# Maintenance of Heterochromatin by the Large Subunit of the CAF-1 Replication-Coupled Histone Chaperone Requires Its Interaction with HP1a Through a Conserved Motif

**Baptiste Roelens,[1] Marie Clémot,[2] Mathieu Leroux-Coyau,[2] Benjamin Klapholz,[3] and Nathalie Dostatni[4]**
Institut Curie, Paris Sciences et Lettres Research University, Centre national de la recherche scientifique, Unité mixte de recherche 3664, 75248, Paris, France and Sorbonne Universités, Université Pierre-et-Marie-Curie, 75005 Paris, France
ORCID ID: 0000-0003-0118-0229 (B.R.)

**ABSTRACT** In eukaryotic cells, the organization of genomic DNA into chromatin regulates many biological processes, from the control of gene expression to the regulation of chromosome segregation. The proper maintenance of this structure upon cell division is therefore of prime importance during development for the maintenance of cell identity and genome stability. The chromatin assembly factor 1 (CAF-1) is involved in the assembly of H3-H4 histone dimers on newly synthesized DNA and in the maintenance of a higher order structure, the heterochromatin, through an interaction of its large subunit with the heterochromatin protein HP1a. We identify here a conserved domain in the large subunit of the CAF-1 complex required for its interaction with HP1a in the *Drosophila* fruit fly. Functional analysis reveals that this domain is dispensable for viability but participates in two processes involving heterochromatin: position-effect variegation and long range chromosomal interactions during meiotic prophase. Importantly, the identification in the large subunit of CAF-1 of a domain required for its interaction with HP1 allows the separation of its functions in heterochromatin-related processes from its function in the assembly of H3-H4 dimers onto newly synthesized DNA.

**KEYWORDS** heterochromatin; variegation; HP1; CAF-1; *Drosophila*

IN eukaryotic cells, the chromatin is partitioned into two cytologically and functionally distinct structures: heterochromatin and euchromatin. Heterochromatin was initially defined as the part of the genome that remains condensed during the whole cell cycle and stains intensively with DNA dyes. Heterochromatin is generally gene poor; rich in repeated sequences and transposable elements (Hoskins *et al.* 2007). Initially considered to correspond to "junk DNA," heterochromatin contains essential protein-coding genes whose expression depends on the neighboring heterochromatin structure (Schulze *et al.* 2005). It encodes essential chromosomal structures such as centromeres (Sun *et al.* 1997) or telomeres (Mason *et al.* 2008) and is required for essential chromosomal functions such as homolog pairing during meiosis (Dernburg *et al.* 1996; Karpen *et al.* 1996) . While essential for the biology of the genome, many of these structures are not directly encoded in the sequence of these regions and epigenetic mechanisms are likely required for their maintenance through generations.

The chromatin assembly factor-1 (CAF-1) is a heterotrimeric complex first isolated as a histone chaperone able to deposit H3-H4 dimers onto newly synthesized DNA during replication or repair (Smith and Stillman 1989; Gaillard *et al.* 1996). Its large subunit interacts directly with PCNA (Shibahara and Stillman 1999; Moggs *et al.* 2000) and the CAF-1 complex is found *in vivo* at the replication foci (Krude 1995; Taddei *et al.* 1999). The large subunit of CAF-1 has also been associated to the maintenance of heterochromatin: it was shown to be essential for the stable inheritance of gene silencing in


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[1]Present address: Stanford University School of Medicine, Stanford, CA 94305.
[2]These authors contributed equally to this work.
[3]Present address: The Gurdon Institute and Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge CB2 1QN, United Kingdom.
[4]Corresponding author: UMR 3664—Pavillon Pasteur, Institut Curie, Paris Sciences et Lettres Research University et Sorbonne Universités, Université Pierre et Marie Curie, 26 rue d'Ulm, 75005 Paris, France. E-mail: nathalie.dostatni@curie.fr

subtelomeric regions in *Saccharomyces cerevisiae* (Monson *et al.* 1997); its absence in fission yeast led to defective maintenance of silencing at both the centromeres and mating type loci, accompanied with a decrease of HP1 ortholog Swi6p binding in these regions (Dohke *et al.* 2008); and in mice, it is required for the duplication and maintenance of pericentric heterochromatin (Quivy *et al.* 2004, 2008). This function of mouse P150 is independent of the known function of CAF-1 in histone deposition and has been linked to its ability to interact with HP1 proteins (Quivy *et al.* 2008). The large subunit of CAF-1 is therefore an important and conserved factor required for maintenance of multiple levels of chromatin organization. It however remains to be determined whether the two apparently separate biochemical activities of CAF-1 ensure common or independent functions during development.

In *Drosophila*, the large subunit of CAF-1, P180 (Tyler *et al.* 2001; Song *et al.* 2007; Klapholz *et al.* 2009), is essential for larval development (Song *et al.* 2007; Klapholz *et al.* 2009) and is required for the following: (i) proliferation of mitotic and endocycling cells (Tyler *et al.* 2001; Song *et al.* 2007; Klapholz *et al.* 2009), (ii) assembly of nucleosomes on newly synthesized DNA (Klapholz *et al.* 2009; Tyler *et al.* 2001), and (iii) replication of euchromatic regions in larval endocycling cells (Klapholz *et al.* 2009). These properties together with genetic interactions between mutant alleles of *Caf1-180* (hereafter referred to as *p180*), which encodes P180, and *asf1* (Klapholz *et al.* 2009), encoding the histone chaperone ASF1, suggest that the function of P180 essential for viability in *Drosophila* is related to CAF-1-dependent histone deposition. Whether P180 is also required for proper maintenance of heterochromatic regions was initially less clear: although two mutant alleles of *p180* were shown to act as dominant suppressors of position-effect variegation (PEV) (Song *et al.* 2007), quantitative analysis of the efficiency of the replication of heterochromatic regions did not show, in contrast to euchromatic regions, major defects upon the loss of P180 activity in larval endocycling cells (Klapholz *et al.* 2009). More recently, an RNA interference approach showed that P180 regulates, in a dose-dependent manner, the structure of pericentric heterochromatin by affecting H3K9Me and H4K20Me levels together with the recruitment of HP1a on polytene chromosomes; thereby conclusively showing the conservation of this function in flies (Huang *et al.* 2010). Moreover, as the artificial targeting of HP1a to chromosomes induces the accumulation of P180 at these ectopic positions (Huang *et al.* 2010), it was proposed that the role of CAF-1 in heterochromatin maintenance in flies was also likely mediated by an interaction between P180 and HP1a.

In this study, we identify a conserved domain in the *Drosophila* CAF-1 large subunit required for its *in vitro* interaction with HP1a. We show that this domain is not essential for viability but is required for proper heterochromatin maintenance in germ cells and participates in two processes that require proper heterochromatin structure in flies: PEV and persistence of pairing between heterochromatic chromosomal regions in developing oocytes. Our results therefore demonstrate that the interaction between the large subunit of CAF-1 and HP1a is not essential for *Drosophila* larval development and viability, but participates in the maintenance of heterochromatin organization.

## Materials and Methods

### *Fly stocks*

Fly stocks include the *p180*$^{3}$ [*caf1-180*(*3*)] (Klapholz *et al.* 2009), *Su*(*var*)*205*$^{5}$ [*Su*(*var*)*205*(*5*)] (Eissenberg *et al.* 1992), and *Mei-W68*$^{1}$ [*Mei-W68*(*1*)] (McKim *et al.* 1998) alleles; the *Dp*(*1*;*Y*)*B*[*S*]-marked *Y* chromosome; the *P*{*mw*,*HSBGHb3Bcd3-lacZ*} insertion on the *X* chromosome (Simpson-Brose *et al.* 1994); the *FM7i*, *P*{*ActGFP*}*JMR3*, *TM3,Sb*, *TM3,Sb Ser* and *SM6b* balancer chromosomes; the *T*(*2*;*3*)*Sb*$^{V}$ (*T*(*2*;*3*)*Sb*[*V*]) translocation; and the *C*(*2*)*EN* and *C*(*4*)*RM*, *ci*[*1*], *ey*[*R*] compound chromosomes. Ubiquitous expression was obtained using the Gal4/UAS system (Brand and Perrimon 1993) with *P*{*Act5C-Gal4*}*25FO1* insertion as a Gal4 driver. Upstream activation sequence (UAS) transgenes include *UAS-p180* (Klapholz *et al.* 2009) and *UAS-p180*$^{\Delta HIM}$, which were obtained by germline transformation of the *pUASp* vector (Rorth 1998) containing the *p180* or *p180*$^{\Delta HIM}$ complementary DNA (cDNA) (see the *Vectors and cloning* section).

### *Genetics*

PEV was assessed by analyzing the progeny of females that were or were not carrying mutant alleles of *p180* and *Su*(*var*)*205* crossed with *Sb*$^{V}$/*TM3,Sb,Ser* (Figure 4C) or *Sb*$^{V}$/*TM3, Ser* (Supplemental Material, Figure S3) males. The 12 macrochaetae in notum and scutellum of *Sb*$^{V}$ offspring were scored to quantify the Sb phenotype.

Meiotic segregation defects of *X* chromosomes were monitored using dominant markers located on the paternal *X* (*mw* from P{*mw*,*HSBGHb3Bcd3-LacZ*}) and *Y* (*Bar* from *Dp*(*1*;*Y*)*B*[*S*]) chromosomes as described in McKim *et al.* (2009). Briefly, females that were or were not carrying mutant alleles of *p180* were also heterozygous for the *y* marker to allow the discrimination of meiosis I from meiosis II segregation defects. When *y*$^{+}$,*p180*$^{+or-}$/*FM7*,*y*,*B*$^{1}$ females were crossed to *y*,{*mw*}/*B*$^{S}$*Y* males, regular progeny include females with normal or mildly Bar (*B*$^{1}$ heterozygotes) eyes and males with severe Bar (*B*$^{S}$) eyes. *X* chromosome segregation defects during female meiosis generate oocytes with two *X* chromosomes or none: the corresponding exceptional progeny are females with severe Bar eyes (*XXY*, *B*$^{S}$ female) or males with normal or mildly Bar eyes (*X*O male). Segregation defects during meiosis I lead to nonyellow-bodied *XXY* females that are heterozygous for the *y* marker, whereas segregation defects in meiosis II lead to an equal number of yellow-bodied and nonyellow-bodied *XXY* females. Because of increased meiotic segregation defects in *XXY* females when compared to wild type (Xiang and Hawley 2006), each female parent and each exceptional progeny was tested by PCR to detect the presence of the *Y* chromosome *Pp1-Y2* gene (primers Pp1-Y2$_1$, GCGAATTATTGACACTCGCCG; and Pp1-Y2$_2$, ATCGTTGTCGCTCCATCCCAT) and of the paternal *X*-linked transgenic sequences (primers HSBG407, CAGCGCTGACTTT GAGTGGAA; and LacZ-seq, AGACCAATGCCTCCCAGACC).

The frequency of segregation defects was normalized to account for unviable *XXX* and O*Y* genotypes, and calculated as 2×(exceptional progeny)/[2× (exceptional progeny) + (regular progeny)].

### Statistical tests

The low level of exceptional progeny observed did not allow us to use the classical $\chi^2$ test. Rates of segregation defects were therefore compared using Fisher's exact test which is more suitable to low effectives. For all tests, a two-tailed *P*-value of lower than 5% was considered statistically significant. A recent study provided tools to compare rates of segregation defects of the *X* chromosome using a multinomial Poisson hierarchy model (Zeng *et al.* 2010). Treatment of our data with the Fisher's exact test or the Poisson hierarchy model reached similar conclusions.

### Sequence alignment and conservation

Sequences were aligned using the MUSCLE algorithm (Edgar 2004a,b). Conservation at any given position was computed as the average of a numerical index reflecting the conservation across the selected species of physico-chemical properties (Livingstone and Barton 1993) on 11 residues centered on the position: for example the value at position 15 reflects the conservation of residues 10–21. When <11 values were available for a given position, conservation was computed on the available values within the +5/−5 distance.

### Vectors and cloning

A cDNA library of *Drosophila* embryos (Clontech) was used to generate the three *Drosophila* HP1a GST constructs using three pairs of primers: ssHP1-a (TTAAGAATTCATGGGCAAGAAAATC GACAACCC) and ssHP1-b (TTAAGAATTCGCTCGCCTCGTACT GCTGG) to amplify the sequence encoding the first 72 residues of HP1a; ssHP1-a and ssHP1-c (TTAAGAATTCGCCGCGATCGAATC CGGTAGATCC) to amplify the sequence encoding the first 146 residues of HP1a; ssHP1-a and ssHP1-d (TTAAGAATT CATCTTCAATATCAGAGTACCAGG) to amplify the full-length HP1a coding sequence. These PCR products were then cloned into the *Eco*RI restriction site of the *pGEX-4T-1* plasmid (GE Healthcare). The wild-type and mutant P180-His proteins were produced in bacteria using the *pET30A*+ plasmid (Novagen), in which the corresponding cDNA was cloned in frame with the sequence encoding the histidine tag. The various cDNAs encoding the truncated P180 proteins used to identify the HP1-interacting motif (HIM) were obtained using standard cloning methods as follows: for $p180^{\Delta Nt}$, ligation of LG1 (TATGCACGCTGGCG TAGTTG) and LG2 (GATCCAACTACGCCAGCGTGCA) oligonucleotides in the *p180* cDNA digested with *Nde*I and *Bam*HI; for $p180^{\Delta Nt\Delta(883–1101)}$, ligation of PSKP1 (GCAGGAG TTCGCTGATGCG) and PSKP2 (GTACCGCATCAGCGAACTCC TGCTGCA) oligonucleotides in the $p180^{\Delta Nt}$ cDNA digested with *Pst*I and *Kpn*I; for $p180^{\Delta Nt\Delta(1110–1183)}$, ligation of KPNO1 (GTACCAGCCGCCTCGCCCGC) and KPNO2 (GTACCGCA TCAGCGAACTCCTGCTGCA) oligonucleotides in the $p180^{\Delta Nt}$ cDNA digested with *Not*I and *Kpn*I; for $p180^{\Delta Nt\Delta(883–959)}$, self-ligation of the $p180^{\Delta Nt}$ cDNA digested with *Pst*I; for $p180^{\Delta Nt\Delta(883–986)}$, ligation in the $p180^{\Delta Nt}$ cDNA digested with *Pst*I and *Kpn*I of the product obtained by PCR amplification of the *p180* cDNA with the PSKP DELTA2959 (CCGCTGCAGGAG TACCTGAAAACCCAAGCGA) and p180-3′*Kpn*I (GCGGCTGGTA CCGCATCCGCTG) primers; for $p180^{\Delta Nt\Delta(883–1010)}$, ligation in the $p180^{\Delta Nt}$ cDNA digested with *Pst*I and *Kpn*I of the product obtained by PCR amplification of the *p180* cDNA with the PSKP DELTA3030 (CCGCTGCAGAAATTCGACGAGCTTGCCAG) and p180-3′*Kpn*I (GCGGCTGGTACCGCATCCGCTG) primers; for $p180^{\Delta Nt\Delta(883-1040)}$, ligation in the $p180^{\Delta Nt}$ cDNA digested with *Pst*I and *Kpn*I of the product obtained by PCR amplification of the *p180* cDNA with the PSKP DELTA3130 (CCGCTGCAGAAACCGAAGAA GCGCCTCTG) and p180-3′*Kpn*I (GCGGCTGGTACCGCATCC GCTG) primers; for $p180^{\Delta Nt\Delta HIM}$, ligation in the *Pst*I digested $p180^{\Delta Nt\Delta(883–986)}$ cDNA of the fragment obtained by *Pst*I digestion of the *p180* cDNA; for $p180^{\Delta(959–986)}$, ligation in the *p180* cDNA digested with *Pst*I and *Kpn*I of the product obtained by PCR amplification of the *p180* cDNA with the PSKP DELTA2959 (CCGCTGCAGGAGTACCTGAAAACCCA AGCGA) and p180-3′*Kpn*I (GCGGCTGGTACCGCATCCG CTG) primers; and for $p180^{\Delta HIM}$, ligation of the fragment obtained by *Pst*I digestion of the *p180* cDNA in the $p180^{\Delta(959–986)}$ cDNA digested with *Pst*I. The P180-His was produced in bacteria using the pET30A+ plasmid (Novagen), in which the *p180* cDNA was cloned in frame with the sequence encoding the histidine tag.

### Co-immunoprecipitation on Schneider S2 cells

Schneider S2 cells were incubated for 15 min on ice in a lysis buffer containing 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml pepstatin, and 15 μg/ml aprotinin. NP-40 was added to a final concentration of 0.5% and the cells were strongly agitated. Nuclei were centrifuged for 5 min at 100 × *g*. The nuclear pellet was resuspended in nuclear extraction buffer (NEB) 0.42 (20 mM HEPES, 25% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml pepstatin, and 15 μg/ml aprotinin) with 0.42 M NaCl and incubated for 20 min at 4° on a wheel before a centrifugation of 30 min at 20,000 × *g*. Immunoprecipitation (IP) was performed on the supernatant with a 1:100 dilution of a polyclonal rabbit anti-P180, kindly provided by Tyler *et al.* (2001), and Sepharose-Protein A beads (Upstate) in NEB 0.2 (same as NEB 0.42 but containing 0.2 M NaCl). The detection of co-immunoprecipitated proteins was performed by western blots using a 1:8000 dilution of the same anti-P180, a 1:1000 dilution of polyclonal rabbit anti-P105 kindly provided by Tyler *et al.* (2001), or a 1:25,000 dilution of a mouse monoclonal anti-HP1a antibody (#C1A9, Developmental Studies Hybridoma Bank). Secondary antibodies were either a 1:50,000 dilution of an HRP-conjugated donkey anti-rabbit (#711-036-152, Jackson ImmunoResearch Laboratories) or a 1:10,000 dilution of a goat anti-mouse (#115-036-062, Jackson ImmunoResearch Laboratories).

### Recombinant proteins and GST pull downs

GST-fusion and His-tagged proteins were produced in *Escherichia coli* BL21-DE3 upon induction with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside. Pellets were suspended in buffer *Y* (1× PBS, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml pepstatin, and 10 μg/ml aprotinin) and sonicated. Lysates were centrifuged at 20,000 × *g* for 30 min to remove aggregates. GST fusions were purified using Glutathione Sepharose 4B beads (GE Healthcare). After washes in buffer *Y*, bound proteins were suspended in buffer (PBS/EDTA, 0.5 M, pH 8.0). The relative concentration of proteins was estimated by Coomassie staining of SDS-PAGE for GST fusion and by western blotting for His-tagged proteins. For GST pull-down assays, beads of Glutathione Sepharose bound to GST fusions were suspended in immuno-precipitation (IP) buffer (50 mM Tris-HCl, pH 7.0, 0.25 mM EDTA, 0.03% NP-40, 5 μg/ml leupeptine, 2 μg/ml pepstatine, and 10 μg/ml aprotinine) and incubated with lysates containing the His-tagged proteins at 4° for 3 hr under gentle agitation. The Sepharose beads were collected by centrifugation at 800 × *g* and washed four times with IPTAG buffer containing 150 mM NaCl. Pulled-down proteins were detected by western blotting with a 1:500 dilution of a His-probe mouse monoclonal antibody (H-3, #sc-8036, Santa Cruz Biotechnology), a 1:10,000 dilution of a secondary goat peroxidase-conjugated anti-mouse antibody (#115-036-062, Jackson ImmunoResearch), and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

### Detection of P180 in larval extracts

Larval protein extracts were prepared as described in Klapholz *et al.* (2009). P180 proteins were detected using an antibody raised in rabbit against a peptide corresponding to the residues 198–421 fused to GST. As this peptide is located in the amino-terminal region of the protein, the deletion of the HIM is unlikely to affect recognition of this epitope in denaturing conditions.

### Immunodetection on salivary gland polytene chromosomes

Staining was performed as described in Paro (2000). P180 was detected using a FLAG-tag fused to the P180 protein in the *UAS-p180* transgene using either a monoclonal mouse (M2, Sigma Aldrich, Figure 2B) or a polyclonal rabbit antibody (F7425, Sigma-Aldrich, Figure 2, A and C). Using the M2 antibody, a nonspecific staining was recurrently observed at the chromocenter, even in flies not expressing the P180-Flag protein. The other antibodies used were: mouse monoclonal PC10 (Dako) specific for PCNA, rabbit polyclonal R20/12 specific for acetylated H4K12 (Turner *et al.* 1992), and mouse monoclonal C1A9 (Developmental Studies Hybridoma Bank) detecting HP1a.

### Immunodetection on ovaries

Ovaries of 4- to 5-day-old females were dissected in PBS and fixed in 4% paraformaldehyde for 15 min. Samples were then permeabilized in PBS with 0.2% Triton X-100 (PBT) for at least 30 min, incubated at 4° overnight with primary antibodies diluted in PBT (rabbit anti-P180, 1:1000, described in the section *Detection of P180 in larval extracts*; and mouse anti-HP1 C1A9, Developmental Studies Hybridoma Bank, 1:100), washed three times for 15 min in PBT, incubated for 2 hr with secondary antibodies diluted in PBT (Alexa-568 conjugated anti-rabbit and Alexa-488 conjugated anti-mouse, Life Technologies, 1:1000), and washed three times for 15 min in PBT. For DNA staining, samples were subsequently stained with DAPI (0.8 μg/ml in PBT) for 10 min. Samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA).

Imaging of germaria and egg chambers was performed with a Carl Zeiss (Thornwood, NY) LSM780 confocal microscope using identical acquisition settings for all analyzed genotypes. Image projections were performed with six to eight images containing the upper half of the germarium or five to six images containing the oocytes for the egg chambers. Images were separated by 0.8–1 μm along the *z*-axis.

### Quantification of HP1a signal in oocytes

The total HP1a signal in oocytes was evaluated using Fiji, by (i) identifying the stacks containing the oocyte nucleus based on the DAPI staining, (ii) performing a sum-intensity projection of the typically five to six stacks containing the oocyte's nucleus, and (iii) summing the value of every pixel contained within a region of interest designed to encompass the oocyte nucleus.

### FISH staining and imaging

The 359-bp repeat probe was obtained by PCR amplification of genomic DNA using primers (359 bp-1, CGGTCATCAAATAAT CATTTATTTTGC; and 359 bp-2, CGAAATTTGGAAAAACA GACTCTGC) designed from the published sequence (Hsieh and Brutlag 1979). Probes were digested with AluI and then tailed with digoxigenin using the DIG-oligonucleotide tailing kit (Roche). FISH was performed as described (Dernburg *et al.* 1996). After hybridization, probes were detected using a sheep anti-digoxigenin primary antibody (1:1500, Roche) and the Alexa Fluor 568 donkey anti-sheep (1:1000, Molecular Probes, Eugene, OR), DNA was stained with DAPI, and the samples were mounted in Vectashield. Image acquisitions and processing were performed using a Delta Vision microscope (GE Healthcare Life Sciences) with 40× and 100× objectives and the SoftWorx software. The settings for acquisition and deconvolution were constant for each staining. Each quantification was performed on at least two independent experiments. Image projections were performed with 4–10 images separated by 0.2 μm along the *z*-axis.

### Data availability

Strains are available upon request. Table S1 contains accession numbers for P180 orthologs used for conservation analysis.

## Results

### The Drosophila large subunit of CAF-1 and HP1a interact directly

P180 and HP1a have been shown to genetically interact in *Drosophila* and to co-immunoprecipitate in embryonic extracts (Huang *et al.* 2010). Given the potential importance

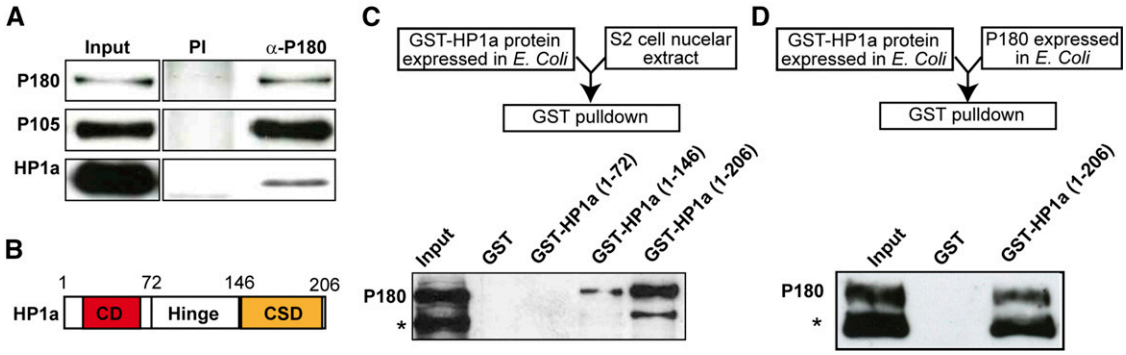


**Figure 1** P180 and HP1a interact directly. (A) IP of P180 in nuclear extracts from cultured Schneider S2 cells. The large (P180) and medium (P105) subunits of CAF-1, as well as the *Drosophila* HP1a protein, were detected by western blot on nuclear extracts (Input), after IP with a preimmune serum (PI) or with an anti-P180 antibody (α-P180). (B) Representation of the HP1a protein, carrying a chromodomain (CD, red) in the N-terminal and a chromo shadow domain (CSD, orange) in the C-terminal. The positions of the first and last HP1a amino acids contained in the GST-fusion proteins used hereafter are indicated. (C) GST pull-down assay in nuclear extracts from cultured Schneider S2 cells, using different forms of HP1a fused to GST in N-terminal. Bottom panel: western blot anti-P180 on the nuclear extract (Input) and after the pull-down assay using the different fusion proteins. (D) GST pull-down assay using recombinant proteins expressed in *E. coli*. The presence of P180 in the bound fraction was analyzed by western blotting against a poly-histidine motif fused to its C-terminal. * designates a putative degradation product of the P180 protein.

of this interaction in the maintenance of heterochromatic structure, we aimed to characterize its molecular details. We first recapitulated this interaction by performing an IP of P180 from a nuclear extract of *Drosophila* Schneider S2 cells. P180 coprecipitated with P105, the medium subunit of CAF-1, and, in agreement with a study by Jiao and colleagues (Huang *et al.* 2010), with HP1a (Figure 1A). Additional GST pull-down assays on S2 nuclear extracts with truncated forms of HP1a (Figure 1B) fused at their N-terminal end to the GST indicated that (a) P180 does not interact with the chromodomain of HP1a, (b) the hinge of HP1a is able to interact weakly with P180, and (c) the full interaction between P180 and HP1a requires both the hinge and the chromo shadow domain of HP1a (Figure 1C). Finally, GST pull-down assays using recombinant P180 and GST-HP1a proteins showed that HP1a was able to pull-down P180 on its own (Figure 1D) and that the interaction between the two proteins was therefore direct.

### *Colocalization between P180 and HP1a is only observed on replicating polytene chromosomes*

In the experiment described Figure 1A, we noticed that only a minor fraction of HP1a was coprecipitated with P180. This could be due to the fact that HP1 proteins interact with other partners that could potentially compete with P180 for binding to HP1 in nuclear extracts (Kellum 2003). In addition, as CAF-1 is mostly required during *S* phase of the cell cycle, this interaction might also be restricted during this phase of the cell cycle *in vivo*. We tested this hypothesis by analyzing the localization of both P180 and HP1a on salivary gland polytene chromosomes. As described previously (James and Elgin 1986; James *et al.* 1989), HP1a was detected at the chromocenter, a DAPI-dense region containing the pericentric and centric regions (Figure 2A). Codetection of P180 using a FLAG-tagged transgene indicated that localization of P180 only poorly correlated with the binding of HP1a but was detected on polytene chromosomes also stained by PCNA (Figure 2B) or showing high levels of newly incorporated histones (H4K12Ac, Figure 2C); two markers of chromatin undergoing replication. Accordingly, polytene chromosomes not undergoing replication and therefore not stained by PCNA, or with lower signals of H4K12Ac, were poorly bound by P180. This suggests that binding of P180 at the HP1a bound loci might occur in a replication-dependent manner and raises the possibility that the interaction between these two factors might be cell-cycle regulated.

### *A 27-residue domain is required for the direct interaction between recombinant P180 and HP1a proteins*

We next searched for a peptide motif in the P180 protein sequence which could mediate its interaction with the chromo shadow domain of HP1a. Surprisingly, P180 lacks the canonical consensus PXVXL motif found in many HP1-interacting proteins, including vertebrate CAF-1 large subunits (Murzina *et al.* 1999; Smothers and Henikoff 2000). P180 also does not contain the HP1-interacting peptide motif of the heterochromatin protein HP2 (Stephens *et al.* 2005), indicating that the direct interaction between P180 and HP1a in *Drosophila* was mediated through a domain not previously described. As we were able to detect a direct interaction between recombinant P180 and HP1a, we performed additional GST pull-down experiments on truncated versions of P180 expressed in bacteria and identified, in the C-terminal, a 27-amino acid motif likely involved in this interaction (Figure S1 and Figure 3A). Further experiments using a full-length P180 protein lacking only the identified 27 residues confirmed that this domain, the HIM domain, was required for P180 to interact with HP1a *in vitro* under stringent conditions (450 mM NaCl) (Figure 3, A and B). Of note, these stringent conditions are commonly used to detect specific interactions with HP1 proteins (Maison *et al.* 2002). Furthermore, many

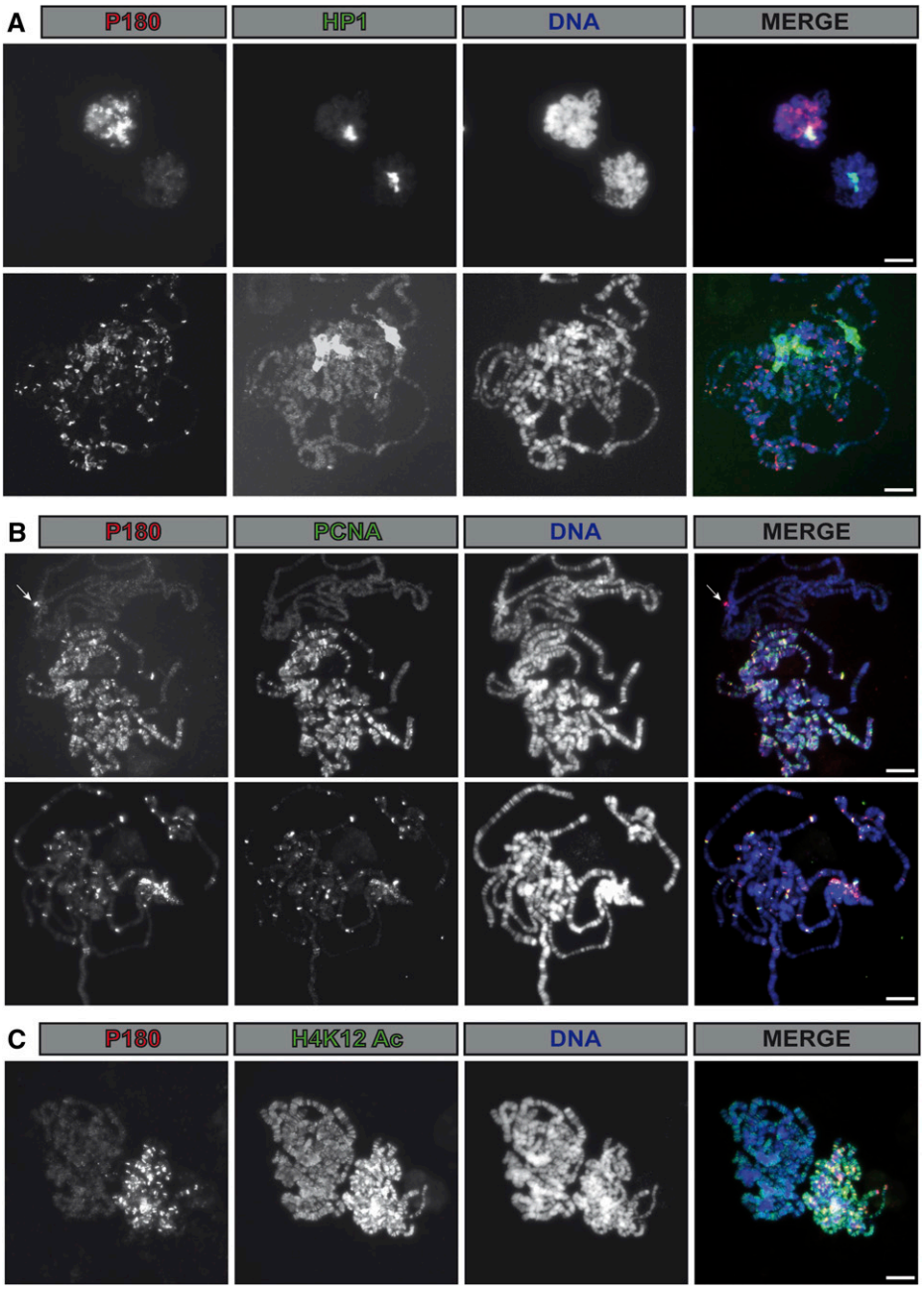


**Figure 2** Binding of P180 on polytene chromosomes occurs in a replication-dependent manner and only poorly correlates with HP1a localization. (A–C) Immunofluorescent staining allows detection of (A–C) P180-FLAG fusion protein, (A) HP1a, (B) PCNA, or (C) H4 acetylated on lysine 12 on salivary gland polytene chromosome in *p180$^3$ /p180$^3$; Act-Gal4,UAS-p180-FLAG/CyO*. Under the staining conditions used for (B), a nonspecific staining (arrow) was recurrently observed at the chromocenter, even in flies not expressing the P180-Flag protein (see *Materials and Methods* for details). The two genomes shown in each panel exemplify the diversity of staining observed and likely represent different replication timings. Scale bar on all panels represents 10 μm.

cells and tissues have been reported to have a high nuclear sodium and potassium concentrations, up to 250 mM NaCl and 280 mM KCl in frog oocyte nuclei (Naora *et al.* 1962; Hooper and Dick 1976; Moore and Morrill 1976), and such stringent salt conditions can therefore be achieved in the nucleus. Altogether, these observations indicate that the robust interaction we observed *in vitro* between the CAF-1 *Drosophila* large subunit and HP1a involves both the hinge and chromo shadow domain of HP1a and requires the HIM domain, a short domain of 27 residues localized in the C-terminal part of the CAF-1 large subunit.

### *The HIM is conserved in vertebrates and insects*

We next assessed the HIM conservation by aligning available sequences of CAF-1 large subunits in various species (Figure S2 and Table S1). We noticed that the HIM was well conserved in insects and vertebrates (Figure 3C). The MIR domain, which contains the PXVXL motif and was identified as the domain mediating the interaction between the murine large subunit of CAF-1 and HP1β protein (Murzina *et al.* 1999), is conserved in mammals and the chicken (Takami *et al.* 2007), but is lacking in *Xenopus* or zebrafish (Figure 3D). In *Drosophila* species, the MIR domain is either completely lacking (*Drosophila grimshawi*) or highly divergent (*D. willistoni* and *D. melanogaster*). Interestingly, when conservation was assessed along the whole length of the protein using a method computing the conservation of physico-chemical properties in the alignment (Livingstone and Barton 1993), the HIM domain clearly corresponded to a local maximum of

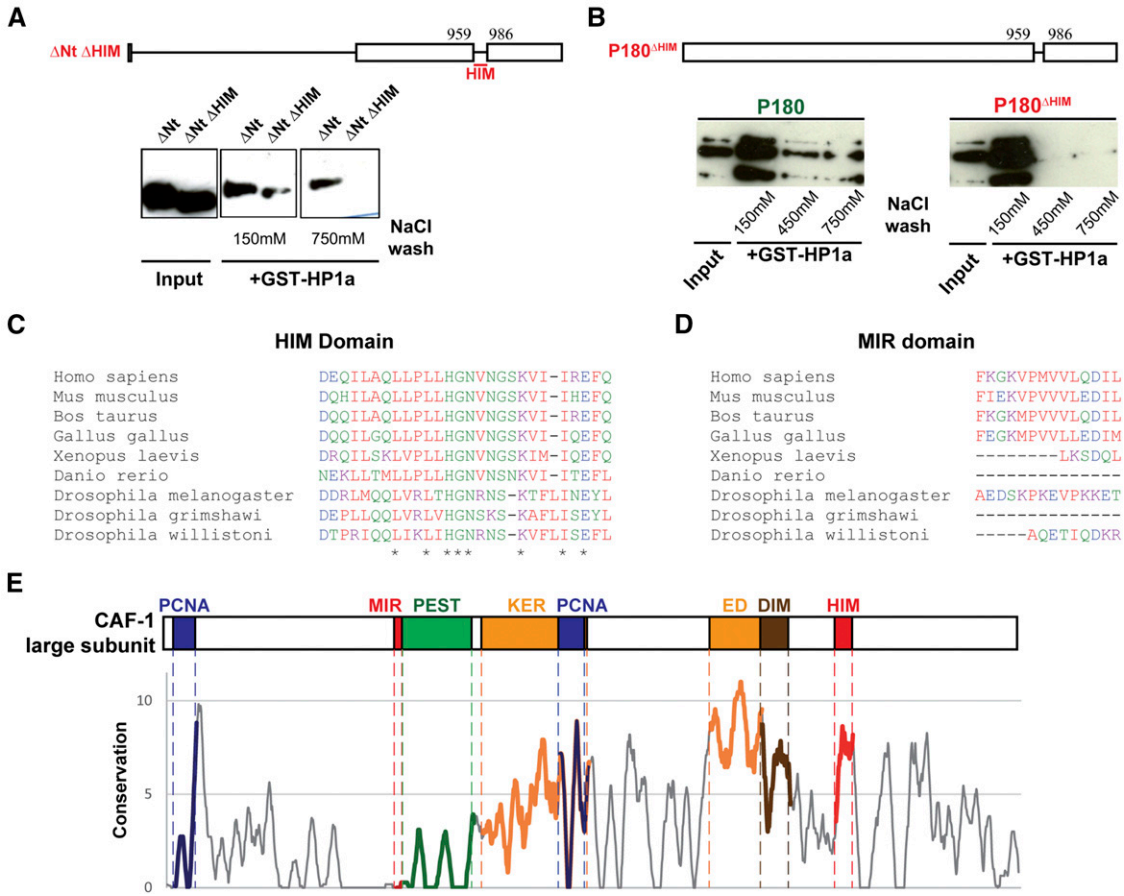


**Figure 3** A conserved 27-residue domain is required for CAF-1 large subunit interaction with HP1a. (A) The same GST pull-down strategy described in Figure 1D was used to identify the residues critical for P180 interaction with HP1a. Truncation of the HIM, comprising residues 959–986, abolished the interaction between the C-terminal part of P180 and GST-HP1a after washing at high salt concentration. (B) The specificity of the interaction between GST-HP1a and the full length P180 with or without the HIM was then challenged by increasing the concentration of NaCl (150, 450, and 750 mM) in the washes. The presence of wild-type and mutant P180 proteins containing a poly-histidine tag at their C-terminal was determined in the bound fraction by western blotting using an anti-polyhistidine antibody. (C and D) Alignment of the (C) HIM and (D) MIR domains in three mammalian, three vertebrate nonmammalian, and three *Drosophila* species. Alignment was initially calculated on the whole orthologous protein sequences using the MUSCLE algorithm (Edgar 2004a,b). Coordinates were determined based on reported coordinates in *Mus musculus* for the MIR domain (Murzina *et al.* 1999) and *D. melanogaster* for the HIM (this study). (E) Conservation was computed across the selected species mentioned in (C and D) by averaging a numerical index reflecting the conservation of physico-chemical properties (Livingstone and Barton 1993) on an 11-residue-wide sliding window centered on the plot *x* coordinate. Coordinates of the domains identified in CAF-1 large subunit are based on the human sequence for the PEST, KER, ED (Kaufman *et al.* 1995), and dimerization domains (Quivy *et al.* 2001); on the mouse sequence for the MIR domain (Murzina *et al.* 1999) and PCNA interacting motifs (Rolef Ben-Shahar *et al.* 2009); and on *D. melanogaster* sequence for the HIM (this study).

conservation with a score comparable to other domains previously identified as important for the function of the large subunit of CAF-1 in replication-coupled nucleosome assembly, such as the acidic KER and ED domains, which are thought to interact with the histones (Kaufman *et al.* 1995); the dimerization domain (Quivy *et al.* 2001); or the PCNA interacting motifs (Shibahara and Stillman 1999; Moggs *et al.* 2000) (Figure 3E). In contrast, in this same analysis, the MIR domain was not highlighted, confirming its poor conservation.

### *The HIM domain of P180 is not essential for viability*

We then intended to test the functional significance of the HIM *in vivo* and constructed, using the Gal4/UAS system (Brand and Perrimon 1993), a strain expressing a mutant version of P180, P180$^{\Delta HIM}$, deleted for the 27 residues identified previously (Figure 4A). Interestingly, ubiquitous expression of P180$^{\Delta HIM}$ using an Act5C-Gal4 driver rescued the viability of *p180*$^{3}$ mutant males at least as efficiently as the wild-type P180 (Figure 4B). We had previously reported that maternally provided P180 is undetectable by the end of embryonic development (Klapholz *et al.* 2009) and therefore the transgene is the sole source of P180 in the rescued *p180*$^{3}$ hemizygous males at least from larval development onwards. Importantly, the rescued individuals showed no obvious developmental defects and in particular none that could resemble the defects previously described upon downregulation of any of the CAF-1 subunits (Huang *et al.* 2010; Yu *et al.* 2013). These observations indicate that the HIM domain of P180 is largely dispensable for *Drosophila* larval development and supports a model in which the ability to interact with HP1a

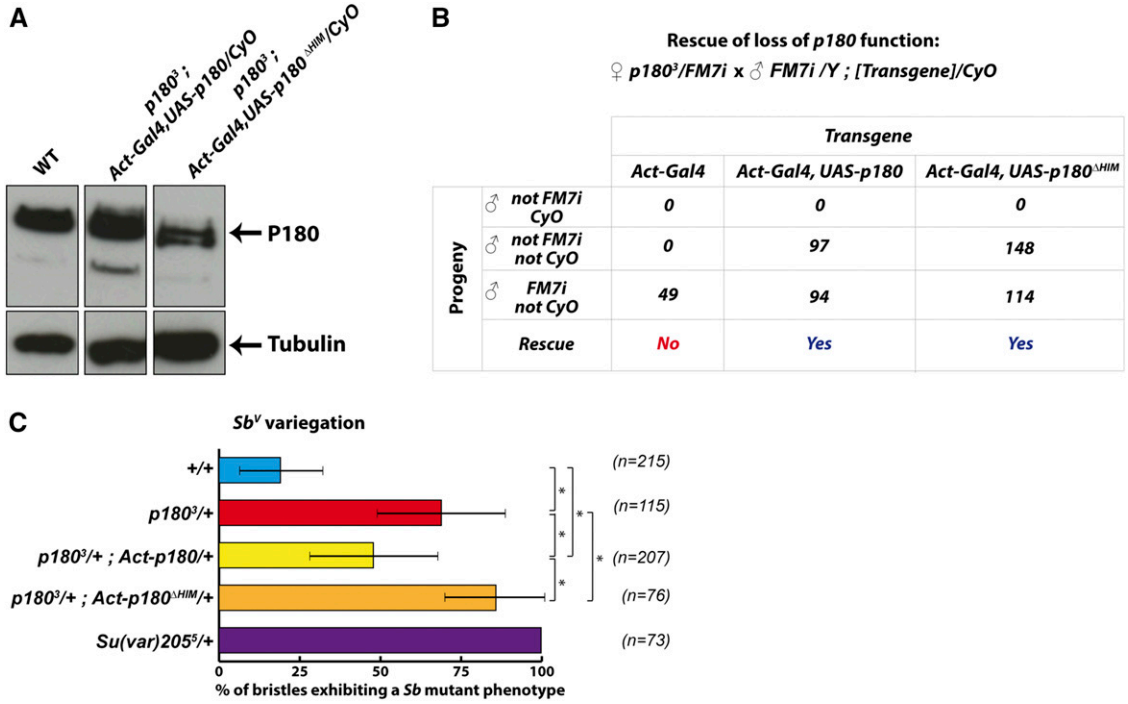


**Figure 4** The HIM domain is not essential for viability but contributes to *p180*-induced PEV. (A) Expression of P180 in larvae of the indicated genotype assessed by western blotting on larval extracts using an anti-tubulin antibody and an α-P180 antibody raised against a peptide covering residues 198–421 of P180. WT, wild type. (B) Rescue efficiency of the various transgenes was quantified using a test cross between $p180^3$/*FM7i* females and *CyO* males carrying the indicated combination of transgenes. Rescue was evaluated in the progeny of the test cross by the presence of rescued males (non-*FM7*, non-*CyO*) only expressing the transgenic P180. (C) Rescue of $Sb^V$ variegation induced by $p180^3$ heterozygosity by transgenic expression of P180. Flies heterozygous for the $Sb^V$ chromosomal aberration and expressing either the wild-type or the HIM-deleted P180 were scored for the number of macrochaetae showing a *Sb* phenotype in $p180^{3/+}$ background. *n* specifies the number of flies analyzed for each genotype and * indicates a significant difference ($P \leq 0.0001$) using Mann–Whitney–Wilcoxon test.

is not an essential feature of P180, at least after embryogenesis. The $P180^{\Delta HIM}$ construct can therefore be used to directly test *in vivo* the role of the interaction between the large subunit of CAF-1 and HP1a during these stages, without affecting CAF-1 essential functions.

### *The HIM domain of P180 is required for P180-induced modification of PEV*

In a previous study, mutant alleles of *p180* were shown to act as dominant suppressors of PEV, thereby highlighting the role of P180 in the maintenance of heterochromatin (Huang *et al.* 2010). We therefore wondered whether transgenic expression of P180 or its mutant version, $P180^{\Delta HIM}$, was able to counteract this effect. As a reporter of PEV, we used the $T(2;3)Sb^V$ translocation (hereafter referred to as $Sb^V$) which juxtaposes the dominant $Sb^1$ mutation and a portion of the centric heterochromatin of the second chromosome. This induces mosaic flies with short (stubble) or long (normal) bristles which can be rigorously quantified. Changing the genetic context from wild type to $p180^3/+$ in $Sb^V$ flies induced a significant increase in the frequency of short bristles (Figure 4C, compare the blue and the red histograms), indicating that this allele also behaves as a dominant suppressor of PEV. Interestingly, this suppression of PEV was rescued, albeit partially, by Gal4-induced expression of wild-type P180 (Figure 4C, compare red and yellow histograms), while Gal4-induced expression of the $P180^{\Delta HIM}$ mutant did not rescue and indeed further suppressed the variegation in a $p180^3/+$ background (compare the red and orange histograms). In contrast, expression of either the wild-type or the HIM-depleted versions of P180 in flies with two wild-type copies of *p180* induced only a mild suppression of $Sb^V$ variegation of similar magnitude (Figure S3). Altogether, these observations support a role for the HIM domain of P180 in $p180^3$-induced suppression of variegation.

### *The HIM domain of P180 contributes to heterochromatin-mediated pairing in oocytes*

In *Drosophila* oocytes, homologous chromosomes remain associated along their pericentromeric regions throughout meiotic prophase and, when homologs have failed to form crossovers during meiotic recombination, this persisting association promotes their correct segregation according to the homologous achiasmate segregation system (Dernburg *et al.* 1996; Karpen *et al.* 1996). This pairing process relies on the integrity of pericentric regions that are heterochromatic and, accordingly, mutations in components of heterochromatin have altered levels of achiasmate segregation (Verni *et al.* 2000; Peng and Karpen 2009; Subramanian and Bickel 2009). More specifically, a recent study was able to demonstrate that partial

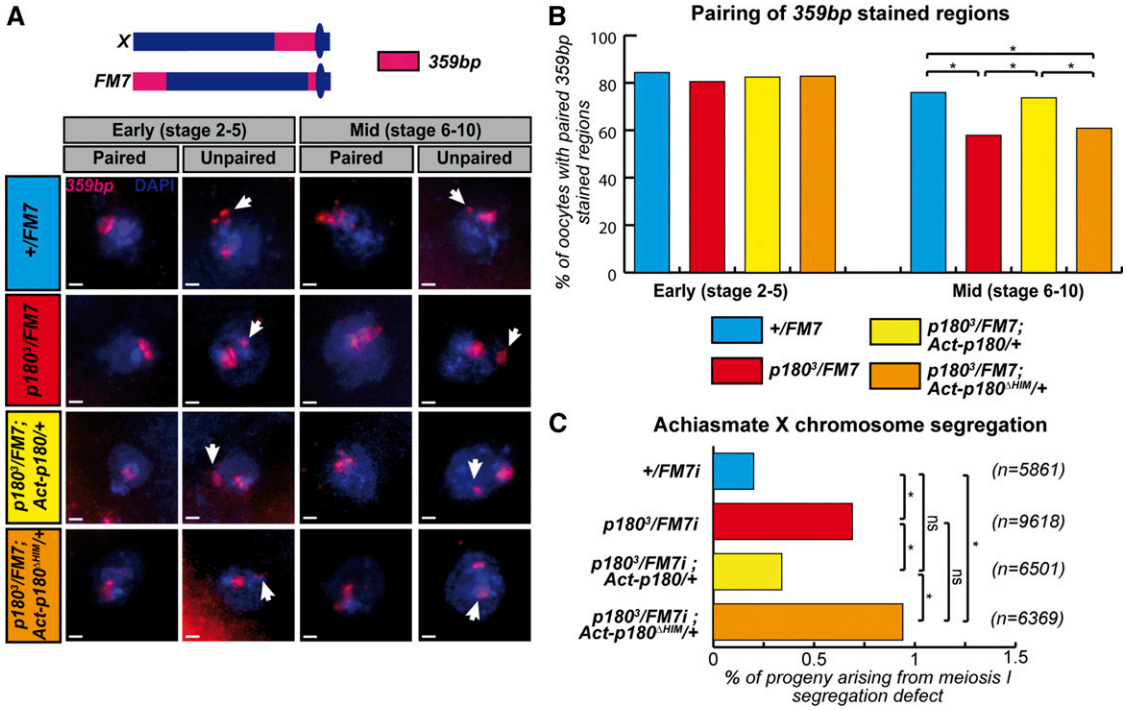


**Figure 5** The HIM domain participates in heterochromatin-mediated pairing in germ cells. (A) FISH staining on oocytes allows the detection of *X* pericentric regions (359 bp) and total DNA (DAPI) in females of the indicated genotype. Based on previously described detection of synaptonemal complex components (Resnick *et al.* 2009), two different regions of the ovariole were analyzed: the early region from vitelline stage 2–5 in which the synaptonemal complex, maintaining aligned homologs, is fully assembled; and the middle region containing egg chambers from stage 6–10, in which the synaptonemal complex is undergoing disassembly and therefore likely requires additional mechanisms to maintain pericentric interactions. Scale bar represents 1 μm. (B) Pairing of 359-bp regions of homologous *X* chromosomes was quantified in females of the indicated genotype. Numbers of oocytes scored for stages 2–5 (early): *+/FM7i*, 64; *p180³/FM7i*, 68; *p180³/FM7;Act-p180/+*, 80; and *p180³/FM7i;Actp180ΔHIM/+*, 126. Number of oocytes scored for stages 6–10 (late): *+/FM7i*, 75; *p180³/FM7i*, 63; *p180³/FM7;Act-p180/+*, 57; and *p180³/FM7i;Actp180ΔHIM/+*, 99. (C) Rate of exceptional oocytes arising from *X* chromosome-segregation defects during meiosis I, measured in females of the indicated genotype. In this experiment, due to heterozygosity of the females for the *FM7i* balancer chromosome, *X* chromosomes fail to recombine and therefore segregate according to the homologous achiasmate system. Differences are indicated to be statistically significant (*) or not significant (ns) using Fisher's exact test ($P \leq 0.05$).

depletion of HP1a, or its paralog Rhino in germ cells, induced defects in maintenance of pericentric associations of a pair of achiasmate *X* chromosomes, which resulted in elevated segregation defects (Giauque and Bickel 2016). We therefore aimed to test the functional impact of the loss of the HIM domain of P180 in this process and analyzed the pairing status of heterochromatic repeats of an achiasmate pair composed of a wild-type *X* and a *FM7* balancer chromosome in females expressing various doses of wild-type or HIM-deleted versions of P180.

We first compared pairing efficiencies between wild-type and *p180³* heterozygous females. FISH using a probe specific for the pericentric heterochromatin sequences of the wild-type *X* chromosome (359-bp repeats) indicated that oocytes produced by females carrying one wild-type and one *p180³* allele were significantly impaired in maintaining the pairing of *X* chromosome-heterochromatic 359-bp sequences during oogenesis when compared to their wild-type counterparts (Figure 5, A and B, compare red and blue histograms in midstage egg chambers). In the *FM7* chromosome, the heterochromatic 359-bp repeats are split in a large subtelomeric region and a smaller pericentromeric region (Figure 5A). The defects in pairing could thus involve only one of those sequences or both. By assessing the size of the unpaired sequence, which allows distinguishing defects in pairing involving either the subtelomeric (large FISH signal) or the pericentric sequences (small FISH signal), we observed that the defects in pairing observed upon reduction in the dose of P180 affect both sequences in proportions correlated to their respective sizes (see Figure S4 for quantification).

As some of these pairing events have been proposed to contribute to the correct segregation of homologous achiasmate chromosomes, we analyzed this process in *p180³* heterozygous females. In agreement with their pairing defects, these females produced oocytes with a modest but significant increase in the segregation defects of this pair of nonrecombining chromosomes (Figure 5C, compare red and blue histograms). These segregation defects are specific to nonrecombining chromosomes: similar defects were observed with achiasmate chromosomes II and IV but were not observed with chiasmate chromosomes II (Figure S5).

Importantly, ubiquitous expression of wild-type P180 but not P180$^{\Delta HIM}$, using the Gal4/UAS system, was able to rescue both the pairing maintenance defects and segregation defects of oocytes produced by *p180³* heterozygous females

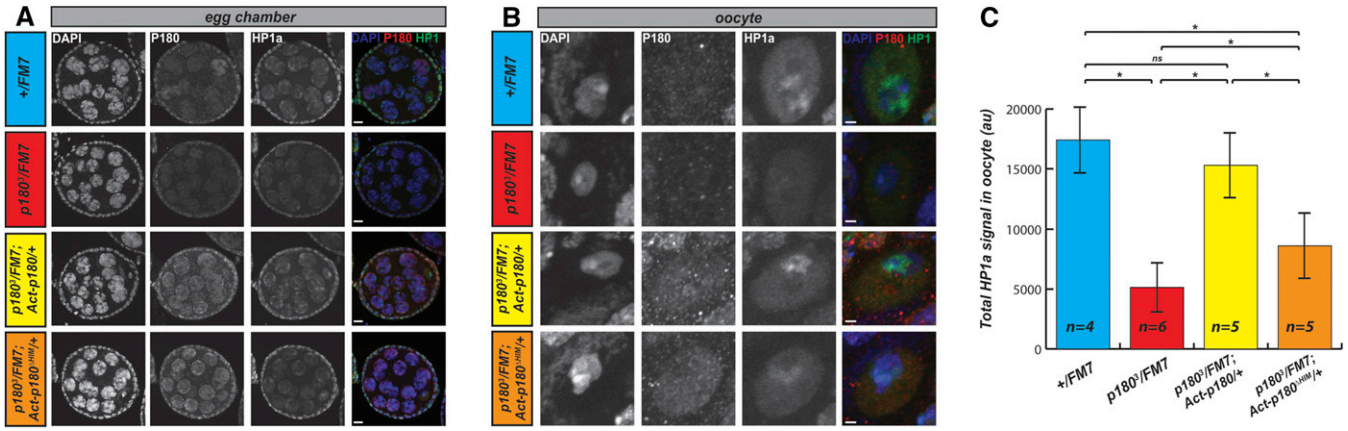


**Figure 6** Altered loading of HP1a in germ-cells of females expressing either low dose of P180 or P180$^{\Delta HIM}$. (A and B) Immunodetection of P180, HP1a, and DNA (DAPI) on (A) egg chambers and with higher magnification on the (B) oocytes obtained from females of the indicated genotype. Images are maximum intensity projections of all the stacks containing the oocyte. Scale bar represents 5 μm (A) and 1 μm (B). (C) Quantification of total HP1a signal in *n* oocytes nuclei of the indicated genotype. Settings for acquisition and processing were similar between all analyzed genotypes and fluorescence is therefore quantified using a scale of arbitrary unit (au) that is identical across all genotypes. Differences are indicated to be statistically significant (*) using Fisher's exact test ($P < 0.05$) and Holm *P*-value correction for multiple comparisons. ns, nonsignificant.

(Figure 5, A–C, compare yellow and orange histograms). This argues that the HIM domain, which is central to the direct interaction between HP1a and the large subunit of CAF-1, is also important for the maintenance of pericentric pairing during meiotic prophase; a process relying on proper HP1a loading and function (Giauque and Bickel 2016).

### *The HIM domain of P180 contributes to the loading of HP1 at the oocyte chromocenter*

One possible way P180 could contribute to heterochromatin maintenance would be by regulating the loading of HP1a to chromosomes. As we identified the HIM as an important domain for the interaction between P180 and HP1a, we tested this possibility by analyzing the *in vivo* distribution of P180 and HP1a in egg chambers isolated from females expressing a wild-type dose of *p180*. We observed a robust P180 signal in nurse cell nuclei (Figure 6A, second column, blue genotype) but not in oocytes (Figure 6B, second column, blue genotype). As nurse cells are actively replicating and oocytes are postreplicative and undergoing the meiotic prophase program, this observation is consistent with staining on salivary gland polytene chromosomes indicating that P180 localizes to the chromatin during *S* phase. In contrast, P180 expression was detected at similar levels in oocytes from females expressing the wild-type or the HIM-depleted transgene (Figure 6B, second column, yellow and orange genotypes); indicating that at least some degree of P180 accumulation during *S* phase is due to transcriptional regulation, which is not recapitulated by the ubiquitous *Act5C-Gal4* driver. As the samples were prepared, stained, and imaged under identical conditions, we could also compare the overall levels of HP1a accumulated in the oocyte nucleus in the different genotypes. We could robustly observe that levels of HP1a were reduced in oocytes of *p180$^3$* heterozygous females when compared to females with two wild-type alleles of *p180* (Figure 6, B and C, third column, compare blue and red genotypes). This decrease was rescued by transgenic expression of the wild-type P180 (Figure 6, B and C, third column, yellow genotype) whereas expression of the HIM-depleted P180 protein resulted in only partial rescue (Figure 6, B and C, third column, orange genotype). Similar trends were observed on the relative level of HP1a in nurse cells, depending on the genotype (Figure S6). Thus the HIM-depleted P180 appears defective in loading HP1 in the DAPI-dense regions of the oocyte nucleus. Altogether, these experiments indicate that P180 is required for the loading or maintenance of HP1a in germ cells and that the HIM domain contributes to this effect.

## Discussion

The CAF-1 complex was originally isolated and characterized as a histone chaperone that assembles histone H3-H4 dimers onto newly synthesized DNA (Smith and Stillman 1989). Further studies showed that the interaction between the large subunit of CAF-1 and the HP1 proteins was required for the proper duplication and maintenance of heterochromatin regions in mitotically dividing cells (Dohke *et al.* 2008; Quivy *et al.* 2008). These two functions of CAF-1's large subunit, which involve different levels of chromatin organization, are also conserved in *Drosophila*. First, the large subunit of CAF-1 is involved in H3-H4 dimer deposition during DNA replication (Tyler *et al.* 2001): this function was shown to be essential for larval development and viability (Song *et al.* 2007; Klapholz *et al.* 2009). Second, CAF-1's large subunit interacts with HP1a (Huang *et al.* 2010). We have shown that this interaction is direct and have identified the HIM domain in CAF-1's large subunit required for this interaction. Interestingly, flies expressing only a mutant form of CAF-1's large subunit deleted for the HIM are viable. This indicates that the ability of CAF-1's large subunit to interact

with HP1a is not strictly required for viability in flies and that additional mechanisms, such as the one involving the interaction between HP1a and the H3.3 chaperone XNP (Bassett *et al.* 2008), might contribute to proper heterochromatin formation or maintenance and ensure successful development. As this function is essential in mammalian cells (Houlard *et al.* 2006; Quivy *et al.* 2008), *D. melanogaster* represents a unique model to understand its role during development.

Interestingly, although we could obtain individuals, males and females, expressing only the HIM-deleted forms of CAF-1 large subunit, we realized that the HIM deletion resulted in female sterility (Marie Clémot, unpublished data). This could indicate either that the HIM domain is strictly required for some aspect of fly reproduction, either via its role in heterochromatin maintenance or via some other functions of CAF-1's large subunit, or that the HIM is required during embryogenesis for the initial formation of heterochromatin structure, a possibility we could not directly test because of the important maternal contribution and the inability of $p180^{3}$ germline clones to develop into mature oocytes (Klapholz *et al.* 2009).

Our work also provides new insights into the mechanisms of heterochromatin maintenance: previous studies in *S. pombe* and mammalian cells had led to propose a model in which CAF-1 is recruited to the replication forks through its interaction with PCNA and transfers HP1 to the replicated chromatin at these sites to locally maintain the chromatin structure (Quivy *et al.* 2004; Dohke *et al.* 2008; Quivy *et al.* 2008). Consistently, we only detected colocalization of CAF-1's large subunit with HP1a on salivary gland polytene chromosomes upon replication of the HP1a-enriched regions. However, although it is now clearly established that CAF-1 participates in the maintenance of heterochromatin in eukaryotes, very little is known on the contribution of the different domains to this function. In mammals, the MIR domain, which contains a PXVXL motif, is essential for heterochromatin maintenance (Quivy *et al.* 2004; Dohke *et al.* 2008; Quivy *et al.* 2008). The MIR domain is not conserved in the yeasts *S. cerevisiae* and *S. pombe*, although a role in heterochromatin maintenance has been reported for CAF-1's large subunits (Monson *et al.* 1997; Dohke *et al.* 2008). Similarly, in chicken cultured cells, the MIR domain does not seem to be involved in heterochromatin maintenance since a point mutant in the PXVXL motif could support normal cell proliferation with no detectable defects in heterochromatin structure (Takami *et al.* 2007). In this study, we coupled biochemistry, genetics, and cytology to identify the first domain of *Drosophila* CAF-1's large subunit involved in heterochromatin maintenance. Although our data suggest a similar role for the MIR and the HIM domains in transferring the HP1 protein upon replication of heterochromatin regions, further studies are required to elucidate the precise molecular mechanisms by which the HIM domain contributes to the maintenance of heterochromatin and to determine whether it is functionally conserved in other species.

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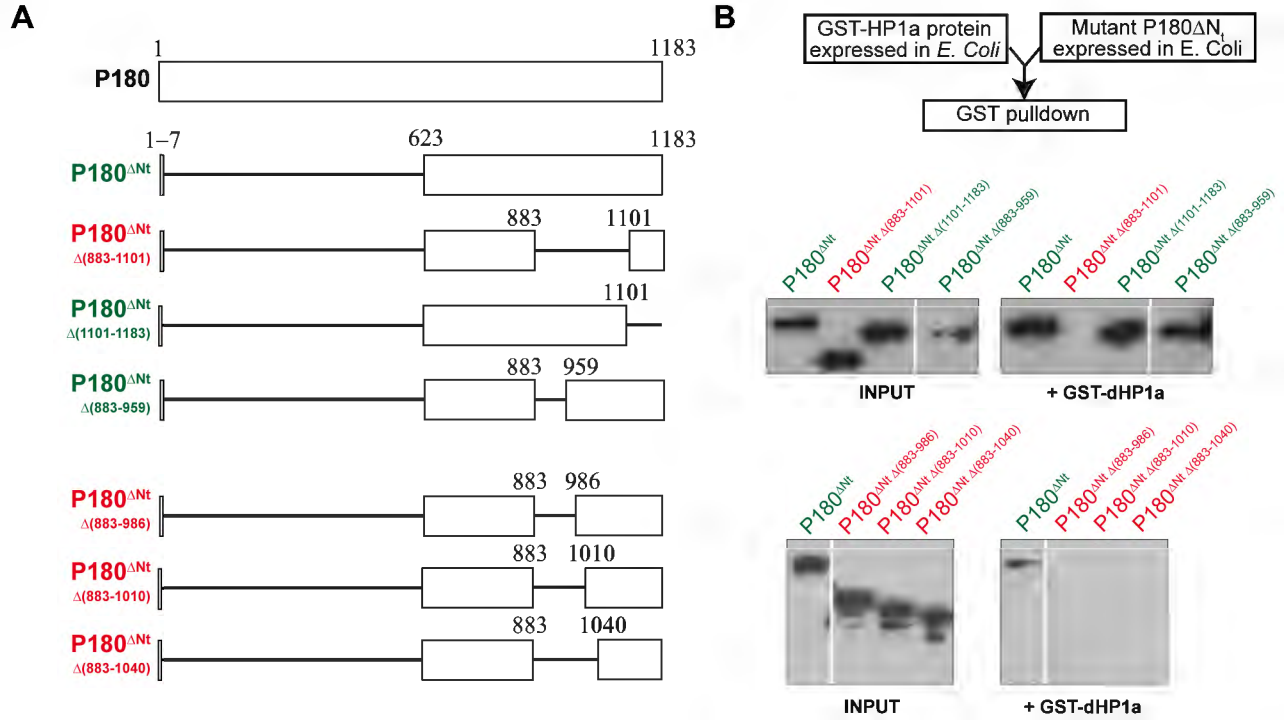
A
1
1183
P180
1–7
623
1183
P180ΔNt
883
1101
P180ΔNt
Δ(883-1101)
1101
P180ΔNt
Δ(1101-1183)
883
959
P180ΔNt
Δ(883-959)
883
986
P180ΔNt
Δ(883-986)
883
1010
P180ΔNt
Δ(883-1010)
883
1040
P180ΔNt
Δ(883-1040)
B
GST-HP1a protein expressed in E. Coli
Mutant P180ΔN1 expressed in E. Coli
GST pulldown
P180ΔNt
P180ΔNt Δ(883-1101)
P180ΔNt Δ(1101-1183)
P180ΔNt Δ(883-959)
INPUT
+ GST-dHP1a
P180ΔNt
P180ΔNt Δ(883-986)
P180ΔNt Δ(883-1010)
P180ΔNt Δ(883-1040)
INPUT
+ GST-dHP1a

```
Drosophila melanogaster          DDRLMQQLVRLTHGNRNSKTFLINEYL
Drosophila sechellia             DDRLMQQLVRLTHGNRNSKMFLINEYL
Drosophila yakuba                DDRHMQQLVRLTHGNRNSKMFLINEYL
Drosophila erecta                DDRLMQQLVRLTHGNRNSKMFLVNEYL
Drosophila ananassae             DERLLQQLVRLTHGNRNSKVFLINEYL
Drosophila pseudoobscura         DERLLQQLVRLTHGNRNAKAFLISEYL
Drosophila willistoni            DTPRIQQLIKLIHGNRNSKVFLISEFL
Drosophila mojavensis            DEQLLQQLVRLIHGNSNAKMFLIAEYL
Drosophila virilis               DEQLLQQLVRLIHGNSNAKAFLISEYL
Drosophila grimshawi             DEPLLQQLVRLVHGNSKSKAFLISEYL
Aedes aegypti                    VDEGVKELITLIHGSALNRKFLIKEFL
Anopheles gambiae                TDEAVCDLARLVHGNVNNRKFLVREFH
Tribolium castaneum              REDLIPAFLKLIQGNVNKRKMIVDEFI
Danio rerio                      NEKLLTMLLPLLHGNVNSNKVIITEFL
Xenopus laevis                   DRQILSKLVPLLHGNVNGSKIMIQEFQ
Gallus gallus                    DQQILGQLLPLLHGNVNGSKVIIQEFQ
Mus musculus                     DQHILAQLLPLLHGNVNGSKVIIHEFQ
Bos taurus                       DQQILAQLLPLLHGNVNGSKVIIREFQ
Homo sapiens                     DEQILAQLLPLLHGNVNGSKVIIREFQ
Caenorhabditis elegans           --------------------------
Caenorhabditis briggsae          --------------------------
Caenorhabditis remanei           --------------------------
Arabidopsis thaliana             PSNSKAKIIP------DSDLLTVVSTI
Saccharomyces cerevisiae         NSDLQAQTASQSQSPEKKQKAMITDPM
Schizosaccharomyces pombe        PSEDIPKFIEYVRNSHDNKVFLIENLR
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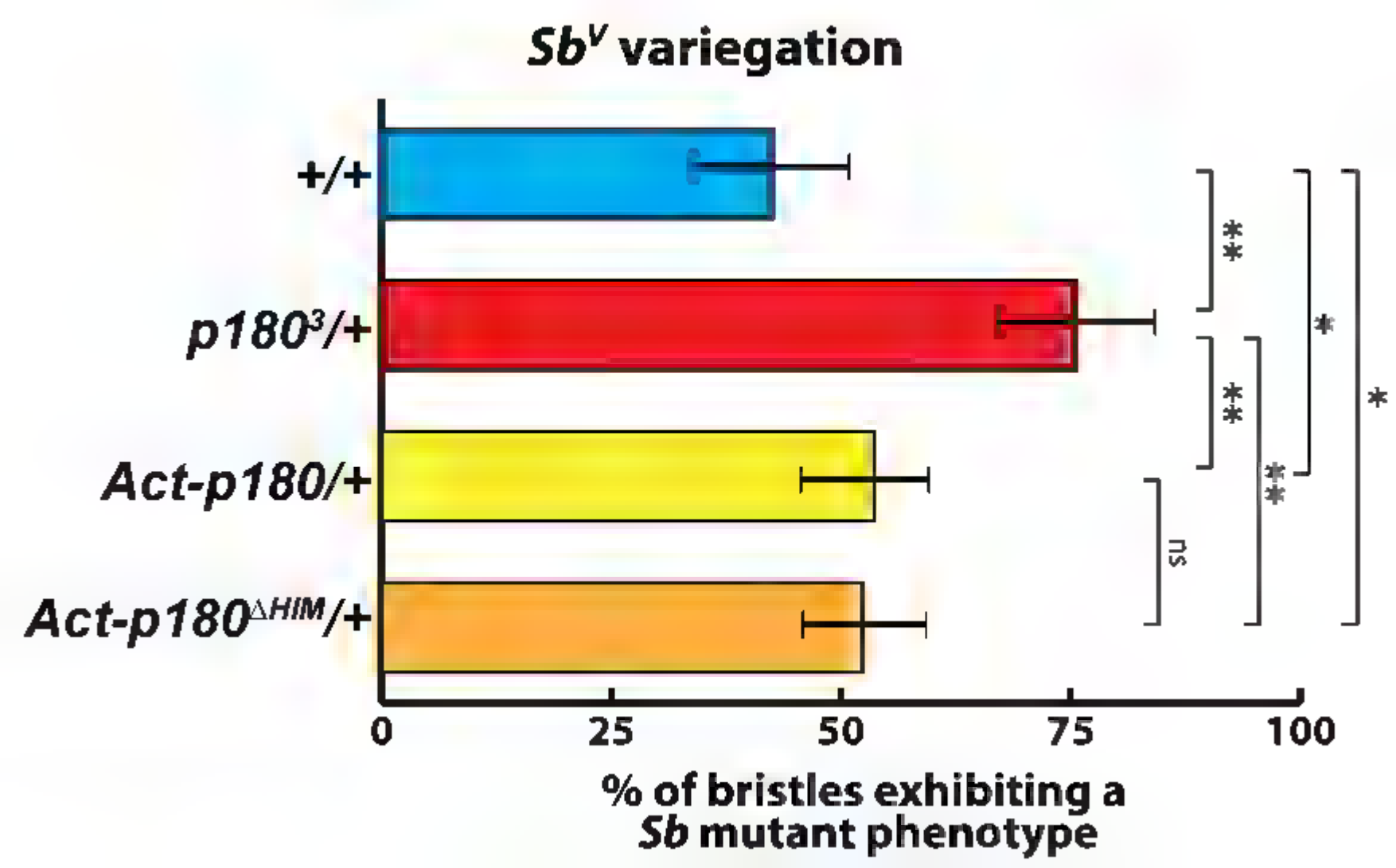
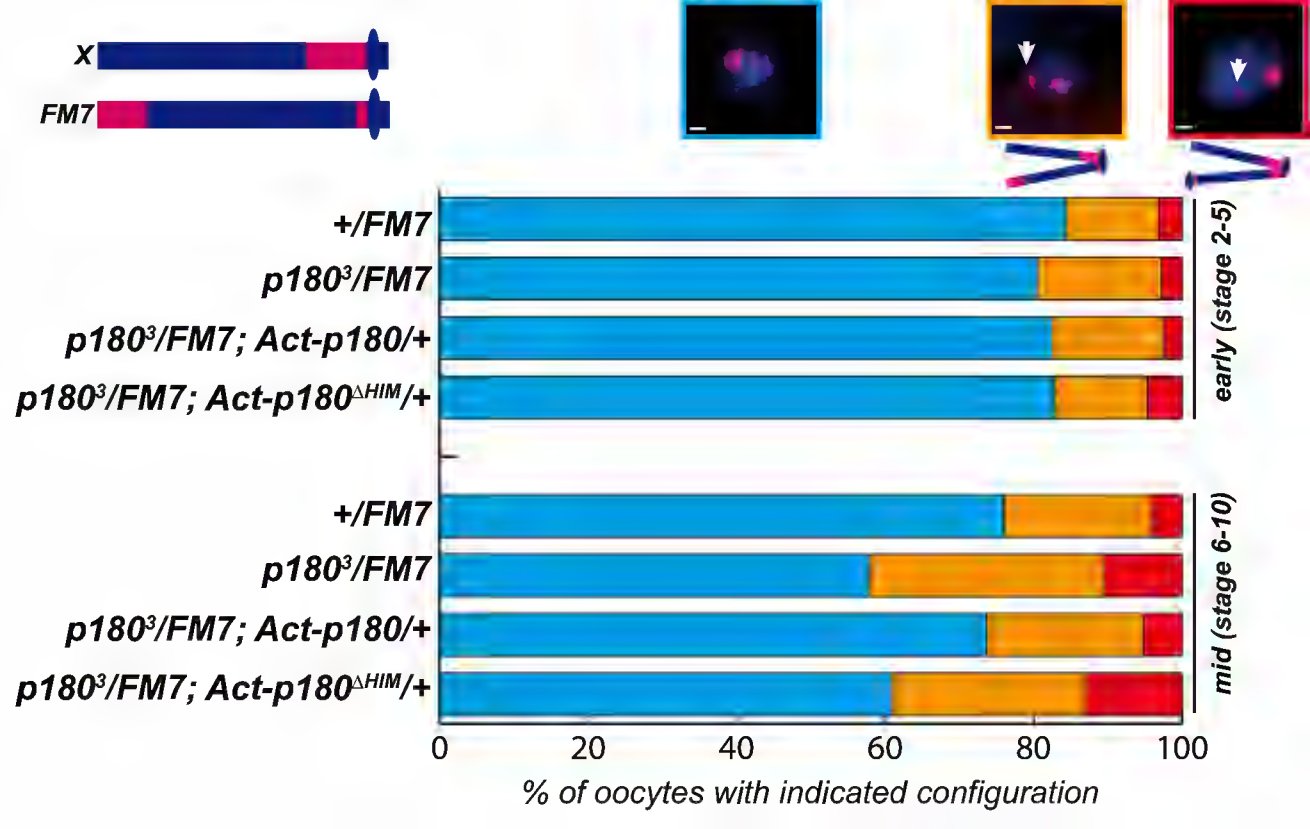
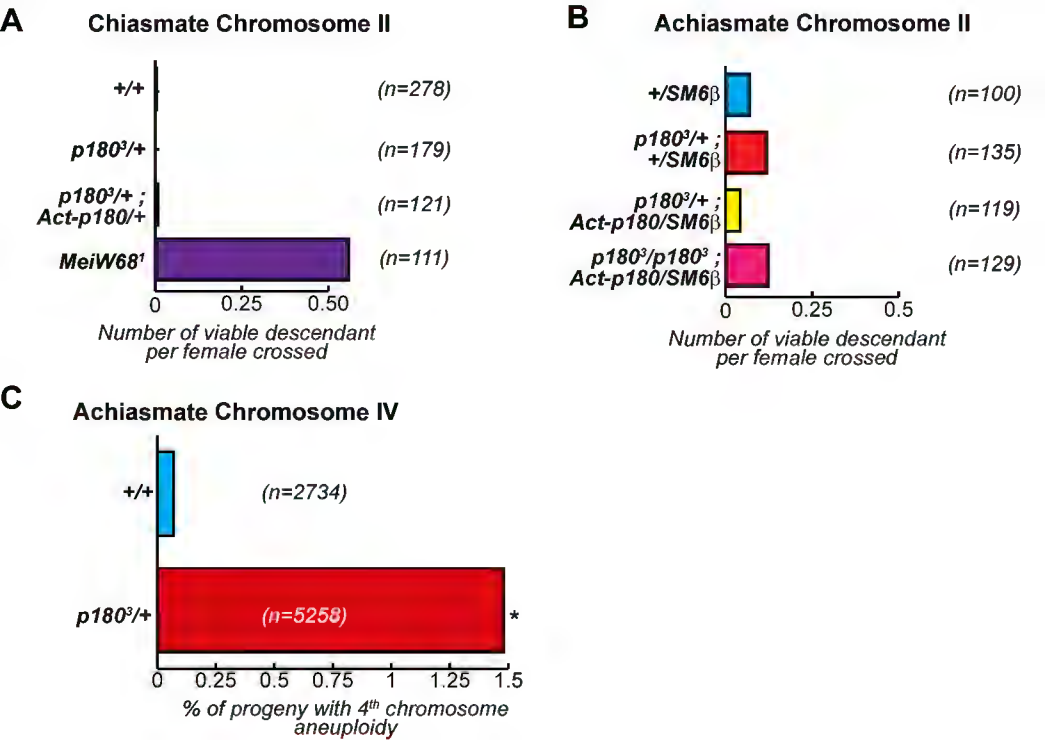

Sb^V variegation
+/+
p180^3/+
Act-p180/+
Act-p180^ΔHIM/+
**
*
**
***
*
ns
0
25
50
75
100
% of bristles exhibiting a
Sb mutant phenotype


X
FM7
+/FM7
p180³/FM7
p180³/FM7; Act-p180/+
p180³/FM7; Act-p180^ΔHIM/+
+/FM7
p180³/FM7
p180³/FM7; Act-p180/+
p180³/FM7; Act-p180^ΔHIM/+
early (stage 2-5)
mid (stage 6-10)
0
20
40
60
80
100
% of oocytes with indicated configuration


A
Chiasmate Chromosome II
+/+
p180³/+
p180³/+ ;
Act-p180/+
MeiW68¹
(n=278)
(n=179)
(n=121)
(n=111)
0
0.25
0.50
Number of viable descendant
per female crossed
B
Achiasmate Chromosome II
+/SM6β
p180³/+ ;
+/SM6β
p180³/+ ;
Act-p180/SM6β
p180³/p180³ ;
Act-p180/SM6β
(n=100)
(n=135)
(n=119)
(n=129)
0
0.25
0.5
Number of viable descendant
per female crossed
C
Achiasmate Chromosome IV
+/+
p180³/+
(n=2734)
(n=5258)
*
0
0.25
0.5
0.75
1
1.25
1.5
% of progeny with 4th chromosome
aneuploidy

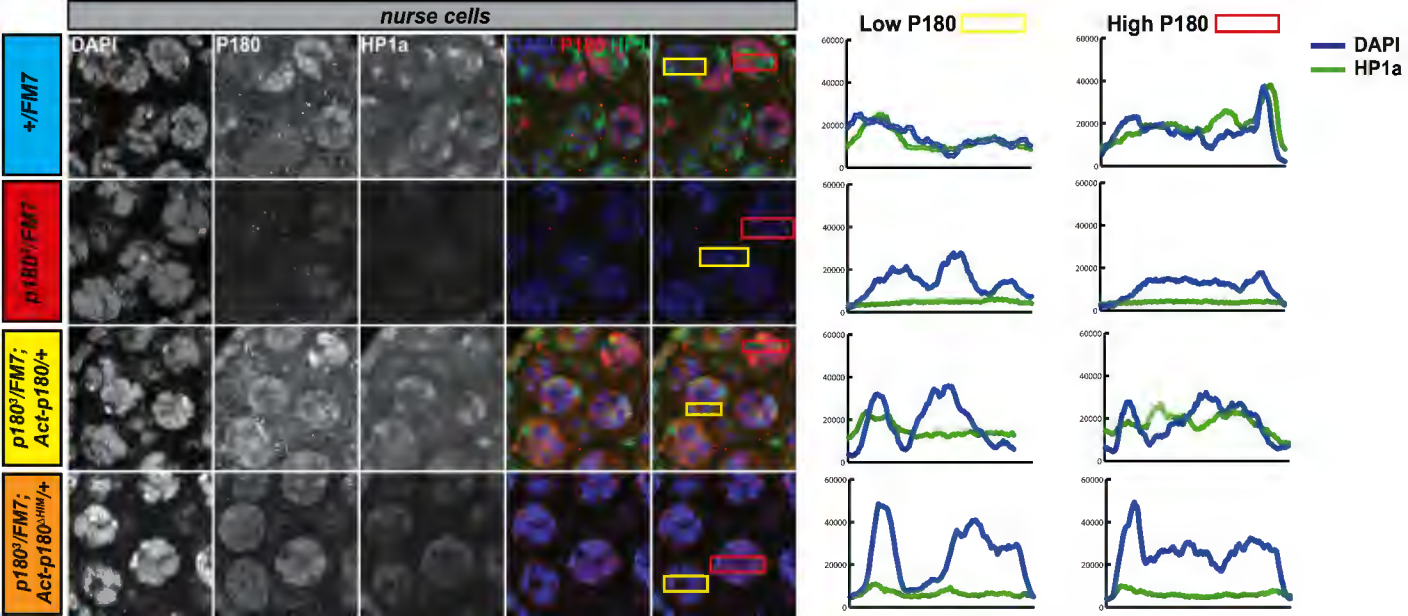

nurse cells
DAPI
P180
HP1a
P180 HP1a
+/FM7
p180³/FM7
p180³/FM7; Act-p180/+
p180³/FM7; Act-p180^HMW/+
Low P180
High P180
DAPI
HP1a
60000
40000
20000
0

Table S1:

| **Species** | **GenBank Accession number** |
|---|---|
| *Aedes aegypti* | XP_001662838 |
| *Anopheles gambiae* | EAA01417 |
| *Arabidopsis thaliana* | BAA77811 |
| *Bos taurus* | AAI47897 |
| *Caenorhabditis briggsae* | CAP31419 |
| *Caenorhabditis elegans* | CAB63306 |
| *Caenorhabditis remanei* | XP_003112214 |
| *Culex quinquefasciatus* | EDS42701 |
| *Danio rerio* | AAI25917 |
| *Drosophila ananassae* | EDV35238 |
| *Drosophila erecta* | EDV46344 |
| *Drosophila grimshawi* | EDV92135 |
| *Drosophila melanogaster* | AAF46399 |
| *Drosophila mojavensis* | EDW06763 |
| *Drosophila sechellia* | EDW51345 |
| *Drosophila virilis* | EDW65561 |
| *Drosophila willistoni* | EDW86482 |
| *Drosophila yakuba* | EDX02228 |
| *Drosophila_pseudoobscura* | EAL32030 |
| *Gallus gallus* | CAG31858 |
| *Homo sapiens* | AAH67093 |
| *Mus musculus* | AAH53740 |
| *Saccharomyces cerevisiae* | DAA11444 |
| *Schizosaccharomyces pombe* | CAB44771 |
| *Tribolium Castaneum* | EFA13257 |
| *Xenopus laevis* | AAK31811 |
| | |

Supporting Information:

Figure S1: **Identification of the HIM domain**. **A:** Structure of P180 deleted constructs used to identify the HIM. **B:** The GST pull-down strategy described in Figure 1D was used to identify the residues critical for this interaction. Truncation of the residues 959 to 986 abolished the interaction between the carboxy-terminal part of P180 and GST-HP1a after washing with 450mM NaCl. These blots were performed similarly as in Figure 2A.

Figure S2: **Conservation of the HIM domain.** Available sequences of CAF-1 large subunits orthologs were collected (see table S1 for accession numbers) and alignment was calculated on whole protein sequences using the MUSCLE algorithm (EDGAR 2004b; EDGAR 2004a). Only the sequences aligned with *D. melanogaster* HIM domain are shown here. The sequence of this domain seems well conserved across eukaryotes and especially across metazoans with some exceptions: the nematodes *C. elegans, C. briggsae and C. remanei* and the fly *D. simulans*. In these four species, the predicted protein sequence seems to have lost all of the carboxy-terminus when compared to orthologous sequences. As the carboxy-terminal region of CAF-1 large subunit has been shown in human to be responsible for the interaction with the medium subunit of the complex (KAUFMAN *et al.* 1995), loss of these sequences would likely strongly impair CAF-1 function in replication-coupled histone deposition. As a consequence, we think that the absence of the C-terminal sequences in these species is either due to a defective genome sequence or genome annotation or to evolution of a different interaction platform between the medium and the large subunits of CAF-1. However, as these C-terminal sequences seem conserved in both *D. sechellia* and *D. erecta,* which are also very closely

related to *D. melanogaster*, we rather support the hypothesis of defective genome annotation for *D. simulans*.

Figure S3: **Both the wild-type and the HIM-depleted P180 are mild suppressors of PEV.** Flies heterozygous for the $Sb^{V}$ chromosomal aberration and expressing either the wild-type or the HIM-deleted P180 were scored for the number of macrochaetes showing a *Sb* phenotype in a $p180^{+/+}$ background. Because of the sensitivity of the assay, $Sb^{V}$ variegation was recorded both in flies expressing the transgenes and, as a reference, in flies expressing one or two doses of *p180*. Number of flies analyzed (n), *+/+*, 117; $p180^{3}/+$, 133; *Act-Gal4,UAS-p180/+*, 118 and *Act-Gal4,UAS-*$p180^{\Delta HIM}$, 72. Differences are indicated to be statistically significant (*, $p < 0.05$; **, $p<0.0001$) or not (ns, $p > 0.05$) using the Mann-Whitney-Wilcoxon test.

Figure S4: **Pairing defects observed between the *359bp* sequences of a wild-type X and a *FM7* balancer involve both the subtelomeric or the pericentric *359bp* repeats of the FM7 chromosome.** In the highly rearranged *FM7* chromosome, the pericentric *359bp* repeats are split in two blocks: a small one remains in a pericentric positon and a larger one is located in a subtelomeric position. As a consequence, defective association of all *359bp* sequences as observed by FISH in Figure 6A does not necessarily reflect defective maintenance of pericentric association. This can however be assessed by analyzing the distribution of the FISH signal as described in (SUBRAMANIAN AND BICKEL 2009): the presence of two FISH signals of the same size likely reflect association between the X and FM7 pericentric regions and exclusion of the sub-telomeric 359bp block (orange) whereas the presence of two FISH signals of very different size and intensity likely reflect association between the X chromosome pericentric region with the subtelomeric 359bp repeat block of the FM7 with exclusion of the FM7 pericentric region (red). Only the latter configuration with defective maintenance of pairing between pericentric regions is thought to have functional consequences in the homologous

achiasmate segregation system (DERNBURG *et al.* 1996; KARPEN *et al.* 1996). The dataset used for this quantification is the same as in Figure 5B.

Figure S5: **Dose-dependent requirement of P180 for the proper segregation of achiasmate chromosomes**. **A and B:** Segregation efficiency of 2nd chromosomes was estimated as described in (MCKIM *et al.* 2009), using a cross between males carrying the compound *C(2)EN* chromosomes and females of the indicated genotype. In such cross, the viable descendants can only arise from maternal 2nd chromosome segregation defect. The total number of viable descendants was normalized by the number of females crossed. In panel B, most 2nd chromosomes are achiasmate due to heterozygosity for the *SM6β* balancer chromosome. **C**: Rate of exceptional progeny arising from 4th chromosome segregation defect in females of the indicated genotype also homozygous for the $sv^{spa\text{-}pol}$ 4th chromosome recessive marker. Meiotic segregation defects were assayed on the 4th chromosome using a classical test cross between females, carrying or not mutant alleles of *p180* and homozygous for the $sv^{spa\text{-}pol}$ recessive mutation, and males, carrying the compound 4th chromosome *C(4)RM*, which consists of two covalently linked 4th chromosomes carrying the $ey^{R}$ and $ci^{1}$ recessive mutations. Regular progeny $sv^{spa\text{-}pol}$/*C(4)RM* with three 4th chromosomes have normal eyes and wing venation whereas exceptional progeny with two 4th chromosomes, $sv^{spa\text{-}pol}/sv^{spa\text{-}pol}$ have rough eyes and normal wing venation and *C(4)RM* exhibit wing venation defects. Individuals with no 4th chromosome are not viable and flies with four 4th chromosomes are both rare and indistinguishable from regular progeny. Flies with one 4th chromosome have a *Minute* phenotype and were not included in our calculations. The frequency of segregation defects was therefore calculated as (exceptional progeny)/[(normal progeny + exceptional progeny)]. In every crosses, due to lethality of $p180^{3}$ hemizygous males, normal progeny number was adjusted by estimating that the number of $p180^{3}$/Y males was equal to their +/Y siblings. In panels A and B, n indicates the number of females crossed for the indicated genotype and in panel C, n indicates the number of progeny adjusted for unviable genotypes as described in Materials and Methods. Differences are indicated to be statistically significant (*, $p \leq 0.05$) using Fisher's exact test.

Figure S6: **Altered level of HP1a in nurse cells of females expressing either low dose of P180 or P180$^{\Delta HIM}$.** Distribution of fluorescent DAPI, HP1a and P180 signals were analyzed in nurse cells from similarly staged egg chambers, by averaging for each position on the horizontal axis of the depicted boxes the values of all pixels of the region of interest located at the vertical of this position. Analyzed images are maximum intensity projections of an identical number of slices. As at this stage, nurse cell nuclei have different P180 levels, likely dependent on their cell-cycle state, analysis was performed on two nuclei of the same egg-chamber with either relatively low (yellow box) or relatively high (red box) levels of P180.

Table S1: **Accession numbers of P180 orthologs.**