

General, negative feedback mechanism for regulation of *Trithorax-like* gene expression *in vivo*: new roles for GAGA factor in flies

Jordi Bernués*, David Piñeyro and Ana Kosoy

Institut de Biologia Molecular de Barcelona-CSIC, Parc Científic de Barcelona, Josep Samitier, 1-5, 08028 Barcelona, Spain

Received March 29, 2007; Revised June 22, 2007; Accepted July 18, 2007

ABSTRACT

Expression of every gene is first regulated at the transcriptional level. While some genes show acute and discrete periods of expression others show a rather steady expression level throughout development. An example of the latter is *Trithorax-like* (*Trl*) a member of the *Trithorax* group that encodes GAGA factor in *Drosophila*. Among other functions, GAGA factor has been described to stimulate transcription of several genes, including some homeotic genes. Here we show that GAGA factor is continuously down-regulating the expression of its own promoter using a negative feedback mechanism *in vivo*. Like its expression, repression by GAGA factor is ubiquitous, prevents its accumulation, and takes place throughout development. Experimental alteration of GAGA factor dosage results in several unexpected phenotypes, not related to alteration of homeotic gene expression, but rather to functions that take place later during development and affect different morphogenetic processes. The results suggest that GAGA factor is essential during development, even after homeotic gene expression is established, and indicate the existence of an upper limit for GAGA factor dosage that should not be exceeded.

INTRODUCTION

Drosophila GAGA factor is encoded in the single copy gene *Trithorax-like* (*Trl*) and is present in two isoforms, GAGA₅₁₉ and GAGA₅₈₁ generated by alternative splicing that only differ at the C-terminus (1,2). They share a POZ/BTB domain, responsible for the formation of homo- and hetero-oligomers (3–5), a DNA-binding domain (DBD) containing a single zinc finger flanked by three basic

regions that confer specificity for binding GA-rich sequences (6,7), and a domain (X) of largely unknown structure and function(s) between the former two that interacts with some nuclear factors, NURF301 and FACT, and directs GAGA to the nucleus [(8,9) Regué, L. and J.B., unpublished data, for a review see (10)]. At the C-terminal part the two isoforms present a glutamine-rich domain (Q-domain) that, although different in sequence and in length, is similar in amino acid composition. Both Q-domains have the ability to stimulate transcription of several reporter genes in transiently transfected cells. Both isoforms are modified post-translationally, and recently phosphorylation at the DBD has been reported (11). GAGA factor is present in all cell types of the fly and it is strictly nuclear [(2); our unpublished data].

GAGA factor belongs to the *Trithorax* group of genes and is involved in maintaining large regions of chromatin in an open state, in particular those regions of certain homeotic genes. *Trl* mutants behave as enhancers of position effect variegation (1). Recently, GAGA factor has been shown to prevent heterochromatin spreading by directing histone H3.3 replacement in association with FACT (12). GAGA factor collaborates with NURF in chromatin remodeling *in vitro* and also stimulates transcription of many genes both *in vitro* and *in vivo* [(13–19), for a review see (20)]. GAGA factor presents maternal effect, and null mutants are embryonic lethal. Although a few adult flies can develop with low levels of GAGA factor, homozygous hypomorphic *Trl*^{13C} embryos present major defects in nuclear divisions at early stages of embryonic development and strong embryonic lethality. Severe defects in expression of *en* and *ftz* genes were also reported (18). Homozygous *Trl*^{R85} null mutant embryos (from heterozygous females) showed reduced levels of some homeotic genes (*Ubx* and *en*), but not of others (*Scr*, *Antp*, *Abd-A* and *Abd-B*) indicating that adequate regulation of some homeotic genes can still be observed in developing embryos despite

*To whom correspondence should be addressed. Tel: 34934034960; Fax: 34934034975; Email: jmbmc@ibmb.csic.es
Present address:

Ana Kosoy, Ludwig Institute for Cancer Research, 605 Third Avenue, New York, NY 10158 USA

a lack in the maternal contribution. During larval development, loss of function clones also suggest that *Trl* function is not required for homeotic gene expression (21).

In transient transfection experiments, GAGA was found to down-regulate its own expression by binding to the *Trl* promoter in S2 cells. This repression was very efficient, dose-dependent, and did not require either the Q-domain or the POZ/BTB domain but was strictly dependent on the integrity of the DBD (22). Here we show *in vivo* that *Trl* gene is self-regulated by its own product GAGA factor in a negative way. This repression appears to be general during development and is dose-dependent. Alteration of local levels of GAGA factor protein, by forced expression and depletion by RNAi, resulted in a variety of new phenotypic defects that appeared after homeotic gene expression is already established.

MATERIALS AND METHODS

Transgenic flies

Transgenic fly lines were generated by microinjection of a P-element based vector construct bearing a white marker (pCasper or pUAST) along with a construct source of transposase in 0–45 min *Drosophila* embryos (*w* or *yw*) (23). UAS-GAGA line was kindly supplied by Dori Huertas (IBMB). RNAi GAGA lines contain two copies of a fragment of GAGA coding sequence (from +263 to +927), coding for a C-ter region of the POZ domain and the entire X domain, inserted in pWIZ in inverted orientations at AvrII and NheI sites (construct kindly provided by M^a Lluïsa Espinás, IBMB). To generate *Trl*-GFP fly lines a GFP-pCasper4 vector was prepared by inserting a GFP coding sequence at NotI/BamHI sites in the pCasper4 polylinker. Then a long *Trl* promoter fragment (NheI/PstI from previous constructs) was inserted between XbaI and PstI sites in the polylinker just upstream of GFP coding sequence (to obtain 'long' series). For the minimal ('min') and null *Trl* promoter series a similar strategy was followed but fragments were obtained by digestion with *Asp*718 and *Bpu*1102I, blunt-ended with T4 DNA polymerase and inserted at the StuI in the polylinker of the GFP-pCasper4 vector. UAS-GAGA₅₁₉ΔBΔKO and UAS-GAGAΔQ constructs were prepared in pUAST vector from constructs previously described (22). All constructs were checked by restriction analysis and sequenced.

Immunostaining and microscopy

Embryos were collected and dechorionated in sodium hypochlorite for 2.5 min, exhaustively washed with 0.1% Triton X-100 and fixed by the slow formaldehyde fix procedure (23). Imaginal disks were dissected from 3rd instar larvae in ice-cold PBS and immediately fixed in 4% *p*-formaldehyde in PBS for 20 min at room temperature, followed by extensive washing with PBS–0.3% Triton X-100. Disks were either directly used or stored in this buffer at 4°C until use. For immunostaining, all washes and incubations were in PBS–0.3% Triton X-100–2% BSA. Embryos or imaginal disks were incubated

overnight at 4°C with primary antibodies with agitation. Embryos and disks were then extensively washed and secondary antibodies labeled with fluorophores were added and incubated in the dark for 2 h with agitation at room temperature. Samples were then extensively washed and DNA stained with 20 ng/ml DAPI in PBS for 5 min. After extensive washing with PBS–0.3% Triton-X-100, the tissues were mounted in Mowiol. Primary antibodies were used as follows: rat α-GAGA (1:100), rabbit α-GFP (1:1000) (Molecular Probes), rabbit α-GAL4 (1:600) (Santa Cruz). Secondary antibodies were always used at 1:600 and were α-rabbit IgG-Cy2 and α-rabbit IgG-Cy3 and α-rat IgG-Cy3. Images were recorded on a Leica confocal microscope. α-GAGA antibodies were raised in rats following conventional protocols.

Adult wings were prepared from flies kept in 75% ethanol, 25% glycerol solution for at least 24 h at room temperature. Flies were washed in PBS, wings dissected and immediately mounted in Fauré's medium under gentle pressure. If required, after dissection wings were treated with 10% KOH for 10–20 min at 110°C, washed with PBS and mounted as before. Images were recorded using a Nikon E-600 microscope equipped with a DXM 1200F camera.

Cuticles were prepared from 1st–3rd instar larvae by treatment with sodium hypochlorite and washing with Triton X-100 as above and then vigorously shaken for 30 s in a mix of heptane/methanol v/v. Liquid was removed, larvae were washed twice with 0.1% Triton X-100 for 5 min and mounted on a drop of Hoyer's-lactic and incubated at 60°C overnight (23). Dark-field pictures were taken on a Nikon E-1000 microscope equipped with a cool-snap camera.

RESULTS

GAGA represses expression of its own promoter *in vivo*

In a previous study, GAGA was found to down-regulate its own expression by binding to the *Trl* promoter in *Drosophila* cells (22). To study this negative regulation and its consequences *in vivo*, transgenic flies carrying different versions of the *Trl* promoter fused to green fluorescent protein (GFP) coding sequences as a reporter were generated. Three constructs containing 3.47 kb, 345 bp and 49 bp long sequences corresponding to the longest, minimal and null promoter described previously, plus 737 bp of 5' UTR region, were selected (Figure 1A). Several independent transgenic lines were obtained for each construct. None of them showed any visible defect and stocks grew normally. Characterization of these transgenic lines indicated that the long and the minimal *Trl* promoter constructs expressed GFP indistinguishable to endogenous GAGA expression, and defined a compact *Trl* promoter that, to the extent analyzed, did not show tissue-specific or development-specific regulation. The null *Trl* promoter construct did not express GFP at all, as expected (data not shown).

To manipulate the levels of the GAGA factor, and to direct specific GAGA factor over-expression or depletion via RNAi, the GAL4-UAS system was used.

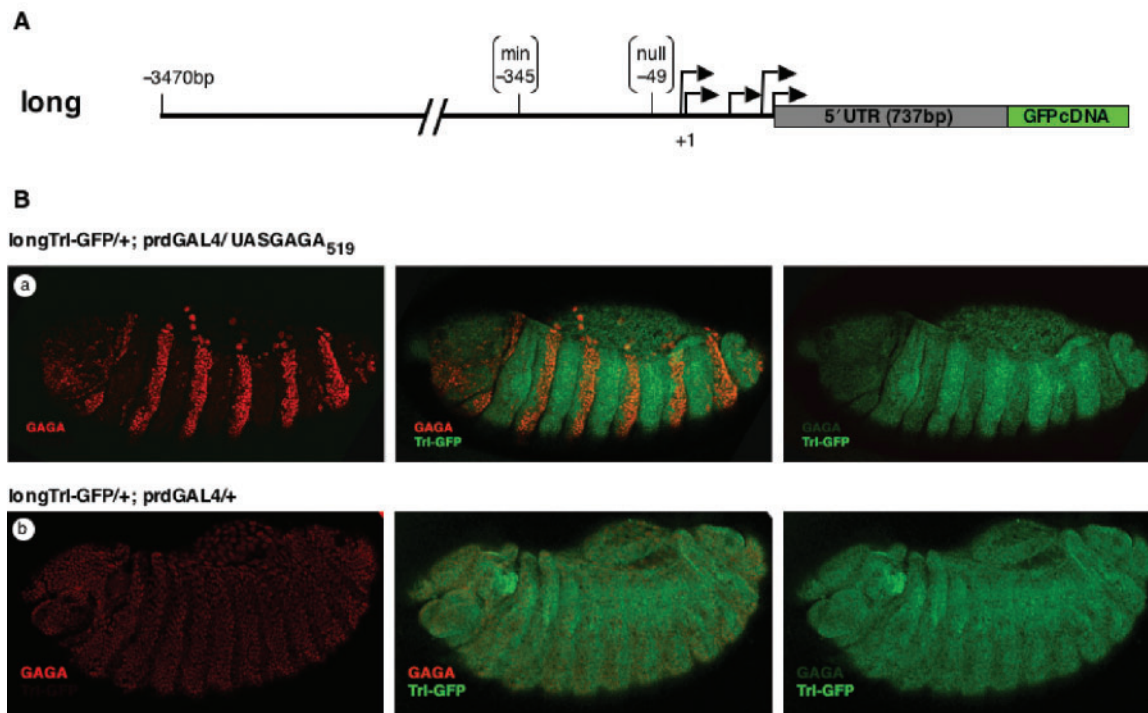


Figure 1. GAGA₅₁₉ can partially repress *Trl* transcription in embryos. (A) Diagram of the long *Trl* promoter constructs used to generate transgenic flies. The 5' position of the minimal- and null-*Trl* construct series are indicated inside brackets. (B) Confocal microscopy images of *Drosophila* embryos stained with α -GAGA (in red) and α -GFP (in green) antibodies. Central panels show merge images. a: repression on a long *Trl* promoter-GFP reporter by over-expressing GAGA₅₁₉ under *prdGAL4* control; b: control embryo carrying a long or a short *Trl* promoter-GFP but not over-expressing GAGA₅₁₉. Samples were obtained at 18°C.

Over-expression of GAGA₅₁₉, from a UAS-GAGA₅₁₉ construct, was tested by crossing with several GAL4 drivers and expression was checked by antibody staining (data not shown). We next proceeded to study the effect of GAGA₅₁₉ over-expression on the activity of the *Trl*-GFP. Because flies carrying long and minimal *Trl* promoter constructs produced identical results, only results obtained with the long construct are presented.

GAGA₅₁₉ over-expression experiments showed high lethality with all GAL4 drivers (even at 18°C, see below and Table 1). Among them *prdGAL4* (at 18°C) was selected because it allowed the study of effects in the embryos (that further develop to reach larval stage) and also because GAL4 protein was expressed in alternating stripes thus providing convenient internal negative controls. Over-expression of GAGA₅₁₉ resulted in a pattern of seven bands of high expression of GAGA₅₁₉ alternating with bands of background GAGA expression from the endogenous *Trl* gene (Figure 1B, a). In segments where GAGA₅₁₉ over-expression took place (in red) a reduction in GFP signal (in green) was observed. The intensity of this reduction was variable among embryos while no reduction was observed in control stripes adjacent to the GAGA₅₁₉ over-expression domain. GAL4 expression on its own had no effect on GFP levels (Figure 1B, b). GAGA₅₁₉ over-expression had no effect on flies carrying a null promoter *Trl*-GFP construct and no GFP signal appeared, as expected (data not shown).

Table 1. Lethality observed in GAGA over-expression experiments. Unless specified lethality was 100%

Condition	Lethality
<i>ptcGAL4/UASGAGA₅₁₉</i>	Larval (at 18°C)
<i>prdGAL4/UASGAGA₅₁₉</i>	Larval (at 18°C)
<i>dppGAL4/UASGAGA₅₁₉</i>	Just before hatching, some escapers (at 18 and 25°C)
MS1096GAL4/UASGAGA ₅₁₉	Pupal/some escapers (at 18°C)
<i>ApGAL4/UASGAGA₅₁₉</i>	Larval/pupal (at 18°C)
<i>ptcGAL4/UASGAGAΔQ</i>	Larval (at 18°C)
<i>prdGAL4/UASGAGAΔQ</i>	No lethality (at 25°C)
MS1096GAL4/UASGAGA Δ Q	Larval/pupal (at 18°C)
<i>ptcGAL4/UASGAGA₅₁₉DBDKO</i>	No lethality (at 25°C)
<i>prdGAL4/UASGAGA₅₁₉DBDKO</i>	No lethality (at 25°C)
MS1096GAL4/UASGAGA ₅₁₉ DBDKO	No lethality (at 25°C)
<i>dppGAL4/UASGAGA₅₁₉DBDKO</i>	No lethality (at 25°C)

Embryos shown in Figure 1 corresponded to stages 13–14. Although expression of GAL4 protein from the paired promoter could be detected earlier (and GAGA₅₁₉ over-expression was easily detected in embryos from stages 9–11) no effect on GFP expression could be observed at these earlier stages (data not shown). Perhaps, the short time elapsed between forced GAGA expression and assay (a few hours before collection) combined with the long GFP half-life in flies (>24h)

makes it technically impossible to detect changes in GFP protein content at these early stages. This may also explain why GFP reduction was less pronounced in embryos when compared to imaginal disks (see below and compare Figures 1 and 2).

GAGA factor repression of *Trl* was further studied in 3rd instar larval imaginal disks. Flies carrying several GAL4 drivers for expression in wing and haltere imaginal disks were crossed to long*Trl*-GFP/UASGAGA₅₁₉ transgenic lines. GAGA₅₁₉ over-expression under MS1096GAL4 (Figure 2A and B in red) resulted in a clear reduction of GFP expression (in green) in the area of GAGA₅₁₉ over-expression both in wing and haltere imaginal disks. Without GAGA₅₁₉ over-expression GFP expression was not affected (Figure 2C). When dppGAL4 was used equivalent results were obtained. In this case, equally efficient repression of the *Trl*-GFP reporter was also observed in leg disks where dppGAL4 could also direct UASGAGA₅₁₉ expression (data not shown). With the different GAL4 drivers used, repression of *Trl*-GFP was variable in intensity (depending on the GAL4 driver) but never complete, suggesting a dose dependence. Figure 2D shows that repression can also take place when a C-terminal deletion encompassing the Q domain (the transactivation domain) is expressed. However, repression is fully abolished when a GAGA factor is expressed carrying a single point mutation at the DB domain that disrupts the zinc finger (Figure 2E and F). Additionally, with this point mutant there is no lethality at all and drivers like ptcGAL4 can be used to reveal expression of this GAGA mutant throughout development (an example is its expression in the wing disk, Figure 2E).

We conclude that GAGA₅₁₉ repression of *Trl* expression is a general mechanism apparently operating throughout fly development—likely at all cell types—that takes place through interaction with DNA sequences.

Depletion of GAGA factor stimulates *Trl* transcription

A logical consequence of the negative feedback model is that depletion of GAGA factor should result in stimulation of *Trl* transcription. To test this hypothesis, a complementary set of experiments was carried out to deplete GAGA factor. Two transgenic lines carrying a UASRNAi_{GAGA} construct were obtained (4-2 and 7-2 on chromosomes II and III, respectively) and conditions to obtain substantial depletion of GAGA factor with several GAL4 expressing lines were determined. Depletion was always more efficient with UASRNAi_{GAGA}4-2 although results were equivalent at 29°C (results with line 7-2 are not shown). In general, GAL4-driven expression of RNAi constructs showed no lethality. In embryos only moderate depletion, not sufficient for our purposes, was obtained (even at 29°C, data not shown). In wing imaginal disks (and also haltere, data not shown) of 3rd instar larvae expression of RNAi_{GAGA} under ptcGAL4 control resulted in a clear GAGA depletion in a central stripe defining the anterior–posterior axis (Figure 3A, in red) that corresponded to ptcGAL4 expression domain. On its own ptcGAL4 expression did not affect GAGA

expression (data not shown). GAGA depletion resulted in enhanced expression of *Trl*-GFP reporter constructs (Figure 3A, in green) exactly in the area of GAGA depletion. In the absence of RNAi_{GAGA} expression, neither GAGA nor GFP expression were altered (Figure 3B, in red and green, respectively). GAGA depletion was higher at 29°C than at 25°C and *Trl*-GFP expression was also more intense at 29°C than at 25°C (data not shown). Note that in these experiments, RNAi_{GAGA} knocked down both GAGA isoforms (see Materials and Methods section). These results show that *Trl*-GFP expression *in vivo* is stimulated by GAGA depletion in a dose-dependent manner and, therefore, that GAGA factor is keeping *Trl* promoter partially repressed *in vivo*.

Phenotypic consequences of altering GAGA factor dosage

Analysis of the high lethality observed in GAGA₅₁₉ over-expression experiments (Table 1) revealed a remarkable amount of morphological defects, usually local even though GAGA₅₁₉ was over-expressed in a larger area. These defects affected different body parts and were observed independently of the presence of the GFP reporters.

When MS1096GAL4 was used to over-express GAGA₅₁₉ only a few escapers hatched and reached adult stage at 18°C (none at 25°C). These flies showed a severe wing phenotype with only some remnants of the wing vein pattern apparent, complete loss of the wing border identity and a clear separation of the two dorsal/ventral cell layers. The rest of the body looked normal in these flies (Figure 4A). When ApGAL4 was used, lethality was absolute and earlier (larval, data not shown). GAGA₅₁₉ over-expression under ptcGAL4 control resulted in a major abnormality in the T1–T3 segments affecting the larval cephalopharyngeal skeleton at 18°C (Figure 4B, red circle). While GAGA₅₁₉ is similarly over-expressed in the other segments in the embryo (data not shown), no other morphological defects could be observed. These animals died before reaching 3rd instar larvae. When dppGAL4 was used, a large majority of the animals completed metamorphosis at 25°C without hatching. The few that hatched showed all their legs reduced to two apparently normal segments (femur and tibia) whereas the five tarsal segments and tips were reduced to a stump (Figure 4C, central panels). In males, some remnants of the sex combs could still be appreciated abnormally developing on the stump (arrow in Figure 4C). In more severe cases, legs were further reduced and could be completely absent and these animals could not hatch (see the lower three panels on the right column in Figure 4C showing increasing phenotype indicated with an arrow from – to +). This same phenotype could also be observed at 18°C although in the less severe forms.

Over-expression of GAGAΔQ resulted in a variation of all the phenotypes that depended on the driver used. Thus, when it was over-expressed with MS1096 no animals hatched from pupae at 18°C. However, GAGAΔQ over-expression with prdGAL4 did not result in any lethality. Importantly, over-expression of GAGA₅₁₉DBDKO did

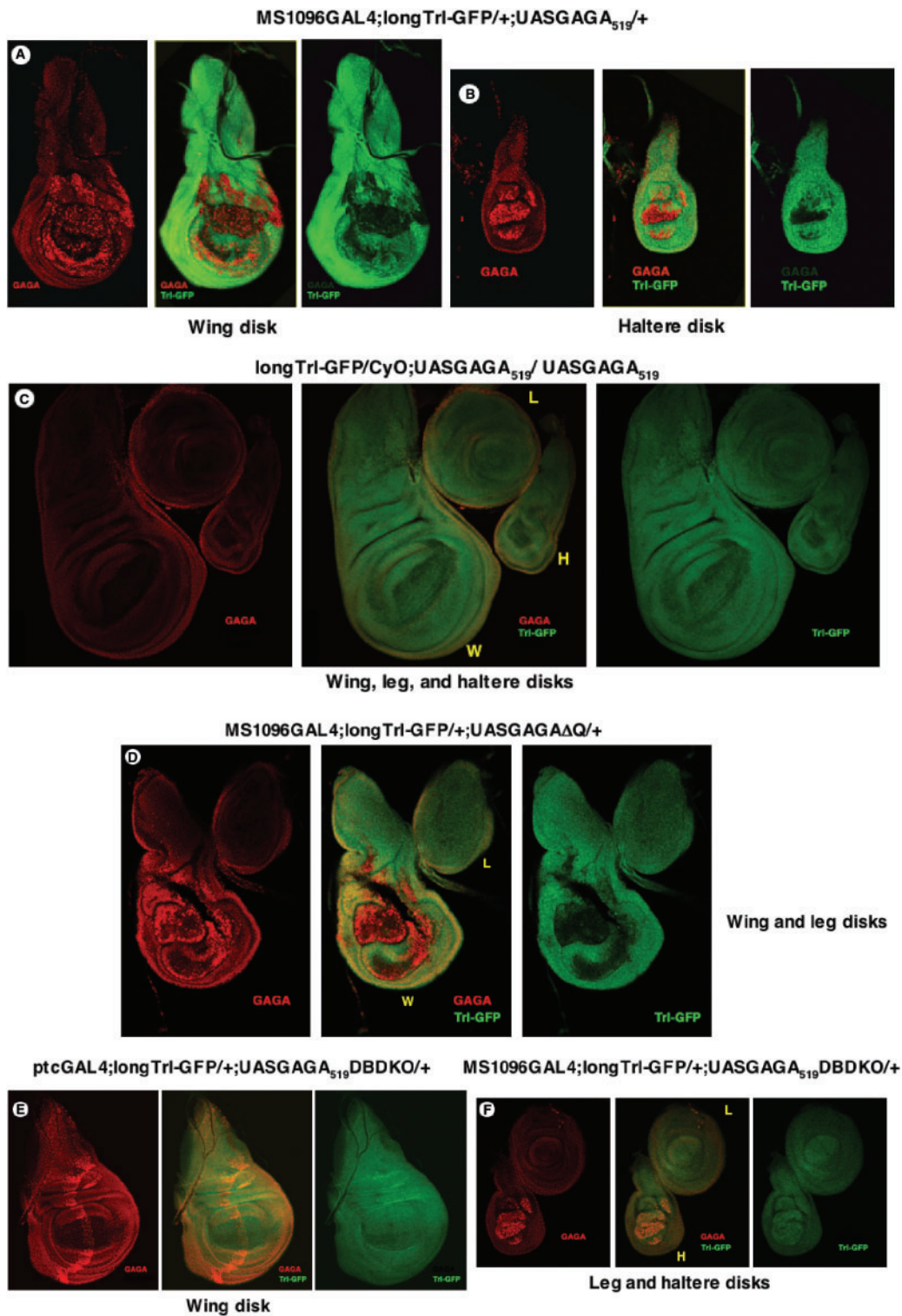


Figure 2. GAGA₅₁₉ can strongly repress *Trl* transcription in imaginal disks. Confocal microscopy images of *Drosophila* imaginal disks from 3rd instar larvae stained with α -GAGA (in red) and α -GFP (in green) antibodies. Central panels show merge images. (A and B) Wing and haltere imaginal disks carrying a long *Trl* promoter-GFP reporter and over-expressing GAGA₅₁₉ under MS1096 control, respectively; (C) control imaginal disks carrying a long *Trl* promoter-GFP reporter but not over-expressing GAGA₅₁₉; (D) wing imaginal disk carrying a long *Trl* promoter-GFP reporter and over-expressing GAGA Δ Q under MS1096 control. Samples were obtained at 18°C. (E and F) Wing and haltere imaginal disks carrying a long *Trl* promoter-GFP reporter and over-expressing GAGA₅₁₉DBDKO under *ptc* and MS1096 control, respectively. Samples were obtained at 25°C. (W: wing, H: haltere, L: leg).

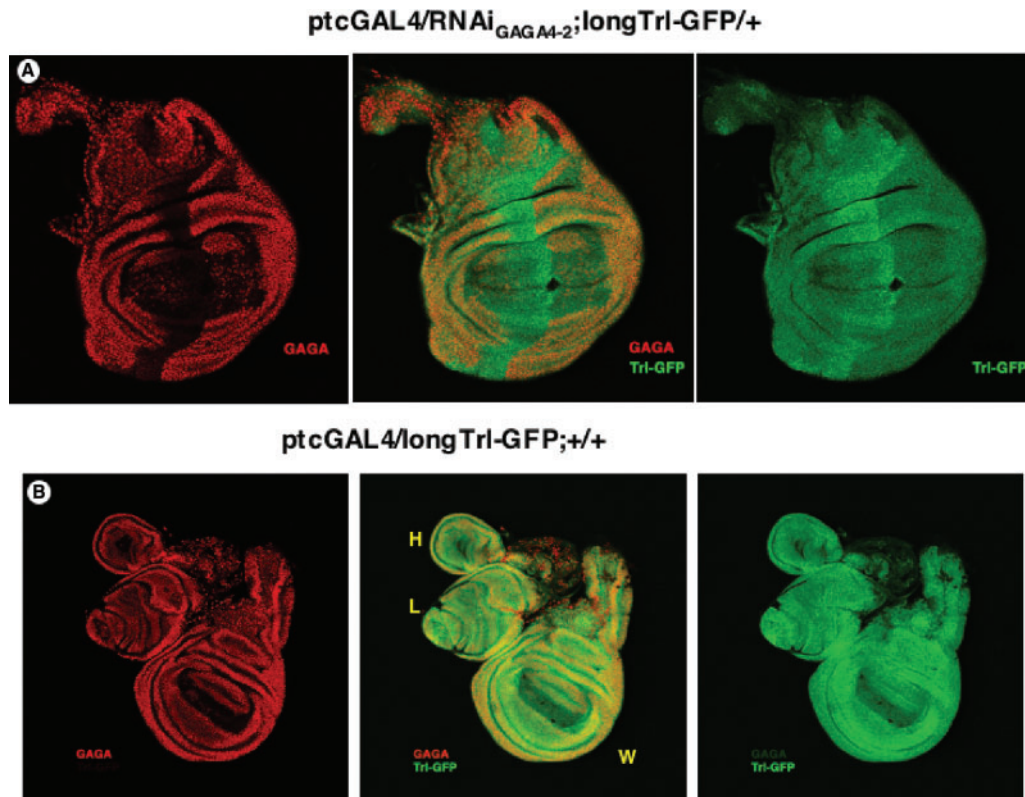


Figure 3. *Trl* is activated upon GAGA factor depletion in imaginal disks. Confocal microscopy images of *Drosophila* imaginal disks from 3rd instar larvae stained with α -GAGA (in red) and α -GFP (in green) antibodies. Central panels show merge images. (A) Wing imaginal disk carrying a long *Trl* promoter–GFP reporter and expressing RNAi_{GAGA4-2} under ptcGAL4 control at 29°C (identical results were obtained with haltere imaginal disks, data not shown). (B) Control imaginal disks carrying a long *Trl* promoter–GFP reporter that do not express RNAi_{GAGA4-2}. Sample obtained at 25°C (W: wing, H: haltere, L: leg).

not result in any lethality nor did we detect any phenotypic defects with this mutant (Table 1 and data not shown).

Flies expressing RNAi_{GAGA} also showed some abnormal phenotypes with the use of some GAL4 drivers. Defects were absent in embryos and larvae, probably because RNAi-based depletion likely generates a hypomorphic situation (see the previous section). Hypomorphic GAGA mutants are known to show a wt phenotype even in homozygosity, and the sole defect observed is that females are sterile and can only lay a few eggs (18).

GAGA depletion with ptcGAL4 resulted in a reduction of the L3–L4 intervein territory in wings of adult flies (Figure 5, compare wt and GAGA depleted, A and B, respectively). This phenotype was of high penetrance (~90%) at 25°C (100% at 29°C, data not shown) and frequently ($\geq 50\%$) included a loss of the anterior cross-vein (acv). GAGA depletion with MS1096GAL4 resulted in a frequent appearance of deltas (denoted by asterisks in Figure 5) at the tip of veins L3, L4 and L5 and even some branching (Figure 5, black arrowhead) and a complete or partial loss of acv (with a lower incidence than above). To a lower extent, these abnormalities were also seen in MS1096GAL4 control flies as a result of the insertion of the GAL4 sequence itself as already described (Figure 5, compare black arrowhead in D with

gray arrowhead in C) (24). In addition, ectopic veins were frequently observed (Figure 5D, arrow). In this case, no reduction of the L3–L4 intervein territory was observed (Figure 5A, C and D). When ApGAL4 directed depletion of GAGA factor adult wings displayed a curled phenotype, the distal parts of veins L2 to L5 frequently showed deltas (completely absent at L2 with MS1096) and also acv was often split although complete loss was rare. Neither L3–L4 intervein territory was reduced, nor ectopic veins were observed (Figure 5E and F). All depletion defects were enhanced at 29°C and were independent of the presence of *Trl*-GFP reporters (data not shown). Although the drivers used also direct expression in the halteres, our experiments never resulted in any haltere phenotype, in agreement with similar results obtained by other techniques (1,21). In principle, *Ubx* is described to be under GAGA control in halteres because of an observed low frequency of enhancement (0.27–6%, depending on experimental conditions) of the *Ubx* haplo-insufficient phenotype in *Trl*^{R85} (or *Trl*^{13C})/*Ubx*¹³⁰ flies (1,21). However, regulation of *Ubx* by GAGA has not been demonstrated by other means.

DISCUSSION

Transcriptional regulation is the first point of control of gene expression and often plays a key role among other

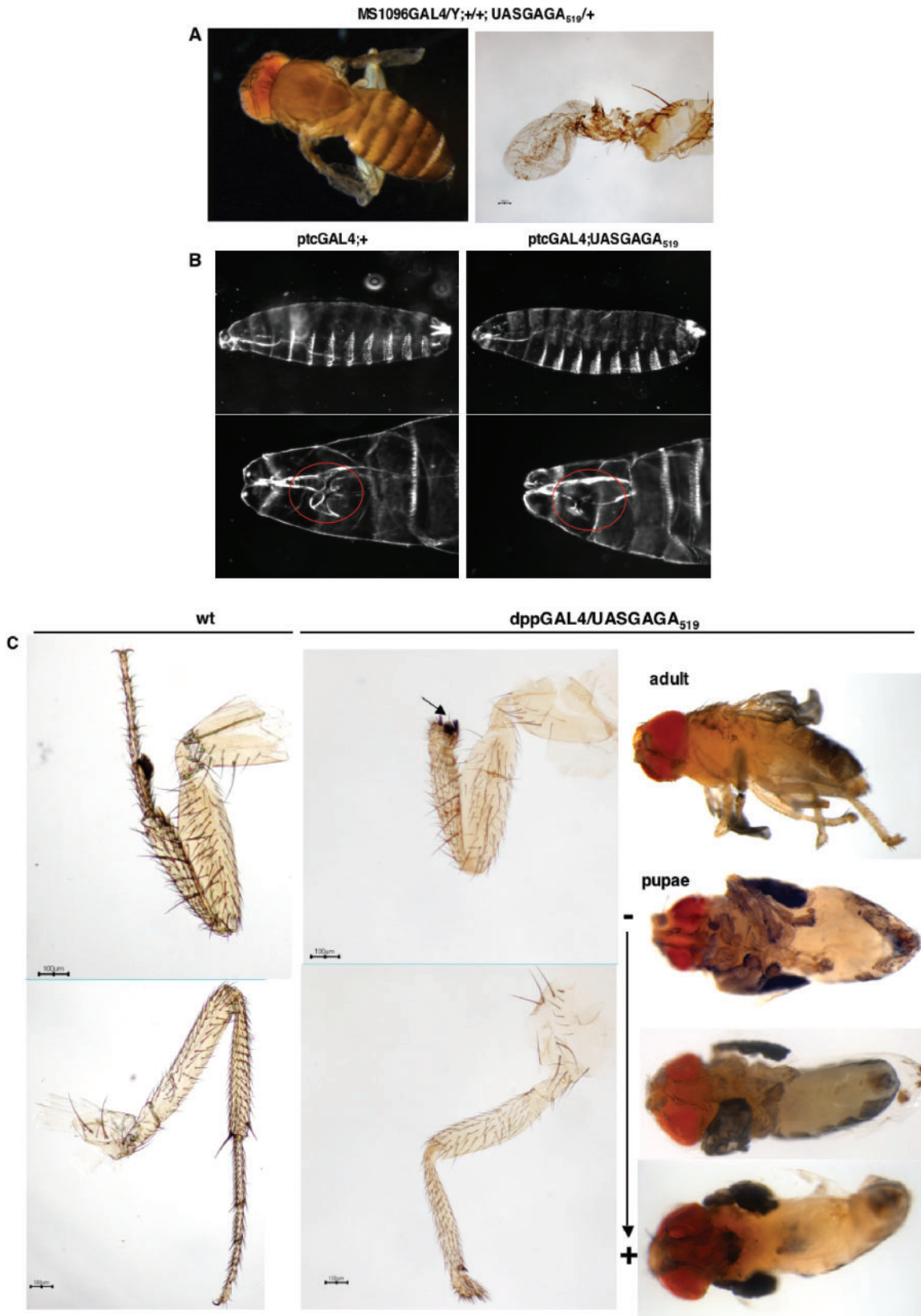


Figure 4. Phenotypes associated to GAGA₅₁₉ over-expression. (A) GAGA₅₁₉ over-expression under MS1096 control. Left panel: adult displaying strong abnormalities in wings while the rest of the body looks normal. Right panel: wing from the adult in left panel at higher magnification. Scale bar is 100 μm. (B) Wild type (left panels) and over-expressing GAGA₅₁₉ under ptcGAL4 control larvae (right panels) observed at 18°C. Magnification of the anterior region is shown below each, respectively (defects are indicated by a red circle). (C) GAGA₅₁₉ over-expression under dppGAL4 control. Left panels: legs from wt flies. Central panels: legs from flies over-expressing GAGA₅₁₉ under dppGAL4 control. Arrow points

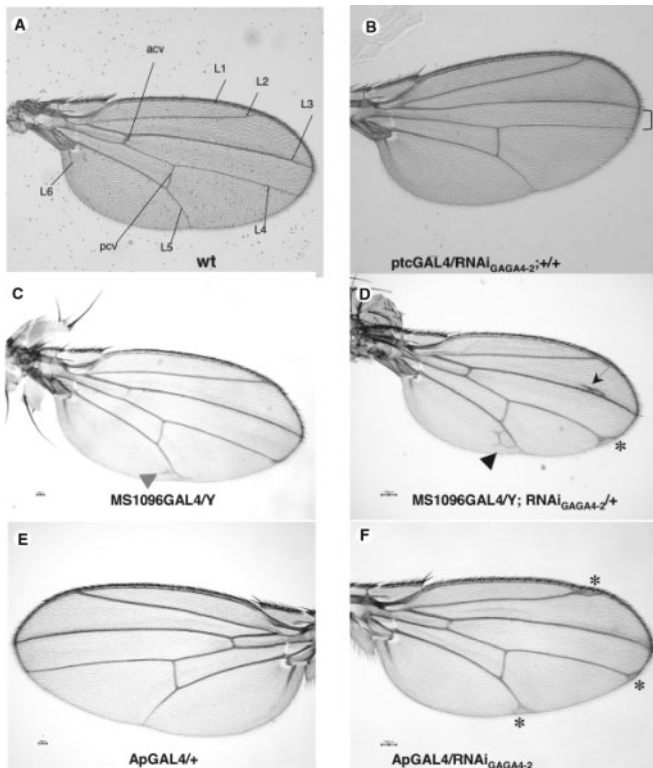


Figure 5. Phenotypes associated with GAGA factor depletion in wings. (A) Wild-type wing (all veins are indicated); (B) wing from a fly expressing $\text{RNAi}_{\text{GAGA4-2}}$ under ptcGAL4 control. Note that L3–L4 intervein territory is reduced (indicated by a bracket); (C) wing from a MS1096/Y control fly. Note the weak vein branching caused by MS1096 insertion (gray arrowhead); (D) wing from a fly expressing $\text{RNAi}_{\text{GAGA4-2}}$ under MS1096 control (arrow indicates ectopic vein appearance, black arrowhead shows a branched vein, indicating enhancement of the MS1096 phenotype); (E) wing from a control ApGAL4 fly; (F) wing from a fly expressing $\text{RNAi}_{\text{GAGA4-2}}$ under ApGAL4 control. Asterisks indicate the appearance of deltas at the tip of veins. All samples were obtained at 25°C.

regulatory levels in eukaryotes. Such regulation includes how a promoter is activated and how it is repressed but also the expression rates at which it is transcribed. While some genes show acute and discrete periods of expression, others like *Trl* show a steady expression level throughout development. While in oocytes *Trl* mRNA is already deposited by the mother, zygotic expression begins ~4 h after egg laying, peaks around 8 h and decreases remarkably and reaches a basal plateau after 16–20 h (18). Interestingly, GAGA factor protein is present at a relatively similar level from 0 to 3 h embryos to adult flies only showing a reduction from 2nd instar larval stage to late 3rd instar larval stage (2).

A negative feedback mechanism for *Trl* expression was described in S2 cells whereby GAGA factor directly repressed in a dose-dependent manner, its own transcriptional unit by binding to the *Trl* promoter. The results showed that *Trl* promoter was always under GAGA negative modulation in transiently transfected S2 cells (22).

Here we show that this mechanism is operative *in vivo* and generally used throughout development. Thus, *Trl* expression is actively balanced and kept between certain limits by a negative action of GAGA factor. Remarkably, a reduction in GAGA factor is much better tolerated than an increase in general terms, suggesting the existence of a critical upper threshold that must not be exceeded.

GAGA₅₁₉ over-expression experiments revealed that negative autoregulation of *Trl* could be observed during embryogenesis and more clearly at larval stages. In all cases, reduction in GFP signal strictly co-localized with GAGA₅₁₉ over-expression indicating a direct cause–effect. Although GAGA₅₈₁ was not over-expressed in these experiments, we speculate that these results can be extended to this isoform, because the GAGA domain(s) involved in down-regulation of *Trl* expression are shared by the two isoforms (22). RNAi depletion experiments affected both isoforms and gave exactly the complementary result, i.e. an increased expression of *Trl*-GFP in the regions where GAGA protein factors were depleted. In combination with the results obtained with GAGA Δ Q over-expression and the results obtained in S2 cells (22) they provide additional evidence in favor of a similar repressive activity of *Trl* promoter by GAGA₅₈₁ isoform. Taking into account that largely overlapping functions have been reported for the two isoforms in flies this is not surprising (25). In any case, GAGA₅₈₁ over-expression experiments are in progress to confirm this point.

In the course of over-expression experiments, a high lethality was observed (even at 18°C) essentially with all GAL4 drivers tested. Some phenotypes have been described here and include morphological defects occurring at different developmental stages that frequently are local, despite the fact that GAGA₅₁₉ over-expression takes place in a larger area. For example, even though GAGA₅₁₉ was expressed in all along embryonic segments using ptcGAL4 , morphological defects are only seen in T1–T3 thoracic segments. Similarly, GAGA₅₁₉ over-expression directed by dppGAL4 resulted in important defects only in legs. While this phenotype affected the most distal parts of the legs, expression of *distal-less* (*dll*) gene was not affected at all (data not shown). At present these results could be explained in different ways and may involve cell growth, cell proliferation, cell migration and/or apoptosis. Over-expression of GAGA₅₁₉ using MS1096GAL4 resulted in major and rather general wing disorders in the few flies that could still hatch at 18°C. MS1096 is a rather late driver, expressing GAL4 in the whole dorsal wing blade and part of the ventral wing blade. On the other hand, despite the fact that ApGAL4 is expressed much in the same area (except for the ventral wing blade) when it was used to drive over-expression of GAGA₅₁₉, lethality was absolute and earlier (larval) at 18°C. The differences observed between ApGAL4 and MS1096 may be due to the earlier expression of the first

to sex combs abnormally developing on a stump of the tarsal segments. Scale bar is 100 μm . Right panels: general view of GAGA₅₁₉ over-expressing flies that hatched spontaneously (upper one) or dissected pupae (lower three) showing increasing degrees of severity (indicated – to +, respectively). Samples were obtained at 25°C.

in tissues other than wing [e.g. in central nervous system where Ap is known to be active (26)].

Some results suggest that the high lethality are related to the activation/repression activities of GAGA, because while over-expression of GAGA₅₁₉ directed by prdGAL4 is 100% lethal, over-expression of GAGAΔQ (a mutant unable to transactivate) with the same driver is not. Interestingly, over-expression of this mutant with MS1096GAL4 is still highly lethal suggesting that in some contexts GAGA wt and GAGAΔQ behave similarly (Table 1). These results suggest that GAGA factor might be able to act both as an activator and as a repressor *in vivo*. In any case, interaction with DNA is absolutely required, as demonstrated by the lack of any effect from over-expression of GAGA₅₁₉DBDKO (a GAGA mutant unable to interact with DNA).

While GAGA over-expression resulted in strong phenotypes, GAGA depletion using RNAi was difficult to study because only moderate reduction of GAGA protein levels were often achieved. This resulted in the lack of effects on *Trl*-GFP expression in imaginal disks when certain GAL4 drivers were used (e.g. dppGAL4, ApGAL4). On the other hand, GAGA depletion was efficient with the use of ptcGAL4 and with other drivers showing high GAL4 expression at later stages in wing development, like MS1096GAL4 (data not shown). A possible explanation takes into account a considerably long half-life for GAGA factor protein. In this respect, we have estimated a GAGA half-life of ~3 days from inducible expression experiments of stably transfected S2 cells (data not shown). In addition, because of the negative feedback mechanism described above, removal of GAGA factor protein would increase *Trl*-GAGA mRNA production, thus making it even more difficult to deplete GAGA factor protein. In any case, ptcGAL4 allowed us to observe GAGA depletion in imaginal disks, likely because it is highly expressed since embryonic stages. Phenotypes from the other GAL4 drivers could appear later in the adults because GAGA depletion continued beyond 3rd instar. Results obtained with ptcGAL4, MS1096GAL4 and ApGAL4 drivers show a variety of defects in venation that suggest that GAGA factor is involved in establishing the correct wing vein pattern, likely by regulating the expression of different genes. While MS1096 and ApGAL4 expression largely overlaps on the dorsal part of the wing disk, ectopic veins appeared with MS1096 but never with ApGAL4. This result may be explained by the different expression timing of these two drivers and/or because of the ventral wing expression of MS1096. In addition, the intensification of the phenotype that MS1096 produces on its own suggests that GAGA depletion is interacting with MS1096 and enhancing its effects. The curling of the wings observed with ApGAL4 together with the reduction of the L3–L4 intervein territory observed with ptcGAL4 driver may indicate a growth defect. In this sense, we have noted a moderate reduction in wing size (6.2–8.45% with ApGAL4 and MS1096) and a remarkable reduction of the L3–L4 intervein territory with ptcGAL4 (~25%) that correlates with a reduction in the cell number (data not shown). These results are consistent with

some previous studies indicating that GAGA depletion in cultured cells affects cell growth and/or viability, that imaginal disks are consistently smaller in larvae trans-heterozygous for hypomorphic and null alleles (*Trl*^{L3c}/*Trl*^{R85}), and that severe GAGA loss of function results in many nuclear cleavage cycle defects in embryos (18,21,27).

Our results suggest that GAGA factor may be regulating the expression of genes other than homeotics, either by activation or repression, during larval to adult development. In fact, GAGA is likely controlling some essential function(s) in the larva, because general depletion of GAGA factor using actinGAL4 or armadilloGAL4 is absolutely lethal (data not shown). We suggest the phenotypes observed are likely due to effects on different genes rather than effects on a common gene or on an essential cellular function. In fact, a genome-wide analysis of GAGA factor target genes in *Drosophila* Kc cells has shown that the number of genes under GAGA factor regulation can be remarkably high (at least 250) and that GAGA factor can affect many cellular pathways (28).

ACKNOWLEDGEMENTS

We wish to thank Steve Cohen (EMBL) for kindly supplying anti-dll antibody, Esther Fuentes, Lidia Bardia and Mònica Pons for excellent technical assistance, and Marta Llimargas, Marco Milán, Héctor Herranz, Dori Huertas and Antonio Tugores for providing *Drosophila* stocks and protocols. Thanks also to all of them, people of the group and many others at the Parc Científic de Barcelona, especially Ferran Azorín, Lluïsa Espinàs, Marc Furriols and Enrique Martín-Blanco for many fruitful discussions. We also thank Daniel Shaye for a careful revision of the manuscript. D.P. was supported by a predoctoral fellowship from ‘Ministerio de Educación y Ciencia’. This work was supported by Grants BMC2003-00616 and BFU2006-09761 from the Spanish ‘Ministerio de Educación y Ciencia’ and 2005SGR 00678 from the ‘Agència de Gestió d’ajuts Universitaris i de Recerca (AGAUR)’ of the Generalitat de Catalunya. This work was carried out in the frame of the ‘Centre de Referència en Biotecnologia’ of the Generalitat de Catalunya. Funding to pay the Open Access publication charges for this article was provided by the Spanish Ministerio de Educación y Ciencia Grant BFU2006-09761 (to J.B.).

Conflict of interest statement. None declared.

REFERENCES

1. Farkas, G., Gausz, J., Galloni, M., Reuter, G., Gyurkovics, H. and Karch, F. (1994) The *Trithorax-like* gene encodes the *Drosophila* GAGA factor. *Nature*, **371**, 806–808.
2. Benyajati, C., Mueller, L., Xu, N., Pappano, M., Gao, J., Mosammaparast, M., Conklin, D., Granok, H., Craig, C. *et al.* (1997) Multiple isoforms of GAGA factor, a critical component of chromatin structure. *Nucleic Acids Res.*, **25**, 3345–3353.
3. Espinàs, M.L., Jiménez-García, E., Vaquero, A., Canudas, S., Bernués, J. and Azorín, F. (1999) The N-terminal POZ-domain of GAGA mediates the formation of oligomers that bind DNA with high affinity and specificity. *J. Biol. Chem.*, **274**, 16461–16469.

4. Katsani, K.R., Hajibagueri, M.A.N. and Verrijzer, C.P. (1999) Co-operative DNA binding by GAGA transcription factor requires the conserved BTB/POZ domain and reorganizes promoter topology. *EMBO J.*, **18**, 698–708.
5. Pagans, S., Piñeyro, D., Kosoy, A., Bernués, J. and Azorín, F. (2004) Repression by TTK69 of GAGA-mediated activation occurs in the absence of TTK69 binding to DNA and solely requires the contribution of the POZ/BTB-domain of TTK69. *J. Biol. Chem.*, **279**, 9725–9732.
6. Pedone, P.V., Ghirlando, R., Clore, G.M., Gronenborn, A.M., Felsenfeld, G. and Omichinski, J.G. (1995) The single cys₂-his₂ zinc finger domain of the GAGA protein flanked by basic residues is sufficient for high affinity specific DNA binding. *Proc. Natl Acad. Sci. USA*, **93**, 2822–2826.
7. Omichinski, J.G., Pedone, P.V., Felsenfeld, G., Gronenborn, A.M. and Clore, G.M. (1997) The solution structure of a specific GAGA factor-DNA complex reveals a modular binding mode. *Nat. Struct. Biol.*, **4**, 122–132.
8. Xiao, H., Sandaltzopoulos, R., Wang, H.-M., Hamiche, A., Ranallo, R., Lee, K.-M., Fu, D. and Wu, C. (2001) Dual functions of largest NURF subunit NURF301 in nucleosome sliding and transcription factor interactions. *Mol. Cell*, **8**, 531–543.
9. Shimojima, T., Okada, M., Nakayama, T., Ueda, H., Okawa, K., Iwamatsu, A., Handa, H. and Hirose, S. (2003) *Drosophila* FACT contributes to *Hox* gene expression through physical and functional interactions with GAGA factor. *Genes Dev.*, **17**, 1605–1616.
10. Lehman, M. (2004) Anything else but GAGA: a nonhistone protein complex reshapes chromatin structure. *Trends Genet.*, **20**, 15–22.
11. Bonet, C., Fernández, I., Aran, X., Bernués, J., Giralt, E. and Azorín, F. (2005) The GAGA protein of *Drosophila* is phosphorylated by CK2. *J. Mol. Biol.*, **351**, 562–572.
12. Nakayama, T., Nishioka, K., Dong, Y.-X., Shimojima, T. and Hirose, S. (2007) *Drosophila* GAGA factor directs histone H3.3 replacement that prevents the heterochromatin spreading. *Genes Dev.*, **21**, 552–561.
13. Biggin, M.D. and Tjian, R. (1988) Transcription factors that activate the *Ultrabithorax* promoter in developmentally staged extracts. *Cell*, **53**, 699–711.
14. Soeller, W.C., Poole, S.J. and Kornberg, T. (1988) In vitro transcription of the *Drosophila engrailed* gene. *Genes Dev.*, **2**, 68–81.
15. Read, D., Nishigaki, T. and Manley, J.L. (1990) The *Drosophila* even-skipped promoter is transcribed in a stage-specific manner in vitro and contains multiple, overlapping factor-binding sites. *Mol. Cell. Biol.*, **10**, 4334–4344.
16. Soeller, W.C., Oh, C.E. and Kornberg, T.B. (1993) Isolation of cDNAs encoding the *Drosophila* GAGA transcription factor. *Mol. Cell. Biol.*, **13**, 7961–7970.
17. Tsukiyama, T., Becker, P.B. and Wu, C. (1994) ATP-dependent nucleosome disruption at a heat-shock promoter mediated by binding of GAGA transcription factor. *Nature*, **367**, 525–532.
18. Bhat, K.M., Farkas, G., Karch, F., Gyurkovics, H., Gausz, J. and Schedl, P. (1996) The GAGA factor is required in the early *Drosophila* embryo not only for transcriptional regulation but also for nuclear division. *Development*, **122**, 1113–1124.
19. Okada, M. and Hirose, S. (1998) Chromatin remodeling mediated by *Drosophila* GAGA factor and ISWI activates *fushi tarazu* gene transcription in vitro. *Mol. Cell. Biol.*, **18**, 2455–2461.
20. Granok, H., Leibovitch, B.A., Shaffer, C.D. and Elgin, S.C.R. (1995) Ga-ga over GAGA factor. *Curr. Biol.*, **5**, 238–241.
21. Bejarano, F. and Busturia, A. (2004) Function of *Trithorax-like* gene during *Drosophila* development. *Dev. Biol.*, **268**, 327–341.
22. Kosoy, A., Pagans, S., Espinás, M.L., Azorín, F. and Bernués, J. (2002) GAGA factor down regulates its own promoter. *J. Biol. Chem.*, **277**, 42280–42288.
23. Sullivan, W., Ashburner, M. and Hawley, R.S. (2000) *Drosophila Protocols* Cold Spring Harbor Laboratory, New York, USA.
24. Milan, M., Diaz-Benjumea, F.J. and Cohen, S.M. (1998) Beadex encodes an LMO protein that regulates Apterous LIM-homeodomain activity in *Drosophila* wing development: a model for LMO oncogene function. *Genes Dev.*, **12**, 2912–2920.
25. Greenberg, A.J. and Schedl, P. (2001) GAGA factor isoforms have distinct but overlapping functions in vivo. *Mol. Cell. Biol.*, **21**, 8565–8574.
26. Lundgren, S.E., Callahan, C.A., Thor, S. and Thomas, J.B. (1995) Control of neuronal pathway selection by the *Drosophila* LIM homeodomain gene apterous. *Development*, **121**, 1769–1773.
27. Boutros, M., Kiger, A.A., Armknecht, S., Kerr, K., Hild, M., Koch, B., Haas, S.A., Consortium, H.F.A., Paro, R. et al. (2004) Genome-wide RNAi analysis of growth and viability in *Drosophila* cells. *Science*, **303**, 832–835.
28. van Steensel, B., Delrow, J. and Bussemaker, H.J. (2003) Genomewide analysis of *Drosophila* GAGA factor target genes reveals context-dependent DNA binding. *Proc. Natl Acad. Sci. USA*, **100**, 2580–2585.