Supplementary Material

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

Anton Golovnin, Larisa Melnikova, Ilya Volkov, Margarita Kostuchenko,
Alexander V. Galkin & Pavel Georgiev

Results

Protein interactions

Mutant ModΔQ and ModΔC proteins were tested for their ability to interact with Su(Hw) in the yeast two-hybrid system (Table S1). We used a truncated variant of the Su(Hw) protein that contained only the Mod(mdg4)-67.2 -interacting domain [$Su(Hw)^{MID}$]. $Su(Hw)^{MID}$, ModΔQ, ModΔC, and wild-type ModWT were fused in-frame with either the yeast GAL4 DNA binding domain (GAL4BD) or the activation domain (GAL4AD). ModWT and ModΔQ showed strong interactions with $Su(Hw)^{MID}$ in the reciprocal two-hybrid tests, while ModΔC failed to interact with Su(Hw). The interaction between ModΔQ and Su(Hw) was also confirmed by co-IP (Fig S1). FLAG×3 tagging did not affect any Mod(mdg4) function tested *in vitro* or *in vivo* (not shown).

Interacting proteins		Strength of interaction
GAL4BD	**GAL4AD**	
ModWT	$Su(Hw)^{MID}$	+++
$Su(Hw)^{MID}$	ModWT	+++
$Su(Hw)^{MID}$	ModΔC	–
ModΔC	$Su(Hw)^{MID}$	–
$Su(Hw)^{MID}$	ModΔQ	+++
ModΔQ	$Su(Hw)^{MID}$	+++
ModΔC	ModWT	+++
ModWT	ModΔC	++
ModΔC	ModΔC	+++
ModΔQ	ModWT	+++
ModWT	ModΔQ	++
ModΔQ	ModΔQ	+++

Table S1 Summary of yeast two-hybrid analysis of interactions between proteins. The relative strength of two-hybrid interaction is shown by the number of "+" signs, with "–" indicating no interaction. Equivalent expression of the chimeric proteins in yeast was confirmed by immunoblotting with GAL4 BD or AD monoclonal antibodies (data not shown).

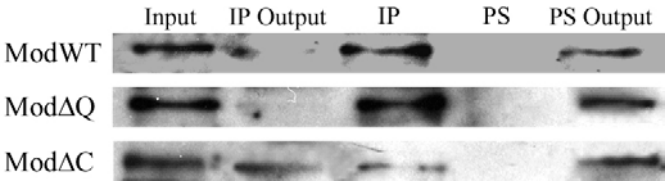


Fig S1 Su(Hw) co-immunoprecipitation with FLAG-tagged ModWT, ModΔQ or ModΔC from transformed S2 cells. The immunoprecipitated complexes were washed with 500 mM and 100 mM KCl-containing buffers before SDS-PAGE followed by Western blot analysis with anti-Su(Hw) antibodies. **Input**, the input fraction; **IP Output**, the supernatant fluid after immunoprecipitation with anti-Su(Hw) antibodies; **IP**, immunoprecipitate; **PS**, preimmune serum; **PS Output**, the supernatant fluid after immunoprecipitation with preimmune serum.

Functional tests

To determine the *in vivo* effects of mutant Mod(mdg4) proteins, we additionally used *gypsy*-induced alleles in the *Achaete-Scute* Complex (AS-C) adjoining the *yellow* gene (Modolell and Campuzano, 1998). A complex pattern of *ac* and *sc* expression is mediated by the action of site-specific enhancer-like elements distributed over approximately 90 kb of the AS-C cluster (Campuzano *et al*, 1985; Modolell and Campuzano, 1998). Several AS-C alleles were tested. The sc^{D1} mutation is caused by an insertion of *gypsy* 20 kb downstream of the *sc* gene that blocks the communication between many bristle-specific enhancers and the *sc* promoter. The $mod(mdg4)^{u1}$ mutation only partially suppresses the *sc*-mutant phenotype, indicating that Mod(mdg4)-67.2 is not critical for the block of the *sc* enhancers by the *gypsy* insulator (Georgiev and Kozycina, 1996). In the sc^{ms1} and sc^{ms2} mutants (Fig S3A), the 1A2 insulator was duplicated between the *sc* gene and its enhancers (Golovnin *et al*, 2003). In contrast to its effects on the sc^{D1} allele, $mod(mdg4)^{u1}$ almost completely suppresses the mutant phenotype of the sc^{ms} alleles, providing evidence for the role of Mod(mdg4)-67.2 in the enhancer-blocking activity of the 1A2 insulator (Fig S3B).

Finally, we used the $In(1)sc^{v2}$ mutation (Fig S3A) that carries an inversion with one breakpoint very close to the 3' end of the *ac* coding region and the second in centric heterochromatin (Campuzano *et al*, 1985). Despite the close proximity to centric heterochromatin, $In(1)sc^{v2}$ causes only a weak mutant phenotype. However, in the $mod(mdg4)^{u1}$ background, this inversion strongly enhances the *ac* and *sc* phenotypes, suggesting that the Mod(mdg4)-67.2 protein blocks heterochromatin-mediated repression (Fig S3B).

Once again, we found that $mod(mdg4)^{T6}$ had the same effect on the *sc* mutations as did $mod(mdg4)^{u1}$, further confirming that the ModΔC protein is not functional (Fig S3B). In contrast, expression of ModΔQ in the $mod(mdg4)^{u1}$ background completely restored the blocking of the *sc* enhancers by the 1A2 and *gypsy* insulators and suppressed the heterochromatin-mediated repression in $In(I)sc^{V2}$ flies.

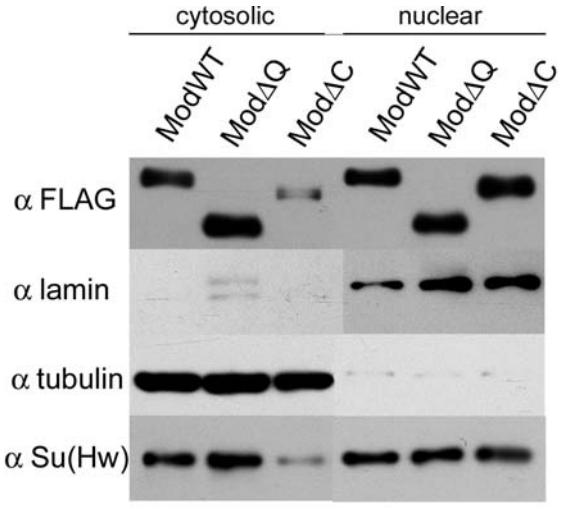
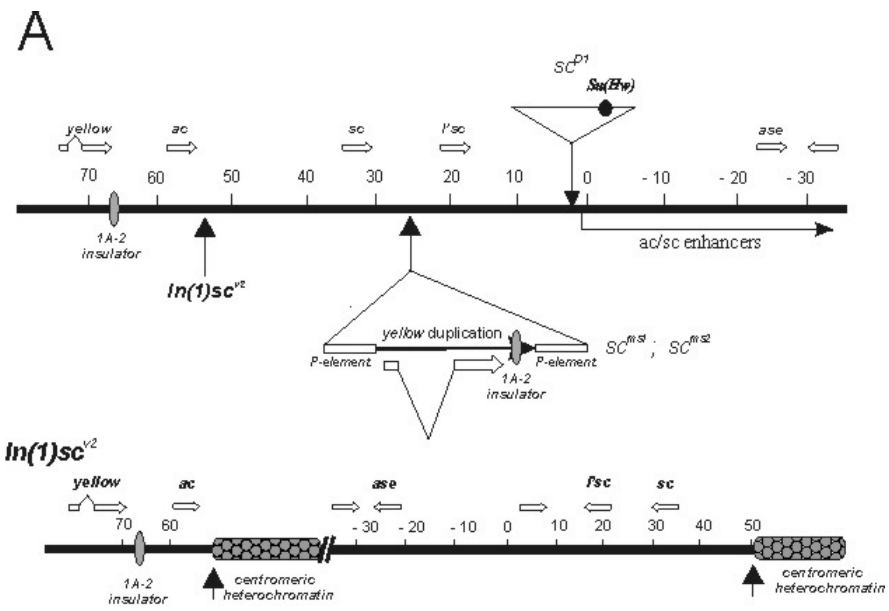


Fig S2 Subcellular fractionation of transfected S2 cells. The presence of FLAG-tagged ModWT, ModΔQ, or ModΔC proteins and Su(Hw) in the cytoplasmic and the nuclear fractions was assessed by Western blotting with antibodies (α) against FLAG (Mod variants), Su(Hw), tubulin (cytosolic marker), and lamin (nuclear marker). The deletion derivatives occupy accordingly lower positions in the FLAG blot. This experiment serves only for qualitative demonstration; though all nuclear FLAG and Su(Hw) spots appear roughly similar, the amount of nuclear material in the ModΔQ lane was about threefold greater than in the ModWT lane as inferred from the lamin spots, but the imprecision of the assay made formal normalisation inexpedient.



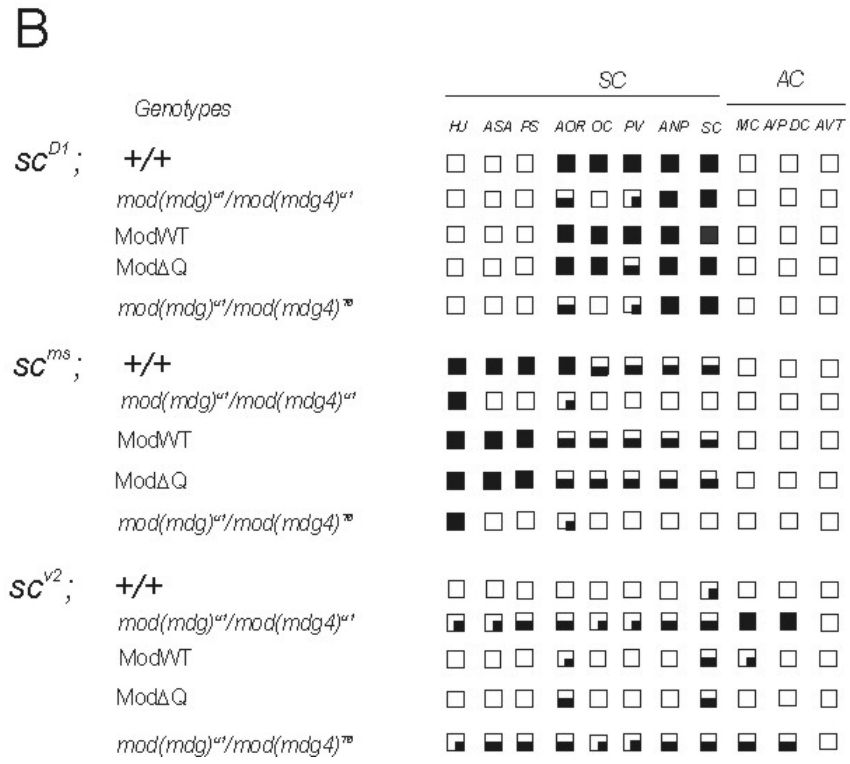


Fig S3 Effects of mutant Mod(mdg4) proteins on the activities of the 1A2 and Su(Hw) insulators inserted in the Achaete-Scute complex. (**A**) Scheme of the *yellow/ac/sc* region in $sc^{D1}$and sc^{ms}. The coordinates of the AS-C region are as defined by Campuzano et al. (1985). Vertical arrows indicate the position of chromosomal breakpoints associated with the $In(I)sc^{v2}$ mutation. Arrows with triangles show insertions of *gypsy* (sc^{D1}) and *P* elements (sc^{ms}) associated with duplication of the *yellow* sequences. Thick horizontal white arrows show the positions and direction of *yellow* and AS-C gene transcripts. Filled ovals indicate the endogenous Su(Hw) insulator and the Su(Hw) binding sites in *gypsy*. (**B**) The effect of Mod(mdg4) variants on the male phenotypes of mutations in AS-C. ModΔQ and ModWT were expressed in the $mod(mdg4)^{u1}$ background. The standard nomenclature for each bristle is as follows: HU, humeral; AOR, anterior orbital; PS, presutural; ASA, anterior supra-alar; OC, ocellar; PV, postvertical; ANP, anterior notopleural; SC, scutellar; ADC, anterior dorsocentral; PDC, posterior dorsocentral; AVT, anterior vertical; MC, rows of microchaetae on the notum. Only the bristles affected in the *ac* and *sc* mutations are shown. Empty boxes indicate that the corresponding bristles are present (wild-type phenotype). One-quarter-filled, half-filled, and fully filled boxes indicate the absence of the corresponding bristle(s) in more than 10, 50, or 90% of the flies, respectively. For scutellars, the respective boxes indicate the presence of 3–4, 2–3, or 0–1 scutellar bristles (average number among ca. 100 flies examined).

Table S2 Primers used for PCR reaction

Locus	Direction	Sequence
gypsy	Fw	5’ CACGTAATAAGTGTGCGTTGA 3’
	Rev	5’ TTCTCTAAAAAGTATGCAGCACTT 3’
actin	Fw	5’ GGCACCACACCTTCTACAATGAGC 3’
	Rev	5’ GAGGCGTACAGCGAGAGCACAG 3’
ras	Fw	5’ GAGGGATTCCTGCTCGTCTTCG 3’
	Rev	5’ GTCGCACTTGTTACCCACCATC 3’
1A2	Fw	5’ ACCACACATCAGTCATCGTGT 3’
	Rev	5’ CTTCGTCTACCGTTGTGC 3’
87E	Fw	5’ TTTGCGTTTCGGCTGCTGTC 3’
	Rev	5’ GGATGTTACATTGAGAGTGCTTAGG 3’
66E	Fw	5’ AACTCCATTCCATTCACCTGTCTC 3’
	Rev	5’ GCTGCTGATCCTCGCTTTCC 3’
62D	Fw	5’ TGATACCAGGCGAACAGAAATC 3’
	Rev	5’ TTTGGGCTTGGTGAGAACAG 3’
50A	Fw	5’ TTGATAAATAGTCCAGCACGCATAC 3’
	Rev	5’ ATACAAAGTGGTTTCAGCCAAGAAG 3’
24D	Fw	5’ GCAATTCTATGGAGATTAAAGGTG 3’
	Rev	5’ ACAGCATGAGAACTTCTTAGG 3’

Methods

Drosophila strains, germ line transformation and genetic crosses. All flies were maintained at 25°C on standard yeast medium. The construct together with P25.7wc, a *P* element with defective inverted repeats used as a transposase source, was injected into *y ac* w^{1118} preblastoderm embryos as described (Karess *et al*, 1984). The resulting flies were crossed with *y ac* w^{1118} flies, and the transgenic progeny were identified by their eye and/or cuticle color. Chromosome localisation of various transgene insertions was determined by crossing the transformants with the *y ac* w^{1118} balancer stock carrying dominant markers: *In(2RL),CyO* for chromosome 2 and *In(3LR)TM3,Sb* for chromosome 3.

Generation of transgenic lines and introduction into the *mod(mdg4)*^{u1} or *mod(mdg4)*^{T6} background were described previously (Georgiev and Kozycina, 1996). To express transgenes regulated by the *UAS* promoter, flies homozygous for the construct were crossed with the Act5-GAL4 driver strain. The effects of the Mod(mdg4) variants produced from homozygous expression vectors were scored independently by two authors. In order to determine the *yellow*, *cut,* and *sc* phenotypes, we examined 3–5-day-old males developing at 25°C. For *yellow* phenotypes, wild-type expression in abdominal cuticle, wings, and bristles was assigned an arbitrary score of 5, while the absence of *y* expression was ranked 1. Flies with the previously characterized *y* allele were used as reference. The representative abdomens and wings were photographed.

Transgenic constructs. To prepare *P{w*$^{+}$*;UAS-ModΔQ}* and *P{w*$^{+}$*;UAS-ModWT}*, Mod(mdg4)-67.2 cDNA in pGEX2T (kindly provided by D. Dorsett) was digested with *Blp*I, filled in with Klenow fragment, and self-ligated. The resulting mutant and original cDNAs were subcloned at *Bam*HI and filled-in *Eco*RI in pCaSpeR2 digested with *Bgl* II and *Stu*I. To construct transposons, mutant Mod(mdg4) was cloned in pCaSpeR4 under control of the UAS promoter. Vectors were digested with *Eco*RI and *XbaI* and ligated with the *Eco*RI–*Xba*I fragment of Mod(mdg4)-67.2 containing mutations described above.

To generate pAc5.1ModWT-FLAG and pAc5.1ModΔQ-FLAG, the full-length or mutant cDNA was cloned from pCaSpeR2-Mod(mdg4)-67.2 or pCaSpeR2-ModΔQ at *Eco*RI and *Bam*HI in pAc 5.1NF.

To generate pAc5.1ModΔC-FLAG, the protein-coding sequence was PCR-amplified from pCaSpeR2-Mod(mdg4)-67.2 with primer 5' ATTGGATCCAATCTCAAACTCCTCG 3' and a primer from the 3’ end of the pCaSpeR2 *P*-element.

Nuclear extracts and immunoprecipitation were described previously (Georgieva *et al,* 2001). Subcellular fractionation of S2 cells was performed with the Qproteome Cell Compartment Kit (Qiagen), with subsequent Western analysis.

Yeast two-hybrid assays were carried out using yeast strain pJ694A, with plasmids and protocols from Clontech (Palo Alto, CA). For growth assays, plasmids were transformed into yeast strain pJ694A by the lithium acetate method as described by the manufacturer and plated on media without tryptophan and leucine. After 2 days of growth at 30°C, the cells were plated on selective media without tryptophan, leucine, histidine or adenine, and the results were compared. Liquid culture assays were performed according to the yeast protocols handbook (Clontech).

References

Campuzano, S., Carramolino, L., Cabrera, C.V., Ruiz-Gomez, M., Villares, R., Boronat, A., and Modolell, J. (1985). Molecular genetics of the *achaete-scute* gene complex of *D. melanogaster*. Cell *40*, 327-338.

Karess, R.E., and Rubin, G.M. (1984). Analysis of *P* transposable element functions in Drosophila. Cell *38*, 135-146.

Modolell, J., and Campuzano, S. (1998). The *achaete-scute* complex as an integrating device. Int. J. Dev. Biol. *42*, 275-282.