000; Gause et al., 2001; Gerasimova et al., 1995; Georgiev and Kozycina, 1996; Ghosh et al., 2001; Pai et al., 2004; Kurshakova et al., 2007). Mod(mdg4)-67.2 and CP190 are BTB/POZ domain proteins that are recruited to chromatin by the Su(Hw) protein. The BTB domain identifies a large family of proteins in different organisms, from yeast to humans, and functions as a protein interaction domain facilitating homodimer or tetramer formation as well as oligomerization (Stogios et al., 2005). Mod(mdg4)-67.2 is one of isoforms produced by the *mod(mdg4)* gene, also known as *E(var)3-93D*, which encodes a large set of proteins containing the BTB domain at the N-terminus and different C-terminal domains (Büchner et al., 2000). Mod(mdg4)-67.2 interacts with the enhancer-blocking domain of the Su(Hw) protein through an isoform-specific C-terminal acidic domain (Gause et al., 2001; Ghosh et al., 2001; Golovnin et al., 2007). This domain is affected in two viable mutations, $mod(mdg4)^{u1}$ and $mod(mdg4)^{T6}$.

The Su(Hw), Mod(mdg4)-67.2 and CP190 proteins colocalize in discrete foci, named insulator bodies, in the *Drosophila* interphase cell nucleus (Gerasimova et al., 2000; Pai et al., 2004). Hence, it has been asserted (Gerasimova et al., 2000) that the insulator bodies arise via association of individual Su(Hw)-containing nucleoprotein complexes located at distant chromosomal sites. Hypothetically, a number of Su(Hw) insulators coalesce into an insulator body owing to interactions between insulator proteins Mod(mdg4)-67.2 and CP190. The interacting insulators form chromatin loop domains that, by unknown mechanisms, block communication between regulatory

elements separated by the insulators. However, the results of our recent study (Golovnin et al., 2008) show that the insulator bodies are aggregates of insulator proteins that resemble well-known promyelocytic leukemia nuclear bodies (PML-NB) comprising many unrelated proteins (for a review, see Bernardi and Pandolfi et al., 2007).

Mod(mdg4)-67.2 and CP190, but not Su(Hw), conjugate to small ubiquitin-like modifier (SUMO) in vivo and in vitro (Capelson and Corces, 2006). Conjugation to SUMO is a post-translational modification that regulates the activity of many nuclear factors (Johnson, 2004; Meulmeester and Melchior, 2008; Ulrich, 2008). SUMO attachment (sumoylation) is essential for a variety of functional outputs, including regulation of transcription activity, subnuclear targeting and formation of nuclear compartments (Gill, 2005; Kerscher et al., 2006; Stielow et al., 2008; Riising et al., 2008). In particular, sumoylation of proteins is essential for the formation of PML bodies (Heun, 2007; Lin et al., 2006; Shen et al., 2006). SUMO is covalently attached to target proteins by a cascade of enzymes, including the activating enzyme E1, the conjugating enzyme E2 (also known as Ubc9), and a variety of the specificity-enhancing E3 ligases (Gareau and Lima, 2010).

Here, we demonstrate that the assembly of insulator bodies is determined by CP190 and SUMO. Sumoylation of Mod(mdg4)-67.2 is essential for incorporation of this protein and Su(Hw) into the insulator bodies but is dispensable for the stability of CP190-dependent insulators bodies. The recruitment of Mod(mdg4)-67.2 and Su(Hw) proteins to the insulator bodies facilitates the formation of relevant complexes on the insulators but is not necessary for their functional activity.

Results

SUMO is essential for the assembly of insulator bodies in *Drosophila*

The nuclei of S2 cells derived from *Drosophila* embryos showed speckles with positive staining for Mod(mdg4)-67.2, Su(Hw) and CP190 (Fig. 1A, left column). These speckles were similar in size, number and arrangement to insulator bodies reported in flies (Gerasimova et al., 2000; Golovnin et al., 2008; Pai et al., 2004). To assess the presence of SUMO in these structures, we stained S2 cells with antibodies to SUMO and to Mod(mdg4)-67.2, Su(Hw) or CP190. SUMO was visualized as numerous dots (Fig. 1A, middle column), many of them showing colocalization with the insulator bodies (Fig. 1A, right column). In general, all insulator bodies (speckles) proved to colocalize with SUMO.

To estimate the role of SUMO in organization of insulator bodies, we performed double-stranded RNA-mediated interference (RNAi) assays. Western blot analysis showed that the amount of *Drosophila* SUMO was significantly reduced by RNAi depletion (Fig. 1B). Inactivation of SUMO resulted in a diffuse distribution of the Su(Hw), Mod(mdg4)-67.2 and CP190 proteins (Fig. 1C). Thus, SUMO is required for organization of insulator proteins into insulator bodies.

CP190 organizes insulator proteins into insulator bodies

To assess the role of insulator proteins in the assembly of nuclear speckles, S2 cells were depleted of individual insulator proteins by means of RNAi treatment. Western blot analysis showed that the amount of each protein, compared with the control, was reduced to less than 50% after 24 hours (not shown) and to less than 10% after

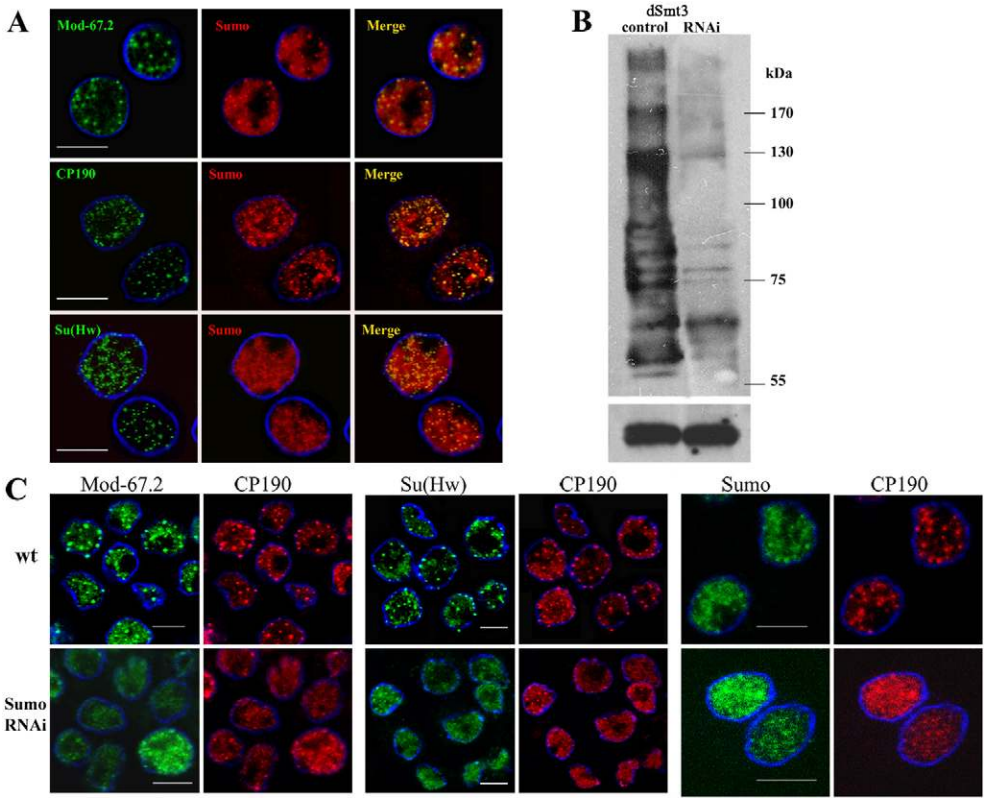

Fig. 1. SUMO is crucial for the assembly of insulator bodies. (**A**) Immunofluorescent localization of SUMO in insulator bodies. Here and in C, the nuclear envelope is stained with lamin (blue). (**B**) Western blot analysis of dSmt3 protein in wild-type and RNAi-treated S2 cells. Tubulin was used as a loading control. (**C**) Sumoylation of insulator proteins provides for their maintenance in the nuclear foci. Upper row: insulator bodies formed by major protein components of the insulator complex in wild-type (wt) S2 cell line. Lower row: dSmt3 RNAi treatment interferes with nuclear coalescence of insulator proteins. Scale bars: 5 μm.

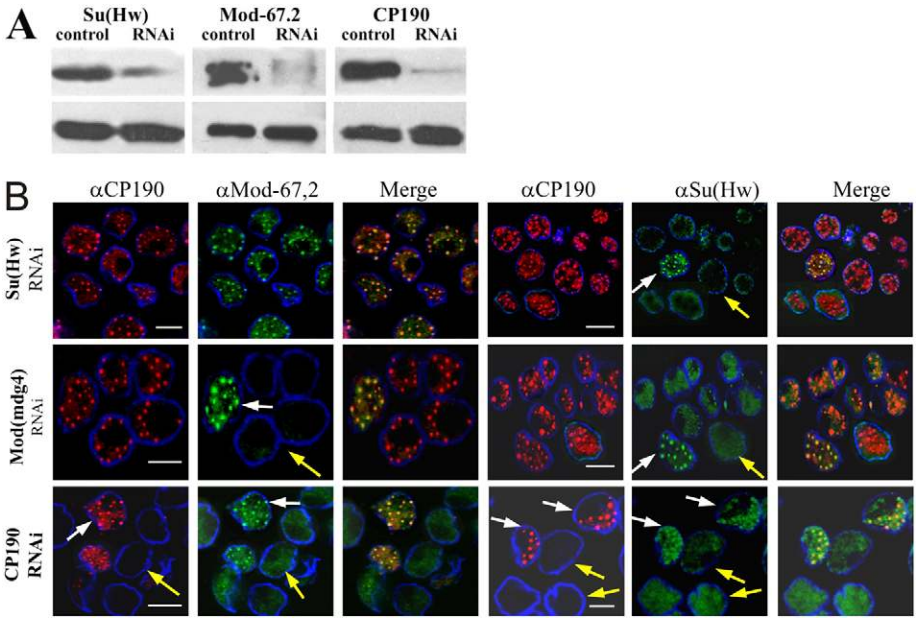

Fig. 2. CP190 is a major component responsible for the formation of all kinds of insulator bodies. (**A**) Western blot analysis of test proteins in wild-type (control) and RNAi-treated S2 cells. More than 70% reduction in the amount of these proteins was observed after treatment. Tubulin was used as a loading control. (**B**) Series of cells RNAi-depleted of different insulator complex components. White arrows indicate cells with normal localization of a test protein and and yellow arrows indicate cells depleted of this protein. The nuclear envelope is stained with lamin (blue). Scale bars: 5 μm.

48 hours of RNAi treatment (Fig. 2A). Immunofluorescence analysis of cells fixed at the 48-hour time-point confirmed that the contents of these proteins were indeed reduced significantly (Fig. 2B, yellow arrows). In some cells, however, the content and localization of the test protein remained normal (Fig. 2B, white arrows), which was due apparently to incomplete efficiency of cell transformation by RNAi treatment. Such cells were used as an internal positive control to compare the structure of insulator bodies in presence or absence of the test protein.

Cell depletion of the Su(Hw) protein had no effect on the assembly of CP190 and Mod(mdg4)-67.2 into the nuclear speckles (Fig. 2B), indicating that Su(Hw) was not required for the formation of insulator bodies. The depletion of Mod(mdg4)-67.2 resulted in a diffuse distribution of Su(Hw) but did not affect CP190 localization in the speckles (Fig. 2B). These results confirm that Mod(mdg4)-67.2 recruits Su(Hw) to the insulators bodies, whereas CP190 can forms speckles independently. It is noteworthy that inactivation of CP190 resulted in a diffuse distribution of both Su(Hw) and Mod(mdg4)-67.2 proteins (Fig. 2B). Therefore, the CP190 protein is crucial for the assembly of insulators bodies.

According to sequence analysis, the CP190 protein contains two potential SUMO-interacting motif (SIM) sites that might be important for the interaction with SUMO. However, we failed to detect any direct interaction between SUMO and either CP190 or Mod(mdg4)-67.2 in the GST pull-down or yeast two-hybrid assays (supplementary material Fig. S1A,B). According to our previous data (Golovnin et al., 2007), Mod(mdg4)-67.2 directly interacts with CP190, and both insulator proteins are efficiently co-immunoprecipitated from the nuclear extract of S2 cells. Here, we found that inactivation of SUMO by RNAi had no significant influence on the efficiency of co-immunoprecipitation between insulator proteins (supplementary material Fig. S1C). These results suggest that additional interactions between insulator proteins, along with SUMO-mediated interactions, are involved in the formation of insulator complexes.

CP190 is redistributed to the nuclear periphery during heat shock, whereas Su(Hw) and Mod(mdg4)-67.2 mostly remain in the nuclear interior

The heat shock response in *Drosophila* has been studied in detail. In particular, it has been shown that exposure of flies to heat shock stress results in disruption of insulator bodies (Gerasimova et al., 2000). We repeated these experiments with S2 cells (Fig. 3A). In the cells heat-shocked for 30 minutes, we observed redistribution of CP190 protein to the nuclear envelope, whereas Su(Hw) and Mod(mdg4)-67.2 mainly remained in the nuclear interior. Thus, heat shock proved to induce dissociation of CP190 from Su(Hw) and Mod(mdg4)-67.2 in the nuclear bodies.

At the same time, we revealed no changes in co-immunoprecipitation of CP190 and Mod(mdg4)-67.2 or in their sumoylation status (Fig. 3B). This suggests that desumoylation of these insulator proteins is not the factor triggering their redistribution in the nucleus under heat shock conditions and that they remain bound into complexes. Moreover, we observed partial colocalization of CP190 with SUMO at the nuclear periphery (Fig. 3A), which indicated that both these proteins were still present in the complexes.

For this reason, we examined the in vivo binding of the Su(Hw), CP190 and Mod(mdg4)-67.2 proteins to the *gypsy* and four endogenous Su(Hw) insulators by chromatin immunoprecipitation (ChIP) analysis of chromatin isolated from S2 cells before and after heat shock treatment (Fig. 3C). In agreement with co-immunoprecipitation experiments, we found that the Su(Hw), Mod(mdg4)-67.2 and CP190 proteins under heat shock could still form stable complexes on the tested insulators. Thus, heat shock treatment affects insulator bodies but not chromatin insulators in S2 cells.

Inactivation of SUMO conjugating sites in Mod(mdg4)-67.2 renders the mutant protein incapable of entering insulator bodies

SUMO conjugation (for a review, see Hay, 2005) has been detected most often within the ΨKXE sumoylation consensus

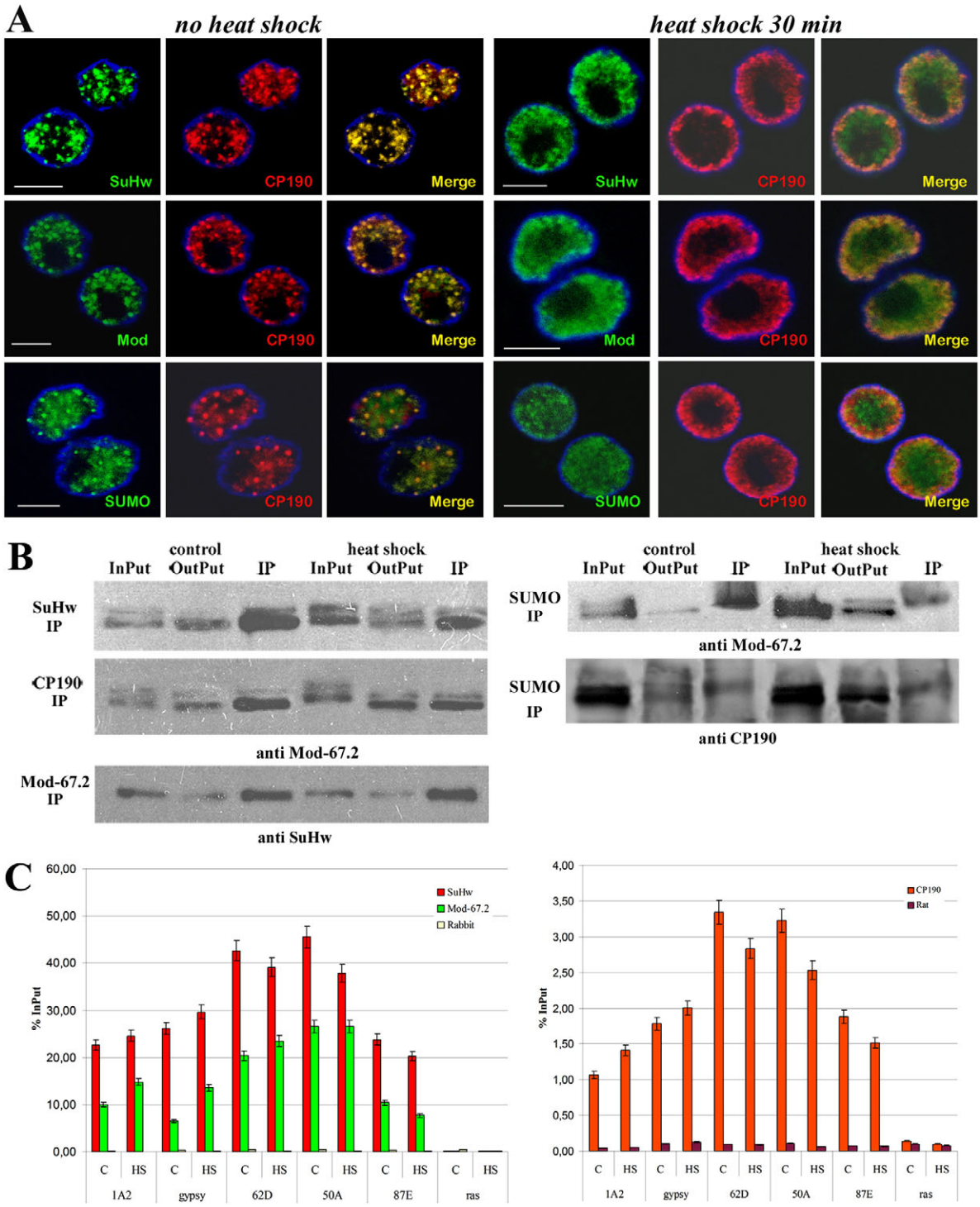

Fig. 3. Heat shock treatment affects insulator bodies but not chromatin insulators in S2 cells. (**A**) Nuclear localization of insulator proteins in S2 cells before and after 30 minutes of heat shock. The upper row shows the distribution of Su(Hw) (green) and CP190 (red) proteins; the middle row, distribution of Mod(mdg4)-67.2 (green) and CP190 (red) proteins; and the bottom row, distribution of SUMO (green) and CP190 (red) proteins. The nuclear envelope is stained with lamin (blue). (**B**) Co-immunoprecipitation between the insulator proteins and their sumoylation status under non-heat-shock (control) and heat shock conditions. Immunoprecipitated complexes were washed with 500 mM KCl-containing buffers before loading on SDS-PAGE for western blot analysis with the indicated antibodies. InPut, input fraction; OutPut, supernatant after immunoprecipitation; IP, immunoprecipitate. (**C**) Results of X-ChIP (percentage of input DNA; $n=3$) of specified chromatin regions from non-heat-shocked (C) and heat-shocked (HS) S2 cell cultures probed with antibodies to Su(Hw) (red bars), Mod(mdg4)-67.2 (green bars) and CP190 (orange bars); *ras* coding regions were used as controls devoid of Su(Hw) binding sites. For a negative control with nonspecific immunoglobulins, rabbit (white bars) or rat (purple bars) pre-immune serum was used. Scale bars: 5 μm.

motif, where Ψ is a large hydrophobic amino acid residue, K is the lysine residue undergoing SUMO attachment, X is any residue and E is glutamic acid. The Mod(mdg4)-67.2 protein contains two sumoylation consensus motifs at positions 160 and 423 (Fig. 4A). To examine the role of sumoylation in the functionality of Mod(mdg4)-67.2, we mutated either one (Mod-160 and Mod-423) or both of these sites (Mod-160/423) by performing lysine-to-arginine exchanges.

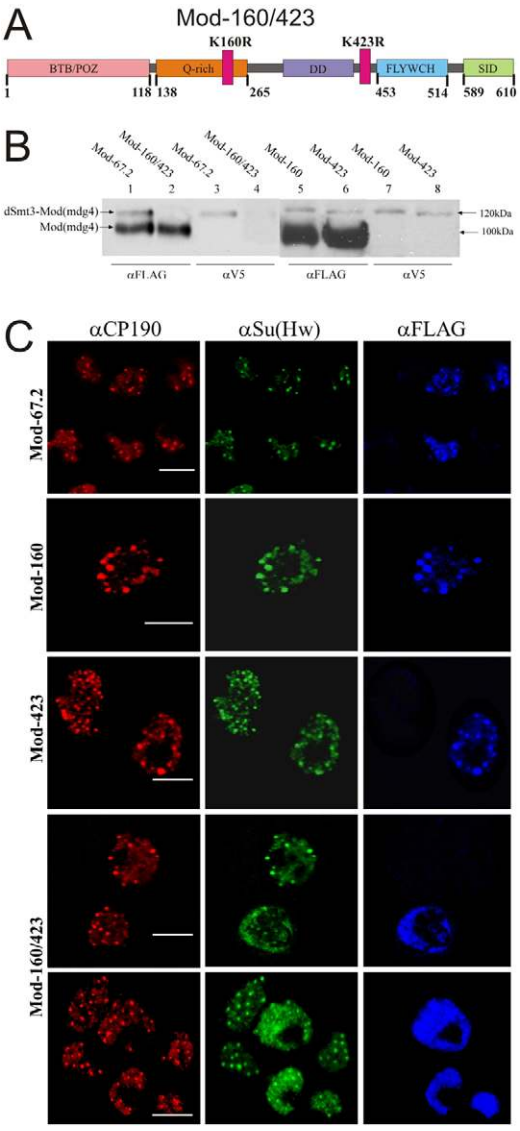

Fig. 4. Mod(mdg4) protein sumoylation. (**A**) Scheme of Mod(mdg4) structural domains showing BTB/POZ domain; Q-rich, glutamine-rich domain; DD, dimerization domain; FYWCH, FYWCH-type Zn finger domain; SID, Su(Hw) interacting domain; and two sumoylation sites (red boxes with figures indicating their amino acid positions). (**B**) Immunoprecipitation with anti-FLAG antibodies of the coexpressed dSmt3-V5 fusion protein and different Mod(mdg4) variants: Mod-67.2–FLAG, Mod 160–FLAG, Mod 423–FLAG or Mod-160/423–FLAG. The western blot was probed with either FLAG or V5 antibodies. Unmodified and sumoylated forms of Mod(mdg4) are visualized as bands at 100 and 120 kDa, respectively. (**C**) Transfected S2 cells expressing FLAG-tagged Mod-67.2, Mod 160, Mod 423 or Mod-160/423 variants. The columns show the distribution of CP190 protein (red), Su(Hw) (green) and transfected FLAG-tagged Mod(mdg4) variants (blue). Non-transfected cells were used as the internal control for CP190 and Su(Hw) distribution. Scale bars: 5 μm.

To test whether SUMO failed to attach to the mutant protein, we made plasmids encoding wild-type (Mod-67.2) or mutant Mod-160/423, Mod-160 and Mod-423 proteins tagged with triple FLAG epitopes and a plasmid encoding dSmt3 tagged with a V5 epitope. S2 cells were co-transfected with either of the Mod(mdg4) proteins tagged with triple FLAG and dSmt3-V5 plasmids. Extracts from these cells were treated with FLAG Sepharose beads, and the precipitates analyzed by western blotting with anti-FLAG or anti-V5 antibodies. In the precipitates from cells transfected with Mod-67.2–FLAG, Mod-160–FLAG or Mod-423–FLAG, the anti-FLAG antibody revealed two bands. The first band corresponded to the major Mod(mdg4)-67.2 isoform with an apparent molecular weight of 100 kDa, and the second band to a larger isoform with an apparent weight of 120 kDa (Fig. 4B, lanes 1, 5, 6). By contrast, in the cells transfected with Mod-160/423–FLAG, only the 100-kDa band was detected (Fig. 4B, lane 2). To confirm that the additional 120-kDa band is associated with SUMO conjugation, FLAG-precipitated extracts were analyzed with anti-V5 antibodies. These antibodies recognized the 120-kDa band in the cells transfected with Mod-67.2–FLAG, Mod-160–FLAG or Mod-423–FLAG, indicating that it corresponds to the sumoylated Mod(mdg4)-67.2 protein (Fig. 4B, lanes 3, 7, 8). The antibodies did not reveal any band in the case of Mod-160/423–FLAG transfection (Fig. 4B, lane 4), indicating that SUMO failed to attach to the mutant Mod-160/423 protein. These results show that Mod(mdg4)-67.2 contains two sites for sumoylation.

We also compared the distribution of CP190, Su(Hw) insulator proteins and Mod(mdg4) proteins (wild-type and mutants) tagged with triple FLAG epitope in transfected S2 cells (Fig. 4C). Overexpression of Mod-67.2–FLAG did not appreciably change the normal staining patterns: anti-FLAG showed exclusively nuclear punctate distribution consistent with that of Su(Hw) or Cp190 (Fig. 4C, top row). Under conditions of Mod-160–FLAG or Mod-423–FLAG overexpression, the staining pattern was similar to that of the wild-type protein (Fig. 4C, middle rows). By contrast, overexpression of Mod-160/423–FLAG resulted in massive, diffuse staining of the protein in the nuclei. At the same time, CP190 still formed nuclear speckles, whereas Su(Hw) was distributed between these speckles and the zone of diffuse Mod-160/423–FLAG staining (Fig. 4C, bottom row). Therefore, Mod-160/423–FLAG apparently failed to efficiently enter the insulator bodies, which suggests that SUMO conjugation plays a crucial role in this process.

In vivo functional testing of the mutant Mod(mdg4)-67.2 protein devoid of sumoylation sites

To assess the functional role of Mod(mdg4)-67.2 sumoylation, we compared the activities of wild-type and mutant proteins in vivo. To this end, we used transgenic lines characterized by UAS-driven expression of the Mod-67.2 and Mod-160/423 genes in a null *mod(mdg4)*^{u1} background.

Phenotypic analysis of the competence of mutant versus wild-type protein in the insulator function was performed in male flies carrying *gypsy*-induced alleles in the *cut* locus. In the *ct*^{6} allele (Fig. 5A), *gypsy* is between the wing margin enhancer and the *cut* promoter, which are 85 kb apart (Gause et al., 2001). The insulator in *ct*^{6} completely blocked this enhancer, producing a cut wing phenotype (Fig. 5B). The null *mod(mdg4)*^{u1} mutation

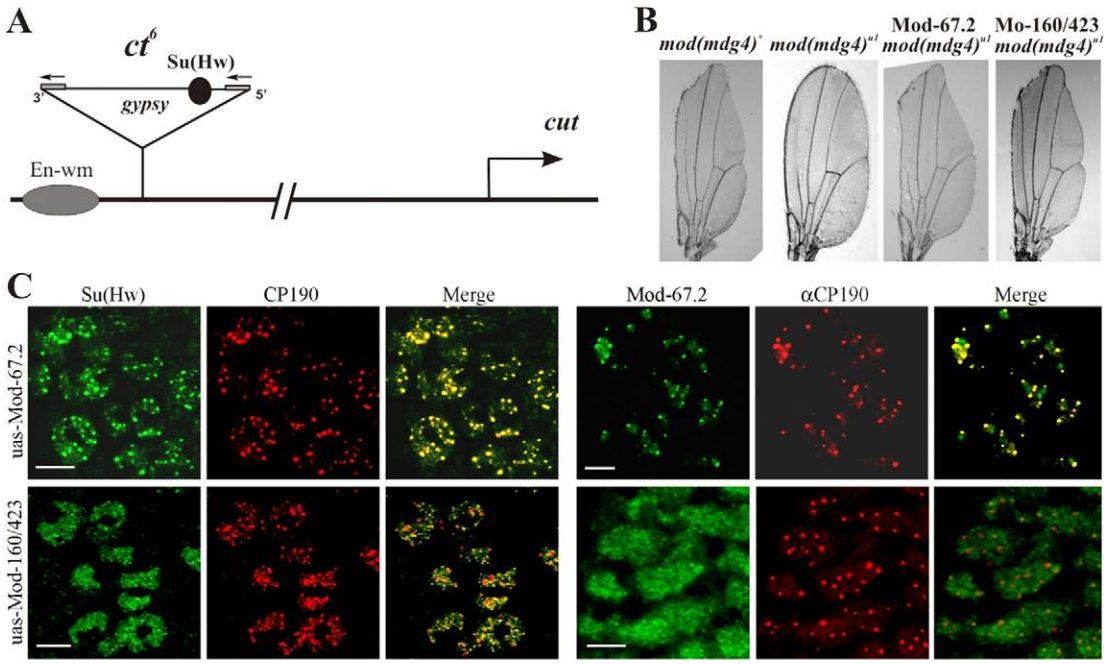

Fig. 5. Phenotypic effects of the Mod(mdg4) proteins. (**A**) Scheme of ct^6 alleles: the bent arrow indicates the start site and direction of *cut* gene transcription, the gray oval is the wing margin enhancer (En-wm), and the triangle shows the insertion of *gypsy* insulator with flanking LTRs and the Su(Hw) insulator (black circle). (**B**) Effects of Mod(mdg4) variants on the cut wing phenotype in the ct^6 allele. Unmodified Mod-67.2 and mutant Mod-160/423 proteins were expressed under *actin* driver in ct^6; $mod(mdg4)^{u1}/mod(mdg4)^{u1}$ flies. (**C**) Immunostaining of diploids cells from larval imaginal discs of $mod(mdg4)^{u1}$ flies expressing either wild-type Mod(mdg4)-67.2 (upper row) or mutant Mod-160/423 protein (bottom row) with antibodies to Su(Hw) (green), Mod(mdg4)-67.2 (green) and CP190 (red). Scale bars: 5 μm.

almost completely suppressed the ct^6 mutant phenotype, indicating that Mod(mdg4)-67.2 is essential for blocking the wing margin enhancer (Fig. 5B). When GAL4 was under control of the strong, ubiquitously expressed *actin* promoter (actin-GAL4), transgenes expressing either Mod-67.2 or Mod-160/423 completely restored the *gypsy* insulator function in the $mod(mdg4)^{u1}$ background (Fig. 5B). Similar results were obtained with other *gypsy*-induced alleles in the *yellow* (y^2) and *scute* (sc^{D1}) loci (data not shown). These results suggest that sumoylation of Mod(mdg4)-67.2 is not essential for the enhancer-blocking activity of the *gypsy* insulator.

Next, we examined immunostaining patterns in imaginal disc cells (Fig. 5C). In accordance with published observations (Gerasimova et al., 2000; Pai et al., 2004), the wild-type nuclei contained multiple speckles including CP190, Mod(mdg4)-67.2 and Su(Hw) proteins. In the $mod(mdg4)^{u1}$ mutant background, Su(Hw) was only weakly associated with the CP190 speckles. As expected, transgenic expression of Mod-67.2 in the $mod(mdg4)^{u1}$ null background restored the wild-type staining pattern. However, Mod-160/423 in the null background failed to enter any nuclear speckles formed by CP190 (Fig. 5C). The Su(Hw) protein partially colocalized with CP190 in the speckles and also with Mod-160/423 in the zone of diffuse staining. These results indicate that the presence of Mod-160/423 in the insulator bodies is not required for the enhancer-blocking activity of the *gypsy* insulator.

Sumoylation reduces the amount of Mod(mdg4)-67.2 required for activity of Su(Hw)-dependent insulators

The UAS-driven promoter under the control of *actin*-Gal4 induces several-fold higher expression of the transgene, compared with the endogenous *mod(mdg4)* gene (Fig. 6A). Thus, it is possible that overexpression of the mutant Mod(mdg4)-67.2 protein compensates for its inability to participate in the assembly of nuclear speckles. To examine this possibility, we used the GAL4 driver under control of the *heat shock 70* (*hsp70*) promoter (*hsp70*-GAL4) in order to regulate the expression of UAS-dependent transgenes. The minimum time of heat shock treatment allowing complete rescue of the mutant $mod(mdg4)^{u1}$ phenotype by the UAS-Mod-67.2 transgene was estimated at 10 minutes (Fig. 6B). However, the same time of heat shock treatment was insufficient for counterbalancing the $mod(mdg4)^{u1}$ mutation by the UAS-Mod-160/423 transgene, although the mutant ct^6 phenotype was slightly enhanced under such conditions (Fig. 6B). In these transgenic lines, the expression of UAS-Mod(mdg4) constructs was approximately 12% of that in transgenic lines with the *actin*-GAL4 driver and 50% of that in lines with the endogenous *mod(mdg4)* gene (Fig. 6A). Enhancement of the UAS-Mod-160/423 expression to the level characteristic of the endogenous *mod(mdg4)* gene (three heat shock treatments of transgenic flies over 30 minutes) resulted in complete rescue of the mutant $mod(mdg4)^{u1}$ phenotype (Fig. 6B). These results suggest that, to restore normal activity of the *gypsy* insulator, the wild-type protein is required in a smaller amount than is the mutant Mod(mdg4)-67.2 protein.

It was shown previously that Mod(mdg4)-67.2 facilitates Su(Hw) binding to insulator sequences (Gerasimova et al., 2000; Golovnin et al., 2007). The in vivo binding of the Su(Hw) and Mod(mdg4)-67.2 proteins to the *gypsy* and four endogenous Su(Hw) insulators (Parnell et al., 2006) was examined by ChIP

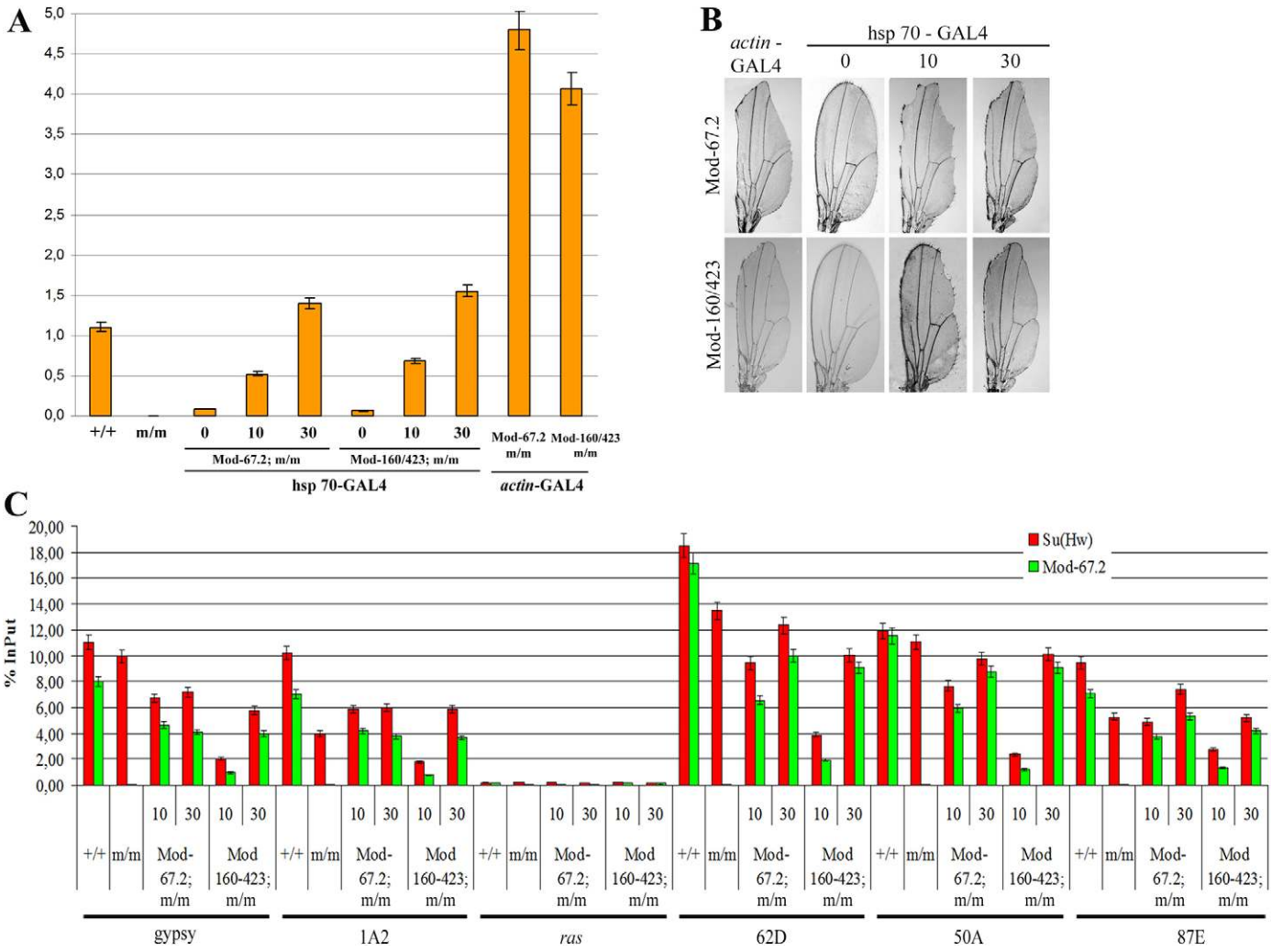

Fig. 6. Higher level of mutant Mod(mdg4) protein compensates for its inability to form insulator bodies. (**A**) Relative expression levels of UAS-Mod-67.2 (Mod-67.2) and UAS-Mod-160/423(Mod-160/423) transgenes depend on driver type and time of heat shock. RNA was isolated from 3-day-old adult flies from lines y^2; +/+ (+/+), y^2; *mod(mdg4)*^{u1}/*mod(mdg4)*^{u1} (m/m) and y^2; *mod(mdg4)*^{u1}/*mod(mdg4)*^{u1}, which expressed either UAS-Mod-67.2 (Mod-67.2; m/m) or UAS-Mod-160/423 (Mod-160/423; m/m) transgenes under *actin*-GAL4 or *hsp70*-GAL4 driver. To express the driver regulated by the *hsp70* promoter, the flies were exposed once to heat shock at 37℃ for either 10 or 30 minutes at the embryonic, second-instar larval and middle pupal stages of development. Symbols +/+ and m/m indicate the level of *mod(mdg4)* gene expression in wild-type and *mod(mdg4)*^{u1}/*mod(mdg4)*^{u1} flies, respectively. For each experiment, duplicate or triplicate reactions were performed and averaged. At least three independent experiments were performed with each primer set, using at least two independent RNA samples. The expression level of each gene was determined using Ras64B as an internal control. For more details, see Materials and Methods. (**B**) Effects of Mod (mdg4) variants on the cut wing phenotype in the ct^6 allele. The UAS-Mod-67.2 and UAS-Mod-160/423 transgenes were expressed under *actin*-GAL4 or *hsp70*-GAL4 drivers in ct^6; *mod(mdg4)*^{u1}/*mod(mdg4)*^{u1} flies. To induce GAL4 expression under control of the *hsp70* promoter (*hsp70*-GAL), transgenic flies were exposed to heat shock for 10 or 30 minutes at the embryonic, second-instar larval and middle pupal stages of development. (**C**) Results of X-ChIP (percentage of input DNA, $n=3$) of specified chromatin regions with antibodies to Su(Hw) (red bars) or Mod(mdg4)-67.2 (green bars). Symbols +/+ and m/m refer to wild-type and *mod(mdg4)*^{u1}/*mod(mdg4)*^{u1} flies, respectively. Analysis was done on *mod(mdg4)*^{u1} (m/m) pupa expressing Mod-67.2 or Mod-160/423 variants under control of heat shock drivers. The *ras* coding regions were used as controls devoid of Su(Hw) binding sites. Immunoprecipitation and washing for X-ChIP analysis were performed as described previously (Golovnin et al., 2008). Precipitated products were analyzed by real time PCR. For input PCRs, 0.1% of DNA applied to a ChIP reaction was used as a template. To determine the percentage of input, PCR products were amplified from at least three separate immunoprecipitation products from at least two different chromatin preparations.

analysis of chromatin isolated from the middle pupa stage (Fig. 6C). Inactivation of the Mod(mdg4)-67.2 protein in the *mod(mdg4)*^{u1} mutant resulted in significant reduction of Su(Hw) binding only to the 1A2 and 87E insulators. This agrees with our previous data that Mod(mdg4)-67.2 is crucial for activity of the endogenous 1A2 insulator (Golovnin et al., 1999). At other sites, weak reduction of Su(Hw) binding might be explained by partial masking of the Su(Hw) epitope by Mod(mdg4)-67.2. The expression of the wild-type Mod(mdg4)-67.2 protein at either low or high level (after heat shock treatment for 10 or 30 minutes, respectively) restored normal binding of the Su(Hw) protein to the insulators. By contrast, when the mutant Mod(mdg4)-67.2 protein was expressed at a low level, Su(Hw) binding was reduced significantly and only higher expression of this protein could provide for effective recruitment of Su(Hw) to the insulators. These results indicate that sumoylation improves

the activity of Mod(mdg4)-67.2 and, therefore, a smaller amount of the sumoylated protein, compared with unsumoylated protein, is sufficient for the functional activity of Mod (mdg4)-67.2 in the insulator complex.

Discussion

Post-translational modification by sumoylation has been shown to regulate subcellular localization of many targets, including RanGAP, PML, SATB2 and others (Pichler and Melchior, 2002; Saitoh and Hinchey, 2000). Here, we present data showing that SUMO is necessary for colocalization of the Su(Hw), Mod(mdg4)-67.2 and CP190 proteins in nuclear speckles, named insulator bodies.

An opposite model was proposed previously, according to which sumoylation of Mod(mdg4)-67.2 and CP190 leads to disruption of insulator bodies (Capelson and Corces, 2006). This model was mainly based on the observation that, in diploid cells from the larval brain, mutations in the gene encoding Ubc9 restored aggregation of the CP190 protein in the *mod(mdg4)*^{u1} background. Here, we found that inactivation of Mod(mdg4)-67.2 did not affect the ability of CP190 to form insulator bodies in S2 cells. Furthermore, the *mod(mdg4)*^{u1} mutation did not affect CP190 incorporation into the insulator bodies in diploid cells of wing and eye imaginal discs (A.G., unpublished results). Thus, the significance of Mod(mdg4)-67.2 for CP190 recruitment to the insulator bodies is confined to diploid cells of the larval brain. Capelson and Corces tested the role of sumoylation in the formation of insulator bodies (Capelson and Corces, 2006) by using the *lwr*^{5} mutation generated by a single amino acid substitution in the Ubc9 region (R104H) located on the loop between strand 7 and helix B (Apionishev et al., 2001). This region of Ubc9 is required for the interaction of its active site with the substrate (Bernier-Villamor et al., 2002). Although untested, it appears that R104H makes the surface of the mutant enzyme (Ubc9^{5}) more hydrophobic, thereby strengthening binding interactions for certain enzyme–substrate pairs. Thus, *lwr*^{5} is not a null-mutation in the gene, and Ubc9^{5} can either increase or decrease sumoylation, depending on the protein substrate. Therefore, additional studies are required to demonstrate the role of Ubc9^{5} in the formation of insulator bodies in the imaginal disks of larvae.

Our data provide evidence for a crucial role of CP190 and a passive role of Su(Hw), a DNA-binding protein, in the formation of insulator bodies. In addition to Su(Hw), CP190 forms complexes with dCTCF that is also colocalized in the insulator bodies (Gerasimova et al., 2007). Thus, it is probable that Mod(mdg4)-67.2 and CP190 proteins recruit DNA-binding dCTCF and Su(Hw) proteins to the insulator bodies.

As shown previously, SUMO is necessary for the formation of PML nuclear bodies (Lin et al., 2006; Shen et al., 2006). These bodies are primarily formed due to the self-assembly ability of the PML N-terminal domain (Reymond et al., 2001). Moreover, SUMO-1 modification of PML was shown to target the protein from the nucleoplasm to the nuclear bodies (Müller et al., 1998). The occurrence of both sumoylation sites and SIMs in the PML protein provides a basis for the network of interactions that constitute the nucleation event for subsequent recruitment of sumoylated proteins and SIM-containing proteins (Bernardi and Pandolfi, 2007; Lin et al., 2006; Zhong et al., 2000). Cells that lack PML are unable to form nuclear bodies, with other nuclear body components remaining diffusely distributed in the nucleus (Ishov et al., 1999). Although analysis of the CP190 sequence suggests the presence of two SIMs, we have not observed a direct interaction between CP190 and SUMO in vitro. However, CP190 and Mod(mdg4)-67.2 contain several protein–protein interaction domains, including BTB/POZ (Golovnin et al., 2007; Pai et al., 2004), that might be involved in direct interaction with many DNA-binding transcription factors, such as Su(Hw) and dCTCF, to facilitate their assembly into the insulator bodies (Gerasimova et al., 2007; Golovnin et al., 2007; Mohan et al., 2007; Pai et al., 2004). It is noteworthy that heat shock has proved to induce redistribution of CP190 to the nuclear periphery, in complex with SUMO. This is evidence that the formation of insulator bodies requires interactions with additional proteins, which are disrupted as a result of heat shock treatment.

Sumoylation is essential for the functional activity of proteins in transcriptional repression, activation and recruitment of modifying complexes (Gill, 2005; Hay, 2005; Heun, 2007). We have demonstrated here that inactivation of sumoylation sites in the Mod(mdg4)-67.2 protein does not affect its functional activity in the insulator complex. This finding is in accordance with the previous observation that only 10% of Su(Hw) binding sites coincide with SUMO on polytene chromosomes (Capelson and Corces, 2006).

Our findings and previously published results (Gerasimova et al., 2000; Golovnin et al., 2007) confirm the role of Mod(mdg4)-67.2 in recruiting the Su(Hw) protein to the insulator bodies and insulators. When the mutant Mod(mdg4)-67.2 protein was expressed at a low level, Su(Hw) binding was reduced, whereas low expression of the wild-type Mod(mdg4)-67.2 protein was sufficient for completely restoring Su(Hw) binding to insulators. Therefore, assembly of the Su(Hw) and Mod(mdg4)-67.2 proteins in insulator bodies is essential for subsequent recruitment of insulator complexes to DNA. A higher level of the mutant Mod(mdg4)-67.2 protein increases the probability of formation of the Su(Hw)–Mod(mdg4)-67.2 complex outside of insulator bodies, thereby providing for more effective binding of the Su(Hw) and mutant Mod(mdg4)-67.2 proteins to the insulators.

Taken together, these results support the model of insulator bodies as a depot of proteins involved in transcription regulation and insulation (Golovnin et al., 2008) (Fig. 7). According to our results, the insulator proteins can interact and form complexes without SUMO. However, partial sumoylation of the Mod(mdg4)-67.2 and CP190 proteins leads to further aggregation of the protein complexes in insulator bodies. The sumoylated Mod(mdg4)-67.2 and CP190 proteins interact with Su(Hw) and recruit it to the insulator bodies. The insulator bodies possibly protect the insulator complex from degradation and facilitate the formation of complexes between Su(Hw)–Mod(mdg4)-67.2–CP190 and other transcription factors. ‘Mature’ insulator complexes might then transiently interact with the chromatin fibril and detach from the insulator bodies by means of desumoylation. As was suggested for PML bodies, proteins deposited in the insulator bodies might be used during cell stress. For example, we found that heat shock treatment induced relocation of CP190 from the insulator bodies to the nuclear periphery but did not affect the insulator complexes bound to DNA. Such an unusual relocation of the CP190 protein resulted in a diffuse distribution of the Su(Hw) and Mod(mdg4)-67.2 proteins. Thus, it appears that insulator proteins might have an as-yet-unknown role in the cell response to heat shock stress.

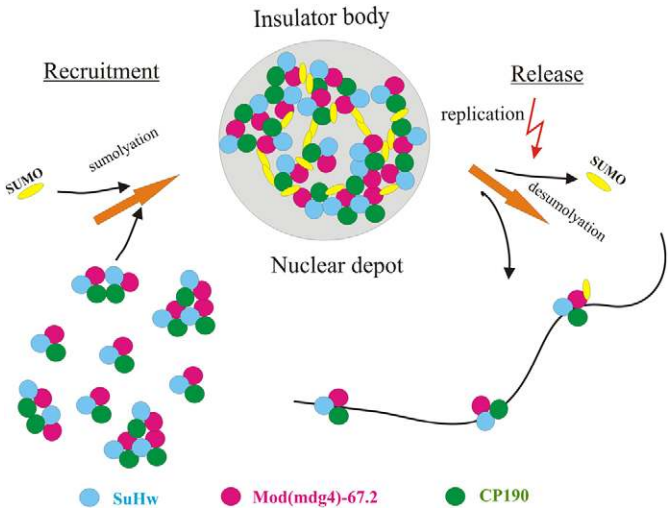

Fig. 7. Model describing the role of the SUMO pathway in organization of insulator bodies. Red, green and blue circles represent Mod(mdg4)-67.2, Su(Hw) and CP190 proteins, respectively; yellow ovals are SUMO molecules; and the curved line shows a chromatin fibril with insulators.

During DNA replication, a large amount of insulator proteins is required for newly synthesized chromosomes. It is possible that desumoylation of insulator bodies during DNA replication results in the release of protein complexes that form functional insulators on the newly synthesized DNA (Fig. 7). Further studies are required to verify this model.

Materials and Methods

Drosophila strains, plasmid constructions, germ line transformation and genetic crosses

All flies were maintained at 25℃ on standard yeast medium. The transposon constructs together with P25.7wc, a *P* element with defective inverted repeats used as a transposase source, were injected into *y ac* w^{1118} preblastoderm embryos (Karess and Rubin, 1984). Transgenic lines were generated and the constructs were introduced into the *mod(mdg4)*^{u1} background as described previously (Georgiev and Kozycina, 1996). The effects of various Mod(mdg4) proteins produced from homozygous expression vectors were scored by two researchers independently. To express the driver regulated by the *hsp70* promoter, the progeny from crosses were exposed once to heat shock at 37℃ for either 10 or 30 minutes at the embryonic, second-instar larval and middle pupal stages of development. The *cut* phenotypes were scored in 3- to 5-day-old males developing at 25℃. Representative wing forms (shown in Figs 6, 7) were selected as 'average' from the series of wings arranged in order of increasing severity of their mutant phenotype.

Transgenic constructs

Mod(mdg4)-67.2 cDNA from pCaSpeR2 (Golovnin et al., 2008) was cloned in pGem3 using *Eco*RI and *Bam*HI. To generate a lysine-to-arginine exchange at position 160 of Mod(mdg4)-67.2, cDNA templates were amplified by PCR with two primer pairs: T7 5′-TAATACGACTCATCACA-3′ and 5′-TCGATCC-TGTAGCGGGCGGA-3′; 5′-GACCGTGGACGACGGCCTGGG-3′ and 5′-TG-TTGCTTGACCACAGCCTTG-3′. The first and second PCR products were digested by *Eco*RI and *Bst*XI, respectively. The resulting fragments of Mod(mdg4) cDNA with the point mutation were joined together in pGem3 Mod(mdg4) digested by *Eco*RI and *Bst*XI (pGem3 Mod-K160R). To generate a lysine-to-arginine exchange at position 423 of Mod(mdg4)-67.2, cDNA templates were amplified by PCR with two primer pairs: 5′-CAAGGCTGTGGTCAAGCAACA-3′ and 5′-AACCCTTGTTTTCGGTCTGCC-3′; 5′-GAGGATCAGACGCCC-AAGCCG-3′ and 5′-CTTGAACAGGTGACCATTGAA-3′. The first and second PCR products were digested by *Bst*XI and *Bst*EII, respectively. Thereafter, both mutations were combined by ligating the corresponding mutant fragments of Mod(mdg4) cDNA in pGem3 Mod-K160R digested by *Bst*EII and *Bst*XI. Thus, the Mod-160/423 mutant was obtained.

To prepare *P{w*$^{+}$*;UAS-Mod-160/423}* or pAc5.1Mod-160/423–FLAG, the Mod-160/423 mutant was cloned in corresponding vectors using *Eco*RI and *Bam*HI. The pAc5.1Mod-160–FLAG and pAc5.1Mod-423–FLAG constructs were obtained by cloning the Mod-160 and Mod-423 mutants in corresponding vectors using *Eco*RI and *Bam*HI.

Preparation of *P{w*$^{+}$*;UAS-Mod-67.2}* and pAc5.1Mod-67.2–FLAG was described previously (Golovnin et al., 2008).

RNA interference treatment and analysis of S2 cells in culture

CP190, Su(Hw), Mod(mdg4) and dSmt3 cDNA templates were amplified by PCR using the following primer pairs: for CP190, 5′-ATGGGTGAAGTCAA-GTCCGTGAAAG-3′ and 5′-GAATTCCTTAACCTCTTCCAAAC-3′; for Su(Hw), 5′-ATGAGTGCCTCCAAGGAGGGCA-3′ and 5′-AGCAGAAGCA-TATGTCCTTCTTC-3′; for Mod(mdg4), 5′-ATGGCGGACGACGAGCAATT-CAG-3′ and 5′-AGGAGGCGGGGGTCAGGG-3′; and for dSmt3, 5′-CGAATTCATGTCTGACGAAAAGAAGGG-3′ and 5′-AGGATCCTGATGGA-GCGCCACCA-3′. The 5′ end of each primer also contained the T7 RNA polymerase promoter site. PCR products were purified using the Gel Extraction Kit (Zymo Research) according to the manufacturer's instructions. Purified PCR products were used to produce double-stranded RNA (dsRNA) using a Megascript T7 transcription kit (Ambion). The RNA was purified according to the manufacturer's instructions, heated at 65℃ for 30 minutes and left to cool at room temperature. Each batch of RNA was analyzed in agarose gel to ensure the quality of dsRNA. *Drosophila* embryonic S2 cells were grown in Schneider's insect medium (Sigma) supplemented with 10% fetal calf serum (FCS, HyClone) at 27℃. The RNAi treatment and subsequent viable cell count analysis of S2 cultures were basically performed as described (Adams et al., 2001; Clemens et al., 2000; Giet and Glover, 2001).

Two-hybrid and in vitro interactions

Two-hybrid assays were carried out with yeast strain pJ694A, using plasmids and protocols from Clontech (Palo Alto, CA). For growth assays, plasmids were transformed into yeast pJ694A cells by the lithium acetate method, as described by the manufacturer, and plated on media without tryptophan and leucine. After 3 days of growth at 30℃, the cells were plated on selective media without tryptophan, leucine, histidine and adenine, and their growth compared after 2–3 days.

For GST pull-down experiments, GST-Mod(mdg4), GST-CP190 or GST alone were incubated with glutathione Sepharose 4B beads in binding buffer (20 mM HEPES–KOH pH 7.6, 200 mM KCl, 2.5 mM MgCl, 10% glycerol and 0.05% NP40) for 2 hours. The beads were then blocked in 5% BSA for 1 hour and incubated with 6His-tagged dSmt3, dSmt3-M, dSmt3δGG or dSmt3δSIM proteins for 3 hours. After incubation, the beads were washed three times in wash buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.2% NP40 and 400 mM NaCl), boiled in Laemmli buffer and separated in separated by SDS-PAGE (8% gels). The proteins were blotted on a PVDF membrane, which was then incubated with antibodies to GST (Amersham) or SUMO (Antibodies-Online).

Transformation

The S2 cells cultured as described previously (Georgieva et al., 2001) were transformed using the Effectene Transfection Reagent (Qiagen) as recommended by the manufacturer.

Nuclear extracts and immunoprecipitation

These experiments were performed as described previously (Georgieva et al., 2001). Primer sequences used in PCR for the ChIP analysis will be provided upon request.

Immunostaining

The S2 cells were grown on coverslips, stained with antibodies against Mod(mdg4)-67.2, FLAG, Su(Hw) and CP190 as described (Golovnin et al., 2007) and examined under a Leica TCS SP2 confocal microscope. Larval diploid cells were treated as described in the protocol (Georgieva et al., 2001).

RNA isolation and real-time PCR analysis

Chromatin was prepared from S2 cells, as described previously (Golovnin et al., 2008), or from the middle pupa stage. The procedure used in the latter case was as follows: The material (150–200 mg) was homogenized in 5 ml of buffer A (15 mM HEPES pH 7.6, 60 mM KCl, 15 mM NaCl, 4 mM $MgCl_2$, 0.5% Triton X-100, 0.5 mM DTT, 10 mM sodium butyrate, EDTA-free protease inhibitors cocktail and 1.8% formaldehyde) at room temperature using first a Potter homogenizer and then a Dounce homogenizer with type A pestle (three strokes). The protease inhibitors cocktail (Roche, Cat #1873 580) was used following the manufacturer's instructions. After 15 minutes (total time starting from beginning of homogenization), glycine solution was added to a concentration of 225 mM and the mixture stirred, incubated for 5 minutes and then centrifuged at 4000 ***g*** and 4℃ for 5 minutes. The pellet was washed three times with buffer A at 4℃ and resuspended in 0.5 ml of lysis buffer (15 mM HEPES pH 7.6, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.5 mM DTT, 0.1% sodium deoxycholate, 0.1% SDS, 0.5% *N*-lauroylsarcosine, 10 mM sodium butyrate and

protease inhibitors cocktail). The mixture was incubated on a rotary shaker at 4℃ for 10 minutes, sonicated on ice with a Branson Sonifier 450 (power 2, duty cycle 100%, time 4 × 30 seconds at 2-second intervals), rotated for another 10 minutes and then centrifuged for 5 minutes at 15,000 ***g***. The supernatant was transferred to a new tube. The pellet was resuspended in 0.5 ml of lysis buffer, rotated for 10 minutes and centrifuged again. The first and second supernatants were pooled and centrifuged twice for 10 minutes at 15,000 ***g***. The resulting chromatin preparation was used for ChIP experiments as described previously (Golovnin et al., 2008).

For real-time PCR experiments, RNA was isolated from 3-day-old adult flies. To express the driver regulated by the *hsp70* promoter, the flies were exposed once to heat shock at 37℃ for either 10 or 30 minutes at the embryonic, second-instar larval and middle pupal stages of development. RNA was isolated from 120 flies per genotype using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Genomic DNA was removed by treatment with DNase I (1 U per 10 μg; Fermentas) followed by purification with a QIAGEN RNeasy kit. RNA was reverse-transcribed into cDNA with a RevertAid H Minus RT Revert Transcriptase (Fermentas) according to the manufacturer's instructions.

Real-time PCR was performed using a Bio-Rad CFX 96 Cycler and SYBR Green. Reactions were performed in a 25-μl volume, using 0.05 μg of cDNA template and 200 nM forward and reverse primers. Primer sequences will be provided upon request. Thermocycler conditions were as follows: 95℃ for 2.5 minutes and then 40 cycles of 95℃ for 15 seconds, 60℃ for 30 seconds and 72℃ for 30 seconds. Following the amplification process, a melt curve was generated between 55℃ and 95℃, with a reading at every 0.5℃ to confirm that a single PCR product was obtained. For each experiment, duplicate or triplicate reactions were performed and averaged. At least three independent experiments were performed with each primer set, using at least two independent RNA samples. The expression level of each gene was determined using Ras64B as an internal control.

Antibodies

Specific antibodies and working dilutions were as follows: mouse anti-Lamin (1:300) from Abnova Corporation, mouse anti-FLAG (1:300) from Sigma and rabbit anti-SUMO (1:100) from Antibodies-Online (cat. #ABIN356801). Rat anti-CP190 (1:500), rabbit anti-Mod(mdg4)-67.2 (1:500) and rabbit anti-Su(Hw) (1:200) were raised in our laboratory. The secondary antibodies were Cy3-conjugated anti-rat, FITC-conjugated anti-rabbit and Cy5-conjugated anti-mouse immunoglobulins, all from Jackson ImmunoResearch and used at 1:500 dilution.

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