Supplementary Materials and Methods

***Ex vivo* drug treatments.** Brains dissected as described above from Oregon R third-instar larvae were carefully transferred to a small drop of 0.5% citrate on a poly-lysine-coated microscope slide. After removing excess solution, the samples were rinsed once in HENMT buffer (10 mM HEPES-NaOH, pH 7.4, 1 mM EDTA, 50 mM NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100) supplemented with thiodiglycol (0.5%). After removing the buffer carefully with the edge of a tissue, the samples were incubated for 2 hours in the absence or presence of P9 in the same buffer, rinsed twice in HENMT and fixed for 10 minutes in 2% PFA in HENMT followed by 10 minutes in 2% PFA, 45% acetic acid. A siliconized coverslip was then placed over the slide, which was immediately immersed in liquid nitrogen after squashing. After removal of the coverslip with a razor blade, samples were processed as described above for fluorescence microscopy. In experiments following entry of fluorescent drugs in permeabilized tissues, samples were fixed for 20 minutes in 2% PFA in HENMT as the fluorescence signal is strongly quenched by acid treatment.

***In vivo* drug treatments.** Dechorionated Oregon R embryos affixed to microscope slides with double-stick tape were bathed for 2 hours in mineral oil and 10% DMSO (100 µl per embryo) in the presence of 10-50 µM P9. Embryos were carefully removed from the slides and fixed in 3.7% paraformaldehyde (PFA) in PBS, 0.1% Triton X-100, processed for immunofluorescence, counterstained with Lex9F (20 nM), P9F (5 nM) or P31TR (2 nM) as described above and examined by epifluorescence microscopy after mounting in DAPI. For drug feeding experiments, Oregon R third-instar larvae were transferred to agar plates containing 100 µg/ml colchicine, 100 µM P9 and yeast paste supplemented with the same concentration of the drugs. The use of colchicine was required to increase the yield of mitotic

chromosomes in eye disks. Eye disks were dissected after 16 hours and processed as described above for immunostaining and fluorescence microscopy.

For the experiment shown in Figure 6, third-instar *In(1)w*^{m4h} (*w*^{m4h}), *Tp(3;Y)BL2* or wild-type Oregon R larvae were transferred to agar plates containing 0, 25, 50, 75 or 100 µM VM26 or 10µM or 25 µM m-AMSA and yeast paste supplemented with the same concentration of the drugs. Treatment with 100 µM VM26 or 50 µM m-AMSA led to > 90% lethality. No lethality (< 10%) was observed following treatment with lower concentrations of the drugs, which led, however to developmental delays during pupation. Eye color was scored on 5-day old adult flies. Eyes were photographed using a Leica MZ-FLIII binocular microscope equipped with a CoolSnap CCD camera and eye pigment levels were measured as described (Aulner et al, 2002).

Fluorescence *in situ* hybridization (FISH) experiments. FISH experiments were performed as described (Pardue, 1986) with the following modifications. Probes used corresponded to the *white* full-length cDNA sequence provided by Benoit Auge (CBD, Toulouse, France), a full-length *Su(f)* cDNA probe provided by Kevin O'Hare (Division of Cell and Molecular Biology, Imperial College, London) and to a full-length rDNA sequence provided by Igor B. Dawid (NCI, NIH, Besthesda, U.S.A.). Probes were labeled using Digoxigenin or Biotin nick translation kits (Roche Applied Science) according to the manufacturer's instructions. Briefly, 1 µg of insert DNA was incubated in a 20 µl volume in nick translation mix for 60 minutes at 15 ºC. Labeling reactions were stopped by addition of EDTA to 25 mM final and heating at 65 ºC for 10 minutes. Labeled probes were checked on a 1% agarose gel and typically yielded a DNA smear of 500-bp average size.

Brains from third-instar larvae were dissected as described in Materials and Methods and fixed for 10 minutes in a small drop of 1:3 acetic acid:ethanol. Brains were then

transferred onto a small drop of 60% acetic acid pre-heated at 40 ºC placed on similarly pre-heated poly-lysine-coated slides. The slides were quickly and gently tilted for 60 seconds to remove excess liquid and air-dried. For subsequent treatments, slides were first incubated in 2X SSPE at 70°C for 30 minutes, transferred to 70% ethanol at room temperature for 10 minutes, washed in 70% ethanol at room temperature for another 10 minutes, in 95 % ethanol for 5 minutes and air-dried. Denaturation was performed by incubating the slides in 0.07 N NaOH for exactly 3 minutes, after which they were immediately transferred to 70% ethanol, incubated for 10 minutes at room temperature, washed twice 10 minutes in 70% ethanol, twice 10 minutes in 95% ethanol and air-dried.

For each slide, 400 ng to 1 µg of the *white* or *Su(f)* probes and 50 ng of the rDNA probe were mixed with 10 µg of sonicated salmon sperm DNA in a 12.5 µl final volume. The excess of *white* or *Su(f)* over rDNA probes allowed for the simultaneous clear detection of these single-copy and 200-fold repeated sequences. The probes solutions were denatured by boiling for 10 minutes, quick-chilled on ice, mixed with 12.5 µl of hybridization solution (8% Dextran sulfate, 2X SSPE, 6M urea) and added to the slides. Hybridization was allowed to overnight at 42 °C in a moist chamber. Slides were washed twice 5 minutes in 2X SSPE at room temperature, twice 5 minutes in 2X SSPE 2X at 55°C and twice 5 minutes in 1X SSPE 55°C. For probe immunodetection, the slides were transferred to a blocking solution containing 4X SSPE and 5% non fat milk in 1X PBS, 1 mM $MgCl_2$, 0,1 % Triton, and incubated for 30 to 60 minutes at room temperature. Incubation with anti-digoxigenin-fluorescein or anti-biotin-Cy3 antibodies (Roche Applied Science) was performed for 60 minutes at room temperature according to the manufacturer's instructions in 1X PBS, 1 mM $MgCl_2$, 0,1 % Triton, 0.4 X SSPE, 0,5 % non fat milk. Slides were washed twice 5 minutes at room temperature in the same solution, twice 5 minutes in 1X PBS, mounted in DAPI and examined as described in Materials and Methods.

DNA binding and DNAse I footprinting assays. Purified bacterially expressed recombinant D1 protein was incubated with 1 ng of 3' end-labeled SAT III DNA in the presence of 250 ng salmon sperm DNA (average size 2 kbp) in a 20 µl volume of 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 5% glycerol for 30 minutes at 28 ºC. Gel shifts were performed as described (Monod et al, 2002). For competition experiments, complexes preformed for 15 minutes were challenged with increasing concentrations of P9, incubated for an additional 15 minutes and processed as above. DNAse I footprinting experiments were performed on similarly prepared samples, except that glycerol was omitted and $MgCl_2$ was added to a final concentration of 5 mM. Samples were digested for 1 minute at 28 ºC with 25 ng DNAse I (20000 units/mg), purified and electrophoresed on sequencing gels. Experiments with purified MATH20 protein were performed as above.

References

Aulner N, Monod C, Mandicourt G, Jullien D, Cuvier O, Sall A, Janssen S, Laemmli UK, Käs E (2002) The AT-hook protein D1 is essential for *Drosophila melanogaster* development and is implicated in position-effect variegation. *Mol. Cell. Biol.* **22**: 1218-1232

Monod C, Aulner N, Cuvier O, Käs E (2002) Modification of position-effect variegation by competition for binding to *Drosophila* satellites. *EMBO Rep.* **3**: 747-752

Pardue ML (1986) In situ hybridization to DNA of chromosomes and nuclei. *Drosophila: a practical approach.*, 111-137