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Functional mammalian homologues of the *Drosophila* PEV-modifier *Su(var)3-9* encode centromere-associated proteins which complex with the heterochromatin component M31

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The chromo and SET domains are conserved sequence motifs present in chromosomal proteins that function in epigenetic control of gene expression, presumably by modulating higher order chromatin. Based on sequence information from the SET domain, we have isolated human (*SUV39H1*) and mouse (*Suv39h1*) homologues of the dominant *Drosophila* modifier of position-effect-variegation (PEV) *Su(var)3-9*. Mammalian homologues contain, in addition to the SET domain, the characteristic chromo domain, a combination that is also preserved in the *Schizosaccharyomyces pombe* silencing factor *clr4*. Chromatin-dependent gene regulation is demonstrated by the potential of human *SUV39H1* to increase repression of the pericentromeric *white* marker gene in transgenic flies. Immunodetection of endogenous Suv39h1/SUV39H1 proteins in a variety of mammalian cell lines reveals enriched distribution at heterochromatic foci during interphase and centromere-specific localization during metaphase. In addition, Suv39h1/SUV39H1 proteins associate with M31, currently the only other characterized mammalian SU(VAR) homologue. These data indicate the existence of a mammalian SU(VAR) complex and define Suv39h1/SUV39H1 as novel components of mammalian higher order chromatin.

Keywords: centromeres/chromo and SET domains/ heterochromatin/mammalian SU(VAR) complex/PEV in *Drosophila*

Introduction

Gene expression in eukaryotes is modulated by positional information and higher order chromatin. Transcriptionally permissive domains (euchromatin) are separated from more restricted, developmentally regulated regions (facultative heterochromatin) and from the structurally heterochromatic telomeres and centromeres (Heitz, 1929; Zuckerkandl, 1974; Karpen and Allshire, 1997). In addition to its role in epigenetic control of gene expression, e.g. in X inactivation, genomic imprinting (for reviews see Efstradiadis, 1994; Brockdorff and Duthie, 1998) or during developmental regulation of the homeotic gene cluster (HOM-C) (reviewed by Orlando and Paro, 1995; Pirrotta, 1996), higher order chromatin is essential for the correct folding and segregation of mitotic and meiotic chromosomes (Dernburg *et al*., 1996b), and for telomere (Hecht *et al*., 1995) and centromere activity (Ekwall *et al*., 1997; Williams *et al*., 1998). Despite these crucial functions in both gene expression and chromosomal architecture, the underlying structural components for the establishment and propagation of higher order chromatin, particularly in mammalian systems, remain largely unknown.

Although changes in the acetylation (Jeppesen and Turner, 1993; O'Neill and Turner, 1995) and phosphorylation (Gurley *et al*., 1978; Hendzel *et al*., 1997) of core histones participate in the generation of eu- or heterochromatin, several non-histone chromosomal proteins have been identified in yeast and *Drosophila* that are centrally involved in the regional organization of chromatin domains and the regulation of chromatin-dependent gene activity. In particular, genetic screens in *Drosophila* have revealed ~120 loci that enhance [E(var) genes] or suppress [Su(var) genes] position-effect-variegation (PEV) (reviewed by Reuter and Spierer, 1992; Weiler and Wakimoto, 1995; Wallrath, 1998), thus implicating E(VAR) proteins in the establishment of active and SU(VAR) proteins in the organization of repressive chromatin domains. Although only ~10% of the corresponding loci have have been isolated to date, *Su(var)3-7* (Reuter *et al*., 1990; Cleard *et al*., 1997), *Su(var)2-5* [which encodes the centromeric heterochromatin protein 1 (HP1); James and Elgin, 1986; Eissenberg *et al*., 1992] and *Su(var)3-9* (Tschiersch *et al*., 1994) all appear to provide key functions, since they display dose-dependent modification of PEV.

In contrast to PEV in *Drosophila*, genetic screens on centromeric position effects in *Schizosaccharomyces pombe* have identified <10 modifying loci (Allshire *et al*., 1995; Ekwall *et al*., 1996). Among these, *swi6* (Lorentz *et al*., 1994) and *clr4* (P.Lord and R.Allshire, personal communication; Ivanova *et al*., 1998) represent the *S.pombe* homologues of *Drosophila* HP1 and *Su(var)3-9*. In addition to their role in epigenetic control of gene expression (Thon *et al*., 1994; Ekwall and Ruusala, 1994), the major function of *swi6* and *clr4* appears to reside in co-regulating centromere activity, because *swi6* and *clr4* mutants display anaphase defects and elevated rates of chromosome loss (Ekwall *et al*., 1996). Moreover, SWI6 has been shown to accumulate at fission yeast centromeres in a *clr4*-dependent manner (Ekwall *et al*., 1995).

Su(var)3-9/*clr4* are the only modifying loci whose gene products combine the two most characteristic sequence motifs of 'chromatin regulators': the chromo (Paro and Hogness, 1991; Aasland and Stewart, 1995; Koonin *et al*., 1995) and SET (Tschiersch *et al*., 1994; Jenuwein *et al*., 1998) domains. Whereas the 40 amino acid chromo domain appears to be a protein-specific interaction motif (Ball *et al*., 1997) that targets hetero- or euchromatic associations (Messmer *et al*., 1992; Platero *et al*., 1995), the function of the 130 amino acid SET domain is currently undefined. Since it is also shared by several proteins of either the Polycomb-group (Pc-G) or the trithorax-group (trx-G), which antagonize transcriptional states of the HOM-C cluster (reviewed by Paro and Harte, 1996), the SET domain may represent a novel protein interaction motif for the assembly of repressing or activating chromatin complexes. Alternatively, the SET domain could be a target for phosphorylation-dependent signals (Cui *et al*., 1998) that may trigger dynamic transitions in chromatin structure.

Despite the important functions that are predicted from the analyses in *S.pombe* and *Drosophila*, almost no Su(var) gene products—with the exception of HP1 homologues (Singh *et al*., 1991; Saunders *et al*., 1993; Wreggett *et al*., 1994; Horsley *et al*., 1996)—have been characterized in mammals. Furthermore, biochemical characterization and possible chromatin association of SU(VAR)3-9 or CLR4 proteins are currently not known. Here, we describe the isolation of human (*SUV39H1*) and mouse (*Suv39h1*) homologues of *Drosophila Su(var)3-9* and demonstrate function of the human *SUV39H1* [for Su(var)3-9 homologue 1] gene in modifying pericentric PEV in transgenic flies. Immunodetection of endogenous Suv39h1/SUV39H1 proteins reveals enriched heterochromatic distributions during interphase that accumulate at centromeric positions on metaphase chromosomes. Importantly, Suv39h1/SUV39H1 associate with M31, a murine and human HP1 homologue, indicating the existence of a mammalian SU(VAR) protein complex. These data define SU(VAR)3-9 related proteins as novel heterochromatic components and implicate Suv39h1/SUV39H1 in the structural organization of mammalian higher order chromatin.

Results

Isolation of mammalian Su(var)3-9 homologues

Based on sequence information from the conserved C-terminal SET domain of *Drosophila Su(var)3-9*, we screened a human B-cell specific cDNA library for mammalian homologues (see Materials and methods). Out of 500 000 plaques, two primary phages with longer inserts were selected. One of the isolates contained a full-length human *SUV39H1* cDNA of 2.7 kb, encoding a protein of 412 amino acids. The *SUV39H1* cDNA sequence contains an in-frame stop codon preceding a consensus ATG, a 1.2 kb open reading frame and a 1.4 kb 3′ untranslated region, followed by a putative polyadenylation signal (data not shown). Sequence similarity searches against DDBJ/EMBL/GenBank sequences (Altschul *et al*., 1997) indicated that the full-length *SUV39H1* cDNA represents an authentic copy of a previously described partial cDNA (MG-44) of a gene that was mapped to the human X chromosome (Geraghty *et al*., 1993). However, preliminary data suggest that mammalian *Su(var)3-9* homologues are represented by at least two distinct loci (D.O'Carroll, A.Lebersorger and T.Jenuwein, in preparation), which encode related proteins of ~55% identity but whose cDNAs fail to cross-hybridize.

Using part of the human *SUV39H1* cDNA as a probe, we next screened a mouse brain cDNA library for the corresponding murine homologue. A DNA primer derived from the longest murine cDNA insert was used for a 5′ RACE amplification of the missing 5′ end. After subcloning, a full-length *Suv39h1* cDNA of 2.8 kb was obtained that encodes an open reading frame of 412 amino acids and displays the same structural characteristics as outlined for the human *SUV39H1* cDNA. Thus, from all available cDNA sequences, mammalian *Su(var)3-9* homologues encode significantly shorter gene products as compared with the 635 amino acids comprising fly protein (Tschiersch *et al*., 1994; see below).

To determine the accurate sizes of the mRNAs encoded by *Suv39h1* and *SUV39H1*, we hybridized RNA blots containing total RNA from several mouse tissues (data not shown) and from various mammalian cell lines (see Figure 6, bottom panel) with a 1.6 kb DNA probe that comprises *Suv39h1*-coding sequences. This probe detected a specific mRNA of ~2.9 kb which is present in all tissues and cell lines analysed and whose size is in good agreement with the 2.8 kb *Suv39h1* and 2.7 kb *SUV39H1* cDNAs.

Sequence conservation of yeast, fly and mammalian SU(VAR)3-9 related proteins

Sequence comparisons of the 412 amino acid human SUV39H1 and murine Suv39h1 proteins indicate overall identities of 95%. Both the human and mouse homologues lack 155 N-terminal amino acids of SU(VAR)3-9 (635 amino acids) (Tschiersch *et al*., 1994). Interestingly, the 490 amino acid CLR4 protein (P.Lord and R.Allshire, personal communication; Ivanova *et al*., 1998) also does not contain the fly-specific N-terminal extension.

Overall, cross-species amino acid identities reach 42% between the fly and the two mammalian proteins, and 38% between CLR4 and SUV39H1/Suv39h1 (Figure 1, top panel). Alignment of all four proteins reveals three regions of sequence identity. Most highly related is the 130 amino acid SET domain core (36% identity), which is followed at the very C-terminal tail by three conserved cysteine residues. N-terminal to the SET domain is a 110 amino acid domain (27% identity) which contains several conserved cysteine residues. Cysteine-rich regions appear specifically associated with the SET domains of most, but not all SET domain proteins (Huang *et al*., 1998). Despite the lack of homology to other well-defined cysteine stretches, such as RING (C_3HC_4)- (Freemont *et al*., 1991) and PHD (C_4HC_3)-fingers (Aasland *et al*., 1995) or LIM domains (C_2HC_5) (Sanchez-Garcia and Rabbits, 1994), this cysteine-rich cluster—and probably also the three C-terminal cysteine residues—may participate in facilitating molecular interactions. The third conspicuous sequence motif is the 40 amino acid chromo domain (20% identity) which is located close to the N-termini of SUV39H1/Suv39h1. Finally, although not present in *S.pombe* CLR4, both mammalian N-termini share a 45 amino acid region (29% identity) with SU(VAR)3-9.

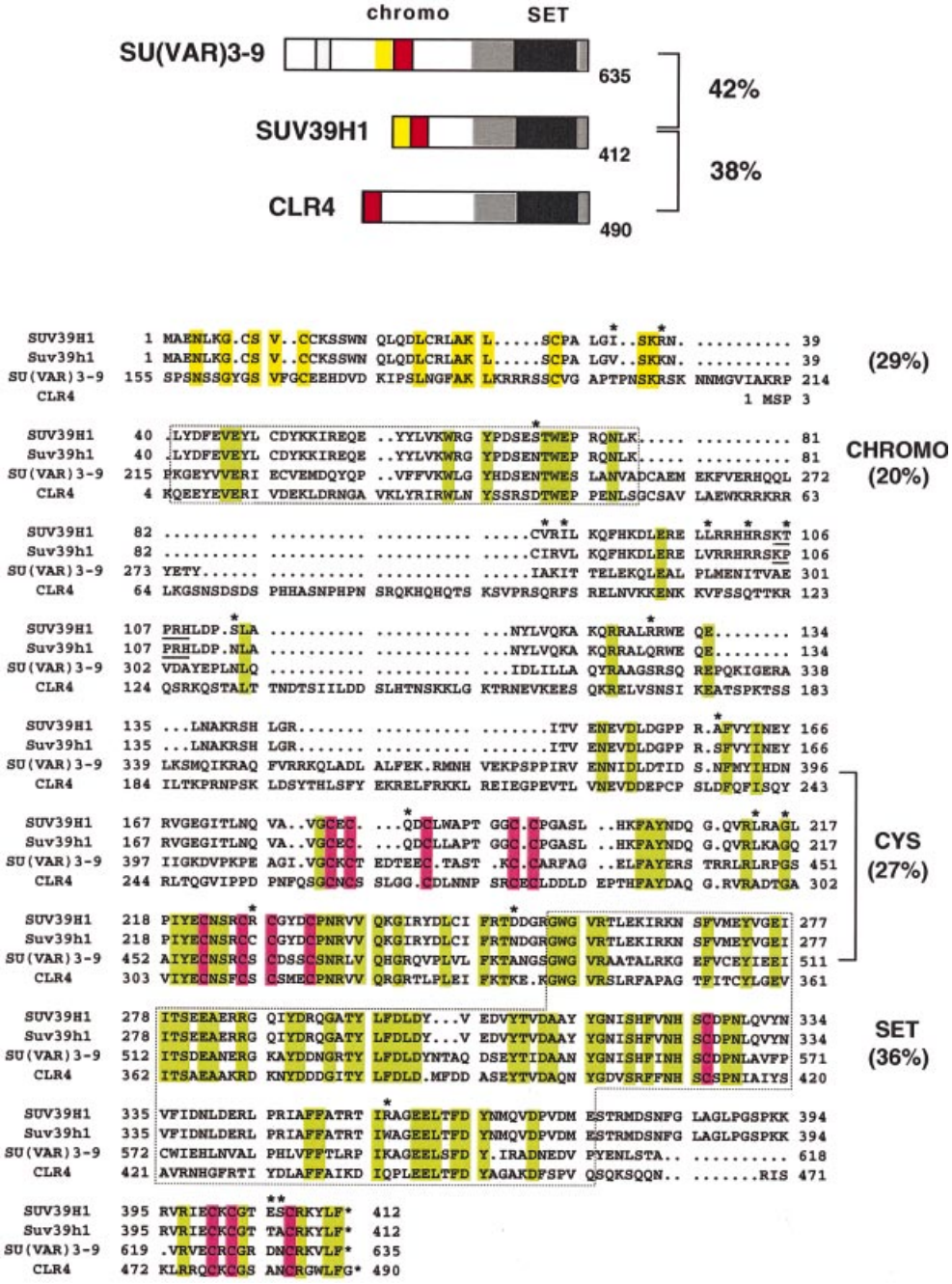

Fig. 1. Conserved domains of *S.pombe*, *Drosophila* and mammalian SU(VAR)3-9 related proteins. A schematic representation of the *S.pombe* CLR4 (490 amino acids), *Drosophila* SU(VAR)3-9 (635 amino acids) and human SUV39H1 proteins (412 amino acids) is shown on top. Over the entire length of the mammalian protein, SUV39H1 displays 42% identity with SU(VAR)3-9 and 38% identity with CLR4. Conserved domains are highlighted, and include the chromo (red) and SET (black) domains and cysteine-rich clusters (grey). A putative GTP-binding domain (Tschiersch *et al*., 1994), present in the N-terminal third of the fly protein, is indicated by a light-shaded box. Amino acid sequences of human SUV39H1, mouse Suv39h1, *Drosophila* SU(VAR)3-9 and *S.pombe* CLR4 proteins were aligned using the PILEUP programme of the GCG software package. Amino acids that are identical in all four proteins are shown in green, and conserved cysteine residues are highlighted by a pink colour. Three regions of high sequence similarity are indicated to the right, together with the respective amino acid identities calculated from conserved positions in all four proteins within these individual domains. The chromo and SET domain cores are boxed by a dashed line, and putative nuclear localization signals in SUV39H1 and Suv39h1 are underlined. Both mammalian N-termini share a 45 amino acid, 'SU(VAR)3-9 specific' region (29% identity; highlighted in yellow) with SU(VAR)3-9 that is not conserved in *S.pombe* CLR4. Human SUV39H1 and mouse Suv39h1 are 95% identical, and variant amino acid positions between these two proteins are indicated by an asterisk above the SUV39H1 sequence.

Broad expression profile of Suv39h1 during mouse development

The *Su(var)3*-9 gene is ubiquitously expressed and displays highest abundance from early to mid-embryogenesis in *Drosophila* (Tschiersch *et al*., 1994). To analyze the temporal and tissue-specific expression profile of *Suv39h1* during mouse development, we performed an RNase protection analysis with a 500 bp riboprobe that is specific for the SET domain of *Suv39h1*. *Suv39h1*-specific transcripts were detected throughout mouse embryogenesis

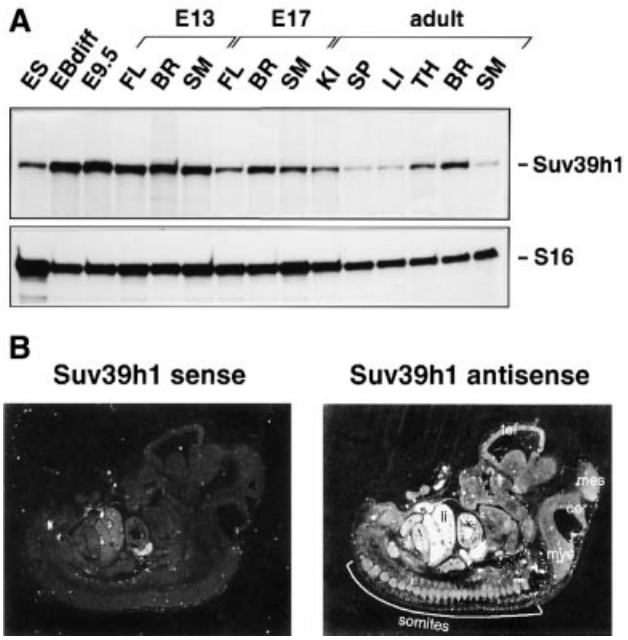

Fig. 2. Temporal and spatial expression of *Suv39h1* during mouse development. (**A**) RNase protection analysis to detect *Suv39h1* transcripts in 10 mg of total RNA prepared from undifferentiated D3 embryonic stem (ES) cells, embryoid bodies derived after retinoic acid-induced *in vitro* differentiation of D3 cells (EBdiff), whole embryos at day E9.5 and fetal liver (FL), brain (BR) and skeletal muscle (SM) of day E13 or day E17 129/Sv fetuses. In addition, total RNA was prepared from adult 129/Sv tissues, including kidney (KI), spleen (SP), liver (LI), thymus (TH), brain (BR) and skeletal muscle (SM). As a control for the quality of the RNA, RNAs were co-protected with a riboprobe that is specific for murine S16 rRNA sequences. (**B**) *In situ* hybridization on a sagittal section of day E12.5 mouse C57/Bl6 fetuses with *Suv39h1*-specific sense and antisense RNA probes. Broad expression is visualized in tissues derived from all three germ layers that include telencephalon (tel), mesencephalon (mes), cerebellum (cer), myelencephalon (mye), somites, ventricle (ve), liver (li) and stomach (st).

(Figure 2A), and their relative abundance reaches 2- to 3-fold higher levels between day E9.5 and day E13 of development. A similar increase was also observed after retinoic acid-induced *in vitro* differentiation of embryonic stem cells. In contrast, *Suv39h1* transcripts remained at reduced levels during later stages of embryogenesis and in adult tissues.

To investigate the spatial expression profile of *Suv39h1*, we also performed *in situ* hybridizations with a *Suv39h1*-specific riboprobe (see Materials and methods) on sagittal sections of day E12.5 total mouse fetuses. Whereas no signals are visualized with a *Suv39h1* control sense probe, the *Suv39h1* antisense probe reveals a rather uniform expression throughout the entire fetus, with *Suv39h1* transcripts being present in tissues derived from all three germ layers (Figure 2B). In comparison with neuroectodermal structures, *Suv39h1* expression is slightly elevated in the mesoderm-derived somites and reaches highest levels in fetal liver. *Suv39h1* expression is also detected in heart, stomach and many other organs. Together with the RNase protection analysis shown above, these data indicate broad expression of *Suv39h1* during embryonic and adult stages of mouse development.

Human SUV39H1 enhances PEV in Drosophila

Su(var)3-9 is a dominant dose-dependent modifier, and extra gene copies significantly enhance silencing of different PEV marker genes (Tschiersch *et al*., 1994). Using this 'triplo-enhancer effect' as an experimental assay, we established transgenic fly lines that carry the human *SUV39H1* or a $(myc)_3$-tagged variant cDNA under the control of the heat shock promoter hsp70. From a total of 14 transformed fly lines, nine insertions in the second and third chromosome were selected, and basal activity of the transgene was confirmed by expression analysis (data not shown). As controls, we used transgenic flies carrying *Su(var)3-9* cDNAs or a genomic fragment comprising the *Su(var)3-9* locus (Tschiersch *et al*., 1994). All transgenic lines were crossed into the $In(1)w^{m4h}$ indicator strain (see Materials and methods), which contains an inversion placing the *white* marker gene adjacent to pericentric X heterochromatin. In this strain, heterochromatin-mediated, variegated *white* gene expression can be easily detected as red (active transcriptional state) or white (repressed transcriptional state) patches in the *Drosophila* eye.

Visual inspection of progeny derived after crossing $In(1)w^{m4h}$; transgenic females into $In(1)w^{m4h}$ males indicated that all *Su(var)3-9* and *SUV39H1* transgenes induced a significant increase in the proportion of unpigmented areas in the eyes, therefore demonstrating repression of w^{m4h} gene activity (Figure 3, right panel). This 'triplo-enhancer effect' was largely independent of heat shock treatment and correlated with basal transcription of the preselected transgenes (see above). In contrast, ongoing studies reveal that partial rescue of the 'haplo-suppressor effect' of *Su(var)3-9* heterozygotes by human *SUV39H1* requires the correct developmental expression of the transgene already from very early embryogenesis (G.Schotta, V.Krauss, A.Fischer, S.Kuhfittig, R.Dorn and G.Reuter, in preparation).

To quantify the degree of PEV enhancement, we next crossed $In(1)w^{m4h}$; transgenic males into the 'sensitized' $In(1)w^{m4h}$; *Su(var)2-1* indicator strain (Dorn *et al*., 1986) (see Materials and methods), which allows a more accurate measurement of red-eye pigments as in the $In(1)w^{m4h}$ strain (Figure 3, left panel). Eye pigments were extracted from male progeny and pigment absorbance at 480 nm was determined. The results of these quantitations (Figure 4) show that one extra gene copy of genomic *Su(var)3-9* induced a pronounced (8- to 14-fold) reduction in the concentration of red-eye pigments, which was reflected by 8- to 28-fold reduced levels in the three lines carrying *Su(var)3-9* cDNAs. Importantly, the nine lines with *SUV39H1* cDNAs also displayed a 2- to 7-fold reduction in red eye pigmentation. Despite some variation among the transgenic lines and although a significant fraction of transgenic flies carrying *Su(var)3-9* and *SUV39H1* cDNAs display paternal effects (data not shown), these results demonstrate functional homology between human and fly *Su(var)3-9* genes, and indicate that *SUV39H1* is capable of repressing gene activity in the vicinity of heterochromatin.

The transgenic SUV39H1 protein localizes to the chromocentre of Drosophila polytene chromosomes

To examine its role in heterochromatin-mediated gene repression in more detail, we next analysed the distribution of the transgenic human $(myc)_3$-SUV39H1 protein at *Drosophila* polytene chromosomes. Third instar larvae of transgenic line mA were heat shocked for 2 h to increase

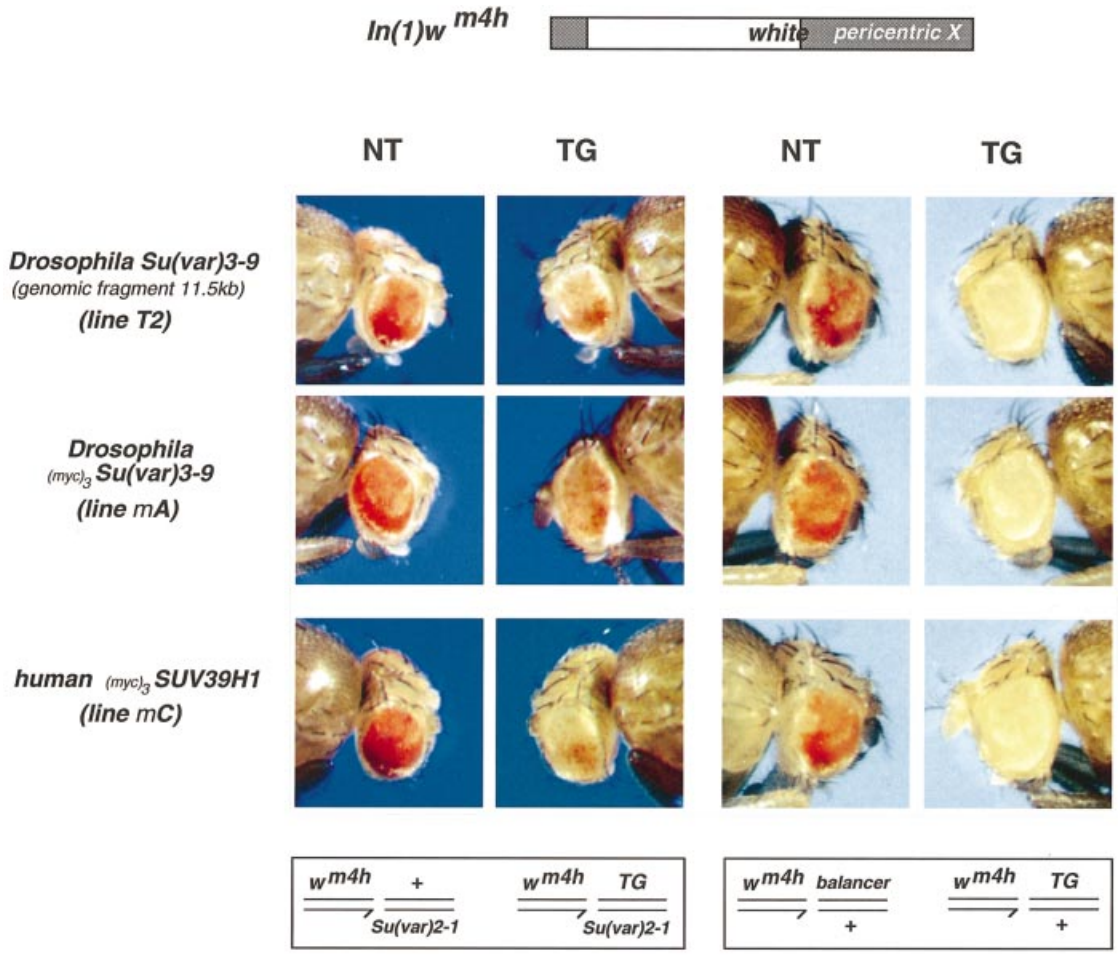

Fig. 3. Human *SUV39H1* enhances PEV in *Drosophila*. Transgenic flies carrying an extra gene copy of the *Su(var)3-9* locus (line T2) or hsp70-driven cDNAs encoding full-length $(myc)_3$-SU(VAR)3-9 (line mA) or $(myc)_3$-SUV39H1 (line mC) were crossed (see Materials and methods) into indicator strains that contain a pericentromeric, X-linked PEV allele of *white* [*In(1)w*^{m4h}; see diagram on top] (right panel) or, in addition, a strong suppressor mutation [*Su(var)2-1*] (left panel). PEV enhancement was observed independently of heat shock. Transgene-mediated repression of *w*^{m4h} is visualized in male offspring and is reflected by increased proportions of unpigmented areas in the eyes. NT, non-transgenic offspring; TG, transgenic offspring.

expression of the hsp70-driven transgene, and localization of $(myc)_3$-SUV39H1 protein was detected with monoclonal α-myc (9E10) antibodies at polytene chromosomes that were prepared 30 and 60 min after heat shock.

Immunolocalization of $(myc)_3$-SUV39H1 30 min after heat shock reveals a pronounced staining of the chromocentre and of the fourth chromosome (Figure 5B, arrow), indicating a preferred association with heterochromatin. No signal is present for non heat shocked control preparations (Figure 5A). At a later timepoint (60 min), staining at the chromocentre appears enhanced and several discrete euchromatic sites are visualized (Figure 5C). A similar distribution of transgenic $(myc)_3$-SUV39H1 has also been observed in other lines (data not shown) and is even reflected by ectopic SU(VAR)3-9 proteins that contain green fluorescent protein as a tag (G.Schotta, V.Krauss, A.Fischer, S.Kuhfittig, R. Dorn and G.Reuter, in preparation). These data demonstrate preferred binding of SU(VAR)3-9 related proteins to the chromocentre and thus support their direct involvement in regulating heterochromatin-mediated gene repression of pericentric marker genes.

Characterization of mammalian Suv39h1/SUV39H1 proteins

To characterize the endogenous Suv39h1/SUV39H1 proteins in mammalian cells, we generated a polyclonal rabbit antiserum that was raised against a bacterially expressed, glutathione *S*-transferase (GST) fusion product comprising amino acids 82–412 from the murine Suv39h1 protein. Western blot analysis of *in vitro* translated SUV39H1 indicated that the α-Suv39h1 antiserum also recognises the almost identical (95%) human protein (see below), but not the related Suv39h2 gene product (data not shown). Following affinity-purification (see Materials and methods), this polyclonal α-Suv39h1 antiserum was used to detect endogenous proteins in Ponceau S-adjusted nuclear extracts derived from a variety of human and mouse cell lines. In all eight cell lines tested, the α-Suv39h1 antiserum recognizes a specific endogenous protein of ~48 kDa (Figure 6, top panel) that co-migrates with *in vitro* translated SUV39H1 and whose size is in good agreement with products predicted from the coding sequences of the respective mammalian cDNAs. In addition to the endogenous proteins, the α-Suv39h1 antiserum

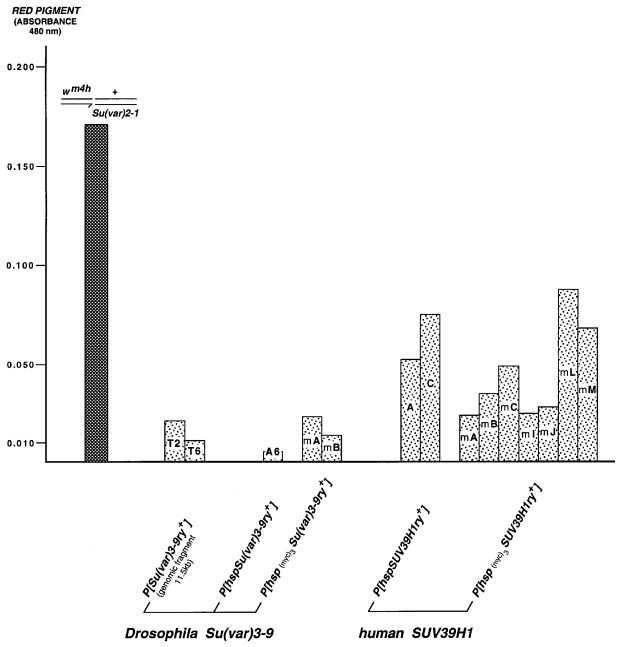

Fig. 4. Quantitation of PEV enhancement. Only transgenic lines with transgene insertions in the second [$(myc)_3$-*Su(var)3-9*, line mA and $(myc)_3$-*SUV39H1*, lines mL and mM) or third chromosome (all other lines) were selected for the PEV analysis. Balanced *In(1)*w^{m4h}; transgenic male stocks were back-crossed with *In(1)*w^{m4h}*; Su(var)2-1* females (see Materials and methods). Pooled red eye pigments extracted from the eyes of 10 individual siblings representing male transgenic [w^{m4h}/*Y*; +/ap^{Xa}*Su(var)2-1*^{01}/TG or w^{m4h}/*Y*; TG/ap^{Xa}*Su(var)2-1*^{01}/*ry*+; stippled bars] progeny were analysed for their absorbance at 480 nm. The values given are the mean of five to six independent measurements with a standard variation of <10%. Unmated *In(1)*w^{m4h}; *Su(var)2-1*/+ male control flies exhibit an average pigment absorbance of 0.170 (dark column on the left). The enhancer effect on PEV (reflecting repression of w^{m4h}) was measured at room temperature, independent of heat shock.

also detects ectopic $(myc)_3$-SUV39H1 (~55 kDa; shown by arrow in Figure 6, top panel) that is overexpressed in 'stably' transfected HeLa-B3 cells. No other proteins are visualized, demonstrating the specificity of this α-Suv39h1 antiserum, which is similarly efficient in detecting both mouse Suv39h1 and human SUV39H1. Protein abundance largely correlates (with the exception of NIH 3T3 cells) with the levels of endogenous *Suv39h1*/*SUV39H1* mRNAs (Figure 6, lower panel), indicating broad expression in mammalian cell lines.

Suv39h1 significantly co-localizes with M31 at heterochromatic foci in mouse interphase nuclei

To investigate the subnuclear localization of Suv39h1/SUV39H1 proteins, we first analysed their distribution in interphase nuclei of several of the above-mentioned mouse and human cell lines (data not shown). In contrast to the dispersed human interphase chromatin, mouse nuclei contain cytologically visible blocks of heterochromatin that can be highlighted with 4′-6′-diamidino-2-phenylindole (DAPI), which preferentially stains A/T-rich repeat sequences of constitutive heterochromatin. In addition, we also performed co-localization analyses with rat monoclonal α-M31 antibodies, which have been shown to define heterochromatic foci (Wreggett *et al*., 1994), and with human auto centromeric antibodies (hACA) which specifically decorate centromeric positions (Earnshaw and Rothfield, 1985; Sullivan *et al*., 1994).

Indirect immunofluorescence of Triton X-100-extracted (see Materials and methods) mouse Cop8 cells with the α-Suv39h1 antiserum indicates concentration of Suv39h1 protein at several (7–10) nuclear patches (Figure 7B and F) which overlap with the bright DAPI counterstaining (Figure 7A and E) and with the focal distribution of M31 (Figure 7C). The merged image of the Suv39h1 and M31 staining patterns demonstrates significant but not complete co-localization of these proteins (Figure 7D). In addition to the prominent heterochromatic foci, some weakly staining areas are detected in which Suv39h1 and M31 may only partly coincide. This subnuclear distribution of Suv39h1 protein has been confirmed in other mouse cell lines, whereas no specific signals were visualized with the Suv39h1 pre-immune serum (data not shown, but see control stainings on human metaphase chromosomes in Figure 8A).

In contrast to M31, the hACA serum detects many discrete dots (Figure 7G), most of which do not overlap with the Suv39h1 staining pattern (Figure 7F). However, the majority of these hACA positions appears enriched at the periphery of Suv39h1 foci (Figure 7H, insert), consistent with the clustering of centromeres around heterochromatic regions in interphase. We conclude that endogenous Suv39h1 protein significantly co-localizes with M31 and preferably associates with heterochromatin in mouse interphase nuclei.

SUV39H1 is concentrated at centromeric heterochromatin of human metaphase chromosomes

We next investigated a possible association of the SUV39H1 protein with mitotic chromatin in several human cell lines. Logarithmically growing cells were treated with colcemid, resulting in metaphase arrest of ~20% of the cells. Distribution of endogenous SUV39H1 protein along unfixed metaphase chromosomes (see Materials and methods) was then analysed by indirect immunofluorescence with the α-Suv39h1 antiserum and, as a comparison, with hACA antibodies.

Interestingly, endogenous SUV39H1 protein in HeLa metaphase spreads is detected at centromeric positions in a staining that resolves into the classical two-dotted pattern (Figure 8B and F), which reflects the centromeres of sister chromatids. Higher magnification of the characteristic blocks of pericentromeric heterochromatin, which can be visualized by staining with distamycin A-DAPI (DA-DAPI) and which are prominent, for example, in human chromosome 1, demonstrates that SUV39H1 is specifically concentrated at the centromeres, but does not decorate the adjacent heterochromatic domain (see insert of merged DA-DAPI and SUV39H1 stainings in Figure 8D). Furthermore, co-localization analysis with the hACA serum indicates a very similar, yet distinct distribution between the SUV39H1 signals and the hACA staining (Figure 8G). Indeed, higher magnification of the merged images (see insert in Figure 8H) illustrates that SUV39H1 is concentrated at the outer region of the centromeres, whereas hACA epitopes appear more internal. At this level of resolution, the extent of partial overlap between SUV39H1

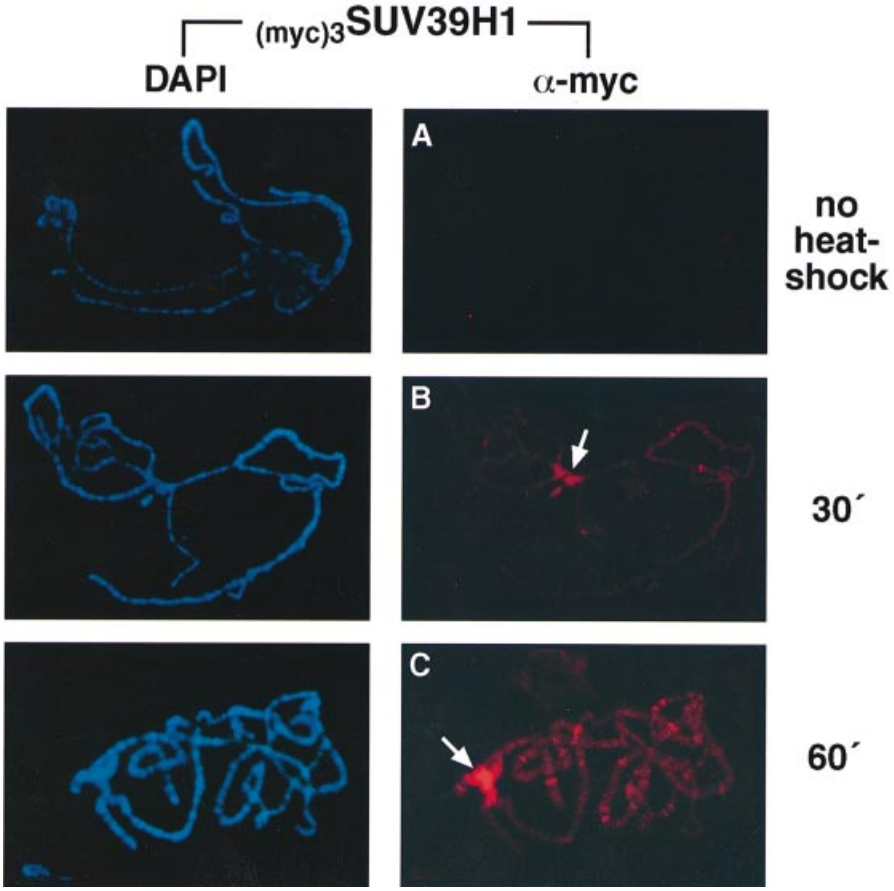

Fig. 5. Preferred localization of transgenic human $(myc)_3$-SUV39H1 protein with the chromocentre of *Drosophila* polytene chromosomes. Transgenic third instar larvae of the $(myc)_3$-*SUV39H1* line mA were reared at room temperature and heat shocked by incubation for 2 h at 37°C. Polytene chromosomes prepared from the salivary glands 30 min (**B**) and 60 min (**C**) after heat shock were processed for indirect immunofluorescence with myc-epitope specific monoclonal 9E10 and secondary, Texas Red-conjugated antibodies. DNA was counterstained with DAPI. Preferred localization of $(myc)_3$-SUV39H1 with the chromocentre and the heterochromatic fourth chromosome is indicated by an arrow. As a control, indirect immunofluorescence of non heat shocked polytene chromosomes (**A**) indicates undetectable levels of $(myc)_3$-SUV39H1 prior to induction.

and hACA epitopes in a common centromeric region is difficult to define.

Centromere-specific localization of SUV39H1 has been confirmed on metaphase spreads of other human cell lines, and is also observed at acrocentric mouse metaphase chromosomes (data not shown). Together, these data classify endogenous SUV39H1/Suv39h1 as novel centromere-associated proteins in mammalian mitotic chromatin.

SUV39H1 co-immunoprecipitates with M31

The significant co-localization with M31 suggested that Suv39h1/SUV39H1 and M31 may be components of a heterochromatic protein complex *in vivo*. To address this notion directly, we performed co-immunoprecipitations (co-IPs) with nuclear extracts from murine Cop8 cells, human HeLa cells and HeLa-B3 cells that 'stably' over-express $(myc)_3$-SUV39H1 (see Figure 6). The HeLa-B3 cells were chosen, because higher amounts of protein can be immunoprecipitated with monoclonal α-myc (9E10) antibodies. To detect possible complex formation between endogenous Suv39h1/SUV39H1 and M31, we also performed co-IPs with monoclonal α-M31 antibodies (Wreggett *et al*., 1994). Since the sizes of SUV39H1 (48 kDa), $(myc)_3$-SUV39H1 (55 kDa) and M31 (25 kDa) largely co-migrate with either the heavy or the light chain of immunoglobulins, we covalently coupled α-myc and α-M31 antibodies to protein G–Sepharose beads (see Materials and methods). Following IP with these antibody beads, immunoprecipitates were separated by SDS–PAGE, transferred to nitrocellulose membranes and probed with α-myc, α-Suv39h1, α-M31 and hACA antibodies.

The results of these co-IPs show that the α-myc beads specifically immunoprecipitate $(myc)_3$-SUV39H1 from nuclear extracts of HeLa-B3 cells but, as expected, not from HeLa or Cop8 cells (Figure 9, top two rows of left panel). Interestingly, M31 is present in the precipitated material, indicating complex formation with ectopically expressed $(myc)_3$-SUV39H1. In contrast, CENP-A (19 kDa), which is a crucial hACA epitope of the inner centromeric region (Warburton *et al*., 1997), does not co-immunoprecipitate with $(myc)_3$-SUV39H1. Using the α-M31 beads in the converse co-IPs, similar amounts of endogenous M31 are enriched from nuclear extracts of all three cell lines (Figure 9, third row of right panel). Importantly, endogenous SUV39H1 or Suv39h1 is co-immunoprecipitated from HeLa or Cop8 nuclear extracts. In addition, $(myc)_3$-SUV39H1 appears over-represented in co-IPs from HeLa-B3 nuclear extracts, suggesting that

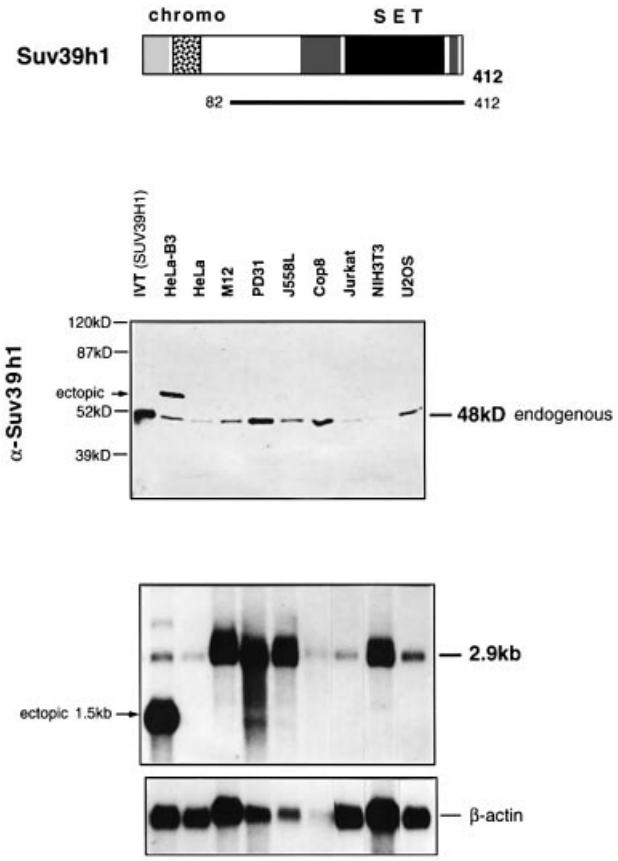

Fig. 6. Detection and size of endogenous Suv39h1/SUV39H1 proteins. A polyclonal rabbit antiserum was raised against a bacterially expressed GST-fusion protein that comprises amino acid positions 82–412 of mouse Suv39h1 (see top diagram). (Middle panel) Fifty microgrammes of Ponceau S-adjusted total protein prepared from nuclei of each of the indicated human (HeLa, Jurkat, U2OS) or mouse (M12, PD31, J558L, Cop8, NIH 3T3) cell lines was resolved by SDS–PAGE and electro-transferred to nitrocellulose for immunoblotting with the α-Suv39h1 antibodies. Suv39h1/SUV39H1-specific antigens are detected at M_r ~48 kDa (endogenous), and co-migrate with a product derived from *in vitro* translated (IVT) *SUV39H1* cDNA. Protein extracts from HeLa-B3 cells, which overexpress $(myc)_3$-tagged SUV39H1 (see Materials and methods), contain an additional specific antigen of 55 kDa (ectopic). (Bottom panels) RNA blot analysis to detect Suv39h1/SUV39H1 transcripts in 5 μg of poly(A)$^+$ RNA prepared from the indicated cell lines. The RNA blot was rehybridized with a murine β-actin DNA probe.

the ectopic protein can efficiently compete with the lower abundant endogenous SUV39H1 (Figure 9, second row of right panel) for putative M31 interaction surfaces. Finally, CENP-A is again absent in the α-M31 co-IPs.

SUV39H1 partly co-sediments with M31

The above data demonstrate complex formation between SUV39H1 and M31, and provide the first evidence for the existence of a mammalian SU(VAR) protein complex. To characterize the approximate size of this complex, we next sedimented HeLa nuclear extracts the same as used for the co-IPs by velocity centrifugation in a 10–40% sucrose gradient. Twenty fractions were collected and subsequently analysed by Western blotting with the hACA, α-Suv39h1 and α-M31 antibodies. As an internal size standard, the blots were also stained with antibodies that are specific for the p32 subunit of the 20S proteasome (Peters *et al*., 1994). Whereas CENP-A is distributed over a broad range, other hACA epitopes (CENP-B) are enriched in the lower molecular mass fractions. In contrast, although a minor portion is detected towards the top of the gradient, the majority of SUV39H1 protein was found in fractions 8–11, which overlap with the M31 peak (fractions 7–9) and co-sediment with the 20S proteasome (Figure 10).

M31 (also called HP1β) represents one of several mammalian HP1 isoforms (reviewed by Wallrath, 1998). We therefore also probed the same protein blots with antibodies that are specific for HP1α or M32 (also called HP1γ). However, both M32 and HP1α peak in fractions 2–3, with the euchromatic M32 protein (Horsley *et al*., 1996) being restricted to the low molecular mass range, whereas the heterochromatic HP1α protein (Nicol and Jeppesen, 1994) extends into higher fractions (Figure 10, bottom panels). These results indicate distinct sedimentation profiles for the three different mammalian HP1-related proteins and, together with the co-IPs shown above, provide supporting evidence that M31 is the most likely partner for endogenous SUV39H1 to be present in a multimeric mammalian SU(VAR) protein complex, which sediments at ~20S.

Discussion

Our functional analysis of human (*SUV39H1*) and mouse (*Suv39h1*) homologues of the *Drosophila* PEV modifier *Su(var)3-9* characterizes *SUV39H1* as the first mammalian Su(var) gene to be shown to modulate chromatin-dependent gene activity. Suv39h1/SUV39H1 are chromosomal proteins that are enriched at heterochromatic foci in interphase and which accumulate at centromeres of metaphase chromosomes. Moreover, Suv39h1/SUV39H1 associate with M31, providing direct evidence for the existence of a mammalian SU(VAR) protein complex. These data define Suv39h1/SUV39H1 as novel heterochromatic components and implicate these proteins in both epigenetic gene control and the structural organization of mammalian higher order chromatin.

SU(VAR)3-9-related proteins and gene silencing

The preferred affinity of endogenous Suv39h1/SUV39H1 for heterochromatic regions (discussed below) and of ectopic $(myc)_3$-SUV39H1 for the polytenic chromocentre in *Drosophila* (see Figure 5) suggests a direct role in the organization of repressive chromatin domains and the regulation of heterochromatin-dependent gene silencing. For example, variegation and the clonal nature of gene repression have been explained by the variable and co-operative (Locke *et al*., 1988) extension of heterochromatin from the chromocentre along the chromosome (‘*cis*-silencing’). However, variegation at centromere-distal positions, like repeat-induced silencing (Dorer and Henikoff, 1994), or even ‘*trans*-inactivation’ across homologous chromosomes is also modulated by Su(var) gene dosage (Csink and Henikoff, 1996). Furthermore, centromeric heterochromatin appears to be able to selectively recruit repressed genes into transcriptionally inactive subnuclear compartments (Csink and Henikoff, 1966; Dernburg *et al*., 1996a; Brown *et al*., 1997). Thus, a more general model has been proposed, in which the nucleation of repressive

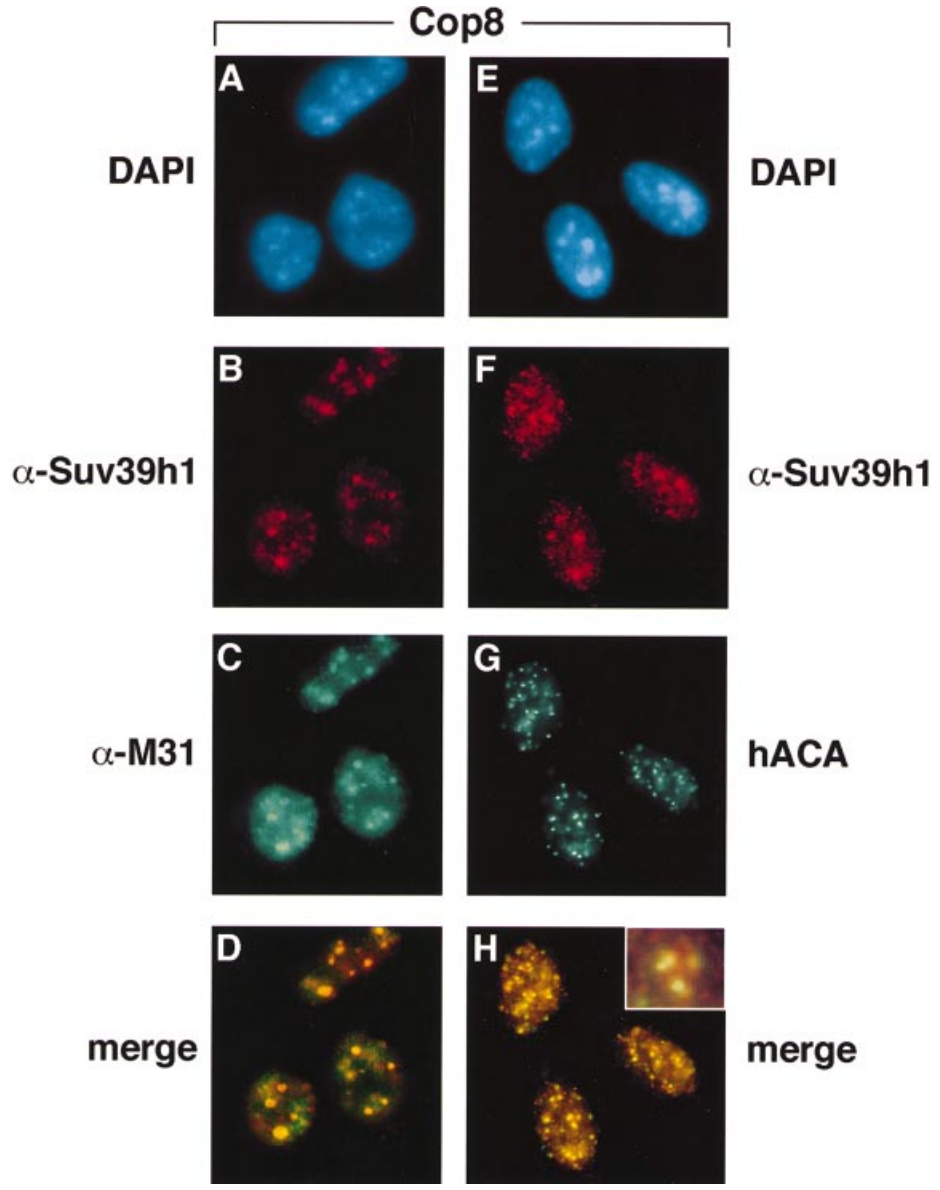

Fig. 7. Significant co-localization of Suv39h1 with heterochromatic foci and M31 in mouse interphase cells. Interphase cells of the mouse fibroblastoid Cop8 cell line were Triton X-100 extracted (see Materials and methods), and processed for indirect immunofluorescence with α-Suv39h1 and secondary and tertiary, CY-3 conjugated antibodies [red staining in (**B**) and (**F**)]. DNA was counterstained with DAPI, which highlights A/T-rich repeat sequences present in the prominent heterochromatic foci [bright blue patches in (**A**) and (**E**)] that are characteristic for mouse interphase chromatin. Preparations were sequentially incubated with α-M31 and hACA (SM serum) antibodies, which are visualized by secondary FITC conjugated antibodies [green staining in (**C**) and (**G**)]. Merged images indicate significant but not complete co-localization of Suv39h1 and M31 [yellow staining in (**D**)] that also overlaps with blocks of constitutive heterochromatin. In contrast, hACA epitopes largely do not coincide with the Suv39h1 signals but rather cluster in the vicinity of heterochromatic foci [enlarged insert in (**H**)].

chromatin domains is largely dictated by the pairing or looping potential of target sequences. Repeat-driven looping or pairing may induce an altered structure which is then stabilized and expanded in response to the local concentration of heterochromatin-specific proteins (Henikoff, 1996; Pirrotta, 1996). SU(VAR)3-9-related proteins represent excellent candidates to match most of these required functions and, since Suv39h1/SUV39H1 are components of mitotic chromatin, they could also propagate distinct transcriptional states during cell divisions.

Suv39h1/SUV39H1 are novel components of mammalian heterochromatin

The subnuclear distribution and chromatin association of endogenous Suv39h1/SUV39H1 proteins indicates significant co-localization with heterochromatin-specific M31 during interphase and partial overlap with epitopes recognized by human anti-centromeric autoantibodies (hACA) during metaphase (see Figures 7 and 8). Interphase heterochromatin and mitotic chromatin most probably differ in their condensation levels, and the mitotic restructuring of chromosomes has been proposed to induce dynamic

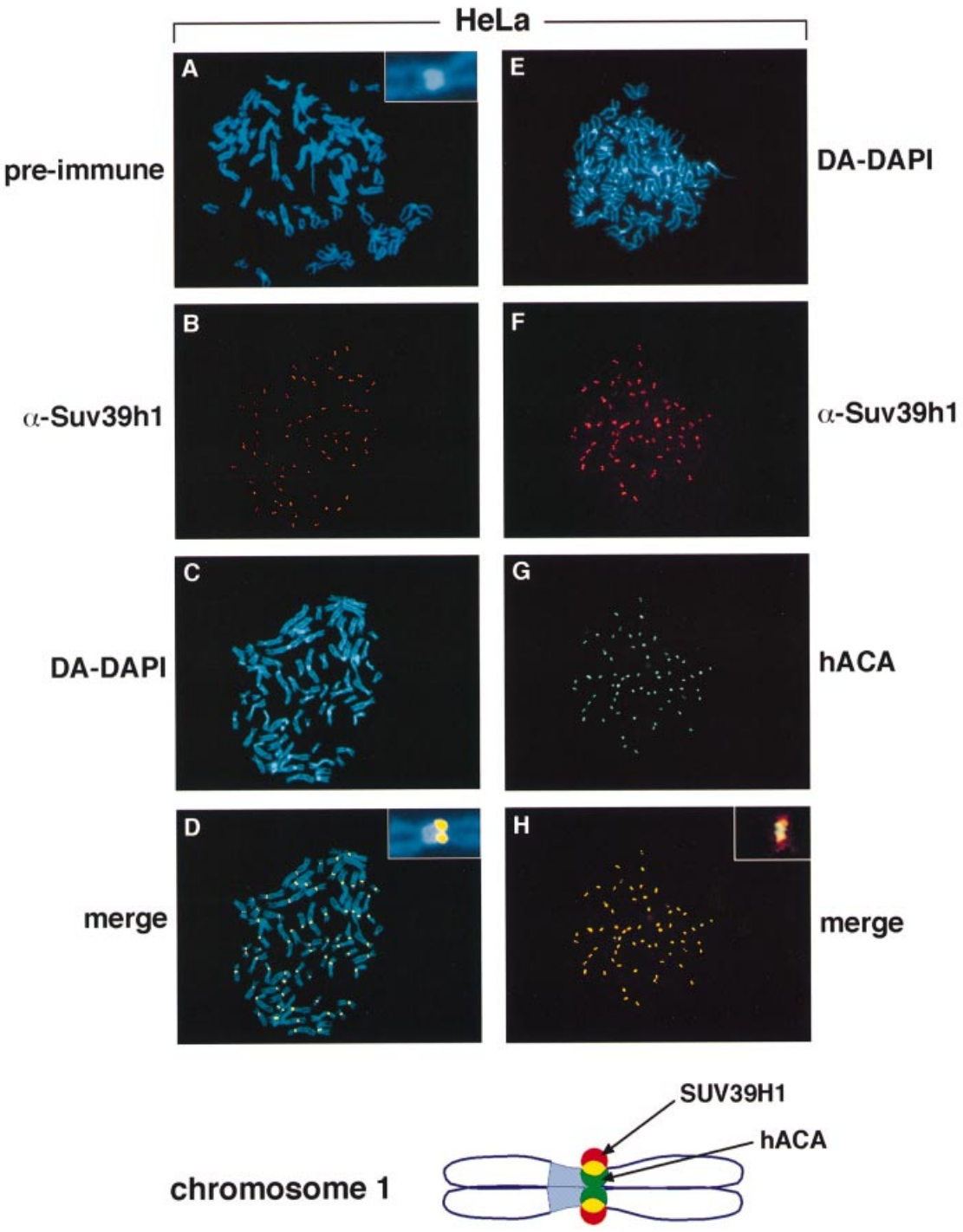

Fig. 8. Specific association of SUV39H1 with centromeric positions at human metaphase chromosomes. Human epitheloid HeLa cells were enriched for metaphase-arrest with colcemid, and unfixed metaphase chromosomes (see Materials and methods) were processed for indirect immunofluorescence with α-Suv39h1 and secondary and tertiary, CY-3 conjugated antibodies [red staining in (**B**) and (**F**)]. As a control for the specificity of the observed metaphase staining, HeLa cells were also processed with precleared pre-immune serum (**A**). DNA was counterstained with DA-DAPI to visualize A/T-rich satellite repeats of centric and pericentromeric heterochromatin [focal bright blue staining in (**C**) and (**E**)]. Merged images [regions of overlap are painted yellow in (**D**)] indicate a specific, two-dotted co-localization with the centromeres of the sister chromatids, which does not extend into the adjacent pericentromeric heterochromatin of e.g. human chromosome 1 [enlarged insert in (D)]. Preparations were also processed for hACA epitopes [green staining in (**G**)], which partly overlap with SUV39H1 signals [enlarged insert of merged image in (**H**) and schematic diagram at the bottom]. The used HeLa cell line is hypo-tetraploid and contains 62(66-68)70 chromosomes.

redistributions for several chromatin regulators (Raff *et al*., 1994; Torok *et al*., 1997; discussed in Csink and Henikoff, 1998). In this respect, we also observe that the localization of Suv39h1 protein during interphase is spatially separated from hACA epitopes, which appear to cluster in the vicinity of heterochromatic foci. On the other hand, SUV39H1 specifically accumulates at centromeric positions of human metaphase chromosomes, but does not decorate pericentromeric heterochromatin (see inserts in Figure 8D and H). Thus, Suv39h1/SUV39H1 resembles

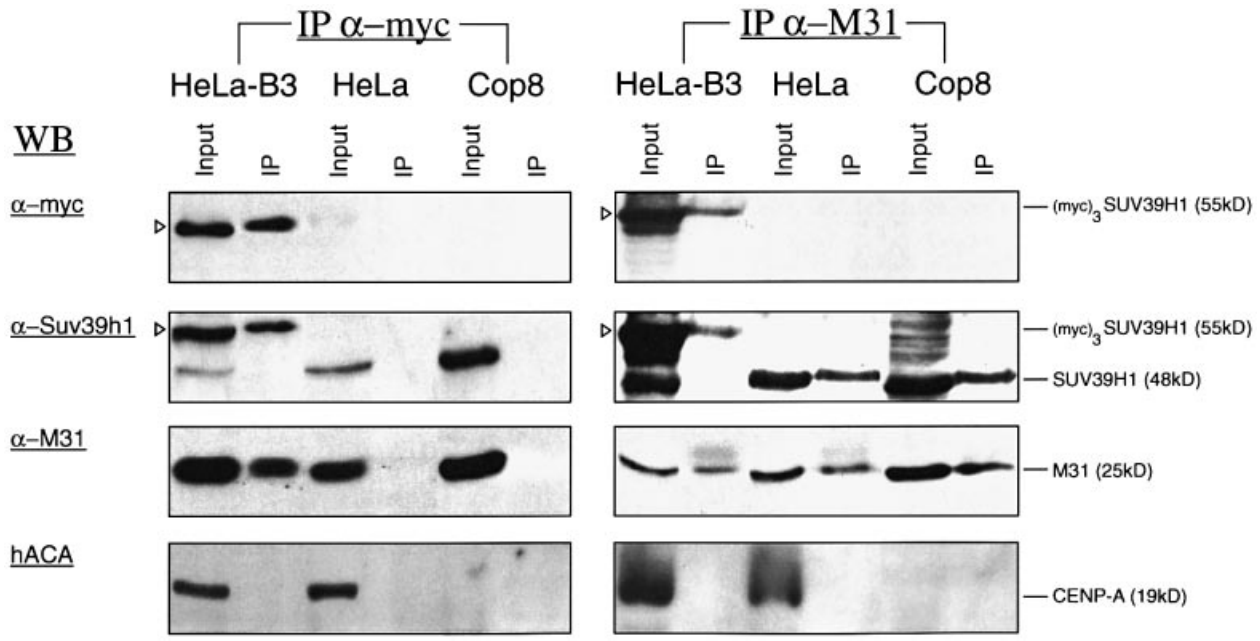

Fig. 9. SUV39H1 and M31 co-immunoprecipitate *in vivo*. Nuclear extracts from the indicated cell lines were immunoprecipitated with α-myc (left panel) or α-M31 (right panel) antibody beads (see Materials and methods), separated by SDS–PAGE, transferred to nitrocellulose membranes and probed with α-myc, α-Suv39h1, α-M31 and hACA antibodies. As input controls, 20–50 μg of nuclear proteins were loaded, representing 2–5% of total nuclear extract that was processed by the IP. HeLa-B3 is a 'stably' transfected cell line that overexpresses $(myc)_3$-SUV39H1 (indicated by arrowhead). The used human hACA serum (SM serum) does not detect CENP-A in mouse Cop8 nuclear extracts, although it is immuno-reactive with other murine CENPs (data not shown), as visualized by the immunofluoresecence shown in Figure 7G.

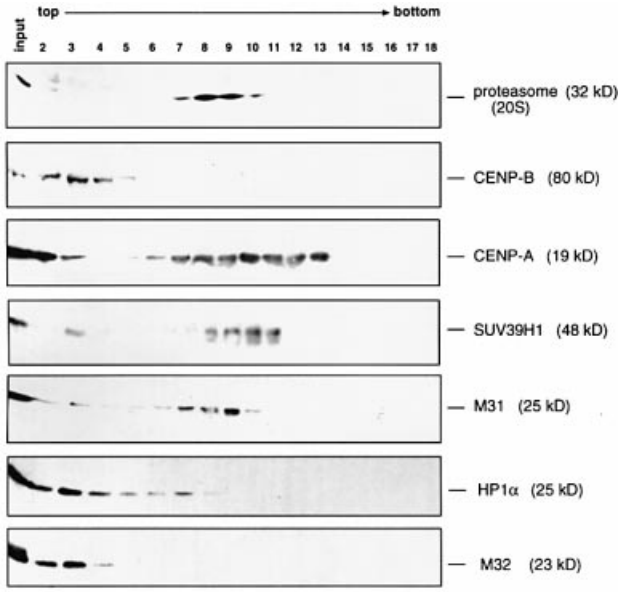

Fig. 10. SUV39H1 and M31 partly co-sediment in a protein complex of ~20S. Five milligrammes of soluble nuclear extract (the same as used for the co-IPs) was prepared from HeLa cells and sedimented in a 10–40% sucrose gradient. Twenty 600 μl fractions were collected, and 50 μl per fraction was analysed on protein blots with α-Suv39h1 and hACA (SM serum) antibodies. Mammalian HP1 isoforms were detected with monoclonal antibodies that are specific for M31 (also called HP1β), HP1α or M32 (also called HP1γ). As an internal size standard, blots were also probed with antibodies that recognize the p32 subunit of the 20S proteasome.

dynamic chromosomal proteins that display highest affinities for non-centromeric, heterochromatic foci during interphase and centromeric heterochromatin at metaphase.

This Suv39h1/SUV39H1 staining pattern is clearly distinct from the interphase distribution of several mammalian Pc-G proteins (Alkema *et al*., 1997; Gunster *et al*., 1997; van Lohuizen *et al*., 1998; Sewalt *et al*., 1998). In addition, only a minor fraction of M33 (PC homologue) and BMI1 (PSC homologue) remains associated with mitotic chromatin (Wang *et al*., 1997; Saurin *et al*., 1998), whereas EZH [E(Z) homologues] proteins do not appear to localize at human metaphase chromosomes (L.Aagaard and T.Jenuwein, unpublished). Direct examination of possible interactions with Pc-G proteins indicated no physical *in vivo* association between SUV39H1 and M33 or EZH2 (M.Schmid and T.Jenuwein, unpublished). These distinct staining and interaction patterns are in agreement with the described differences between Pc-G and Su(var) gene function (Jenuwein *et al*., 1998), despite several common sequence motifs, including chromo and SET domains, that are shared by some PEV modifiers and chromosomal regulators of HOM-C.

SUV39H1/Suv39h1 and centromere function

The high-affinity association with centromeric positions on metaphase chromosomes implicates a direct role for SUV39H1/Suv39h1 in mammalian centromere activity. This interpretation is supported by the functional analysis of *clr4* mutations that result in perturbed chromosome segregation and disrupt localization of the centromere component SWI6 (Ekwall *et al*., 1996), which represents the HP1 homologue in *S.pombe*. However, human *SUV39H1* has so far failed to rescue *clr4*-dependent centromeric gene silencing in *S.pombe* (T.Jenuwein and R.Allshire, unpublished). On the other hand, overexpression of $(myc)_3$-SUV39H1 in HeLa cells appears to perturb chromosome segregation (M.Melcher and T.Jenuwein, in preparation). Since SUV39H1 is specifically localized at

the outer region of the centromere (see Figure 8H), and because *clr4*-dependent segregation defects are synergistically enhanced by β-tubulin mutations (Ekwall *et al*., 1996), deregulated SUV39H1 function could probably interfere with kinetochore assembly.

In mammals, several centromere-specific proteins (CENPs) have been identified (reviewed in Choo, 1997), of which CENP-A appears to be a crucial component of active centromeres (Warburton *et al*., 1997). CENP-A resembles a histone H3-variant (Sullivan *et al*., 1994) that is cell cycle regulated and which has been implicated to target assembly of $(CENP\text{-}A/H4)_2$ tetramers to centromeric heterochromatin, specifically during late replication (Shelby *et al*., 1997). Despite the apparent similarities in centromeric localization (see above) and partly overlapping sedimentation profiles (see Figure 10), we have been unable to detect physical association between SUV39H1 and CENP-A, or other hACA epitopes (CENP-B and CENP-C) (see Figure 9; data not shown). Instead, SUV39H1 is present in a complex with M31 (see below). According to current models, centromere function is likely to be co-regulated at multiple levels: whereas CENP-A containing tetramers may induce an altered nucleosomal array, higher order chromatin appears to be required to 'imprint' active centromeres (Karpen and Allshire, 1997; Warburton *et al*., 1997; Williams *et al*., 1998). Based on our data, we propose that Suv39h1/SUV39H1 are involved in the organization of such a higher order chromatin structure at mammalian centromeres.

SUV39H1/Suv39h1 and M31 define a mammalian SU(VAR) protein complex

In interphase, SUV39H1/Suv39h1 significantly co-localize and co-immunoprecipitate with M31 (see Figures 7 and 9). In addition, ectopic $(myc)_3$-SUV39H1 also associates with M31 *in vivo* (see Figure 9). We have, however, been unable to detect binding between *in vitro* co-translated SUV39H1 and M31 or retention of endogenous M31 on affinity columns that contain bacterially expressed GST–Suv39h1 (data not shown), suggesting that possible direct interactions are dependent on post-translational modifications. The sedimentation profiles of SUV39H1 and mammalian HP1 isoforms are most consistent with SUV39H1 and M31 being present in a common, multimeric complex of ~20S (see Figure 10). In contrast, the also heterochromatic HP1α (Nicol and Jeppesen, 1994) or the euchromatic M32 (Horsley *et al*., 1996) are restricted to lower molecular mass fractions. These results underscore the specificity of the SUV39H1–M31 complex and are in agreement with described differences in interacting partners for M31 and HP1α that have been identified through yeast two-hybrid screens (Le Dourain *et al*., 1996).

However, although SUV39H1 and M31 share part of their peak fractions, they do not entirely co-sediment, raising the possibility that both proteins may also participate in more promiscuous interactions and in the formation of additional complexes. A variety of heterogeneous partners implicated in transcriptional regulation, replication and subnuclear architecture have been described for HP1-related proteins (reviewed in Wallrath, 1998). Although these interactions would be consistent with the proposed molecular nature of HP1 as an 'adaptor protein', direct biochemical interactions *in vivo* have been difficult to define. Recently, physical association between *Drosophila* SU(VAR)3-7 and HP1 has been reported (Cleard *et al*., 1997). Since *Su(var)3-9*, *Su(var)3-7* and *Su(var)2-5* (HP1) are all dose-dependent modifiers of PEV (see Introduction), this finding predicts the putative mammalian SU(VAR)3-7 homologue(s) as another likely candidate to be present in the SUV39H1–M31 protein complex. In summary, our analysis of mammalian SU(VAR)3-9-related proteins characterises SUV39H1/Suv39h1 as novel heterochromatic components and provides an entry point to dissect the structural principles that underlie the formation and function of mammalian higher order chromatin.

Materials and methods

Molecular cloning of mammalian Su(var)3-9 cDNAs

A DNA probe encoding amino acids 476–635 of SU(VAR)3-9 was amplified by PCR from the *Drosophila Su(var)3-9* cDNA clone M4 (Tschiersch *et al*., 1994). Human *SUV39H1* cDNAs were obtained after reduced stringency hybridizations of a B-cell specific (BJAB) λgt10 cDNA library (kindly provided by M.Busslinger) with this SET-specific DNA probe as described previously (Laible *et al*., 1997). One of the isolates (λ48) contained the full-length 2.7 kb *SUV39H1* cDNA. Mouse cDNA clones were isolated from a Balb/c brain λgt11 cDNA library (Clontech) by screening, under reduced stringency as above, with a 0.9 kb *Pst*I–*Bgl*II cDNA fragment that encodes amino acids 1–290 of human SUV39H1.

Full-length coding murine Suv39h1 cDNA, 5′ RACE and sequence analysis

Missing 5′ sequences of the murine *Suv39h1* cDNA were generated by RACE amplification (Marathon cDNA amplification kit; Clontech) of 1 μg $poly(A)^+$ RNA from the murine B-cell line J558L. After cDNA synthesis, 5′ RACE products were obtained by nested PCR using the gene-specific primers *Suv39h1*-520 (5′-CCAGGTCTACCTCATTCT-CCACGGTGATC) and *Suv39h1*-480 (5′-GGTGGCTGCGCTTGGCAT-TGAGCTCTTG). The resultant 0.6 kb PCR product was trimmed with *Not*I and *Hin*dIII to generate a 120 bp fragment comprising the starting ATG codon and combined with a pBluescript-based 1.4 kb cDNA subclone (λ48m3), which contains coding sequences for amino acids 6–412 plus 290 bp of the 3′ untranslated region.

The complete human *SUV39H1* and murine *Suv39h1* cDNAs were sequenced by primer walking on an automated sequencer (Applied Biosystems). Sequence data were analysed using the University of Wisconsin Genetics Computer Group sequence analysis software package (Devereux *et al*., 1984). Sequence similarity searches were performed with the BLAST network service (Altschul *et al*., 1997) and also included comparisons with mammalian ESTs (Basset *et al*., 1995).

RNA isolation and analysis

Total RNA was isolated by Trizol (Gibco-BRL) homogenization. $Poly(A)^+$ RNA was purified using a PolyATrack kit (Promega). For RNA blot analysis, 5 μg of $poly(A)^+$ RNA were fractionated on formaldehyde–agarose gels, transferred to GeneScreen nylon membranes in 20× SSC and baked for 2 h at 80°C. The membrane was sequentially hybridized under stringent Church conditions (Sambrook *et al*., 1989) with a 1.6 kb *Eco*RI cDNA fragment comprising nearly all *Suv39h1* coding sequences, and with a DNA probe that is specific for murine β-actin sequences (Laible *et al*., 1997).

For RNase protection analysis (Sambrook *et al*., 1989), 10 μg of total RNA were co-incubated at 60°C with riboprobes that specifically protect 340 bp of the *Suv39h1* SET domain or, as an internal control, 156 bp of murine S16 rRNA sequences (Laible *et al*., 1997). The murine *Suv39h1* SET domain riboprobe was derived from a 350 bp PCR-amplified *Eco*RI–*Sac*I fragment (encoding amino acids 248–364 of Suv39h1) that had been subcloned into pGEM-3Zf (Promega). After linearization with *Pvu*II, antisense RNA was internally labelled by transcription with SP6 RNA polymerase in the presence of $[^{32}P]GTP$, and the full-length riboprobe of 500 bp was purified from a denaturing urea-polyacrylamide gel.

In situ hybridizations of day E12.5 mouse fetuses

Development of C57/Bl6 mouse fetuses was timed by appearance of the vaginal plug and set as day E0.5. *In situ* hybridizations were performed

as described (Gaunt *et al*., 1988) on 8 μm sagittal sections of paraffin-embedded fetuses. Sections were hybridized O/N at 55°C with *Suv39h1* antisense or sense RNA probes, washed under high-stringency, processed in photoemulsion (Kodak NTB-2) and developed after 18 days.

The *Suv39h1*-specific RNA probe was derived from a 395 bp PCR-amplified *Sal*I–*Bam*HI fragment (encoding amino acids 113–237 of Suv39h1) that had been subcloned into pGEM-3Zf. *In situ* RNA probes were internally labelled with [^{35}S]CTP by transcription with SP6 RNA polymerase (antisense probe of *Eco*RI linearized plasmid) or T7 RNA polymerase (sense probe of *Bam*HI linearized plasmid).

(myc)$_3$-H$_6$ epitope-tag and expression plasmids

A *Not*I site was inserted just downstream of the start codon in *SUV39H1* by PCR mutagenesis with the modifying oligonucleotide 5′-atagg-atccGGGGAAAGATGGgcggccgcGAAAATTTAAAAGGCTGCAGC-GTG (start codon underlined). Overlapping oligonucleotides encoding an in-frame triple myc (EQKLISEEDLN)$_3$ epitope preceding six histidines were ligated, cleaved with *Not*I and inserted into the *Not*I-digested *SUV39H1* derivative. The resulting (myc)$_3$-H$_6$ epitope extends the N-terminus by 48 amino acids. For protein expression studies, this (myc)$_3$-*SUV39H1* variant was transferred as a 1.4 kb *Sal*I-*Eco*RI fragment into the polylinker of pKW2T (derivative of pRK7; Gentech) which allows *in vitro* transcription by the SP6 RNA polymerase or directs *in vivo* over-expression in mammalian cells driven by the cytomegalovirus (CMV) enhancer/promoter.

For epitope-tagging of *Drosophila Su(var)3-9* constructs, a derivative plasmid was generated by mutagenising (gcggacgc) the ATG-proximal *Not*I site, resulting in a vector which now contains a unique in-frame *Not*I cloning site immediately following the N-terminal tag [CMV-(myc)$_3$-H$_6$*Not*I]. A PCR-amplified DNA fragment was inserted into this unique *Not*I site which encodes nearly full-length SU(VAR)3-9 (amino acids 4–635). For *Drosophila* expression plasmids, (myc)$_3$-*SUV39H1* and (myc)$_3$-*Su(var)3-9* were converted by PCR amplification into Asp718 fragments [partial digest required for the *Su(var)3-9* construct] and inserted into the Asp718 site of the P-element, *rosy*$^+$ vector pHT4 (Schneuwly *et al*., 1987), which directs expression from a minimal hsp70 promoter. Similarly, PCR-amplified *Not*I fragments containing the untagged coding sequences for *SUV39H1* and full-length *Su(var)3-9* were transferred into the *Not*I site of pNHT4 (Schneuwly *et al*., 1987). The genomic *Su(var)3-9* sequences are expressed from the natural promoter and the respective construct has been described (Tschiersch *et al*., 1994). For all of the mammalian and fly expression plasmids, the 5′ and 3′ boundaries of the cDNA inserts were confirmed by sequencing.

Drosophila genetics

All *Drosophila* stocks were raised under standard culture conditions. A description of chromosomes and mutations used in this study can be found in Lindsley and Zimm (1992). Transgenic lines (see Figures 3 and 4) expressing the various *Su(var)3-9* and *SUV39H1* cDNAs under the control of a minimal hsp70 promoter (Schneuwly *et al*., 1987) were generated by P element-mediated germline transformation (Rubin and Spradling, 1982), using a *ry*^{506} fly strain as recipient. Transformed ry$^+$ lines were selected for transgene insertions in the second or third chromosome, and *w*^{m4h}*; Cy* or *w*^{m4h}*; TM3, Ser* balanced transgenic stocks were established. All transgenic lines were confirmed by PCR-genotyping and transgene expression was examined by whole-mount *in situ* hybridization of embryos (data not shown). Transgenic lines carrying a 11.5 kb genomic DNA fragment which contains the *Su(var)3-9* locus have been described (Tschiersch *et al*., 1994). PEV enhancement was initially analysed in offspring derived from crosses between *w*^{m4h}/*w*^{m4h}*; Cy/ry*+TG or *w*^{m4h}/*w*^{m4h}*; TM3/ry*+TG transgenic females and *w*^{m4h}*/Y; +/+* males (Figure 3, right panel).

To quantify PEV enhancement, transgenic males from *w*^{m4h} balanced stocks were first crossed into *w*^{m4h}/*w*^{m4h}*; Cy/T(2;3) ap*^{Xa}*Su(var)2-1*^{01}*/Sb* females. Transgenic male offspring (*w*^{m4h}*/Y; Cy/+; Sb*/TG or *w*^{m4h}*/Y;* Cy/TG; *Sb/ry*+) was then back-crossed into *w*^{m4h}/*w*^{m4h}*; Cy/T(2;3) ap*^{Xa}*Su(var)2-1*^{01}*/Sb* females. Male non-transgenic (*w*^{m4h}*/Y; Cy/ap*^{Xa} *Su(var)2-1*^{01}*/Sb*) and transgenic (*w*^{m4h}*/Y; +/ap*^{Xa}*Su(var)2-1*^{01}/TG or *w*^{m4h}*/Y;* TG/*ap*^{Xa}*Su(var)2-1*^{01}*/ry*+) offspring from this last back-cross are shown in Figure 3, left panel. The PEV enhancer effect was analysed at room temperature in the absence of heat shock and quantified by measuring the absorbance (at 480 nm) of red eye pigments that had been extracted from the eyes of ten individual male siblings of these indicated genotypes.

Heat shock induction and immunostaining of polytene chromosomes

Third instar larvae were raised on standard *Drosophila* medium at RT, incubated for 2 h in a water bath at 37°C and dissected 30 and 60 min after heat shock. Preparation of polytene chromosomes and immunostaining was performed as described (Silver and Elgin, 1978), with the following modifications: salivary glands were isolated in 0.7% NaCl, fixed for 10 min and squashed in a solution containing 45% acetic acid/2% formaldehyde. Polytene chromosomes were incubated with mouse monoclonal α-myc antibodies (1:100 dilution of an IgG-purified 9E10 hybridoma supernatant) at 4°C O/N, followed by incubation with a secondary, Texas Red-conjugated goat α-mouse antibody (Jackson Immuno Research Laboratories). DNA was counterstained for 3 min with 4′,6′-diamidino-2-phenylindole (DAPI) (Sigma) at 0.1 μg/ml in phosphate-buffered saline (PBS). Preparations were mounted in sodium carbonate buffer pH 9.5, containing 87% glycerol and 5% *N*-propyl-gallate.

Mammalian cell culture and 'stably' transfected HeLa-B3 cells

Information on tissue culture conditions and mammalian cell lines used in this study can be found in the ATCC (cell culture and hybridoma) catalogue. The *in vitro* differentiation of feeder-independent mouse embryonic stem cells (D3) has been described previously (Laible *et al*., 1997).

For generation of a cell line that gives rise to long-term over-expression of (myc)$_3$-SUV39H1, ~2×10^5 HeLa cells were co-transfected using Lipofectase (Gibco-BRL) with 10 μg CMV-driven (myc)$_3$-*SUV39H1* and 1 μg of a plasmid conferring G418 resistance. Out of 25 individual G418^r colonies analysed by immunofluorescence, 10 clones displayed low to moderate expression, and only one clone (HeLa-B3) showed significant overexpression of (myc)$_3$-SUV39H1 in ~80% of clonal cells. Since the presence of (myc)$_3$-SUV39H1 rapidly declined with increasing cell divisions of HeLa-B3 cells and could not be stabilized by further subcloning, aliquots of early passage cells were stored in liquid nitrogen and recultivated immediately prior to use.

Generation and purification of rabbit polyclonal α-Suv39h1 antibodies

Suv39h1 coding sequences comprising amino acids 82–412 were converted into a 1050 bp *Bam*HI–*Sma*I DNA fragment by PCR amplification and combined in-frame with N-terminal GST in the *Escherichia coli* expression vector pGEX-2T (Pharmacia). Recombinant protein was produced in the protease-attenuated *E.coli* strain BL21. To minimize retention in inclusion bodies, production was induced with 100 mM IPTG at reduced temperature (30°C) for only 25 min, resulting in 0.6–1.0 mg of soluble GST–Suv39h1 protein per 3 l of culture. Cell pellets were carefully broken up with glass beads in RIPA500 buffer [10 mM Tris pH 7.5, 500 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA and a full set of protease-inhibitors (Boehringer Mannheim)], and soluble fusion-protein was purified over a glutathione–Sepharose column (Pharmacia). Two rabbits were initially immunized with 250 μg of purified GST–Suv39h1 antigen each, followed by three to four boost injections with 150 μg. An IgG fraction was prepared from the crude serum of rabbit #7592, and α-Suv39h1 antibodies were affinity-purified by incubation with the GST–Suv39h1 antigen, immobilized to nitrocellulose (Harlow and Lane, 1988) or to glutahione–Sepharose beads. Following elution with 100 mM glycine pH 2.5, the purified antibodies were dialysed O/N against PBS. To enrich further the specificity of the α-Suv39h1 antiserum, antibodies were precleared over an affinity-column (Bio-Rad) containing tissue extracts of *Suv39h1*$^{-/-}$ mice (D.O'Carroll and T.Jenuwein, unpublished). The affinity-purified, precleared antibodies were used at a 1:300 dilution for Western blot analysis or at a 1:10 dilution for indirect immunofluorescence. Accordingly, crude pre-immune serum of rabbit #7592 was purified with immobilized GST–Suv39h1 antigen prior to its use in control immunofluorescence analyses.

Nuclear extracts of mammalian cells and protein blot analysis

Nuclei were prepared (see below) from ~1×10^6 cells of the respective cell lines and directly lysed in Laemmli sample buffer (Harlow and Lane, 1988), followed by 10 min boiling. Total protein concentrations were adjusted by Ponceau S staining, and ~50 μg of nuclear proteins were separated by SDS–PAGE and transferred to nitrocellulose. Protein blots were blocked in 5% non-fat dry milk containing 0.2% Tween 20, incubated with the purified α-Suv39h1 antibodies O/N at 4°C, washed 5× 10 min at room temperature in PBS containing 0.1% Tween 20 and stained by a 30 min incubation at room temperature with secondary donkey α-rabbit antibodies (Jackson Immuno Research Laboratories)

that had been conjugated to horseradish peroxidase. Peroxidase staining was visualized using Enhanced ChemiLuminescence (ECL) (Amersham).

To generate SUV39H1 protein by *in vitro* translation (IVT), 1 μg of plasmid DNA was incubated for 2 h at 30°C with 50 μl of completed reaction mix from the coupled transcription-translation TNT reticulocyte lysate kit (Promega) in the presence of SP6 RNA polymerase. For protein blot analysis, 1.5 μl of reaction products were used.

Immunofluorescence of mouse interphase chromatin

For interphase staining, cells were grown directly on coverslips. Cells were immersed in stabilization buffer (100 mM HEPES pH 6.9, 1 mM EGTA, 4 M glycerol), extracted for 2 min in stabilization buffer containing 0.5% TritonX-100 (Compton *et al*., 1991), and subsequently fixed for 15 min at room temperature in 2% formaldehyde in PBS. Preparations were blocked for 1 h at room temperature in 10% fetal calf serum (FCS), and sequentially incubated with purified α-Suv39h1, secondary CY-3 conjugated goat α-rabbit and tertiary CY-3 conjugated donkey α-goat antibodies (both Jackson Immuno Research Laboratories) in a light-protected humid chamber. All antibodies were diluted in serum-containing medium, and cells were washed 3× 5 min in PBS containing 0.05% Tween 20 between different incubation steps.

For co-localization studies, preparations were first triple-stained for Suv39h1 epitopes as outlined above, followed by incubation with rat α-M31 (Wregget *et al*., 1994) or human hACA (SM serum; kindly provided by K.Sullivan, The Scripps Research Institute, La Jolla, CA) antibodies. M31 or hACA primary antibodies were then visualized with FITC-conjugated goat α-rat (M31) or FITC-conjugated donkey α-human (hACA) secondary antibodies (both Jackson Immuno Research Laboratories). Immunolocalization of hACA epitopes detected by the SM serum in mouse interphase nuclei (see Figure 7G) has been confirmed with three additional hACA sera (kindly provided by Gunter Steiner, AKH, Vienna). DNA of mouse cells was counterstained for 1 min with DAPI (0.2 μg/ml). Samples were mounted in Vectashield medium containing AntiFade (Vector Laboratories), analysed on a Zeiss Axiophot microscope and processed with a CCD camera (Photometrics) and Adobe Photoshop 3.0.

Immunofluorescence of unfixed human metaphase chromosomes

Logarithmically growing cells were incubated for 1 h with colcemid (0.1 μg/ml) (Gibco-BRL), which resulted in metaphase arrest of ~20% of the cells. Mitotic cells were harvested by shake-off, hypotonically swollen for 30 min at room temperature in 75 mM KCl, spread by spinning them at 2000 r.p.m. for 10 min onto microscope slides in a Cytospin 3 (Shandon) and immediately immersed in KCM-buffer (120 mM KCl, 20 mM $MgCl_2$, 10 mM Tris pH 8.0, 0.5 mM EDTA, 0.1% Triton X-100) (Wregget *et al*., 1994). Spreaded chromosomes were blocked for 30 min with 10% FCS in KCM-buffer, and incubated for 1 h at room temperature with purified α-Suv39h1 antibodies, followed by the secondary and tertiary, CY-3 conjugated antibodies. Sequential co-localization with primary hACA antibodies (SM serum) and secondary FITC-conjugated antibodies was performed as decribed above. All antibodies were diluted with 10% FCS in KCM buffer. After the final washes, chromosomes were fixed in 4% formaldehyde in KCM buffer for 10 min and processed for documentation. Human DNA was counterstained for 10 min with DA 0.2 mg/ml (Sigma), followed by a 10 min incubation with DAPI, to highlight A/T-rich repeat sequences of constitutive heterochromatin.

Co-immunoprecipitations with antibody beads

Fifteen millilitres of crude rat hybridoma (MAC353; α-M31) (Wreggett *et al*., 1994) supernatant or 3.6 mg IgGs (in 10 ml PBS) which had been purified from mouse hybridoma (9E10; α-myc) supernatant were incubated at 4°C O/N with 1.25 ml protein G–Sepharose beads (Pharmacia). Following cross-linking for 30 min at room temperature with 57 mg dimethylpimelidate (DMP) (Sigma) in 0.2 M sodium borate buffer pH 9.0, DMP was repeatedly inactivated in 0.2 M ethanolamine pH 8.0, beads were cleared by a 2 min incubation with 100 mM glycine pH 2.5, washed and resuspended in 1 ml PBS that had been supplemented with 0.02% NaN_3. Approximately 90% of antibodies could be covalently coupled, resulting in bead preparations that contain ~1.5 mg IgG per ml of a 50:50 slurry. Forty microlitres of these antibody-beads were subsequently used for co-immunoprecipitations.

Nuclei were prepared from ~2×10^7 cells by gentle centrifugation through 20% Ficoll Paque (Pharmacia) in NI buffer (100 mM Tris pH 7.4, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 2% NP-40, 1.6% Triton X-100, 0.1% DMSO). Nuclei were washed in PBS, lysed in IP buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 1% NP-40, 1 mM EDTA, 10% glycerol, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM $NaVO_3$, 1 mM DTT and full set of protease inhibitors), sonicated twice for 10 s, and insoluble material was removed by a 20 min centrifugation at 15 000 r.p.m. Protein concentration of cleared nuclear extracts was adjusted to 1 mg/ml, and 20–50 μg of soluble protein was used as input controls. For co-IPs, 900 μg were incubated at 4°C O/N with 40 μl of antibody-beads (50:50 slurry). Bound proteins were washed 5× in IP buffer, eluted with 1.5% SDS in 60 mM Tris pH 6.8, equilibrated with Laemmli sample buffer, separated by SDS–PAGE and transferred to nitrocellulose membranes. Protein blots were cut into halves and the upper parts were sequentially probed with α-Suv39h1 and α-myc antibodies. Lower parts were probed with α-M31 antibodies (10% SDS–PAGE) or with the different hACA sera (12% SDS–PAGE). In addition, some blots were also incubated with a CENP-A specific rabbit polyclonal antiserum (R8; kindly provided by K.Sullivan, The Scripps Research Institute, La Jolla, CA) (data not shown).

Sucrose gradients

Soluble nuclear extracts were prepared as described for the co-IPs. A 10–40% sucrose gradient in modified IP buffer (only 0.1% NP-40 and lacking glycerol) was established with a Gradient Master (Biocomp). Five milligrammes of total protein was loaded onto the gradient and sedimented at 4°C for 19 h at 28 000 r.p.m. in a SW40 rotor (Beckman). After centrifugation, twenty 600-μl fractions were collected in a density gradient fractionator (ISCO), and 50 μl per fraction was analysed on protein blots with α-Suv39h1, α-M31 and hACA antibodies as outlined above. For detection of mammalian HP1 isoforms, monoclonal rat α-M32 (Horsley *et al*., 1996) and monoclonal mouse α-HP1α (kindly provided by Pierre Chambon, CNRS, Illkirch) antibodies were used. As an internal size standard, blots were also probed with mouse monoclonal antibodies (mAB26S-161) that are specific for the p32 subunit of the 20S proteasome (Peters *et al*., 1994).

DDBJ/EMBL/GenBank accession numbers

The human *SUV39H1* (accession No. AF019968) and murine *Suv39h1* (accession No. AF019969) cDNA sequences have been deposited in DDBJ/EMBL/GenBank.

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References

Aasland,R. and Stewart,A.F. (1995) The chromo shadow domain, a second chromodomain in heterochromatin-binding protein1, HP1. *Nucleic Acids Res.*, **23**, 3168–3173.

Aasland,R., Gibson,T.J. and Stewart,A.F. (1995) The PHD finger: implications for chromatin-mediated transcriptional regulation. *Trends Biochem. Sci.*, **20**, 56–59.

Alkema,M.J., Bronk,M., Verhoeven,E., Otte,A., van't Veer,L.J., Berns,A. and van Lohuizen,M. (1997) Identification of Bmi1-interacting proteins as constituents of a multimeric mammalian Polycomb complex. *Genes Dev.*, **11**, 226–240.

Altschul,S.F., Madden,T.L., Schaffer,A.A., Zhang,J., Zhang,Z., Miller,W. and Lipman,D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.*, **25**, 3398–3402.

Ball,L.J. *et al*. (1997) Structure of the chromatin binding (chromo) domain from mouse modifier protein 1. *EMBO J*., **16**, 2473–2481.

Basset,D.R.,Jr, Boguski,M.S., Spencer,F., Reeves,R., Goebl,M. and Hieter,P. (1995) Comparative genomics, genome cross-referencing and XREFdb. *Trends Genet*., **11**, 372–373.

Brockdorff,N. and Duthie,S.M. (1998) X chromosome inactivation and the Xist gene. *Cell. Mol. Life Sci*., **54**, 104–112.

Brown,K.E., Guest,S.S., Smale,S.T., Hahm,K., Merkenschlager,M. and Fisher,A.G. (1997) Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. *Cell*, **91**, 845–854.

Choo,K.H.A. (1997) Centromere proteins of higher eukaryotes. In Choo,K.H.A. (ed.), *The Centromere*. Oxford University Press, Oxford, UK, pp. 143–253.

Cleard,F., Delattre,M. and Spierer,P. (1997) SU(VAR)3-7, a *Drosophila* heterochromatin-associated protein and companion of HP1 in the genomic silencing of position-effect variegation. *EMBO J*., **16**, 5280–5288.

Compton,D.A., Yen,T.J. and Cleveland,D.W. (1991) Identification of novel centromere/kinetochore-associated proteins using monoclonal antibodies generated against human mitotic chromosome scaffolds. *J. Cell Biol*., **112**, 1083–1097.

Csink,A.K. and Henikoff,S. (1996) Genetic modification of heterochromatic association and nuclear organisation in *Drosophila*. *Nature*, **381**, 529–531.

Csink,A.K. and Henikoff,S. (1998) Something from nothing: the evolution and utility of satellite repeats. *Trends Genet*., **14**, 200–204.

Cui,X., De Vito,I., Slany,R., Miyamoto,A., Firestein,R. and Cleary,M.L. (1998) Association of SET domain and myotubularin-related proteins modulates growth control. *Nature Genet*., **18**, 331–337.

Dernburg,A.F., Broman,K.W., Fung,J.C., Marshall,W.F., Philips,J., Agard,D.A. and Sedat,J.W. (1996a) Perturbation of nuclear architecture by long-distance chromosome interactions. *Cell*, **85**, 745–759.

Dernburg,A.F., Sedat,J.W. and Hawley,R.S. (1996b) Direct evidence for a role of heterochromatin in meiotic chromosome segregation. *Cell*, **86**, 135–146.

Devereux,J., Haeberli,P. and Smithies,O. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res*., **12**, 387–395.

Dorer,D.R. and Henikoff,S. (1994) Expansions of transgene repeats cause heterochromatin formation and gene silencing in *Drosophila*. *Cell*, **77**, 993–1002.

Dorn,R., Heymann,S., Lindigkeit,R. and Reuter,G. (1986) Suppressor mutation of position effect variegation loci in *Drosophila melanogaster* affecting chromatin properties. *Chromosoma*, **93**, 398–403.

Earnshaw,W.C. and Rothfield,N. (1985) The identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. *Chromosoma*, **91**, 313–319.

Efstradiadis,A. (1994) Parental imprinting of autosomal mammalian genes. *Curr. Opin. Genet. Dev.*, **4**, 265–280.

Eissenberg,J.C., Morris,G.D., Reuter,G. and Hartnett,T. (1992) The heterochromatin-associated protein HP-1 is an essential protein in *Drosophila* with dosage-dependent effects on position-effect variegation. *Genetics*, **131**, 345–352.

Ekwall,K. and Ruusala,T. (1994) Mutations in *rik1*, *clr2*, *clr3* and *clr4* genes asymmetrically derepress the silent mating-type loci in fission yeast. *Genetics*, **136**, 53–64.

Ekwall,K., Javerzat,J.-P., Lorentz,K., Schmidt,H., Cranston,G. and Allshire,R. (1995) The chromo domain protein Swi6: a key component at fission yeast centromeres. *Science*, **269**, 1429–1431.

Ekwall,K., Nimmo,E.R., Javerzat,J.-P., Borgstrøm,B., Egel,R., Cranston,G. and Allshire,R. (1996) Mutations in the fission yeast silencing factors *clr4*+ and *rik1*+ disrupt the localisation of the chromo domain protein Swi6p and impair centromere function. *J. Cell Sci*., **109**, 2637–2648.

Ekwall,K., Olsson,T., Turner,B.M., Cranston,G. and Allshire,R.C. (1997) Transient inhibition of histone deacetylation alters the structural and functional imprint at fission yeast centromeres. *Cell*, **91**, 1021–1032.

Freemont,P.S., Hanson,I.M. and Trousdale,J. (1991) A novel cysteine-rich sequence element. *Cell*, **64**, 483–484.

Gaunt,S.J., Sharpe,P.T. and Duboule,D. (1988) Spatially restricted domains of homeo-gene transcripts in mouse embryos: relation to a segmented body plan. *Development Suppl*. **104**, 169–178.

Geraghty,M.T., Brody,L.C., Martin,L.S., Marble,M., Kearns,W., Pearson,P., Monaco,A.P., Lehrach,H. and Valle,D. (1993) The isolation of cDNAs from OATL1 at Xp11.2 using a 480kb YAC. *Genomics*, **16**, 440–446.

Gunster,M.J., Satijn,D.P.E., Hamer,K.M., den Blaauwen,J.L., de Bruijn,D., Alkema,M.J., van Lohuizen,M., van Driel,R. and Otte,A.P. (1997) Identification and characterisation of interactions between the vertebrate Polycomb-group protein BMI1 and human homologs of Polyhomeotic. *Mol. Cell. Biol*., **17**, 2326–2335.

Gurley,L.R., D'Anna,J.A., Barhan,S.S., Deaven,L.L. and Tobey,R.A. (1978) Histone phoshorylation and chromatin structure during mitosis in chinese hamster cells. *Eur. J. Biochem*., **84**, 1–15.

Harlow,E. and Lane,D. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hecht,A., Laroche,T., Strahl-Bolsinger,S., Gasser,S.M. and Grunstein,M. (1995) Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast. *Cell*, **80**, 92–95.

Heitz,E. (1929) Heterochromatin, Chromocentren, Chromomeren. *Ber. Dtsch. Bot. Ges*., **47**, 274–284.

Hendzel,M.J., Wei,Y., Mancini,M.A., van Hoose,A., Ranalli,T., Brinkley,B.R., Bazett-Jones,D.P. and Allis,C.D. (1997) Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G_2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma*, **106**, 348–360.

Henikoff,S. (1996) Position-effect-variegation in *Drosophila*: recent progress. In Russo,V.E.A., Martienssen,R.A. and Riggs,A.D. (eds) *Epigenetic Mechanisms of Gene Regulation*. CSHL Press, New York, NY, pp. 319–334.

Horsley,D., Hutchings,A., Butcher,G.W. and Singh,P.B. (1996) M32, a murine homologue of *Drosophila* heterochromatin protein 1 (HP1), localises to euchromatin within interphase nuclei and is largely excluded from constitutive heterochromatin. *Cytogenet. Cell Genet*., **73**, 308–311.

Huang,N., vom Baur,E., Garnier,J.-M., Lerouge,T., Vonesch,J.-L., Lutz,Y., Chambon,P. and Losson,R. (1998) Two distinct nuclear receptor interaction domains in NSD1, a novel SET protein that exhibits characteristics of both corepressors and coactivators. *EMBO J*., **17**, 3398–3412.

Ivanova,A.V., Bonaduce,M.J., Ivanov,S.V. and Klar,A.J.S. (1998) The chromo and SET domians of the Clr4 protein are essential for silencing in fission yeast. *Nature Genet*., **19**, 192–195.

James,T.C. and Elgin,S.C.R. (1986) Identification of a nonhistone chromosomal protein associated with heterochromatin in *Drosophila* and its gene. *Mol. Cell. Biol*., **6**, 3862–3872.

Jenuwein,T., Laible,G., Dorn,R. and Reuter,G. (1998) SET-domain proteins modulate chromatin domains in eu- and heterochromatin. *Cell. Mol. Life Sci*., **54**, 80–93.

Jeppesen,P. and Turner,B.M. (1993) The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. *Cell*, **74**, 281–289.

Karpen,G. and Allshire,R. (1997) The case for epigenetic effects on centromere identity and function. *Trends Genet*., **13**, 489–496.

Koonin,E.V., Zhou,S. and Lucchesi,J.C. (1995) The chromo superfamily: new members, duplication of the chromo domain and possible role in delivering transcription regulators to chromatin. *Nucleic Acids Res*., **23**, 4229–4232.

Laible,G., Wolf,A., Dorn,R., Reuter,G., Nislow,C., Lebersorger,A., Popkin,D., Pillus,L. and Jenuwein,T. (1997) Mammalian homologues of the *Polycomb*-group gene *Enhancer of zeste* mediate gene silencing in *Drosophila* heterochromatin and at *S.cerevisae* telomeres. *EMBO J*., **16**, 3219–3232.

Le Dourain,B., Nielsen,A.L., Garnier,J.-M., Ichinose,H., Jeanmougin,F., Losson,R. and Chambon,P. (1996) A possible involvement of TIF1α and TIF1β in the epigenetic control of transcription by nuclear receptors. *EMBO J*., **15**, 6701–6715.

Lindsley,D.L. and Zimm,G.G. (1992) *The Genome of Drosophila melanogaster*. Academic Press, San Diego, CA.

Locke,J., Kotarski,M.A. and Tartof,K.D. (1988) Dosage dependent modifiers of position-effect variegation in *Drosophila* and a mass action model that explains their effect. *Genetics*, **120**, 181–198.

Lorentz,A., Ostermann,K., Fleck,O. and Schmidt,H. (1994) Switching gene *swi6*, involved in repression of silent mating-type loci in fission yeast, encodes a homologue of chromatin-associated proteins from *Drosophila* and mammals. *Gene*, **143**, 139–143.

Messmer,S., Franke,A. and Paro,R. (1992) Analysis of the functional role of the *Polycomb* chromo domain in *Drosophila melanogaster*. *Genes Dev*., **6**, 1241–1254.

Nicol,L. and Jeppesen,P. (1994) Human autoimmune sera recognize a conserved 26 kD protein associated with mammalian heterochromatin that is homologous to heterochromatin protein 1 of *Drosophila*. *Chromosome Res.*, **2**, 245–253.

O'Neill,P.A. and Turner,B.M. (1995) Histone H4 acetylation distinguishes coding regions of the human genome from heterochromatin in a differentiation-dependent but transcription-independent manner. *EMBO J.*, **14**, 3946–3957.

Orlando,V. and Paro,R. (1995) Chromatin multiprotein complexes involved in the maintenance of transcription patterns. *Curr. Opin. Genet. Dev.*, **5**, 174–179.

Paro,R. and Hogness,D.S. (1991) The Polycomb protein shares a homologous domain with a heterochromatin-associated protein of *Drosophila*. *Proc. Natl Acad. Sci. USA*, **88**, 263–267.

Paro,R. and Harte,P.J. (1996) The role of Polycomb group and trithorax group complexes in the maintenance of determined cell states. In Russo,V.E.A., Martienssen,R.A. and Riggs,A.D. (eds), *Epigenetic Mechanisms of Gene Regulation*. Cold Spring Harbor Laboratory Press, New York, NY, pp. 507–528.

Peters,J.-M., Franke,W.W. and Kleinschmidt,J.A. (1994) Distinct 19S and 20S subcomplexes of the 26S proteasome and their distribution in the nucleus and the cytoplasm. *J. Biol. Chem.*, **269**, 7709–7718.

Pirrotta,V. (1996) Stable chromatin states regulating homeotic genes in *Drosophila*. In Russo,V.E.A., Martienssen,R.A. and Riggs,A.D. (eds), *Epigenetic Mechanisms of Gene Regulation*. Cold Spring Harbor Laboratory Press, New York, NY, pp. 489–505.

Platero,J.S., Harnett,T. and Eissenberg,J.C. (1995) Functional analysis of the chromo domain of HP-1. *EMBO J.*, **14**, 3977–3986.

Raff,J.W., Kellum,R. and Alberts,B.M. (1994) The *Drosophila* GAGA transcription factor is associated with specific regions of heterochromatin troughout the cell cycle. *EMBO J.*, **13**, 5977–5983.

Reuter,G. and Spierer,P. (1992) Position-effect variegation and chromatin proteins. *BioEssays*, **14**, 605–612.

Reuter,G., Giarre,M., Farah,J., Gausz,J., Spierer,A. and Spierer,P. (1990) Dependence of position-effect variegation in *Drosophila* on dose of a gene encoding an unusual zinc- finger protein. *Nature*, **344**, 219–223.

Rubin,G.M. and Spradling,A.C. (1982) Genetic transformation of *Drosophila* with transposable element vectors. *Science*, **218**, 348–353.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanchez-Garcia,I. and Rabbits,T.H. (1994) The LIM domain: a new structural motif found in zinc-finger-like proteins. *Trends Genet.*, **10**, 315–320.

Saunders,W.S. *et al*. (1993) Molecular cloning of a human homologue of *Drosophila* heterochromatin protein HP1 using anti-centromere autoantibodies with anti-chromo specificity. *J. Cell Sci.*, **104**, 573–582.

Saurin,A.J., Shiels,C., Wiliamson,J., Satijn,D.P., Otte,A.P., Sheer,D. and Freemont,P.S. (1998) The human Polycomb group complex associates with pericentromeric heterochromatin to form a novel nuclear domain. *J. Cell Biol.*, **142**,887–898.

Schneuwly,S., Klemens,R. and Gehring,W.J. (1987) Redisigning the body plan of *Drosophila* by ectopic expression of the homeotic gene *Antennapedia*. *Nature*, **325**, 816–818.

Sewalt,R.G., van der Flag,J., Gunster,M.J., Hamer,K.M., den Blaauwen,J., Satijn,D.P., Hendrix,T., van Driel,R. and Otte,A.P. (1998) Characterisation of interactions between the mammalian Polycomb-group proteins Enx1/EZH2 and EED suggests the existence of different mammalian Polycomb-group protein complexes. *Mol. Cell. Biol.*, **18**, 3586–3595.

Shelby,R.D., Vafa,O. and Sullivan,K.F. (1997) Assembly of CENP-A into centromeric chromatin requires a cooperative array of nucleosomal DNA contact sites. *J. Cell Biol.*, **136**, 501–513.

Silver,L.M and Elgin,S.C.R. (1978) Immunological analysis of protein distributions in *Drosophila* polytene chromosomes. In Busch,H. (ed.), *Chromatin. Part B: The Cell Nucleus.* Vol. 5. Academic Press, New York, NY, pp. 215–262.

Singh,P.B., Miller,J.R., Pearce,J., Kothary,R., Burton,R.D., Paro,R., James,T.C. and Gaunt,S.J. (1991) A sequence motif found in a *Drosophila* heterochromatin protein is conserved in animals and plants. *Nucleic Acids Res.*, **19**, 789–794.

Sullivan,K.F., Hechenberger,M. and Masri,K. (1994) Human CENP-A contains a histone H3 related fold domain that is required for targeting to the centromere. *J. Cell Biol.*, **127**, 581–592.

Thon,G., Cohen,A. and Klar,A.J.S. (1994) Three additional linkage groups that repress transcription and meiotic recombinaiton in the mating-type region of *Schizosaccharomyces pombe*. *Genetics*, **138**, 29–38.

Torok,T., Harvie,P.D., Buratovich,M. and Bryant,P.J. (1997) The product of the *proliferation disrupter* is concentrated at centromeres and required for mitotic chromosome condensation and cell proliferation in *Drosophila*. *Genes Dev.*, **11**, 213–225.

Tschiersch,B., Hofmann,A., Krauss,V., Dorn,R., Korge,G. and Reuter,G. (1994) The protein encoded by the *Drosophila* position-effect variegation suppressor gene *Su(var)3-9* combines domains of antagonistic regulators of homeotic gene complexes. *EMBO J.*, **13**, 3822–3831.

van Lohuizen,M., Tijms,M., Voncken,J.W., Schumacher,A., Magnuson,T. and Wientjens,E. (1998) Interaction of mouse Polycomb-group (Pc-G) proteins Enx1 and Enx2 with Eed: indication for separate Pc-G complexes. *Mol. Cell. Biol.* **18**, 3572–3579.

Wallrath,L.L. (1998) Unfolding the mysteries of heterochromatin. *Curr. Opin. Genet. Dev.*, **8**, 147–153.

Wang,G., Horsley,D., Ma,A., Otte,A.P., Hutchings,A., Butcher,G.W. and Singh,P.B. (1997) M33, a mammalian homologue of *Drosophila* Polycomb localises to euchromatin within interphase nuclei but is enriched within the centromeric heterochromatin of metaphase chromosomes. *Cytogenet. Cell Genet.*, **78**, 50–55.

Warburton,P.E. *et al*. (1997) Immunolocalisation of CENP-A suggests a distinct nucleosome structure at the inner kinetochore plate of active centromeres. *Curr. Biol.*, **7**, 901–904.

Williams,B.C., Murphy,T.D., Goldberg,M.L. and Karpen,G.H. (1998) Neocentromere activity of structurally acentric mini-chromosomes in *Drosophila*. *Nature Genet.*, **18**, 30–37.

Wreggett,K.A., Hill,F., James,P.S., Hutchings,G.W. and Singh,P.B. (1994) A mammalian homologue of *Drosophila* heterochromatin protein1 (HP1) is a component of constitutive heterochromatin. *Cytogenet. Cell Genet.*, **66**, 99–103.

Zuckerkandl,E. (1974) A possible role of 'inert' heterochromatin in cell differentiation. *Biochimie*, **56**, 937–954.

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