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Review

Chromatin Structure with Respect to Histone Signature Changes during Cell Differentiation

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ABSTRACT. **Here, we would like to point out important milestones in the study of nuclear radial positioning and gene expression during differentiation processes. In addition, changes in the histone signature that significantly precede various differentiation pathways are reviewed. We address the regulatory functions of chromatin structure and histone epigenetic marks that give rise to gene expression patterns that are specific to distinct differentiation pathways. The functional relevance of nuclear architecture and epigenetic traits is preferentially discussed in the context of *in vitro* induced enterocytic differentiation and pluripotent or differentiated embryonic stem cells. We especially focus on the recapitulation of nuclear events that have been characterized for some genes and proto-oncogenes that are important for development and differentiation.**

Key words: chromatin/gene expression/differentiation/enterocytes/chromosomes

Introduction

In eukaryotic cells, interphase nuclei have a non-randomly organized genome, with chromosomes existing in an arrangement that is thought to be important for nuclear functions (summarized by Cremer and Cremer, 2001; Lanctôt *et al.*, 2007). This idea has been supported by measurements of the nuclear radial positions of chromosomes and their sub-regions in three-dimensional (3D) space, and is particularly striking when 3D radial distributions are compared with gene expression or density (Kurz *et al.*, 1996; Verschure *et al.*, 1999; Croft *et al.*, 1999; Bártová *et al.*, 2000; Zink *et al.*, 2004; Williams *et al.*, 2006). Studies of nuclear radial arrangements of individual genomic elements began with Popp *et al.* (1990) and/or Höfers *et al.* (1993) describing the calculation of radial distributions of genetic elements within interphase nuclei. Later, Kozubek *et al.* (1999) introduced the concept of data normalization to the local nuclear radius allowing several groups to precisely calculate radial positions in 3D space (Kozubek *et al.*, 2002; Scheuermann *et al.*, 2004; Goetze *et al.*, 2007, summarized by Lanctôt *et al.*, 2007). The 3D-radial positions of transcription sites were first measured by Harničarová *et al.* (2006). Transcripts of the mono-allelically expressed *c-myc* gene (Wilson *et al.*, 2002; Levsky *et al.*, 2002) were found to be positioned in more interior regions of interphase nuclei (~40% of nuclear radius) relative to the average location of *c-myc* coding sequences (~70% of nuclear radius) (Harničarová *et al.*, 2006). These experiments, as well as others (e.g., Xing *et al.*, 1993, 1995; Smith *et al.*, 1999; Takizawa *et al.*, 2008a; Bártová *et al.*, 2008a; Royo *et al.*, 2009), asked whether transcription sites are associated with regulatory domains, such as nuclear speckles, nucleoli, or PML bodies. An important discovery was that transcriptionally active genes, from distinct chromosome territories, are positioned in RNA polymerase II-positive regions, called "transcription factories" (Iborra *et al.*, 1996; Hirose *et al.*, 1999, summarized by Chakalova *et al.*, 2005), which are homogenously dispersed throughout the nucleus but are absent from nucleoli (Wansink *et al.*, 1993). In some cells, the nuclear periphery lacks RNA polymerase II-positive regions (Harničarová *et al.*, 2006; Bártová *et al.*, 2008a), implying that transcription preferentially occurs within the nuclear interior (Chuang *et al.*, 2006; Finlan *et al.*, 2008, summarized by Fedorova and Zink, 2009). This idea is

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Abbreviations: CT, chromosome territory; FISH, fluorescence *in situ* hybridization; HAT, histone acetyltransferase; HDAC, histone deacetylase; HP1, heterochromatin protein 1; HSA, Homo sapiens autosome; NaBt, sodium butyrate; PML, promyelocytic leukaemia; 5'HS4, a constitutive DNase I-hypersensitive site 5' of the chicken β-globin locus; hMLH1, human mut L homolog 1; USF, upstream stumulatory factor; GFAP, glial fibrillary acidic protein; 3D, three dimensional; LCR, locus control region.

consistent with the finding that gene silencing occurs at the telomere-dense nuclear periphery in yeast (Andrulis *et al.*, 1998). In contrast, some active genes have been found in close proximity to the nuclear periphery, associated with the nuclear pore complex, which is likely related to RNA processing and transport (Casolari *et al.*, 2004). These loci interact with the nuclear lamina, a prominent structure formed by lamins at the nuclear periphery, which underlines the inner nuclear membrane and binds to a variety of proteins, such as emerins and lamina-associated polypeptides (LAP2β). In lamin B1 deficient murine cells, some chromosomes dissociate from the nuclear periphery (Malhas *et al.*, 2007). Similarly, one allele of the *CFTR* gene is associated with a high concentration of LAP2β at the acetylation-poor nuclear periphery. Hovewer, when the chromatin is hyperacetylated following treatment with the deacetylase inhibitor TSA, the *CFTR* locus re-locates closer to the nucler interior (Zink *et al.*, 2004). These studies showed that the decreased expression of some genes located at the nuclear periphery is dependent on the activity of histone modifying enzymes (Finlan *et al.*, 2008; Fedorova and Zink, 2008). Interestingly, the stable radial positioning of mammalian genes, including *Rb1*, *Oct3/4*, and *c-myc* may be regardless of the transctiptional status of the gene (Bártová *et al.*, 2002; Wiblin *et al.*, 2005; Bártová *et al.*, 2008b and our recent observations). On the other hand, the stably expressed *Abl* gene is repositioned closer to the nuclear envelope during myeloid differentiation (Bártová *et al.*, 2002). This implies that nuclear radial positioning is gene-specific and can be influenced by complex differentiation processes (summarized by Takizawa *et al.*, 2008b). Thus, the importance of nuclear radial positioning in transcription is unclear.

Here, we review the molecular and structural aspects of chromatin organization during cell differentiation, focusing on the differentiation of intestinal stem cells into mature enterocytes and embryonic stem cells. Knowledge of how cells differentiate may lead to a better understanding of differentiation disorders that result in tumorigenesis.

Higher Order Chromatin Organization of the Interphase Nucleus

In the cell nucleus, the organization of chromatin fibers undergoes cell cycle-dependent changes. A large proportion of the chromatin in interphase nuclei is packaged into stable, large-scale structures above the level of the 30-nm chromatin fiber (Manuelidis and Chen, 1990; Belmont and Bruce, 1994; Gilbert and Ramsahoye, 2005). There are several models of chromatin organization above this level. According to the chromonema model, the 30-nm fiber can be further organized into distinct chromonema fibers, with diameters of 60–80 nm (Belmont and Bruce, 1994). The coiling of the chromonema forms the 100- to 130-nm large chromonema fiber. In addition, there exist local regions where chromonema fibers are unfolded to individual 30-nm chromatin structures (Belmont and Bruce, 1994; Belmont *et al.*, 1999). Unfolding of the chromatin at the level of the 30-nm chromatin fiber is believed to be important in the regulation of gene expression (Felsenfeld, 1992; Gilbert and Ramsahoye, 2005). Prior to cell division, a progressive condensation of chromatin fibers is observed. The chromatin fibers fold, twist, and kink into condensed prophase chromatids with a diameter of 200–400 nm, and then coil to form the metaphase chromosome, which measures 700 nm in width (Manuelidis and Chen, 1990; Belmont and Bruce, 1994; Belmont *et al.*, 1999).

The radial loop model of interphase chromatin organization, derived from mitotic chromosome structures, assumes that the 10- to 30-nm chromatin fibers in the range 30 to 100 kb of DNA are shaped as large chromatin loops. The loops are attached via scaffold attachment regions (SARs) at their bases to dispersed remnants of the metaphase chromosome scaffold, which, in the interphase nucleus, is called the nuclear matrix (Stack and Anderson, 2001). Additional associations of the chromatin loops with the nuclear matrix, created by matrix attachment regions (MARs), further organize the chromatin loops into subdomains that are important for gene regulation (Paul and Ferl, 1999). The scaffold/matrix anchoring region is created from segments of DNA, which likely represent AT-rich sequences. However, the existence of the nuclear matrix is still being debated and the possibility of chromatin self-organization must be considered owing to the results of several experimental and mathematical approaches (Misteli, 2001; Rajapakse *et al.*, 2009; Cook and Marenduzzo, 2009).

Types of Chromatin and Its Folding

Chromatin fibers of increasing thickness, for example the 10- and 30-nm fibers and the 60- to 130-nm chromonema fibers, are not randomly distributed throughout the nucleus, but are rather hierarchically grouped into chromosomes. In the interphase nucleus, each chromosome occupies a spatially well-defined subvolume, called a chromosome territory (CT). Some authors have suggested that chromatin fibers create small-scale chromatin loops of ~50–200 kbp that are essential building blocks of the interphase chromosome. Using base computer simulations, Münkel *et al.* (1999) developed the multiple-loop compartment model, which assumes the folding of chromatin fibers into 120-kbp sized loops and the arrangement of these loops into rosette-like subcompartments. Different subcompartments are connected by small chromatin fragments, and the lengths of loops do not overlap to a large extent. Other researchers have used multiple distance measurements of FISH-labelled genomic sequences to determine that chromosome territories are built up from intermingling giant loops composed of

2–3 Mbp of DNA, called the random walk/giant loop model. Within each giant loop, chromatin is randomly folded and specific loop attachment sites are arranged to form backbone-like structures (Sachs *et al.*, 1995; Yokota *et al.*, 1995).

Folding and the level of condensation determine the basic types of chromatin: Euchromatin is characterized as gene-rich regions that are less-condensed within interphase nuclei, replicate earlier, and are more actively transcribed relative to heterochromatin (summarized by Misteli, 2005). Heterochromatin domains are inaccessible to DNA binding factors and a variety of reagents, particularly nucleases (Elgin and Grewal, 2003). These regions remains condensed throughout the cell cycle (Craig, 2005) and, in interphase nuclei, are located at the nuclear periphery and near nucleoli (Weierich *et al.*, 2003). Heterochromatin is further divided into constitutive (permanent) and facultative heterochromatin (gained during development). Constitutive heterochromatic domains including centromeres, their adjacent pericentromeric regions, and telomeres, are enriched in DNA repetitive sequences and are surrounded by other functional components of chromosomes (Grewal and Moazed, 2003). In many cell types, it has been observed that pericentromeric heterochromatin regions conspire to form clusters known as chromocenters (Alcobia *et al.*, 2003). Constitutive heterochromatin often causes the silencing of active genes that are moved into its vicinity by chromosomal rearrangement or insertion (Brown *et al.*, 1997, 1999; Bártová *et al.*, 2002). In contrast, facultative heterochromatin is formed by the stable inactivation of primarily active genes during development and cellular differentiation. A good example is the inactivation of one of the two X chromosomes in female mammalian cells at an early stage of embryogenesis (summarized by Elgin and Grewal, 2003). Several components are involved in the process of forming facultative heterochromatin, such as DNA methylation, specific histone signatures, and binding of specific proteins and noncoding RNAs (summarized by Craig, 2005).

Chromosome Territories and the Nuclear Distribution of Euchromatin and Heterochromatin

Over the last two decades, multiple studies have confirmed that the cell nucleus is structurally and functionally compartmentalized and that chromosome territories are the basic structural components (Cremer and Cremer, 2001; Parada *et al.*, 2003) (Fig. 1). The organization and architecture of chromosome territories, with respect to the functional aspects of the nucleus, have been described by several authors. The original interchromosome domain (ICD) model was based on the finding that the RNA transcripts of an integrated human papilloma virus genome and the components of the transcription machinery are preferentially excluded from the interiors of chromosome territories and accumulate in the surrounding space. This observation led to the idea that distinct chromosome territories have a rather smooth surface and are separated from each other by the structural and functional interchromosome domain compartment (Zirbel *et al.*, 1993). A similar model with respect to the interchromosome domains was published by Bridger *et al.* (1998), who observed that an intranuclear array of vimentin filaments is situated outside of chromosome territories and colocalizes with nuclear structures, such as specific RNAs, coiled bodies, and promyelocytic leukemia (PML) bodies (Fig. 1). Because the factors of the transcription machinery are found outside of chromosome territories, it is thought that transcriptionally active genes are localized on the surfaces of chromosome territories (Zirbel *et al.*, 1993). Among the first experiments to measure the positions of genomic DNA sequences in relation to their respective chromosome territories were those carried out by Kurz *et al.* (1996), which supported this model. Subsequent studies of the relationship between the structure and function of chromosomes have confirmed that transcription is not limited to the periphery of chromosome territories, but is also scattered throughout the chromosome interior (Verschure *et al.*, 1999).

From observations made using light microscopy and electron microscopy, it has been shown that chromosome territories are partially organized as condensed chromatin, but dispersed chromatin on the chromosome periphery extends into the interchromatin space that is largely devoid of DNA (Visser *et al.*, 2000). The interchromatin space forms interconnecting channels running through and around chromosome territories. This channel-like structure facilitates access of regulatory factors to sequences both at the outer surface and within the chromosome territory. At the nuclear periphery, these channels are connected to nuclear pores (Visser *et al.*, 2000). These attributes have been included in the chromosome teritory-interchromatin compartment (CT-IC) model (for reviews see Cremer and Cremer, 2001; Cremer *et al.*, 2006). The term interchromatin compartment is used as a synonym for the inter-chromatin space, but with some exceptions. The interchromatin compartment starts at the nuclear pore, expands between chromosome territories, extends into the chromosome interior (Fig. 1A), and possibly ends with small domains of chromatin loops between ~100 kb and ~1 Mb. Notably, by definition, the inter-chromatin compartment does not encompass the additional interchromatin space. The authors of this model refer to the functional significance of the interchromatin compartment, which is important for transcription. According to the CT-IC model, regulatory and coding sequences of active genes can interact with the transcription machinery only when they are positioned at the surfaces of chromatin domains that line the interchromatin compartment, or on chromatin loops that extend into the IC space (Cremer and Cremer, 2001).

The boundaries of chromosome territories are not clearly

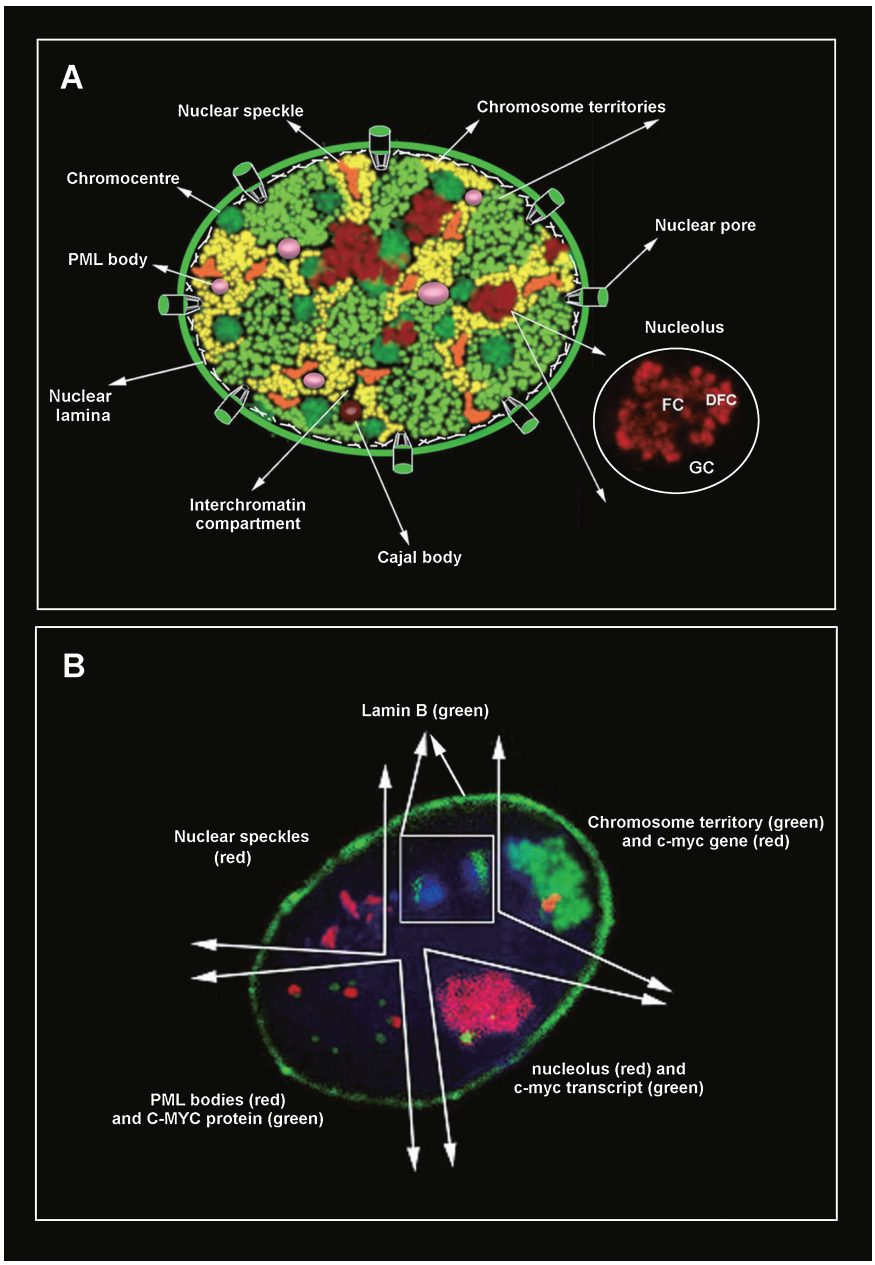

Fig. 1. An illustration of the compartmentalization of interphase nuclei. (A) A representation of the arrangement of chromatin and chromatin-related domains within interphase nuclei. The following structures are indicated: chromosome territories (yellow and green), clusters of centromeres called chromocentres (dark green), interchromatin compartment (black), nuclear lamina (green rim and white lines), nuclear pores (green) nuclear speckles (orange), PML bodies (pink), Cajal body (brown), nucleolus (red), which consists of fibrilar centers (FC involves rDNA genes), dense fibrilar component (DFC; rDNA is transcribed to rRNA at the border of FC and DFC; DFC are depicted as red according to staining by antibody against nucleolar protein fibrillarin), and granular component (GC is the site of rRNA processing and assembly of pre-ribosomal particles). (B) Individual nuclear structures visualized by DNA/RNA FISH and/or by immunocytochemistry combined with confocal microscopy were compiled into one imaginary nucleus. The presence of lamin B (green) at the nucler periphery and lamin B associated with a more centrally located chromocenres (blue) is documented, similar to the location of the *c-myc* gene (red) within the chromosome 8 territory (difuse green). Positioning of *c-myc* transcripts (green) at the periphery of the nucleolus (red). An example of the association of PML bodies (red) with C-MYC protein (green) and pattern of nuclear speckles (red). Images were created and adapted using Adobe Photoshop 5.0LE. Individual nuclear structures (in panel B) were acquired by Nipkow disc-based confocal microscopy.

defined. In some experiments, chromatin fibers were observed to be extended outwards from the surface of CTs (Volpi *et al.*, 2000; Chambeyron and Bickmore, 2004; Bártová *et al.*, 2008b). In contrast, it has also been shown that chromosome territories can be in close contact, with little or no intermingling of their DNA (Visser and Aten, 1999; Visser *et al.*, 2000). This observation was made by Branco and Pombo (2006), who saw extensive intermingling of chromatin, both within a given chromosome territory and between neighbouring chromosome territories. Albiez *et al.* (2006) undertook to resolve these conflicting views. Using experimental manipulations of the nuclear architecture, these authors obtained results supporting the CT-IC model, but they did not confirm the conclusions of the interchromosomal network model published by Branco and Pombo (2006). On the other hand, a certain degree of intermingling between neighbouring territories and their physical distance may be important for the occurrence of chromosome translocations in tumor cells.

Chromosome territories can adopt irregular shapes, with numerous protrusions and invaginations forming as a result of chromatin mobility. The size of the chromosome territory is roughly determined by its DNA content, but is influenced by other factors, such as transcriptional state (Mahy *et al.*, 2002). The two X chromosome territories of female somatic nuclei provide a particularly useful model for exploring the differences in higher order organization of individual chromosomes with distinctly different transcriptional activities. According to the Lyon hypothesis, during the female development, one X chromosome becomes transcriptionally inactivated, while the other remains transcriptionally active (Lyon, 1961). Eils *et al.* (1996) studied the 3D morphology of both X chromosome territories and demonstrated that the territory of the transcriptionally active X chromosome (HSA Xa) has a flatter shape and exhibits a larger and more irregular surface than the transcriptionally inactive X chromosome territory (chromosome Xi).

By staining metaphase chromosomes with intercalating dyes, it is possible to observe light and dark-staining bands, called R- and G-bands (Zink *et al.*, 1999; Cremer and Cremer, 2001). These bands differ in GC and AT nucleotide composition and correlate to early or late replicating chromatin (Tajbakhsh *et al.*, 2000; Zink *et al.*, 1999). A close structural relationship was found between the organization of mitotic chromosomes and chromosome territories. The arm and band domains of metaphase chromosmomes are maintained during interphase and occupy discrete regions within a chromosome territory (Dietzel *et al.*, 1998; Tajbakhsh *et al.*, 2000; Cremer and Cremer, 2001). Moreover, a specific spatial distribution of nucleotide sequences within a chromosome territory was observed. The AT rich DNA is frequently localized towards the interior, while GC-sequences are more variable in their intraterritorial position (Tajbakhsh *et al.*, 2000). Early and late replicating chromatin, observed as R- and G-bands, generally corresponds to the euchromatin and heterochromatin domains of interphase nuclei, respectively (Craig and Bickmore, 1993). Each of these is associated with a distinct level of chromatin condensation and post-translational modifications, such as methylation and acetylation, predominantly on lysine residues of histones H3 and H4 (Grewal and Moazed, 2003; Misteli, 2005; Kouzarides, 2007).

Histone Signatures

Histone acetylation of H3 and H4 amino-terminal tails is thought to function as a signal for active chromatin by enhancing the accessibility of the transcription machinery. On the other hand, histone deacetylation is associated with repressive chromatin configurations. The activity of chromatin is also determined by the methylation of specific lysine residues on histone H3. This important epigenetic mark is mediated by the histone methyltransferases (HMTs). Conversely, the recently described LSD1 histone demethylase is selectively responsible for demethylation of lysine 4 of histone H3 (Shi *et al.*, 2004; for review see Martin and Zhang, 2005; Bannister and Kouzarides 2005). Generally, H3K4 methylation is considered to be an epigenetic mark associated with transcriptionally active chromatin, and LSD1 mediates its silencing. The functions of several families of enzymes responsible for histone modification have been described by Allis *et al.* (2007), showing the pro-transcriptional and silencing potential of these enzymes. The evidence collectively indicates that actively transcribed euchromatin has high levels of acetylation, reduced levels of DNA methylation, and is methylated at H3 lysine 4 H3(K4), H3(K36), and H3(K79). Meanwhile, transcriptionally silent heterochromatin is commonly characterized by low levels of acetylation, abundant DNA methylation, and high levels of methylation at H3(K9), Polycomb group (PcG)-associated H3(K27) methylation, and H4(K20) methylation (summarized by Kouzarides, 2007; Hublitz *et al.*, 2009). The quantity of methyl-groups also specifies distinct patterns of gene expression. For example H4K20 monomethylation (me1) correlates with ongoing transcription, whereas H3K20 trimethylation (me3) is responsible for heterochromatin-mediated silencing (summarized by Hublitz *et al.*, 2009). Dimethylation of histone H3 at arginine 2 (H3R2me2) maintains transcriptional silencing by inhibiting Set1-mediated trimethylation of H3K4 in yeast. Conversely, H3R2me1 does not inhibit H3K4 methylation; thus, it correlates with active transcription (Kirmizis *et al.*, 2009). Moreover, Lennartsson and Ekwall (2009) summarized the effects of specific combinations of several histone marks that are responsible for high, intermediate, and low gene expression in human T-cells, human embryonic stem cells, and budding yeast. For example, high gene expression is associated with the presence of the following histone modification backbone: H2AK9Ac,

H2BK5me1, H3K79me1, H3K79me2, H3K79me3, H4K12Ac, H4K16Ac, H4K20me1, H2AK9Ac, H2BK5me1, H3K79me1, H3K79me2, H3K79me3, H4K16Ac, H4K20me1, H2BK5me1, H3K79me1, H3K79me3, H3K79me3, H4K16Ac, H4K20me1, while low gene expression was observed according to backbone: H2AZ, H3K4me1, H3K4me2, H3K4me3, H3K9me1, H3K27me3, H2AZ, H3K4me2, H3K4me3, H3K9me1, H3K27me3, H2AZ, H3K4me3, H3K27me3, H3K4me3, H3K27me3, H3K4me3, H3K27me3, H3K4me3, H3K27me3, H3K4me3, H3K27me3 (Wang *et al.*, 2008, summarized by Lennartsson and Ekwall, 2009). Another significant mark of transcriptionally silent chromatin and constitutive heterochromatin is the presence of heterochromatin protein 1 (HP1), the variants of which (HP1α, HP1β and HP1γ) are key components of heterochromatin formation and maintenance. HP1 variants bind to methylated H3K9 and are preferentially involved in gene silencing (Eissenberg and Elgin, 2000). For example, it has been demonstrated by Ondřej *et al.* (2006) that transgenes move to the nearest HP1 focus in order to become silenced. On the other hand, HP1β and HP1γ were found to be responsible for the transcription of ribosomal genes (Yuan *et al.*, 2007; Harničarová Horáková *et al.*, 2009). In the case of HP1γ, Yuan *et al.* (2007) showed that CSB (Cockayne syndrome protein B)—mediated activation of rDNA transcription requires the G9a histone methyltransferase responsible for H3K9me1 and H3K9me2 and facilitates HP1γ binding. Taken together, these results indicate that epigenetic states of histones, histone modification backbone, and histone-binding proteins have defined roles in gene expression regulation.

Nuclear Topographies of Chromosome Territories and "Chromosome Kissing"

The fluorescence *in situ* hybridization (FISH) technique combined with high-resolution confocal microscopy and image processing systems has made it possible to visualize and analyze defined domains of the interphase nucleus. Several studies of nuclear topography have confirmed that chromosome territories and their related genetic regions are non-randomly arranged in three dimensionally-preserved interphase nuclei (Kozubek *et al.*, 1997, 1999; Croft *et al.*, 1999; Skalníková *et al.*, 2000; Bártová *et al.*, 2001; Boyle *et al.*, 2001; Cremer *et al.*, 2001, summarized in Razin, 2004; Bolzer *et al.*, 2005; Bártová *et al.*, 2008b). Chromosome territories, including locus-specific subregions, adopt specific positions in the nuclear space, which are conserved in different cell types and at various stages of the cell cycle (Kozubek *et al.*, 1999; Skalníková *et al.*, 2000). A convenient indicator of the position of a chromosome or locus within the interphase nucleus is the radial position, which is defined as the location of a given genetic element in relation to the nuclear center or periphery (Kozubek *et al.*, 1997; Skalníková *et al.*, 2000; Bártová *et al.*, 2001; Boyle *et al.*, 2001; Misteli, 2001; Finlan *et al.*, 2008; Bártová *et al.*, 2008b).

Many authors have shown that chromosome territories are spatially organized by parameters, such as gene density and DNA content (Croft *et al.*, 1999; Sun *et al.*, 2000, summarized in Cremer *et al.*, 2001; Bolzer *et al.*, 2005). A striking example of the radial non-random distribution of mammalian chromosomes has been shown for human chromosomes 18 and 19. Gene-dense chromosome 19 is positioned in the central part of lymphocyte nuclei, while the similarly sized, but gene-poor chromosome 18 is found at the nuclear periphery (Croft *et al.*, 1999). Likewise, the inactive female X chromosome resides near the nuclear periphery (reviewed in Chubb and Bickmore, 2003). Other studies have shown the existence of chromosome-size dependence (DNA content) for positioning within human interphase nuclei (Sun *et al.*, 2000). In flat-ellipsoid and spherical nuclei of diploid human cells, "large" chromosomes occupy the nuclear periphery, while "small" territories tend to reside towards the center of nuclei (Cremer *et al.*, 2001).

Aside from being organized by gene density or size, chromosomes also form non-random distributions relative to each other. In the cells of healthy individuals, certain chromosomes tend to always be located in close proximity. Hovewer, Kozubek *et al.* (1999) and Parada *et al.* (2002) have illustrated that the tight association of chromosomes increases the risk of possible translocation during tumor cell transformation (Cafourková *et al.*, 2001). In addition, translocation must preferentially occur between two proximally positioned double strand breaks rather than spatially distant chromosome lesions (Soutoglou and Misteli, 2007).

The intranuclear proximity of genes from different chromosomes has also been discussed with respect to transcriptional regulation. For example, the term "chromosome kissing" (Kioussis, 2005), which is a chromatin property that permits contact between distinct chromosome regions, has been debated from different angles. A concept discussed by Kioussis (2005) suggests that the close proximity of genes from different chromosomes can influence gene function. For example, intrachromosomal interactions have been observed for the gene encoding *IFN-γ*, on chromosome 10 and the regulatory regions of the *T(H)2* cytokine locus on chromosome 11 (Spilianakis *et al.*, 2005). These authors observed that these two genes remain associated via chromatin loops that extend away from distinct chromosomes until developmentally regulated signals split the genes apart. One gene moves to the heterochromatin region, which causes gene silencing, while the other relocates to a euchromatin area. Transient pairing was also observed for X-inactivation centres (XIC) in mouse female ES cells (Bacher *et al.*, 2006), illustrating the importance of loci proximity in transcriptional regulation. In the so-called "looping-scannig model", the Locus Control Region (LCR) regulates several genes. Proteins binding to the LCR can scan through large

portions of DNA. The intervening LCR region is looped out until it finds the target gene promoter (summarized by Kioussis, 2005). Other insights into the functional relevance of "gene kissing" have been introduced through studies of PcG-mediated gene silencing. Polycomb-group (PcG) and trithorax-group (trxG) proteins maintain the memory of chromatin states through binding to *cis*-regulatory elements, called PcG response elements or cellular memory modules. Lanctôt *et al.* (2007) discussed examples of "gene kissing" for copies of the *Fab7* regulatory element in *Drosophila melanogaster* that are presented on two different chromosomes. *Fab-7* is a well-defined cellular memory module involved in the regulation of the homeotic gene, Abdominal-B (*Abd-B*). 3D-FISH experiments have shown that the *Fab-7* element leads to the association of transgenes with each other or with the endogenous *Fab-7*, even when inserted into different chromosomes (Bantignies *et al.*, 2003). These long-distance interactions enhance PcG-mediated silencing, which is supported by the observation that the co-localization of the two loci is not detected when Polycomb-related proteins are deficient. This observation leads to the argument that endogenous PcG target genes likely undergo physical association at the site of nuclear PcG body accumulation (summarized by Lanctôt *et al.*, 2007). Conversely, the term "gene kissing" can be used in terms of the transcriptional activation that occurs in "transcription factories", which are RNA polymerase II-positive sites abundant on de-condesed genes originating from different chromosome territories (Iborra *et al.*, 1996; Chakalova *et al.*, 2005).

Mathematical Approaches to Studying Chromosomal Topologies

The chromosome territories adopt a polar arrangement within interphase nuclei. GC-rich chromosome sub-domains preferentially occur internally, whereas the GC-poor regions are observed closer to the nuclear envelope (Saccone *et al.*, 2002; Küpper *et al.*, 2007). A polarized organization of chromosome territories has also been documented by Amrichová *et al.* (2003), who found that telomeres are positioned on the opposite side of the territories relative to centromeres. Additionally, in the radial geometry of the nucleus, both telomeres of a given chromosome were much nearer to the center of the cell nucleus than its centromere. In addition, q-telomeres were, on average, more centrally localized than p-telomeres (Amrichová *et al.*, 2003). Moreover, genes were observed to be located mostly in the nuclear interior relative to peripherally located centromeres (Kozubek *et al.*, 1997; Skalníková *et al.*, 2000).

Changes in chromatin arrangement have also been solved by more mechanistic insights by Cook and Marenduzzo (2009), and Rajapakse *et al.* (2009). These authors have documented that specific local forces are responsible for the non-random distribution of chromosomes. Using Monte Carlo simulations, Cook and Marenduzzo (2009) showed that nonspecific (entropic) forces are responsible for chromosome positioning. Using different types of polymers, these authors simulated the affinity of gene-rich chromosomes for the nuclear interior and of gene-poor chromosomes for the periphery. Flexible polymers, representing gene-rich regions, tended to intermingle less frequently than others, and thus, represent poor translocation partners. Moreover, polymers with large terminal beads (representing centromeric heterochromatin) accumulated into peripherally located centromeric clusteres called chromocenters. Finally, Cook and Marenduzzo (2009) summarized that entropic forces probably participate in the chromosome self-organization that has been described by Misteli (2001) as a set of components assembled into a steady-state, dynamic structure. Conversely, the phenomenon of self-assembly involves the physical association of molecules that form static structures reaching thermodynamic equilibrium (Misteli, 2001). Self-organization is an attractive model for understanding how nuclear arrangement is integrated with chromatin function. In particular, during cell differentiation, coherence or self-organization between chromosome structure and gene regulation could lead to the stabilization of higher-order chromatin structures (Rajapakse *et al.*, 2009).

Gene Positioning and Transcriptional Activity

Many chromatin studies have uncovered an apparent correlation between the intranuclear localization and the transcriptional status of genes. Several experiments have looked at the relationship between gene expression and the structure of chromosome territories in interphase nuclei. In many cases, it was observed that actively transcribed genes are positioned closer to the nuclear interior (Zink *et al.*, 2004; Wiblin *et al.*, 2005; Williams *et al.*, 2006) and/or preferentially at the chromosome periphery (Kurz *et al.*, 1996; Dietzel *et al.*, 1999; Scheuermann *et al.*, 2004). Other reports have indicated that active gene are located on chromatin loops that extend away from compact interphase chromosomes (Volpi *et al.*, 2000; Mahy *et al.*, 2002; Williams *et al.*, 2002; Galiová *et al.*, 2004). The transcription-dependent spatial arrangement of select adjacent loci (*GASZ*, *CFRT*, and *CORTBP2*) on human chromosome 7q31 was published by Zink *et al.* (2004) and showed that inactive genes preferentially associate with the nuclear periphery and with perinuclear heterochromatin. On the other hand, actively transcribed loci preferentially associate with euchromatin in the nuclear interior. Zink *et al.* (2004) have suggested that transcriptional activity affects nuclear positioning, but not vice versa. Similar examples were found by Scheuermann *et al.* (2004) for the intranuclear topography of nontranscribed sequences and genes. In the majority of cases, the nontranscribed sequences were found

at the nuclear periphery or at the nucleoli, and genes tended to localize to interior chromosome surfaces. Kurz *et al.* (1996) analyzed the gene topography of actively transcribed or inactive genes [human dystrophin (Xp21.3-p21.2), β-myosin (14q11.2-q13), and β-globin (11p15.5) genes] relative to non-expressed anonymous sequences in the corresponding chromosome territory. Active and inactive genes were preferentially positioned at the periphery of the chromosomes territories, whereas the non-expressed anonymous sequences mapping to 11p14 were found randomly distributed or localized most often to the territorial interior. In another study, the nuclear localization of *ANT2* (Xq24-q25) and *ANT3* (Xp22.3) was determined with respect to active and inactive X chromosome territories (Xa and Xi). The *ANT2* gene was transcriptionally active only in Xa, while the *ANT3* gene was transcriptionally active in both the Xa and Xi chromosome territories. When expressed, *ANT2* and *ANT3* were observed to be more peripherally localized within their chromosome territories than the inactive *ANT2* locus (Dietzel *et al.*, 1999). Other experiments have shown that the up-regulation of genes in the major histocompatibility complex mapping to 6p21.3 was induced by interferon-γ and the formation of loops extending from the chromosome 6 territory was observed (Volpi *et al.*, 2000). Similarly, the genes of the epidermal differentiation complex (EDC) appear to extend outside of the corresponding interphase chromosome in keratinocytes, where the genes are highly expressed, but not in lymphoblasts, where they are silent (Williams *et al.*, 2002). Ragoczy *et al.* (2003) have suggested that genes that loop out from their chromosome territory may be in a poised state for transcriptional activation, as described above. On the other hand, there are conflicting results for whether changes of gene localization in the chromosome periphery are essential for transcriptional activity (Brown *et al.*, 2006). Similarly, there are examples of both ubiquitously expressed and tissue-specific genes that are not confined to the chromosome periphery, suggesting that the basal transcription machinery and transcription factors have access to the interior of interphase chromosomes (Verschure *et al.*, 1999; Mahy *et al.*, 2002). Furthermore, the results of Scheuermann *et al.* (2004) indicate that active/inactive genes and putatively nontranscribed sequences are predominantly in the periphery of the respective chromosome territories, independent of their transcriptional status and GC content.

Taking all the available information into account, there is reason to believe that transcriptional activity is not solely confined to loci positioned at the outer surfaces of chromosome territories. This is consistent with the observation that transcription sites are scattered throughout the chromosome territory (Verschure *et al.*, 1999). Recent comprehensive studies have indicated that several parameters, including specific histone modifications, the degree of chromatin decondensation, local gene density, and the surrounding chromatin environment, have a greater impact on higher-order chromatin structure than the activity of individual genes (Mahy *et al.*, 2002; Chambeyron and Bickmore, 2004; summarized by Bártová and Kozubek, 2006; Küpper *et al.*, 2007).

Structural and Functional Significance of Colaborations between Promoters and Regulatory Sequences

The regulation of gene expression is not only mediated by RNA polymerase II binding to a promoter, but also by important regulatory sequences that map to regions up to several Mb away, called enhancers, locus control regions (LCR), or insulators. Many experiments have been performed to analyze the function of these genomic elements, which are highly abundant in genomes and likely represent important factors in the regulation of gene expression (Bulger and Groudine, 2009). Enhancers, LCRs, isulators, and other *cis*-regulatory DNA sequences are called promoter-distal regulatory elements that regulate transcription apart from *trans*-acting proteins. The term insulator represents two distinct sub-classes of activity, including barrier elements and enhancer-blocking elements (Bulger and Groudine, 2009). Barrier elements are only effective when they flank the transgene on either side, while LCRs function any orientation with respect to a transgene. Taken together, barrier elements serve to block the attachment of repressive chromatin structures from a given integration site, while LCRs actively function to establish a chromatin structure that is more conducive to gene expression. The most well-characterized barrier element is 5'HS4 of the chicken β-globin locus. A specific sequence within this element is bound by USF transcription factors and has been shown in multiple studies to insulate transgenes from position effects (Huang *et al.*, 2007). On the contrary, enhacer-blocking elements have the ability to block the function of an enhancer on a linked promoter, but only when located between the enhancer and promoter. Enhancers are characterized by DNase I hypersensitivity and contain combinations of bindings sites for various transcription regulators resonsible for cell-type specific gene expression (summarized by Ram and Meshorer, 2009). Moreover, the functions of regulatory distal-elements is intimately connected with specific histone signatures (Heintzman *et al.*, 2009). For example, H3K4 monomethylation, H3K27 acetylation, and the association of specific factors, such as HATs and transcriptional coactivator p300, are significant epigenetic markers of enhancers, but not promoters. On the other hand, H3K4me3 associates with transcription start sites, but not with enhancers (summarized by Bulger and Groudine, 2009). These observations demonstrate how histone signature regulates functional events related to chromatin arrangement and gene expression (Heintzman *et al.*, 2009).

The "Mediator Complex"

Another component of the transcription machinery is the "mediator complex", which likely transfers regulatory information from enhancers and other control elements to the basal RNA polymerase II transcription machinery. In *Saccharomyces cervisiae*, the "mediator complex" was first described as a factor necessary for activator-dependent stimulation of RNA polymerase II (Kelleher *et al.*, 1990 and Flanagan *et al.*, 1991). Moreover, post-translational modifications, especially phophorylation (Kim *et al.*, 1994), likely influence the function of "mediator". Thus, this epigenetic event is responsible for transcription (Björnklund and Gustafsson, 2005). Purified "mediator" possesses histone acetyltransferases (HATs) and can interact directly with free nucleosomes (Lorch *et al.*, 2000). The HAT activity is associated with the Nut1 subunit of "mediator" and has an important regulatory role for "mediator" function (Gustafsson and Samuelsson, 2001). Transcriptional activation has been studied in bacteria using catabolite activator protein (CAP), whose functions at promoters involves a recruitment mechanism that facilitates the binding of RNA polymerase II to the promoter, but does not influence later stages, such as open complex transition or promoter escape. "Mediator" seems to influence the recruitment of the RNA polymerase II transcription machinery to promoters and facilitates isomerization of the pre-initiation complex to initiate active transcription. The C-terminal domain of the largest subunit of RNA polymerase II likely plays an important role in the function of "mediator". Another posibility is that "mediator" influences other steps of gene transcription and processing, such as elongation, splicing, chromatin remodeling, and mRNA export (Björnklund and Gustafsson, 2005). However, the exact mechanism of "mediator"-dependent regulation of transcription remains elusive. At present, "mediator" represents a dynamic connection between gene-specific regulatory proteins and the conserved RNA polymerase II-related transcription machinery. Moreover, "mediator" is considered as a bridge between enhancers and promoters in eukaryotic cells (Kuras *et al.*, 2003)

Changes in Chromatin Structure That Accompany Differentiation Processes: A Focus on Enterocytic and ES Cell Differentiation

The most pronounced changes in the organization of whole chromosomes, centromeres, and genes in the cell nucleus have been observed during differentiation processes (Chaly and Munro, 1996; Bártová *et al.*, 2002; Chambeyron *et al.*, 2005; Meshorer and Misteli, 2006). Cell differentiation is accompanied by the reprogramming of gene expression, which leads to the progression of progenitor cells towards terminally differentiated stages that block the cell in the G0 phase of the cell cycle (Fig. 2A). This process is related to chromatin re-arrangement and seems to be differentiation pathway-specific (summarized by Francastel *et al.*, 2000; Bártová and Kozubek, 2006). Adipocyte differentiation is characterized by a closer proximity of chromosome territories (Kuroda *et al.*, 2004). In granulocyte and erythroid like cells, the condensation of chromosome territories has been described and the peripheral repositioning of some chromosome territories and centromeric heterochromatin was observed (Bártová *et al.*, 2001; Galiová *et al.*, 2004). Furthermore, the association of centromeres into chromocenters during the differentiation of monocytes, lymphocytes, and granulocytes has been found (Chaly and Munro *et al.*, 1996; Alcobia *et al.*, 2000). During leukemic and embryonic stem cell (ES) differentiation, some, but not all, genes change their nuclear location (Bártová *et al.*, 2000, 2002; Wiblin *et al.*, 2005; Bártová *et al.*, 2008c). Some experiments have demonstrated strikingly specific nuclear and territorial arrangements of transcriptionally active genes compared to inactive ones, especially during differentiation processes. For example, Takizawa *et al.* (2008a) showed that the functionally distinct alleles of the monoallelically expressed astrocyte-specific marker, *GFAP*, occupy distinct radial positions within the cell nucleus, which was confirmed by Harničarová *et al.* (2006) for the *c-myc* proto-oncogene. Furthermore, the transcription sites of several genes are associated with specific nuclear domains, including nuclear speckles (involving splicing factors, Fig. 1A and 1B) and/or some transcripts can be observed in close proximity to nucleoli (Xing *et al.*, 1995; Bártová *et al.*, 2008a; Takizawa *et al.*, 2008a; Royo *et al.*, 2009, illustration in Fig. 1B). Interestingly, during enterocytic cell differentiation induced by the HDAC inhibitor, sodium butyrate (Fig. 2B), the nuclear arrangement of *c-myc* transcripts was conserved and, therefore, independent of variability in the level of differentiation-specific *c-myc* gene expression or of global morphological changes. However, after the induction of differentiation, the single copy *c-myc* gene of normal chromosome 8 occupied a more internal territorial location, while *c-myc* amplicons (observed within derivative chromosome 8 in HT29 cells) were characterized by conserved territorial topography (Harničarová *et al.*, 2006). Moreover, enterocytic differentiation induced by NaBt seems to be a highly specific differentiation pathway, from the view of chromatin structure and especially histone signature (Fig. 2C). In contrast to various ES cell differentiation pathways (Fig. 2D), enterocytic differentiation is characterized by completely different epigenetic profiles, such as increased H3K9 acetylation, H3K4 dimethylation, and H3K9 dimethylation (Bártová *et al.*, 2005, Fig. 2C). The level of all HP1 subtypes was significantly reduced after NaBt treatment of human colon cancer cells induced to differentiate (Bártová *et al.*, 2005, illustration in Fig. 2C). Similarly, HP1 binding to H3K9 trimethylation was decresed when *hMLH1* gene transcription was induced in colorectal cancer cells (McGarvey *et al.*, 2006). Contrary to enterocytic differenti-

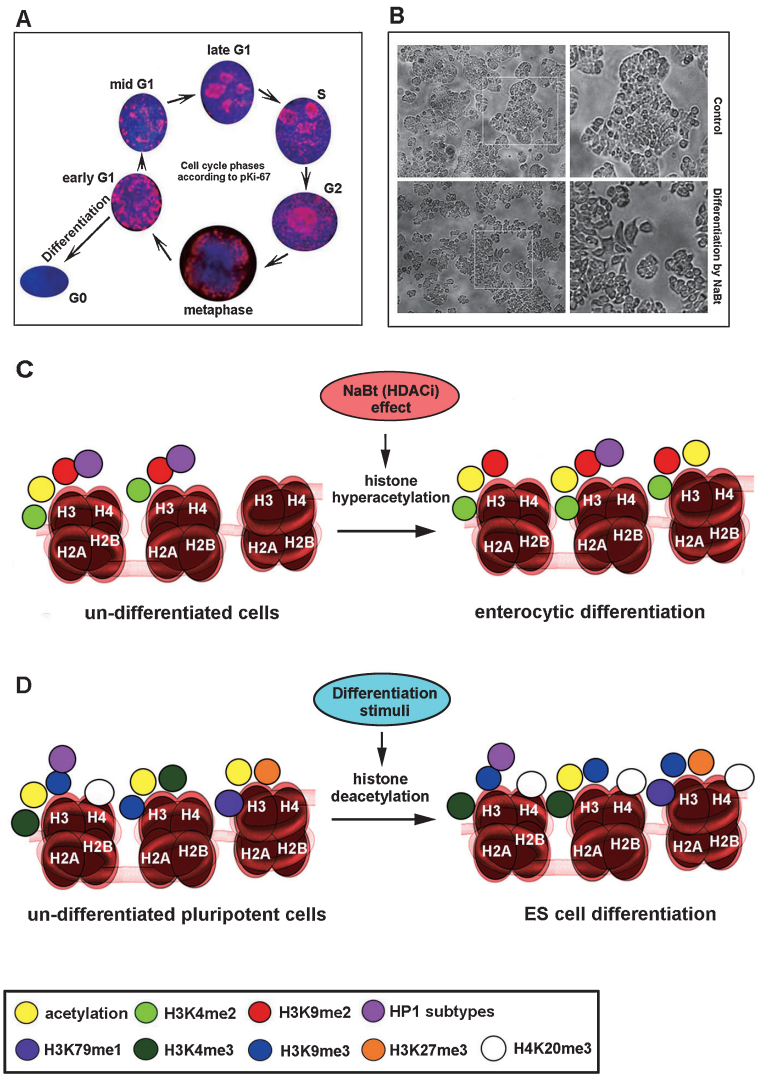

Fig. 2. An illustration of cells in various cell cycle phases and epigenetics of selected differentiation pathways. (A) The cell cycle phases of individual cells (blue) were determined on the basis of the pattern of proliferation antigen pKi-67 (red). The cells undergoing differentiation are characterized by an absence of pKi-67. (B) An example of enterocytic differentiation induced by sodium butyrate (NaBt) in human colon adenocarcinoma HT-29 cells. Micrographs, obtained by light microscopy, show HT-29 morphology in the presence (control) or in the absence of NaBt. (C) A schematic of the chromatin modifications caused by HDAC inhibitor butyrate (NaBt). (D) Epigenetic changes accompanying differentiation of ES cells. In panels C and D, we show epigenetic changes published by several authors.

ation, distinct epigenetic changes were observed during retinoic acid-induced differentiation of human ES cells, which was accompanied by partially decreased levels of HP1γ, but not HP1α or HP1β (recent unpublished data of our group). Moreover, decreased histone acetylation (Krejčí *et al.*, 2009) and enhanced H3K9me3, H4K20me3 was observed in differentiated ES cells (summarized by Lennartsson and Ekwall, 2009). Conversely, no change in H3K4me3, H3K27me3 or H3K79me1 was observed (Bártová *et al.*, 2008c). An important epigenetic marker of differentiation processes is H4K20 methylation, which is associated with gene down-regulation and is important for DNA damage control (Sanders *et al.*, 2004). However, H4K20 methylation patterns vary during cell differentiation. For example, in proliferating neural cells from mouse embryos, the level of H4K20me1 is high and H4K20me3 is significantly low, while H4K20 trimethylation increases throughout development (Biron *et al.*, 2004).

The functional, epigenetic, and structural aspects of chromatin have been addressed by many authors (Brown *et al.*, 1997; Bártová *et al.*, 2002; Galiová *et al.*, 2004; Wiblin *et al.*, 2005; Williams *et al.*, 2006; Bártová *et al.*, 2008b, and others). Wiblin *et al.* (2005) found that a pluripotent gene, such as *Nanog,* adopts a more central location in pluripotent

human embryonic stem (hES) cells compared to terminally differentiated B-cells. A similar example was found for the nuclear location of the proneural regulatory gene, *Mash1,* which is located at the nuclear periphery when transcriptionally repressed in hES cells. In contrast, after the induction of neural differentiation, the up-regulated *Mash1* gene is repositioned to the nuclear interior (Williams *et al.*, 2006). In some cases, the extra-territorial positioning of genes on chromatin loops that extend away from the compact chromosome territory has been found. This organization has been shown for the β-globin gene cluster after the induction of differentiation by interleukin-3, which leads to the erythroid-like lineage (Galiová *et al.*, 2004). A similar chromatin configuration has been observed for the transcriptionally active *Oct-3/4* gene in pluripotent hES cells (Wiblin *et al.*, 2005), while in hES cells differentiated by retinoic acid, this gene has been observed on the periphery of the corresponding chromosome territory (Bártová *et al.*, 2008b). In addition, the dynamic repositioning of certain genes into the vicinity of heterochromatin foci has been associated with silencing in mouse lymphocytes and during the differentiation of leukemia cells (Brown *et al.*, 1997; Bártová *et al.*, 2002).

Conclusion

Despite the invention of advanced methodological approaches, the functional importance of the non-random organization of chromosome territories and their subdomains remains unclear. The relationship between nuclear radial distribution and gene expression is influenced by aspects of various experimental methods (e.g., 2D vs. 3D fixation, way of confocal imaging, and different mathematical algorithms, such as those used for image de-convolution). Similarly, data derived from DNA-FISH and RNA-FISH can differ significantly, due to the denaturation step in the DNA-FISH protocol, and to the fact that many mono-allelically and bi-allelically expressed genes undergo a long period of quiescence preceding transcription (Osborne *et al.*, 2004). Moreover, the majority of studies that show a correlation between chromatin structure and gene expression were performed in model cell differentiation systems, and where thus influenced by additional cell maturation processes. As noted by Brown *et al.* (2006), all of this implies that data related to the effects of chromatin organization on gene expression, and vice versa, must be interpreted with consideration of the methodological caveats and limitations.

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