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'Insulator bodies' are aggregates of proteins but not of insulators

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Chromatin insulators are thought to restrict the action of enhancers and silencers. The best-known insulators in *Drosophila* require proteins such as Suppressor of Hairy wing (Su(Hw)) and Modifier of mdg4 (Mod(mdg4)) to be functional. The insulator-related proteins apparently colocalize as nuclear speckles in immunostained cells. It has been asserted that these speckles are 'insulator bodies' of many Su(Hw)–insulator DNA sites held together by associated proteins, including Mod(mdg4). As we show here using flies, larvae and S2 cells, a mutant Mod(mdg4) protein devoid of the Q-rich domain supports the function of Su(Hw)-dependent insulators and efficiently binds to correct insulator sites on the chromosome, but does not form or enter the Su(Hw)-marked nuclear speckles; conversely, the latter accumulate another (C-truncated) Mod(mdg4) mutant that cannot interact with Su(Hw) or with the genuine insulators. Hence, it is not the functional genomic insulators but rather aggregated proteins that make the so-called 'insulator bodies'.

Keywords: *Drosophila*; Mod(mdg4); nuclear speckles; Su(Hw) insulator

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INTRODUCTION

Insulators are genomic regulatory elements that are defined by two properties: these nucleoprotein complexes can block enhancer action on a promoter when interposed between them, and can protect the transgenes that they flank from chromosomal position effects (for reviews, see Kuhn & Geyer, 2003; Brasset & Vaury, 2005; West & Fraser, 2005; Gaszner & Felsenfeld, 2006). The most studied insulator in *Drosophila* is the one found in the *gypsy* retrotransposon (mdg4). It contains 12 degenerate repeats of the binding motif for the zinc-finger protein Suppressor of Hairy wing (Su(Hw)), which is essential for its function (Holdridge & Dorsett 1991; Geyer & Corces 1992). Among the numerous potential Su(Hw)-binding sites dispersed throughout the wild-type genome, rarely three or more motifs occur within reasonable proximity (Parnell *et al*, 2006; Ramos *et al*, 2006). However, the 1A2 insulator downstream of the *yellow* gene, with only two Su(Hw)-binding sites, shows both insulator functions in standard transgene assays (Golovnin *et al*, 2003; Parnell *et al*, 2003).

Two more proteins, Modifier of mdg4 (Mod(mdg4)) and Centrosomal protein 190kD (CP190), are required for the *gypsy* insulator function (Gerasimova *et al*, 1995; Georgiev & Kozycina, 1996; Pai *et al*, 2004). Mod(mdg4) is a BTB/POZ protein capable of oligomerization; the Mod(mdg4)-67.2 isoform interacts with Su(Hw) by its unique carboxy-terminal domain (Buchner *et al*, 2000; Gause *et al*, 2001; Ghosh *et al*, 2001).

A decade ago, it was reported by Gerasimova & Corces (1998) that Su(Hw) and Mod(mdg4) colocalized in discrete foci observed by microscopy in the *Drosophila* interphase cell nucleus. Exclusively on the basis of the disappearance of such immunofluorescent foci and concurrent weakening of a gypsy insulator after a Mod(mdg4)-affecting mutation, these nuclear speckles were named 'insulator bodies'. Furthermore, it was stated by the same team (Gerasimova *et al*, 2000; Ghosh *et al*, 2001; Pai *et al*, 2004; Capelson & Corces, 2004, 2005; Lei & Corces, 2006) that these bodies represent nuclear matrix-fixed congregations of many genomically remote Su(Hw)–insulator DNA complexes, somehow brought together and held by interactions through Mod(mdg4) and CP190, thereby establishing 'separate chromatin loop domains' and thus controlling the higher order organization and function of the genome.

In fact, the presumed clustering of distinct insulator DNA sequences within an 'insulator body' has not been verified over the years. Here, we show, by expressing altered forms of the

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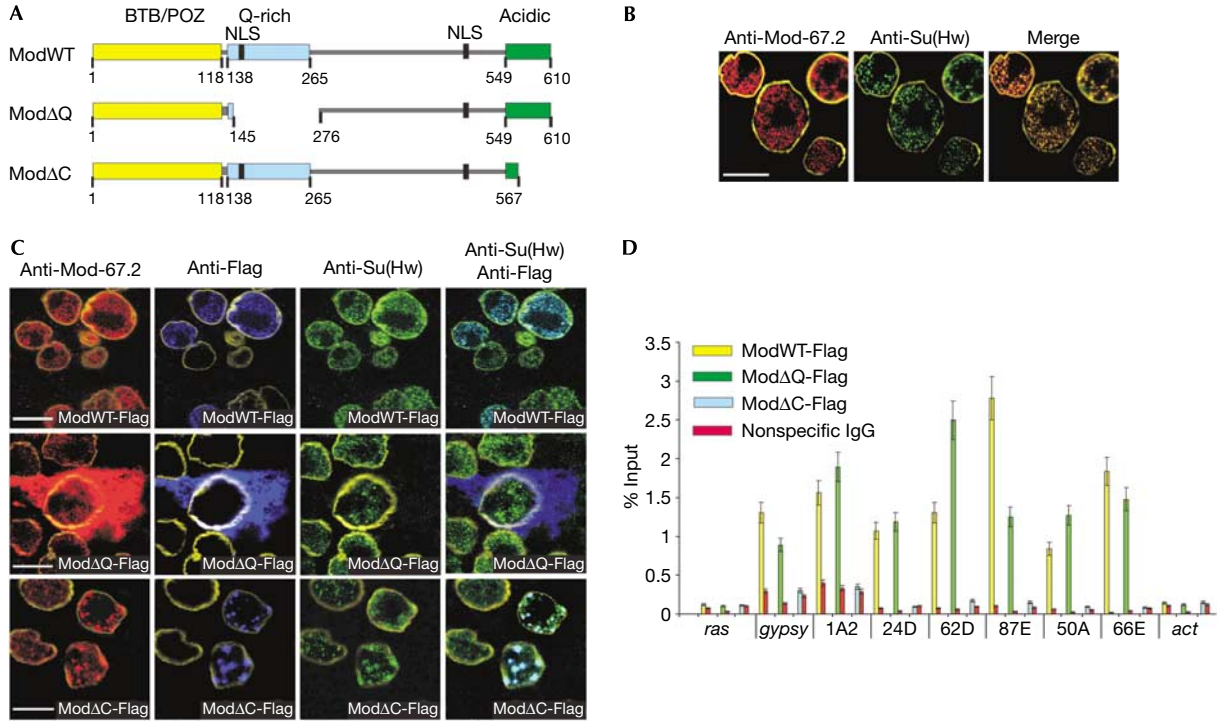

Fig 1 | Mod(mdg4) proteins and their distribution in S2 cells. (**A**) Schematics of Mod(mdg4)-67.2 and its deletion derivatives: the wild-type protein (ModWT), ModΔQ, which lacks the Q-rich domain, and ModΔC, which lacks most of the Su(Hw)-binding domain. (**B**) Immunostained control cells; scale bar, 5 μm. (**C**) Transfected cells expressing Flag-tagged Mod variants specified in (**A**) are the upper three cells in the top row, the central one in the middle row and the right two in the bottom row; scale bar, 5 μm. All images in (**B**) and (**C**) include staining for lamin to demarcate the nuclei. (**D**) Crosslinking chromatin immunoprecipitation of specified chromatin regions with the Mod variants (percentage of input DNA, $M \pm m$, $n = 3$); *actin* and *ras* coding regions are controls devoid of Su(Hw)-binding sites. Mod(mdg4), Modifier of mdg4; NLS, nuclear localization signals; Su(Hw), Suppressor of Hairy wing.

indispensable Su(Hw)–insulator component Mod(mdg4) in the same objects, that such nuclear speckles are irrelevant to genuine insulators or their function.

RESULTS AND DISCUSSION

Structure and properties of the Mod(mdg4) mutants

We designed deletions in the Mod(mdg4)-67.2 protein on the basis of published data (Buchner *et al*, 2000; Gerasimova *et al*, 2000; Gause *et al*, 2001; Ghosh *et al*, 2001; Golovnin *et al*, 2007). The wild-type protein (ModWT; Fig 1A) has an amino-terminal BTB/POZ domain, an adjacent glutamine(Q)-rich domain and a C-terminal acidic domain that binds to Su(Hw) (Buchner *et al*, 2000); sequence analysis predicts two nuclear localization signals (NLS). Two Mod derivatives were used in the present study: in ModΔQ, deletion of residues 145–276 removed the Q-rich domain and one NLS; in ModΔC, deletion of 43 C-terminal residues removed most of the Su(Hw)-binding domain (Fig 1A).

ModΔQ has been shown to be able to self-associate and interact with ModWT and Su(Hw), as evidenced by the yeast two-hybrid assay and co-immunoprecipitation from transfected S2 cells (supplementary Table S1 and Fig S1 online). As expected, ModΔC could also oligomerize by itself as well as with ModWT, but had completely lost the ability to interact with Su(Hw) in the two-hybrid assay. Nonetheless, Su(Hw) was partly co-precipitated with ModΔC from the S2 nuclear extract (supplementary Fig S1 online)—that is, both proteins were present in some type of agglomerate, although they were incapable of direct binding.

Localization of Mod(mdg4) variants in S2 cells

The nuclei of S2 cells derived from *Drosophila* embryos showed speckles that stained for Mod(mdg4) and Su(Hw) (Fig 1B). These speckles were similar in size, number and disposition to those reported in flies and named 'insulator bodies' (Gerasimova & Corces, 1998; Gerasimova *et al*, 2000). To assess the distribution of the Mod(mdg4) variants, these cells were transfected with plasmids encoding ModWT, ModΔC and ModΔQ tagged at the C termini with triple Flag epitopes. Thus, the plasmid expression of the Flag-tagged wild-type or mutant protein was superimposed on the basal genomic expression.

The results of immunostaining are summarized in Fig 1C. By way of an internal control, each panel shows transfected cells (distinguished by Flag staining and specified in the legend) and nontransfected cells.

Overexpression of ModWT-Flag did not appreciably change the 'normal' staining patterns: anti-Flag shows exclusively nuclear 'punctated' (Gerasimova *et al*, 2000) deposition, incident with that of Su(Hw). Overall, the Mod + Su(Hw) speckles might be more abundant than in the control.

By contrast, intense expression of ModΔQ-Flag gave rise to massive diffuse staining of the protein and Flag in the cytoplasm,

but not in the nucleus. The few nuclear speckles stained with anti-Su(Hw) and anti-Mod represent the 'basal deposits' (or rather those that existed before transfection) and none of these was stained with anti-Flag. Such a pattern could be expected for ModΔQ, which retains the ability to interact with proteins but is handicapped in nuclear targeting: its accumulation/oligomerization in the cytoplasm can further reduce the amount delivered to the nucleus and also trap a considerable amount of the 'basal' Mod(mdg4) and Su(Hw). The overexpression of ModΔQ clearly shows the marked difference between the Mod variants in their intracellular distribution. The minor drawback is that no cytoplasmic deposition of Su(Hw) can be discerned by simultaneous immunostaining among such overwhelming amounts of Mod, so the cytoplasm appears 'empty' in the ModΔQ-Flag/anti-Su(Hw) panel. However, co-deposition of Su(Hw) and ModΔQ in the cytoplasm was clearly seen in transgenic flies discussed below, in which the amounts of the two proteins are naturally much closer.

Expression of ModΔC-Flag again resulted in exclusive nuclear deposition of the mutant protein together with Su(Hw); these speckles were somewhat fewer but larger than in the control or with ModWT. Notably, all Flag-positive speckles were also Su(Hw) positive, whereas some (most likely, the pre-existing ones) contained the Mod protein and Su(Hw), but not ModΔC-Flag. The colocalization of ModΔC and Su(Hw)—which cannot interact directly—is in line with their partial co-precipitation (see the end of preceding section) and is not at all surprising; the speckles perhaps also include other proteins that can link them, for example, CP190 is known to interact with Su(Hw) and Mod(mdg4) *in vitro* (Pai *et al*, 2004; Golovnin *et al*, 2007).

It must be noted that among these four cases, the nuclear speckles might vary or not in number and/or dispersion; such variability is not consistent with the crucial structural and functional role proposed for 'insulator bodies'.

Importantly, the lack of ModΔQ-Flag staining in the nucleus does not mean that there is no protein; it only means that the intranuclear ModΔQ did not aggregate or stick to the existing speckles. Standard subcellular fractionation and Western blotting (supplementary Fig S2 online) clearly showed ModΔQ-Flag and Su(Hw) in the nuclei (although rough estimates 'per nucleus' were 3–4 times lower than for the ModWT case, as expected). Still more pertinent were the crosslinking chromatin immunoprecipitation (X-ChIP) data (Fig 1D), showing that, overall, the same amounts of ModWT and ModΔQ were bound to the chromatin regions known to contain Su(Hw)-dependent insulators and similar motifs (Golovnin *et al*, 2003; Parnell *et al*, 2003, 2006; Ramos *et al*, 2006). By contrast, the ModΔC level throughout was indistinguishable from the background.

To summarize, ModΔQ, which retains all the properties essential to insulator function, is delivered into *Drosophila* cell nuclei, perhaps owing to the single NLS, and associates with the chromatin Su(Hw)–insulator sites no less efficiently than the wild-type protein, but it does not form any nuclear speckles or join the existing ones. Conversely, ModΔC, which cannot interact with Su(Hw), completely fails to bind to the correct insulator sites in chromatin but instead consistently colocalizes (perhaps aided by other proteins) with Su(Hw) in the nuclear speckles.

In vivo functional testing of Mod(mdg4) mutants

Next, we compared the functional effects of ModΔQ and ModΔC in flies. The source of ModΔQ was a transgenic line providing UAS-driven ModΔQ expression in a null *mod(mdg4)^{u1}* background. Its counterpart expressing ModWT was used as a reference in addition to the wild type. The source of ModΔC was the previously described (Ghosh *et al*, 2001) *mod(mdg4)^{T6}* mutation, which generates the same protein lacking the 43 C-terminal residues.

Phenotypic analysis of the competence of Mod variants in insulator function was performed in male flies carrying *gypsy*-induced alleles in the *yellow* and *cut* loci, as in the studies furthering the idea of 'insulator bodies' (Pai *et al*, 2004; Capelson & Corces, 2005; Lei & Corces, 2006). *Yellow* expression determines the cuticular pigmentation and is controlled by several tissue-specific enhancers. In the y^2 mutation (Fig 2A), a *gypsy* element is interposed between the *yellow* promoter and the wing and body enhancers (Geyer *et al*, 1986), therefore its insulator blocks these enhancers but not the bristle enhancer in the *yellow* intron (Geyer *et al*, 1986; Geyer & Corces, 1992). Phenotypically, this gives a pale abdomen with dark bristles (leftmost panel). The *mod(mdg4)^{u1}* mutation alters the y^2 phenotype, repressing *yellow* expression in bristles (Gerasimova *et al*, 1995; Georgiev & Kozycina, 1996) and partly weakening the *gypsy* insulator, which results in variegated *yellow* expression in the body cuticle (Gerasimova & Corces, 1998), as shown by the second-left dappled abdomen with pale bristles. Expression of ModWT as well as ModΔQ completely overrides the *mod(mdg4)^{u1}* effect on both traits, indicating that ModΔQ substitutes for the wild-type protein in this insulator-related function. Conversely, the *mod(mdg4)^{T6}* mutation expressing ModΔC yields exactly the same phenotype as the null *mod(mdg4)^{u1}* mutation, indicating that ModΔC is nonfunctional.

In the ct^6 and ct^k alleles (Fig 2B), *gypsy* is between the wing margin enhancer and the *cut* promoter, which are 85 kb apart (Hoover *et al*, 1992; Gause *et al*, 2001). In ct^6, the insulator completely blocked this enhancer, producing a cut wing phenotype (leftmost in the upper row). The null *mod(mdg4)^{u1}* (middle left) and the *mod(mdg4)^{T6}* (rightmost) mutations clearly suppressed the ct^6 mutant phenotype, indicating that Mod(mdg4)-67.2 is essential for blocking the wing margin enhancer and that ModΔC does not compensate for its loss. By contrast, ModΔQ completely restored the *gypsy* insulator function in the *mod(mdg4)^{u1}* background, similar to ModWT (Fig 2B). The *gypsy* insulator was weaker in ct^k than in ct^6, perhaps because it has only 7 instead of 12 Su(Hw) sites (Hoover *et al*, 1992), and is also more sensitive to the level of Mod(mdg4)-67.2 (Gause *et al*, 2001): it produced an intermediate cut wing phenotype (leftmost in the bottom row) that was almost completely suppressed with a single dose of *mod(mdg4)^{u1}* (second left). ModΔQ restored the activity of this insulator in the null background similar to ModWT, confirming that sufficient amounts of the functional ModΔQ protein bind to insulator sites. Again, ModΔC is ineffective (the *mod(mdg4)^{T6}* and null *mod(mdg4)^{u1}* wing phenotypes were identical).

Exactly the same pattern of responses was obtained in another system (AS-C; supplementary information and Fig S3 online), which allowed testing the functionality of Mod variants with both *gypsy* and endogenous Su(Hw)-dependent (1A2) insulators.

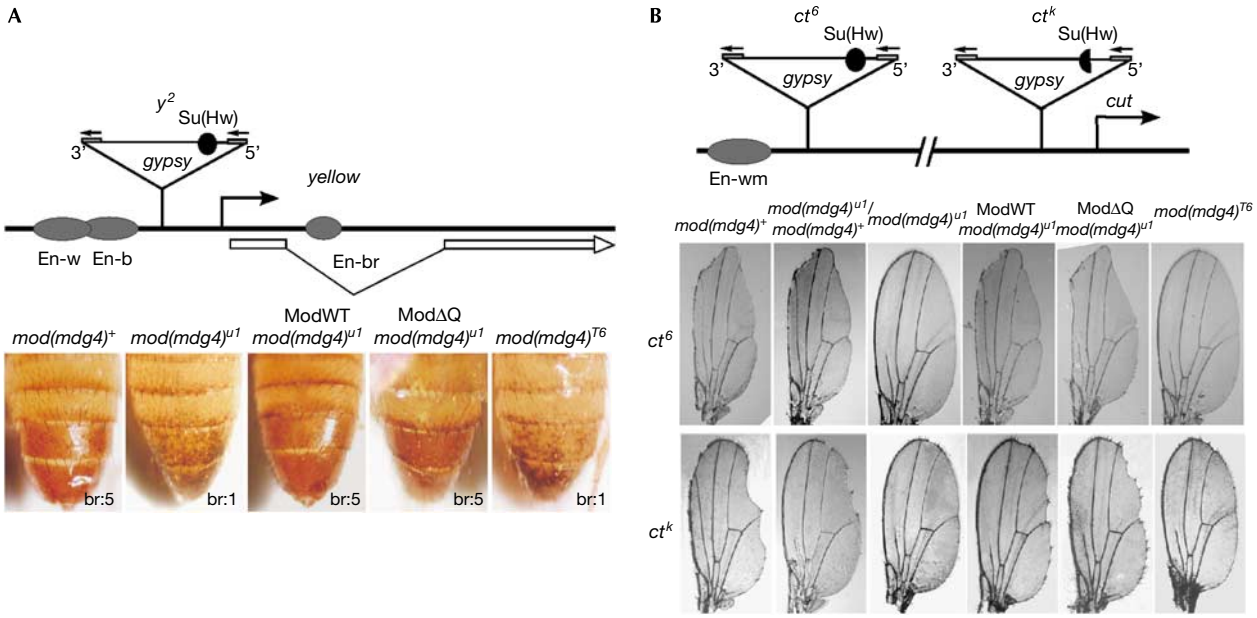

Fig 2 | Testing of Mod(mdg4) variants for insulator function in y^2 ct^6 male flies. Schematics show the structure of the (**A**) y^2 and (**B**) ct^6 or ct^k alleles; beginnings and direction of the *yellow* and *cut* genes are shown by arrows; ovals denote wing (w), body (b), bristle (br) and wing margin (wm) enhancers (En); triangles show insertions of *gypsy* with flanking long terminal repeats and the Su(Hw) insulator as a black (semi)circle. Photographs collate (**A**) the body cuticle (bottom-right, bristle pigmentation: 5 for wild type, 1 for none) and (**B**) the wing phenotypes; ModWT and ModΔQ refer to transgenic expression of the variant specified in Fig 1A; $mod(mdg4)^{T6}$ itself produces ModΔC; $mod(mdg4)^{u1}$ is the null mutation. Mod(mdg4), Modifier of mdg4; Su(Hw), Suppressor of Hairy wing; y^2, *yellow* mutation associated with *gypsy* insertion.

These data prove that, in agreement with the properties established *in vitro*, ModΔQ is functionally equivalent to the wild-type Mod(mdg4)-67.2 protein at the authentic insulators in *Drosophila*, whereas ModΔC is totally incompetent.

Localization of the Mod(mdg4) mutants in larvae

The binding of mutant Mod(mdg4) to insulator sites was analysed by immunostaining on polytene chromosomes, in which ModWT was shown to colocalize with Su(Hw) (Gerasimova & Corces, 1998). Two well-defined locations of Su(Hw) insulators are the *gypsy* inserts in the y^2 and sc^{D1} loci at the tip of the X chromosome (Gerasimova *et al*, 1995). The two corresponding intense bands for Su(Hw) in the y^2sc^{D1} strain are indicated in Fig 3A. As expected, ModΔQ bound to polytene chromosomes exactly as the wild-type protein did, in particular, at the *gypsy* bands in y^2 and sc^{D1}. By contrast, ModΔC ($mod(mdg4)^{T6}$) decorated a considerably smaller number of places that did not coincide with the Su(Hw) insulators. These results further show that ModΔQ, but not ModΔC, interacts with authentic Su(Hw) insulators.

Finally, we examined the immunostaining patterns in imaginal disc cells (Fig 3Bi–vi). In accordance with the published observations (Gerasimova *et al*, 2000) and our results in S2 cells (Fig 1), the wild-type nuclei contained multiple Mod + Su(Hw)-positive speckles (i). Again, only a cloudy Su(Hw) backdrop was barely visible in the $mod(mdg4)^{u1}$ cells (ii). As expected, transgenic expression of ModWT in this null background (iii) restituted the wild-type staining pattern.

However, ModΔQ in the null background—already shown to restore all tested Su(Hw) insulator functions and to bind to all correct insulator sites—did not form or enter any nuclear speckles (iv), although we saw pronounced co-deposition of Mod and Su(Hw) in the cytoplasm. Interestingly, the $mod(mdg4)^+$ background for ModΔQ (v) largely restored the double-positive nuclear speckles, attenuated the cytoplasmic Mod staining and virtually abolished cytoplasmic Su(Hw).

Conversely, in the $mod(mdg4)^{T6}$ cells (vi), the ModΔC variant—which cannot functionally support the Su(Hw) insulators or associate with the corresponding chromatin sites—was seen to colocalize with Su(Hw) in nuclear speckles (which were fewer than in wild type but comparable with ModΔQ/$mod(mdg4)^+$).

Conclusion

We reproduced the basic features and behaviour of putative 'insulator bodies' (see Introduction) using the same or equivalent objects and experimental approaches. However, on analysis of the structural and functional data obtained using different forms of the essential insulator protein Mod(mdg4) in various genetic environments, we must conclude that the very presence of such bodies in the nucleus (or their absence, let alone their number, size or disposition) is irrelevant to the organization and function of authentic Su(Hw)-dependent genomic insulators, and thus cannot be regarded as evidence for insulator clustering.

It was not the aim of this study to scrutinize the composition, properties or actual purpose of these nuclear inclusions. The various bodies that can be visualized by microscopy in the nucleus (apart from the nucleolus) often appear to be depots for spare components. By analogy to the well-known promyelocytic leukaemia nuclear bodies comprising many unrelated proteins

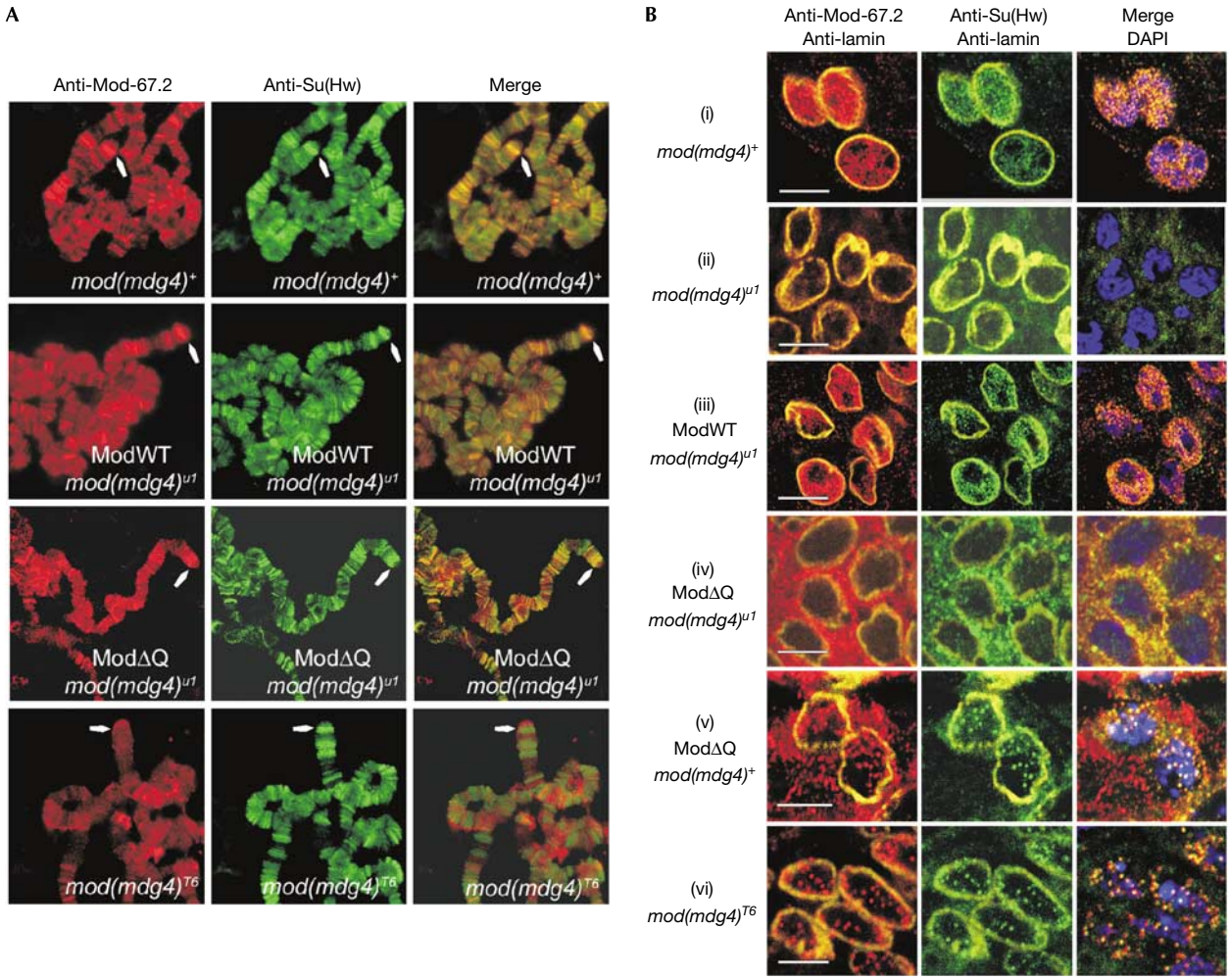

Fig 3 | Localization of Mod(mdg4) variants in *Drosophila* larvae. (**A**) Polytene chromosomes of y^2sc^{D1} larvae; arrows indicate *gypsy* inserts at the X-chromosome tip. (**B**) Imaginal disc cells; scale bar, 5 μm. The *mod(mdg4)* genotype designations are as in Fig 2. DAPI; 4,6-diamidino-2-phenylindole; Mod(mdg4), Modifier of mdg4; Su(Hw), Suppressor of Hairy wing.

(Bernardi & Pandolfi, 2007), the so-called 'insulator bodies' in *Drosophila* are perhaps aggregates of surplus proteins not immediately engaged in any function, and most certainly include proteins other than Mod(mdg4), Su(Hw) and CP190. We have preliminary data that the *Drosophila* analogue of the vertebrate CTC-binding factor (dCTCF), another zinc-finger protein required for activity of another type of insulator (Mohan *et al*, 2007), is also present in the same nuclear speckles. The findings recounted here—especially when viewed together with the well-known facts that elimination of Su(Hw) protein affects only female fertility and that the null $mod(mdg4)^{u1}$ mutation does not apparently affect any trait in fly development—defy the idea of such 'insulator bodies' as organizers of genome structure and function, notwithstanding the general plausibility and expedience of its higher order organization and management.

METHODS

Transformation. The S2 cells cultured as described previously (Georgieva *et al*, 2001) were transformed using the Effectene Transfection Reagent as recommended by Qiagen (Hilden, Germany). The constructs, *Drosophila* strains, transgenic manipulations and phenotypic analyses are described in the supplementary information online.

Chromatin immunoprecipitation. The S2 cell suspension was treated with 1% formaldehyde at 20 °C for 10 min. The nuclei were washed and lysed, and chromatin was sheared to an average length of 400 bp by sonication. X-ChIP was carried out as recommended by Upstate Biotechnology (Lake Placid, NY, USA), with 4 μg of antibodies against Flag (Sigma, St Louis, MO, USA). The negative control was 4 μg of nonspecific IgG from preimmune serum. The PCR primers are listed in supplementary Table S2 online.

Immunostaining. The S2 cells were grown on coverslips, stained with antibodies against Mod(mdg4)-67.2, Flag, Su(Hw) and lamin as described by Kyrshakova *et al* (2007), and examined using a Leica TCS SP2 confocal microscope. Squashed salivary gland specimens were prepared and immunostained as described by Platero *et al* (1996) and co-stained with 4,6-diamidino-2-phenylindole (DAPI). Diploid larval cells were treated according to Gerasimova *et al* (2000).

Antibodies. The specific antibodies and working dilutions were as follows: chicken anti-Mod(mdg4)-67.2 (1:500), a gift from P. Geyer; mouse anti-Flag (1:300) from Sigma; rabbit or mouse anti-lamin (1:500) and mouse anti-tubulin (1:2,000), gifts from P. Fisher; and rabbit antibodies against the Su(Hw) N-terminal domain (1:200), raised in our laboratory. The secondary antibodies were Cy3-conjugated anti-chicken (Amersham, Little Chalfont, UK), Alexa Fluor 488 anti-rabbit and Cy-5 anti-mouse (Invitrogen, Carlsbad, CA, USA) goat IgG, all used at a dilution of 1:500.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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