Commentary

Epigenetics of heterochromatin

The term "heterochromatin" was coined by Heitz (1928) to describe certain regions along the longitudinal axis of mitotic chromosomes in a variety of moss species, which displayed differential staining (heteropycnosis) after aceto-carmine application and boiling following his specially devised squashing method. The heterochromatic regions of specific chromosomes or the heterochromatic chromosomes, did not get reorganized at telophase and thus were distinctly identifiable even in the non-dividing nucleus. By contrast, euchromatin ('chromatin proper') regions were those that got structurally altered at telophase so that their individuality was no more visible in the non-dividing (interphase) nucleus. To be sure that the differential staining or heteropycnosis displayed by the heterochromatic chromosomes or their parts on using his special staining method was not an artifact, Heitz (1928) demonstrated the presence of heterochromatin in live cells as well. He concluded, "The cause of heteropycnosis can only lie in the concerned chromosomes themselves". Subsequent identification of comparable heterochromatic regions or chromosomes in *Drosophila* by Heitz and correlation of genetic maps with specific segments of polytene chromosomes provided the first evidence of heterochromatin being devoid of, or poor in, "genes" (see Zacharias 1995).

Heterochromatin has since been identified, with or without special staining methods, in nuclei of almost all eukaryotes. Although sometimes ignored as a "junk" or "selfish" component of the genome because of its general lack of protein-coding "genes", heterochromatin continues to pose a challenge to cytologists, geneticists and molecular biologists since, in spite of numerous studies, the molecular basis and significance of heterochromatin has remained elusive. This is because different mechanisms may exist for differential staining or condensation of the underlying chromatin in different cases. Brown (1966) distinguished between "constitutive" and "facultative" heterochromatin, the former being represented by those chromosome regions or chromosomes which always (constitutively) displayed a more condensed and genetically inactive state on both the homologs in a nucleus. The latter category included those regions which were capable of changing from a potentially active, euchromatic and less condensed state to an inactive and condensed state especially on only one of the two homologs in a given cell type or lineage. Subsequently, however, this distinction between different classes of heterochromatin has often been ignored and any region of chromatin that displays a more condensed and/or transcriptionally silent state has been considered as "heterochromatin". This has resulted in some confusion about definition of heterochromatin itself.

1. Methylated histones as molecular markers of "heterochromatin"

Classically, heterochromatin has been identified by its condensed state, heteropycnosis, late replication and genetic inertness. Combination of genetic and molecular studies on position effect variegation in *Drosophila* and other organisms identified the heterochromatin associated protein HP1 and the histone methyl transferase (HMT) like SU(VAR)3-9 whose loss of function suppresses position effect variegation. These and related proteins soon established themselves as markers of a distinct molecular organization of heterochromatin (see reviews by Fischle *et al* 2003; Vaquero *et al* 2003; Zhimulev and Belyaeva 2003a,b). A variety of post-translational modifications of histones, like acetylation, phosphorylation, methylation, ubiquitination, ADP-ribosylation, sumouylation, etc. have been extensively studied in recent decades (for recent reviews see Fischle *et al* 2003; Vaquero *et al* 2003; Zhimulev and Belyaeva 2003a,b; Cao and Zhang 2004; Tariq and Paszawoski 2004; Grewal and Rice 2004) and such modifications seem to provide a "histone code" in the context of gene regulation and the higher order structures of chromatin (Lachner *et al* 2003). Several of these histone modifications are reversible and thus provide for a dynamic regulation of gene activity. However, modifications like methylation

of H3 and H4 histones, specifically at arginine (R) or lysine (K) residues, are longer-lasting and thus are important in epigenetically modifying and maintaining the higher order structures of chromatin regions through cell generations. Since the N-terminal tails of histones H3 and H4 protrude out of the compact nucleosome, methylation of the lysine and arginine residues in these regions provides a convenient mechanism for the recruitment of additional proteins and other molecules for establishing the higher order structure of chromatin. Lysines at positions 4, 9, 27 and 36 of histone H3 (K4H3, K9H3, K27H3 and K36H3, respectively) and at position 20 of histone H4 (K20H4) can be mono-, di- or tri-methylated (Me1-, Me2- or Me3-, respectively). Since each of these methylations affects transcriptional activity/silencing or chromatin condensation, these modifications have attracted special attention in recent years (Lachner *et al* 2003; Vaquero *et al* 2003; Cao and Zhang 2004; Tariq and Paszawoski 2004; Grewal and Rice 2004). Like the methylation of DNA, methylated histones are generally present on transcriptionally silent or repressed chromatin; an exception is the K4H3 methylation, which is associated with gene activation (Lachner *et al* 2003; Vaquero *et al* 2003; Zhimulev and Belyaeva 2003a; Cao and Zhang 2004). Thus methylated H3 and H4 histones have generally become markers for inactive chromatin and heterochromatin.

A family of conserved HMTs carrying the SET domain (named after the common domain shared by the Suppressor of variegation, Enhancer of zeste and Trithorax proteins of *Drosophila*) methylates the different lysine residues in H3 and H4. Nearly 50 SET domain genes have been identified in mammals (Vaquero *et al* 2003; Fischle *et al* 2003; Cao and Zhang 2004; Tariq and Paszawoski 2004; Grewal and Rice 2004).

Significant insights into the cellular roles of the different histone methylations have been obtained through immunolocalization and chromatin immunoprecipitation (CHIP) studies using antibodies against specific methylated lysine residues of H3 and H4 and the proteins that modify the histones. Using these and other methods, it has been shown that K9H3 methylation is a distinct marker for heterochromatin as well as other transcriptionally repressed chromatin and both these classes also generally show binding of the HP1 protein (Maison *et al* 2002; Zhimulev and Belyaeva 2003a; Grewal and Rice 2004; Tariq and Paszkowski 2004). These common features have sometimes led to the belief that the cytologically identifiable heterochromatin need not be distinguished from the molecularly defined repressed genes or chromatin domains. However, a careful analysis of the observations reported in some recent papers, as discussed below, reinforces the belief that all chromatin regions that share properties like transcriptional inactivity, late replication, condensed state and so on are really not similar, either in terms of the patterns of histone modifications or with regard to other associated proteins.

2. Methylation of K20H4 adds more variety to chromatin organization

Methylation of K20H4 has been added to the growing list of histone modifications important for chromatin organization and function. Fang *et al* (2002) and Nishioka *et al* (2002) identified nucleosomal histone K20H4 specific methyl-transferases named SET8 and PR-SET7, respectively. However, the two methyltransferases may refer to the same protein since, at least in *Drosophila melanogaster*, same gene has been identified for SET8 and PR-SET7. K20H4 methylation occurs in higher eukaryotes but not in *Saccharomyces cerevisiae* and *Tetrahymena* (Fang *et al* 2002; Nishioka *et al* 2002). In higher eukaryotes, methylated K20H4 seems to be more widespread in its chromatin distribution than methylated K9H3. In polytene chromosomes of *Drosophila* most of the condensed (transcriptionally inactive) bands and the chromocentric heterochromatin show high levels of di-methylated K20H4 while puffs and interbands show very little or no immunostaining with anti-K20H4 antibodies (Fang *et al* 2002; Nishioka *et al* 2002). In mitotic mammalian cells, the level of di-methylated K20H4 varies in a cell cycle dependent manner, increasing during S-phase and being maximal during mitosis. Acetylation of K16H4, a marker for transcriptionally active chromatin, inhibits methylation of K20H4. Thus it appears that methylation of K20H4 directs condensation of chromatin, be it during interphase or during mitosis (Fang *et al* 2002; Nishioka *et al* 2002; Rice *et al* 2002).

As with H3 methylation, the level of methylation (mono-, di- or tri-) of K20H4 has significantly different consequences. Julien and Herr (2004) revealed an important switch between mono- and di-

methylated K20H4 during mitosis in mammalian cells, a disruption of which affects proper movement and separation of chromosomes. Julien and Herr (2004) suggest that the abundant chromatin-associated HCF-1 (herpes simplex virus host-cell-factor 1) coordinates cell division, possibly through regulation of the PR-SET7 dependent di-methylation of K20H4 during mitosis. Mitotic cells and a subset (possibly G2) of interphase cells displayed high levels of mono-methylated K20H4. Depletion of the HCF-1$_c$ subunit through RNAi, resulted in a significantly increased level of di-methylated K20H4 and decreased level of mono-methylated K20H4 in mitotic chromosomes and consequent abnormalities in chromosome alignment and segregation during mitosis. On the other hand, HCF-1$_c$ subunit depletion affected neither the levels of mono-, di- or tri-methylated K20H4 in interphase cells nor of tri-methylated K9H3 in mitotic chromosomes (Julien and Herr 2004). The authors suggest that mono-methylated K20H4 may facilitate the binding of as yet unknown protein factors (to histone H4) that regulate chromosome dynamics during mitotic division.

An extensive study by Kourmouli *et al* (2004) on the distribution of tri-methylated K20H4 in mammalian, *Drosophila* and mealy bug cells has revealed an intriguing pattern of distribution of tri-methylated K20H4 in different cell types and chromosome regions. In the mouse, the DAPI-positive and late replicating heterochromatic centromeric regions of different chromosomes, but not the Y-chromosome or the inactive X-chromosome, showed a strong presence of Me3-K20H4 in interphase and mitotic cells. Intriguingly, different interphase nuclei showed significantly varying levels of immunofluorescence, which might suggest cell cycle dependent regulation. It will be interesting to examine if this cell cycle dependent variation in the presence of Me3-K20H4 is in some ways linked to regulation of mono- and di-methylation of K20H4 during the mitotic cycle (Julien and Herr 2004). During meiosis in the male mouse, Me3-K20H4 was found at centromeric regions of all but one pair of autosomes, some telomeres and on the pseudo-autosomal region (PAR) of X and Y chromosomes. It is interesting that at leptotene and zygotene stages, the PAR showed the presence of Me3-K20H4 as well as Me3-K9H3, but at pachytene stage, while Me3-K9H3 continues to be present at the PAR of the X-Y body, Me3-K20H4 was no longer detectable (Kourmouli *et al* 2004). These authors also found a very interesting difference in male and female pronuclei immediately after fertilization of the mouse egg with respect to the presence of Me3-K20H4. While neither the mature sperm nor the male pronucleus within the fertilized egg had any Me3-K20H4, the polar body nuclei and the maternal pronucleus showed distinct Me3-K20H4 staining. This difference in the presence of Me3-K20H4 in maternal and paternal chromosomes continues till early G1 of the 2-cell stage. The authors suggest a relationship between demethylation of DNA during early mouse embryogenesis and disappearance of Me3-K20H4.

In polytene nuclei of *Drosophila*, the anti-Me3-K20H4 antibody decorated the chromocentric heterochromatin as well as many bands in the euchromatic chromosome arms. Kourmouli *et al* (2004) find that several bands on polytene chromosomes do not stain with the antibody fully across their entire width, and suggest that this may be related to the salivary glands being derived from younger stages of flies: it has been reported earlier (Sarg *et al* 2002) that the Me3-K20H4 level increases with ageing. However, this is unlikely, since polytene nuclei in larval salivary glands do not survive beyond the larval stage therefore the age-related changes that occur in mammalian cells may not be applicable to polytene cells in salivary glands of *Drosophila* larvae. On the other hand, the partial immunostaining of some of the bands on polytene chromosome arms may be due to the homologous chromosomes carrying allelic differences in some of the repetitive sequences, which may be associated with Me3-K20H4. This needs further analysis.

Mealy bugs (coccids) have interesting chromosome behaviour, the lecanoid system (Brown 1966; Bongiorni and Prantera 2003). Mealy bug males and females both are diploid and chromosomally similar, but the paternal set of chromosomes becomes condensed and transcriptionally repressed early in all somatic cells of male embryos. The inactivated paternal set of chromosomes in male mealy bugs and the inactive X-chromosome in somatic cells of female mammals are classical examples of facultative heterochromatin (Brown 1966). Kourmouli *et al* (2004) now show that with respect to the presence of Me3-K20H4, these two cases of epigenetically inactivated and condensed chromosomes differ. As opposed to the absence of Me3-K20H4 on the heterochromatinized X-chromosome in female mammalian cells, the heterochromatinized paternal set of chromosomes in male mealy bugs is enriched in Me3-K20H4.

Kourmouli *et al* (2004) report that both the Me3-K9H3 and Me3-K20H4 modifications in mouse are dependent upon the HMT activity of SU(VAR)3-9 and since the distribution of these two types of modified histones in mouse chromosomes is generally similar, they suggest epigenetic cross-talk between these two modifications in regulating centromeric heterochromatin structure. Another independent recent paper (Schotta *et al* 2004) also documents a specific enrichment of Me3-K9H3 and Me3-K20H4 in the pericentromeric heterochromatin of mouse. Schotta *et al* (2004) in addition provide a clear evidence for a cross-talk between K9H3 and K20H4 and suggest a mechanism that effects the trimethylation of K20H4. Schotta *et al* (2004) show that experimental knockdown of SU(VAR)3-9 by RNAi abolished trimethylation of K9H3 as well as K20H4 but a similar RNAi dependent inactivation of SU(VAR)4-20, a novel member of the nucleosome specific HMT family conserved in *S. pombe*, *Drosophila* and mammals, affects trimethylation of K20H4 but not of K9H3. Apparently, Me3-K9H3 is prerequisite for trimethylation of K20H4 by SU(VAR)4-20. Schotta *et al* (2004) suggest a stepwise model of methylation of K9H3 and K20H4: in the first step, an as yet unidentified HMT monomethylates K27H3 and K9H3 on repeat rich pericentromeric heterochromatin regions so that SU(VAR)3-9, in association with HP1 (and other related proteins) and the RNAi machinery, trimethylates K9H3 which then permits trimethylation of K20H4 by SU(VAR)4-20 family HMT(s).

3. Histone methylation patterns provide variety to "heterochromatin" and transcriptionally repressed chromatin regions

Classical heterochromatin and transcriptionally inactive or repressed regions of "euchromatin" generally share several common features like condensed state, trasncriptional inactivity, late replication, methylation of DNA, hypoacetylation of H3 and H4 histones, methylation of specific lysine (except K4H3) and/or arginine residues on H3 and H4 histones and association of certain proteins like HP1, HP2, SU(VAR)3-7, SU(VAR)3-9, Set07/Set08, SUUR etc. (Zhimulev and Belyaeva 2003a,b). Another unexpected similarity has come to light in recent years. This is the role played by RNA interference or RNAi in the silencing of individual genes, epigenetic condensation, inactivation of euchromatic regions and the formation of constitutive heterochromatin blocks (see Grewal and Moazed 2003; Grewal and Rice 2004; Heard 2004). RNAi is emerging as a widely used means of regulation of gene activity through small RNA molecules (for recent reviews, see Hannon 2002; Murchison and Hannon 2004; Tijsterman and Plasterk 2004).

In the context of these similarities exhibited by transcriptionally repressed parts of genome, it is to be noted that the patterns and levels of methylation of specific lysine residues on H3 and H4 histones provide a vast repertoire (Lachner *et al* 2003), which genomes can exploit, on the one hand for regulating the gross chromatin structure in relation to its activity, and on the other for fine-tuning of an individual gene's states of activity. A closer examination of the available information on different kinds of condensed and transcriptionally repressed chromatin reveals that they differ in subtle but significant mechanistic and functional aspects from each other. It will be instructive to consider a few examples that reveal the diversity of organization, sometimes contradictory, of condensed and inactive chromatin in eukaryotes.

The facultatively heterochromatinized inactive X chromosome in female mammalian cells displays, like the other peri-centric and telomeric heterochromatin regions, cytosine methylated DNA, hypoacetyled histones H3 and H4, di-methylated K9H3 and non-methylated K4H3 (see Heard 2004). However, the inactive X chromosome, in addition to K9H3 methylation, also carries tri-methylated K27H3, which is not common in other heterochromatic regions (Lachner *et al* 2003). Interestingly, the di-methylated K9H3 and tri-methylated K27H3 are present in overlapping but distinct regions of the inactive X-chromosome (Rougeulle *et al* 2004; Heard 2004). Di-methylated K9H3 is considered to provide the platform essential for binding of HP1 protein and thus bring about condensation and transcriptional repression of chromatin. However, the inactive X-chromosome in mitotic cells in mouse as well as human females, in spite of carrying di-methylated K9H3, is not associated with HP1 (Zhimulev and Belyaeva 2003a; Chadwick and Willard 2003). On the other hand, Chadwick and Willard (2003) report that in human female interphase cells HP1 is enriched on the inactive X only when a Barr body is detectable. Apparently, association of HP1 with the inactive X-chromosome is dependent

upon formation of the Barr body and is limited to interphase nuclei. Furthermore, the methylation of K9H3 on the inactive X chromosome is through the activity of HMT other than SU(VAR)3-9 (Maison *et al* 2002). Inactive X chromosome carries Me1-K20H4 while the pericentromeric constitutive heterochromatin regions show Me3-K20H4 (Kourmouli *et al* 2004; Schotta *et al* 2004). Another difference between pericentric heterochromatin and the inactive X-chromosome in mouse cells relates to some aspect of their higher order structure since the former, but not the later, is sensitive to RNAse treament (Maison *et al* 2003).

The heterochromatinized paternal set of chromosomes in male mealy bugs shares many properties with the inactive mammalian X-chromosome, but differs from it in being enriched in methylated K9H3 as well as HP1 (Bongiorni and Prantera 2003; Zhimulev and Belyaeva 2003a). Furthermore, as already mentioned, the inactive X chromosome and the paternal set of chromosomes in male mealy bugs also differ with respect to the presence of Me3-K20H4 (Kourmouli *et al* 2004).

Seemingly going against the well known silencing function of the HP1 protein, several typical euchromatic regions in *Drosophila* also show binding of HP1 (Piacentini *et al* 2003; Zhimulev and Belyaeva 2003a,b). More significantly, binding of HP1 has been shown to be essential for activation, rather than repression, of heat shock and some other developmental puffs in polytene nuclei of *Drosophila* (Fanti *et al* 2003; Kellum 2003). Obviously, classifying a chromatin region as transcriptionally silent or as heterochromatin just because HP1 protein is present, can be misleading. It will be interesting for future studies to examine the role of HP1 and other proteins in regulating activities of those genes that normally reside within typical "heterochromatin" and whose activity actually gets repressed when rearranged to a "euchromatin" domain.

Instances are also known where different regions identified as "constitutive heterochromatin" differ in histone modifications, in the same cell at that. For example, the Y-chromosome and the peri-centric heterochromatin on one pair of autosomes in mouse cells are not associated with Me3-K20H4 (Kourmouli *et al* 2004).

Most of the earlier studies only looked at methylation, irrespective of the level of methylation, of a given H3 or H4 histone lysine residue and consequently, subtle differences in methylation patterns of different chromatin regions were not distinguished. However, as the results of Julien and Herr (2004), Kourmouli *et al* (2004) and Schotta *et al* (2004) demonstrate, levels of methylation of specific lysine residues are indeed important determinants. Use of more specific antibodies that distinguish between mono-, di- and tri-methylated states at the different lysine residues will make it possible to unravel the enormous combinatorial diversity of lysine methylation that may exist. With about 50 or more SET domain genes already known in mammals, the possible combinations of methylated lysines (specific residues and the level of methylation) in H3 and H4 histones in different chromatin regions can indeed be very large.

Apparently, any specific set of features cannot be universally and invariantly applied to either the constitutive or the facultative heterochromatin or to the transcriptionally repressed euchromatin (like the many dark bands of polytene chromosomes or the G-band regions of mitotic cells). Each class is characterized by some over-lapping and some unique subsets of properties. Biological systems are well known to display enormous diversity in means to achieve a common end. Heterochromatinization and transcriptional silencing are no exception to this. For example, the two yeast species, *S. cerevisiae* and *Schzosaccharomyces pombe*, carry functionally comparable heterochromatin regions but differ in their histone methylation patterns: SET domain HMTs and methylation of K9H3, K27H3 and K20H4 are generally absent in *S. cerevisiae*, while *S. pombe* displays HMTs and H3 and H4 histone methylation patterns which are generally reminiscent of those in higher eukaryotes (Schotta *et al* 2004). Given the diversity of histone modifications and the vast repertoire of chromosomal proteins that can interact with the variously modified histone moieties, the coming years will quite likely be disappointing to those seeking a unified and definitive property of heterochromatin. Although Heitz (1928), said that the basic property of "heteropycnosis can only lie in the concerned chromosomes themselves", for the chromatin region to be or not to be identifiable as "heterochromatin" depends on various epigenetic modifications. As our knowledge about these modifications and the combinations of these modifications that occur in different situations becomes more detailed and refined, it increase, it will be increasingly unlikely that a unified "code" for heterochromatin can be defined. But will have a better appreciation of what it is.

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