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Chromosomal Territories, Higher-Order Chromatin Remodeling and the Control of Gene Expression in Keratinocytes

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Abstract

Three-dimensional (3D) organization of transcription in the nucleus and mechanisms controlling the global chromatin folding including spatial interactions between the genes, non-coding genome elements, epigenetic and transcription machinery are essential for the establishment of lineage-specific gene expression programs during cell differentiation. Spatial chromatin interactions in the nucleus involving gene promoters and distal regulatory elements are currently considered as one of the major forces that drive cell differentiation and the genome evolution in general, while such interactions are substantially re-organized during many pathological conditions. During terminal differentiation of the epidermal keratinocytes, the nucleus undergoes programmed transformation from highly active status, associated with execution of the genetic program of epidermal barrier formation, to fully inactive condition and finally becomes a part of the keratinized cells of the cornified epidermal layer. This transition is accompanied by marked remodeling of the 3D nuclear organization and micro-anatomy including changes in the spatial arrangement of lineage-specific genes, nuclear bodies and heterochromatin. This mini-review highlights the important milestones in accumulation of the current knowledge on three-dimensional organization of the nucleus, spatial arrangement of the genes and their distal regulatory elements, and provides an update on the mechanisms that control higher-order chromatin remodeling in the context of epidermal keratinocyte differentiation in the skin.

Three-dimensional (3D) organization of transcription in the nucleus and mechanisms controlling the global chromatin folding including spatial interactions between the genes, non-coding genome elements, epigenetic and transcription machinery are essential for the establishment of lineage-specific gene expression programs during cell differentiation (Bickmore, 2013; Chakalova and Fraser, 2010; Cremer *et al.*, 2015; Gomez-Diaz and Corces, 2014; Sequeira-Mendes and Gutierrez, 2015). During the last decade, a tremendous progress has been achieved in understanding of the functional micro-anatomy of the nucleus as a dynamic structure, in which actively transcribed or repressed genes are spatially compartmentalized into the distinct domains and frequently form preferential intra- and inter-chromosomal interactomes, which provide functional and structural frameworks for

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cell-specific transcription (Dekker *et al.*, 2013; Schoenfelder *et al.*, 2010; Sexton and Cavalli, 2015).

The cell nucleus is highly complex organelle that consists of the nuclear membrane, individual chromosomes occupying the distinct territories, as well as of a number of nuclear bodies (nucleoli, Cajal bodies, promyelocytic leukaemia (PML) bodies, nuclear speckles, Polycomb bodies, etc.) facilitating an execution of gene expression programs and other nuclear functions (Hubner and Spector, 2010; Lanctot *et al.*, 2007; Mao *et al.*, 2011; Misteli, 2007; Pederson, 2011). Serving as a central hub in the establishing adaptive cell behavior, the nucleus integrates the signals coming from the extracellular space and transforms them into specific gene expression programs to assist cells to survive and generate an appropriate response to changes in the microenvironment.

During terminal differentiation of the epidermal keratinocytes, the nucleus undergoes programmed transformation from highly active status, associated with execution of the genetic program of epidermal barrier formation, to fully inactive condition and finally becomes a part of the keratinized cells of the cornified epidermal layer. This transition is accompanied by marked remodeling of the 3D nuclear organization and micro-anatomy including changes in the spatial arrangement of lineage-specific genes, nuclear bodies and heterochromatin (Gdula *et al.*, 2013). This mini-review highlights the important milestones in accumulation of the current knowledge on three-dimensional organization of the nucleus and provides an update on the mechanisms that control higher-order chromatin remodeling in the context of epidermal keratinocyte differentiation in the skin.

Chromosomes and chromosomal territories

Chromosomes are the largest units of the genome organization occupying distinct territories in the interphase nucleus (Cremer *et al.*, 2001; Cremer and Cremer, 2011; Cremer *et al.*, 2015) (Fig. 1a). In the chromosomes, DNA is compacted up to several thousand fold and organized into DNA-protein complex (chromatin) that allow the genome to be transcribed, replicated and repaired (Hemberger *et al.*, 2009; Ho and Crabtree, 2010; Sequeira-Mendes and Gutierrez, 2015). Each chromosome contains a centromer (pericentromeric chromatin enriched in α-satellite repetitive sequences), chromosome arms containing the gene-rich and gene-poor domains enriched in the GC- and AT-sequences and visualized as the light and dark bands by Gimsa staining, respectively, as well as the telomeres (Fukui, 2009). Chromosomes are visualized by three-dimensional fluorescence hybridization (3D-FISH) technique with specific paints that allow defining their positioning in the nucleus (Cremer and Cremer, 2001; Solovei and Cremer, 2010).

The term “chromosomal territory” was first introduced by Theodor Boveri in 1909 (reviewed in (Cremer and Cremer, 2006a, b). Research in Thomas Cremer’s laboratory performed during last three decades has brought a tremendous progress into our understanding of the spatial organization of the genes and chromosomes in the interphase nucleus [for reviews, see (Cremer and Cremer, 2001; Cremer and Cremer, 2011; Cremer *et al.*, 2015)]. Confocal microscopy analyses of tissue sections or isolated cells by using the whole chromosome 3D-FISH probes demonstrated that in interphase nucleus the relative

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positioning of the chromosomes within 3D nuclear space is not random and depends on many factors including the cell type, differentiation stage, chromosome size and their gene-rich or gene-poor status (Cremer and Cremer, 2010). Data obtained from the mouse skin *in situ* show that in basal epidermal keratinocytes, the chromosome 3 harboring the Epidermal Differentiation Complex (EDC) locus is always located at the nuclear periphery (Fig. 1a, c), and its positioning does not change during embryonic and post-natal development, as well as during terminal differentiation and keratinocyte transition to the spinous and granular epidermal layers (Fessing *et al.*, 2011; Gdula *et al.*, 2013; Mardaryev *et al.*, 2014). However, the chromosomes 11 and 15 harboring the Keratin type I and type II loci, respectively, occupy predominantly central positions in the keratinocyte nuclei (Botchkarev *et al.*, 2012).

In the interphase nucleus, positioning of the chromosomes is controlled through several mechanisms that include the interactions between specialized lamina-associated domains (LADs) and nuclear lamina, as well as through association of the chromosomes bearing the nucleolar-organizing region domains with nucleoli [reviewed in (Joffe *et al.*, 2010; Kind and van Steensel, 2014; McKeown and Shaw, 2009; Misteli, 2007)]. Distinct chromosomes may be arranged in the nuclei of differentiated cells in a cell lineage-specific manner, which explain an increased frequency of translocations between the distinct chromosomal parts in the corresponding tumors (Brianna Caddle *et al.*, 2007; Khalil *et al.*, 2007; Parada *et al.*, 2004; Roix *et al.*, 2003). However, it is unclear whether genes from neighboring chromosomes may share common regulatory mechanisms required for their transcription (Cremer and Cremer, 2011).

Introduction of the super-resolution confocal microscopy allowed improving the resolution of the fluorescence images up to the 20–100 nm and served as an important next step in the analyses of the nuclear architecture (Cremer *et al.*, 2015; Schermelleh *et al.*, 2010). Super-resolution confocal microscopy revealed that each chromosome territory resembles a sponge-like structure and consists of the chromatin domains permeated by interchromatin channels connected with a network of larger channels and lacunas separating distinct chromosomes and harboring a number of nuclear bodies (Markaki *et al.*, 2011). Inter-chromatin channels are also serving as a reservoir for macromolecular complexes, transcription factors, regulators of splicing, replication, and repair, as well as for exporting the mRNA-containing ribonucleoprotein complexes (Cremer *et al.*, 2015). The network of interchromatin channels starts at nuclear pores and expands throughout the nuclear space, while chromatin domains in each territory are separated from the interchromatin channels by a 100–200-nm layer of decondensed chromatin, called the perichromatin and enriched by nascent DNA and RNA, RNA polymerase II (RNA Pol II), as well as by the H3K4me3 histone modification specific for transcriptionally active chromatin (Cremer *et al.*, 2015; Markaki *et al.*, 2011).

These observations were further developed into a model that suggests a presence of the active and inactive nuclear compartments inside of each chromosome territory that harbor trancriptionally active or inactive genes, respectively (Cremer *et al.*, 2015) (Fig. 1b). This model also suggests a large degree of flexibility in the positioning of distinct chromatin domains inside of each chromosome territory, which is correspond well to the fact that some gene loci (IFN-γ and T_H2 cytokine loci in T_H lymphocytes, globin genes in erythroid cells,

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Nanog locus in iPS cells) may change their positioning relatively to other loci or the corresponding chromosomal territories associated with either gene activation or silencing (Spilianakis et al., 2005; Schoenfelder et al., 2010; Jost *et al.*, 2011). During epidermal morphogenesis and differentiation of the basal epidermal progenitor cells, the lineage-specific EDC locus shows marked remodeling of its higher-order chromatin structure and relocates away from the peripheral part of the chromosomal territory 3 towards its internal part, which is associated with an increase in the transcriptional activity of genes involved in the control of terminal keratinocyte differentiation and epidermal barrier formation (Mardaryev *et al.*, 2014) (Fig. 1c). Such developmentally-regulated relocation of the EDC towards the nuclear interior is a keratinocyte-specific event, which does not occur in dermal cells, and it is maintained during adulthood despite the many cycles of cell division that occur in this rapidly proliferating and self-renewing epithelial tissue (Mardaryev *et al.*, 2014).

These data are generally consistent with previous observations showing the looping out from chromosome territory 1 of the EDC locus in cultured human keratinocytes, which suggest that the positioning of this genomic domain within the nucleus is quite flexible (Williams *et al.*, 2002). Developmentally-regulated relocation of the EDC locus into the nuclear interior is associated with an increase in the number of SC-35 nuclear speckles present within the vicinity of the EDC, suggesting that this nuclear compartment may provide a “permissive environment” for the efficient transcription and maintenance of the high expression levels among genes activated during keratinocyte differentiation (Mardaryev *et al.*, 2014).

Nuclear speckles are considered to be the sites of association between active genes within the nucleus (Brown *et al.*, 2008; Mao *et al.*, 2011; Popken *et al.*, 2014; Spector and Lamond, 2011; Szczerbal and Bridger, 2010) and contain important constituents of the pre-mRNA processing machinery, such as polyadenylation and splicing factors including small nuclear ribonuclear proteins (snRNPs), as well as poly-A^+ RNA and other splicing-related proteins (Spector and Lamond, 2011). Many of these factors are either recruited to transcription sites from the speckles or are involved in mRNA processing in the speckles (Spector and Lamond, 2011). Nuclear speckles are also considered as the sites of accumulation of the non-coding RNAs including MALAT1, which interacts with RNA-binding proteins and target pre-mRNAs at sites of active transcription (Engreitz *et al.*, 2014). However, the impact of distinct speckle components in the control of gene expression within the EDC and other keratinocyte-specific gene loci remains to be further determined.

Systematic analyses of the remodelling of nuclear architecture during terminal keratinocyte differentiation in mouse epidermis demonstrate that terminally differentiated keratinocytes show marked differences in micro-anatomical organization of the nucleus compared to basal epidermal cells including: i) Decrease of the nuclear volume; ii) Decrease in expression of the markers of transcriptionally-active chromatin; iii) Internalization and decrease in the number of nucleoli; iv) Increase in the number of pericentromeric heterochromatic clusters; v) Increase in the frequency of associations between nucleoli, pericentromeric clusters and chromosomal territory 3 (Gdula *et al.*, 2013) (Fig. 1b). These changes are likely to contribute to the global changes in the transcriptional landscape in terminally differentiating keratinocytes and transition of the keratinocyte nucleus from a metabolically active status to

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an inactive condition (Gdula *et al.*, 2013). These data also suggest the nucleoli and pericentromeric clusters as important elements of the nuclear architecture which may control the local "transcriptional micro-environment" of the distinct chromatin domains by modulating the processes of chromosome tethering and regulating their positioning, folding and/or orientation.

Spatial proximity of the genes and chromosomes in the nucleus play an important role in the occurrence of chromosomal translocations during neoplastic transformation: neighboring chromosomes show higher frequencies of translocations compared to distal chromosomes, and translocations are formed predominantly between proximal chromosome breaks (Roukos and Misteli, 2014). In basal cell carcinoma, *SHH* gene shows translocation between chromosomes 7 and Y, which might contribute to its abnormal activation in the absence of the *PTCH1* and *SMO* mutations (Gomez-Ospina et al., 2012). Thus, it appears to be important to carefully dissect how topological organization of the genome in keratinocytes is changed in pathological skin conditions including epidermal tumors or the disorders of epidermal differentiation (such as psoriasis), and how such changes contribute to the alterations in the transcriptional landscape of keratinocytes underlying these diseases.

Chromatin conformation capture analyses of 3D genome organization

Chromatin conformation capture (3C and its variations 4C, 5C and Hi-C) technologies were developed by Job Dekker and his laboratory (Dekker *et al.*, 2002) and are based on the formaldehyde-mediated cross-linking between the closely located chromatin domains and multi-protein complexes followed by the DNA digestion with the restriction enzymes and the ligation at high dilution to facilitate the formation of intra-molecular but not inter-molecular products (Dekker *et al.*, 2013; Lajoie *et al.*, 2015). These techniques allowed defining the chromatin interactions between two distinct genomic sites (3C or "one-versus-one") or between the genomic site of interest and the genome globally (4C or "one-versus-all"), as well as assessing the complex interactions within the distinct genomic locus (5C or "many-versus-many") or global interactions within the whole genome (Hi-C or "all-versus-all") (de Laat and Dekker, 2012).

Hi-C analyses of the global chromatin interactions revealed that the genes and chromatin domains from the same chromosomes show the higher frequency of interactions compared to the genes from other chromosomes, which confirmed the presence of chromosome territories on the molecular levels (Lieberman-Aiden *et al.*, 2009). Furthermore, these analyses demonstrated an existence of at least two types of sub-chromosomal compartments, in which actively transcribed or transcriptionally silenced chromatin domains are segregated (Lieberman-Aiden *et al.*, 2009). Such sub-chromosomal compartments were subsequently identified by applying the 3D structural illumination microscopy that revealed presence of the active and inactive sub-chromosomal compartments enriched either by the elongating form of PolII and H3K4me3 or by the H3K9me3 histone modifications, respectively (Popken *et al.*, 2014).

Most importantly, Hi-C analyses also revealed an existence of another level of chromatin folding and presence of the Topologically Associating Domains (TADs) on each interphase

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chromosome, which size varies from several hundred Kb to 1–2 Mb (Dekker and Heard, 2015). TADs are characterized by much higher interaction frequencies between the distinct elements within the TAD (intra-TAD interactions) compared to the interactions between different TADs (inter-TAD interactions) (Dekker and Heard, 2015). Interestingly, TAD's borders are conserved between the humans and mice and are not changed during cell differentiation, while TADs are lost alongside the inactive X-chromosome, as well as during the mitosis (Dixon *et al.*, 2012; Naumova *et al.*, 2013; Nora *et al.*, 2012).

TAD borders in the mammalian genome are enriched in the binding sites for a number of architectural proteins including the CTCF and cohesin (Dixon *et al.*, 2012; Gomez-Diaz and Corces, 2014). CTCF and cohesin binding sites also exist within TADs, in which CTCF is involved in organizing the smaller-sized (100–200 kb) intra-TAD chromatin loops (Rao *et al.*, 2014) and in mediating the enhancer-promoter contacts (Dekker and Heard, 2015). Satb1 is another chromatin architectural protein that binds specialized DNA regions with an ATC-sequence context and folds chromatin into loops involving tissue-specific gene loci (T_{H2}-cytokine and MHC class I loci, globin locus, etc.) (Cai *et al.*, 2003; Cai *et al.*, 2006; Kohwi-Shigematsu *et al.*, 2012). Satb1 also targets chromatin remodelers/transcription factors to gene loci and plays a unique role in the execution of lineage-specific gene expression programs by integrating high-order chromatin organization with regulation of gene expression (Kohwi-Shigematsu *et al.*, 2012; Kohwi-Shigematsu *et al.*, 2013).

Chromatin conformation capture analyses allowed substantiating our knowledge on the enhancer-promoter interactions as a major driving force facilitating execution of lineage-specific differentiation programs (Dowen *et al.*, 2014). Enhancers are the sequence modules that are preferentially located in the non-coding part of the genome at various distances from their target genes or even at different chromosomes (de Laat and Duboule, 2013). In normal differentiating cells, interactions between the gene promoters and their enhancers occurring via chromatin looping are very important for execution of lineage-specific gene expression programs (de Laat and Duboule, 2013; Dowen *et al.*, 2014). In keratinocytes, an epidermal-specific regulatory enhancer 923 is present within the EDC locus and interacts with multiple EDC gene promoters, while some of these interactions are regulated by AP-1 transcription factor (Oh *et al.*, 2014). Also, calcium stimulation in differentiating keratinocytes results in increased physical proximity of the enhancer and the promoter regions of the peptidylarginine deiminase 3 gene that control metabolism of the filaggrin (Adoue *et al.*, 2008). However, the role of CTCF, cohesin, Satb1 and other chromatin architectural proteins in regulation of the enhancer-promoter interactions during establishment and maintenance of epidermal differentiation program in keratinocytes remain to be clarified.

Higher-order chromatin remodeling and the control of gene expression in keratinocytes

Establishment of the functional epidermal barrier is one of the major goals of the epidermal differentiation program, which includes a tightly regulated process of keratinocyte proliferation, terminal differentiation, apoptosis and shedding. The program of epidermal development and keratinocyte differentiation is governed by coordinated involvement of

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several transcription factors (p63, AP-1, Klf4, Arnt, etc.), signalling pathways (Wnt, Bmp, Hedgehog, EGF, Notch, FGF, etc.) and epigenetic regulators (DNA/histone-modifying enzymes, Polycomb genes, higher-order and ATP-dependent chromatin remodelers, non-coding RNAs) that control expression of lineage-specific genes [reviewed in (Botchkarev *et al.*, 2012; Fessing, 2014; Frye and Benitah, 2012; Perdigoto *et al.*, 2014)].

Epigenetic regulators exhibit both *activating and repressive effects on chromatin* in KCs: histone demethylase Jmjd3, ATP-dependent chromatin remodeler Brg1 and genome organizer Satb1 promote terminal KC differentiation, while DNA methyltransferase DNMT1, histone deacetylases HDAC1/2, Polycomp components Bmi1 and Ezh1/2 stimulate proliferation of progenitor cells via repression of the genes encoding cell-cycle inhibitors, as well as inhibit premature activation of terminal differentiation-associated genes (reviewed in (Benitah and Frye, 2012; Botchkarev *et al.*, 2012; Fessing, 2014; Perdigoto *et al.*, 2014).

Our recent studies revealed that transcription factor-dependent and epigenetic regulatory mechanisms in keratinocytes are highly connected, and p63 transcripton factor, operating as a master regulator of epidermal development (Koster and Roop, 2007; Kouwenhoven *et al.*, 2015b; Vanbokhoven *et al.*, 2011, Botchkarev, 2014 #2114), plays a hitherto unrecognized role in the higher-order chromatin remodeling of the EDC locus via direct control of the genome organizer *Satb1* and ATP-dependent chromatin remodeler *Brg1* (Fessing *et al.*, 2011; Mardaryev *et al.*, 2014). Satb1 is expressed in basal epidermal KCs and promotes cell differentiation via establishment of specific conformation of the EDC locus, while its ablation in mice results in the marked elongation of the EDC central domain associated with alterations in expression of the EDC genes and in epidermal morphology (Fessing *et al.*, 2011).

ATP-dependent chromatin remodeler *Brg1*, on the other hand, promotes developmentally-regulated relocation of the EDC locus from the nuclear periphery towards nuclear interior into the compartment enriched by nuclear speckles, which is associated with marked increase in expression of the EDC genes (Mardaryev *et al.*, 2014). Importantly, conditional ablation of Brg1 in the epidermis results in failure to form a functional barrier, thus partially resembling phenotype of p63 KO mice (Indra et al., 2005). These data suggest that chromatin remodeling genes represent a novel cohort of p63 targets that mediate its effects on execution of lineage-specific gene expression program in KCs (Botchkarev *et al.*, 2012; Fessing, 2014).

Recent data revealed that in human keratinocytes, about 50% of the p63 binding sites are co-localized with H3K27ac histone modification specific for active enhancers (Kouwenhoven *et al.*, 2015a). Interestingly, p63 binding alone was not sufficient for the regulation of gene transcription, while the gene expression dynamics correlated better with the H3K27ac signal at p63 binding sites than with p63 binding itself (Kouwenhoven *et al.*, 2015a). Apparently, other co-regulators, such as RUNX1, are involved in the control of expression of p63 target genes (Kouwenhoven *et al.*, 2015a). These data suggest that p63-mediated regulation of the epidermal differentiation program is far more complex than previously appreciated and include the control of enhancer-promoter interactions of the p63 target genes (Kouwenhoven *et al.*, 2015b).

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Conclusions

Spatial chromatin interactions in the nucleus involving gene promoters and distal regulatory elements located in the non-coding genomic domains are currently considered as one of the major forces that drive evolution of the mammalian genome (de Laat and Duboule, 2013). Genome-wide association studies (GWAS) demonstrate that many human diseases show the single nucleotide polymorphisms (SNPs) in the intergenic regions and suggest that such defects might perturb normal gene expression programs by affecting the activity of distal gene regulatory elements (Maurano *et al.*, 2012). Furthermore, the global chromatin landscape and spatial arrangements between different genes and their regulatory elements are substantially re-organized in malignant cells and are functionally important for their growth (Gondor, 2013; Kohwi-Shigematsu *et al.*, 2013; Zane *et al.*, 2014).

Clearly, at present, we have only a limited knowledge of the mechanisms that control the spatial folding of the genome in keratinocytes in healthy and diseased skin (Fessing, 2014), while additional efforts are required to fully understand the complexity of interactions between distinct transcription factors and epigenetic regulatory machinery in the control of epidermal development, regeneration and stem cell activity. Recently, a number of molecules that are capable of modulating distinct components of the epigenetic machinery have been developed, and some of them are already approved for treatment of the distinct neoplastic conditions or under clinical trials (Tough *et al.*, 2014). Thus, understanding of the complexity of spatial genome organization as a part of epigenetic regulatory program controlling epidermal differentiation and skin stem cell activity and their alterations in different pathological skin conditions will help to further progress in this exciting area of research towards the development of a novel cohort of epigenetic drugs for the management of skin disorders.

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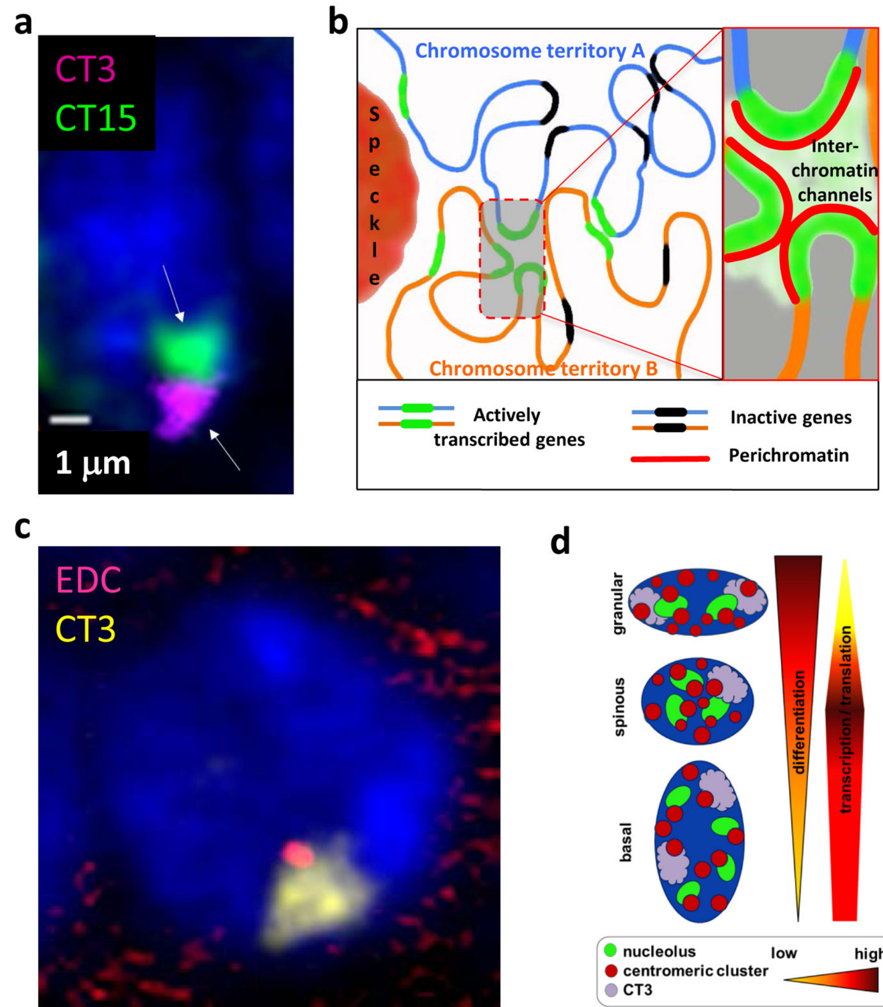

Figure 1. Changes in the spatial organization of the keratinocyte nucleus during epidermal development and differentiation

A – 3D-FISH image of the nucleus of murine basal epidermal keratinocyte showing the positioning of the chromosomes 3 and 15 (arrows). Chromosome territory 3 (CT3, pink/violet) occupy more peripheral positioning in the nucleus, while the chromosome territory 15 (CT15, green) show more central positioning (courtesy of I. Malashchuk).

B - Chromosomes occupy distinct territories, in which distinct chromatin domains are permeated by interchromatin channels connected with a network of larger channels and lacunas separating distinct chromosomes and harboring different nuclear bodies including speckles (see Cremer et al., 2015, for details).

C – 3D-FISH image of the nucleus of murine basal epidermal keratinocyte showing the chromosome territory 3 (CT3, yellow) with EDC locus located at the internal part of the CT3 (red) (courtesy of I. Malashchuk).

D – Scheme illustrating the remodeling of 3D nuclear organization during terminal keratinocyte differentiation in the epidermis (see Gdula et al., 2013, for details).