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REGULATION OF CHROMATIN TRANSITIONS IN THE 3D ARCHITECTURE OF THE NUCLEUS

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Regulation of chromatin transitions in the 3D architecture of the nucleus

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To my family

Practice is the sole criterion of truth !

ABSTRACT

The thesis explores the dynamic features and functions of chromatin crosstalk in the 3D nucleus space, with a focus on the regulation of chromatin transitions. In Paper I we employed the circular chromosome conformation capture assay (4C) and Chromatin in situ Proximity (ChrISP) technique to uncover an inter-chromosomal chromatin fiber interactome comprising both transcriptionally active circadian genes and repressed lamina-associated domains (LADs). Moreover, we documented that this interactome was under the control of the circadian clock. Synchronization of circadian transcriptional oscillations by external time cues thus involved the rhythmic mobility of clock-controlled genes between the transcriptionally permissive nuclear interior and the repressive environment of the lamina. The transient interactions between LADs and circadian genes were regulated by rhythmic complex-formation between the 3D genome organizers PARP1 and CTCF, which not only served as molecular ties of the chromatin fiber network, but also regulated chromatin mobility to and from the lamina and, as a consequence, circadian gene expression.

In Paper II, we described a novel principle regulating *MYC* expression in colon cancer cells. Using an innovated method, Nodewalk, and ChrISP, we thus found that the oncogenic colorectal super-enhancer (OSE) regulated *MYC* expression at the post-transcriptional level by facilitating "gene-gating". OSE/*MYC* complexes were dynamically tethered to the nuclear pores specifically in cancer cells by interactions between ELYS/AHCTF1 that connects chromatin to the NUP107 nuclear pore subcomplex and the ß-catenin-TCF4 complex. Tethering to the nuclear pores facilitated the nuclear export of *MYC* transcripts into the cytoplasm where the stability of *MYC* mRNAs is several-fold higher than in the nucleus, resulting in a several-fold increase of cellular *MYC* mRNA levels in human colon cancer cells. WNT signalling thus regulates pathological *MYC* mRNA export post-transcriptionally through ß-catenin-TCF4-ELYS complex formation.

In summary, this thesis describes new principles of gene regulation in the 3D nuclear architecture, including circadian transcriptional regulation and OSE-facilitated gene gating. They open up new avenues for our understanding of the function and dynamics of the 3D nuclear architecture and genome organization, and provide new directions for cancer treatment.

LIST OF SCIENTIFIC PAPERS

I. *PARP1- and CTCF-Mediated Interactions between Active and Repressed Chromatin at the Lamina Promote Oscillating Transcription. Mol Cell, 2015. 59(6): p. 984-97.*

Zhao H*, Sifakis EG*, Sumida N*, Millán-Ariño L*, Scholz BA, Svensson JP, Chen X, Ronnegren AL, Mallet de Lima CD, Varnoosfaderani FS, Shi C, Loseva O, Yammine S, Israelsson M, Rathje LS, Németi B, Fredlund E, Helleday T, Imreh MP, Göndör A.

II. *WNT signaling and AHCTF1 promote oncogenic MYC expression through super-enhancer-mediated gene gating. Nature Genetics, Accepted 23 October 2019.*

Barbara A Scholz#, Noriyuki Sumida#, Carolina Diettrich Mallet de Lima, Ilyas Chachoua, Mirco Martino, Ilias Tzelepis, Andrej Nikoshkov, **Honglei Zhao**, Rashid Mehmood, Emmanouil G Sifakis, Deeksha Bhartiya, Anita Göndör* and Rolf Ohlsson*

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

3C	chromatin conformation capture
3D	three dimensional
4C	circular chromatin conformation capture
4C-Seq	circular chromatin conformation capture sequencing
5C	chromosome conformation capture carbon-copy
5-EU	5-ethynyl uridine
AHCTF1	AT-Hook containing transcription factor 1
ALK	activin receptor-like kinase
BAC	bacterial artificial chromosome
bHLH-PAS	HLH (helix-loop-helix)-PAS (period-arnt-single-minded)
BMAL1	brain and muscle arnt-like 1
CCG	clock controlled gene
ChIA-PET	chromatin interaction analysis by paired-end tag sequencing
ChIP	chromatin immunoprecipitation
ChIP-chip	chromatin immunoprecipitation on chip
ChIP-loop	chromatin immunoprecipitation loop
ChIP-Seq	chromatin immunoprecipitation sequencing
ChrISP	chromatin in situ proximity
CLOCK	circadian locomotor output cycles kaput
Co-IP	co-immunopurification
CRY	cryptochromes
CT	chromosome territory
CTCF	CCCTC-binding factor
DamID	DNA adenine methytransferase identification
DAPI	4,6-diamidino-2-phenylindole
DBP	D-box binding proteins
DEC1 and 2	differentially expressed in chondrocytes 1 and 2
DNA	deoxyribonucleic acid
FISH	fluorescence in situ hybridization
GFP	green fluorescent protein
HCEC	human colon epithelial cell
HAT	histone acetyltransferase
HDAC	histone deacetylase
HEB	human embryoid body
HESC	human embryonic stem cell
HMT	histone methyltransferase
eRNA	enahncer RNA
ICC	immunocytochemistry
ICR	imprinting control region
IFN-γ	interferon gamma
IGF2	insulin-like growth factor 2
IgG	immunoglonulin G
iPS	induced pluripotent stem
ISPLA	in situ proximity ligation assay

LAD	lamina-associated domain
LBR	Lamin B receptor
LCR	locus control region
LOCK	large organized chromatin K9 modification
MED12	mediator subunit 12
MEF	mouse embryonic fibroblast cell
MIZ	Msx-interacting-zinc finger
MYC	myelocytomatosis viral oncogene
NAD	nucleolus-associated domain
NET	nuclear envelope transmembrane protein
NPC	nuclear pore complex
NUP	nucleoporin
NuRD	Mi-2/nucleosome remodelling and deacetylase
OCT4	octamer-binding transcription factor 4
OSE	oncogenic super-enhancer
PAR	poly ADP ribose
PARG	poly (ADP-ribose) glycohydrolase
PARP1	poly (ADP-ribose) polymerase 1
PARylation	poly (ADP-ribosyl)ation
PCR	polymerase chain reaction
PER	period
PRC	polycomb repressive complex
RCA	rolling circle amplification
rDNA	ribosomal DNA
REV-ERBα/β	reverse erithroblastosis α and β
RNAi	RNA interference
RNA POL II	RNA polymerase II
ROR	retinoic acid receptor-related orphan receptor
RRE	ROR/ REV-ERB -binding element
sciATAC-seq	single-cell combinatorial indexed ATAC-seq
sciHi-C	single-cell combinatorial indexed Hi-C
SCN	suprachiasmatic nuclei
SIRT1	NAD-dependent deacetylase sirtuin-1
TAD	topologically associating domain
TCF4	transcription factor 4
TF	transcription factor
TGF-β	transforming growth factor- β
TNF-α	tumor necrosis factor α
TSS	transcription starting site
YY1	yin yang 1

1 INTRODUCTION

1.1 EPIGENETIC REGULATION

The sequence of an organism's genome does not directly determine how the genome is used to build the organism. A second, more complex regulatory code is encrypted in the chromatin structure and 3D nuclear organization of chromosomes. This epigenetic information, or the epigenome, not only provides an essential cue to allow a cell to interpret the genome but can also be transmitted through cell division to preserve cellular identity. Epigenetic mechanisms thus converge on chromatin features to manifest specific changes in gene expression in response to developmental and environmental cues [1]. To effectuate this principle, the chromatin structure provides an essential platform for the collaboration between transcription factors and specific chromatin modifications [1]. Chromatin modifications are generated by the synergism between DNA and histone modifications, three-dimensional (3D) genome organizers, as well as non-coding RNAs [2, 3] in response to environmental cues. Once established, such features are generally stable to ensure the generation of robust phenotypes during development [4, 5]. However, the loss of this robustness to generate metastable and hence more plastic epigenetic states is considered a key factor underlying the development of complex diseases, such as cancer [5]. The molecular mechanisms underlying epigenetic plasticity and its deregulation in diseases remain poorly understood, although they are likely reflected by a change in the stability of physical interactions between distant regulatory elements, such as promoters and enhancers, crosstalk between different epigenetic marks, formation of transcriptional memory as well as compartmentalization of active and inactive chromatin domains [3, 6-10].

1.2 CHROMATIN CROSSTALK IN 3D

Although dynamically responding to extra-cellular cues, each chromatin is not randomly organized in the nucleus and is under development control [6, 11, 12]. The genome is packaged in the confines of the nuclear space in a highly dynamic while regulated manner and forms higher-order chromatin conformations. The 3D organization of the genome in the nucleus space influences and is influenced by genomic functions, including transcription, replication and DNA repair [6, 11]. The nuclear architecture and 3D genome organization, moreover, influence chromatin crosstalk and modulate the stability of chromatin states[6, 11]. Various methods have been evolutionally developed to assess genome-wide chromatin fiber interactions and close spatial proximities in the 3D architecture of the nucleus.

1.2.1 The innovation of techniques to explore the 3D genome

During the last two decades, mainly two types of approaches have been innovated to explore 3D chromatin structures: chromosome conformation capture (3C)-based technologies more recently combining with high-throughput sequencing analyses and fluorescence imaging techniques, such as fluorescence in situ hybridization (FISH) [13, 14]. Those techniques, which have their pros and cons discussed below, have been widely used for the spatial visualization of chromatin features and how these features relate to genome functions and cellular phenotypes [15-17].

The 3C technique, which was initially invented by Job Dekker [13], was subsequently replaced by improved derivatives, such as 4C, 5C and Hi-C techniques [15]. The common denominator between all these "C" techniques is that chromatin structures are cross-linked in the living cell with formaldehyde. Following purification of chromatin DNA, digestion using restriction endonucleases and ligation, the proximity between distal chromatin regions could be determined by either PCR or high throughput DNA sequencing analyses [13] (schematically shown in Fig. 1a). Based on this principle, the circular 3C (4C) technique was established, combining high throughput sequencing, to simultaneously capture multiple ongoing intra-chromosomal and inter-chromosomal interactions genome wide with high resolution from a known locus [18]. Later, 3C- carbon copy (5C) was established by introducing ligation-mediated PCR amplification to cover all possible ligation combinations from a 3C library within a defined distance, typically around one million base pairs, followed by deep sequencing [19-21]. Hi-C was subsequently invented to capture theoretically all to all interaction genome wide based on capturing the biotin-labeled ligation sequences bridging two different interacting DNA regions [22, 23]. ChIP-loop [24] and ChIA-PET [25] techniques represented other versions of 3C-based techniques, which are built on the 3C technique integrated with Chromatin Immunoprecipitation (ChIP). These methods are thus capable to detect chromatin fiber interactions when binding a particular protein, such as transcription factors. The "C" techniques listed above are based on cell populations, usually more than 10 million cells. To adapt Hi-C to single-cell analysis, Takashi and colleague modified the protocol by involving in-nucleus ligation, which enables the isolation of single nuclei carrying Hi-C ligated DNA [15, 26]. This single cell Hi-C approach suffers, however, from a limited sequencing coverage like several of the other "C" techniques. Recently, single-cell combinatorial indexed ATAC-seq (sciATAC-seq) [27] and single-cell combinatorial indexed Hi-C (sciHi-C) [28] were invented based on the improved Hi-C protocol. A major drawback of all of these techniques is an extensive use of exponential PCR amplifications,

which introduce serious biases. To overcome this drawback, a new variant of the 3C technique termed Nodewalk was developed (Paper II) [29].

Nodewalk is identical to the classical "C" techniques up to the ligation of digested DNA. To further fragment the ligated DNA and equip it with suitable primer sequences, transposases are used to tagment the ligated DNA. In the next step, primers containing a T7 RNA polymerase promoter are used to amplify the ligated DNA within 5-7 cycles, allowing the production of large amounts of RNA from small amounts of input material. These chimeric RNA sequences are subsequently converted to DNA for high throughput sequencing using cDNA primers strategically positioned close to the ligated restriction site. This technique allows the reproducible identification of stochastic chromatin interactions in input material corresponding to at least 7 cells [29] (schematically shown in Fig. 1b).

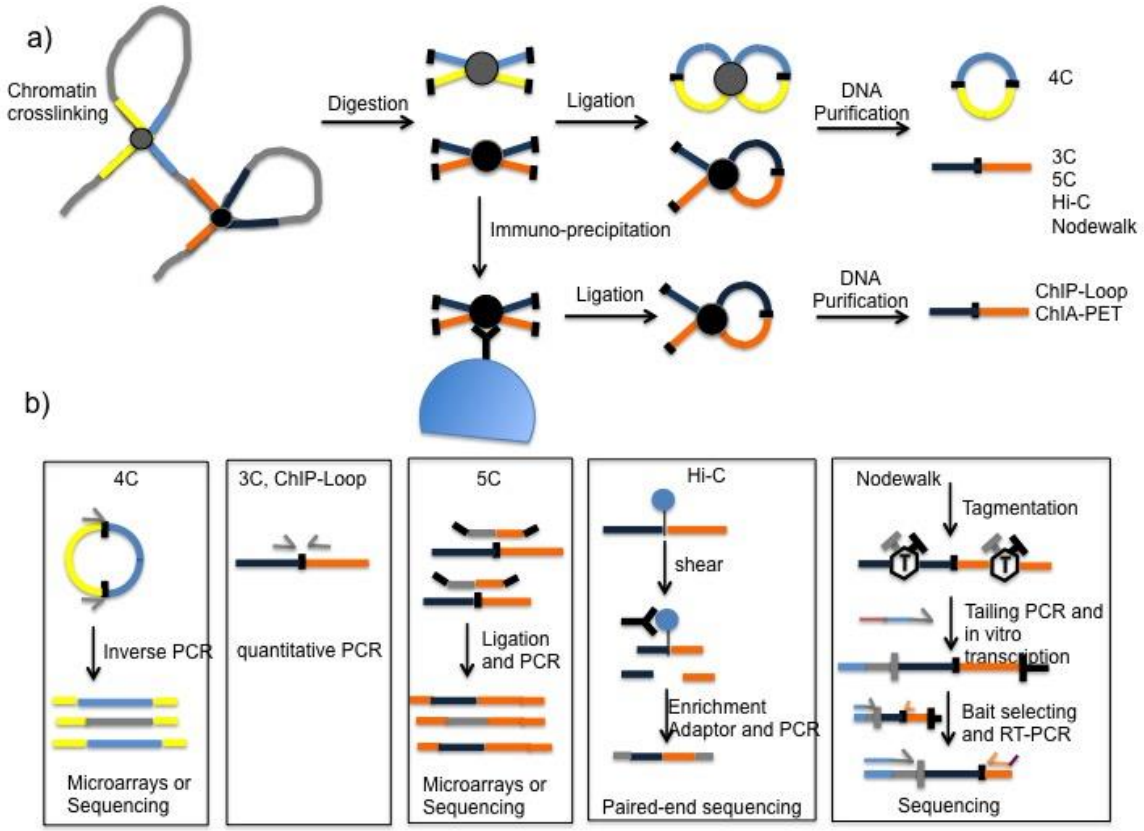

Figure 1. Schematic illustration of different types of 3C-based techniques

Although the "C" techniques provide direct evidence of interactions between chromatin fibers, they are generally poor in assessing the frequencies of such interactions and their positions within the nuclear architecture. These shortcomings are neutralized by confocal microscopic analysis, which can then be combined with 3D FISH to provide information

about the location of proximities between different chromatin regions in single cells. Image-based approaches benefit from the preserved natural spatial context of individual loci to enable direct analysis of the physical distance between regions of interest. However, the 3D-DNA FISH approach suffers from the limitation of the light microscope. Moreover, although the automation of FISH protocol and advancing of imaging techniques have made it possible to map the position and proximity of multiple endogenous loci in single cells [30-32], it still suffers from a relatively low output in comparison with the "C" techniques.

While the resolution of 3C-based methods is determined by the length of fixation reagent (5,9Å for monomeric formaldehyde) and thus rather high, these techniques offer only average information of chromatin fiber interactions. The resolution of 3D DNA FISH is limited by the wavelength of the fluorophores, which is much lower than 3C-based methods, while provides a powerful visualization of chromatin organization in single nuclei. To fill this gap between 3C-based and 3D DNA FISH techniques, an *in situ* technique termed chromatin in situ proximity (ChrISP) was developed and successfully used to quantitatively detect chromatin proximity in single cells with a resolution exceeding those of super-resolution microscopes [33] (schematically shown in Fig. 2).

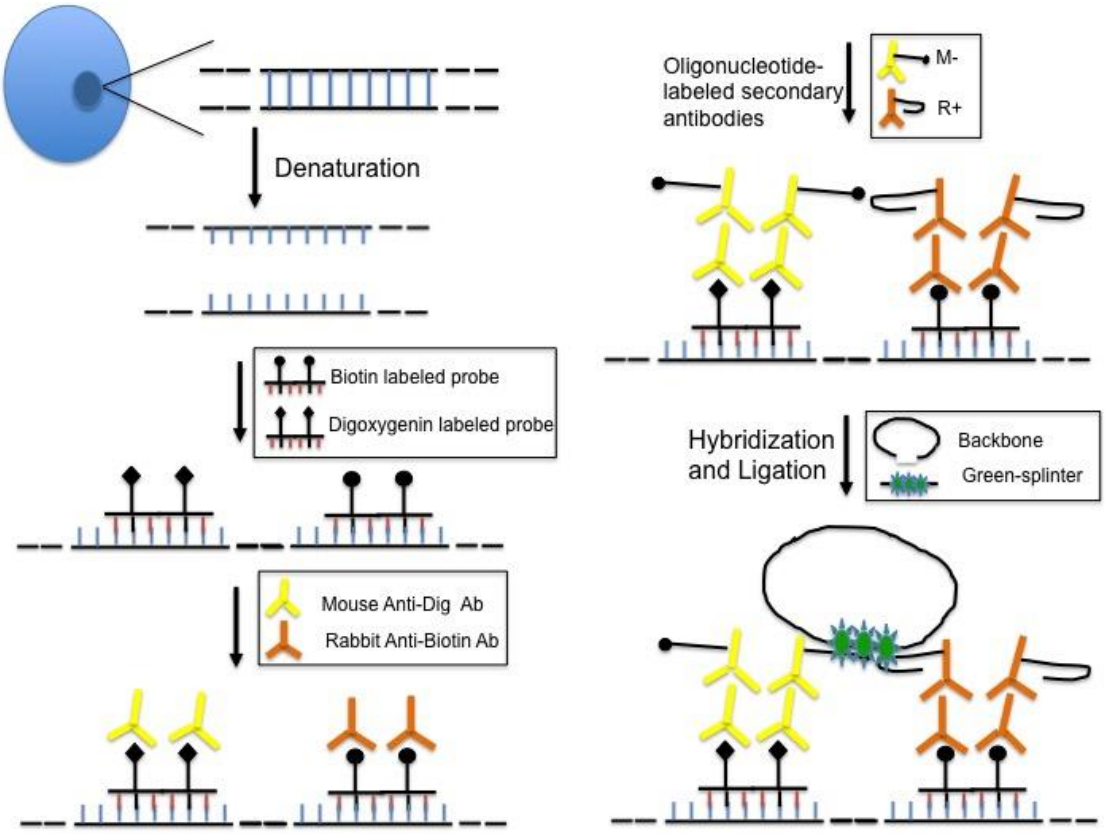

Figure 2. Chromatin in situ proximity

Combining the "C" techniques with ChrISP analyses thus provides an unprecedented insight into 3D chromatin features and their relationships to hallmarks of the nuclear architecture.

1.2.2 The genome organizer CTCF and its PARP1 partner

Using the "C" technologies it was observed that the communication between enhancers and promoters could be regulated by so-called insulator elements when placed in between the gene and its related enhancer [34]. The *H19* imprinting control region (ICR) is so far the only well-characterized insulator in the mammalian genome, which regulates the monoallelic expression of a pair of imprinted genes, *H19* from the maternal allele and *Igf2* from the paternal allele [7, 35]. This feature is manifested by a cluster of binding sites of CCCTC-binding factor (CTCF) at the *H19* ICR, positioned between a downstream enhancer and upstream *Igf2*, which is methylated on the paternal but not the maternal allele. The maternal-specific binding of CTCF to the *H19* ICR thus ensures the monoallelic expression of *Igf2* [35, 36]. This feature has a critical role in differential regulation of higher order chromatin conformations [34, 37, 38].

CTCF is an eleven-zinc factor that binds to its targets by the combinatorial usage of a subset of the zinc fingers [39]. Mutations in the 11 zinc finger domains [40-43] thus inhibit CTCF binding to specific target sites [43]. Mutations, disruption in the associated modifications, or structural changes in CTCF binding sites adjacent to oncogenes and cancer-related genes frequently occurred in cancer resulting in deregulation of cancer associated genes, as well as the local and long-range chromatin structure in cancer cells [44-49]. It is not surprising therefore that CTCF has been linked with the regulation of cancer cell proliferation and clonogenicity [40, 50, 51]. Based on these and other features (see below) CTCF has been coined as the master regulator of the genome [52].

It is not known how CTCF imparts chromatin insulator functions, although it involves the enzymatic activity of PARP1. PARP1 enzymatically poly(ADP-ribosy)lates (PARylates) itself and its protein partners, including CTCF, and affects a number of cellular and biologic outcomes by mediating the genotoxic stress response, DNA repair, regulating genome integrity, chromatin structure and transcription [53, 54]. Inhibition of the PARP1 function thus counteracted the insulator function of CTCF to generate biallelic IGF2 expression [55]. Of note, we show in Paper I that CTCF can activate PARP1 enzymatically to provide a plausible explanation for how PARP1 and CTCF collaborate to manifest the chromatin insulator function. It is, moreover, of interest that the potential for interactions between CTCF and PARP1 is under circadian control (Paper I) to raise the tantalizing possibility that the

function of chromatin insulators oscillates to diversify enhancer-gene communications in rhythmic manners.

1.2.3 Compartmentalization of nuclear functions in 3D

During interphase, individual chromosomes occupy certain nuclear space, the so-called chromosome territory (CT), and display radial orientation [6]. AT-rich sequence elements tend to localize to the nuclear periphery while GC-rich and gene-rich domains move towards the interior of the nucleus. However, specific regions have the probability to loop out from their corresponding territory to intermingle in inter-chromosomal contacts to influence and coordinate nuclear functions [6]. Active and inactive chromatin domains are separated spatially in the nuclei; this is one of the most striking features of the nuclear architecture [11, 15]. In the vast majority of differentiated mammalian cells studied, FISH along with 3C-based analyses thus show that transcriptionally active or poised chromatin has a tendency to be located in the nuclear interior, whereas repressed chromatin states containing gene deserts as well as genes repressed in a cell type-specific manner tend to tether at the nuclear periphery, or cluster around the nucleolus [12, 56, 57].

The spatial separation between active and inactive chromatin states likely reflects the maintenance of stable cellular memories by reducing transcriptional noise [4]. As chromatin modifications are reversible, the nuclear environment is expected to have an influence on the dynamics and stability of histone modifications. For example, the re-localization of developmentally regulated genes to and away from the nuclear periphery has been linked to transcriptional repression or activation, respectively. Similarly, artificial tethering of certain loci to the lamina has been shown to induce repression, although there are examples where localization does not affect transcription [12]. Paper I demonstrates that circadian genes exploit their dynamic juxtaposition to inactive domains at the nuclear periphery to effectuate transient repression.

1.2.4 The active compartments: nuclear interior

Chromatin fiber interaction occurs often within active chromatin states in the nuclear interior. Using high-throughput assays that are capable to map long-range transient chromatin fiber interactions, dynamic physical contacts between regulatory elements that are located far apart from each other in linear distance have been captured [58]. Chromatin fiber interactions are most frequent within the same chromosome to form chromatin loops in *cis*. It can also, however, involve large-scale movements and form transient contacts between different

chromosomes in *trans.* Such enhancer-promoter and gene-gene communications have been liked with transcriptional coordination [59].

1.2.4.1 Topologically associating domains (TADs)

Hi-C revealed that mammalian genomes are compacted into evolutionary conserved self-interacting chromatin domains in mega base scale. Such regions, named topologically associating domains (TADs) [60], may influence gene expression by constraining chromatin movements to involve only neighboring enhancer-promoter contacts [61]. Disruptions of TADs lead to *de novo* enhancer-promoter interactions and mis-expression [62]. Despite the underlying mechanisms of TAD formation remain to be obscured, some evidence pointed that architectural proteins or genome organizers, such as CTCF, can participate in the formation of TAD boundaries [61, 63, 64]. Preventing CTCF binding to a TAD boundary thus disrupt TAD integrity and lead to mis-expression of limb developmental associated genes causing limb developmental defects [62].

1.2.4.2 Enhancer usage in 3D

Enhancer-promoter interactions are cell type- and differentiation stage-specific to manifest developmental decisions. The underlying molecular mechanisms include the stochastic chromatin fiber movements and transient stabilization of chromatin fiber interactions, which form the basis of transcriptional regulation [65]. Such functional contacts between enhancers and promoters, which are constrained by the 3D organization of the chromosome, are thus influenced by the binding of transcription factors (TFs) and stabilized by the interaction between TFs and chromatin architectural proteins or genome organizers, such as CTCF[62, 66-71]. Enhancer-promoter contacts could also be influenced by chromatin mobility, which is constrained by the 3D organization of the nearby chromatin context[62, 69]. It has been shown that intra-TADs CTCF binding sites stabilizes enhancer–promoter interactions and maintains robust gene expression, so as to reduce cell-to-cell variation of gene expression [71]. The cohesin complex plays an important role in this process is exemplified by the observation that OCT4 (octamer-binding transcription factorÊ4) expression was lost when one of its members was down regulated [72]. Enhancer RNAs (eRNAs), a class of long noncoding RNAs that transcribed by and characterize active enhancers, have also been shown to take part in the regulation of enhancer-promoter contacts through promoting the recruitment and enhancing the kinase activity of the Mediator complex [73]. Furthermore, mutations of the mediator subunit 12 (MED12) abolished the interaction between eRNAs and MED12 to cause developmental defects. The frequency of enhancer–promoter interactions

might therefore regulate the frequency and/or duration of productive transcription, and modulate the variability of gene expression in a cell population.

Multiply enhancer elements covering tens or hundreds of kilo bases tend to form clusters, so-called super-enhancers, to ensure robust expression of cell fate–determining genes and to maintain cellular phenotypes during development and cancel evolution [74, 75]. However, such regions can be formed *de novo* during cancer development and integrate several different signaling pathways, such as the WNT pathway, to form oncogenic super-enhances (OSEs). This feature is thought to cause unscheduled activation of cancer genes, such as *MYC* [75, 76]. Transcriptional regulators and genome organizers, such as CTCF [76] and the Mediator complex [67], influence the formation and dissolution of super-enhancers. CTCF often occupies the promoter of oncogenes and facilitates their docking with essential enhancers [76]. The Mediator complex is an essential regulator of transcription by affecting enhancer-promoter contact, chromatin remodeling, as well as RNA Pol II activity [38].

While 3D genome organization regulate cell type-specific enhancer-promoter interaction [69, 70], transcription activation in turn influence subnuclear positioning of local chromosome [77]. The interplay between gene expression and chromatin conformation is a driving force for cell-fate decisions [69]. It is still not clear, however, how the physical constrains of 3D genome organization govern cell type- and differentiation stage-specific crosstalk between enhancers and promoters. This issue is compounded by the observations that some enhancers have restricted access to only a gene in their neighborhood, while other enhances regulate the expression of several different genes located far apart [10]. To understand how enhancer-associated chromatin state transitions could manipulate gene expression during differentiation, 3D maps of dynamic chromatin fiber interactions between enhancer and promoter, as well as between enhancers have been elaborated to uncover a new feature of genome organization.

1.2.5 The inactive nuclear compartments

As noted above, the compartmentalization within the nucleus plays a key role in the regulation of transcriptional activity [12, 57, 78]. Long-range heritable repressive epigenetic modifications contribute to the establishment of stable repressive chromatin domains during differentiation [6, 7]. Such repressed domains, which are positioned at the nuclear periphery and/or at the nucleolus, are functionally and physically separated from transcriptionally active regions by boundary elements.

1.2.5.1 Formation of repressive compartment at the nuclear periphery

The inner nuclear membrane, the nuclear lamina and the lamina-associated proteins constitute the nuclear periphery. The repressive environment at the nuclear periphery is based on the regulated interaction between the inner nuclear membrane and silenced chromatin [79]. Repressive histone modifications [15, 80] and specific genome organizers [57] coordinately drive dynamic interactions between genomic loci and the transcriptionally repressive environment at the nuclear periphery.

The genomic regions that directly associated with nuclear lamins are termed LADs. These regions are approximately 10 Kb to a few megabases in size, covering around 40 % of the genome. LADs contain the AT-rich, repressed portion of the genome and include the so-called constitutive LADs (cLADs) that are present at lamina in at least two different cell types the facultative LADs (fLADs) that cover developmentally silenced genes which are recruited to the periphery in a cell type-specific manner. Around 30% of LADs are positioned at the periphery at any given time, dynamically contacting with the nuclear lamina [81]. In mammalian cells, LADs might correspond to large blocks of peripheral heterochromatin that were first observed by electron microscopy [82, 83] and detected by native ChIP [81]. LADs thus substantially overlap with large domains that are enriched in repressive histone modifications, rich in Histone 3 Lysine 9 di- and tri-methylation (H3K9me2 and me3) [78, 80], the so-called “Large Organized Chromatin K9-modifications” (LOCKs), which emerge during differentiation [12, 78].

Lamin proteins, such as Lamin A/C and Lamin B1, have been shown to regulate tethering of LADs at the nuclear lamina [84, 85], while the exact role of lamin proteins in chromatin organization is not clear. Murine embryonic stem cells (mESCs) with triple knockout of lamin proteins are still viable and DamID detected that the organization of LADs remains unchanged in these cells [86], suggesting that tethering of LADs to the nuclear lamina does not need the presence of lamins in mESCs. Removal of both the Lamin B receptor (LBR) and lamin A/C proteins, on the other hand, inverts the architecture of the nucleus and causes heterochromatin to accumulate in the center of the nucleus, suggesting that LBR is involved in mediating interactions between LADs and the repressive nuclear periphery at least in certain cell types [87]. The inner nuclear membrane protein emerin, in complex with histone deacetylase 3 (HDAC3) or other nuclear envelope transmembrane proteins (NETs), has also been implicated in mediating interactions between LADs and the nuclear periphery. Indeed, some of these NETs were shown to control the positioning of individual chromosomes

relative to the nuclear periphery [88].

Since the formation of repressive environment at the nuclear periphery involves the acquisition of large blocks of repressive histone modifications during cell differentiation, it is not surprising that factors depositing repressive histone marks have been shown to promote LAD-lamina interactions. For example, the histone methyltransferases (HMTs) MET-2 and SET-25 are responsible for regulating the localization of heterochromatin to the nuclear periphery in *C. elegans* [80]. In mammalian cells, the loss of K3K9 mono-methylation (H3K9me1) results in the disruption of the nuclear lamina [89]. Furthermore, methylation of H3K9 by G9a, a histone methyltransferase, has been shown to regulate the recruitment of LADs to the nuclear lamina [81]. Ying Yang 1 (YY1), in collaboration with lamin A/C, Histone 3 Lysine 27 tri-methylation (H3K27me3) and H3K9me2/3, has also been observed to promote the maintenance of lamina-proximal positioning[90]. CTCF, a major 3D genome organizer [91, 92], is enriched at the border between active and inactive domains [36] as well as at LAD boundaries [93]. In *Drosophila*, down regulation of CTCF reduces H3K27me3 levels within inactive domains, indicating that CTCF is required for the maintenance of repression, however its involvement in maintaining LAD-lamina interactions has not been examined [94].

1.2.5.2 The heterochromatic compartment at the nucleolus

Genomic regions in addition to ribosomal DNA (rDNA) surround the nucleoli are termed nucleolus-associated domains (NADs) [95]. NADs have a relatively high density of AT-rich regions, low density of gene-rich domains and enriched in transcriptionally repressed genes [95]. Moreover, NADs substantially overlap with LADs suggesting that silenced regions can be dynamically repositioned between the nuclear periphery and nucleolus [95, 96]. Indeed, certain silent chromatin loci are recruited to the nuclear periphery or to the nucleolus in a dynamic and stochastic manner after each cell division, suggesting a substantial mobility between these two compartments [81]. The silenced compartment seems to be established first during differentiation around the nucleolus and then promote the expansion of H3K9me2 LOCKs at NADs and LADs, raising the question that perhaps chromatin movements between these two compartments is necessary for the establishment of differentiated phenotypes [82, 97, 98]. Nuclear lamina and the nucleolus might thus contribute to the global 3D organization of the genome by constituting two alternative locations for repressed genomic domains.

1.3 NUCLEOPORINS AND THE GENE GATING PRINCIPLE

Although the nuclear periphery provided a well-documented transcriptionally repressed environment, it was early observed that heterochromatin patches were intermingled with euchromatin staining at the nuclear periphery and recruitment of genes to the periphery [99] provided the basis of the so-called "gene gating hypothesis". This fact prompted the formulation of the so-called "gene gating" hypothesis by Günter Blobel already in 1985. This principle posits that yeast nuclear pore complexes (NPCs) and their components coordinate transcription, mRNA processing and nuclear export, serving as gene-gating organelles[100].

This hypothesis is supported by studies focusing on nuclear pore complexes and their components in yeast and mammalian cells during the last decades. NPCs are large uniform transmembrane complexes (approximately 50 MDa) consisting of multiple copies of about 30 distant conserved proteins called nucleoporins (NUPs) [100-102] (Fig 3. NPC structure and molecular composition). Each fully assembled NPC thus consists of 500 to 1000 nucleoporin molecules [101, 103, 104]. NPCs non-randomly span the nuclear envelope with a large degree of structural and compositional conservation [101]. In humans, the NPC is structurally constituted by cytoplasmic filaments, nuclear basket formed by NUP153 and TPR [105], the central pore formed by Nup93/Nup205 and two rings composed of the NUP107/NUP160 complex flanking the central pore in the vertical plane [106].

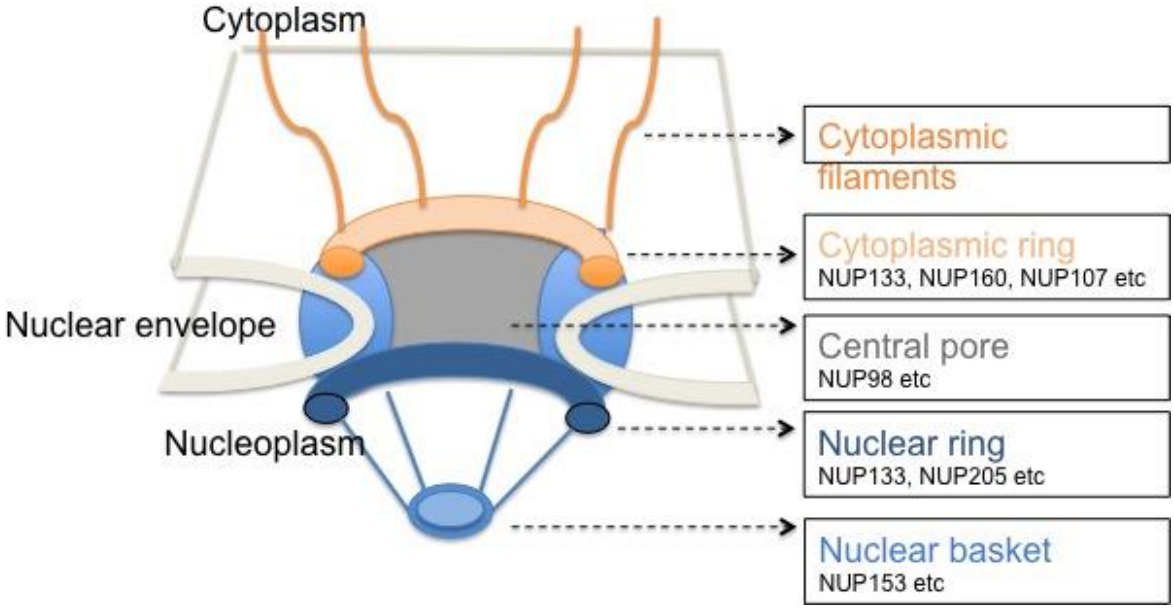

Fig 3. NPC structure and molecular composition

NUPs have been involved in the regulation of both transcriptional activation and repression in a transport-independent manner, as exemplified by NUP98 in transcriptional activation and NUP153 in transcriptional repression [107, 108]. Accumulated evidence indicated that certain inducible genes are frequently relocated to the NPCs upon transcriptional activation, while maintaining or increasing their transcriptional activity at the nuclear periphery [109-111]. NUP133 and NUP155 can moreover mediate the transport of a target plasmid DNA molecule from an intra-nucleoplasmic position to the nuclear pores [112]. Studies among yeast and mammals have implicated NUPs in the regulation of gene expression through accumulating specific factors to form evolutionary conserved protein interaction hubs. Those hubs maintain the chromatin-to-pore and nascent mRNA-to-pore interactions to regulate gene expression at different stages during development and adaptation to the environment [99].

The recruitment of nuclear pore sub-complex NUP107-160 to chromatin is regulated by interaction between AHCTF1 and chromatin [113]. AHCTF1 interacts with chromatin directly *via* its AT-hook domain and is essential for compartmentalization of chromosomal DNA [113]. In line with all the functions listed above, NUPs are essential not only for the regulation of pluripotency and differentiation, but also the plasticity of phenotypes during adaption to environmental cues [107, 114]. It is not surprising therefore that deregulation of NUP expression or mutations of NUPs have been found during aging as well as in variety of tissue specific and systemic human diseases [115-117].

NPCs and NUPs interacting with chromatin have been suggested to form a transcriptional memory of previous gene activation processes in yeast [118, 119]. This is a phenomenon called epigenetic transcriptional memory that persists through several cell generations [118, 120]. NUP98 in human and NUP100 in yeast interact with promoters to modulate H3K4me2 resistance and poised Pol II binding after expose to the prior stimuli [118]. Formation of gene loops between the promoter and 3′end of the responsive genes through interaction with the nuclear pore complex is also involved in the maintenance of transcriptional memory [121]. Such chromatin loops at the nuclear pore complex were suggested to provide a platform for the fast of RNA Pol II recruitment the re-initiation of transcription and thereby for facilitating the RNA processing [121]. NUP98 was also shown recently to promote enhancer-promoter looping of ecdysone-inducible silent genes in *Drosophila* cells and tissues [119]. Apart from their role in nucleocytoplasmic transport, NUPs has also been shown to contribute to the 3D genome organization, maintenance of memory gene loops and genome stability [119, 122, 123].

1.4 REGULATION OF CIRCADIAN TRANSCRIPTION IN THE COMPARTMENTALIZED NUCLEUS

Almost all the organisms sensitive to light display behavioral and biochemical oscillations with an approximately 24h period, referred to as circadian rhythms [124]. Circadian (Latin; *circa*-, ‘‘approximately’’, -*diem*, ‘‘day’’) rhythm is a cell-autonomous and evolutionarily conserved timing system govern the oscillation of a large variety of physiological and behavioral reactions [124]. Consequently, many processes, such as body temperature, blood pressure, sleep–wake cycles, glucose and lipid metabolism, and behavior, e.g. food intake, are all under circadian control. Such rhythms in phenotype require flexible, rhythmic gene expression patterns at a subset of genes, which is governed by circadian chromatin transitions within the 3D nuclear architecture.

Circadian systems are composed of three major components: the input pathways that receive environmental cues (mostly known ones are light, temperature and food) and entrain the oscillator; the central oscillator that ensures endogenous rhythmicity; and the output pathways that drive rhythmic biological processes, such as sleep–wake cycles, body temperature and metabolism. This endogenous clock system is a free-running system, which maintains the circadian oscillation even when the external cue is absence, while it is entrained or synchronized by external time cues to adjust to the geophysical time.

1.4.1 The central and peripheral clocks

Mammalian circadian rhythms are controlled by endogenous biological oscillators, that include a central pacemaker in the hypothalamic suprachiasmatic nuclei (SCN) [125] and other oscillators referred as the peripheral oscillators. Peripheral oscillators reside in other tissues in the brain, such as hypothalamic nuclei [126], in peripheral tissues, like liver and adipose tissue [127, 128], and even in cell lines maintained in culture [128, 129]. As the central clock system, the SCN is responsible for the biological rhythms in peripheral organs and is influenced by both internal and external cues[125]. Peripheral tissues contain self-sustained oscillators with a molecular composition similar to that of SCN neurons [130-133].

1.4.2 The entrainment of circadian rhythm by external time cues

To maintain the synchrony between endogenous clocks and the environment, the clock system is readjusted daily by external time cues through inducing alteration in gene expression level of clock genes and stability of clock proteins, during the so-called entrainment process [134]. External time cues are termed as “Zeitgebers”, which is German

for “giving time”. A Zeitgeber is any exogenous or environmental cue that entrains or synchronizes an organism's biological rhythms.

The most dominant synchronizer or Zeitgeber of the SCN is light: the retina perceives photic cues and transmits the signal to SCN through the retino-hypothalamic tract [135], the SCN receives external light signals to synchronize all the oscillators and is highly resistant to phase perturbations [136]. Other cues, such as food availability, strongly influence the clock system of peripheral cells [137, 138]. The phase of peripheral clocks in various tissues is synchronized and reset by signaling from the SCN directly, by the cooperation of neural, humoral and other signals indirectly, as well as by feeding-fasting cycles and humoral signals independent of the central clock [139, 140]. Food intake is the best-documented time cue for the periphery clocks for in particular the liver [139, 141, 142]. As the humoral signals, transforming growth factor- β (TGF-β) or activin signals induces the resetting of the cellular clock through activation of activin receptor-like kinase (ALK) independently of light-input signaling pathway in mice [143]. Furthermore, high concentration of horse serum could transiently synchronize circadian transcription of various genes in mammalian tissue culture cells [129].

1.4.3 The clock machinery: driving circadian transcription

The circadian clock, operating as a cell-autonomous molecular oscillator, is built on conserved negative transcriptional and translational feedback loops [144] (Figure 4). CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle arnt-like 1, also known as ARNTL) as core clock proteins belong to the basic HLH (helix-loop-helix)-PAS (period-arnt-single-minded) (bHLH-PAS) transcription factor family. CLOCK and BMAL1 form heterodimers and work as the positive limb of the feedback loop. The heterodimeric CLOCK:BMAL1 binds to E-box regulatory elements and drives transcription of clock-controlled genes (CCGs), such as Period-encoding genes (*Per1-3*) and Cryptochrome-encoding genes (*Cry1-2*) [145, 146]. PER and CRY proteins assemble into one or more protein complexes (PER complexes) [147, 148] in the cytoplasm. Upon translocation into the nucleus, PER complexes establish the negative limb by interfering with the BMAL1 and CLOCK function. The PER complexes thus suppress transcription of their own genes [149-151]. Once the levels of the PER complexes have been lowered, the BMAL1/CLOCK complex will be re-activated to reform the positive limb.

To further adjust the precision of the central system, other regulatory feedback loops, such as transcription factors RORs (retinoic acid receptor-related orphan receptors) and REV-

ERBα/β (reverse erithroblastosis α and β), have been evolved [152-155]. RORs and REV-ERBα/β bind to the ROR/ REV-ERB-binding element (RRE) on the *Bmal1* promoter to promote or inhibit its transcription, respectively [152-156]. *Ror* and *Rev-erbα/β* are also CCGs transcriptionally regulated by CLOCK:BMAL1 [152-155]. Rhythmic transcription of REV-ERBα and RORα drive oscillations in BMAL1 expression while the CLOCK:BMAL1 heterodimer feeds back on the *Ror* and *Rev-erbα/β* genes to form an "accessory" loop.

This feedback loop also mediates rhythmic expression of several other transcription factors, including DBP (D-site binding protein), HLF (hepatic leukemia factor), TEF (thyrotroph embryonic factor), E4BP4 (E4 promoter–binding protein 4), as well as the bHLH transcription factors, DEC1 (also known as BHLHB2, STRA13, or SHARP2) and DEC2 (BHLHB3 or SHARP1)) [137]. DBP, HLF, TEF, and E4BP4 bind to D-boxes in the genome and function in the circadian output pathways that drive rhythmic biological processes. These interconnected feedback loops, together with yet other regulatory factors, drive multiple patterns of transcriptional oscillations [157].

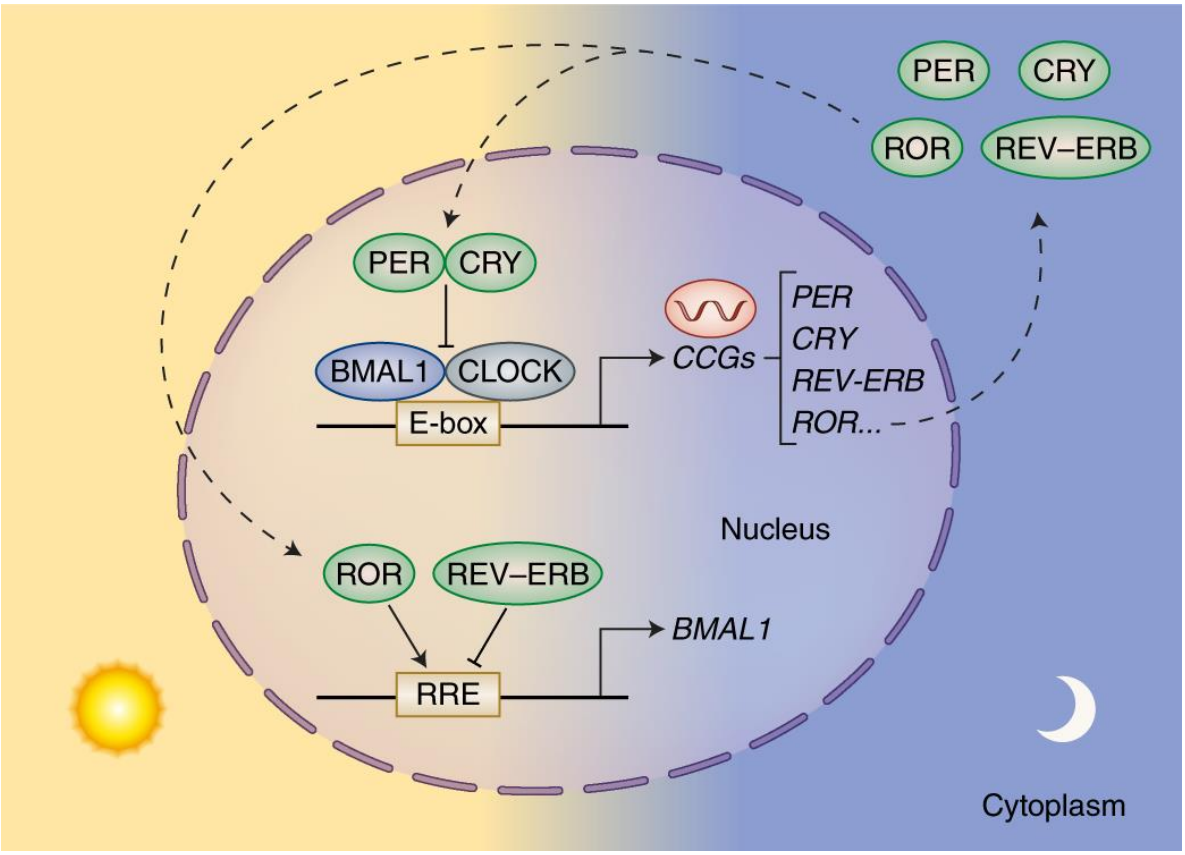

Figure 4: The molecular components of the mammalian circadian clock. Adapted from Masri, S., Sassone-Corsi, P. The emerging link between cancer, metabolism, and circadian rhythms. Nat Med 24, 1795–1803 (2018). Reprinted with permission from publisher.

1.5 CIRCADIAN CHROMATIN TRANSITIONS

As accounted for above, the core clock molecular machinery consists of transcription factors and regulators, both activators and repressors, which drive circadian expression of a fraction of the genome. Depending on the tissue or cell type, approximately 3–30% of the transcripts are under circadian regulation [158-160]. As transcription of a circadian gene, like that of any other gene, is influenced by its chromatin environment [65], the chromatin transitions enabling plasticity and specificity of circadian transcription are likely controlled by members of the positive and negative limbs of the circadian oscillator.

1.5.1 The establishment of active chromatin states by the positive limb

To initiate the transcription, the central circadian regulators BMAL1 and CLOCK act in combination with histone acetyltransferases (HATs) CREB binding protein (CBP) and p300, respectively, to acetylate histones and assist accessible chromatin state at promoters of CCGs [161-163]. Most likely, CLOCK itself functions as a HAT on H3K9 and H3K14 [164], both chromatin marks are associated with an active chromatin state. CLOCK: BMAL1 heterodimer has been shown to interact also with other regulatory factors that have been associated with transcriptional activation, including histone methyltransferase (HMT) MLL [165], Jarid1a [166], Trap150 [167], and P300/CBP-associated factor PCAF [161]. Moreover, in the mouse liver, recruitment of RNA polymerase II (Pol II) to promoters was reported to be rhythmic and chromatin marks H3K4me3 and H3K36me3 linked with active transcription are highly dynamic and globally remodeled during the 24-hour period [168].

1.5.2 The establishment of repressed chromatin states by the negative limb

During the transition from the transcriptionally active state to the repressed state, the CLOCK: BMAL1 complex interacts with the PER complex at the beginning of the night as the levels of PER and CRY accumulate and translocate to the nucleus. The PER complex acts as a platform for recruitment of chromatin modifiers that can suppress transcription, e.g., acetylation is counterbalanced by a number of histone deacetylases (HDACs). SIN3A-HDAC1 [169] rhythmically deacetylates histone H3K9 at the *Per1* promoter to contribute to the feedback repression of the clock. Moreover, the NAD+ - dependent enzyme SIRT1 targets and deacetylates histone H3K9 and K14 on the promoter sites of *Bmal1* and *Per2*, whereas SIRT6 deacetylates histone H3K9 and is involved in the recruitment of the circadian transcriptional machinery (CLOCK and BMAL1) to E-box containing CCG promoters [170-

172].

To further perform the transition from activation to repression, histone methylations are recruited to the target promoters of circadian genes. The HMT Hp1γ-Suv39h, which regulates circadian di- and tri-methylation of H3K9 at the *Per1* promoter, is recruited to the *Per1* and *Per2* promoters approximately 4 hours after recruitment of HDAC1 [173]. Recruitment of PER complexes could also repress the transcriptional re-initiation through inhibiting action of SETX and other helicases [147].

Very little is known about how such circadian chromatin transitions are integrated into the compartmentalized 3D architecture of the nucleus. Most of the studies exploring the circadian dynamics of higher order chromatin states thus focus on circadian enhancer-promoter interactions or interactions between circadian genes transcribed in the same phase [174-178] without exploring the role of nuclear hallmarks and compartments in oscillating gene expression.

1.5.3 Crosstalk between the positive and negative limb of the clock machinery during chromatin transitions

As mentioned above, post-translational modifications represent an important level of regulation of circadian expression. This is exemplified by the observation that the CLOCK:BMAL1 heterodimer recruits the Ddb1–Cul4 ubiquitin ligase to *Per*, *Cry* and other CCGs. Ddb1 and Cul4 are E3 ubiquitin ligases, which monoubiquitinate H2B and possibly other histones on neighboring nucleosomes. This process stabilizes the PER complex with DNA-bound CLOCK:BMAL1 at *Per* genes to reinforce its negative feedback function [179].

Interestingly, the Mi-2/nucleosome remodeling and deacetylase (NuRD) transcriptional corepressor complex interacts with both the CLOCK:BMAL1 and PER complexes [180]. Thus, the CLOCK:BMAL1 heterodimer complexes with two NuRD subunits, MTA2 and CHD4, the latter of which promotes the transcriptional activity of CLOCK:BMAL1 on CCGs. Only when the PER complex carries the remaining complementary NuRD subunits to the DNA-bound CLOCK:BMAL1 complex, the NuRD complex could be rebuilt as an active corepressor that is important for the negative limb of circadian regulation. Thus, achieving the full repressor activity of the PER complex requires its successful targeting of CLOCK:BMAL1-occupied genomic loci [180].

In another example, entrainment of circadian clock by feeding is initiated by binding of PARP1 to CLOCK:BMAL1 heterodimers. Poly(ADP-ribosyl)ation (PARylation) of CLOCK

at the beginning of the light phase evicts it from chromatin, due to the introduction of negative charges provided by Poly(ADP-Ribose)(PAR) chains, to inhibit transcription of CCGs. Conversely, induced loss of PARP1 enhances the binding of CLOCK:BMAL1 to DNA and leads to a phase-shift of the interaction of CLOCK:BMAL1 with PER and CRY repressor proteins. It is not surprising therefore that the entrainment of liver clocks to inverted feeding is significantly delayed in the absence of PARP1 [141].

PARP1 is a factor with a more general involvement in the regulation of chromatin structure, which acts either by direct protein-protein interactions or by the NAD+-dependent PARylation process [181, 182]. While it is associated with DNA damage response pathways, being activated by damaged DNA ends [183], its activity can be modulated by other factors. This is exemplified by CTCF, which is not only a major factor of transcriptional regulation in 3D [91, 92], but also a main activator of DNA damage-independent PARP1 activity. We envisage therefore, that CTCF and PARP1 are dynamic partners of the DNA-bound CLOCK:BMAL1 complex to regulate the rhythmic activity of CLOCK.

1.6 CIRCADIAN CLOCK, CELLULAR METABOLISM AND COMPLEX DISEASES

Although features of clock-resetting factors and how they synchronize peripheral oscillators have not yet been fully understood, mounting evidences showed that circadian clocks in peripheral tissues are not only frequently linked to cellular metabolism, but also that the disruption of circadian rhythms might contribute to complex diseases, such as tumorigenesis, *via* perturbation to metabolic states [184, 185].

As early as in 1998, Balsalobre and Schibler already demonstrated that horse serum shock could entrain circadian gene expression in mammalian tissue culture cells [129]. The components of the serum responsible for resetting the rhythm and the underling mechanisms remains to be elucidated, although Kon et al has shown that the resetting of cellular clocks can be induced by the activation of ALK, triggered by TGF-beta, activin or alkali signals. This process occurred independently of PER induction and was mediated by DEC1 [143]. Moreover, Yamajuku et al established a real-time monitoring cell culture system and could demonstrate that insulin directly regulates the phase entrainment of hepatocyte circadian oscillators [186].

Although it is known that metabolic processes regulate circadian patterns, while the underlying molecular mechanisms are still not yet fully explored. However, cellular

NAD+/NADH levels, mirroring metabolic states, appear to regulate the function of the circadian oscillator [187]. Meanwhile, loss of circadian function might increase NAD+ levels in cancer cells because of higher rate for glycolysis [185, 188]. Moreover, the mammalian sirtuins, particularly SIRT1, which forms complex with CLOCK, has been shown to regulate circadian transcription through the NAD+-dependent deacetylation of circadian transcription factors and chromatin-associated proteins [170, 171]. Similarly, PARP1, a NAD+-dependent ADP-ribosyltransferase, plays essential roles in the entrainment of peripheral clocks to food intake [141].

The close association between circadian rhythms and cellular metabolism likely and underlies its link with a number of pathologies such as metabolic syndrome, diabetes, obesity, sleep disorders, and some tumorigenic processes [171]. Therefore, it is of great importance to understand the molecular mechanisms underlying clock function and how these can be perturbed pathologically.

2 AIMS

The overall aim of this thesis was to explore the regulation of chromatin transitions in the 3D architecture of the nucleus in relationship to biological processes. To this end, two different studies were implemented to address the role of the nuclear architecture in transcriptional regulation with the following specific questions:

- How do external time cues reset the phase of circadian transcription in the compartmentalized 3D architecture of the nucleus? Specifically, does synchronization of circadian chromatin transitions upon entrainment involve communication between transcriptionally repressive and permissive sub-nuclear environments?
- How do enhancer-promoter interactions collaborate with the compartmentalized 3D nuclear architecture in the regulation of gene expression? More specifically, does the gene gating principle exist in human cells, and if so what is the underlying mechanism?

3 METHODS AND MATERIALS

3.1 CELL CULTURES AND TREATMENTS

Female hESCs (HS181) were maintained on irradiated male feeder fibroblasts, and HEBs were generated as described previously [189]. HCT116 cells were maintained in complete growth medium (McCoy's 5A modified medium (Thermo Fisher Scientific, 26600023) supplemented with 10 % Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, 16141079) and 1% penicillin-streptomycin. HCECs were maintained in Colonic Epithelial Cell Medium (HCoEpiC, ScienCell, 2950). Cells were cultured at 37 °C under 5 % CO2 and routinely tested mycoplasma contamination using EZ-PCR Mycoplasma Test Kit (Biological Industries, 20-700-20).

Serum shock treatments were performed as described previously [129]. Briefly, HCT116 cells were cultured with serum-rich medium (McCoy's 5A modified medium, supplemented with 50% horse serum (Thermo Fisher Scientific, 16050122)) for 2 hours. Cells were cultured with complete growth medium subsequently for indicated periods.

HCT116 cells were transfected with 20 nM of CTCF siRNA (h) (sc-35124) or GFP siRNA (sc- 45924); 20nM PARP1 siRNA (h) (sc-29437) or GFP siRNA (sc- 45924); 50 nM of ELYS siRNA (h) (sc-77266) or GFP siRNA (sc- 45924) from Santa Cruz Biotechnology cells using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, 13778150) following the manufacturer's instructions respectively. Cells were harvested after 48h incubation. qRT-PCR analysis, immunofluorescence staining and/or western blot were performed to detect the efficiencies.

HCT116 cells were treated with Olaparib (0.3 mM final concentration) for 24 hours, Flavopiridol (2 mM final concentration) for 8 hours, or 0.5 μM G9a enzymatic inhibitor BIX 01294 trihydrochloride hydrate (Sigma-Aldrich, B9311) for 72 hours before harvesting as described in paper I.

HCT-116 cells were treated with 10 μM β-Catenin/TCF Inhibitor V, (BC21) (Merckmillipore, 219334), or an equivalent amount of the solvent DMSO for 16 hours in paper II. Recombinant human Wnt3a (R&R Systems, 5036-WN) was reconstituted in PBS containing 0.1% BSA and added directly to the cell culture medium for indicated lengths of time.

3.2 RNA/DNA FISH ANALYSES

H19/IGF2, TLK1, VAT1L, PARD3, TARDBP, LADs and 4C interactors in Paper I and *MYC* probes in Paper II were generated based on bacterial artificial chromosome/clone (BAC). The *MYC* exon/intron probe and enhancer probes was generated from a pool of 4 PCR products spanning the *MYC* promoter and its gene body (chr8:128,746,000-128,756,177 (GRCh37). The BACs and PCR products were sonicated to 500-2000 bps range and followed by labelling with Biotin-16-dUTP (Roche, 11093070910) using Bioprime Array CGH kit (Life technologies, 18095-011). A mixture of equal amounts of each labelled PCR product was used as FISH probe. The single-stranded intron 1 probe was prepared by generating double-stranded PCR fragments spanning *MYC* intron1 (chr8:128,749,271-128,750,480).

RNA FISH was performed on cells cultured on chamber slides (Thermo Fisher Scientific, 154534) were crosslinked with 3 % formaldehyde for 15 minutes at room temperature (RT). The crosslinked slides were stored in 70% Ethanol at -20°C until further use. The ribonuclease inhibitor Ribonucleoside Vanadyl Complex (NEB, S1402S) was added to the buffers at all steps. Cells were rehydrated in 2 x sodium salt citrate (SSC), and permeabilised with 0.5 % Triton X-100 in 2 x SSC for 10 minutes at room temperature. The FISH probe was mixed with a 10-fold excess of human *Cot-1* DNA (Thermo Fisher Scientific, 15279011) and hybridised to the slides in a buffer containing 2 x SSC, 50% formamide and 10 % dextran sulphate overnight at 37°C. Cells were washed twice with 2 x SSC/ 50% formamide for 15 minutes at 40°C and with 2 x SSC for 15 minutes at 40°C, followed by mounting with Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Labs, H-1200).

DNA FISH analyses were performed on cells that were crosslinked and permeabilised as described for RNA FISH. After denaturation in 2 x SSC/ 50% formamide for 40 minutes at 80°C, cells were kept in ice cold 2 x SSC for 5 minutes. The following hybridization and washing steps were prepared as described for RNA FISH.

3.3 IN SITU PROXIMITY LIGATION ASSAY (ISPLA)

ISPLA was performed on cells that were fixed with 1% formaldehyde to detect proximities between different proteins: CTCF-PARP1, CTCF-CTCF, PARP1-PARP1 in Paper I; ß-catenin-TCF4, ß-catenin-ELYS, TCF4-ELYS in Paper II. Modified antibodies (termed R+ and M-) were added to the slides following incubation with primary antibodies, followed by hybridization of backbone and splinter oligo DNAs, ligation and rolling-circle amplification,

as described [190].

3.4 CHROMATIN IN SITU PROXIMITY (CHRISP)

ChrISP assay was performed and quantitated as previously described [191]. Briefly, cells were crosslinked and permeabilised as described for RNA FISH. Following hybridization of the FISH probes and incubation with primary antibodies, the cells were incubated with modified antibodies (termed R+ and M-), hybridized with backbone and green splint, and ligated with T4 ligase, as described [191].

For the proximity analysis between the Digoxigenin-labelled probes annealing to either the *MYC* promoter/gene body or the OSE and the nuclear pore component NUP133, a Tyramide signal amplification step (TSA™ Kit with Biotin-XX Tyramide, Thermo Fisher Scientific, T20931) was included to increase the concentration of biotin molecules in the vicinity of the NUP133 epitopes. The TSA reaction was performed as described in the manufacturer's protocol. In brief, after hybridization of the FISH probes and incubation with the primary antibodies anti-NUP133 (Abcam, ab155990) and anti-Digoxigenin (Roche, 11333062910), cells were incubated with anti-HRP antibody, treated with the TSA-working solution and then incubated with anti-biotin antibody (Abcam, ab53494).

3.5 GRID WIDE-FIELD MICROSCOPY

Cell imaging and generation of optical section in 3D were carried out on Leica DMI 3000B fluorescent microscope with OptiGrid device (Grid confocal) using Volocity software (Quorum Technologies Inc). Stacks were taken at 0.3 μm intervals in the Z-axis. On average, 150-300 alleles were counted for distance measurements and/or ChrISP and ISPLA signal intensity in each case. RNA FISH signals were determined by subtracting the intensity of the background in the immediate surroundings.

3.6 CHROMATIN NETWORKS AND INTEGRATION ANALYSES

3.6.1 Circular chromatin conformation capture sequencing (4C-Seq)

Using the human *H19* ICR region as targeting bait, 4C-seq was performed in both HESCs and HEBs as previously described [192]. Briefly, formaldehyde crosslinking of hESCs and hEBs was performed with/without the presence of Olaparib (0.3 mM final concentration) in the presence of 4 mM (final concentration) Ribonucleoside Vanadyl Complex. Upon PARG treatment, crosslinked chromatin was treated with recombinant PARG (25 ng/ml final concentration) (catalog no. 4680-096-01, Trevigen) in the presence of 2 mM (final

concentration) DTT in BglII restriction buffer at 25°C prior to BglII digestion for 24 hours. Chromatin was digested with BglII for 2 weeks at 37°C in the presence of 1 U/ml RNasin Plus (Promega). RNase A (0.8 mg/ml final concentration) was added during the period of BglII digestion for RNase treatment. After digestion, intra-molecular ligation, reversal of crosslink and DNA purification were performed.

3.6.2 Nodewalk

The identification of chromatin networks impinging on MYC and flanking enhancers was done as has been previously described [29].

3.7 RNA ANLYSES

3.7.1 Pulse labeling of RNA

Newly synthesized RNA samples were generated by incubating the cells with 0.5 mM (final concentration) 5-ethynyl uridine (EU, Thermo Fisher Scientific, E10345) for 15 or 30 minutes. For pulse chase, cells were washed with 5xPBS after the labeling with EU and then incubated with pre-warmed normal growing medium for indicated periods.

3.7.2 The nuclear RNA export assay

To determine the ratio between exported cytoplasmic and nascent nuclear RNA, EU-labelled nuclear and cytoplasmic RNA were fractionated, processed to cDNA and analysed for the presence of intronic and exonic regions of *MYC* by QPCR analysis. The separation of the nuclear and cytoplasmic fraction and the RNA isolation was performed by using the Ambion® PARIS™ system (Thermo Fisher Scientific, AM1921) according to the manufacturer's protocol. Briefly, 300 µl of Fractionation buffer was used to lyse the cells. The nuclear and cytoplasmic fractions were separated following centrifugation at 500 g for 5 minutes. Labelled RNA or total RNA was purified with RNeasy Mini kit (Qiagen, 74014). EU-labelled RNAs were captured using Click-iT Nascent RNA capture kit (Thermo Fisher, C10365) following the manufacturer’s instruction before their conversion into cDNA using SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific, 11754050).

3.7.3 mRNA decay analyses

To block elongation of transcription, cells were incubated with 5 µg/ml Actinomycin D (Sigma Aldrich, A1410) for 0, 0.5, and 1 hour. Cytoplasmic and nuclear fractions were separated and total RNA purified as described above. An additional DNA digestion step was included to remove residual DNA (TURBO™ Dnase, Ambion, AM1907). The samples were

normalized against the total recovery of the nuclear and cytoplasmic fractions, respectively.

3.7.4 RT-QPCR analysis of transcription

The quality of purified RNA samples was assessed before cDNA synthesis (SuperScript VILO cDNA Synthesis Kit, Life Technology, 11754050) using Bioanalyzer 2100 (Agilent). All the QPCRs were performed using 10-fold diluted cDNA and iTaq Universal SYBR Green Supermix (Bio-Rad, 1725125) on RotorGene 6000 (Corbett Research).

4 RESULTS

4.1 PAPER I: PARP1- AND CTCF-MEDIATED INTERACTIONS BETWEEN ACTIVE AND REPRESSED CHROMATIN AT THE LAMINA PROMOTE OSCILLATING TRANSCRIPTION

4.1.1 Interactome connecting circadian loci and LADs

To uncover the dynamic feature and function of chromatin crosstalk in the 3D space of the nucleus, we took the advantage of the 4C technique to capture more than two ongoing chromatin fiber interactions simultaneously. Using a well-characterized epigenetically regulated imprinting region *H19* ICR as the bait, 4C was performed and 518 regions were observed that reproducibly interacted with the bait region in Human embryonic stem cells (hESCs), as well as in the derived embryoid bodies (hEBs) in a developmentally regulated manner. Approximately two thirds of regions detected by the 4C analyses formed a highly modular and approximately scale-free network, which covered both intra- and inter-chromosomal chromatin fiber interactions. The detected network represents the sum of interactions from a cell population, where the individual interactions are dynamic and variable at the single cell level. Thus, only regions with more frequent interactions reproducibly emerged as central nodes in two or more samples within the network.

The networks were validated by 3D DNA FISH analysis by measuring the physical distance between two loci identified by 4C. Unsurprisingly, it turned out that interactors with high read counts in the 4C library were more proximal to the bait than nodes with low read counts. The central nodes with higher connectivity were thus proximal to each other much more frequently than regions further apart in the topology of the network. To further validate this observation, we employed a chromatin hub connected to every module of the *H19* ICR interactome, i.e. the *VAT1L* locus (coding for a vesicle amine transport 1 homolog-like protein), as a new 4C bait. Apart from reproducing the interaction between *VAT1L* and *H19* ICR, we also identified regions interacting both with *VAT1L* and *H19* ICR, as well as loci that interacted with *VAT1L* independently of the *H19* ICR. These results reinforced the topology of the network and that the existence was independent of *H19* ICR.

We further analyzed the feature of the network based on chromatin states. Surprisingly, the interactome impinging on *H19* ICR covered various chromatin states ranging from transcriptionally permissive to transcriptionally repressive without either segregation away

from each other, or preferred interactions in between active loci in both hESCs and hEBs. This is striking since it did not follow the generally believed hallmark of the nuclear architecture that active and inactive chromatin domains are dynamically and spastically separated [11, 15].

4.1.2 Molecular ties connecting circadian loci to LADs

The reproducibility of the network suggested that the encounters between the nodes were regulated and that there might be dynamic molecular ties connecting different nodes. Since long-range insulation by the maternal *H19* ICR requires PARylated CTCF and our data showed that PARP1 bound to the same, maternal allele as CTCF [35, 36], we predicted that PARylation and/or PARP1 together with CTCF contributed to the chromatin crosstalk. This hypothesis was confirmed: the depletion of PAR chains from cross-linked chromatin by PAR glycohydrolase (PARG) collapsed the chromatin network between *H19* ICR and its interactors in both hESCs and hEBs. This observation demonstrates that PAR chains were present between the interacting nodes at the time of interaction, although it does not explain if PARylation was the cause or consequence underlying the network formation.

PAR chains are generated by the enzymatic activity of PARP1, which could be activated by CTCF independently of DNA damage [193]. Reduction of CTCF expression by small interfering RNA (siRNA) indeed reduced cellular PAR levels in HCT116 cells, indicating that PARylation of chromatin complexes and hence the network might be caused by functional CTCF-PARP1 interactions. This deduction was supported by two different experiments. First, ChIP-loop assays revealed that PARP1 is part of the H19 ICR-VAT1L complex. Second, treating hESCs with Olaparib, an inhibitor of PARP1 enzymatic activity [194], for 24 hours not only significantly reduced cellular PAR levels, but also affected chromatin movements and disrupted chromatin fiber interactions impinging on the *H19* ICR. Furthermore, ChIP analyses showed that binding of PARP1 to *VAT1L*, *H19* ICR, and other chromatin hubs, as well as indirect binding of CTCF to several interactors of *H19* ICR were disrupted by a 24-hour Olaparib treatment. Conversely, direct binding of CTCF to the *H19* ICR was insensitive to Olaparib treatment to suggest that the CTCF-PARP interaction was directly or indirectly underlying the formation of the chromatin network. Finally, 4C analyses illustrated that incubation with Olaparib during a mere 10-min during the formaldehyde crosslinking step, could disassemble the majority of interactions in hESCs despite that it did not remove already existing PAR chains. Taken together, the results indicated that the interaction between CTCF and PARP1 was essential for the connection between *H19* ICR

and its interacting chromatin hubs in the initial phase. The *in situ* generated PAR chains could then regulate the stability of the CTCF-PARP1 interaction and provide a platform for interaction with other proteins to further diversify the network.

Given that transcriptionally active and repressed genes communicated with each other extensively within the network, we next addressed the underlying reason. One clue was provided by the findings that PARP1 activity is rhythmic and that PARP1 participates in the entrainment of circadian rhythm in response to feeding in the mouse liver [141]. Enrichment analysis showed that the network was enriched in potential circadian control genes, particularly in the flanking regions located 10-kb or less distal to the site of interaction, indicating a *cis*-acting regulatory role of the interactors. The phenomenon that four chromatin hubs were proximal to LADs at the nuclear periphery frequently raised the idea that chromatin fiber interactions in the *H19* ICR interactome was established at this compartment. Indeed, 3D DNA FISH combined with Olaparib treatment confirmed the frequent proximities between circadian loci and LADs in untreated control cells, as well as the involvement of PARP1 activity or protein interactions in the recruitment of circadian genes to LADs. Moreover, nearest neighbor analysis illustrated that the circadian genes that were active in hESCs and hEBs interacted with LADs more frequently than with other circadian genes.

4.1.3 The role of the nuclear periphery in circadian transcriptional attenuation

In contrast to the general dogma that active and repressed domains were segregated to different compartments [11, 13, 15], we considered the possibility that there might be an oscillating re-positioning of active circadian loci to the repressed compartment at the nuclear periphery. This perception was reinforced by the observation that repressive chromatin modifiers that contribute to the regulation of the repressive sub-compartment at nuclear periphery [195] are also involved in the regulation of circadian transcription by acting in company with factors that regulate the negative limb of the circadian feedback loop [173, 180]. To explore this issue, serum shock was used to synchronize circadian gene expression in cultured cells [129]. Using this method, we managed to synchronize or reset the circadian rhythm of the human colon cancer cell line HCT116. Strikingly, in situ proximity ligation assay (ISPLA) revealed that CTCF and PARP1 were in close physical proximity to each other primarily at the nuclear periphery and the interaction displayed a circadian rhythm. Similarly, 3D DNA FISH analyses showed that the *IGF2/H19, VAT1L, PARD3* and *TARDBP* loci co-localized with the lamina in a rhythmic manner following serum shock. Moreover, the phase of recruitment was dependent on transcriptional activity in HCT116 cells, as loci with

low transcriptional activity, such as *IGF2/H19* and *VAT1L*, peaked at 0 and 24 hours upon serum shock, while active loci, such as *PARD3* and *TARDBP* peaked around 8 hours later. This deduction was reinforced by the observation that inhibiting transcriptional elongation by Flavopiridol [196] accelerated the recruitment of *PARD3* and *TARDBP* to the lamina by about 8 hours upon serum shock. Finally, both Olaparib treatment and knocking down of either CTCF or PARP1 not only abolished the rhythmic tethering of circadian loci to the lamina, but also the rhythmic transcription of *PARD3*. Therefore, both protein levels and protein interaction of CTCF and PARP1 as well as PARP1 activity were critical for the entrainment of circadian transcription and for the rhythmic recruitment of circadian loci to the nuclear periphery.

Interestingly, although transcription level of PARD3 peaked at the time when it was recruited to the nuclear periphery in the highest level, its activity dropt notably a few hours later at this sub-compartment, documenting that its juxtaposition to the nuclear periphery indeed preceded its transcriptional attenuation. Since transcriptional repression is not immediate and the periphery is enriched in repressive chromatin modifiers as well as in H3K9me2 LOCKs, enrichment of H3K9me2 while not H3K27me3 at PARD3 was documented by ChIP sequencing analysis in unsynchronized cell populations. ChrISP analysis was performed to further explore the presence of H3K9me2 at PARD3 alleles at the single cell level. Oscillating acquisition of the repressive H3K9me2 mark at PARD3 peaked at the time of transcriptional attenuation upon serum shock. Moreover, inhibiting the HMT activity of G9a/Glp [197-199] to deplete the H3K9me2 mark not only abolished the juxtaposition of PARD3 to the nuclear periphery, but also reduced its rhythmic transcription. Overall, the data are consistent with the observation that circadian transcriptional attenuation of PARD3 took place at the nuclear periphery requiring a time-dependent acquisition of the repressive H3K9me2 modification.

4.1.4 Summary: novel principles in the entrainment of circadian transcription

Taken together, in Paper I we have uncovered a chromatin network regulated by the genome organizers PARP1 and CTCF. The network is organized mainly by inter-chromosomal interactions connecting transcriptionally active loci enriched in circadian genes to repressed LADs at the nuclear periphery. Serum-shock induced entrainment of the circadian rhythm involves the rhythmic recruitment of clock controlled genes to the repressive environment at the nuclear periphery, followed by the time-dependent acquisition of the repressive H3K9me2 mark, leading to circadian transcriptional attenuation. PARP1 and CTCF not only

facilitate the recruitment of circadian loci to the lamina in an oscillating manner, but also promote rhythmic transcription upon serum shock (Figure 5, Model explaining the oscillating recruitment of circadian loci to the nuclear periphery).

The circadian system uncovered in this paper is likely to interplay with the core clock machinery. The NAD+-dependent PARP1 activity has been shown to be rhythmic in mouse liver and regulated by feeding [141]. PARP1 has been shown to bind to and PARylate CLOCK in a circadian manner, and this PARylation prevents the binding of negative limb proteins to CLOCK, as well as evicts CLOCK:BMAL1 from the E-box elements [141]. Thus, PARP1 modulates the temporal interactions between CLOCK:BMAL1 and PER complexes to potentially regulate transcriptional attenuation. Our findings are in line with the observation that PARP1 collaborate with CTCF to modulate the circadian chromatin transition from active to repressive chromatin states. PARP1 and CTCF thus interact with each other in a circadian manner to regulate and promote the rhythmic recruitment of active circadian loci to the repressive nuclear periphery. This in turn ensured the gradual attenuation of circadian transcription upon entrainment by serum shock. This finding opens up new avenues for our understanding of the complex role of PARP1 in the entrainment of peripheral circadian clocks to feeding.

In summary, Paper I describes a novel principle of the entrainment of circadian transcription that involves rhythmic chromatin mobility between active and repressive sub-nuclear compartments. This observation demonstrates the critical role of nuclear architecture in circadian regulation. Given the tight link between circadian rhythms, cellular metabolism and pathologies such as metabolic syndrome, diabetes, and some tumorigenic processes [170], Paper I might have an impact on our understanding of the molecular mechanisms underlying complex diseases and how these can be perturbed. Erosion of the repressive domains might thus counteract circadian transcription to perturb metabolic states in complex diseases.

Questions not addressed here include how the circadian loci are released from the repressive periphery and how PARP1- and CTCF-regulated circadian oscillations collaborate with the core clock machinery. Since CTCF-PARP1 interaction is essential for the recruitment of circadian genes to the lamina, we predict that dissociation of this complex probably contribute to the release process. Factors under circadian control might influence the PARP1 activity [200] to rhythmically destabilize the CTCF-PARP1 complexes. The interplay with the central clock machinery needs to be further analyzed using other model systems. Finally, the observation that circadian genes remain active at the lamina for several hours after their

initial recruitment suggests that they might land in an environment that is transcriptionally permissive. Such an environment might be represented by the nuclear pores, raising the possibility that rhythmic chromatin mobility to the lamina is coordinated with the rhythmic nuclear export of circadian gene products.

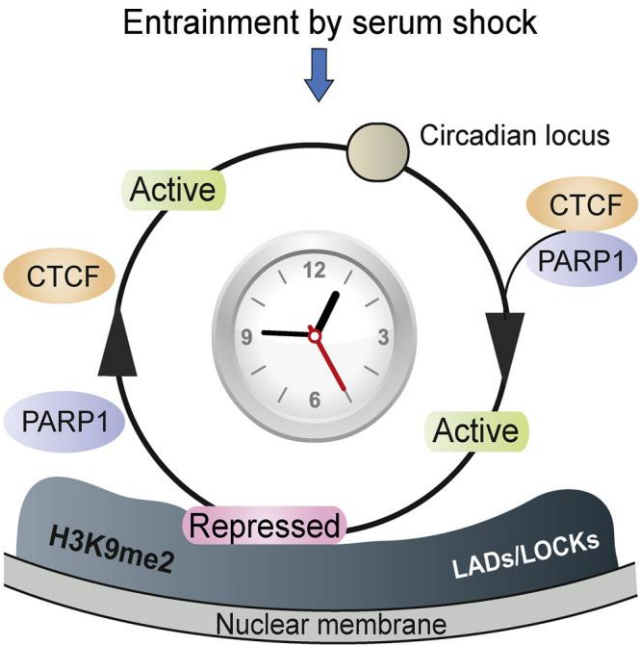

Figure 5, Model explaining the oscillating recruitment of circadian loci to the nuclear periphery. Adapted from H.Zhao et al. PARP1- and CTCF-Mediated Interactions between Active and Repressed Chromatin at the Lamina Promote Oscillating Transcription. Mol Cell. 17;59(6):984-97 (2015). Reprinted with permission from publisher.

4.2 PAPER II: WNT SIGNALING AND AHCTF1 PROMOTE ONCOGENIC MYC EXPRESSION THROUGH SUPER-ENHANCER-MEDIATED GENE GATING

Enhancers and super-enhancers stabilize the expression of cell fate-determining genes during normal development. In some instances, abnormal super-enhancers can be formed during neoplasia to drive pathologically high levels of expression of oncogenes, such as *MYC* [68, 74]. As *MYC* plays key roles in cell cycle progression and apoptosis [201], the emergence of an oncogenic super-enhancer (OSE) promotes uncontrolled cell proliferation.

4.2.1 Regulation of *MYC* transcription in 3D

To identify the enhancer network impinging on *MYC* gene in HCT116 cells, we have performed earlier a novel 3C-based technique named Nodewalk [29], which could comprehensively detect stochastic and dynamic interactions with high resolution and ultra-sensitivity using small input material. Using several enhancer regions as baits in HCT116 cells and human colon epithelial cells (HCECs), we have uncovered an extensive network covering active regions enriched in H3K27ac marks and inactive regions represented by cLADs located primarily at the nuclear periphery [81]. Although the nuclear periphery provides a well documented transcriptionally repressed environment [81, 85] and recruitment of genes to the periphery could promote silencing, peripheral localization leads not only to silencing [103]. Recruitment of genes to the NPCs can be associated to both transcription activation and repression [107, 108]. Some nucleoporins could bind to enhancers to form clusters and could also interact with lamins directly [202, 203] to provide a potential link between active genes and cLADs. We could show that NUP153-binding enhancer regions cluster with high connectivity impinging on *MYC* (within two TADs flanking *MYC*), which is in line with the notion that NUP153 generally associates with enhancer domains preferentially localized at NPCs [203].

In contrast to NUP153, another nucleoporin, NUP133, showed little or no binding to the *MYC* promoter in either HCT116 or primary cultures of normal human colon epithelial (HCEC) cells. However, ChIP analyses showed a broad occupancy of NUP133 within both the hematopoietic super-enhancer region and the colorectal super-enhancer region, the OSEs of HCT116 cells, with markedly less binding to the region corresponding to the OSE in HCECs. Taken together, the OSE has both NUP153 and NUP133 occupancy, while *MYC* is occupied primarily by NUP153. Given that NUP153 and NUP133 contribute to the formation of nuclear basket/cage and ring structure respectively, there might be a division of labor

between the OSE and the *MYC* gene at the nuclear pore.

We next determined the sub-nuclear location of NUP133 and OSE interaction by using the ChrISP technique [33]. This technique allows the detection of the NUP133 epitope in the proximity of the OSE with a resolution less than 16.2 nm. Compared with the negative control, lacking one of the secondary antibodies, we could document specific ChrISP signals between NUP133 and the OSE in HCT116 cells, while rarely in HCECs. Importantly, 73% of the OSE alleles juxtaposed to the nuclear periphery are proximal to the NUP133 epitope. There is also a fraction of OSE-NUP133 ChrISP signals within the nucleus, which is in line with the presence of NUP133 in the intra-nuclear space [112]. Conversely, *MYC* itself was rarely in direct physical contact with NUP133, thus independently validating the low NUP133 occupancy at *MYC* promoter detected by the ChIP assay.

To explore the relationship between NUP133 occupancy and OSE-*MYC* interaction, we first measured the physical distance between *MYC* or OSE and the nuclear periphery by 3D DNA FISH analysis in both HCT116 cells and HCECs. The OSE was observed to be generally closer to the nuclear periphery than *MYC* in HCT116 cells. Intriguingly, the *MYC* and OSE regions appeared more proximal to each other only when the OSE region was within 1 μm from the periphery. A similar tendency was observed in HCECs. To independently validate this observation, we scored for ChrISP signals between *MYC* and OSE in relation to the nuclear periphery. The results confirmed that the proximity between the OSE and *MYC* directly correlated with their proximity to the nuclear periphery. To rule in or out that this pattern was specific to the OSE region, we compared this data using another enhancer region (EnhD) more proximal to *MYC* and not binding NUP153, as identified by Nodewalk and ChIP analyses. Indeed, although its proximity to *MYC* was generally prominent, these two regions were rarely proximal to each other at the nuclear periphery. The results therefore suggest that the EnhD region loops out when OSE-*MYC* approach the nuclear periphery.

By analogy to the ability of the nuclear pore to coordinate transcription, mRNA processing and nuclear exports in lower model systems [100], we considered the possibility that the nuclear pore provides a platform for *MYC* expression by recruiting the OSE when interacting with *MYC*. First, the presence of transcriptionally active *MYC* at the nuclear periphery was assessed through 3D RNA FISH analysis using both probes targeting intron 1 and probes covering both exons and introns. In order to identify the *MYC* mRNA signal, the RNA FISH analyses were followed by denaturation and DNA FISH using a large BAC probe to identify the corresponding genomic location of *MYC*. The results showed that most of the *MYC* alleles

juxtaposed to the nuclear periphery/pore were transcriptionally active. However, those *MYC* transcripts were largely devoid of intron 1 sequences, suggesting that mRNA processing is being completed when *MYC* approaches the periphery.

When we compared the ratio of the *MYC* mRNA FISH signals generated by the intron 1 and intron/exon probes, it turned out that the transcriptionally active alleles could be discerned into two different populations: one more proximal to the nuclear periphery (within 0.3-0.7 μm) and the other more distal (within distance bin 1.1-1.3 μm). This biphasic pattern is strikingly similar to the distribution of the OSE-*MYC* or EnhD-*MYC* ChrISP signals. Thus, the distribution of EnhD-*MYC* proximities was similar to the intron 1 RNA FISH signals while the distribution of the OSE-*MYC* proximities showed an emphasis on the intron/exon RNA FISH signals. Furthermore, there were frequently more than one OSE and *MYC* alleles in each cell that were within 0.7μm from the nuclear periphery, indicating that there is a dynamic repositioning of *MYC* alleles to the nuclear periphery.

4.2.2 Contribution of gene gating to *MYC* mRNA accumulation

In a next step we compared the expression levels of both nascent and total *MYC* mRNA levels in HCT116 cells and HCECs by RT-QPCR analysis. Although cytoplasmic mRNA levels in HCT116 cells were 3-fold higher than in HCECs; the nascent transcripts in the nucleus were paradoxically lower in HCT116 cells than in HCECs. To explain this discrepancy, we considered the scenario that the proximity of *MYC* to the nuclear pore would facilitate nuclear export of processed mRNA. We therefore developed a 5-ethynyl uridine (5-EU) pulse-chase analysis technique in HCT116 cells and HCECs. Total RNA was extracted from the nuclear and cytoplasmic fractions followed by purification of newly synthesized RNA by immunopurification. Levels of newly exported versus newly transcribed *MYC* RNA were determined by RT-QPCR. Visualizing the efficiency of nuclear export by cytoplasmic/ nuclear ratios showed that the nuclear export of *MYC* mRNA is on average 5-fold more efficient in HCT116 cells than in HCECs. This result is in line with our observation that the OSE region or corresponding region is juxtaposed to the nuclear pore in HCT116 cells, but not in HCECs.

However, this process would contribute to higher total *MYC* mRNA levels only if the stability of the *MYC* transcripts is lower in the nucleus than in the cytoplasm. We addressed this issue by inhibiting transcriptional elongation using Actinomycin D, followed by RT-QPCR analyses of the levels of nuclear and cytoplasmic *MYC* transcripts during a time course. While there was no difference in overall *MYC* mRNA stability between HCT116 and

HCEC cells, there was in both cell types a several-fold difference in the decay rates between the nuclear and cytoplasmic *MYC* transcripts. The main difference in cytoplasmic *MYC* mRNA levels between HCT116 and HCEC cells therefore likely reflects the more than 3-fold difference in decay rates between the nuclear and cytoplasmic compartments in combination with the facilitated export in HCT116 cells.

Considering that the assembly of NUP107/160 nuclear pore sub-complex on chromatin requires the transcription factor AHCTF1 [112, 113], we analyzed the effects of knock down of *AHCTF1* expression on the binding of NUP133 to *MYC* and the OSEs and the export ratio of *MYC* transcripts. Indeed, ChrISP analyses showed that knockdown of *AHCTF1* expression reduced the binding of NUP133 to the OSE at, or close to the nuclear pores. Conversely, it did not interfere with the polarized anchoring of the OSE-*MYC* complex to the nuclear pores. These results document that AHCTF1 controls the anchoring of the OSE to nuclear pores but is not involved in the migration of OSE/*MYC* to the nuclear periphery/pore. Importantly, as the attenuation of *AHCTF1* expression reduced the nuclear export of *MYC* transcripts, we argue that AHCTF1 is essential for both the anchoring of super-enhancer to the nuclear pores and the facilitated nuclear export of *MYC* transcripts.

4.2.3 The role of WNT in the super-enhancer mediated gene gating of *MYC*

Tumorigenesis has been associated with hyperactivity of the WNT/β-catenin pathway [204]. WNT/β-catenin pathway frequently target to *MYC* expression in the majority of colorectal cancers [205, 206]. As the DNA binding protein TCF7L2/transcription factor TCF4, which forms a complex with β-catenin in the nucleus of cells with an activated canonical WNT signaling pathway, binds to the OSE at the *MYC* locus in HCT116 cells [75], we hypothesized that the WNT/β-catenin pathway might be the upstream regulator of OSE-mediated *MYC* gene gating. To address this possibility, we treated HCT116 cells with BC21, which specifically interferes with the complex formation between TCF4 and β-catenin [207]. Both ISPLA and, in particular co-immunoprecipitation (co-IP) assays showed that AHCTF1 and ß-catenin bound to each other in a BC21-independent manner, while AHCTF1 binding to TCF4 was BC21-dependent. TCF4-ß-catenin complex formation is therefore likely needed for AHCTF1 to be able to interact with the OSE at the *MYC* locus in HCT116 cells, which was subsequently confirmed by ChIP analyses.

To further examine whether the interaction between AHCTF1 and TCF4 is linked to the regulation of the anchoring of the OSE to the nuclear periphery/pore, we performed ChrISP analyses between the OSE, which has TCF4 occupancy, and NUP133, which is recruited to

chromatin *via* AHCTF1. The results confirmed that BC21 treatment indeed suppressed both the binding of NUP133 to the OSE and the recruitment of OSE to the nuclear periphery/pore. Given that *MYC* is a WNT/β-catenin target gene and AHCTF1 physically interacts with ß-catenin, we further analyzed the presence of AHCTF1 at one prominent *MYC* WNT-responsive DNA element (WRE) WRE-520 [208] by ChIP. The results showed that AHCTF1 binding to this site was attenuated in BC21-treated HCT116 cells. Of note, the conditions we used for the BC21 treatment reduced cytoplasmic *MYC* mRNA without any significant effect on the levels of *MYC* transcription. Taken together, our results are consistent with that WNT signaling regulates pathological *MYC* mRNA export post-transcriptionally by tethering the OSE-*MYC* complex to the nuclear pores mediated by AHCTF1/ß-catenin.

While the gene gating principle has been documented in yeast and Drosophila systems, it was not well understood in mammals [209]. Although the results in Paper II agree with the basic features of this principle and extend its validity to humans, they have also uncovered novel features. In particular, the OSE-mediated, post-transcriptional increase of cytoplasmic levels of *MYC* mRNA in response to extra-cellular cues is novel. Given that *MYC* is a key regulator of diverse biological processes particularly in cancer, targeting WNT-regulated gating of *MYC* may provide an alternative way for cancer therapy.

5 DISCUSSIONS

5.1 THE ROLE OF NUCLEAR PERIPHERY IN THE REGULATION OF GENE EXPRESSION

The nuclear periphery provides environments for both transcriptional activation and repression. LADs and LOCKs form the repressive components at the periphery. Repressive histone modifications [15, 210] and specific genome organizers [34] thus coordinately drive dynamic interactions between genomic loci and the transcriptionally repressive environment at the nuclear periphery. While the nuclear pores supply a platform for both transcription activation and repression in various organisms, nuclear pore complexes (NPCs) and their components (NUPs) coordinate transcription, mRNA processing and nuclear export in lower eukaryotes [100].

Paper I described a novel principle of circadian transcriptional regulation involving the rhythmic recruitment of active clock controlled genes to the nuclear periphery and following a time-dependent transcriptional attenuation. This observation not only demonstrated a critical role of the nuclear architecture, the nuclear periphery in particular, in circadian regulation, but also suggested that transcriptional attenuation from activation was not immediate, and involved a delayed acquisition of the repressive H3K9me2 modification at the lamina during the circadian cycle. Circadian genes thus maintained their activity for several hours after their recruitment to the nuclear periphery. Taken together with the results of Paper II, showing that the colorectal super-enhancer facilitates the accumulation of *MYC* transcripts in the cytoplasm by anchoring *MYC* to the nuclear pore in colon cancer cells, we speculate that CTCF and PARP1 recruit active genes to the nuclear pore prior to reaching the repressive environment at the periphery.

5.2 MYC AND THE CIRCADIAN CLOCK

MYC and perturbed circadian rhythm are frequently linked to tumorigenesis [211]. While whether altered *MYC* expression in cancer could be mechanistically related to deregulated circadian rhythm either directly or indirectly remains to be established. While E-box elements exist in *MYC* gene itself [212], *MYC* and MYC-target genes have been demonstrated to be regulated by CLOCK-BMAL1 at both transcriptional and posttranscriptional levels in mammals [213, 214]. Of note, the circadian repressor CRY2 promotes ubiquitylation and degradation of the MYC protein, suggesting that circadian disruption might promote tumorigenesis in part through MYC protein stabilization [215].

Alternatively, given the master role of MYC in cell proliferation and metabolism, a hyper-activated *MYC* could interfere with the clock system and thus change circadian oscillation of metabolism in cancer cells. Altman and colleagues have documented that an overexpressed *MYC* could alter the expression of the negative limb members of the core clock machinery, e.g. PER2 and CRY1, *via* direct binding to E-boxes [216]. Moreover, direct activation of REV-ERBs repressed *BMAL1* expression thus suspend the molecular clock *in vitro* [216]. Shostak *et al* further identified that MYC mediated circadian repression requires interaction with MIZ1 and suggested that MYC/MIZ1complex-dependent gene repression inversely coordinates the circadian clock [217]. The observation that *MYC* overexpression profoundly disrupted oscillation of glucose metabolism [216] suggests that pathological expression of MYC promotes tumor growth by disrupting circadian gene expression and cell metabolism. Given these observations it is conceivable that a too efficient gating of *MYC* might provide a negative feed-back to the clock machinery, a deduction in line with our observation that only a subpopulation of *MYC* alleles are subjected to gene gating (Paper II).

The observations that factors involved in oncogenic pathways, such as MYC, are strongly regulated by the circadian machinery suggest there is an interdependent relationship between MYC and circadian rhythms. Both oncogenic alterations and circadian rhythms are involved in the regulation of many cellular processes, including metabolism, and metabolism itself could also regulate circadian patterns. The close association between circadian rhythms and oncogenic alterations might be changed during tumorigenesis, while the underlining mechanism has not been explored. Combining the findings from Paper I and II, one new opening is that PARP1 and CTCF complexes formation facilitates the recruitment of circadian genes, such as *MYC*, first by anchoring them to the nuclear pore followed by a lateral transition to repressive LADs.

5.3 ADAPTATION TO THE ENVIRONMENT

5.3.1 WNT signaling

The WNT signaling cascade is a critical and highly evolutionally conserved regulatory pathway that has been implicated in a wide range of processes such as embryonic development, tissue regeneration and carcinogenesis. It mediates metabolic reprogramming of both normal and tumor cells by directly regulating downstream signaling pathways such as TCF/LEF, MYC [218] and negative regulation of tumor repressor proteins, such as p53 [219]. In addition, WNT can regulate the expression and activity of enzymes involved in metabolic pathways and oncogenes to promote metabolic changes.

WNT signaling is, moreover, involved in mechanisms sensing and translating environmental cues into complex cellular programs during both normal and pathological proliferative states. The expression level of β-catenin is thus sensitive to changes in glucose levels in macrophage cell lines in a hexosamine-dependent manner conceivably linking the canonical WNT/β-catenin pathway to glucose availability [220]. Moreover, the WNT modulator Dapper1/Frodo1 (Dact1) coordinated expression of WNT ligands WNT10b, WNT3a and WNT antagonists in response to *in vivo* adipogenesis, and nutritional cues, as well as during the development of dietary and genetic obesity [221].

5.3.2 Circadian entrainment

Circadian entrainment is a necessary adaptation of organisms to a changing environment. To maintain the synchrony between endogenous clocks and the environment, the clock system has to be readjusted by external time cues or "Zeitgebers" by inducing alterations both in the expression levels of clock genes and in the stability of clock proteins, this is what entrainment stand for [134]. In the presence of an external time cue or a Zeitgeber, an organism's biological clock readjusts its amplitude, phase and period every day to the geophysical time cycle.

The most dominant synchronizer or Zeitgeber of the SCN is light, SCN receives external light signals to synchronize all the oscillators and is highly resistant to phase perturbations [136]. The clock system of peripheral cells in various tissues are synchronized and reset by signaling from the SCN directly or indirectly by the cooperation of neural, humoral, and other signals. The clock system of peripheral clocks can also, however, be strongly influenced by other cues, such as food availability [137, 138]. The phase of peripheral clocks can therefore be entrained or reset by feeding-fasting cycles and humoral signals independent of the central clock [139, 140]. Food intake is thus the most documented time cue for the periphery clocks for in particular the liver [141, 142]. Moreover, humoral TGF-β or activin signals can induce the resetting of the cellular clock through activation of ALK independent of light-input signaling pathway in mice [143]. Given the central role of CTCF and PARP1 in mediating circadian transcriptional oscillation, we speculate that several external cues regulate circadian transcription by targeting the formation of this complex.

5.3.3 Nucleoporins and the transcriptional memory

The transcriptional memory, maintained by epigenetic/chromatin marks, endows cells to

quickly respond to environmental cues to change the transcription repertoire. The juxtaposition of chromatin regions to the NPC provides one of such memory to increase the rate of responses to external signals by accelerating RNA Pol II recruitment in yeast and *Drosophila* [121]. For example, Nup98 binds to ecdysone-inducible genes and mediates their enhancer-promoter looping upon activation in *Drosophila* cells and tissues [119]. Such a transcriptional memory could be regulated by modulating NUP98 levels without affecting ongoing transcription [119]. Paper II uncovered another principle that how NUPs can confer transcriptional memory by showing that the OSE promotes the anchoring of *MYC* to the nuclear pore to facilitate the nuclear export of *MYC* mRNA in colon cancer cells.

5.4 CTCF AND PARP1 IN COMPLEX DISEASES

5.4.1 CTCF in disease

CTCF is a versatile factor linked to inhibition of cancer cell proliferation and clonogenicity [40, 50, 51]. Mutations in the 11 zinc finger domains [40-43] inhibit CTCF binding to specific target sites [43]. Deletion of CTCF or mutations in the 11 zinc finger domains [40-43] to inhibit CTCF binding to specific target sites [43], has been observed in leukaemia [42, 222], Wilms' tumour [43], as well as breast [47, 51, 223] and prostate cancers [47]. Mutations, disruption in the associated modifications, or structural changes in CTCF binding sites adjacent to oncogenes and cancer-related genes result in the deregulation of cancer associated genes, as well as in the changes of the local and long-range chromatin structures in cancer cells [44-49]. For example, epimutations of CTCF binding sites within the *H19* ICR in patients suffering from the Beckwith–Wiedemann syndrome, predispose to paediatric cancer development [224, 225]. Mutation in the *isocitrate dehydrogenase (IDH)* gene resulted in loss of CTCF binding and the disruption of TAD organization in human gliomas [226]. Furthermore, disruption of CTCF-associated TAD boundary domains rewired long-range enhancer-promoter interactions, and the ensuing mis-expression resulted in malformation syndromes [62]. These diverse observations indicate that deregulation of CTCF or its DNA occupancy play important roles in cancer development.

5.4.2 PARP1 in disease

PARP1 mediates the genotoxic stress response, DNA repair, gene integrity, chromatin structure and transcription by PARylating itself and its protein partners to affect a number of cellular and biologic outcomes [53, 54]. PARP1 participates in transcriptional regulation in diverse manners, including physical and functional interactions with genomic DNA and chromatin, with chromatin modifying proteins and transcription factors [227, 228] partly

through the synthesis of PAR at its targets. These processes underlie pathological conditions, including cancer, inflammation-related diseases, and metabolic dysregulation [229].

At a low level of DNA damage, PARP1 acts as a survival factor and initiates repair through recruiting factors involved in DNA repair pathways. Conversely, when DNA damage is high, PARP1 promotes cell necrosis [230], suggesting that the available levels of its NAD+ substrate influences the decision between cell survival and cell death [231]. Inhibition of PARP1 activity or genetic deletion of PARP1 thus prevents animals from aberrant cell death caused by DNA damage [232, 233].

PARP1 has also been shown to interact with TCF-4/β-catenin complex and works as a co-activator of their transactivation [234-237]. Inhibition of PARP1 activity thus represses β-catenin signaling to reduce the expression of β-catenin, *Myc*, cyclin D1 and matrix metalloproteinase (MMP)-7. The PARP1 activity playing a role in colorectal [234, 237] ad cervical carcinogenesis [235]. Accordingly, silencing PARG function suppressed cancer development induced Benzo(a)pyrene by in mice [238]. Auto-PARylation of the PARP-1 protein induced by DNA damage inhibited the functional interaction of PARP-1 with TCF-4 [237]. The interplay between PARP1, the DNA damage response and WNT signaling may thus constitute a new option for the diagnosis and treatment of cancer.

It will be of importance to explore whether the consequences of altered CTCF or PARP1 function in the above mentioned diseases involve a perturbation of the circadian clock and/or the gene gating process.

6 SUMMARY

This thesis focuses on exploring the interplay between epigenetic regulation, enhancer-promoter interaction, 3D genome organization and circadian homeostasis in the 3D architecture of the nucleus.

In Paper I, we uncovered a novel principle of circadian transcriptional regulation: coordination of circadian transcriptional oscillations by external time cues involves the rhythmic mobility of circadian genes between transcriptionally permissive and repressive sub-nuclear compartments. The transient localization of clock-controlled genes to the lamina promoted the formation of dynamic inter-chromosomal chromatin fiber interactions between circadian genes and LADs, prior to the gradual attenuation of their transcriptional activity and the subsequent release of clock-controlled genes from the lamina to the nuclear interior. These LAD-circadian gene interactions were regulated by rhythmic complex-formation between the 3D genome organizers PARP1 and CTCF, which not only served as molecular ties of the chromatin fiber network, but also regulated chromatin mobility to and from the lamina.

In Paper II, we described the discovery that the OSE regulates *MYC* expression post-transcriptionally by facilitating its gating to the nuclear pore in colon cancer cells. This cancer cell-specific tethering of OSE/*MYC* complexes to the nuclear pores was regulated by AHCTF1, resulting in several-fold increase of cytoplasmic *MYC* mRNA levels in human colon cancer cells, but not in normal cell counterparts. Finally, we showed that WNT signaling regulates this process by promoting the binding of AHCTF1 to the OSE, as mediated by ß-catenin-TCF4-AHCTF1 complex formation.

These findings provide new perspectives to understand not only the function of the dynamic 3D nuclear architecture and genome organization, but also how to antagonize cancer cells in therapeutic strategies.

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