Review

Heterochromatin and the molecular mechanisms of 'parent-of-origin' effects in animals

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Twenty five years ago it was proposed that conserved components of constitutive heterochromatin assemble heterochromatin-like complexes in euchromatin and this could provide a general mechanism for regulating heritable (cell-to-cell) changes in gene expressibility. As a special case, differences in the assembly of heterochromatin-like complexes on homologous chromosomes might also regulate the parent-of-origin-dependent gene expression observed in placental mammals. Here, the progress made in the intervening period with emphasis on the role of heterochromatin and heterochromatin-like complexes in parent-of-origin effects in animals is reviewed.

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Abbreviations used: 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; ADD, ATRX-DNMT3-DNMT3L domain; ATRX, Alpha Thalassemia/Mental Retardation Syndrome X-Linked; CAF-1, chromatin assembly factor 1; CD, chromodomain; CE, controlling element; CF, chromocenter formation; CGIs, CpG islands; CSD, chromo shadow domain; DamID, DNA adenine methyltransferase identification; DAXX, Death Domain associated protein; DNMT1, maintenance DNA methyltransferase 1; DNMT3A, *de novo* DNA methyltransferase 3A; DNMT3B, *de novo* DNA methyltransferase 3B; DNMT3L, DNA methyltransferase 3L; ES, embryonic stem; G9a, K9H3 HMTase; GLP, G9a-like protein K9H3 HMTase; gDMRs, germline differentially methylated regions; H3K9me2, *di*-methylated lysine 9 on histone H3; H3K9me3, *tri*-methylated lysine 9 on histone H3; H3S10P, phosphorylation of serine 10 on histone H3; H4K20me3, *tri*-methylated lysine 20 on histone H4; HMTases, histone methyltransferases; HDACs, histone deactylases; HR, hinge region; HP1, Heterochromatin Protein 1; ICR, imprinting control region; IPS, induced pluripotent stem cell; KAP1, KRAB-associated protein 1; Kb, kilobases; KRAB-ZNPs, Krüppel-associated box (KRAB) domain zinc-finger proteins; KRAB-ZNF, KRAB domain-zinc finger; LINE, long interspersed elements; lncRNA, long non-coding RNA; LTR, long terminal repeat; Mb, megabases; MEFs, murine embryonic fibroblasts; MuERV-L, murine endogenous retrovirus L; NOR, nucleolar organizer region; Np95, Nuclear protein 95; NuRD, Nucleosome Remodelling histone Deacetylase; ORC, origin of replication complex; PCHET2, *Plannococcus citri* heterochromatin protein 2; PCNA, proliferating cell nuclear antigen; PGCs, primordial germ cells; PHD, plant homeodomain; PEV, position-effect variegation; pN, pico-Newtons; PN, pro-nuclear stage; PNBs, peri-nucleolar bodies; PxVxL, Proline/Any/Valine/Any/Leucine penta-peptide motif; RBCC, Ring-finger B Box-Coiled Coil domain; reverse majSat, reverse major satellite sequence; RNF12/RLIM, Ring Finger protein LIM Domain interacting; RRR, reprogramming resistant regions; SCNT, somatic cell nuclear transfer; SETDB1, SET Domain Bifurcated 1 K9H3 HMTase; siRNA, small interfering RNA; SMARCAD1, SWI/SNF-Related, Matrix-Associated Actin-Dependent Regulator of Chromatin, Subfamily A, Containing DEAD/H Box 1; SUMO2, Small ubiquitin-related modifier 2; SUV39H1/2, mammalian suvar K9H3 HMTase 1 and 2; Tet, Ten-eleven translocation dioxygenase; UBE2i, Ubiquitin conjugating enzyme 2i; X_p, paternal X-chromosome; X_m, maternal X chromosome; ZGA, zygotic genome activation; Zscan4, Zinc Finger and SCAN domain-containing 4.

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1. Introduction

It was with some satisfaction that Gregor Mendel wrote in his seminal work that ushered in the discipline of Genetics: '…it is perfectly immaterial whether the dominant character belongs to the seed-bearer or to the pollen parent; the form of the hybrid remains identical in both cases' (Mendel 1866). As we know, the significance of Mendel's work remained unnoticed by the scientific world during his own lifetime and the circumstances surrounding its rediscovery by Correns, de Vries and Tschermak in 1900 have been well documented (for example, Bateson 1909). Less well known is that within the first few decades of the twentieth century Mendel's claim that reciprocal crosses are equivalent had been brought into question by the finding of parent-of-origin effects.

Early genetic and cytogenetic analyses in insects revealed remarkable parent-of-origin-specific behaviour of chromosomes and chromosome sets. In the lecanoid chromosome system found in a diverse group of coccid families, including the mealy bugs, an entire haploid chromosome set becomes heterochromatic when inherited from one parent while, in the same nucleus, the homologues remain euchromatic. In the fungus gnat, *Sciara coprophila*, programmed elimination of whole chromosomes occurs during various stages of development. It was careful study of the chromosome eliminations in *Sciara* that led to the discovery of parent-of-origin-specific behaviour of chromosomes by Charles Metz in the 1920s and prompted him to ask: 'How do the chromosomes know which parent they come from?' (cited in Chandra and Brown 1975). Here, I aim to provide a tentative answer using an evolutionary approach that consists of two steps. First, I describe studies on parent-of-origin effects in the classical insect systems alluded to above. These studies have shown that heterochromatin plays a key role in parent-of-origin-specific behaviour of chromosomes. Second, I describe how conserved components of heterochromatin are likely to be part of the mechanism that specifies the 'imprint' in placental mammals, thereby proffering an answer to Metz's almost century old query.

2. Heterochromatin and the parent-of-origin effect in coccids: Conservation of the H3K9me3:HP1:H4K20me3 pathway

Heterochromatin is the largest differentiated chromatin compartment in eukaryotic nuclei. It is either constitutive or facultative (Brown 1966). Constitutive heterochromatin is found surrounding the centromeres, at the telomeres and at the nucleolar organizer regions (Heitz 1928; Yunis and Yasmineh 1971). These chromosomal regions can be stained with simple dyes and shown to be condensed throughout the cell cycle in different cells types. Facultative heterochromatin is developmentally-regulated and was first described in coccids (superfamily Coccideae), especially the *Pseudococcidae* family of mealy bugs (reviewed by Hughes-Schrader 1948); facultative heterochromatin was later observed in female mammals with the phenomenon of X-chromosome inactivation (Lyon 1961). The mealy bug system is an epigenetic *tour-der-force* (reviewed in Brown and Nur 1964; White 1954). In mealy bugs the chromosomes within the newly-fertilized zygote are euchromatic and remain so during the early embryonic cleavages. It is at the seventh to eighth cleavage that a cytological difference in the parental chromosomes becomes distinct and then only in embryos destined to become male (Bongiorni *et al.* 2001). In a male embryo, an entire haploid set of chromosomes becomes heterochromatic and that set is paternal in origin; in his sons the maternal set he inherited becomes heterochromatic; both sets are euchromatic in females (Figure 1A). These observations indicate that the parental origin of the chromosome sets must be molecularly distinguishable at the syncytial blastoderm stage in presumptive males, whereupon there is specific facultative heterochromatinization of paternally derived chromosomes.

Attempts to identify the 'imprint' that enables discrimination at blastoderm have focussed on the epigenetic signature of paternal chromatin. This has included analysis chromatin structure (Khosla *et al.* 1996, 1999), histone modification (Bongiorni *et al.* 2009) and DNA methylation (Bongiorni *et al.* 1999; Buglia *et al.* 1999; Mohan and Chandra 2005). Approximately 10% of sperm chromatin is nuclease resistant (Khosla *et al.* 1996). The sperm and the pro-nucleus it forms in the zygote are enriched in *tri*-methylated lysine 9 of histone H3 (H3K9me3; Bongiorni *et al.* 2009). There have been conflicting results with DNA methylation, with one study showing that paternal DNA is hypomethylated in both females and males (Bongiorni *et al.* 1999), a second that there is no significant difference in DNA methylation between parental chromosomes (Buglia *et al.* 1999) and a third that methylation of paternal DNA is often found to be greater in males than in females (Mohan and Chandra 2005). Because all sperm are likely to share the same epigenetic characteristics, and give rise to both sons and daughters, the sperm epigenotype cannot specify whether or not the paternal chromosomes will be subject to heterochromatinization at blastoderm. That choice must be under maternal control, a conclusion that was drawn some time ago. For example, the sex-ratio in coccids fluctuates from female to female and is markedly influenced by the mother's age (Nelson-Rees 1960). Indeed, there is evidence that imprinting is exclusively under maternal control without any contribution of the father. This comes from the facultative deuterotoky observed in *Pulvinaria hydrangea*, a soft scale related to

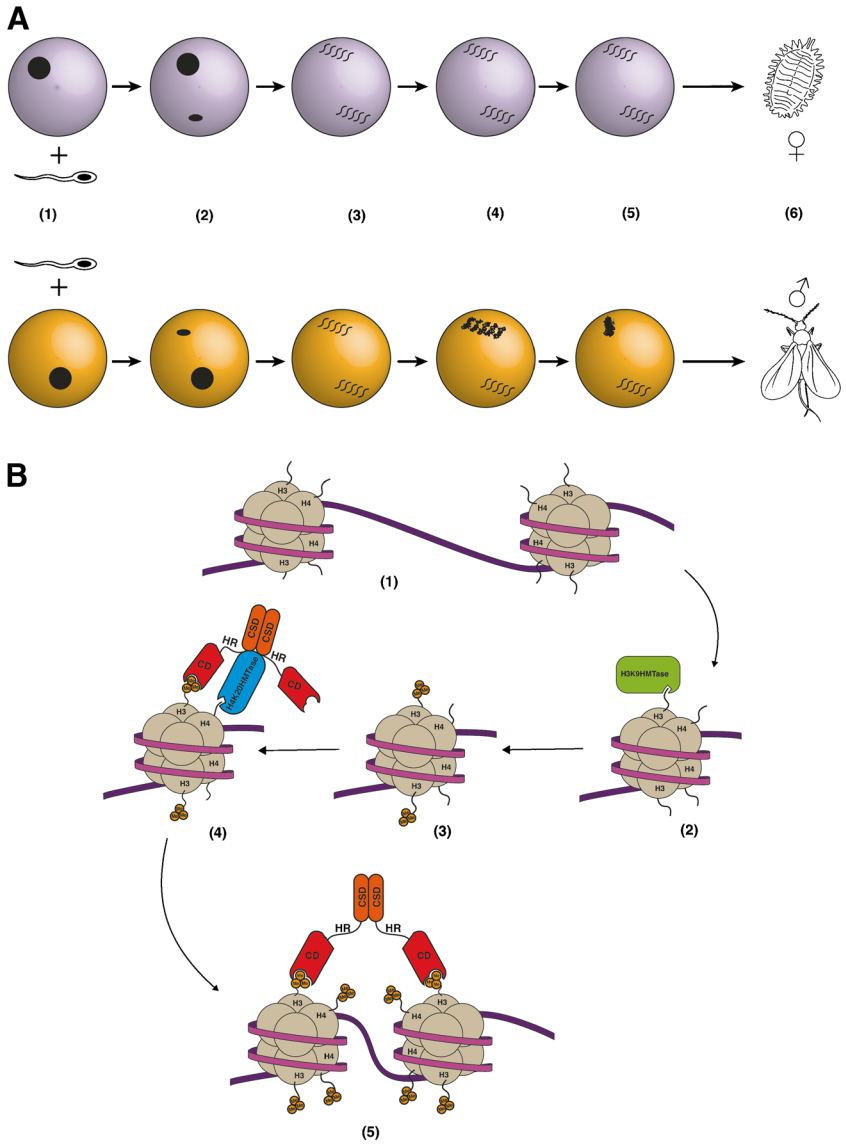

Figure 1. (**A**) Maternal regulation of parent-of-origin-specific heterochromatinization in the mealy bug *Plannococcus citri.* (1) Sperm fertilize two different types of egg. Those that are conditioned by the mother to direct female development (purple) or those that are conditioned to direct male development (orange). (2) In 'male-conditioned' eggs the maternal ooplasm 'imprints' one of the two parental genomes such that the paternal chromosome set is heterochromatinized at the blastoderm stage. (3) During the early cleavage divisions both parental sets of chromosomes are cytologically indistinguishable. (4) In the syncytial blastoderm, at around the seventh to eighth embryonic cleavage, heterochromatinzation of the paternal chromosomes becomes distinct in presumptive males. (5) In the adult male most of the tissues possess a heterochromatic 'chromocenter' that results from the aggregation of the heterochromatinized paternal chromosomes; the maternal set in males remains euchromatic. Both sets are euchromatic in females. (6) Males and females exhibit a profound sexual dimorphism, which reflects the chromosomal dimorphism observed in their nuclei. (**B**) The H3K9me3:HP1:H4K20me3 pathway. (1) Nucleosomal organization of DNA. For sake of clarity the tails for only four histones are shown. (2) H3K9 HMTase(s) methylate lysine 9 of histone H3. (3) The H3K9 HMTase(s) generate the tri-methylated form of lysine 9 on histone H3 (H3K9me3). (4) H3K9me3 provides a high-affinity binding site for HP1 proteins which are composed of two sequence-related structures called the chromo domain (CD) and the chromo shadow domain (CSD) that are connected by a flexible hinge region (HR). The CD binds H3K9me3 while the CSD dimerises. HP1 recruits H4K20 HMTase(s). (5) The H4K20 HMTase(s) *tri*-methylate lysine 20 on histone H4. The association of H3K9me3:HP1:H4K20me3 pathway with parent-of-origin-specific regulation of gene expression was initially described in mealy bugs (Cowell *et al.* 2002; Kourmouli *et al.*, 2004; Bongiorni *et al.*, 2007). The H3K9me3:HP1:H4K20me3 pathway is conserved and is associated with paternal inactivation of the X-chromosome in marsupials (Rens *et al.* 2010) and the gDMRs of autosomally-imprinted genes in the mouse (Pannetier *et al.* 2008; Regha *et al.* 2007).

coccids, where impaternate males (with a heterochromatic set) and females (both sets euchromatic) result from the fusion of the two haploid division products of the female pro-nucleus (reviewed in Brown and Nur 1964). As cogently argued by Chandra and Brown (1975) it is unlikely that a new mechanism would evolve to produce unnecessary males so the system in *P. hydrangea* evolved from what is already present in zygogenetic species and concluded that imprinting was strictly under maternal control and takes place during the time when the maternal and paternal pro-nuclei lie separately within the ooplasm. There was no need to posit any imprint carried on the sperm that might direct heterochromatinization. Nevertheless, the identification of epigenetic modifications or lack thereof in paternal chromatin (Khosla *et al.* 1996, 1999; Buglia et al. 1999; Mohan and Chandra 2005; Bongiorni *et al.* 1999, 2009) is noteworthy, albeit their relationship to an imprint that causes heterochromatinization at blastoderm has yet to be proven. Putting the case for a paternal epigenetic imprint at its highest, depending on the conditioning of the ooplasm by the mother, an imprint on the sperm may be registered at blastoderm leading to heterochromatinization and male development, ignored, or even erased, leaving the chromosomes euchromatic thereby instructing female development (Figure 1A).

The facultative heterochromatinization that takes place at blastoderm has been studied in the coccid, *Plannococcus citri*. Staining of male and female embryonic nuclei with antibodies to H3K9me3 and *tri*-methylated lysine 20 of histone H4 (H4K20me3) showed that both modifications localized to the heterochromatic chromosome set in males (Figure 1B) (Cowell *et al.* 2002; Kourmouli *et al.* 2004). There is known to be epigenetic 'cross-talk' between these two modifications that is effected by binding of HP1 to H3K9me3 and, once bound, HP1 recruits H4 K20 histone methyltransferases (HMTases) to generate H4K20me3 (Figure 1B) (Kourmouli *et al.* 2004; Schotta *et al.* 2004; Kourmouli *et al.* 2005). The function of the H3K9me3:HP1:H4K20me3 pathway has been tested by siRNA-mediated 'knockdown' of the *P. citri* HP1 homologue PCHET2 (Epstein *et al.* 1992), which caused a precipitous de-condensation of the paternal heterochromatic set with loss of both H3K9me3 and H4K20me3 staining (Bongiorni *et al.* 2007). Developmental reversal of the H3K9me3:HP1:H4K20me3 pathway can be observed during the de-heterochromatinzation that takes place in some adult male tissues, where H3K9me3 remains associated with the de-condensing paternal chromosome set, HP1 becomes loosely associated and H4K20me3 is completely dissociated and found distributed through the cytoplasm (Bongiorni *et al.* 2007). The H3K9me3:HP1:H4K20me3 pathway is conserved and associated with parent-of-origin-specific regulation of chromosomes and genes in other species. In marsupials, an H3K9me3:HP1:H4K20me3 pathway likely operates to silence the paternal X-chromosomes, which are also DNA hypomethylated (Rens *et al.* 2010). Both H3K9me3 and H4K20me3 modifications also localize to germline differentially methylated regions (gDMRs) within imprinted clusters and genes (Regha *et al.* 2007; Henckel *et al.* 2009; McEwen and Ferguson-Smith 2010; Strogantsev *et al.* 2015) and, where it has been investigated, co-localize with HP1 proteins (Regha *et al.* 2007; Pannetier *et al.* 2008).

3. Heterochromatin and parent-of-origin effects in *Sciara*: The 'controlling element'

Parent-of-origin-specific behaviour of chromosomes was first observed in classical studies on chromosome eliminations in the fungus gnat, *Sciara coprophila*. Chromosome loss occurs at various stages of development, namely during: (i) the early embryonic divisions, (ii), germ-line development and, (iii), male meiosis (for reviews, see Metz 1938 and Gerbi 1986). It was after a careful analysis of meiosis in the male that the term 'chromosome imprinting' was coined by Helen Crouse (Crouse 1960). Male meiosis is highly aberrant; each primary spermatocyte gives rise to only one sperm due to the unequal nature of both meiotic divisions. Briefly, in meiosis I a monopolar spindle is formed and there is complete selective segregation of the autosomes and sex-chromosomes of maternal origin from those of paternal origin. The latter move away from the pole, are extruded and degenerate. In meiosis II the maternal X-dyad undergoes nondisjunction and both chromatids pass into what will become the sperm nucleus. The remaining chromosomes, now at the opposite pole, degenerate. Crouse noted: 'the "imprint" a chromosome bears is unrelated to the genic constitution of the chromosome and is determined only by the sex of the germ line through which the chromosome has been inherited' (Crouse 1960). While the term 'chromosome imprinting' was coined with regard to the behaviour of the chromosomes during male meiosis it is study of the early embryonic eliminations that has provided more recent mechanistic insight and the discussion here will focus on the parental control of these early eliminations (Figure 2). Accordingly, the extraordinary pattern of segregation during male meiosis produces sperm that contain two X chromosomes. Since meiosis in the female is conventional

and gives rise to haploid eggs, fusion of the male and female pro-nuclei in the zygote produces a nucleus containing three X chromosomes ($X_pX_pX_m$); two from the double-X sperm (X_pX_p) and one from the egg (X_m). Sex in fungus gnats is determined by the selective elimination of the paternal X-chromosomes in the soma during the seventh to ninth cleavage of embryonic development (Figure 2A). Both paternal X-chromosomes are eliminated giving rise to males, whereas elimination of one paternal X chromosome gives females. Completing the picture one paternal X chromosome is eliminated in the germ-lines on the first day of larval life. At the end of the eliminations sex chromosome constitution in the soma is typical, being XO for male and XX for female, while both germ-lines are XX.

As with coccids, the 'imprint' is heritable through the early cleavage divisions and manifests itself only at the seventh to ninth cleavage of embryonic development, whereupon one or two paternal X chromosomes are eliminated (Figure 2A). The search for Crouse's germline 'imprint' has centred upon DNA methylation. This is largely because of the observation that salivary gland polytene chromosomes in fourth instar *Sciara* larvae stain positively with anti-5-methyl cytosine antibodies, where DNA methylation correlates with gene activity (Eastman *et al.* 1980; Wei *et al.* 1981). However, studies using anti-5-methyl cytosine (5mC) antibodies and restriction enzyme isoschizomers Msp I and Hpa II have failed to detect 5mC in spermatocytes and testes DNA respectively (cited in Gerbi 1986). It seems that DNA methylation is an unlikely candidate for a sperm-specific imprint, although the role of DNA methylation in imprinting the paternal X chromosomes warrants re-investigation in light of more recent advances, which have shown that diploid cells in *Diptera* contain both DNA methylation and methyltransferase (Dnmt2) enzyme activity (Field *et al.* 2004). The analysis of histone methylation and acetylation also needs to be investigated as possible 'imprints' that cause the embryonic eliminations because parent-of-origin-specific differences in the chromosomal distributions of these histone modifications have been observed during the germ-line and meiotic eliminations (Goday and Ruiz 2002; Greciano and Goday 2006). The possession of polytene chromosomes in fourth instar larvae has nevertheless been pivotal in providing insight into the nature of the imprint. Along with the skilful interpretation of the behaviour of X:autosome translocations, careful inspection of said translocations in polytene chromosomes has enabled the precise cytological mapping of the genetic locus that causes the elimination of the paternal X chromosomes (Crouse 1960, 1977, 1979). First, it was shown that the terminal heterochromatin adjacent to the X centromere contains the element, termed the 'controlling-element' (CE), which controls the selective elimination of the paternal X chromosomes (Crouse 1960). Second, the terminal X heterochromatin was further subdivided into three heterochromomeres, H1, H2 and H3, of which H2 was necessary for CE activity (Figure 2B) (Crouse 1977; Crouse 1979). Sequence analysis has shown that the heterochromomeres harbour rDNA sequences (Crouse et al 1977), with H2 containing an additional 30 kb of non-rDNA sequence that may represent the *cis*-acting CE (cited in Gerbi 2007). The CE is the equivalent of a mammalian 'imprinting control region' (ICR; Ferguson-Smith 2011; Kelsey and Feil 2013) since it has been shown in a functional assay to control a parent-of-origin effect. The connection between heterochromatin and a defined genetic element that controls a parent-of-origin effect was so forged.

Notwithstanding the search for a sperm-specific imprint(s), it had been demonstrated already that the oocyte cytoplasm determines the eliminations of paternal X chromosomes. This was shown easily because *Sciara coprophila* is monogenic (reviewed by Metz 1938). A given female gives rise to a brood all of which are of the same sex. Gynogenic mothers produce families that contain daughters and differ genetically from androgenic mothers that produce exclusively sons. The difference is in the X chromosome of which there are two types, X′ and X; the X′ chromosome possesses a large para-centric inversion whose breakpoints have been mapped (Crouse 1977). Mothers that are X′X, produce exclusively daughters and XX mothers will have sons only (Figure 2A). So when a male inseminates two females, one X′X and the other XX, the outcomes are very different. This is because X′- and X-chromosomes in the mother condition the cytoplasm of the fertilized egg to cast aside the appropriate number of paternal X-chromosomes during the seventh to ninth cleavage of embryonic development. As with the coccid system it seems that the site and timing of imprinting likely takes place within the pro-nuclei of the newly fertilized zygote. If there is an imprint carried into the egg by the sperm its ability to cause later X chromosome elimination(s) depends strictly upon the genetic constitution of the mother.

Investigation of the mechanism by which the CE brings about the elimination of paternal X chromosomes during the seventh to ninth cleavage divisions has revealed that the CE does not affect the X centromere, which attaches to the spindle and separates without hindrance (de Saint Phalle and Sullivan 1996). Rather the elimination results from the inability of the X-chromosome arms to separate; there is a failure of sister chromatid separation (Figure 2B). The arms of the sister X chromatids retain high-levels of the histone modification

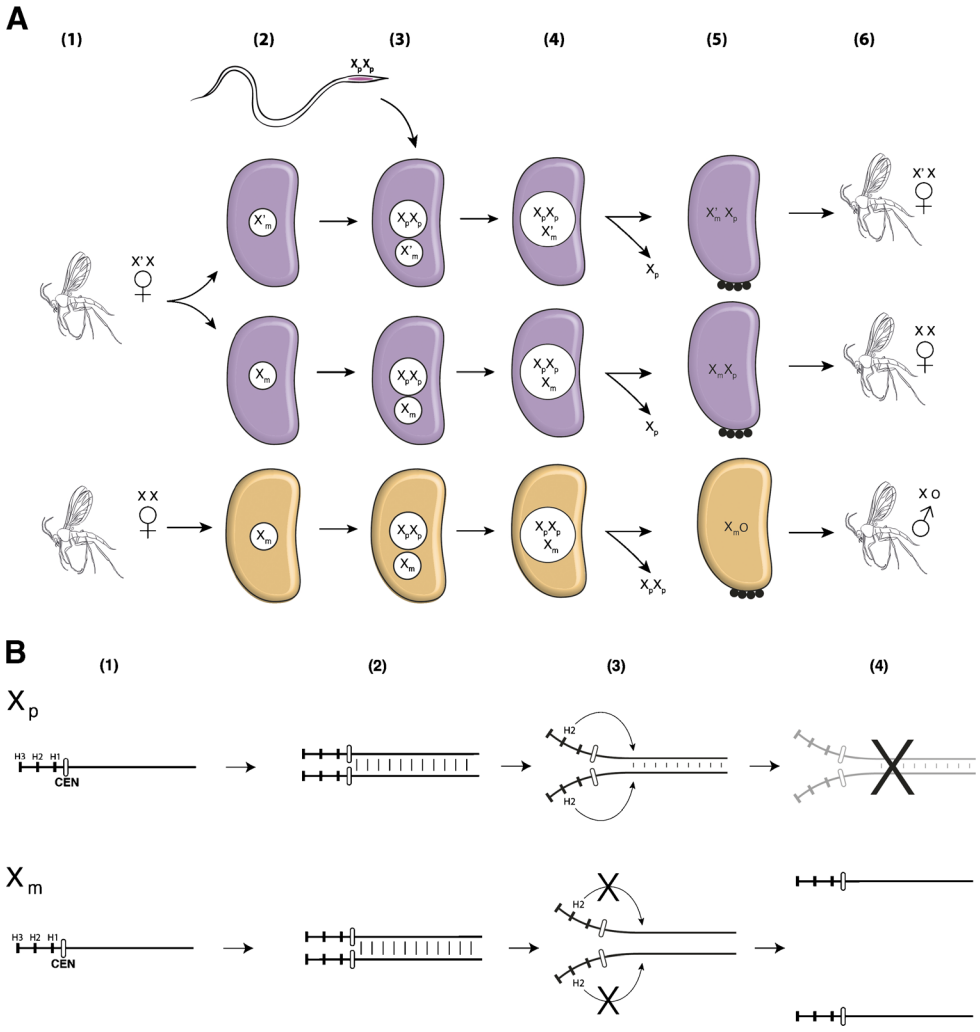

Figure 2. (**A**) Maternal regulation of parent-of-origin-specific elimination of X-chromosomes during embryogenesis in *Sciara coprophila*. (1) Gynogenic mothers are X′X and produce eggs that that are conditioned to give rise to daughters only (purple). Androgenic mothers are XX and produce eggs that that are conditioned to give rise to sons only (orange). (2) The eggs produced by X′X mothers are either X'_m or X_m, whilst androgenic mothers give rise to eggs that are X_m. (3) The sperm carries two X-chromosomes, X_pX_p, and forms a paternal pro-nucleus containing X_pX_p. Conditioning of the egg cytoplasm by X′X mothers leads to the later elimination of one of the two X_p chromosomes. In eggs conditioned by XX mothers both X_p's will be eliminated. (4) After fusion, the zygotic nuclei each possess three X-chromosomes - $X'_mX_pX_p$ or $X_mX_pX_p$ zygotes in eggs laid by X′X mothers and $X_mX_pX_p$ in eggs laid by XX mothers. (5) During the seventh to ninth embryonic cleavage one X_p is eliminated in embryos conditioned by X′X mothers, while both X_pX_p chromosomes are eliminated in embryos conditioned by XX mothers. (6) The eliminations result in gynogenic X′X females that will give rise to daughters only, androgenic XX females that will produce sons and XO males. The pole cells (black filled in circles) contain the germs cells which lose one X_p on the first day of larval life in both males and females. Note: In the interests of clarity, the autosomes and the germ-line 'limited' (L) chromosomes are not shown. For a complete description of the extraordinary chromosome behaviour in *Sciara* see Metz (1938) and Gerbi (1986). (**B**) The 'controlling element' in heterochromomere II (H2) acts in *cis* and over long distances. (1) The terminal X-heterochromatin adjacent to the centromere (open oval; CEN) contains the 'controlling element' (CE) (Crouse 1960). The terminal X heterochormatin can be further sub-divided into three cytologically-distinct heterochromomeres, H1, H2 and H3 of which H2 is necessary for CE function (Crouse 1977; Crouse 1979). (2) After DNA replication the sister chromatids remain aligned and connected before congression to the metaphase plate in preparation for separation to the poles at anaphase. (3) During anaphase the X-centromeres are fully functional and separate on both X_m and X_p. However, on the X_p the H2 heterochromomere is active and affects that ability of the sister chromatids to separate and they remain physically bound together. H2 is not active on X_m and the sister chromatids separate normally. (4) Because of the failure to separate, X_p remains on the metaphase plate and is eliminated. The X_m separates and each X_m chromatid enters the daughter nuclei.

H3S10P compared to other chromosomes (Escribá and Goday 2013), but it is not known if the H3S10P observed is a cause or consequence of the failure to separate.

Distinct functional characteristics can be ascribed to the CE. The CE, which is embedded in heterochromatin, acts *in cis*. It also acts at a distance; indeed, the X centromere lies between the CE and the arms that fail to separate (Figure 2B). The CE can affect chromosomes other than the X as evidenced by reciprocal translocations, which have shown that CE function can be translocated to an autosome and that H2 is necessary for that function (Crouse 1960; Crouse 1977; Crouse 1979). The imprint at the CE is heritable through several cell divisions before elimination of the paternal X chromosomes. These characteristics of the CE are very familiar because they are shared with a well-known heterochromatin-associated phenomenon called position-effect variegation (PEV). PEV was discovered in *Drosophila melanogaster* and has been studied for many decades, and several excellent discussions about its genetics and molecular biology are found in the literature (e.g., Lewis 1950; McClintock 1951; Baker 1968; Spofford 1976; Weiler and Wakimoto 1995; Elgin and Reuter 2013). In essence, classical PEV is a disruption of the boundary between euchromatin and heterochromatin by chromosomal rearrangement where one or both breakpoints lie within or near heterochromatin. In the new arrangement heterochromatin can 'spread' from within its normal confines and cause variable repression of euchromatic genes newly juxtaposed to heterochromatin leading to their phenotypic variegation. Findings from the study of variegating breakpoints bear on the characteristics described for the CE. PEV, like the CE acts *in cis* (Baker 1968) although, exceptionally, PEV does act *in trans* such as in the case of $brown^{Dominant}$ (bw^{D}) variegation (Slatis 1955). Practically any gene can be subject to PEV and once affected the change in expression can be propagated through many cell divisions (Baker 1963). PEV is also subject to parent-of-origin effects (Spofford 1959; Spofford 1961; reviewed in Singh 1994). The heterochromatic characteristics shared by the CE and PEV indicate that the mechanisms involved are likely to be similar.

Heterochromatin is the leitmotif connecting the imprinting systems in coccids, *Sciara* and paternal X chromosome inactivation. When this was recognized some 25 years ago, it was suggested that isolation of evolutionarily-conserved components of heterochromatin could provide mechanistic insight into parent-of-origin phenomena and that such components could be identified based on discoveries made with PEV in *Drosophila* (Singh *et al.* 1991). Using cross-species hybridisation cDNAs encoding mammalian HP1 proteins were isolated and related sequences were identified in a variety of animal and plant species (Singh *et al.* 1991); mammalian SUV39H proteins were isolated using a similar approach some time later (Aagard *et al.* 1999). Along with the demonstration that HP1 proteins were conserved two different, but related, hypotheses were put forward (Singh *et al.* 1991; Singh 1994). First, that during evolution components of constitutive heterochromatin had been recruited to sites within euchromatin where they formed "heterochromatin-*like*" complexes, which bring about heritable changes in gene expressibility. Second, as a special case of the first, that difference in the assembly of heterochromatin-like complexes on homologous chromosomes could regulate the parent-of-origin-dependent gene expression observed in placental mammals.

4. Mammalian HP1 proteins and heterochromatin-like complexes

Mammalian HP1 proteins, termed HP1α, HP1β and HP1γ, are small ~25 kD molecules that have two structured domains, an N-terminal chromodomain (CD) and a sequence-related C-terminal chromo shadow domain (CSD), linked by an unstructured, flexible, hinge region (HR) that can interact with nuclei acid (Muchardt *et al.* 2002; Meehan *et al.* 2003; Maison and Almouzni 2004). The CD binds H3K9me, with the highest affinity for the *tri*-methylated form (H3K9me3; Nielsen *et al.* 2002). The CSD dimerises to form a 'hydrophobic pocket', which can bind a penta-peptide motif, PxVxL, found in many HP1-interacting proteins (Smothers and Henikoff 2000; Thiru *et al.* 2004). As a consequence of the plethora of interacting partners, HP1 proteins exist in different kinetic populations within the nucleus, from highly mobile to tightly bound, immobile, species (Cheutin *et al.* 2003; Festenstein *et al.* 2003; Schmiedeberg *et al.* 2004). They have many functions, such as in gene repression, DNA repair, transcriptional elongation and RNA splicing (Hediger and Gasser 2006; Kwon and Workman 2008; Dinant and Luijsterburg 2009; Smallwood *et al.* 2012; Yearim *et al.* 2015). In biochemical assays HP1 isotypes are interchangeable and can form heterodimers (Nielsen *et al.* 2001). Despite this they are not functionally redundant as evidenced by their different mutant phenotypes (Aucott *et al.* 2008; Brown *et al.* 2010; Singh 2010). Immuno-localization studies have shown that HP1α and HP1β are enriched within constitutive heterochromatin, while HP1γ has a more euchromatic distribution (Minc *et al.* 1999). The fine 'punctate' distribution of HP1γ (then known as M32) throughout euchromatin was interpreted as direct visualization of heterochromatin-like complexes in mammalian cells (Horsley *et al.* 1996). Genome wide studies have greatly extended this initial observation, showing that HP1-containing heterochromatin-like complexes are widespread and can be large in size, reaching Mb size domains (Table 1). Considerable progress has also been made in understanding how such complexes are targeted to specific sites within the

Table 1. Major structural and enzymatic constituents, along with histone/DNA modifications, associated with mammalian constitutive heterochromatin, which are shared with heterochromatin-like domains/complexes.

Constitutive Heterochromatins	**Size**	**H3K9MTase**	**H3K9me3**	**H4K20MTase**	**H4K20me3**	**HP1**	**DNMTase**	**5mC**	**Np95**	**ATRX/DAXX**	**H3.3**	**KAP1**
Peri-centric	Human ~0.2 – 20Mb [1] Mouse ~6Mb [2]	✓ SUV39H1/2 [3,4] SETDB1 [5] G9a/GLP [6]	✓ [7]	✓ SuvH4201/2 [8]	✓ [8,9]	✓ HP1α [10,11] HP1β [11,12] HP1γ [11,13]	✓ DNMT1 [14] DNMT3A [15] DNMT3B [16]	✓ [17,18]	✓ [19,20]	✓ [21,22,23]	✓ [21,22,23]	✓ [5]
Telomeric plus sub-telomeric	Human ~10-300Kb [1] Mouse ~5Mb [24]	✓ SUV39H1/2 [25]	✓ [25,26]	✓ SuvH4201/2 [26,27]	✓ [26,27,28]	✓ HP1α HP1β [26,27,28]	✓ DNMT1 DNMT3A DNMT3B [26,27,28]	✓ [26,27,28]	NK	✓ [21,22,23]	✓ [21,22,23]	NK
NOR plus peri-nucleolar	Human ~250Kb - 6.5Mb [29,30] Mouse NK	✓ SUV39H1 [31]	✓ [32,33]	✓ SuvH420/2 [34]	✓ [33]	✓ HP1α HP1β HP1γ [35]	✓ DNMT1 DNMT3A DNMT3B [35,36,37]	✓ [35,36]	NK	✓ [38]	✓ [38]	NK
Heterochromatin-like domains	**Size**	**H3K9MTase**	**H3K9me3**	**H4K20MTase**	**H4K20me3**	**HP1**	**DNMTase**	**5mC**	**Np95**	**ATRX/DAXX**	**H3.3**	**KAP1**
Odorant receptors	Human 0.1Mb to 1Mb [39] Mouse 1 to 5Mb [40]	✓ G9a/GLP [41]	✓ [41,42]	✓ SuvH4201/2 [41]	✓ [41,42]	✓ HP1β [43]	NK	NK	NK	NK	NK	NK
KRAB-ZNF gene clusters	Human and mouse. Up to 4Mb [44]	✓ SUV39H1 [44]	✓ [44,45]	NK	NK	✓ HP1β [44,45]	NK	NK	NK	NK	vNK	✓ [45]
SCNT Reprogramming Resistant Regions (RRRs)	Human NK Mouse Up to 2Mb [46]	✓ SUV39H1 [46]	✓ [46]	NK	NK	NK	NK	NK	NK	NK	NK	NK
Topologically associated domains (TADs)	Human and mouse ~1Mb [47,48]	NK	✓ [47]	NK	NK	✓ HP1γ [47]	NK	NK	NK	NK	NK	NK
Heterochromatin-like complexes	**Size**	**H3K9MTase**	**H3K9me3**	**H4K20MTase**	**H4K20me3**	**HP1**	**DNMTase**	**5mC**	**Np95**	**ATRX/DAXX**	**H3.3**	**KAP1**
3′ end of KRAB-ZNF genes	Human and mouse ~2Kb [44]	✓ SETDB1 [49,50]	✓ [49,50]	NK	NK	✓ HP1β [44,45]	NK	NK	NK	✓ [51]	✓ [51]	✓ [45,50,52]
IPS reprogramming resistant regions	Human NK Mouse NK	✓ SETDB1 [53,54]	✓ [53,54,55]	NK	NK	✓ HP1γ [55]	NK	NK	NK	NK	NK	NK
gDMRs of imprinted genes	Human NK Mouse ~2-6Kb [56]	✓ SETDB1 [57]	✓ [56,58,59]	✓ SuvH4201/2 [59]	✓ [56,58,59]	✓ HP1α HP1β [56] HP1γ [56,59,60]	✓ DNMT1 DNMT3A DNMT3B [60,61]	✓ [62,63,64]	✓ [60]	✓ [65]	✓ [65]	✓ [60,66]

✓= present. NK = not known. References are given in square brackets and are as follows: **1**, International Human Genome Sequencing, 2004; **2**, Choo 1997; **3**, Peters *et al*. 2001; **4**, Lehnertz *et al*. 2003; **5**, Loyola *et al*. 2009; **6**, Rowbotham *et al*. 2011; **7**, Cowell *et al*. 2002; **8**, Schotta *et al*. 2004; **9**, Kourmouli *et al*. 2004; **10**, Saunders *et al*. 1993; **11**, Minc *et al*. 1999; **12**, Wreggett *et al*. 1994; **13**, Horsley *et al*. 1996; **14**, Leonhardt *et al*. 1992; **15**, Jurkowska *et al*. 2011; **16**, Bachman *et al*. 2001; **17**, Lubit *et al*. 1976; **18**, Baumann *et al*. 2008; **19**, Papait *et al*. 2007; **20**, Liu *et al*. 2013; **21**, Drane *et al*. 2010; **22**, Goldberg *et al*. 2010; **23**, Wong *et al*. 2010; **24**, Chambers *et al*. 2013; **25**, García-Cao *et al*. 2004; **26**, Blasco 2007; **27**, Gonzalo *et al*. 2005; **28**, Benetti *et al*. 2007; **29**, Stults *et al*. 2008; **30**, McStay 2016; **31**, Murayama *et al*. 2008; **32**, Chakrabarti *et al*. 2015; **33**, Guetg *et al*. 2010; **34**, Bierhoff *et al*. 2014; **35**, Santoro *et al*. 2002; **36**, Gagnon-Kugler *et al*. 2009; **37**, Schmitz *et al*. 2010; **38**, Rapkin *et al*. 2015; **39**, Mombaerts 2001; **40**, Zhang *et al*. 2007; **41**, Lyons *et al*. 2014; **42**, Magklara *et al*. 2011; **43**, Clowney *et al*. 2012; **44**, Vogel *et al*. 2006; **45**, Groner *et al*. 2010; **46**, Matoba *et al*. 2014; **47**, Le Dily *et al*. 2014; **48**, Dixon *et al*. 2012; **49**, Frietze *et al*. 2010; **50**, Iyengar *et al*. 2011; **51**, Valle-Garcia *et al*. 2016; **52**, O'Geen *et al*. 2007; **53**, Bilodeau *et al*. 2009; **54**, Chen *et al*. 2013; **55**, Sriharan *et al*. 2013; **56**, Regha *et al*. 2007; **57**, Leung *et al*. 2014; **58**, Strogantsev *et al*. 2015; **59**, Pannetier *et al*. 2008; **60**, Quenneville *et al*. 2011; **61**, Zuo *et al*. 2012; **62**, Li *et al*. 1993; **63**, Smallwood *et al*. 2011; **64**, Kobayashi *et al*. 2012; **65**, Voon *et al*. 2015; **66**, Messerschmidt *et al*. 2014.

genome and replicated from one cell generation to the next. It is to these advances I now turn (sections 4.1 to 4.3) before going on to discuss the role of heterochromatin-like complexes in regulating parent-of-origin-dependent gene expression in placental mammals (section 5).

4.1 *Characterization of HP1-containing heterochromatin-like complexes and larger domains*

The number of HP1γ binding sites in the human genome is in the region of 6,000 to 8,500 depending on the cell line investigated, with the majority of sites being associated with gene activity (Smallwood *et al.* 2012). In murine ES cells there is a strong correlation of HP1β with H3K9me3 throughout the genome (Hiragami-Hamada *et al.* 2016), with 2,100 genes being associated with H3K9me3 that include key developmental regulators (Bilodeau *et al.* 2009). Notably, H3K9me3 sites generated by the HTMase SETDB1 are a barrier to efficient generation of IPS cells from MEFs (Chen *et al.* 2013; Sridharan *et al.* 2013), in part because of the recruitment of HP1γ to pluripotency genes (Sridharan *et al.* 2013). Somatic cell nuclear transfer (SCNT) studies have also identified 222 reprogramming resistant regions (RRRs) that are resistant to reprogramming at the 2-cell stage when the murine embryo undergoes major zygotic genome activation (ZGA) (Matoba *et al.* 2014). RRRs possess characteristics of constitutive heterochromatin, being gene-poor and enriched in H3K9me3 generated by the Suv39h1/2 HMTases (Matoba *et al.* 2014). RRRs can be up to 2Mb in size and are replete with specific LINE and LTR repeat sequences. The genes resident in RRRs encode products that are likely to be involved in mRNA processing and transcription. Within the latter group are genes that encode Krüppel-associated box (KRAB) domain zinc-finger proteins (KRAB-ZNPs) (Matoba *et al.* 2014). Significantly, studies on KRAB-ZFPs have provided considerable insight into the biology of heterochromatin-like complexes.

DamID mapping of the human genome has shown that HP1β and the K9 HMTase SUV39H1 have a preference for large KRAB domain-zinc finger (KRAB-ZNF) gene clusters (Vogel *et al.* 2006). KRAB-ZNF genes represent one of the largest families of transcriptional regulators in mammals. In man, there are 423 genes that encode 742 different KRAB-ZFPs (Lupo *et al.* 2013). High-resolution DamID mapping of KRAB-ZNF clusters on human chromosome 19, where the majority are to be found, showed that HP1β-containing heterochromatin-like complexes can form large domains from 0.1 to 4Mb in size (Vogel *et al.* 2006). HP1β binding is elevated throughout the KRAB-ZNF clusters compared to regions outside the clusters, and analysis of a specific cluster on chromosome 19, encompassing the ZNF77 and ZNF57 genes, has shown that HP1β binding is co-extensive with H3K9me3 (Groner *et al.* 2010). However, there are significant variations along a cluster, with enrichment of HP1β at 3′ end of KRAB-ZNF genes and depletion in the 5′ promoter regions (Vogel *et al.* 2006; Groner *et al.* 2010). Intriguingly, the heterochromatin-like complexes are targeted to the 3′ end of the KRAB-ZNF genes by the KRAB-ZNPs themselves (O'Geen *et al.* 2007; Frietze *et al.* 2010).

Central to the assembly of the heterochromatin-like complexes at the 3′ end of the KRAB-ZNF genes is the KRAB-associated protein 1, KAP1 (also known as Tif1β, TRIM28 or KRIP1) (Friedman *et al.* 1996; Le Douarin *et al.* 1996; Moosmann *et al.* 1996). KAP1 is a modular protein, which is tethered to the 3′ ends of the KRAB-ZNF genes through the binding of its RBCC domain to the KRAB domain of a KRAB-ZNP (Frietze *et al.* 2010) (Figure 3). One molecule of KAP1 in turn recruits a dimer of HP1 molecules through the PxVxL motif in KAP1 called the HP1-box (Ryan *et al.* 1999; Lechner *et al.* 2000) (Figure 3). KAP1 binds equally well to the HP1α/β/γ isotypes in biochemical assays (Ryan *et al.* 1999; Lechner *et al.*, 2000). There is interdependence between KAP1 and HP1 since KAP1 mutants lacking the HP1-box show reduced binding to KRAB-ZNFs genes (Iyengar and Farnham 2011). KAP1 and HP1 may mutually re-inforce their binding to chromatin because KAP1 is a homo-trimer (Peng *et al.* 2000) and would enhance the stoichiometry of HP1-H3K9me3 interactions by cross-linking adjacent nucleosomes. H3K9me3 is generated by the SETDB1 HMTase that is recruited to the 3′ sites by KAP1 (Iyengar and Farnham 2011). SETDB1 binds the sumoylated form of the of KAP1 bromodomain; sumoylated KAP1 is the active, most repressive, form of the molecule (Ivanov *et al.* 2007). Sumoylation is mediated intra-molecularly. The KAP1 PHD domain is an E3 ligase that co-operates with UBE2i (also known as UBC9) to transfer SUMO2 (Yang *et al.* 2015) to the KAP1 bromodomain (Figure 3).

The function of the heterochromatin-like domains that encompass the KRAB-ZNF clusters has been enigmatic because the KRAB-ZNF genes within the domains remain expressible, with some showing activity, while others are repressed (Groner *et al.* 2010; Iyengar and Farnham 2011). This presents a problem because transcription within the domains is likely to 'turn-over' repressive histone modifications thereby disrupting heterochromatin-like domains. Fortunately, there is a specific mechanism to prevent this. The heterochromatin-like complexes at the 3′ end of the KRAB-ZNFs act as a focal point for the machinery that incorporates the replacement histone H3.3 into chromatin (Valle-García *et al.* 2016) and in this way the repressive histone modifications are replenished while transcription is ongoing. Specifically, KAP1 binds to DAXX (Elsässer *et al.* 2015), which is a H3.3 specific chaperone that, along with the chromatin remodeller ATRX, is known to target histone H3.3 to peri-

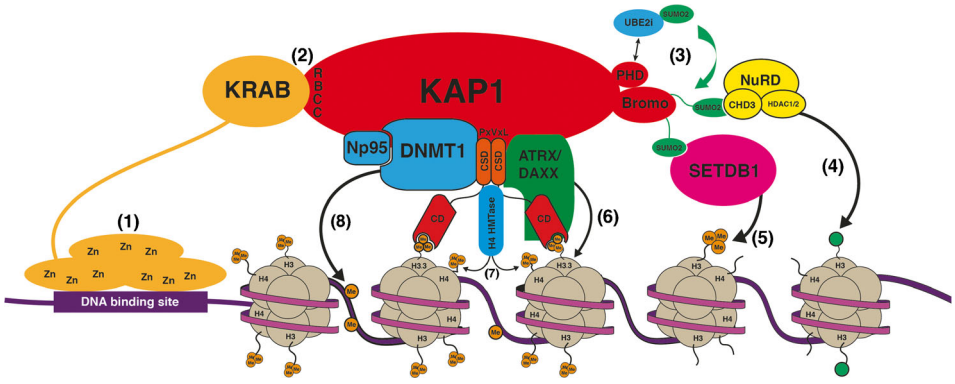

Figure 3. Nucleation of a heterochromatin-like complex by a KRAB-ZNP. The diagram is based on the KAP1 and HP1 interactomes. (1) The KRAB-ZNP binds to its DNA binding site through its zinc-fingers (Zn). (2) The KRAB domain of the KARB-ZFP interacts with the RBCC domain of KAP1 (Frietze *et al.* 2010). KAP1 is a homo-trimer (Peng *et al.*, 2000) although only one molecule of KAP1 is shown. A HP1 CSD dimer binds to one molecule of KAP1 through the PxVxL motif (the HP1-box). The HP1 CD binds to H3K9me3. (3) The PHD domain of KAP1 is an E3 ligase that co-operates with UBE2i to sumoylate the KAP1 bromodomain (Ivanov *et al.* 2007; Yang *et al.* 2015). (4) The sumoylated bromodomain is bound by the NuRD complex that deacetylates acetylated histones (green circle) in preparation for histone methylation (Schultz *et al.* 2001). (5) SETDB1 H3K9 HMTase interacts with the sumoylated bromodomain (Ivanov *et al.* 2007) and generates H3K9me3 (orange circles). (6) The ATRX/DAXX complex is bound to KAP1, HP1, and H3K9me3. ATRX/DAXX incorporates replacement histone H3.3 into chromatin thereby ensuring the maintenance of repressive histone modifications (Voon *et al.* 2015; Valle-García *et al.* 2016). (7) HP1 recruits a H4K20 HMTase that generates H4K20me3 (orange circles). This is the H3K9me3:HP1:H4K20me3 pathway. (8) The maintenance DNA methylase DNMT1 binds to KAP1 (Quenneville *et al.* 2011; Zuo *et al.* 2012). Np95 – the co-factor of DNMT1– is also recruited by KAP1 (Quenneville *et al.* 2011). Np95 activity may be tightly regulated during de novo CF (see text for details). DNMT1 maintains cytosine methylation at the site of assembly (Quenneville *et al.* 2011; Zuo *et al.* 2012). Not shown are DNMT3A and DNMT3B, which can interact with KAP1 (Quenneville *et al.* 2011; Zuo *et al.* 2012). Note: Depicted is nucleation of the heterochromatin-like complex. It is known that the heterochromatin-like complexes typically extend 2 to 6 kB at the site of assembly (Vogel *et al.* 2006; Regha *et al*. 2007; Table 1). Modified from Ivanov *et al*. (2007).

centric and telomeric constitutive heterochromatin (Drané *et al.* 2010; Goldberg *et al.* 2010; Wong *et al.* 2010), where H3.3 is tri-methylated on lysine 9 by a SUV39H HMTase (Voon and Wong 2016). Histone H3.3 is also incorporated at the heterochromatin-like complexes at the 3' end of the KRAB-ZNF genes (Valle-García *et al.* 2016), where it can be tri-methylated at K9H3 by SETDB1 (Frietze *et al.* 2010). Binding of the ATRX-DAAX complex is enhanced by the known interaction of ATRX with both H3K9me3 and HP1, the former through the ADD domain and the latter through an LxVxL motif, and both interfaces are known to combine to localize ATRX to heterochromatin (Eustermann *et al.* 2011). Notably, when HP1 is artificially repositioned within the nucleus both SETDB1 and ATRX are relocated along with it (Kourmouli *et al.* 2005), indicating that the complex containing SETDB1-HP1-ATRX is stable under this *in vivo* manipulation.

The use of artificial constructs, where a regulatable KRAB domain is targeted to a synthetic sequence that drives a reporter gene, has enabled the identification of the histone modifications and molecular machinery associated with KRAB-ZNP-directed heterochromatin-like complexes (Schultz *et al.* 2001, 2002; Ayyanathan *et al.* 2003; Sripathy *et al.* 2006). In such a system, the reach of repression after assembly of KAP1 along with SETDB1 and HP1 is short, extending ~1.2 kb from the promoter and beyond this distance, but a few nucleosomes, there is no detectable 'spreading' of HP1 (Ayyanathan *et al.* 2003). The nucleosomes also show an increase in H4K20me3 as well as H3K9me3 (Sripathy *et al.* 2006) indicating the presence of the H3K9me3:HP1:H4K20me3 pathway. KAP1 recruits the Nucleosome Remodelling histone Deacetylase (NuRD) complex (Schultz *et al.* 2001). There are also increased levels of DNA methylation at the reporter genes (Ayyanathan *et al.* 2003) (Figure 3), which is consistent with biochemical assays showing that KAP1 interacts with all three DNA methyltransferases and the DNMT1 co-factor Np95 (Quenneville *et al.* 2011; Zuo *et al.* 2012); HP1 also interacts with all three DNA methyltransferases (Fuks *et al.* 2003; Smallwood *et al.* 2007).

The KAP1-SETDB1-HP1 recruited to the 3′ end of the KRAB-ZNF genes (Iyengar and Farnham 2011) may operate in synergy with the SUV39H1-HP1 system that 'spreads'

along the KRAB-ZNF gene clusters (Vogel *et al.* 2006; Groner *et al.* 2010). In this scenario, the KAP1-SETDB1-HP1 nucleates the assembly of the heterochromatin-like domain that is then extended by the SUV39H1-HP1 complex. HP1 is known to bind to the N-terminal 44 amino-acids of SUV39H1 in nuclear extracts (Melcher *et al.* 2000) and FRET analysis shows that this interaction takes place *in vivo* (Krouwels *et al.* 2005). As explained, the function of heterochromatin-like domains at the KRAB-ZNF clusters is unclear because the KRAB-ZNF genes within the clusters remain expressible. It has been suggested that the domains 'protect' the KRAB-ZNF gene repeats by preventing illegitimate recombination (Vogel *et al.* 2006). The KRAB-ZNF gene repeats, which are interspersed with repetitive LINE elements, are also thought to strengthen cooperative recruitment of the SUV39H1-HP1 complex, which continues to extend the heterochromatin-like domain (Vogel *et al.* 2006); LINE repeats are also a feature of RRRs (Matoba *et al.* 2014).

A comparison of major protein/enzymatic components and epigenetic modifications found in constitutive heterochromatin (peri-centric, telomeric and peri-nucleolar) with heterochromatin-like domains and complexes is given in Table 1. While there are undoubtedly gaps there is a striking similarity between constitutive heterochromatin and the heterochromatin-like complexes found at imprinted gDMRs. A pattern that may form the basis of a classification of heterochromatin-like domains and complexes is also emerging. Larger heterochromatin-like domains that can be Mb in size are associated with either SUV39H1/2 (SUV39H-directed) or G9a/GLP (G9a/GLP-directed), while smaller complexes that are a few Kb in size are associated with SETDB1 (SETDB1-directed).

4.2 *Regulation of genome organization by heterochromatin-like domains and complexes*

Early immunofluorescence studies showed that in the majority of mouse nuclei KAP1 has the same nuclear distribution as HP1γ, with a small percentage of nuclei showing KAP1 localization to constitutively heterochromatic foci that are positive for HP1β (then known as M31) (Ryan *et al.* 1999). Not long afterwards it was shown that KAP1 (TIF1β) can translocate from euchromatin to constitutive heterochromatin during differentiation of F9 teratocarcinoma cells and the translocation was dependent upon the KAP1-HP1 interaction (Cammas *et al.* 2002). Repression is enhanced by the HP1-dependent re-localization to constitutive heterochromatin (Matsuda *et al.* 2001). Moreover, despite the limited size of the heterochromatin-like complexes artificially generated by targeted KRAB domains, they too preferentially relocated to constitutive heterochromatin (Ayyanathan *et al.* 2003). The role of each of the HP1 isotypes in translocation to constitutive heterochromatin was addressed *in vivo* using FRET imaging and fusions of TIF1β(KAP1)-CFP and HP1α/β/γ-YFP (Cammas *et al.* 2007). In non-differentiated cells the KAP1 found in euchromatin interacts with both HP1β and HP1γ but not HP1α. In differentiated cells, KAP1 is translocated to constitutive heterochromatin through a specific interaction with HP1β; the KAP1-HP1γ complex appeared to be 'repulsed' from constitutive heterochromatin (Cammas *et al.* 2007). It would seem that HP1β is the HP1 isotype that directs the relocation of heterochromatin-like complexes to constitutive heterochromatin. Notably, differentiation is not an absolute requirement for translocation. The imprinted MEST gene preferentially relocates to constitutive heterochromatin in undifferentiated F9 cells *via* a mechanism that is dependent upon the KAP1-HP1 interaction (Riclet *et al.* 2009).

Constitutive heterochromatin is a nuclear compartment that is enriched in many enzymatic activities related to gene repression, particularly during S-phase. These include the *de novo* DNA methyltransferases (Bachman *et al.* 2001), the maintenance methylase DNMT1 and its co-factor Np95 (Leonhardt *et al.* 1992; Sharif *et al.* 2007), KAP1 (Loyola *et al.* 2009), methyl-DNA-binding domain proteins (Hendrich and Bird 1998), HMTases Suv39h1 (Aagaard *et al.* 1999) and SETDB1 (Loyola *et al.* 2009), histone deacetylases (HDACs; Kim *et al.* 1999; Francastel *et al.* 2001), and chromatin remodeling factors (NuRD; Chadwick *et al.* 2009) and ATRX/DAXX (Drané *et al.* 2010; Goldberg *et al.* 2010; Wong *et al.* 2010). The repressive milieu of constitutive heterochromatin could stabilize and help propagate the repressive state of a translocated heterochromatin-like complex, as has been proposed in models for bw^D variegation (Csink and Henikoff 1996). The mechanism by which heterochromatin-like complexes are translocated to constitutive heterochromatin is unknown. Nevertheless, once translocated, stabilization of HP1β-containing heterochromatin-like complexes is likely to take advantage of the known flexibility of the CD and HR of HP1β, which enables HP1β to interact with H3K9me3 on the same or different nucleosomes (Figure 4; Hiragami-Hamada *et al.* 2016). Constitutive heterochromatin has high concentrations of H3K9me3 in condensed oligonucleosomes that are bound by HP1β. The HP1β bound to heterochromatin-like complexes brought into contact with constitutive heterochromatin could then form inter-fibre interactions (Hiragami-Hamada *et al.* 2016), thereby stabilizing the compacted state of the complexes (Figure 4).

4.3 *Replication of heterochromatin-like domains and complexes*

Whatever the size, both domains and complexes must, like constitutive heterochromatin, be inherited through DNA

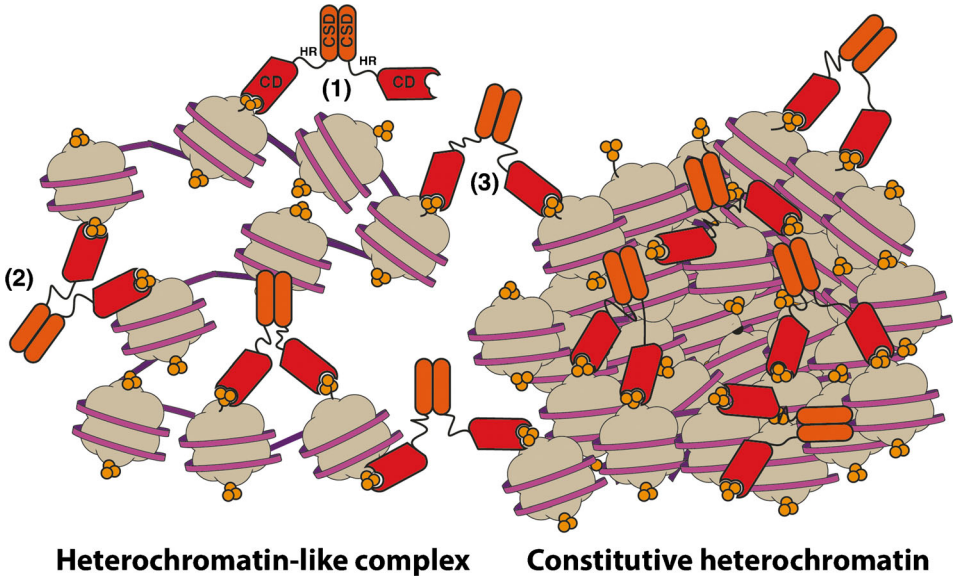

Figure 4. Interaction of a HP1β-containing heterochromatin-like complex with a block of constitutive heterochromatin. On the left is a heterochromatin-like complex that has trans-located into the vicinity of a block of constitutive heterochromatin. HP1β consists of a CD that can bind H3K9me3, a CSD that can dimerise and a hinge region that connects the CD to the CSD. Due to the flexibility of the HR and binding of the CD to H3K9me3 (orange circles) HP1β makes intra-nucleosomal (1) and inter-nucleosomal intra-fibre interactions (2). When in close apposition to a block of constitutive heterochromatin HP1β can make inter-nucleosomal inter-fibre interactions (3). The high density of H3K9me3 in constitutive heterochromatin increases the residence time of HP1β and thereby stabilizes the compacted state of the heterochromatin-like complex. For the sake of clarity, the H4K20me3 modification is not shown, which would be present and would also help stabilize the complex. Larger heterochromatin-like domains would interact in the same way as the depicted smaller complex. Modified from Hiragami-Hamada *et al.* (2016).

replication in order to be propagated from one cell generation to the next. [For the remaining discussion I use the term heterochromatin-like domains to refer to both the domains and complexes. Where I use the term heterochromatin-like complexes I refer to some characteristic that is peculiar to the smaller complexes]. There have been many studies on the replication of constitutive heterochromatin (reviewed in Alabert and Groth 2012). It is a multi-stage process and, notably, HP1 is involved in two of the key stages, namely initiation of replication of heterochromatin and its reassembly after passage of the replication fork (Jones *et al.* 2000). The mechanisms by which replication is initiated are treated briefly. I will focus mainly on the mechanisms responsible for reassembly of heterochromatin-like domains after passage of the replication fork.

In nuclei of multi-cellular organisms there is no specific sequence to which the origin of replication complex (ORC) binds, but there is evidence in *Drosophila* which suggests that HP1 proteins can recruit the ORC (Pak *et al.* 1997) and specify an origin of replication (Schwaiger *et al.* 2010). This role in the initiation of replication is likely to be conserved in mammals because human HP1 interacts with hexa-meric ORC through ORC subunits ORC1 or 3, and these interactions are mutually re-enforcing (Prasanth *et al.* 2010). The interaction of ORC with HP1-containing heterochromatin is stabilized by an ORC-associated protein, ORC-A, that binds to H3K9me2 and H3K9me3 and recruits a HMTase mega-complex containing SETDB1, G9a, GLP and SUV39H1 (Fritsch *et al.* 2010), which generates H3K9me3 at the origin of replication (Giri *et al.* 2015). After initiation, the next stages include the assembly and activation of the replicative helicase and processivity of the replicative polymerases. These stages are generic to eukaryotic DNA replication and will not be covered here because they have been extensively reviewed elsewhere (Johnson and O'Donnell 2005; Bochman and Schwacha 2009; Alabert and Groth 2012).

Progression of the replication fork requires disruption of the heterochromatin-like domain ahead of it. This most likely occurs as a result of collision with the replicative polymerases, which can generate a mechanical force of around 34pN (Wuite *et al.* 2000), in conjunction with the positive supercoiling that runs ahead of the fork (Gasser *et al.* 1996). Reassembly of heterochromatin-like domains behind the fork is integrated with the reassembly of nucleosomal structure on the daughter strands from recycled parental histones

and newly synthesized histones. There is random segregation of parental $(H3–H4)_2$ tetramers to the two daughter strands and subsequent formation of nucleosomes with either new or old H2A–H2B dimers (Xu *et al.* 2010). Entirely new nucleosomes are formed through the recruitment of tri-meric chromatin assembly factor 1 (CAF-1; made up of three subunits, p150, p60 and p40) (Smith and Stillman 1989) to the replication fork by PCNA (Shibahara and Stillman 1999); PCNA forms a 'landing pad' for many of the proteins involved in replication of heterochromatin. CAF-1 promotes the deposition of histone H3.1–H4 dimers onto DNA (Tagami *et al.* 2004) and the nucleosome is completed by the rapid association with histone H2A–H2B (Annunziato 2012). The nascent chromatin formed at the fork is highly acetylated (Sobel *et al.* 1995). Deacetylation of histones is a pre-requisite for incorporation of repressive histone modifications and re-assembly of the heterochromatin-like domain (Figure 5).

Deacetylation of histones takes place in the context of larger assemblies that contain HDACs (Sirbu *et al.* 2011) of which the best characterized is the SMARCAD1 complex that localizes to sites of replication by an interaction with PCNA (Mermoud *et al.* 2011; Rowbotham *et al.* 2011) (Figure 5). The SMARCAD1 complex integrates ATP-dependent regulation of nucleosome spacing by the nucleosome remodeller SMARCAD1 with histone deacetylation by HDAC1, HDAC2 and histone mono- and di- K9 methylation by G9a/GLP HMTases (Rowbotham *et al.* 2011). The SMARCAD1 complex also contains the now familiar KAP1 and HP1 proteins (Rowbotham *et al.* 2011). The SMARCAD1 complex is unlikely to act alone in re-assembling heterochromatin-like domains because the CAF-1 complex, which we have already met as a general nucleosome assembly factor, has also been shown to be crucial in replicating heterochromatin (Murzina *et al.* 1999; Loyola *et al.*, 2009). The heterochromatic CAF-1 complex contains KAP1 and HP1 like the SMARCAD1 complex, but this time the associated HMTase is SETDB1 (Loyola *et al.* 2009). CAF-1 interacts directly with SETDB1 and SETDB1, in turn, binds KAP1 (Loyola *et al.* 2009). The CAF-1 p150 subunit binds HP1 *via* a PxVxL motif (Murzina *et al.* 1999) (Figure 3). SETDB1 in the CAF-1 complex is thought to mono-methylate H3K9 and therefore plays the same role as the G9a/GLP HTMases in the SMARCAD1 complex. Mono-methylation primes H3K9 for the dedicated di-and tri-K9H3 HMTases SUV39H1/2, thereby generating high-affinity H3K9me3 binding sites for HP1 (Figure 5). Notably, for the smaller heterochromatin-like complexes SETDB1 is the HMTase that generates H3K9me3 without the apparent involvement of the SUV39H1/2 HMTases (Table 1). The association of HP1 with both SMARCAD1 and CAF-1 complexes likely promotes transfer of HP1 proteins to the nucleosomal daughter strands, a function that has already been ascribed to CAF-1 (Loyola *et al.* 2009). Maturation of heterochromatin-like domains will also involve re-establishment of DNA methylation, which is mediated by DNA methyltransferase 1 (DNMT1)–Np95 complex and is known to occur soon after the fork passes. The DNMT1–Np95 complex is recruited to sites of replication by PCNA (Chuang *et al.* 1997) and by the binding of Np95 to hemi-methylated DNA and H3K9me3 (Leonhardt *et al.* 1992; Rottach *et al.* 2010).

The machinery that nucleates the assembly of heterochromatin-like complexes shares molecular characteristics with those that replicate the complexes (Figures 3 and 5). For example, KAP1 and HP1 are found at gDMRs (Figure 3; Table 1) and are also components of the CAF-1 and SCMARCAD1 complexes (Figure 5). Thus mutations that might affect nucleation could, in fact, affect replication of heterochromatin-like complexes. For example, the preservation of methylation at gDMRs of imprinted genes in ES cells requires both SETDB1 (Leung *et al.* 2014) and G9a (Zhang *et al.* 2016). It is likely that the loss of SETDB1, which is part of the CAF-1 complex (Figure 5), or G9a, which is part of the SMARCAD1 complex (Figure 5), results in loss of methylation at imprinted gDMRs because replication of heterochromatin-like complexes has been disrupted.

This brings us in a timeously to the second hypothesis, which represents a special case of the first. Could difference in the assembly of heterochromatin-like complexes on homologous chromosomes regulate parent-of-origin effects observed in placental mammals (Singh *et al.* 1991)?

5. Heterochromatin-like complexes and autosomal imprinting in mice

Compared to the coccid and *Sciara* systems, where entire haploid genomes or whole chromosomes are subject to parent-of-origin effects the genetic complement that is subject to genomic imprinting in mice is small. There are in the region of 150 loci that exhibit a parent-of-origin effect (Williamson *et al.* 2013). Although small in number they constitute the barrier to impaternate development observed in some other vertebrates (Booth *et al.* 2012). The need for both maternal and paternal genetic complements for normal growth and development has been demonstrated by uniparental disomies (Cattanach and Kirk 1985) and pro-nuclear transfer experiments (McGrath and Solter 1984; Surani *et al.* 1984). A number of excellent reviews have been written that emphasize aspects of the biology and molecular mechanisms of genomic imprinting. I draw attention to those that provide a time-line of the major discoveries in the field (Ferguson-Smith 2011), the mechanisms by which CpG islands are *de novo* DNA methylated in the respective germ-lines (Kelsey and Feil 2013; Tomizawa *et al.* 2013) and thereafter retained

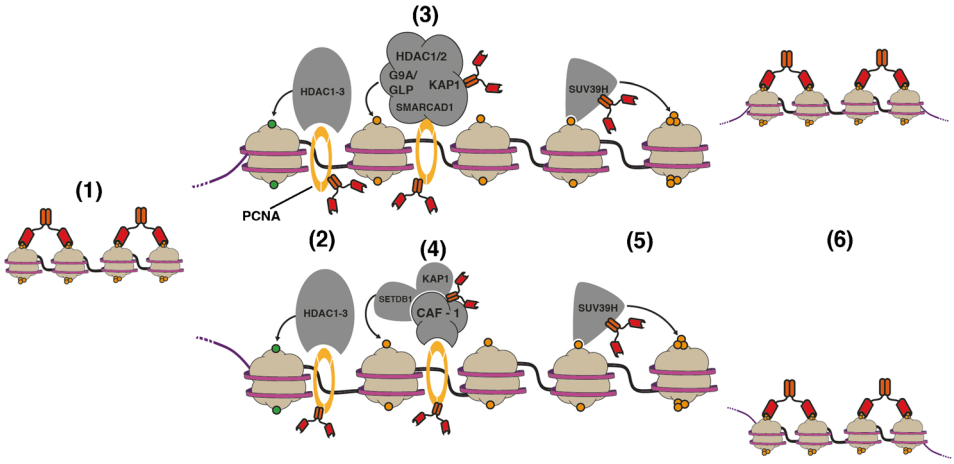

Figure 5. Model depicting replication of heterochromatin-like domains by SMARCAD1 and CAF-1 complexes. (1) A heterochromatin-like domain, which would be considerably larger than that depicted, undergoes replication. (2) Immediately after replication, the histones are highly acetylated (green circle). Deacetylation is a pre-requisite for re-assembly of the heterochromatin-like domain. This is achieved through the recruitment of HDAC1-3 by PCNA to the replication fork. (3) PCNA also recruits the SMARCAD1 complex that also has HDAC activity and contains the G9a/GLP HMTases that mono- methylate H3K9 (yellow circle). (4) A PCNA interaction also recruits the CAF-1 complex, which contains the SETDB1 HMTase that can, like G9a, mono-methylate H3K9. Notably, both the SMARCAD1 and CAF-1 complexes contain KAP1 and HP1 (Loyola *et al.* 2009; Rowbotham *et al.* 2011). (5) Mono-methylated H3K9 is a substrate for the SUV39H HMTases and generates H3K9me3 (three yellow circles). (6) The daughter DNA strands are again assembled into a heterochromatin-like domain after binding of the HP1 dimer to H3K9me3. HP1 binds to PCNA, SMARCAD1, CAF-1 and SUV39H. Note: While SUV39H HMTases are associated with heterochromatin-like domains (Matoba *et al.* 2014; Vogel *et al.* 2006) (Table 1), no such association has been observed with smaller complexes, which likely utilize SETDB1 to *tri*-methylate H3K9 (Frietze *et al.* 2010; Iyengar and Farnham 2011; Schultz *et al.* 2002) (Table 1). Modified from Alabert and Groth (2012).

in the early embryo (Hanna and Kelsey 2014; Messerschmidt *et al.* 2014; Leseva *et al.* 2015), the role of lncRNAs in the regulation of imprinted genes (Barlow and Bartolomei 2014) and the mechanisms whereby epigenetic modifications in primordial germ cells (PGCs) are erased (Hackett and Surani 2013; Reik and Surani 2015). Together, they provide some background for the synthesis presented here.

DNA methylation is necessary for regulation of genomic imprinting in mice. This is founded upon the observation that mutation of the maintenance DNA methyltransferase DNMT1 results in dysregulation of imprinted gene expression (Li *et al.* 1993). DNA methylation has been long-recognized as an epigenetic paradigm for studying the mechanisms that regulate gene expression in *cis* (Bestor *et al.* 2015). In mammalian genomes 5mC occurs at the bulk (~80%) of CpG dinucleotides and is a repressive mark; proper DNA methylation is essential for embryonic viability (Li and Zhang 2014). Within this immensity of methylated DNA there exist small CpG dense regions called CpG islands (CGIs) that are generally associated with gene promoters and remain unmethylated regardless of gene expression (Jaenisch and Bird 2003). Only under certain circumstances do CGIs become methylated, for example during X-chromosome inactivation, when many CGIs on the inactive X chromosome become methylated (Wolf *et al.* 1984). Another example is when CGIs associated with maternally and paternally imprinted genes undergo *de novo* methylation during gametogenesis in their respective germlines (Sasaki and Matsui 2008).

Before *de novo* methylation takes place, CGIs undergo an erasure of epigenetic modifications in PGCs and by the time PGCs have migrated to the genital ridge (embryonic day 10.5; E10.5), DNA methylation is reduced to very low levels throughout the genome (Guibert *et al.* 2012). Only then does *de novo* DNA methylation proceed. The CGIs associated with imprinted genes do not stand apart from those associated with non-imprinted genes. CGIs associated with maternally-imprinted genes are conventional with respect to CpG density and GC-richness, while CGIs associated with paternally-imprinted genes fall just short of these criteria, but are not atypical (Kobayashi *et al.* 2006; Schulz *et al.* 2010; Smallwood *et al.* 2011; Kobayashi *et al.* 2012). Consequently, during gametogenesis the *de novo* DNA methylation machinery methylates a large number of CGIs irrespective of whether they are associated with imprinted genes or not. Depending on the study there are around 1-2,000 CGIs in oocytes that are subject to *de novo* methylation by the DNMT3A-DNMT3L complex starting on postnatal day 10

(P10) and finishing on P21 in dictyate stage oocytes (Obata and Kono 2002; Hiura *et al.* 2006; Smallwood *et al.* 2011; Kobayashi *et al.* 2012; Shirane *et al.* 2013). In the male gonad both DNMT3A and DNMT3B contribute to *de novo* methylation in combination with DNMT3L (Kato *et al.* 2007). *de novo* DNA methylation begins in resting prospermatogonia at around E13.5 and DNA methylation patterns are fully established before birth (Davis *et al.* 2000; Seisenberger *et al.* 2012). The patterns are faithfully maintained thereafter through multiple rounds of cell division before the formation of mature sperm. The number of CGIs methylated in sperm has been identified but again depends on the study, with one identifying 185 methylated CGIs in sperm (Smallwood *et al.* 2011) and the other 818 (Kobayashi *et al.* 2012). Taking into account CGI methylation shared by both gametes and where comparison could not be made these analyses have shown, on a conservative estimate, there are around 1500 differentially methylated CGIs between the oocyte and sperm (Smallwood *et al.* 2011; Kobayashi *et al.* 2012). These ~1500 CGIs are germline differentially methylated regions (gDMRs). Notably, only ~25 of them are known to be primary gDMRs associated with imprinted genes that maintain allele-specific methylation during early development (Tomizawa *et al.* 2011; Proudhon *et al.* 2012). Of these ~25 imprinted gDMRs just seven have been shown to be ICRs, defined as having been shown to be necessary for parent-of-origin-specific expression (Ferguson-Smith 2011; Kelsey and Feil 2013; Barlow and Bartolomei 2014). It would seem that *de novo* DNA methylation of CGIs during murine gametogenesis does not provide specificity. A latter-day Charles Metz might be moved to ask: '*How does one know if a locus will behave in a parent-of-origin specific manner based on its CGI methylation status in the gametes?*' Specification of imprints takes place post-fertilization. And the mechanism operates in the context of the global reprogramming and remodelling of the parental genomes, to be described, that is necessary for the acquisition of totipotency in the two cell embryo (Tarkowski 1959) and establishment of pluripotency at the blastocyst stage.

Sperm that enters the mammalian oocyte has one of the most heavily DNA methylated genomes known exhibiting 80%–90% overall CpG methylation (Popp *et al.* 2010). For much of its genome, protamines in the sperm must be replaced by maternally-supplied histones in order to reconstitute the natural chromatin environment required for proper gene function (Torres-Padilla *et al.* 2003; Loppin *et al.* 2005). The maternal genome shows lower, around 40%, global methylation levels, and is already assembled as chromatin (van der Heijden *et al.* 2005). Both genomes are demethylated after fertilization but follow different DNA demethylation kinetics (Mayer *et al.* 2000a; Oswald *et al.* 2000; Santos and Dean 2004). The paternal genome undergoes a precipitous loss in DNA methylation that is due to an active process whose details are still emerging and for which a consensus has not been reached. According to recent work the demethylation in the paternal pro-nucleus is the result of the base-excision repair pathway followed by a wave Tet3-mediated oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) (Amouroux *et al.* 2016). Demethylation of the paternal genome is almost complete by the onset of DNA replication at pronuclear stage 3 (PN3) (Mayer *et al.* 2000a; Oswald *et al.*, 2000). The maternal genome undergoes mainly passive replication-dependent DNA demethylation because the bulk of the DNMT1 is excluded from the nucleus (Howell *et al.* 2001; Ratnam *et al.* 2002) and both genomes are subject to the same after syngamy (Mayer *et al.* 2000a; Oswald *et al.* 2000; Santos and Dean 2004). DNA demethylation continues during pre-implantation development reaching its lowest levels at the blastocyst stage whereupon *de novo* methylation takes place in a lineage-specific fashion (Smith *et al.*, 2016). Methylation of the ~25 gDMRs of imprinted genes is maintained through the demethylation phase by the scant DNMT1 still remaining in the nuclei of early embryos (Hirasawa *et al.* 2008; Kurihara *et al.* 2008). They are also protected from the activity of demethylating Tet dioxygenases (Messerschmidt *et al.* 2014; Leseva *et al.* 2015). It is the mechanism(s) that preserves methylation at imprinted gDMRs in the zygote and preimplantation embryo, at a time when much of the remainder of the genome is being demethylated, that lies at the heart of the imprinting process.

5.1 *Specification of imprints in the mouse occurs around the time of zygotic genome activation and de novo chromocenter formation*

Ongoing demethylation of the parental genomes is not the only upheaval that takes place as mouse embryos enter and traverse the two-cell stage. There is the major phase of ZGA (Schultz and Worrad 1995). ZGA is characterized by a transient burst of transcription from the zygotic genome where global gene expression profiling has shown that many of the transcripts arise from sequences that lie within constitutive heterochromatin and also heterochromatin-like domains scattered around the genome (Ko 2016). Concomitant with ZGA is *de novo* chromocenter formation (CF). In the mouse, chromocenters represent the 'somatic-type' organization of constitutive heterochromatin, where the pericentric major satellite DNA from different chromosomes aggregate (Guenatri *et al.* 2004). In contrast to the situation in somatic cells, in the zygote, paternal and maternal pericentric constitutive heterochromatin surrounds the nucleoli forming peri-nucleolar bodies (PNBs; Aguirre-Lavin *et al.*

2012). PNBs stain positively for HP1β albeit staining is weaker around paternal PNBs (Figure 6; Santos *et al.* 2005; Probst *et al.* 2007; Burton and Torres-Padilla 2010). *de novo* CF results in the re-organization of the HP1β - positive rings of constitutive heterochromatin surrounding the PNBs into the typical 'dot-like' chromocenters (Martin *et al.* 2006; Almouzni and Probst 2011).

de novo CF is essential for mouse development as evidenced by mutational analysis of STELLA, a SAP-domain protein that binds non-specifically to nucleic acid but specifically to H3K9me2 (Nakamura *et al.* 2007, 2012). 97% of embryos lacking maternal STELLA do not develop to the blastocyst stage and by the 2-4 cell stage already exhibit abnormal cleavage and chromosome segregation defects that cannot be rescued by the paternal genome (Nakamura *et al.* 2007). *STELLA*-deficient embryos fail because there is disruption of a pathway required for CF (Arakawa *et al.* 2015) (Figure 6). The proximate lesion is a reduction in the levels of H3.3-specific chaperone DAXX in the pro-nuclei, which has the effect of reducing the incorporation of H3.3 into constitutive heterochromatin surrounding the PNBs and this in turn reduces the burst of reverse major satellite RNA expression in the 2-cell embryos; injection of *DAXX* mRNA into one cell embryos can rescue CF along with H3.3 incorporation and the expression of reverse major satellite RNAs (Arakawa *et al.* 2015). It is known that either incorporation of mutant H3.3 or interference with expression of reverse major satellite RNAs affects CF and blocks development at the 2-cell stage (Santenard *et al.* 2010; Casanova *et al.*, 2013). Notably, DAXX also fails to be incorporated into constitutive heterochromatin at PNBs in *ATRX*-deficient zygotes (De La Fuente *et al.* 2015) indicating that ATRX and DAXX act as a complex during CF, as they do at heterochromatic regions in other cell types (Voon *et al.* 2015).

STELLA not only regulates *de novo* CF but, because of its specificity for H3K9me2, has an additional role in protecting methylated DNA sequences against demethylation. This is demonstrated by the protection of the maternal pronucleus - which is enriched in H3K9me2 compared to the paternal pro-nucleus – from 'active' demethylation (Nakamura *et al.* 2007; Nakamura *et al.* 2012). In the absence of maternal STELLA, loss of 5mC is observed in both pronuclei, concomitant with accumulation of 5hmC in the maternal pro-nucleus (Wossidlo *et al.* 2011). Loss of global protection of the maternal pro-nucleus also leads to hypomethylation of a few maternally-imprinted loci, namely *Peg1, Peg3* and *Peg10* in PN5 embryos (Nakamura *et al.* 2007). Hypomethylation of two paternally imprinted loci *H19* and *Rasgrf1* is also observed in *STELLA*-deficient PN5 embryos (Nakamura *et al.* 2012). Since *H19* and *Rasgrf1* retain H3K9me2-marked chromatin during spermatogenesis and protamine exchange STELLA binds H3K9me2 and protects both genes against demethylation (Nakamura *et al.* 2012). What these data indicate is that STELLA acts globally. It regulates *de novo* CF and along with that essential function it protects the maternal genome and some gDMRs from 'active' demethylation. The global role of STELLA acts in synergy with DNA-sequence specific mechanisms that specify which gDMRs are to be retained during pre-implantation embryogenesis.

A key sequence-specific binding factor that preserves imprinted gDMRs against loss of methylation is the KRAB-ZFP, ZFP57. The most severe phenotype is observed when embryos lack both maternal and zygotic ZFP57 (Li *et al.* 2008). Loss of maternal-zygotic ZFP57 results in demethylation of a number of gDMRs (Li *et al.* 2008), with deletion of ZFP57 in ES cells having the same effect (Quenneville *et al.* 2011; Liu *et al.* 2012). Parent-of-origin-specific binding of ZFP57 is through a hexamer motif TGCCGC found in gDMRs of imprinted genes where the central CpG dinucleotide in the motif is fully methylated (Quenneville *et al.* 2011; Liu *et al.* 2012; Strogantsev *et al.* 2015; Anvar *et al.* 2016). The heterochromatin-like complex 'nucleated' by ZFP57 is typical of KRAB-ZFPs (Figure 3; Table 1). Genome wide studies using ES cells have shown that endogenous full-length ZFP57 protein always follows the methylated allele of gDMRs and ZFP57 binding overlaps with its co-repressor KAP1, the SETDB1 HTMase and with H3K9me3 and H4K20me3 peaks (Quenneville *et al.* 2011; Strogantsev *et al.* 2015). ChIP analysis using ES cell nuclear extracts showed that HP1γ is recruited to gDMRs in ES cells by the ZFP57-KAP1 complex (Quenneville *et al.* 2011) and that KAP1 interacts with all three DNA methyltransferases and Np95 (Quenneville *et al.* 2011; Zuo *et al.* 2012). It is the recruitment of scarce DNMT1 by KAP1 that is likely to maintain methylation at imprinted gDMRs (Messerschmidt *et al.* 2014). Protection of methylation at imprinted gDMRs against Tet deoxygenase activity is also likely to be conferred by the SETDB1-dependent heterochromatin-like complex (Leung *et al.* 2014). As with the heterochromatin-like complexes found at the 3′ end of the KRAB-ZNF genes the complexes assembled at imprinted gDMRs exist in a milieu of ongoing transcription (Barlow and Bartolomei 2014). The problem of the loss of repressive histone marks by transcription is prevented by recruitment of the ATRX/-DAXX complex, as it is at the 3′ end of the KRAB-ZNF genes (Valle-Garcia *et al.* 2016). Accordingly, ATRX/-DAXX complex is recruited to imprinted gDMRs resulting in incorporation of the replacement histone H3.3 that is trimethylated at H3K9 by an HTMase, most likely SETDB1 (Voon *et al.* 2015) (Figure 3).

Nuclear localization of ZFP57 has shown a striking association with constitutive heterochromatin. Using HP1α as a marker of constitutive heterochromatin ZFP57 was shown to co-localize with chromocenters in over 50% of mouse 3T3 fibroblast nuclei analysed (Alonso *et al.* 2004). The ZFP57

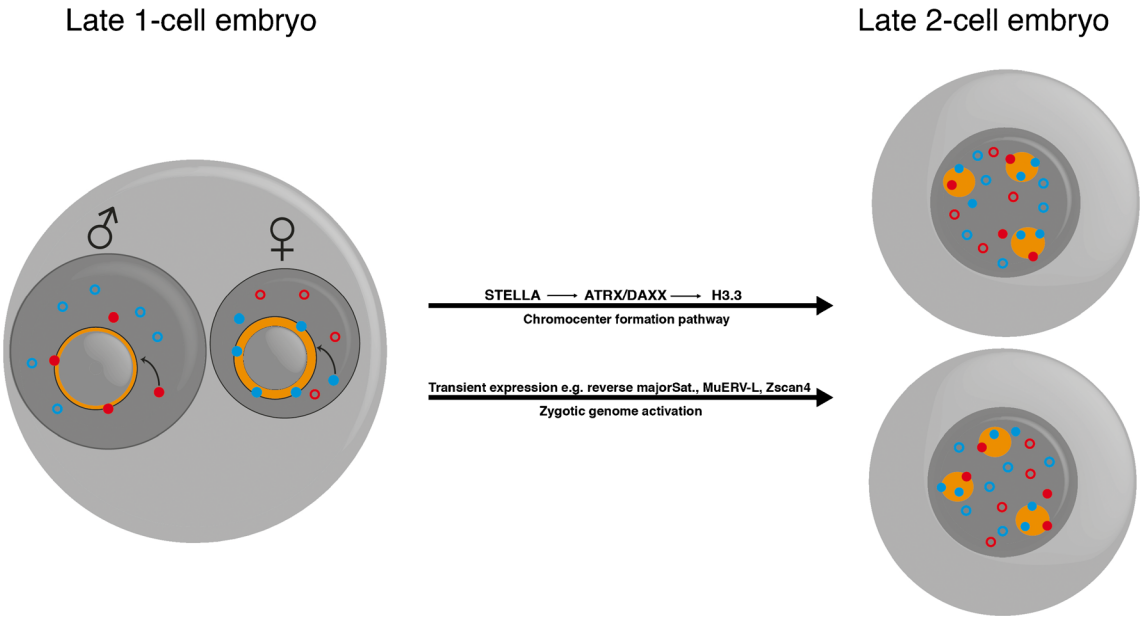

Figure 6. Model depicting preferential recruitment of ZFP57-directed heterochromatin-*like* complexes to constitutive heterochromatin in the early mouse embryo. In the late (PN5) 1-cell embryo on the left the paternal and maternal pro-nuclei are lying separately in the ooplasm. Constitutive heterochromatin that stains positively for HP1β (yellow) is found in a 'ring' surrounding the NPBs in both pro-nuclei, with staining of HP1β around the paternal NPB being noticeably weaker. In the paternal pro-nucleus the paternally-imprinted genes (red filled-circles) that have assembled a ZFP57-directed heterochromatin-like complex, preferentially translocate to the ring of HP1β. In the same pro-nucleus, genes that are maternally imprinted (blue open circles) remain in the nucleoplasm. The reverse takes place in the maternal pro-nucleus where maternally-imprinted genes (blue closed circles), which have assembled a ZFP57-directed heterochromatin-like complex, preferentially translocate to the ring of HP1β the paternally-imprinted genes (open red circles) in the maternal pro-nucleus remain in the nucleoplasm. At the end of the 1 cell stage the CF pathway is triggered and STELLA increases the levels of DAXX, which, as part of the ATRX/DAXX complex, in turn increases the incorporation of histone H3.3 into constitutive heterochromatin (Arakawa *et al.* 2015). Concomitant with CF is ZGA, where there is a transient burst of transcription from the zygotic genome. The transcripts include the reverse major satellite sequence (Casanova *et al.* 2013), MuERV-L (Macfarlan *et al.* 2011) and ZScan4 (Ko 2016) transcripts. In the nuclei of late 2 cell embryos HP1β-positive chromocenters are formed from the aggregation of major satellite sequences from different chromosomes (Guenatri *et al.* 2004). Preferential localization of imprinted genes (red and blue closed circles) at the newly-formed chromocenters is shown. The corresponding paternal and maternal genes which are not imprinted are found in the nucleoplasm (red and blue open circles). Note: The emphasis here is on the preferential translocation of the heterochromatin-like complexes to constitutive heterochromatin. Neither the spatial separation of parental genomes nor Rabl orientation of the centromeres in 2-cell embryos (Mayer *et al.* 2000b) is depicted.

KRAB domain, which binds to the RBCC domain of KAP1 (Figure 3), was necessary for the co-localization (Alonso *et al.* 2004).

5.2 *Specification of imprints: a mouse model*

Based on the above discussion (especially Figures 3 and 4 and Table 1) a model is suggested for specification of imprints around the late 1-cell embryo to late 2-cell mouse embryo. Approaching the late 1-cell stage the global protection against 'active' demethylation by STELLA is largely complete (Nakamura *et al.* 2012). Specificity is conferred by the binding of ZFP57 to the TGCC^{me}GC hexa-nucleotide in imprinted gDMRs, which takes place during the pronuclear stages. The order-of-addition after ZFP57 binding is not known but one possible scenario is that a KAP1 homo-trimer binds to the KRAB domain of ZFP57 and this in turn recruits three molecules of SETDB1 and six molecules of HP1β. SETDB1 generates H3K9me binding sites for HP1β which appears to be the sole HP1 isotype present in the pro-nuclei (Santos *et al.* 2005; Probst *et al.* 2007; Meglicki *et al.* 2012). By the late 1-cell stage, at PN5 there is preferential re-localization of the ZFP57-directed heterochromatin-like complexes to the HP1β-positive rings of constitutive heterochromatin that surround the NPBs (pro-nuclei on left in Figure 6). Notably, SETDB1 is concentrated in distinct foci around the NPBs at PN5 (Cho *et al.* 2012). The ZFP57-directed heterochromatin-like

complexes likely recruit further activities, including DNMT1 and ATRX/DAXX, which would be enhanced by close proximity to constitutive heterochromatin where such activities (Table 1) are concentrated, especially during S-phase. As the embryo enters the 2-cell stage, the CF pathway is triggered, which is concomitant with ZGA (Figure 6). By late 2-cell stage chromocenters can be observed in the nuclei (Santos *et al.* 2005; Martin *et al.* 2006, Probst *et al.* 2007; Probst *et al.* 2010; Santenard *et al.* 2010; Casanova *et al.* 2013). As shown the heterochromatin-like complexes assembled at imprinted gDMRs are preferentially associated with the chromocenters (nuclei on right in Figure 6). A systematic investigation of nuclear localization of imprinted gDMRs in early embryonic (pro-) nuclei has yet to be undertaken. Such a study should take into account any effect of STELLA, which can bind some imprinted gDMRs (Nakamura *et al.* 2012).

de novo CF is likely to be under tight regulation. In particular, precise regulation of Np95 activity in embryonic (pro-)nuclei may be necessary, given that over-expression of Np95 in somatic nuclei is inimical to CF, while removal by siRNA 'knock-down' promotes CF (Papait *et al.* 2008). Np95 may be sequestered by STELLA (Funaki *et al.* 2014) in these early embryonic stages. In this scenario, the interaction of KAP1 with scant nuclear DNMT1 (Quenneville *et al.* 2011; Zuo *et al.*, 2012; Messerschmidt *et al.* 2014) is all the more necessary for maintenance of methylation at imprinted gDMRs. Also of note is that mammalian HP1 proteins can exchange at genomic loci, for example from HP1β to HP1γ (Mateescu *et al.* 2008). This may be the case for imprinted gDMRs, where three studies have shown that HP1γ is present at imprinted gDMRs in MEFs and ES cells (Regha *et al.* 2007; Pannetier *et al.* 2008; Quenneville *et al.* 2011) (Table 1), while only HP1β is present in the pro-nuclei (Meglicki *et al.* 2012). Since the KAP1-HP1γ complex does not translocate to constitutive heterochromatin (Cammas *et al.* 2007) preferential translocation of gDMRs may be reduced or not take place where HP1β has been exchanged for, or makes hetero-dimers with, another HP1 isotype.

6. Comparison of imprinting mechanisms in animals: Metz's query answered?

Where it has been studied, the association of the H3K9me3:HP1:H4K20me3 pathway (Figure 1B) with imprinted chromosomes or genes is conserved. This does not appear to be the case for DNA methylation. For example, the H3K9me3:HP1:H4K20me3 pathway is associated with preferential inactivation of the paternal X-chromosome in marsupials but the DNA of the X chromosome is hypomethylated (Rens *et al.* 2010). The H3K9me3:HP1:H4K20me3 pathway, or an abbreviated form of it, may also be involved in preferential inactivation of the paternal X chromosome in the murine placenta (Tagaki and Sasaki 1975). In the mouse, preferential inactivation of the paternal X is thought to be caused by default due to an 'imprint' on the maternal *Xist* promoter. Specifically, H3K9me3 is enriched at the promoter of the *Xist* gene on the maternal X chromosome where it prevents binding of the *Xist* activator RNF12/RLIM that is provided by the oocyte cytoplasm (Fukuda *et al.* 2014). The paternal *Xist* promoter is bound by RNF12/RLIM resulting in preferential paternal X-inactivation. How RNF12/RLIM is prevented by H3K9me3 from binding the maternal *Xist* promoter is not known, but a clue may have come from the observation that HP1β binding to H3K9me3 prevents access of Polycomb repressive complexes to heterochromatin in the maternal pro-nucleus (Tardat *et al.* 2015). Likewise, HP1β binding to H3K9me3 might inhibit access of RNF12/RLIM to the maternal *Xist* promoter, thereby leading to preferential paternal X-inactivation by default. In any event, this recent work lends weight to the prior suggestion that imprinting of the X-chromosome is under maternal control (Lyon and Rastan 1984): the maternal *Xist* promoter possesses the H3K9me3 'imprint' and the mother also provides RNF12/RLIM, which activates the paternal *Xist* promoter (Fukuda *et al.* 2014).

Study of the germ-line and post-fertilization contributions to the imprinting of chromosomes in the two classical insect systems has shown that the ooplasm is the probable site where imprinting takes place (Chandra and Brown 1975). In coccids imprinting likely results from the differential treatment of the parental chromosomes by the ooplasm while separated within the pro-nuclei – the 'imprint' is conferred on one of the two parental chromosome sets by the mother (Figure 1A). More recent work has shown there are sperm-specific epigenetic modifications, however, proof of their involvement in the process of imprinting is wanting. A germ-line imprint may cause parent-of-origin-specific chromosomal behaviour only if 'licenced' by an appropriately conditioned oocyte cytoplasm (Figure 1A).

The system in *Sciara* mirrors that in coccids, excepting that the nature of the imprint is better defined. The genetic constitution of the mother determines the number of paternal X chromosomes to be eliminated (Figure 2A) and the CE that causes the eliminations is embedded within the terminal X heterochromatin (Figure 2B). In the context of the present discussion, the simplest model would posit that the maternal CE is inexpressible and the conditioning of the ooplasm by an X′X mother regulates assembly of a heterochromatin-like complex at a paternal CE. Accordingly, in eggs laid by X′X mothers a heterochromatin-like complex is assembled at one paternal CE rendering it inexpressible leaving the other paternal CE expressible; assembly likely takes place in the pro-nucleus. In eggs laid by XX mothers both paternal CEs are expressible. At the seventh to ninth cleavage, the expressible paternal CE(s) is activated and causes elimination of the X_p-chromosome(s) in *cis*. A key

question is how the CE can act at a distance (Figure 2B). A possibility is that the heterochromatin-like complex assembled at a CE regulates the expression of a ncRNA(s), perhaps encoded by the 30kb non-rDNA sequence in H2, which acts *in cis* over a long range to inhibit sister chromatid separation. This model would be analogous to the mechanism by which ZFP57-directed heterochromatin-like complexes assembled at ICRs regulate lncRNAs that repress mammalian autosomally-imprinted genes *in cis* and over large distances (Barlow and Bartolomei 2014).

Specificity is a simple matter in *Sciara* because the genetic constitution of the mother determines if one or two paternal CEs are activated at blastoderm leading to elimination of the associated X chromosome(s) in *cis*. The situation in coccids is simpler still and reduces to whether the maternal ooplasm has been conditioned to cause heterochromatinization of the paternal chromosomes at blastoderm. In the mouse the problem of specificity is of a different magnitude altogether. There are around ~1500 gDMRs that are generated by the *de novo* methylation machinery in the respective germ-lines and thereafter find themselves in the pro-nuclei of the newly fertilized zygote. To the end of achieving totipotency at the 2 cell stage and subsequent pluripotency at blastocyst, much of the ~1500 differentially methylated gDMRs are demethylated by 'active' and 'passive' mechanisms (Smith *et al.* 2016). There are around 25 known primary gDMRs that are associated with imprinted genes and retain their DNA methylation in the face of the attrition wrought by the demethylation machinery (Tomizawa *et al.* 2011; Proudhon *et al.* 2012; Strogantsev *et al.* 2015). This brings us to the nub of the matter and an answer to Metz's long-standing query. When taken in the round it seems clear that in the animals under discussion specification of imprints takes place post fertilization. For the mouse, specificity is conferred by interaction of ZFP57 with its cognate recognition sequence, TGCC^{me}GC. It is suggested that this specific interaction leads to assembly of heterochromatin-like complexes (see Figure 3 for possible complex). As a consequence, methylation of imprinted gDMRs is preserved and the associated genes '*know which parent they come from*'.

ZFP57-directed heterochromatin-like complexes are also likely to preserve methylation of imprinted gDMRs during human pre-implantation embryogenesis (Takikawa *et al.* 2013), which undergoes a rapid loss of methylation that is almost complete by the third embryonic division with residual methylation diminishing further by the blastocyst stage (Guo *et al.* 2014; Smith *et al.* 2014). However, ZFP57 is unlikely to be the only factor that specifically preserves methylation at imprinted gDMRs. This was to some extent foreshadowed by the observation that despite global demethylation the number of maternal gDMRs retaining more than 40% methylation was nonetheless around 15% at the blastocyst stage (Smallwood *et al.* 2011), which is somewhat higher than the 150 predicted imprinted loci (Williamson *et al.* 2013) and noticeably greater than the 81 sites in the ES cell genome where ZFP57-KAP1-SETDB1 co-localize with the TGCCGC hexa-nucleotide (Quenneville *et al.* 2011). There is also the observation that the gDMR of the imprinted *Scl38a4* gene does not contain the canonical ZFP57 hexa-nucleotide binding site (Auclair *et al.* 2015; Strogantsev *et al.* 2015). Notably, there is partial loss of methylation at the imprinted *Scl38a4* gDMR in embryos that are homozygous for a null mutation in the gene encoding the G9a HMTase (Auclair *et al.* 2015). One possibility is that the *G9a* mutation affects the nucleation of an alternative heterochromatin-like complex at the *Scl38a4* gDMR, whose replication is further compromised because G9a is a constituent of the SMARCAD1 complex (Figure 5). G9a is known to bind HP1 and the binding is unique amongst HP1-interacting proteins in that both the CD and the CSD are required for interaction with G9a (Nozawa *et al.* 2010). Since the demand for specificity is not relaxed, a G9a-containing complex is likely to be targeted by a sequence-specific binding factor that could be another KRAB-ZNP.

7. Conclusions and perspectives

Evolution is parsimonious. It is no surprise that structural and enzymatic constituents of constitutive heterochromatin have been co-opted to regulate chromatin-templated processes at euchromatic sites. The recruitment of heterochromatin components from constitutive heterochromatin to heterochromatin-like domains is an ancient event, for the latter are found in unicellular eukaryotes. In fission yeast, heterochromatin-like domains silence the donor mating type loci (see Figure 8 in Wang *et al.* 2000). Since the assembly of heterochromatin-like domains is advantageous, especially as a mechanism for epigenetically regulating gene activity, natural selection will have seen to it that the novelty was retained and even spread to other sites within the genome. Indeed, *Polycomb* group complexes are related heterochromatin-like domains, which regulate key developmental pathways (Gaunt and Singh 1990; Paro 1990) and, as a consequence, reprogramming (Onder *et al.* 2012). Evidence of a conserved role for heterochromatin-like complexes in parent-of-origin effects is also burgeoning. In mammals ICR function requires that gDMR methylation is preserved during pre-implantation demethylation and the likely mechanism involves assembly of heterochromatin-like complexes targeted by sequence-specific binding of ZFP57 (Figure 3). ZFP57-directed assembly of the complexes in mice occurs around the time of the global reprogramming and

remodelling of the parental genomes that must take place for the acquisition of totipotency in 2-cell embryos (Figure 6). Thus investigations into the assembly and propagation of heterochromatin-like complexes that regulate genomic imprinting are likely to provide insight into this interesting developmental stage. This is not only important for understanding the biology of heterochromatin-like complexes but also carries considerable practical import.

Heterochromatin-like complexes and larger domains (Table 1) act as barriers to reprogramming by the IPS (Chen *et al.* 2013) and SCNT (Matoba *et al.* 2014) technologies (Becker *et al.* 2016). Recent RNAi screens have identified CAF-1, the SUMO-conjugating enzyme UBE2i, SUMO2, SETDB1, ATRX and DAXX as factors whose inhibition significantly enhances IPS reprogramming efficiency (Cheloufi *et al.* 2015; Borkent *et al.* 2016). All of these proteins are involved in regulation of heterochromatin (Cheloufi *et al.* 2015; Borkent *et al.* 2016) and heterochromatin-like domains (Figures 3 and 5; Table 1). RNAi 'knock-down' of CAF-1 also reduces levels of H3K9me3 at the RRRs (Cheloufi *et al.* 2015) that are regions resistant to reprogramming at the 2-cell stage after SCNT (Matoba *et al.* 2014). Notably, the proportion of totipotent 2C-like cells in ES cell culture is also enhanced by inhibition of CAF-1 (Ishiuchi *et al.* 2015). A molecular signature shared by 2C-like cells and 2 cell embryos is activity of the endogenous retrovirus MuERV-L (Macfarlan *et al.* 2011) whose expression is required for progression beyond the 2 cell stage (Kigami *et al.* 2003), most likely due to the exaptation of ERV LTRs as promoters for essential genes (Rebollo *et al.* 2012). Deletion of either KAP1 or HP1 results in activation of MuERV-L expression (Macfarlan *et al.* 2011; Maksakova *et al.* 2013) indicating that MuERV-L may be regulated directly or indirectly by heterochromatin-like domains. If so, manipulation of heterochromatin-like domains could generate cultures enriched in 2C-like cells that have expanded developmental potential compared to ES cells.

The potential of manipulating constitutive heterochromatin (e.g. telomeres; Table 1; Marión and Blasco 2010), heterochromatin-like domains (e.g. RRRs and topologically associated domains; Table 1; Matoba *et al.* 2014; Parry and Narita 2016) and smaller complexes (e.g. SETDB1-directed; Table 1; Bilodeau *et al.* 2009; Chen *et al.* 2013) lies in disentangling the reprogramming process itself. On the face of it, reprogramming by the IPS and SCNT routes is a seamless process resulting in 'developmental reprogramming', whereby specialized cells are reprogrammed back to an embryonic cell type, and 'age reprogramming' whereby the ageing clock is reset (Singh and Zacouto 2010; Manukyan and Singh 2012). Using HP1β mobility as a measure, it has now been shown that developmental and age reprogramming are in fact separable (Manukyan and Singh 2014). This opens the possibility to reprogramming the age of old cells while maintaining their specialized functions. The benefits to human health of being able to rejuvenate cells *without* de-differentiation are self-evident.

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