

Functional Association between Eyegone and HP1a Mediates *wingless* Transcriptional Repression during Development

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The *eyegone* (*eyg*) gene encodes Eyg, a transcription factor of the Pax family with multiple roles during *Drosophila* development. Although Eyg has been shown to act as a repressor, nothing is known about the mechanism by which it represses its target genes. Here, we show that Eyg forms a protein complex with heterochromatin protein 1a (HP1a). Both proteins bind to the same chromatin regions on polytene chromosomes and act cooperatively to suppress variegation and mediate gene silencing. In addition, Eyg binds to a *wingless* (*wg*) enhancer region, recruiting HP1a to assemble a closed, heterochromatin-like conformation that represses transcription of the *wg* gene. We describe here the evidence that suggests that Eyg, encoded by *eyegone* (*eyg*), represses *wingless* (*wg*) during eye development by association with HP1a. We show that Eyg forms a protein complex with HP1a and both proteins colocalize on salivary gland polytene chromosomes. Using position effect variegation (PEV) experiments, we demonstrated that *eyg* has a dose-dependent effect on heterochromatin gene silencing and identified a genetic interaction with HP1a in this process. We further demonstrated that HP1a binds to the same *wg* enhancer element as Eyg. DNase I sensitivity assays indicated that this enhancer region has a closed heterochromatin-like conformation, which becomes open in *eyg* mutants. In these mutants, much less HP1a binds to the *wg* enhancer region, as shown by ChIP experiments. Furthermore, as previously described for Eyg, a reduction in the amount of HP1a in the eye imaginal disc derepresses *wg*. Together, our results suggest a model in which Eyg specifically binds to the *wg* enhancer region, recruiting HP1a to that site. The recruitment of HP1a prevents transcription by favoring a closed, heterochromatin-like structure. Thus, for the first time, we show that HP1a plays a direct role in the repression of a developmentally regulated gene, *wg*, during *Drosophila* eye development.

One mechanism used by organisms from yeast to mammals to repress gene expression is gene silencing. Silencing inactivates chromosome domains that contain key regulatory genes by packaging them into a specialized chromatin structure that is inaccessible to DNA-binding proteins (14, 32). Several *Drosophila* genes are required for silencing, including the *Su(var)205* gene, which encodes heterochromatin protein 1a (HP1a) (19). HP1a has been shown to bind to methylated histone H3 lysine 9 (H3K9) and to recruit H3K9 methyltransferase to chromatin (1, 24, 41). This sequential process is thought to mediate the spreading of H3K9 methylation and the formation of heterochromatin. HP1-like proteins, in addition to HP1a, have been described in *Drosophila*, but whereas HP1b and HP1c are ubiquitously expressed, HP1d (Rhino) and HP1e are expressed only in the germ line (45).

eyegone (*eyg*) encodes a homeodomain Pax protein (22). Pax proteins, which are present throughout the animal kingdom, are transcription factors that bind DNA through their paired domains (44). In vertebrates, nine Pax genes that fulfill different roles in development have been described to date. Mutations in these genes lead to several diseases, including a variety of cancers (for a review, see references 4 and 31). In *Drosophila*, *eyg* is part of the genetic network activated during eye development (16, 17); it also is involved in the development of the salivary gland ducts (21) and plays a role in the genetic subdivision of the thorax (3). Although the functions of Pax proteins during *Drosophila* development have been studied extensively, very little is known about their molecular modes of action. Nevertheless, in all cases studied so far, they have been shown to act as transcriptional repressors (47).

The most extensively studied aspect of *eyg* is its role in eye development. Proliferative growth in the eye-antennal disc is continuous from the late first-instar to the late second-/early third-instar stages. A key proliferative signal is provided by the Notch

pathway, which is active only along the dorsoventral compartment boundary of the eye disc. It has been shown that the action of Notch in stimulating eye growth is mediated by Eyg (10). Thus, *eyg* is expressed from the second instar in a wedge within the eye primordium straddling the dorsal-ventral boundary; this expression is under the control of Notch and is a downstream requirement for eye growth (10). *wingless* (*wg*) is also expressed at these stages at the anterior-dorsal and anterior-ventral disc margins. Thus, Eyg represses *wg* expression, thereby promoting eye growth and preventing head capsule formation (16, 20). As a result, viable *eyg* alleles develop without eyes and with expanded head tissue (18).

Here, we describe the repression mechanism of Eyg. We show that Eyg acts as a suppressor of variegation, suggesting a role for Eyg in heterochromatin-mediated gene silencing. We show that Eyg colocalizes with HP1a to heterochromatin regions on polytene chromosomes. Eyg and HP1a also colocalize to some euchromatin regions, including the *wg* genomic region. We demonstrate that Eyg and HP1a bind to the *wg* enhancer, where they silence *wg* activity by generating a closed, transcriptionally inactive chroma-

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tin structure. Our data thus support a role for *Eyg* in repressing its targets through a heterochromatin gene-silencing mechanism.

MATERIALS AND METHODS

Eye pigmentation measurement. The heads of 25 female flies (raised at 25°C) of each genotype were homogenized in methanol (1 ml, acidified with 0.1% HCl). Eye pigmentation was expressed as the absorbance of the supernatant at 480 nm.

ChIP assays. Chromatin immunoprecipitation (ChIP) assays were carried out with DNA obtained from 200 *yw* eye-antennal discs or 0.6 g of *yw* *Drosophila melanogaster* embryos and 200 *eyg*^{20MD1/+} heterozygous mutant eye-antennal discs, as previously described (29), with some minor modifications. Homogenized discs were sonicated five times (10-s continuous pulses; power amplitude, 7 μ m) in an MSE Soniprep 150 Sonifier with a microtip probe at 4°C, with 30 s cooling on ice between pulses, yielding predominantly 200- to 700-bp DNA fragments. For immunoprecipitation, the following antibodies were used: a 1:20 dilution of polyclonal guinea pig anti-*Eyg* antibody (3) and its respective preimmune serum, a 1:25 dilution of monoclonal mouse anti-HP1a (Developmental Hybridoma Bank), and anti-2meH3K9 (Cell Signaling) at a 1:25 dilution. The immunoprecipitated DNA was used for PCR amplification of 320 bp of the 1360 satellite sequence using the primers 1360fw (5'-TGT ATC GTT TTT AAA AAA TTG TC-3') and 1360rv (5'-GTG GAC CTG TAA TAT ATG CTC T-3'). A 410-bp *wg* enhancer region was amplified using the primers *wg*1fw (5'-GCG TGT AGT TCG AGG CCT AAG C-3') and *wg*1rv (5'-GCT TGA CGG CCA AAC GGG GCT TG-3'). A 210-bp *wg* promoter region was amplified using the following primers: *wg*promfw (5'-GCG GAA TTA ATC GCA CAA AT-3') and *wg*promrv (5'-TTT ATC TGT TCG ACG GCA CA-3').

The control *labial* region was amplified using the primers *lab*fw (5'-GGC GGG AAG TGC CCC ATC CCA AC-3') and *lab*rv (5'-CGC GTC AAG TAG CGA TTG AAG TGG-3').

Quantitative PCR (qPCR) was performed using Roche Light Cycler equipment and accessories, as described previously (5). To ensure that only a single product was amplified, we evaluated PCR products by agarose gel electrophoresis and performed dissociation curve analyses. For each ChIP assay, the reference sample, termed Mock, corresponds to a ChIP assay performed at the same time without the addition of any specific antibody; negative controls used preimmune serum. Data are presented as the amount of DNA enrichment normalized to the input (100% value) diluted 1:100. All ChIP experiments were performed as three independent events, and the results are presented as the average of each experiment \pm the standard deviation.

DNase I sensitivity assay. DNase I sensitivity assays were performed as previously described (50) with minor modifications. Samples (2 ml each) were analyzed by qPCR using Roche Light Cycler equipment. The cycle threshold (C_T) value for each locus was obtained using Roche Molecular Biochemical-Light Cycler Software (version 3.5). The euchromatin actin (*act5c*) locus was amplified using the primers Ac5Cfw (5'-CAC GGT ATC GTG ACC AAC TG-3') and Ac5Crv (5'-GCC ATC TCC TGC TCA AAG TC-3'), and the heterochromatin locus was amplified using the primers H23fw (5'-CCA AGT TGG CCA GTT TTG AT-3') and H23rv (5'-AGT TCA AGC CCG GGT ATT CT-3'). The *wg* enhancer region was amplified using the primers described above.

Fly strains and genetic crosses. All crosses were carried out at 25°C on standard cornmeal-agar medium, unless otherwise specified. The *yw* *Drosophila* strain was used as the wild-type (WT) strain in this study. For position-effect variegation (PEV) analyses, *P[white⁺]* (from M. Calleja), *DX1* (from L. Wallrath), *Su(var)205* and *His2AV^{S10}* (from Bloomington Stock Center), and *T(2;3)Sb[V]* (from J. A. Birchler) strains were used. The *eyg* mutant lines used were *eyg*^{SA2} and *eyg*^{20MD1} (3, 10). The following transgenes and Gal4 lines were used: *HPIaRNAi* (from Vienna Drosophila RNAi Center), *UAS-shmiR-dpp^{mutant}* (15), *248GAL4* (37), *ywhsFLP*; *act>y⁺>Gal4UAS-GFP* (from Bloomington), and *P[PZ]wg[r0727]* (*wglaCZ*). *UAS* transgenes were expressed in random clones by heat shock-

ing the larvae for 10 min at 34°C and dissecting them at the third-instar larval stage. The *P[PZ]wg[r0727]* line was used for fluorescence *in situ* hybridization (FISH) experiments on polytene chromosomes.

Antibodies and immunostaining. Polytene chromosomes were prepared according to standard procedures (40). The antibodies used were mouse monoclonal anti-HP1a (C1A9; 1:75; Developmental Hybridoma Bank) and guinea pig polyclonal anti-*Eyg* (1:200). Topro3 was used to counterstain nuclei. Combined FISH and immunostaining of polytene chromosomes was performed as described previously (25) using the PZ vector as a biotin-labeled probe. For staining of eye-antennal imaginal discs, third-instar larvae were fixed in 4% paraformaldehyde for 25 min, washed, and blocked in BBT (phosphate-buffered saline [25 mM NaCl] containing 0.1% Triton X-100 and 1% bovine serum albumen). The primary antibodies used were mouse monoclonal anti-Wg (CD4; 1:50; Developmental Hybridoma Bank) and chicken anti- β -galactosidase (1:200); incubations with primary antibodies were performed overnight in BBT at 4°C. After 1-h washes in BBT, the appropriate fluorescent secondary antibody was added and incubated for 1 h at room temperature. After extensive washes in BBT, the imaginal discs were mounted in Vectashield (Vector Laboratories). Images were obtained with a SPE Leica Confocal microscope and subsequently processed using Adobe Photoshop.

Western blotting and immunoprecipitation experiments. For coimmunoprecipitation experiments, salivary gland extracts (1 mg protein) from *yw* larvae were homogenized in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA) containing protease inhibitors (Roche). The cell pellet was discarded, and the protein extract was incubated overnight with 6 mg protein A-Sepharose (Sigma). Pre-cleared lysates were incubated with 6 mg protein A-Sepharose for 2 h, and antibody (1:500 guinea pig anti-*Eyg*, 1:500 guinea pig preimmune serum, 1:75 mouse anti-HP1a, 1:1,000 mouse anti- α -tubulin, or 1:2,000 mouse antihemagglutinin (anti-HA) (HA.11 from Covance) was added and incubated overnight at 4°C. After two washes in lysis buffer, proteins in immunoprecipitated extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and incubated with guinea pig anti-*Eyg* antibody (1:750) and mouse anti-HP1a antibody (1:500). Immunoreactive proteins were detected with the appropriate horseradish peroxidase-conjugated secondary antibodies, and the signal was developed using the ECL Western Blotting Analysis System (Amersham Pharmacia).

Generation of *eyg*^{PSSLN} and stable S2 cells. *eyg*^{PSSLN} was generated by PCR using the primers PSNfw (5'-GGT CCG CCA CCT TCG TCG CTG AAT CAC CCA CTG CAT GGT-3') and PSNrv (5'-ACC ATG CAG TGG GTG ATT CAG CGA CGA AGG TGG CGG ACC-3'). The template vector tubulin>Flag-HA-dAgo1 was kindly provided by S. Cohen (11a). The dAgo1 coding sequence was excised using NotI/XhoI and replaced with *eyg*^{PSSLN} and *eyg*^{PSSLN}. Transgenic Schneider S2 cells were established as described by Easow et al. (11a) with slight modifications. Briefly, an empty pRmHA vector containing a puromycin resistance gene was cotransfected with Flag-HA-*eyg*^{PSSLN} or -*eyg*^{PSSLN}, and puromycin was applied 24 h after transfection. Puromycin-resistant cells were grown to confluence and used for coimmunoprecipitation experiments as described above.

RESULTS

***Eyg* is a suppressor of variegation.** *Eyg*, a Pax protein encoded by the *eyg* gene, has been reported to act as a transcriptional repressor (46–48). We therefore assessed whether *Eyg* might repress its targets through heterochromatic gene silencing. The effects of reducing the *eyg* dosage on heterochromatic gene silencing were examined by PEV using two independent variegated lines: *DX1* and *T(2;3)Sb[V]*. *DX1* contains seven tandem copies of a *P[white⁺]* reporter transgene inserted in a euchromatic region that induces heterochromatin formation at the insertion locus (Fig. 1a) (11). Reducing the *eyg* dosage using a null allele (*eyg*^{SA2}) (see Materials and Methods) strongly suppressed variegation (Fig. 1b), causing a

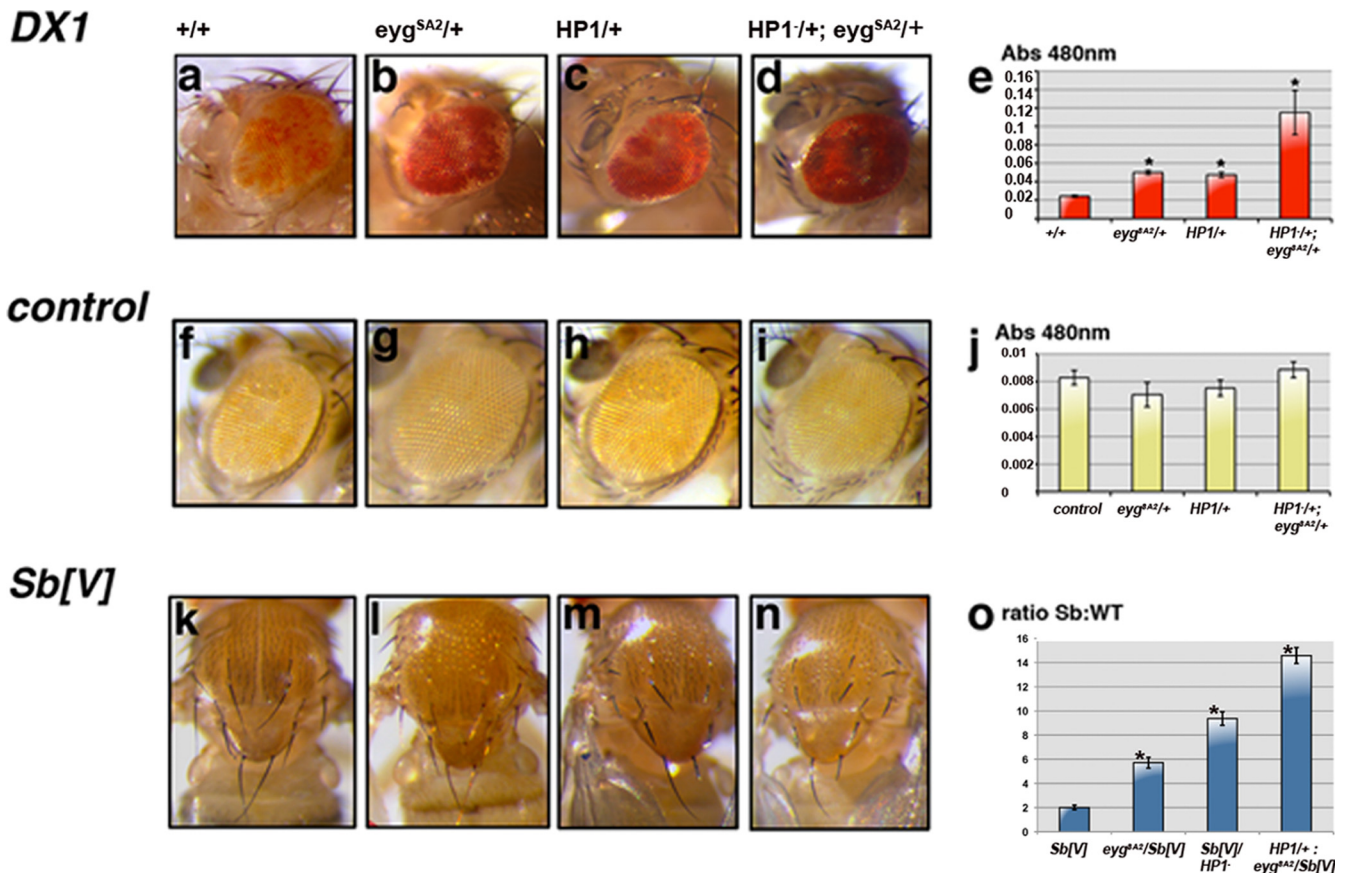


FIG 1 Eyg is required for heterochromatic gene silencing. Effects of mutations in *eyg* and *Su(var)205* on the *white⁺* variegation induced by *DX1* transgene repeats (a to e) or a control transgene (f to j), are shown as changes in red eye pigmentation. Altering the dosage of *eyg* (b) or *HP1a* (c) suppresses variegation in *DX1* flies. Note the increased red eye pigmentation in *DX1* flies (d) heterozygous for both *eyg* and *Su(var)205* genes. (e and j) Changes in pigmentation were determined by measuring absorbance at a wavelength of 480 nm. The data are expressed as means \pm standard errors of the mean (SEM) (*, $P < 0.001$ compared with +/+ control; Student's *t* test; $n = 60$ per group). *control* is a single *P[white⁺]* element inserted in a euchromatic region that does not induce heterochromatin formation. (k to o) Variegation of *Sb* in a *T(2;3)Sb[V]* rearranged chromosome (7) is suppressed by partial depletion of Eyg and/or *HP1a*. (o) Suppression of *Sb* variegation is shown as changes in the *Sb*-to-WT macrochete ratio. The data are expressed as means \pm SEM (*, $P < 0.005$ compared with an *Sb[V]* control; Student's *t* test; $n = 40$ per group).

marked derepression of the *white* gene and a substantial increase in eye pigmentation.

One of the best-studied proteins involved in heterochromatic gene silencing is HP1, encoded by *Su(var)205* in *Drosophila* (8, 23, 26). *HP1* also acts as a dosage-dependent modifier of PEV; therefore, we analyzed its relationship with *eyg*. The suppression of variegation observed in heterozygous *HP1^{-/+}* flies (Fig. 1c) was enhanced when combined with *eyg^{SA2/+}* heterozygous flies in the *DX1* variegated line (Fig. 1d). Derepression of the *white* gene was quantified by measuring red pigment absorbance levels at 480 nm (Fig. 1e) (35a). We found a statistically significant difference under each mutant condition compared to the control situation. Heterozygosity of *eyg* did not affect the expression of a control *P[white⁺]* transgene (compare Fig. 1f with g). The same result was obtained in *HP1^{-/+}* (Fig. 1h) and *HP1^{-/+} eyg^{SA2/+}* flies (Fig. 1i), and quantification of eye pigment levels showed no statistically significant difference from the control situation (Fig. 1j).

We also examined the influence of halving the *eyg* dosage on the *T(2;3)Sb[V]* variegated line. *T(2;3)Sb[V]* is caused by a chromosomal translocation that juxtaposes the *Sb* gene to a heterochromatin region (7). Silencing of *Sb* caused a variegated increase in bristle length toward the wild-type phenotype (Fig. 1k). Flies

heterozygous for *eyg^{SA2/+}* developed more *Sb* bristles in the notum than did control flies (compare Fig. 1k with l). *HP1^{-/+}* heterozygous flies also suppressed variegation of *Sb* (Fig. 1m), and again, double-mutant *HP1^{-/+} eyg^{SA2/+}* flies displayed enhanced suppression of variegation (Fig. 1n). The ratio between *Sb* and wild-type bristles was calculated for all phenotypes and was shown to be statistically significant (Fig. 1o) for each heterozygous mutant condition. Similar results were obtained with the *eyg^{20MD1/+}* allele (not shown).

We did not observe any genetic interaction of *eyg* with *His2Av*, a gene known to be essential for heterochromatin assembly in *Drosophila* (reference 43 and data not shown), suggesting that Eyg acts specifically with *HP1a* and not in general with heterochromatin proteins. Taken together, these results indicate that Eyg is a suppressor of variegation that plays a role in heterochromatin-mediated gene silencing and suggest that Eyg represses gene expression through heterochromatic gene silencing. We also found that *eyg* and *HP1* variegating phenotypes were additive, suggesting possible cooperation in the gene-silencing process.

Eyg colocalizes and coimmunoprecipitates with HP1a. We next examined the localization of Eyg and *HP1a* in wild-type salivary gland polytene chromosomes. Eyg was located at the chro-

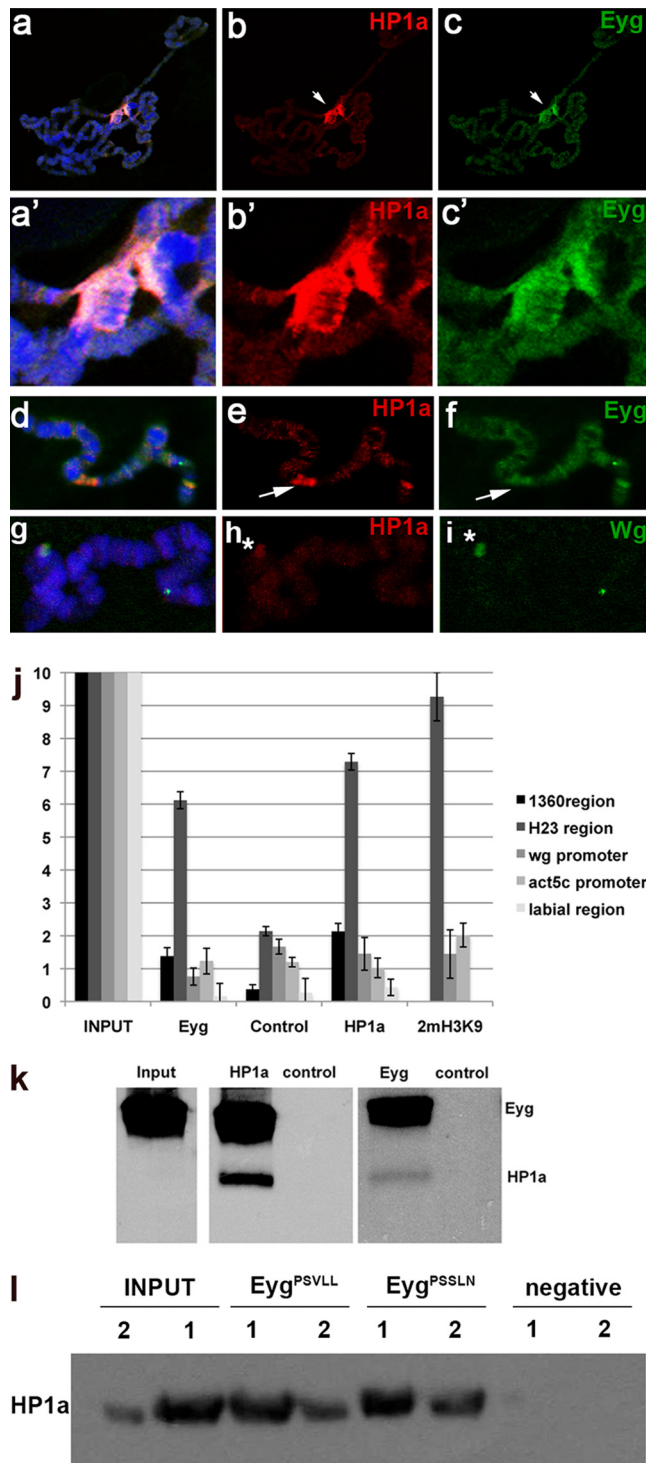


FIG 2 Eyg and HP1a colocalize to heterochromatic regions. (a to c) Eyg and HP1a colocalize to the heterochromatic regions of salivary gland polytene chromosomes (arrows in panels b and c). (a' to c') Enlargement of the chromocenter of the chromosome. (d to f) Eyg and HP1a also colocalize in some interband regions of salivary gland polytene chromosomes (arrows in panels e and f). (g to i) HP1a localizes to the *wg* genomic region, marked by a fluorescent DNA probe (asterisks in panels h and i). (j) Enrichment of constitutive heterochromatic regions *1360* and *H23* when immunoprecipitated with anti-Eyg, expressed as the percentage of input (1:100 dilution) in ChIP experiments. HP1a binding to the same heterochromatic regions was also observed. Control refers to chromatin immunoprecipitated with guinea pig preimmune

serum. An isotype control for mouse antibody gave similar results (not shown). The same immunoprecipitated DNA was amplified with primers for the *labial* genomic region, the *wg* promoter, and the *act5c* promoter region, which were used as negative controls. (k) Eyg and HP1a belong to the same protein complex. Western blot analyses detected HP1a protein in salivary gland extracts immunoprecipitated with anti-Eyg antibody and Eyg protein in salivary gland extracts immunoprecipitated with anti-HP1a antibody. In the control lanes, salivary gland extracts were immunoprecipitated with guinea pig preimmune serum or with a monoclonal anti- α -tubulin antibody (control). (l) An Eyg^{PSSLN} mutant for the PSVLL motif is able to bind HP1a in S2 cells, as does wild-type Eyg (Eyg^{PSVLL}). negative, no antibody. 1 and 2 refer to two independent coimmunoprecipitation experiments.

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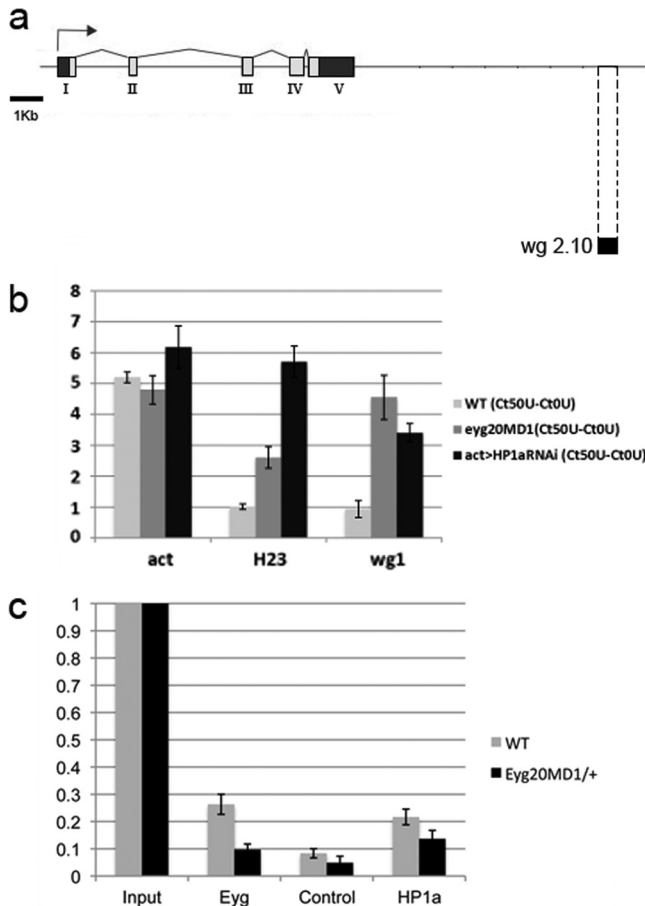


FIG 3 Eyg recruits HP1a and mediates the assembly of a heterochromatic-like structure in the *wg* enhancer region. (a) Schematic view of the *wg* genomic region. The *wg* enhancer region that drives *Wg* expression in the eye imaginal disc is located at the 3' end of the genomic region (*wg* 2.10, black bar). (b) Formation of a DNase I-resistant structure in the *wg* enhancer region. The *act5c* and *H23* loci were used as controls for open and closed genomic regions, respectively. The bars represent changes in C_T values (ΔC_T) in the *wg* enhancer region of wild-type, *eyg*^{20MD1/+}, and *HP1a* mutant *act>HP1aRNAi* eye-antennal disc extracts after treatment with 50 U DNase I. In *eyg*^{20MD1/+} heterozygous discs, as well as in *act>HP1aRNAi*, the *wg* enhancer region became almost as sensitive to DNase I as the euchromatic actin locus. The *H23* locus remained mostly closed for wild-type control and *eyg*^{20MD1/+} and open and DNase I sensitive for *HP1a* mutant extracts. (c) Analysis of HP1a and Eyg binding to the *wg* enhancer region using ChIP. A similar enrichment in binding to the *wg* enhancer region was found in wild-type control chromatin immunoprecipitated with an anti-Eyg or an anti-HP1a antibody. Binding of Eyg and, to some extent, HP1a was reduced in the chromatin of *eyg*^{20MD1/+} eye-antennal disc extracts.

(Fig. 3a, *wg* 2.10). To determine whether *wg* repression by Eyg was mediated by gene silencing and generated changes in its chromatin structure, we performed DNase I sensitivity assays to analyze the chromatin structure of this *wg* enhancer region in eye imaginal discs. Quantitative PCR was performed on native (undigested; 0 U) chromatin samples and on samples digested with DNase I (50 U). The C_T value was calculated as a function of the amount of DNA at the outset (unless otherwise stated, ΔC_T values refer to C_T [50 U] - C_T [0 U]).

For the transcribed region of actin (*act5c*), a constitutively active gene used as a positive control for the “open” chromatin state, the ΔC_T value was around 5 in wild-type, *eyg*^{20MD1/+} heterozygous

and *HP1a* mutant (*act>HP1aRNAi*) eye-antennal disc extracts (Fig. 3b). We used the Gal4-UAS binary system to eliminate HP1a via RNA interference (RNAi), because HP1 mutations are recessive lethal (see below). In contrast, the heterochromatic *H23* region (22,000 to 24,000 of chromosome 2 heterochromatin) (50), used as a negative control for the “closed” chromatin state, was refractory to DNase I treatment, exhibiting a lower ΔC_T value in both wild-type and *eyg*^{20MD1/+} heterozygous eye-antennal disc extracts (Fig. 3b). In *act>HP1aRNAi* extracts, the *H23* region became sensitive to DNase I, supporting a role for HP1a in constitutive heterochromatin formation/maintenance.

We found that in wild-type control eye-antennal imaginal discs, the *wg* enhancer region behaved like heterochromatin, with a ΔC_T between 0 and 2 (Fig. 3b). However, the same genomic region was almost as sensitive to DNase I as the *act5c* locus region in *eyg*^{20MD1/+} and *act>HP1aRNAi* mutant extracts, with a ΔC_T value between 3.5 and 5 (Fig. 3b). Halving the dosage of *eyg* was sufficient to change the sensitivity to digestion by DNase I of the *wg* enhancer region. Consistent with this, in some heterozygous *eyg*^{20MD1/+} eye discs, the change in chromatin structure led to the derepression of *wg* described previously (16, 47) and gave rise to a small-eye phenotype (not shown). This result indicates the high sensitivity achieved with the technique and also suggests that there might be other mechanisms regulating *wg* expression.

Using ChIP experiments, we confirmed that Eyg and HP1a bind similarly to this *wg* region (Fig. 3c), but not to the *wg* promoter region or to the *act5c* promoter used as controls (Fig. 2j). The amount of HP1a protein bound to the *wg* enhancer region in *eyg*^{20MD1/+} was reduced (Fig. 3c), but to a lesser extent than the Eyg protein. This result suggests that not all HP1a binding to this enhancer region depends on Eyg. We believe that Eyg may recruit HP1a to the *wg* enhancer region to form a heterochromatin structure that most likely represses *wg* expression in the eye. To further analyze this hypothesis, we tried to determine the localization of HP1a in the polytene chromosomes of *eyg* mutant larvae. However, *eyg* mutant salivary glands are very small and contain fragile chromosomes, which we were unable to squash.

Loss of HP1a leads to *wg* misexpression in the eye and wing disc. To confirm that HP1a binding to the *wg* enhancer region in the eye is indeed functionally important, we expressed a specific interfering RNA against HP1a in random clones of cells in eye imaginal disc using the actin-Gal4 *Drosophila* line. The Gal4-UAS system combined with the flippase recombination target (FRT) system was used to generate the RNAi-expressing clones. In summary, flies of the genotype *hsflp122;actFRTstopFRTGal4UASGFP/Cyo* were crossed to flies containing a UASHP1aRNAi construct. Second-instar larvae were then heat shocked for 10 min and dissected in the third-instar period to visualize the discs. Because of the FRT cassette, only clones of cells in which recombination has occurred (and that no longer have the cassette) will activate Gal4 and, consequently, express the interfering RNA that reduces or eliminates the HP1a product.

Reduction of HP1a levels resulted in ectopic activation of *wg* (Fig. 4a, b, and f), as previously described for *eyg* mutant eye discs (16).

Ectopic activation of *wg* in eye discs of *HP1aRNAi* clones did not occur in all parts of the disc domain and was more specific for the middle region. We found that 32% of *HP1aRNAi* clones ($n = 278$) were positive for ectopic *wg* activation, and more than 90% of these positive clones showed ectopic expression in the middle portion of the third-instar eye imaginal disc (Fig. 4d). These results

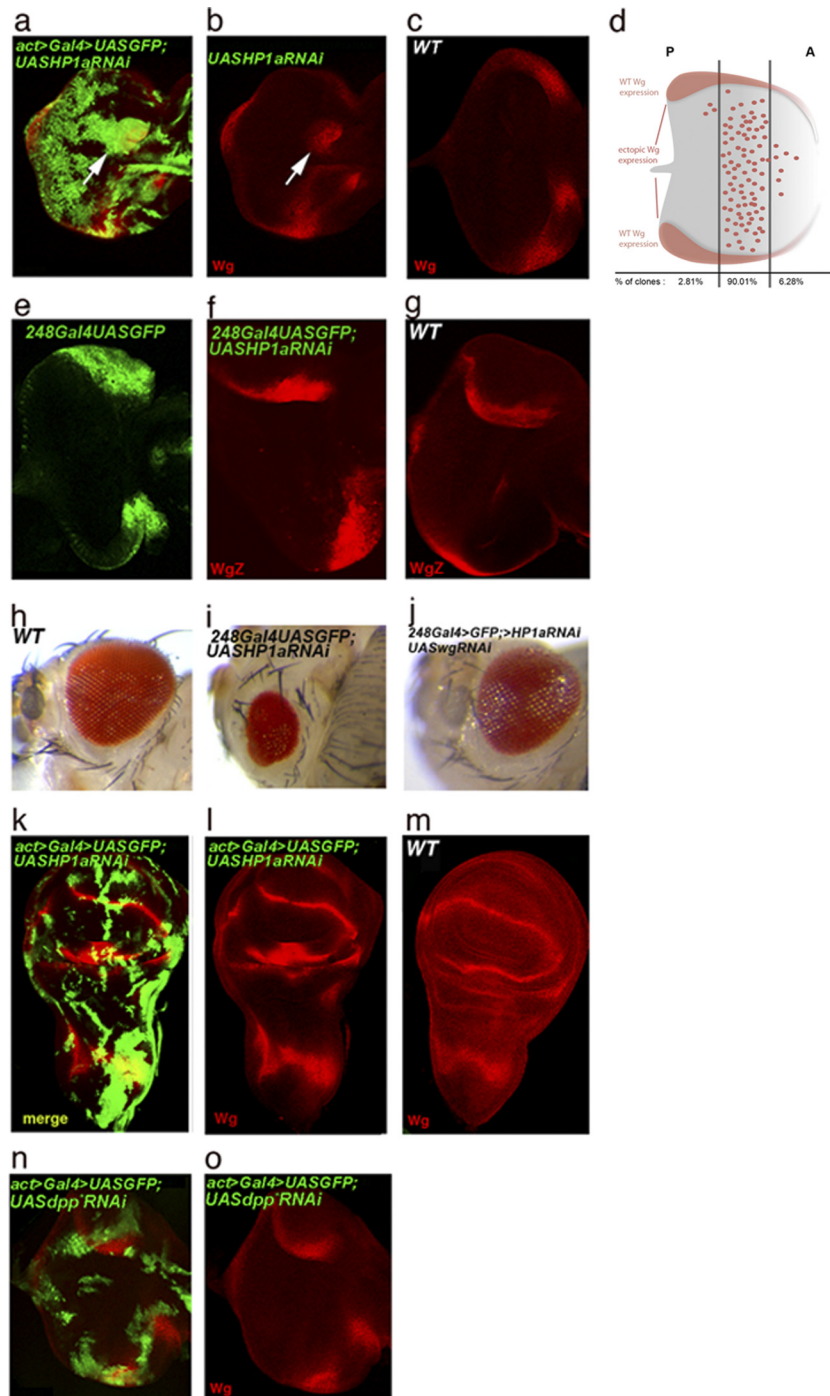


FIG 4 HP1a controls Wg expression in the eye imaginal disc and in the hinge region of the wing disc. (a and b) *wg* expression (red) in third-instar eye imaginal disc clones of cells with reduced HP1a levels induced using the *act>Gal4* line (green). The arrow points to ectopic *wg* activation. (c) Expression of *wg* in a wild-type third-instar eye disc. (d) Schematic depiction of a third-instar eye imaginal disc showing the disc domains in which ectopic activation of *wg* was more frequent after reducing HP1a levels. Of the *UAS-HP1aRNAi* clones that showed ectopic *wg* activation, 90% of ectopic activation sites lay in the middle portion of the disc. (e) Expression domain of the *248Gal4* line in the eye disc (green). (f) *wglacZ* activity in an eye disc of the genotype *248Gal4<UAS-HP1aRNAi*. (g) Expression of *wglacZ* in a wild-type eye imaginal disc. (h) Wild-type eye. (i) Eye phenotype after driving *UAS-HP1aRNAi;UAS-GFP* under the control of the *248Gal4* line. (j) Adult eye of a fly in which both HP1a and Wg levels were reduced in the same domain. Note that the eye size is rescued to almost wild-type levels (compare with panel i). (k to m) Generation of *HP1aRNAi* clones (green) in the wing disc. (l) Reducing HP1a levels in the cells of the wing hinge activated Wg expression (red). However, the distribution of Wg protein was not altered in the notum. (m) Distribution of Wg protein in a wild-type wing disc. (n and o) No ectopic *wg* expression (red) was observed in clones of cells (green fluorescent protein [GFP]-positive cells) expressing a mutated form of *dpp*, *UAS-dpp^{*}RNAi* (15), commonly used as an RNAi control line.

suggest a positional restriction for ectopic *wg* activation in the eye disc, as has also been described for *eyg* mutants (16).

Interestingly, the *HP1RNAi* clones generated in the wing imaginal disc also exhibited derepression of *wg* in the corresponding hinge region, where *eyg* has been shown to regulate *wg* (20) (Fig. 4k and l), but not in the notum, where *wg* expression does not depend on *eyg*.

HP1a has been shown to control gene expression at the post-transcriptional level (34). To determine if the observed regulation of *wg* expression occurs at the transcriptional level, we expressed HP1a RNAi under the control of the *248Gal4* driver (which expresses Gal4 in a discrete domain of the eye disc) by crossing the *UAS-HP1aRNAi* line with the *248Gal4* line in a *wgLacZ* background. Reducing HP1a levels resulted in ectopic *wgLacZ* expression (Fig. 4f), suggesting that *wg* activation actually occurs at the transcriptional level.

To rule out the possibility that derepression of *wg* by HP1a RNAi is a more general effect of the RNAi machinery, we performed a series of experiments in which *shmiR-dpp2HB^{mutant}* (15) was expressed in clones of cells in the eye imaginal disc. In contrast to the effects of expressing *HP1aRNAi*, *wg* was not derepressed by expressing *shmiR-dpp2HB^{mutant}* (Fig. 4n and o), indicating that derepression of *wg* in the eye disc is not a general effect of the RNAi machinery on heterochromatin assembly, but rather a specific effect of reducing HP1a levels.

On the basis of these data, we conclude that HP1a is involved in *wg* repression in the eye imaginal disc and in the hinge region of the wing disc, where *wg* is a target of Eyg. We did not detect any alteration of *wg* expression in the notum region of the wing disc (Fig. 4l), where Eyg is also expressed and was shown to be functional (3) but does not control *wg* expression (20).

We also sought to analyze the phenotypic outcome in adult flies with reduced HP1a levels in the eye disc. To this end, we expressed *UAS-HP1aRNAi* together with *UAS-GFP* (see below) under the control of the above-mentioned *248Gal4* driver, which allows flies to develop to adulthood (Fig. 4e) (see Materials and Methods). Adult flies carrying this genetic combination showed a reduction in eye size (Fig. 4i). To further demonstrate that this was due, at least in part, to ectopic activation of *wg* in the eye field, we performed an experiment in which we coexpressed *UAS-HP1aRNAi* and *UAS-wgRNAi* under the control of the *248Gal4* driver. The flies were allowed to develop at 29°C until adulthood. The transgenes were balanced on different chromosomes, so that we were able to distinguish flies that carried either or both transgenes in the same cross. Flies of the genotype *248Gal4;UAS-wgRNAi;UAS-HP1aRNAi* showed some head defects, but eye size was rescued to almost normal (compare Fig. 4i to j). This result strongly suggests that the reduction in eye size that we observed following reduction of HP1a levels depends on ectopic *wg* activation. Introducing a second UAS transgene (*UAS-GFP*) when expressing *UAS-HP1aRNAi* allowed us to eliminate the possibility of a Gal4 protein titration effect in the experiment.

DISCUSSION

The Pax protein Eyg, a product of the *eyg* gene, is a transcriptional repressor known to function during all developmental stages in *Drosophila*. One known target of Eyg is *wg*. It has been previously reported that Eyg represses *wg* in the eye and that this repression is a critical function of Eyg (16). In this work, we investigated the molecular mechanism underlying the repressive function of Eyg,

examining repression of *wg* using the 3' *cis*-regulatory region known to control *wg* expression in the *Drosophila* eye as an assay to study Eyg's mode of action. On the basis of our findings, we conclude that Eyg acts through heterochromatic gene silencing.

PEV experiments demonstrated that Eyg is a suppressor of variegation that is required for heterochromatic gene silencing and suggested that Eyg represses its target genes through this mechanism. We also demonstrated a genetic interaction between *eyg* and *HP1a* in the modification of PEV, suggesting that the products of these two genes act together in the gene-silencing process. Consistent with this interpretation, we found that Eyg colocalized with HP1a protein on polytene chromosomes in the chromocenter, on chromosome 4, in telomeres, and in some discrete bands along the chromosomes. The localization of Eyg on polytene chromosomes suggests that the two other ubiquitously expressed HP1-like proteins, HP1b and HP1c, are not Eyg partners because we did not detect Eyg protein on the many euchromatic bands where HP1b and HP1c localize on polytene chromosomes (13, 49a) (Fig. 2c).

Eyg contains a PSVLL motif at residues 472 to 476 located in the C-terminal domain of the protein. This is similar to the conserved pentapeptide motif present in the HP1 chromodomain and in other known HP1-interacting proteins (6, 38). However, when this domain was mutated in the Eyg protein, the interaction with HP1a was not affected, suggesting that the PSVLL motif in Eyg is dispensable for interaction with HP1a. This consensus sequence is absent in other well-known HP1 interactors, like INCENP (2), Su(var)3-9 (1), and the lamin B receptor (49; for a review, see reference 39). Eyg could therefore be another example of an HP1 interactor that engages HP1 differently from proteins that contain the consensus; alternatively, it could interact with HP1a indirectly. This result is consistent with a previous report by Yao and Sun, who showed that the C-terminal domain of Eyg is not essential for the repression of *wg* in the eye disc (47). The Eyg protein domain responsible for HP1a binding and the identity of the scaffold protein(s) that complexes Eyg and HP1a are still to be determined.

The results of ChIP experiments designed to determine if Eyg and HP1a bind to the same DNA sequences *in vivo* showed that both proteins bound to the same terminal inverted-repeat 1360 element, which initiates heterochromatic domains (9, 42); to the *H23* locus, a region of heterochromatin of chromosome 2 (Fig. 2j); and to the *wg* enhancer region known to be responsible for *trans*-activation of the *wg* gene in the eye (and where binding sites for Pax-type paired-domain transcription factors have been previously reported [33]). However, both chromatin domains behaved differently in the absence of Eyg. Using DNase I sensitivity assays, we showed that, in *eyg* mutants, the 1360 element remained considerably more refractory to DNase I treatment and thus more closed (although not as closed as in the wild type) (data not shown). It should also be noted that, for all the "closed" genomic regions tested in our DNase I sensitivity assays, none remained as closed in *eyg* mutants after DNase I treatment as they did in the wild type; even the *H23* locus was more open in the mutant background than in the wild type (Fig. 3b). This result points to a more general role for Eyg in heterochromatin formation and/or maintenance that warrants further analysis. In this context, Eyg function does not seem to depend on HP1a, because eliminating HP1a results in a ΔC_T value of 5 to 6 in the DNase I sensitivity assay on the *H23* locus (Fig. 3b).

The *wg* enhancer region behaves differently. In *eyg* mutants, it

adopted an open chromatin state (with ΔC_T values between 4 and 5), as it does in the absence of HP1a (Fig. 3b). In this region, Eyg and HP1a cooperate to silence gene expression. Binding of Eyg and HP1a is not a general feature of the *wg* locus, because we did not observe any binding to the *wg* promoter.

HP1a binding to the *wg* enhancer region was greatly reduced in *eyg* heterozygous mutant extracts, but to a lesser extent than it was the Eyg protein, suggesting that not all of the HP1a binding to that region depends on Eyg. However, the derepression of *wg* was evident in the eye imaginal disc of heterozygous *eyg* mutants, resulting in a small-eye phenotype in the adult fly (not shown). One possible explanation for this observation is that the HP1a that remains bound to the *wg* locus is insufficient to repress *wg* expression or that it has another, as yet undetermined role.

In yeast, it has been demonstrated that HP1 proteins form distinct complexes with different heterochromatin-related functions (27). In *Drosophila*, HP1-mediated silencing has been reported to operate in both a Su(Var)3-9-dependent and -independent manner (8). HP1 silencing of up to 1.9 kb has been shown to be Su(Var)3-9 independent, whereas long-range silencing depends upon the presence of Su(var)3-9. We propose that HP1-dependent *wg* silencing is Eyg dependent but Su(Var)3-9 independent, whereas for the heterochromatinization of other regions (e.g., *H23* or the *1360* element), the presence of Eyg is not crucial, but H3K9 methylation is. Indeed, HP1a and 3mH3K9 colocalize to constitutive heterochromatin regions in salivary gland nuclei of wild-type larvae (12) and still do so in *eyg* mutant larvae (not shown). However, 3mH3K9 is never detected on the chromosomal bands where HP1a and Eyg colocalize (reference 12 and our observations).

HP1a has recently been shown to have a role in the posttranscriptional upregulation of many euchromatic genes (34) owing to the association of its chromodomain with different transcripts. This unexpected role of HP1a indicates that HP1a is a multifunctional protein whose versatility lies in its capacity to interact with different partners in distinct contexts. It has been proposed that the main function of HP1a is nucleic acid compaction, by interacting either with modified histones or with specific hnRNP proteins (34). Our results point to the same conclusion in that the interaction of HP1a with Eyg leads to compaction of the DNA at the *wg* enhancer region and subsequent silencing of the gene. In this way, the HP1a protein could fulfill at least two different functions: assembly of the constitutive heterochromatin present in telomeres and pericentromeres and on chromosome 4 by binding to methylated histone H3K9 and (once targeted to regulatory regions) specific euchromatic gene repression by interacting with transcription factors to form a repressive chromatin structure. In the latter case, the resulting chromatin structure has a heterochromatin appearance and exerts heterochromatin-like repression.

In conclusion, we describe a physical and functional interaction between Eyg and HP1a that regulates *wg* expression in the eye. Although HP1 has been shown to interact with other transcription factors, like those encoded by *retinoblastoma* (*Rb*) or *Krab* (30, 36), mediating the repression of their targets, a direct role of HP1a in this process has not previously been described. Our experiments show that HP1a is necessary for the repression of *wg* in the eye imaginal disc, endowing HP1a with a restricted function in development. In the same way that Eyg represses *wg* in the eye disc, other repressors could also interact with HP1a or other heterochromatin proteins to mediate spatial and/or temporal repression

of their targets through heterochromatin gene silencing. This possibility opens new avenues that link specific transcription factors with general modifiers of chromatin.

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