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DYNAMICS OF HIGHER ORDER CHROMATIN STRUCTURES

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To my family

"The only source of knowledge is experience"

Albert Einstein

Abstract

During the last few decades, the intensive focus on microscopy observations and genome sequencing analyses has proved that the genomic DNA is packaged in the non-random manner in the nucleus of interphase cells. Accumulated evidence have thus documented that the chromatin organization in 3D plays key roles in central biological processes, such as transcription, replication and DNA repair. In the interphase nucleus, each chromosome is expanded and organized in a manner depending on structural hallmarks of the nucleus. Thus, repressed domains localize to the nuclear periphery to form lamina associated domains (LADs) or large organized chromatin K9 modifications (LOCKs). In addition, prevalent chromatin interactions can be formed from same chromosome (in cis) or different chromosome (in trans). It is still not clear how such dynamic interactions between chromatin fibers control the expressivity of the genome and to what extent these depend on epigenetic chromatin states.

The study in this thesis had focused on the dynamics of higher order chromatin structures, particularly on the relationship between the dynamics of chromatin structure and chromatin states. Since the resolution of current single cell techniques in the chromatin organization research, such as DNA FISH and immuno-staining, are limited by the resolution of the microscopy, we invented a new in situ single cell technique termed ChrISP (paper I). Using this technique we could detect chromatin proximities with a resolution less than 17nm even though the analysis was implemented using the low resolution confocal microscope. In paper II, the scope of the ChrISP technique was extended to include an analysis of chromatin states within a single chromosome in a single cell to document that compacted chromatin at the nuclear periphery depends on the H3K9me2 mark that impinges on the nuclear periphery in finger-like structures. Moreover, upon the removal of these marks the rest of the chromosome showed signs of compaction, potentially related to chromosome condensation. These results are consistent with the interpretation that the H3K9me2 mark regulates pleiotropic features of higher order chromatin structure.

In paper III, we had used the view point of a single locus to explore the dynamics of chromatin interactions in developmental window using the circular chromatin conformation capture (4C) technique. The resulting inter-chromosomal network connected, surprisingly, both active and repressive chromatin domains involving LADs. Moreover, this network depended on the circadian recruitment of active chromatin hubs to the repressed chromatin structures at the nuclear periphery mediated by the physical proximities between CTCF and PARP1. This circadian pattern was required to attenuate transcription of the active chromatin hubs in a rhythmic manner.

In summary, a new high-resolution technique termed ChrISP was invented in this thesis to enable quantitative analyses of dynamic of higher order chromatin structures. This technique could, moreover, be used to visualize specific chromatin marks, notably H3K9me2, within a specific chromosome in relation to structural hallmarks of the nucleus within a single cell. The compact chromatin structure thus identified was discovered to transiently harbor active chromatin hubs, which was recruited to the nuclear periphery in oscillating manner. We show that this feature likely underlies the attenuation of genes under circadian control. These findings open new perspectives to understand the function of dynamics of higher order chromatin structure.

List of Publications

I. **Chen X *,** Shi C *, Yammine S *, Göndör A, Rönnlund D, Fernandez-Woodbridge A, Sumida N, Widengren J, Ohlsson, R.

Chromatin in situ proximity (ChrISP): single-cell analysis of chromatin proximities at a high resolution. Biotechniques. 2014 Mar 1; 56(3):117-8, 120-4.

II. **Xingqi Chen**, Samer Yammine, Chengxi Shi, Mariliis Tark-Dame, Anita Göndör, and Rolf Ohlsson.

The visualization of large organized chromatin domains enriched in H3K9me2 within a single chromosome in a single cell.

Epigenetics, accepted for publication.

III. Anita Göndör, Noriyuki Sumida, Honglei Zhao, Emmanouil Sifakis, J Peter Svensson, Anna Lewandowska, **Xingqi Chen,** Lluís Millán-Ariño, Farzaneh Shahin, Chengxi Shi, Olga Loseva, Moumita Biswas, Li-Sophie Zhao-Rathje, Samer Yammine, Maria Israelsson, Balazs Nemeti, Thomas Helleday, Erik Fredlund, Marta P Imreh and Rolf Ohlsson.

Poly(ADP-ribose)polymerase I and CTCF regulated interactions between active and repressive chromatin domains contribute to circadian plasticity of gene expression.

Manuscript.

* Co-first author

Contents

List of abbreviations

3C	chromatin confirmation capture
4C	circular chromatin confirmation capture
4C-Seq	circular chromatin confirmation capture sequencing
5C	chromosome conformation capture carbon-copy
3D	three dimension
BAC	bacterial artificial chromosome
BCL6	B-cell lymphoma 6
BMAL1	brain and muscle arnt-like 1
BNDF	brain-derived neurotrophic factor
CDKN2A	cyclin-dependent kinase inhibitor 2A
CLOCK	circadian locomotor output cycles kaput
ChIA-PET	chromatin Interaction analysis by paired-end tag sequencing
CHD1	chromodomain helicase DNA binding protein 1
ChIP	chromatin immunoprecipitation
ChIP-chip	chromatin immunoprecipitation on chip
ChIP-Seq	chromatin immunoprecipitation sequencing
ChIP-loop	chromatin immunoprecipitation loop
ChrISP	chromatin in situ proximity
CRY	cryptochromes
CT	chromosome territory
CT-IC	chromosome territory- interchromosome compartment
CTCF	ccctc binding factor
DamID	DNA adenine methytransferase identification
DMR	differentially methylated regions
DNA	deoxyribonucleic acid
FISH	fluorescence in situ hybridization
EI	euchromatic island
EM	electron microscopy
EZH1	enhancer of zeste homolog 1
ES	embryonic stem cells
HEBs	human embryoid bodies
HESCs	human female embryonic stem cells
IC	interchromosome compartment

ICN	inter chromatin network
ICR	imprinting control region
PLA	proximity ligation assay
LCR	locus control region
LOCKs	large organized chromatin K9 modifications
LADs	lamina-associated domains
MEFs	mouse embryonic fibroblast cells
MYC	myelocytomatosis viral oncogene
NL	nucleus lamina
PARylation	poly (ADP-ribosyl)ation
PARG	poly (ADP-ribose) glycohydrolase
PARP1	poly (ADP-ribose) polymerase 1
PRC1	polycomb repressive complex 1
PRC2	polycomb repressive complex 2
RNA	ribonucleic acid
PALM	photo-activated localization microscopy
PER	period
STED	stimulated emission depletion
STORM	stochastic optical reconstruction microscopy
SIRT1	sirtuin 1
TAD	topological-associated domains
TNF-α	tumor necrosis factor α
TSS	transcription starting site
WT1	wilms tumour 1 homologue
YY1	yin yang 1

Introduction

1 Non-random linear organization of the genome

Since DNA was proposed (by Frederick Griffith in 1928) and confirmed (by Oswald Avery in 1944) as genetic information carrier, there has been intense focus on the DNA sequence itself and how the information is coded in the DNA sequence. This focus culminated with the sequencing of the human genome in 2001 [1,2]has dramatically accumulated the information in this perspective. In the current post-genomic era, the emphasis has been placed on how the genome is differentially interpreted to generate robust cell phenotypes. This focus includes a methylation modification of the cytosine when this is followed by a guanosine in the genome. The ChIP-chip[3], ChIP-seq [4] and variant techniques have, moreover, made it possible to map histone modifications or specific DNA-protein complexes at specific regions within the genome. The extensive computation studies on omics data from genome sequencing, ChIP-chip and ChIP-seq have revealed the non-random linear organization of genome sequence.

1.1 The non-random segmentation of the human genome

The human genome is segmented into 22 pairs of chromosomes and XY (male) or XX (female) chromosomes. The gene coding regions and noncoding regions are heterogeneous distributed on each chromosome [5]. Genes localized in gene rich regions contain a higher G/C contents and tend to be active comparing with gene poor regions, which are enriched in the A/T content. Centromere and telomere contain highly repetitive sequences and locate at the center or end of each chromosome respectively. Retrotransposons, i.e. SINE and LINE, are also distributed in the genome unevenly with SINEs preferentially located in gene-rich regions whereas LINEs locate preferentially to gene-poor regions[6], although this division is not absolute.

1.2 Large organized chromatin K9 modifications (LOCKs)

Many different types of histone marks, such as methylation and acetylation, are deposited on the chromatin non-randomly. Using naïve ChIP (non-crosslinked chromatin), Feinberg and colleagues uncovered that large regions of the genome (up to several Mbs) are enriched in the H3K9me2 mark. This study coined the term LOCK for large organized chromatin K9 modifications, which are cell type-specific and highly conserved in mouse and human[7]. Such LOCKs are histone methyltransferase G9a dependent and observed in somatic cells, but not

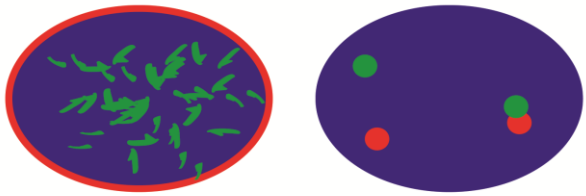
in embryonic stem (ES) cells [7]. Most interestingly, the LOCK regions are anti-related with gene expression and lost in cancer cell lines [7,8].

2 3D organization of the genome

Genomic sequencing and linear maps of chromatin features have increased our knowledge of the genomic code dramatically. However, in order to fully understand the genetic information on the genome and its function, we need understand the 3D organization of the genome in the nucleus. It is currently perceived that the packaging of the mammalian genome must enable for cell type-specific accessibility of general transcription factors, although we remain ignorant about the underlying principles.

2.1 The techniques overview in 3D genome research

a



Immuno-staining 3D DNA-FISH

b

Types of Microscopy	Resolution(nm) X and Y	Z
wide field	~ 200	~ 500
Confocal	~ 100	~ 300
Super-resolution (STED STORM PALM)	~ 30	~100

Figure 1, (a) Overview of in situ techniques in 3D genome research: immuno-staining (left panel, lamina staining = red, protein staining = green, nucleus staining = blue) and 3D DNA FISH (right panel, red and green represent two different gene loci). (b) Overview of the resolution limitations of the different microscopic techniques.

In general, there are two types of techniques available in 3D chromatin organization research, in situ visualization and in vitro biochemical analysis of formaldehyde-fixed cells followed by high throughput sequencing. Both approaches are powerful and widely used although the in situ visualization of single cells and high throughput analysis of cell populations offers a range of pros and cons. Thus while traditional single cell analyses

generates information of frequencies of chromatin fiber proximities at low resolution, the high throughput approach generates an average picture from population cell analyses at a generally high resolution.

The confocal light microscope provides a powerful tool to observe the chromatin location and folding. Thus, confocal microscopic analyses of 3D DNA fluorescence in situ hybridization (3D-FISH) have provided important information of chromatin proximity (schematically shown in Fig. 1a). For the future, real time analysis of the mobility of individual loci in relationship to each other will provide invaluable information [9]. Due to the light diffraction of fluorophores, the resolution of visualization techniques in Z dimension is hampered to 300nm in conventional confocal microscopy [10] (Fig. 1b). More recently, the development of super resolution microscopy, such as STED, STORM and PALM [11], has with a resolution of 20-30 nm in the X and Y plane the potential for much higher resolution of chromatin structures in comparison with the traditional confocal microscope. However, this promise has not yet been realized potentially due to the limitations of analysis in the Z dimension with a resolution only 100 nm [11] and that the choice of colors in super resolution analyses is currently limited to maximally three. To fully understand the organization of the genome it will be essential to overcome such limitations.

The chromatin conformation capture (3C) technique was initially invented by Dekker and colleagues in 2002 and is now widely used to detect chromatin proximity in solution [12]. In the 3C protocol, the intact chromatin fibers are cross-linked in situ with formaldehyde followed by the treatment with 4 or 6 bases restriction endonucleases. The proximal intramolecular chromatin fibers are subsequently ligated together and the final DNA products detected by PCR[12]. Since the 3C quantification is based on PCR primers, the sequences of chromatin, which used for proximity detect in 3C must be guessed beforehand. To avoid this bias, the circular chromatin conformation capture (4C) technique was established [13,14]. Because of the inversing PCR strategy and the high throughput capacity, the 4C is widely used to capture unknown intramolecular chromatin interactions genome wide from a known chromatin sequence, called bait. Later, 3C- carbon copy (5C [15]) was established, which is in principle a 3C technique based on the introduction of multiplex PCR amplification to cover all possible ligation combinations within a defined stretch of DNA, typically around one million bps followed by deep sequencing. Each of these “C” (3C, 4C and 5C) techniques is widely used in chromatin proximity research with different applications (Fig. 2). In order to capture all to all or rather many to many chromatin interaction in a

genome wide scale, the Hi-C was invented [16]. Briefly, this technique is based on the capture of ligated sequences bridging two different DNA regions followed by Hi-seq sequencing (see Fig. 2). Although providing valuable information the current coverage of the genome is limited [6,17-19]. Yet other versions of "C" technique are represented

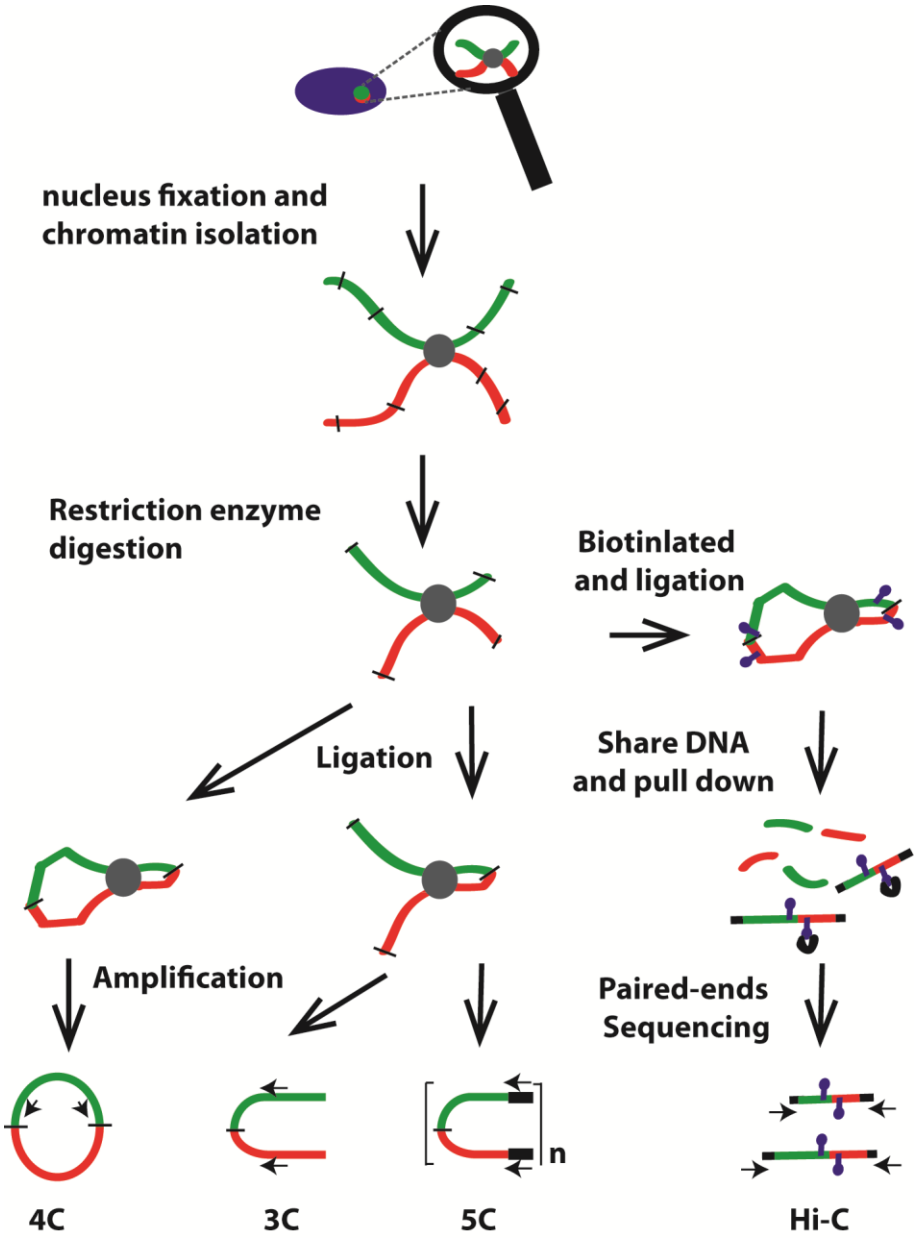


Figure 2, Schematic illustration of the different types of chromatin conformation capture techniques.

by the ChIP-loop[20] and ChIA-PET[21] techniques, which are in both instances built on the 3C technique integrated with ChIP. These techniques are thus able to score for chromatin fiber proximities in the presence of a particular factor.

There are both advantages and disadvantages in these two types of techniques. Since the resolution of 3C based methods is based on the length of the paraformaldehyde, which is around 3 angstrom, 3C based methods offer more precise information of intramolecular chromatin proximity than DNA FISH. However, DNA FISH offers chromatin interaction information at the single cell scale, whereas the "C" techniques can be used only in the context of large cell populations, usually more than 10 million cells. Recently, a single cell Hi-C study [22] shed the light of single cell information from in vitro perspective, but the limited sequencing coverage limited the conclusions [6]. While the "C" techniques and 3D DNA FISH techniques currently complement each other to reveal the genome organization in the nucleus [23-25], there is clearly a need for new techniques to fill the gap between these two approaches.

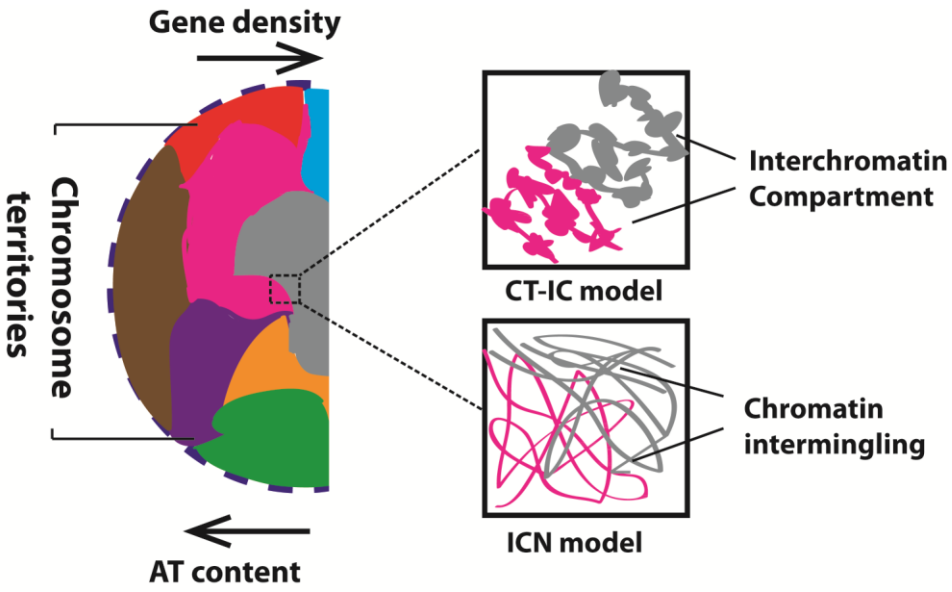


Figure 3, the chromosome territory principle in the interphase nucleus (left panel, each color represents one chromosome territory) and the models of chromatin organizations (right panel).

2.2 Radial chromosome organization and nuclear architecture

With the development and application of new techniques, numerous new concepts in chromatin structure have been introduced with the uncovering of new principles of nuclear organization.

2.2.1 The chromosome territory concept

Theodor Boveri introduced the chromosome territory (CT) concept for the organization of interphase chromosome in 1909, and the concept suggests that each chromosome occupies a particular space in the interphase nucleus [26] (Fig. 3). Only in 1976 using laser-UV-microirradiation experiments[27] did the first evidence for CT emerge. During the 1980s, DNA FISH analysis was performed on mouse and human hybrids, which contains only one or few human chromosome, to visualize individual CT [26,28]. From then on, the CT concept was well accepted. With the achievement of sorting individual human chromosome and the development of specific labeling system for individual chromosome, the chromosome painting protocol was established to observe all CTs within the interphase nucleus [29,30]. The introduction of multicolor DNA FISH using probes specific for sub-chromosomal regions and individual loci dramatically facilitated studies on CT substructures [30]. Currently, the principles of CT organization in interphase cells is believed to be pivotal for the formation of the nuclear architecture in mammalian cells[31].

2.2.2 The nonrandom radial arrangement of CT

The chromosome painting studies revealed that the 3D arrangements of CTs within the interphase nucleus are not random. Thus it was early observed that a gene-rich chromosome (Chr19) prefers to locate at the interior of the nucleus, whereas the gene poor chromosome (Chr18) is preferentially positioned to the periphery of nucleus [32]. This information was independently validated by several labs [33,34], confirming a more general correlation between gene density and radial arrangement in the nucleus [35] throughout evolution [36-38]. Importantly, the use of probes covering parts of chromosomes as well as individual loci extended this conclusion even within an individual chromosome [39]. It is thus generally accepted that the radial position of particular genes within the nucleus is cell type-specific and linked with central biological processes, such as transcription and replication [40] [41,42]. However, this tendency is not absolute, for instance, the chromatin arrangement is totally inversed in the rod photoreceptors of nocturnal animals [43]. This inversion arrangement is believed to be an evolutionary adaption to help the optional vision under the dark condition [43].

While the radial distribution of chromosomes is generally accepted to be non-random it is not clear if the radial proximity between neighbouring chromosomes is stable [26,44-46]. Systematic studies using DNA FISH and living image system have shown that the relative CTs

positions appear stochastic from mother nuclei to daughter nuclei, with the relative proximity between CTs reset in G1 phase [9,47]. Nonetheless, the observation that chromosomal neighbourhoods can be maintained between daughter cells [9,19] suggests that such features are at least metastable.

The report that CT are organized by 1Mb chromatin domains as basic structure units [48] have prompted suggestions that these corresponds to topological associated domain (TAD) which were highlighted from Hi-C data analysis [17,18,49]. Since the concept of 1Mb chromatin is from individual cell observation and TADs represent an average of large cell populations, it is not yet clear if this assumption will survive further scrutiny.

2.2.3 The CT organization and nuclear architecture models

Based on the concept of CTs [26,31], two models for nuclear architecture have been proposed (Fig. 3). The chromosome territory-inter chromatin compartment model (CT-IC) model suggests there are two basic comportments in the nucleus: chromosome territory (CT) and inter chromatin compartment (IC) [26]. The IC has been observed under the light and electron microscopy (EM) [48,50], and is believed to represent a space largely free from chromatin forming contiguous channels [26]. In addition, IC is suggested to harbor different types of non-chromatin nuclear bodies, such as speckles [26,48]. In this model, the perichromatin region (RP) is defined as a boundary layer between IC and the interior chromatin domain [51]. From the EM study, PR was showed to be transcription active region in mammalian nucleus [52].

Since several of other independent studies do not support the CT-IC model [53,54], the inter-chromatin network (ICN) was proposed [53]. The ICN model predicts that the chromatin fibers intermingle with each other within the same chromosome as well as between different chromosomes [53,54], such that chromatin fibers loop out of chromatin domains to meet with the other chromatin partners from the same CT or different CTs to establish crosstalk in cis- or trans [25,26,53,54]. Several lines of evidences have showed the active genes loop out of CTs to meet in transcription factories from C's experiments [54,55]. Moreover, using custom designed repeat-free probes, the FISH experiment showed gene coding regions extensively loop out of its' own CT [56]. It is more believed the ICN is for gene expression regulation. However, the imprinting network prevalent from trans- chromatin interaction was also found in mouse cells, and this ICN correlates with replication timing rather with gene expression [24], which indicates the ICN is not only for gene expression and also for other biological events.

The controversy that resulted from the presentation of two conceptually different models on chromosome organization may at the end of the day reflect limitations in resolution of microscopy as well as different practices in preparing samples for EM. The laser-based light microscopy [57] and the application of super resolution microscopy in chromatin research [11]might in the future help resolve this issue.

2.3 LADs and higher order chromatin structures

In mammalian cells, the inner surface of the nuclear envelope contains a complex filamentous network named nuclear lamina (NL) [58]. The NL mainly includes nuclear lamin A, B and C based on their molecular weights [58]. Since lamins A and C represent different isoforms from different splicing pattern of the same RNA, the lamins are subdivided into primarily A- and B- types [59]. A-type lamins are absent in embryonic stem cells but emerge during differentiation into different lineages whereas the B- type lamin is expressed in almost all the cell types and essential for cell viability [60]. The nonrandom radial organization of the chromosome is exemplified by the positioning of gene poor regions at the nuclear periphery. This principle likely implies that that the NL directly interacts with chromatin [35,59,61,62]. This conclusion is further support by the demonstrations that the lamins can bind core histones [63] and specific sequence of DNA [64] and that the lamin B binding protein receptor can interact with DNA, H3-H4 histone tetramers and heterochromatin protein HP1 [65]. In addition, the electron microscopy analyses had showed the direct evidence that there is interaction between lamina and chromatin [61,62].

Although all observations pointed to an important role for the NL in genome organization, no specific lamin-associated chromatin sequence could be identified until the introduction of the DamID technique [65,66]. DamID is a molecular technique, in which the fusion protein cDNAs-created by combining the genes for the bacterial DNA adenine methyltransferase (Dam) with that of a targeted protein- are transfected into the cells. Since Dam can methylate adenine in the sequence of GATC, the A-methylation mark is left on genomic sequences when these are directly proximal to the fusion protein targeting site. By cutting the chromatin with methylation sensitive restriction enzymes Dpn I and Dpn II, the protein targeting sites could be identified by microarray or high throughput sequencing [65]. Using DamID fused to emerin, a lamin-interacting protein, van Steensel and colleagues had managed to identify large (100kb-10Mb) lamin-associated regions (LADs). These regions cover nearly 40% of the genome [67] and map to the nuclear periphery [65]. Interestingly, while most of the genes located in LADs are silent [67,68] and the constitutive LADs are enriched in

evolutionarily conserved A/T isochores [69] [70,71], it has been claimed that (GA)n repeats can drive human LAD to the NL[72] even though there is no such a motif enrichment in LADs from DamID [69]. It thus appears that the principles tethering LADs to the nuclear periphery might occur not by one but by many mechanisms. In agreement with the dynamic feature of radial chromosome organization, DamID analysis from differentiated ES cells showed the some of the LAD regions are facultative to emerge in cell type-specific manner [68]. The activation of genes upon their release from the nuclear periphery during differentiation [68] demonstrates the repressed state of LADs. However, this feature does not preclude dynamic mobility within the nuclear periphery compartment. Thus real time imaging showed that the LAD positioning is stochastically reshuffled during cell cycle [73]. The dynamic feature of LAD agrees with the dynamic radial chromatin organization during the cell cycle. In the future, it would be interesting to test whether LADs from two different regions or chromosome could be tethered together in the nucleus.

Interestingly, as most (82%) of LOCKs and LADs overlap, although there remains a controversy with respect to the situation in embryonic stem cells [7,74], to throw some light on the still poorly understood mechanism underlying the recruitment of LADs to the NL. This is exemplified by the fact that knocking down the two H3k9 methyltransferases, MET-2 and SET-25, led to release of peripheral chromatin from the NL in worm cells [75]. Moreover, real time imaging similarly showed that the H3K9me2 methyltransferase G9a is a regulator of the contact between chromatin and NL [73]. However, as this could not be reproduced in mouse cells in two independent studies [76,77], the mechanism underlying the recruitment of chromatin to the nuclear periphery may be species-specific.

2.4 Chromatin crosstalk in 3D

The principle of nonrandom radial organization of the genome represents a major leap in our understanding its function. However, in order to be able to more fully understand the genome architecture in 3D and its function implications within the nucleus, we have to examine such features at the molecular level[25]. The innovation of the "C" techniques has made it possible to perform molecular level scanning of chromatin neighborhood [12,13,15,24], with the emerging consensus that chromatin fibers can interact both within (cis) and between (trans) chromosomes [19,25] (Fig. 4). Such patterns, which are termed chromatin crosstalk, have been implicated in diverse biological processes, such as transcription regulation, DNA repair and replication [18,19,24,25,54,78].

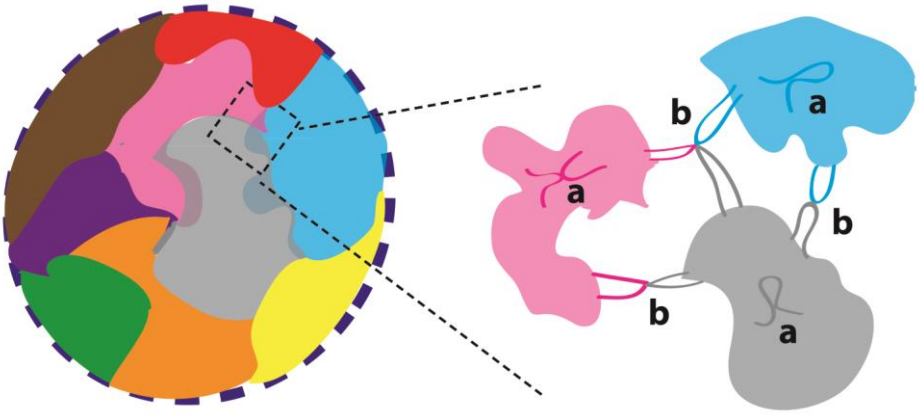


Figure 4, Model of different types of chromatin crosstalk: cis (a) and trans (b). Each color represents one interphase chromosome.

2.4.1 Chromatin crosstalk in cis and in trans

There is long standing hypothesis that gene transcription requires physical contacts between chromatin fibers, in particular the interaction between promoter and regulatory elements [79,80]. This model has only recently been put to the test with the innovation of the 3C techniques [81]. This can be exemplified by the well characterized *Igf2/H19* genes which are separated by the *H19* imprinting control regions (ICR) in mammals. While *Igf2* and *H19* share a common enhancer this activates preferentially the paternal *Igf2* and maternal *H19* alleles. This switch depends on the parent of origin-specific epigenetic states of the *H19* ICR, such that the CTCF-binding maternal *H19* ICR allele insulates the maternal Igf2 promoters from the downstream enhancers by organizing long-range chromatin loops [82,83]. Another similarly well-documented region is represented by the beta-globin domain. Extensive 3C studies in human and murine beta-globin loci had revealed long range cis- chromatin communications between the beta globin locus control region (LCR) and the globin promoters to regulate the transcription of the beta globin genes [84,85].

The ICN model of the nuclear architecture posits that chromatin fibers can intermingle with each other not only within same chromosome, but also between different chromosomes [53]. This hypothesis has been borne out as evidenced by numerous reports using the "C" techniques [86,87]. However, these assays face an inherent problem in distinguishing whether such chromatin networks are the results of pure chance or come with a functional consequence. In at least a few instances it has become clear that these interactomes do not form purely by chance, For example, the *H19* ICR region in mice organizes an imprintome, ie

a network of loci sharing the common feature of being genomic imprinted, which mediates the replication timing [24]. Moreover, it has been observed that stochastic, monoallelic transcription of the IFN-β locus depends on chromatin crosstalk with three identified distinct gene loci dependent to form the enhancosome [88]. Interestingly, genes encoding members of the TNFα signaling pathway form a specialized compartment to coordinate their transcription in trans [89]. Using genome editing techniques together with in situ assay, by analyzing the multi chromatin contact gene complex which is induced by TNF-α pathway, Mhlanga and colleagues had found the chromatin crosstalk in trans- controls hierarchical gene expression coordination [90,91]. Finally, six independent reports uncovered cis- and/or trans chromatin networks, focusing on genes encoding pluripotency factors, which are strongly implicated in the establishment and/or maintenance of pluripotency [92-98]. Taken together, these reports robustly make the case for that chromosomes talk to each other and that the resulting networks are functionally implicated to contribute to biological processes.

2.4.2 Chromatin crosstalk from active regions

The transcription factory concept describes the clustering of several transcription units into one single compartment [99], and it is thought to provide coordination of transcription for numerous genes [6,19,25,100]. Such transcription factories are believed to offer opportunities for the formation of chromatin networks in cis- and in trans. By performing systematic DNA FISH analyses, it was observed the gene-rich regions, but not gene-poor regions tend to cluster together in mega bps structures within the same chromosome [6,101]. These early observations have largely been supported by employing the 4C [14,102] and Hi-C [6,16] techniques. In the latter instance two different spatial chromatin organization status, termed A (open status) and B (closed status) compartments respectively, were identified by mapping human chromosome proximity within a 1 Mbps resolution [6,16]. However, the cluster of active regions is independent on ongoing transcription [103], suggesting that transcriptional units poised for transcription can cluster prior to overt transcriptional activation.

2.4.3 Chromatin crosstalk within and between inactive domains

Since the chromatin is typically separated into euchromatic and heterochromatic compartments in the interphase nucleus, it was believed that also inactive domains could cluster. This supposition was supported by employing derivatives of the “C” technique [14,16]. In comparison with chromatin interactions between active regions, the clustering of inactive domains seems to be restricted to the local context [6,104]. Such a feature might reflect that

condensed chromatin states impair chromatin mobility to establish more long-range interactions. Direct evidence in support of this interpretation is provided in a report showing that the Hox loci are repressed by PRC1/2 complexes to restrict their position within the host chromosome territory in ES cells [105]. However, upon transcriptional activation, the active allele acquires a much higher mobility to enable its looping out of the host chromosome territory [106].

Although repressive chromatin marks, such as polycomb bodies, H3K9me2, and H3K27me3 can be seen as clustered foci at the light microscope level, there is no corresponding conclusion about clustered heterochromatic regions from Hi-C analysis [6,16]. However, such regions that align with H3K9me3 and H3K27me3 marks have been identified using the 5C technique, which examined local interactions within an X-linked 4.5 Mbps domain [107]. The discrepancy between these observations may reflect an inherent problem in analyzing higher order chromatin states in inactive domains: the difficulty to achieve efficient restriction enzyme digestion. As the increased efficiency of enzyme digestion of chromatin DNA might also lead to the increased star activity, a notorious problem with the common enzyme of choice (Hind III), the clustering of inactive domains is likely underestimated in the vast majority of reports using any version of the "C" technique.

2.4.4 Topological associated domains (TAD)

With improved resolution of the Hi-C technique, currently in the range of about 100 kb, several studies had revealed the existence of small domains within larger A and B compartments in human, mouse and Drosophila genomes. These domains, termed topological domains or topological associated domain (TAD) [17,108], reflect long-range chromatin fiber interactions within but not between TADs [17]. Similar conclusions were reached with a more limited 5C analysis focusing on a 4.5 Mbps region including *Xist* [107]. Based on this concept, it has been proposed that the genome is packed into many small domains which are topologically organized within the interphase nucleus [17,18]. Moreover, the sizes of TADs vary from 100 Kbps to 1 Mbps with structures apparently conserved during more than 65 million years of evolution [17]. Since the TAD concept is derived from studies on very large cell populations, it is currently not known if TADs exist within a single nucleus, how stable it is and its function. Indeed, a single cell Hi-C study have shown there are major cell to cell variations in chromosome structure [109] to compound our understanding of the TAD concept. It seems reasonable to assume that the TAD structure is very dynamic to more highlight its adaptation to central processes in the nucleus. This rationale finds support in a

report that combined high resolution DNA FISH with polymer computation simulation to reveal a fluctuating correlation between chromosome conformations and transcription [110].

If the TAD concept will survive the current onslaught in the field it will be important to understand what defines the boundary of TADs and why this boundary exist at all. Extensive mining of ChIP-Seq data and motif computation indicates that transcription starting sites (TSS) and CTCF binding sites are enriched at TAD boundaries [107]. The link to CTCF was experimentally appeared to be verified as the deletion of a 58-kb region including CTCF binding sites located at the border of neighbouring TADs increased their communications [107]. However, the majority of CTCF binding sites are located inside TAD regions, suggesting that CTCF binding sites are not sufficient for maintaining the boundary between TADs [17]. Other arguments have included the cohesin complex which was also found enriched at the TAD boundaries [111]. As the cohesin complex have been associated with numerous long- and short- range chromatin interactions [112-114], it was suggested that CTCF might recruit the cohesin complex to form the TAD boundary [17,107]. However, depletion of either CTCF or the cohesin complex did not affect the TAD boundaries [111] even though TAD organization was affected by the absence of the cohesin complex in postmitotic mouse astrocytes [115]. Taking together, we remain largely ignorant as to how TADs are organized, how the boundaries are established and what the function is.

2.5 CTCF and chromatin organization

Interactions between chromatin fibers in cis and in trans likely influence the principles underlying higher order chromatin organizations within the interphase nucleus. The emerging question is: what kinds of protein factors mediate these complicated chromatin interactors and how do they do it? From traditional biochemistry to “C” applications and high throughput sequencing and computational data mining, CTCF emerges as one of the leading candidates to control such features.

2.5.1 CTCF and its binding regulation

CTCF was first discovered as a factor binding to the *Myc* promoter and acting as a negative regulator of *Myc* transcription in chicken cells [116,117], mouse and human cells [118]. The full length of CTCF contains DNA binding domain, C-terminal and N-terminal. There are eleven zinc fingers in the central DNA binding domain, which displays an almost 100% sequence similarity from chicken to man [117,119], while the C- and N- terminal ends vary slightly among the species [117]. Combining sequential deletion of each zinc fingers with band-shift assays

generated the observation that the zinc finger utilization was DNA sequence-dependent [117]. This observation paved the way for the realization that the underlying DNA sequence determined which zinc fingers were not engaged in direct binding to DNA and hence available in protein-protein interactions. Because of these considerations CTCF was coined as a multivalent factor [118].

The more full extent of the variability of CTCF binding sites was not understood well before the emergence of genome wide ChIP-seq studies. Thus we now know that there are minimally 55 000- 65 000 CTCF binding site in mammalian genomes [120]. Moreover, CTCF binding sites map generally in the linker region between positioned nucleosomes [121,122]. When comparing CTCF binding sites among different cell types, it was established that only around 5000 binding sites are highly conserved [120,123]. The position of the CTCF binding sites in the genome vary considerably. Thus about 50% of the CTCF binding sites are located in intergenic regions, while approximately 15% binding sites are found near promoters and around 40% are enriched in intragenic regions [123]. While CTCF is primarily known as the only established chromatin insulator protein [124], it is intriguing that CTCF binds also to enhancer regions.

An important feature of CTCF is that it is often sensitive to the CpG methylation status of its binding sites. This information was initially observed at the *H19* ICR [125] and has been extended to numerous other sites, such as *CDKN2A* [126], *BCL6* and *BDNF* [127,128] [129]. One study combined CTCF ChIP-Seq with bisulphite sequencing in 19 human cell types, and found that around half of cell type-specific CTCF binding sites regulate their interaction with CTCF depending on the DNA methylation status [130,131]. Interestingly, one study claims that CTCF together with partner proteins PARP1 and DNMT1 is responsible for maintaining the methylation free status of its binding sites [132]. Moreover, different post-translational modifications, such as sumolyation and PARlation, affect the CTCF function as well [133,134]. It has thus been clearly shown that the PARlation on CTCF is essential for CTCF insulator function on mouse *H19 Igf2* domain [134]. Recently, one report showed the insulator CTCF mediated the three dimensional genome organization through PARlation in Drosophila [135]. In addition, RNAs were also found to cooperate together with other protein partners to regulate CTCF binding and function in several independent reports [136,137].

2.5.2 Multi-functions for CTCF in chromatin organization

The CTCF- chromatin insulator connection was first reported for the chicken HS4 element at the 5 end of the β-globin gene [138]. Subsequently, CTCF was found binding on *H19* insulator region in mammalian cells as well [125,139,140] and many more sites identified by using a microarray based insulator assay [129]. However, most of the insulator assays were performed using plasmid transfection systems [141] with the *H19* ICR as the only bona fide in vivo chromatin insulator [141] [142] link between CTCF and the chromatin barrier function, which prevents the spreading of heterochromatin into neighboring regions is not clear. For example, genome wide studies have failed to robustly identify correlations between CTCF binding sites and repressive chromatin states, such as H3K27me3 [121]. It has also been reported that only 9% of the LAD borders contain a CTCF binding site. On the other hand, CTCF binding sites were found to be 8-fold enriched at the flanks of euchromatic islands (EI), which are inside the LOCKs with very low signal density of H3K9me2 signal [143]. Moreover, the idea that CTCF binding sites have a barrier function is partially supported by a recent ChIA-PET study targeting CTCF [144] and some locus-specific analyses [145]. The current controversy between chromatin insulator/barrier functions might at the end of the day reflect that CTCF conformations might be dictated by the underlying DNA sequence via variable zinc finger utilizations.

Increasing evidences had shown CTCF was involved in the chromatin organization by mediating the dominant cis- and trans- chromatin interactions. The most well studies cis-chromatin loop which mediated by CTCT insulator function is from mouse *H19* ICR region. Because of the binding of CTCF, the promoter and enhancer loops are different between paternal and maternal alleles which directly affect the expression of genes [83]. The regulations of long range of chromatin interactions from CTCF were also found in MHC-II loci and β-globin loci [84,146]. It was found CTCF could also mediate the trans- chromatin interaction between *Igf2/H19* and *Wsb1/Nf1* [147]. With 4C experiment, it was found out the *H19* ICR in mice with mutated CTCF binding sites changed the chromatin interaction dramatically [24], which indicates the CTCF plays quite important role in mediating the long range chromatin interaction. Moreover, by targeting the interactions between transcriptional starting sites (TSS) and distal elements in 1% percent of genome, it was found out 79% of long-range chromatin interaction between promoter and regulatory element are presenting with CTCF binding in 5C study [131,148]. This study clearly suggests the CTCF could tether chromatin proximities in genome wide. Some other CTCF partners are also involved in chromatin

organization together with CTCF. For instance, one study had revealed CTCF together with mediator or cohesion complex could mediate different ranges of enhancer-promoter interaction during lineage commitment [95]. It was shown CTCF and cohesion complex could mediate long range enhancer-promoter interactions, while short range enhancer-promoter interactions are bridged by mediator and cohesion complex [95]. Interestingly, several independent genome wide studies had shown the strong cohesion binding sites usually overlap with the CTCF binding sites[149-152].

3 Circadian regulation and genome organization

A wide variety of biological processes oscillates dependent on cycles of light and feeding [153]. In animal cells, such circadian processes encompass 24 hours periods dominated by the feeding behavior as feeding-fasting patterns have the ability to be reset and control circadian rhythms of several tissues, such as the heart and the liver [154]. It is not surprising therefore that the link between metabolism and circadian regulation of transcription is very strong [154,155] involving 10- 20 % of the genes [156]. The central clock machinery involves complicated feed-back controls between the central players, such as CLOCK, BMAL-1, PER and CRY [157,158] with CLOCK and BMAL1 as transcriptional activators (positive limb) and PER and CRY as transcriptional repressors (negative limb) [159].

Accumulated evidences point to a pivotal role of epigenetic modifications in manifesting the circadian rhythms [159]. Thus, CLOCK-BMAL1 and PER-CRY interact with chromatin modifiers such as EZH1, M1L1, P300, JARID1a and SIRT1 in vitro. Moreover, genome wide ChIP-Seq screenings have documented that chromatin marks, such as H3K9ac/H3K14ac and H3K4me3, can be rhythmically established and erased at circadian gene promoters in mouse liver [160]. Moreover, CLOCK acquired PARylation in an oscillating manner probably reflecting that CLOCK and PARP1 can physically interact with each other. This link was functionally reinforced by the observation that the entrainment of the central clock machinery was lost in mice lacking the PARP1 function [161]. Only recently have this system been integrated into the higher order chromatin context. Thus, Sassone-Corsi and colleagues used the 4C technique to explore chromatin networks impinging on the CLOCK regulated gene *Dbp* in mouse embryonic fibroblast cells (MEFs) to make the conclusion that the chromatin neighborhoods of the *Dbp* locus were related to circadian gene expression [159]. This conclusion was reinforced by the observation that the circadian dependent chromatin network could not be documented in BAML1 deficient MEFs [159]. To fully understand the circadian regulation, further work is needed.

Aims

The overall aim of this thesis was to uncover dynamic features of higher order chromatin structure in relationship to biological processes. To this end, two different types of studies were implemented with the following more specific aims:

Study 1: The dynamics of higher order chromatin structure change relevant to epigenetic mark H3K9me2 with single chromosome.

- To establish a new technique chromatin *in situ* proximity (ChrISP) to explore the high order chromatin architecture with a high resolution at the single cell level.
- To investigate the dynamics of higher order chromatin structure relevant to epigenetic mark H3K9me2 using the innovated ChrISP technique.

Study 2: The dynamics of higher order chromatin structures in a developmental window using a single locus as the bait in cell populations.

- To identify dynamic chromatin networks during a developmental window and uncover the mechanism.

Materials and Methods Summary

Materials

Human colon cancer cells (HCT116), Human female embryonic stem cells (HS181) (HESCs) and in vitro differentiated human embryoid bodies (HEBs) from HS181 cells were used as materials in this thesis.

Cell treatments

The G9a function was knocked down using two different strategies. In the first, G9a siRNA was transfected in HCT116 and maintained for 72 hrs as outlined in paper II. In the second, HCT116 cells were treated with 0.5 µM G9a enzymatic inhibitor BIX 01294 trihydrochloride hydrate (B9311, Sigma-Aldrich) for 72 hrs as described in paper III. Similarly, the PARP1 function was knocked down by transfecting a specific PARP1 siRNA, while the enzymatic function of PARP 1 was antagonized by Olaparib (0.3 µM final concentration) treatment in HCT116 cells, as per paper III. CTCF expression was knocked down by siRNA as described in paper III. The inhibition of the activity of CDK8/9 to manipulate the phosphorylation status at the serine 2 position with the large subunit of RNA polymerase II (Flavopiridol treatement) is outlined in paper III. The PARG treatments were performed in both fixed HESCs and HEBs prior to 4C analyses, which were described in paper III. For the serum shock treatment in paper III, HCT116 cells were cultured with serum-rich medium (McCoy5A +penicillin-streptomycin-glutamine (PSG), supplemented with 50% horse serum (26050088, GIBCO)) for 2 hrs. The medium was subsequently replaced with serum-free McCoy5A + PSG and cultured for periods indicated in paper III.

Quantification of cell treatments efficiency

Both Western blotting and immunofluorescence staining analyses were employed to detect the efficiencies of the various treatments as listed in the papers.

DNA probes labeling

Different types of labeling methods were used in this thesis, and the brief information is list as follows. Human Cot-1 DNA (15279-011, Invitrogen) was labeled with Biotin-16-dUTP (11093070910, Roche) or Digoxigenin-11-dUTP (11573152910, Roche) using the Bioprime Array CGH kit (18095-011, Invitrogen). The BAC probes were first sonicated to 500-2000 bps range followed by labeling with fluorescent nucleotides using the Bioprime Array CGH kit. In

papers I and II, the chromosome 11-specific templates were labeled with DOP-PCR as has been described [30].

DNA FISH and RNA FISH

3D DNA FISH analyses enabled to precise position of a chromosome territory (paper I and II), or proximities between chromatin hubs in HESCs, HEBs and HCT116 cells (paper III). RNA FISH was performed to check transcriptional activity in situ (paper III).

In situ proximity ligation assay (ISPLA)

ISPLA [162] was used to detect proximities between repeat regions in DNA (paper I) or proximities between different proteins (paper III).

The innovation and application of ChrISP

In paper I, a new technique termed Chromatin in situ proximity (ChrISP) was innovated to enable the efficient detection of chromatin fiber compaction in single cells. Paper II and III describe its application in some more detail comparing the density of chromatin marks within chromatin fibers. The ChrISP principle is based in the ISPLA technique but combines DNA FISH with immunostaining of chromatin marks/fibers without the use of a rolling amplification circle step. Its principle is based on the inclusion of a fluorescent "splinter" that is able to bridge two different epitopes, such as digoxigenin and biotin, with a very high specificity following ligation to the DNA backbone.

The limits of resolution of the ChrISP technique

In order to detect the resolution limits of ChrISP, DNA fiber FISH and STED microscopy were employed (paper I). In brief, the human genome DNA was stretched out and combed on silane coated slides. The DNA fiber FISH analysis was performed combining biotin and digoxigenin labeled human Cot-1 probes as described [163]. The DNA fiber FISH signals were scanned with STED microscopy, and the total lengths of the DNA fibers analyzed were in each instance encompassing about 14 million bps. The distance between the labeled Cot-1 probes was then determined by a nearest neighbor algorithm where each centers coordinate points was matched to other centers coordinate points [164]. The closest distances were then plotted as histograms and used to create cumulative curve distributions in two independent experiments. The ChrISP analyses between human Cot-1 biotin probes and digoxigenin probes were performed on the stretched DNA fibers, and STED images of the rolling circle amplification (RCA) signals were used to determine frequency of RCA in relation

to the amount of labeled Cot-1 probes. The equation for frequency calculation is as follows: F= (Number of RCA signals) / (Number of Cot-1 probe/2). The frequency was then compared to the cumulative curve distribution in order to determine the resolution of ChrISP, which turned out to be less than 17 nm.

Circular Chromatin Conformation Capture Sequencing (4C-Seq)

Using the human *H19* ICR region as targeting bait, circular chromatin conformation capture sequencing (4C-seq) was performed in both control and PARG treated HESCs and HEBs as previously described [165] (see paper III). Amplified DNA was subjected to Solexa paired-end sequencing and reads aligned to the GRch37/HG19 genome using Bowtie3 and BWA software to generate an interactome of chromatin fibres impinging on the bait.

Results and discussions

Paper I: Chromatin in situ proximity (ChrISP): single-cell analysis of chromatin proximities at a high resolution

Our understanding of the chromatin organization in the nucleus is critical for any further uncovering of principles guiding chromatin condensation or expansion and how these features relate to transcriptional regulation. Till this report, there such endeavor could be addressed by mainly two types of techniques, 3C-based techniques and 3D DNA FISH [19]. 3C-based techniques, such as 3C, 4C, 5C and Hi-C, are ligation-based techniques, which are widely used to check chromatin fiber proximity *in vitro* [12]. The advantage of 3C-based techniques is high resolution, which is determined by the length of fixation reagent. However, these 3C based techniques can only offer the average information of chromatin proximity from couples of million cells in at best a semi-quantitative manner [25]. While 3D DNA FISH provides a powerful tool to visualize chromatin organization in single cells, its resolution is limited by the resolution of the microscopy. For instance, the resolution of confocal microscopy is at best 300 nm in the Z dimension. The general approach to assess chromatin fiber proximities thus is to use 3C-based techniques to uncover chromatin proximities at a high resolution from population cells, and 3D DNA FISH to quantitatively calculate the chromatin proximity frequencies at a low resolution. However, this strategy generates a gap in resolution between these two techniques to compromise interpretations. In order to fill the resolution gap between these two techniques, we had developed a high resolution *in situ* technique termed Chromatin *in situ* Proximity (ChrISP). The resolution of ChrISP to detect chromatin proximity is < 17 nm in all three dimensions independent on the diffusion of fluorophores that compromised the resolution of the conventional confocal microscopy.

The initial strategy of ChrISP was to combine traditional 3D DNA FISH with *in situ* proximity ligation assay (ISPLA). This technique was invented to detect the proximity of two proteins in the cells based on a padlock probe and splinter hybridization followed by rolling cycle amplification (RCA) [162]. In the ISPLA the proximity between two proteins can be assessed by the ligation of a padlock probe and splinter following by rolling circle amplification (RCA) and addition of fluorescence labeled oligos, provided that the two epitopes were sufficient close to each other. This strategy enables the detection of two different protein epitopes based on their distance to each other rather than the resolution of the microscope. Although the principle of ISPLA was incorporated initially when developing the ChrISP technique,

comparing the distance between two different chromatin regions based on their visualization by biotin or digoxigenin probes, respectively, it was found that the RCA step was blocked by dextran sulfate, which is an essential component of the DNA FISH hybridization protocol to increase efficiency. In order to overcome this problem, we replace dextran sulfate with dextran in the hybridization buffer. Using this modification, the proof of principle of ChrISP was established using human Cot1 DNA to visualize the clustering of repeat elements.

As it was considered to be essential to know the resolution limits of the ChrISP technique, we used STED microscopic analysis of DNA fiber FISH samples using the strategy outlined above in the methods section. We found it striking that the resolution of ChrISP is < 17 nm was essentially identical to another estimation using regular ISPLA [166]. Such a resolution compares very favorably to super-resolution fluorescence confocal microscopic techniques, such as PALM, STORM or STED. This improvement was named technique RCA-ChrISP. However, it was subsequently observed that the RCA step is constrained by the nuclear architecture for the RCA-ChrISP Cot-1 signal to emerge primarily at the nuclear periphery. To overcome such problems, the RCA step was omitted and the splinter labeled with green fluorescence to detect the DNA fragment proximities. Critical washing steps were optimized and achieved to keep the circular green fluorescence DNA in place only if the two epitopes were sufficiently close to each other. This final version of ChrISP, was used to determine proximities between Cot-1 repeat elements. In contrast to the RCA-ChrISP the proximity signals could now be observed throughout the nucleus. Using the splinter-ChrISP we had documented chromatin proximities within chromatin 11, visualized by biotin- and digoxigenin-labeled probes. Interestingly, the majority of the splinter-ChrISP signals were located at the boundary of chromosome 11 facing the nuclear periphery. Based on several types of controls, we could show that such signals were highly specific and dependent on the clustering of unique sequences without much contribution from repeat elements. In contrast, such features could not be visualized at all using conventional confocal microscopy.

Taken together, a new single cell technique (ChrISP) was invented to facilitate chromatin organization in unprecedented detail filling the resolution gap between 3C-based and 3D DNA FISH techniques. The versatility of this ChrISP technique that provides yet another benefit in comparison with the 3D DNA FISH technique, it can be used to assess any proximity between any region in the genome and other epitopes, visualized by antibodies to nuclear lamins, transcription as well as any chromatin mark. As ChrISP is a single cell

technique it can also be used to quantitatively assess stochastic events underlying a particular transcription pattern, or the acquisition/erasure of chromatin marks in a cell population. We thus predict that this technique will be very useful in chromatin architecture research that wants to visualize higher order chromatin features in relationship to dynamic process, such as the cell cycle.

Paper II: The visualization of large organized chromatin domains enriched in the H3K9me2 mark within a single chromosome in a single cell

To demonstrate the power of the ChrISP technique, we examined in more detail the features underlying the compact chromosome 11-specific chromatin structures at the nuclear periphery presented in the previous report. The size of the ChrISP signals indicating the involvement of millions of bps of sequences suggested that these structures represented the first visualization of the so-called LOCK structures for Large Organized Chromatin K9 modifications [7]. Initially we performed a new type of ChrISP analysis by combining the presence of H3K9me1/2/3 marks visualized by specific antibodies with a probe specific for the entire chromosome 11. This strategy showed that chromatin hubs could again be visualized using a H3K9me2-specific antibody while the distribution of the H3K9me1/3 marks were chromosome-wide. Moreover, the H3K9me2 enriched chromatin hubs frequently projected towards to the nuclear periphery as finger-like structure from the bulk of chromosome 11 territory – a feature that could not be visualized with normal DNA FISH or immuno-fluorescence staining. Importantly, it was observed that chromatin hubs carrying the H3K9me2 mark revealed by ChrISP differed from the H3K9me2 staining pattern within the nucleus, indicating that the ChrISP patterns emerged only when the chromatin fibers carrying this mark are clustered together at the nuclear periphery.

To independently validate this observation, we reasoned that removal of the function establishing the H3K9me2 mark from its H3K9me1 precursor, the G9/Glp methyltransferase [7], would absolve the compact structures at the nuclear periphery. To test this possibility, HCT116 cells were transfected with a siRNA specific against G9a/Glp followed by ChrISP analysis using chromosome 11-specific digoxigenin/biotin probes. The results showed conclusively that the compacted structures at the nuclear periphery were indeed absent in the relative absence of the G9a/Glp function to document that the H3K9me2 mark is responsible for the clustering of chromatin fibers involving unique sequences at the nuclear periphery. Such an observation is in agreement with an independent report documenting that compact, transcriptionally non-permissive structure of the inactive X chromosome is dependent on the presence of H3K9me2/3 marks and HP1 [167].

Most surprisingly, however, knocking down the G9a/Glp function also significantly increased chromosome-wide chromatin compaction beyond the nuclear periphery. Such an observation could reflect that the clustered chromatin structures at the nuclear periphery

provide physical constraints for the compaction of the rest of the chromosome. Alternative explanations include the activation/repression of chromatin factors induced by the loss of the G9a/Glp functions, or that the newly compacted structures represent premature condensation events. Further work is essential to determine which of these scenarios can be ruled in or out.

Taken together, we have in paper II used the high-resolution ChrISP technique to uncover dynamic changes of higher order chromatin conformation determined by the H3K9me2 mark. Thus chromosome 11-specific chromatin hubs, which are enriched with H3K9me2 marks and located at the nucleus periphery in single cells, could be visualized for the first time. Moreover, reducing the levels of the H3K9me2 marks abolished the appearance of clustered chromatin structures at the nuclear periphery while dramatically increasing the prevalence of compact chromatin structures in the interior portions of chromosome 11. Such dynamic changes of higher order chromatin structure and their regulation have not been recognized before.

Paper III: Poly(ADP-ribose) polymerase I and CTCF regulated interactions between active and repressive chromatin domains contribute to circadian plasticity of gene expression

To uncover the dynamic feature of chromatin conformations, an epigenetically regulated region *H19* ICR region [24]was used as bait in human embryonic stem cells (HESCs) and human derived embryoid bodies (HEBs) 4C analyses. In agreement with previous research in the mouse [24], numerous inter- and intra- chromatin chromatin fibers could be found to interact with the *H19* ICR region in developmentally regulated patterns. Surprisingly, cohesins were not found enriched on the chromatin interactors and excluded as the molecular tie organizing the *H19* ICR interactome. Since the Poly(ADP-ribosyl)ation (PARlation) mark on CTCF from the *H19* ICR has earlier been suspected by us to play a central role in cis chromatin interactions, we examined if this mark underlied the *H19* ICR interactome. To test this possibility, formaldehyde crosslinked chromatin samples were treated with PARG, which degrades the PAR chains, prior to the ligation step in the 4C-seq protocol. The results showed that removing the PARlation mark led to the disassembly of the majority of interactors from the *H19* ICR in both HESCs and HEBs to suggest that this mark is present at the time the *H19* ICR-specific interactome was crosslinked. A unique feature of the 4C-seq method used, to capture more than two simultaneously interacting chromatin fibers, was used to establish a topological network. This network could be validated by 3D DNA FISH analyses showing that well-connected central nodes were on average closer to the H19 ICR than regions that were outliers. Moreover, the distances between central nodes are also closer than to regions further apart in the network topology. A striking feature of this network is that it is organized in modular clusters that mix active chromatin and inactive chromatin within each other. This is surprising as it is generally believed that active chromatin and silence chromatin fibers are physically separated [6].

Since the data suggested that PARylation might mediate the formation of the inter-chromatin interactome, we reproduced the claim that CTCF is able to bind to PARP1 and influence its enzymatic activity without DNA damage [168]. We also showed that Olaparib, a PARP1 inhibitor, abolished not only CTCF-PARP1 interactions in co-immunoprecipitation assays, but also the *H19* ICR interactome. Moreover, ChIP experiments showed that PARP1 interacted with the *H19* ICR and CTCF with the central chromatin hubs in an Olaparib-sensitive manner. As PARP1 was interacting prominently with the central hubs, such results suggested that CTCF binding on the central hubs represented indirect binding mediated via

PARP1. Surprisingly, the same treatment almost completely evicted PARP1 from all the central hubs indicating that also PARP1-DNA interactions are Olaparib-sensitive, potentially due to conformational changes induced by the drug. Taken together, the results indicate that initial CTCF-PARP1 interactions underlie the assembly of the network and that the PARylation emerging as a result of this interaction contributes to the complexity of the trans-interaction network.

To enable an interaction with all autosomes, the bait probably has to emerge from the constraints of its chromosome territory. Indeed, 3D DNA FISH analyses showed that both the *H19* ICR and *VATL1*, which together with the bait constituted the central chromatin in node the network showed such features controlled by PARP1 activity. Moreover, the proximities between the *H19* ICR, *VAT1L* and *PARD3*, another central node, were reduced following Olaparib treatment and reduction of PARP1 expression mediated by siRNA transfection. As down-regulation of CTCF by siRNA transfection dramatically reduced the PARylation levels in living cells, we propose that the mobility of the central node for the inter-chromosome network requires both CTCF and PARP1.

Given that the network mixed active and inactive regions we could not state that it was involved in the segregation of transcriptionally active or repressed parts of the genome. Reasoning that it might rather function by transcriptional attenuation we caught interest in the observation that the network is enriched in genes under circadian control. The possibility that circadian genes interacted with the constitutively repressed regions, represented by LADs [67], for a functional purpose was boosted by two lines of evidence: First, the enrichment of circadian genes was at its highest statistical significance at a distance of 10 kbps indicating a regulatory role of the interacting region. Second, it was recently reported that PARP1, which is also a key regulator of the *H19* ICR-specific network, regulates the entrainment of circadian expression [161]. Indeed, 3D DNA FISH proximity analyses demonstrated that the bait and other central nodes from the network located to the nucleus periphery in an Olaparib-sensitive manner.

To examine this link further, we employed a method to induce synchronization, entrainment, of circadian expression, by serum shock followed by serum starvation. Since this feature could not be achieved in HESCs [169], we used HCT116, which can be induced to efficiently display circadian rhythms [170], to test the hypothesis. Strikingly, we could observe that the proximities between CTCF and PAPR1 are preferentially confined to the nuclear periphery

and show circadian rhythms in serum-shocked HCT116 cells, with peaks at 8 and 24 hours with a period of approximately 24 hours. Similarly, the proximity between *H19 ICR/PARD3/TARDBP* loci and the nuclear periphery showed a similar oscillating pattern with a period of approximately 24 hours, although the *H19* ICR peaked at the nuclear periphery about 8 hours earlier than the *PARD3/TARDBP* loci. We show in the manuscript that these features are linked with transcriptional activity, such that inhibition of *PARD3* transcription accelerated its recruitment to the nuclear periphery. Importantly, nuclear *PARD3* transcription showed a circadian pattern, which peaked at the time its association with the nuclear periphery was most prominent. We reasoned therefore that the attenuation of transcription might follow from the nuclear peripheral association. This assumption was borne out by the demonstration that the attenuation of transcription in *PARD3* was strongly linked to the acquisition of repressive chromatin marks H3K9me2 during its transient association with the nuclear periphery. Moreover, after inhibiting G9a/Glp activity, circadian migration of *PARD3* to the periphery disappeared and circadian transcription stopped. Finally we show that the recruitment of *H19/PARD3* to the nuclear periphery is also sensitive to CTCF and PARP1 levels.

Taken together, we have in paper III uncovered a dynamic and developmentally controlled chromatin network involving direct contacts between active loci and repressed regions. The network arises, at least in part, by the recruitment of central chromatin hubs to the nuclear periphery potentially reflecting oscillating CTCF-PARP1 interactions. Finally, Moreover, the recruitment of the chromatin hubs is essential for the establishment of circadian transcription of the *PARD3* gene by the oscillating acquisition of the repressive H3K9me2 mark. These observations connect circadian regulation and its dynamic shuttle to the nucleus periphery and provide new perspectives for our understanding of the mechanism of circadian regulation of transcriptional plasticity.

Summary and Outlook

The work in thesis has been trying to uncover the nonrandom and dynamic features of the genome in 3D.

In paper I, a new technique, named chromatin in situ proximity (ChrISP), was invented. The successful application of ChrISP has already filled the gap between 3D DNA FISH and 3C-based techniques. The remarkable features of ChrISP include high resolution of chromatin structures at the single cell level. We propose this technique will be widely used in chromatin organization research dedicated to understanding principles of stochastic and dynamic gene expression features and how these relate to chromatin structures within the very same chromosome. In the future, combing the ChrISP technique with super resolution microscopy will likely offer even more information of higher order chromatin structure, especially when combined with splinters of different lengths.

In paper II, the ChrISP technique was applied to examine the function of a chromatin mark, ie H3K9me2, in regulating higher order chromatin structure with a single chromosome in a single cell. We observed that the H3K9me2 mark is enriched in finger-like structures at the nuclear periphery and that removing the epigenetic mark H3K9me2 reduced the appearance of chromatin hubs at the nuclear periphery while increased the compacting chromatin structures in the portions of chromosome 11 in the interior of the nucleus. We proposed that H3K9me2/3 marks regulate the pleiotropic feature of higher order chromatin structure. In the future, it would be very interesting to examine the mechanism of these dynamic changes of higher order chromatin structure relevant to H3K9me2 and any gene expression changes relevant to dynamic changes of chromatin structure devoid of the H3K9me2 mark. It also deserves to test the lamina association in the chromosome 11 and the detail information of chromatin conformation rearrangement from particular loci after removing H3K9me2. Finally, it can be predicted that the effect of knocking down the G9a/Glp function might lead to premature chromosome condensation, as this is a feature that might compromise the integrity of the genome.

In Paper III, we identified a chromatin network impinging on the epigenetically regulated *H19* ICR that depended on the combined function of CTCF and PARP1. The striking observation this interaction could be induced to oscillate in a synchronized manner might underlie the fact that the central chromatin hubs were recruited to the nuclear periphery in circadian patterns. The largely repressive character of the nuclear periphery influenced the

establishment of circadian transcription linked with the oscillating presence of H3K9me2. These observations have compounded our understanding of the mechanism of circadian regulation of transcriptional plasticity. In the future, it would be important to establish in more detail the principles underlying the recruitment of the central chromatin hubs to the nuclear periphery and their subsequent release.

The nuclear periphery and its link to the H3K9me2 mark to form LOCK structures constitute one common denominator of this thesis. This is not surprising given that the nuclear periphery is likely to affect chromosome structures pleiotropically in interphase nuclei. The discovery that the chromatin hubs, identified here as circadian genes, are actually linked to the nuclear periphery provides yet another perspective on this topic. However, the downside of this arrangement is that epi-mutations involving the H3K9me2 mark, for example, can be induced by an irregular life to predispose to complex diseases, such as cancer. It might thus be rewarding to apply the ChrISP technique or any further derivative to examine chromatin structures in biopsies of patients.

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