Chromatin Fibers Are Formed By Heterogeneous Groups Of Nucleosomes In Vivo

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To my family

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THESIS ABSTRACT

Nuclear architecture and chromatin structure, together with the transcriptional network are key players for self-renew, pluripotency and differentiation of embryonic stem cells (ESCs). The architecture of the chromatin fiber, which determines DNA accessibility, remains unknown and, recently, the existence of the 30nm fiber 'in vivo' has been highly debated.

Overcoming the diffraction limit, STORM (Stochastic Optical Reconstruction Microscopy) can reconstruct the intra-nuclear structures with a spatial resolution of ~ 20nm.

Combining quantitative super-resolution microscopy with computer simulations we resolved how nucleosomes are arranged in vivo, identifying a novel model of organization of the chromatin fiber.

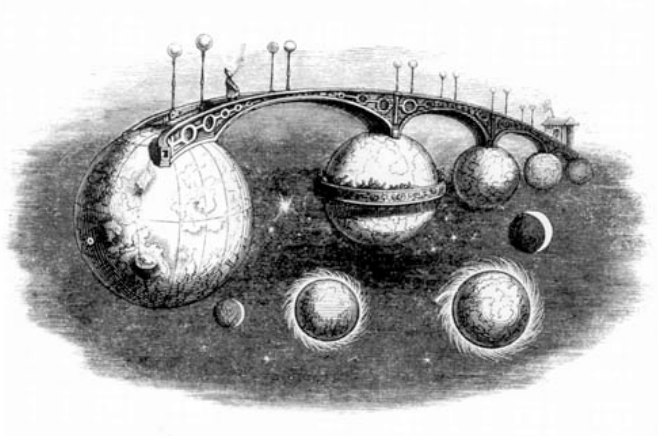
We found that chromatin fiber is formed by groups of nucleosomes of varying sizes, which we term "clutches" and these were interspersed with nucleosome-depleted regions. Moreover the median number of nucleosomes and their compaction inside clutches highly correlated with cellular state. Ground-state pluripotent stem cells had, on average, less dense clutches containing fewer nucleosomes with respect to differentiated cells. These results provide novel insights into chromatin organization at the nanoscale level and open new possibilities for identification of stem cells through the structural organization of their chromatin fibers.

RESUMEN DE LA TESIS

La arquitectura del genoma y la estructura de la cromatina, junto con los factores de transcripción son actores clave para la autorenovación, la pluripotencia y la diferenciación de las células madre embrionarias (ESCs). La arquitectura de la fibra de cromatina, que determina la accesibilidad del ADN, sigue siendo desconocida y, recientemente, la existencia de la fibra de 30 nm 'in vivo' ha sido muy debatida.

Superando el límite dado por la difracción, la técnica STORM (Stochastic Optical Reconstruction Microscopy) puede reconstruir las estructuras dentro del nucleo con una resolución espacial de ~ 20 nm.Combinando una microscopía cuantitativa de super-resolución con simulaciones numéricas hemos sido capaz de definir cómo los nucleosomas están empaquetados en vivo, y hemos identificado un nuevo modelo de organización de la fibra de cromatina.Encontramos que la fibra de cromatina está formada por grupos de nucleosomas de diferentes tamaños, que llamamos "nucleosome clutches" y que estos están intercalados con regiones sin nucleosomas. Además, el número medio de nucleosomas y su nivel de compactación dentro de los clutches, está relacionado con el estado celular. Células madre pluripotentes, tienen en promedio clutches con menos nucleosomas incluidos y de menos densidad con respecto a las células diferenciadas. Estos resultados proporcionan nuevos conocimientos sobre la organización de la cromatina a escala nanométrica y abren nuevas posibilidades para la identificación de las células madre a través de la organización estructural de sus fibras de cromatina.

PREFACE



A Fanciful view of chromatin structure. Olins and Olins, 2003
'Le pont des planètes'

Eukaryotic genome is confined in the nucleus of all the cells of our body. It is made up of 3.2 billion bases of DNA. To fit in the small volume of a nucleus, with about 10 nm of diameter, the DNA is packaged with an equal mass of proteins into chromatin. How the chromatin is organized within the nucleus and how the genetic code is made accessible to execute various cellular functions, nowadays is still a matter of discussion.

Here by using a new high-resolution microscopy we were able to visualize, beyond the diffraction limit, how chromatin fiber is organized in single cells and to correlate nucleosome arrangement to cell state or to the level of pluripotency.

CONTENTS

PART I

INTRODUCTION AND AIMS

Introduction

1. CHROMATIN: THE BEGINNING

The history of chromatin began many years before the discovery of the structure of the DNA (Watson and Crick, 1953) when W.Flemming in 1882 had given the name *chromatin* to a readily stainable material in nuclei (see TIMELINE in Figure 1). Cell biology in the 19th Century was inspired by important technical achievements like the development of light microscopy with minimal optical aberrations, an increased availability of fixatives and stains, an improvement in preparative techniques, and the first chemical characterization of nuclear extract (Olins and Olins, 2003). In the laboratory of F.Hoppe-Seyeler in Tubingen, F.Miesher and A.Kossel, two students, led the groundwork for the characterization of chromatin components. Miesher's work lead to the significant demonstration that the nuclear material consisted of a combination of acidic and basic substances, called '*nuclein*' and '*protamin*'; Kossel subsequently, continued the investigations, and was able for the first time to purify basic proteins, clearly different from Miesher's protamine, which he named '*histon*' (van Holde, 1989). Since then, chromatin has attracted significant interest from biologists, leading to the discoveries of it's structure and it's function.

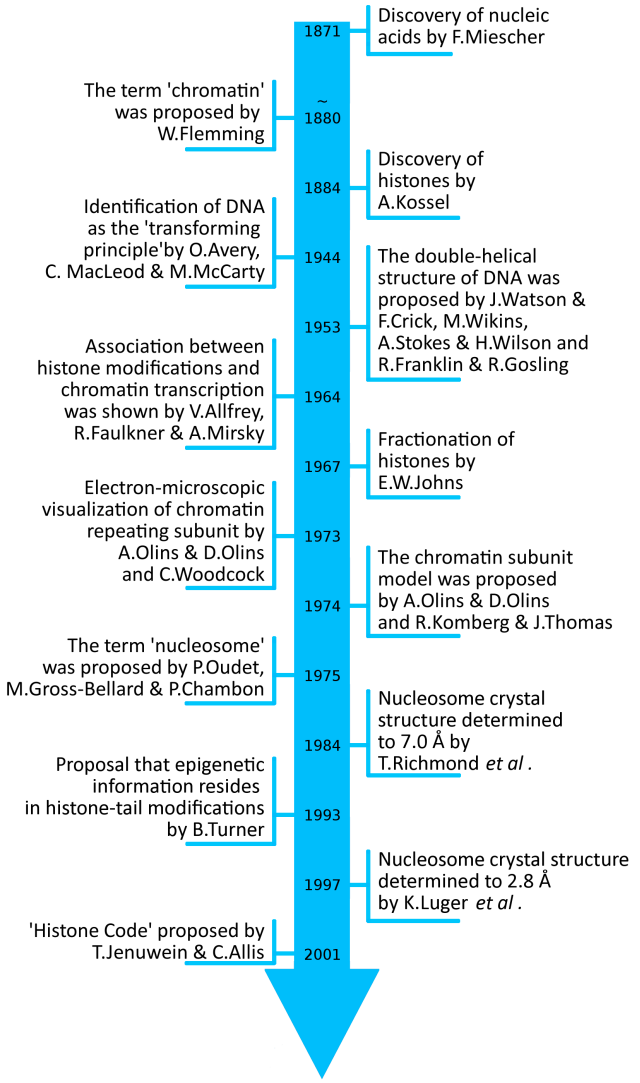


Figure 1. Timeline. History of chromatin. Adapted from Olins and Olins, 2003

2. THE NUCLEOSOME

Chromatin consists of a repeating chain of nucleosomes, which form the fundamental subunits and the first level of packaging of chromosomal DNA. The crystal structure of the nucleosome core particle, NCP is known at atomic resolution (Luger, Mader et al., 1997), ~147 bp of DNA are tightly wrapped around a histone protein octamer in 1.65 left handed super helical turns, leading to a ~ sixfold-length compaction of the DNA. Consecutive NCPs are connected by 'linker' region of DNA making the modular primary structure. Each NCP has a diameter of ~11 nm and a height of ~ 5.5 nm.

2.1 HISTONE PROTEINS

The histone proteins forming the core octamers are highly conserved basic proteins. There are 5 types of mammalian histones namely H2A, H2B, H3, H4 and H1 linker histone. Within a nucleosome, two H3/H4 dimers interact together to form a tetramer, and then two H2A/H2B dimers associate with the H3/H4 tetramer in the presence of DNA (Kornberg, 1974). The formation of nucleosomes involves multiple electrostatic, hydrophobic, and hydrogen bonds between proteins and DNA. The histone H1, or linker histone, is associated with the linker DNA, at the position where the DNA enters and exits the nucleosome core (Ali and Singh 1987). Although H1 exact location remains controversial, H1 has a clear function in the

further compaction of the chromatin structure, by neutralizing remaining DNA negative charges (Finch and Klug, 1976).

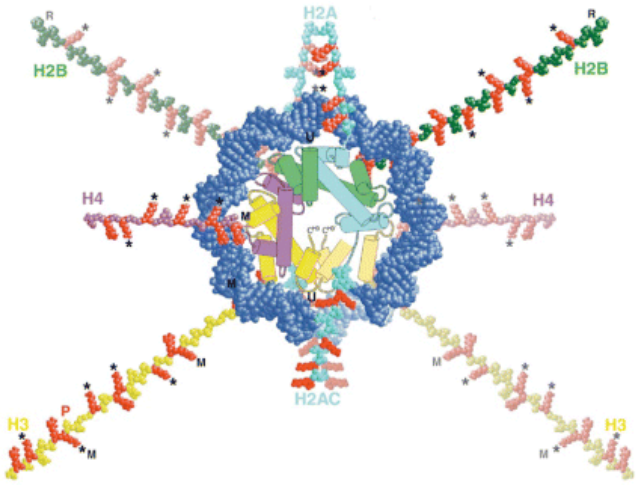


Figure 2. Sites of post-translational modifications within the histone tail domains. The histone tail domains and the nucleosome core are viewed along the superhelical DNA axis. The tail domains are modeled as fully extended polypeptide chains to show the approximate length of these domains with respect to the largely α-helical histone fold domains (columns). Tail sequences are positioned according to the X-ray crystal structure of a nucleosome core (Luger et al., 1997). The top and bottom superhelical turns of core DNA are colored blue and light blue, respectively. H2A, H2B, H3 and H4 are colored cyan, green, yellow and magenta, respectively, while arginine and lysine residues in the tails are colored in red. Sites of acetylation on lysine (*) methylation (M), phosphorylation (P) ribosylation (R) and ubiquitination (U) are indicated. Adapted from Wolffe and Hayes, 1999.

Histone proteins comprise the 'histone fold domain' including a structural motif called ‘helix-turn-helix' that consists of 3 alpha helices (α1, α2 and α3) connected by loops (L1 and L2). Each histone forms a heterodimer with the counterpart that structurally

gives an appearance of a hand-shake (Khare SP et al., 2011). All histones have flexible N-terminal tails, rich in lysine and arginine, protruding out of the compact structure (Figure 2). N-terminal tails contribute to the formation of higher order structure which mediate internucleosomal interactions that promote intra-fiber or inter-fiber associations (Luger et al., 1997).

Histone's tails might be subjected to a variety of post-translational modifications (PTM) such as acetylation, methylation, phosphorylation, ubiquitination etc. (see Table 1) that play important roles in regulating cellular processes like transcription, replication, cell cycle control, etc. PTMs directly regulate chromatin high ordered structure, by affecting histone-DNA interactions or nucleosome-nucleosome interactions (Kornberg and Lorch, 1999) and serve as markers for the recruitment of other architectural and transcriptional factors. PTM are recognized by effector proteins, through which they exert their functions (Bannister and Kouzarides, 2011). Some PTMs such as histone Acetylation or H3K4me3 for example, are marks of regions where DNA is accessible for transcription, the so called 'euchromatin domain', meanwhile other modifications such as H3K9me3 keep the chromatin inaccessible and mark the 'heterochromatin' domains (Bing Li et al., 2007). Over 60 different residues were identified as possible site of modifications, but considering that methylation at lysines or arginines may be one of the three different forms: mono-, di-, or trimethyl, the possible combination of modifications that can take place on a histone is very high (Kouzarides, 2007). The term "histone code" has been used to describe the role of modifications to enable DNA functions (Strahl and Allis, 2000).

Chromatin Modification	Residues Modified	Functions Regulated
Acetylation	K-ac	Transcription, Repair, Replication, Condensation
Methylation (lysines)	K-me1 K-me2 K-me3	Transcription, Repair
Methylation (arginines)	R-me1R-me2a R-me2s	Transcription
Phosphorylation	S-ph T-ph	Transcription, Repair, Condensation
Ubiquitylation	K-ub	Transcription, Repair
Sumoylation	K-su	Transcription
ADP Ribosylation	E-ar	Transcription
Deimination	R > Cit	Transcription
Proline Isomerization	P-cis>P-trans	Transcription

Overview of different classes of modification identified on histones. Adapted from Kouzarides, 2007.

Table 1. Different Classes of Modifications Identified on Histones.

All the five major histones (H1, H2B, H2A, H3, H4) have non-allelic variants whose primary sequence differs mostly in their N-terminal region (Henikoff, Furuyama, Ahmad, 2004). Histone variants have key roles during transcription, development, and DNA repair, and also in the maintenance of ESCs pluripotency and in somatic cell reprogramming (Skene and Henikoff, 2013).

Variant	Species	Chromatin Effect	Function
H1^0	Mouse	Chromatin condensation	Transcription repression
H5	Chicken	Chromatin condensation	Transcription repression
SpH1	Sea urchin	Chromatin condensation	Chromatin packaging
H1t	Mouse	Open chromatin	Histone exchange, recombination?
MacroH2A	Vertebrate	Condensed chromatin	X-chromosome inactivation
H2ABbd	Vertebrate	Open chromatin	Transcription activation
H2A.X	Ubiquitous	Condensed chromatin	DNA repair/ recombination/ transcription repression
H2A.Z	Ubiquitous	Open/closed chromatin	Transcription activation/ repression, chromosome segregation
SpH2B	Sea urchin	Chromatin condensation	Chromatin packaging
CenH3	Ubiquitous		Kinetochore formation/function
H3.3	Ubiquitous	Open Chromatin	Transcription
The species distribution and likely function of major histone variants are shown. Adapted from Kamakaka and Biggins, 2005.			

Table 2. Histone variants and their function

For example, the histone variant H2A.Z has been mapped into enhancers and promoters in embryonic stem cells (ESCs) using

Chip-Seq analysis (G. Hu et al. 2013). H2A.Z increases chromatin accessibility, which promotes binding of Oct4 at pluripotency genes, thereby maintaining self-renewal. Incorporation of H3.3 is also required to maintain pluripotency of ESCs (Meshorer at el., 2006). Some variants exchange with the preexisting histones and have specialized functions just in precise stages during development and differentiation (Kamakaka and Biggins, 2005). (See Table 2 for the major histone variants with known function).

3. CHROMATIN FIBER STRUCTURE

In 1970 what became the classical view of the chromatin structure was delineated. First Roger Kornberg identified the organizing principle of the nucleosome. He initially discovered the H3-H4 tetramer structure, then by X-ray diffraction experiments using recombinant histone oligomers with DNA, he proposed that the histone octamer was associated with about 200 bp of DNA wrapped around it. The observation that almost half amount of H1 with respect to the other histones was present in the chromatin in addition to the characteristic X ray diffraction pattern of chromatin, obtained also in absence of H1, suggested that 1 molecule of H1 was bound to the outside of the nucleosome (Kornberg 1974, Kornberg and Thomas 1974).

The idea of chromatin as oligomers of histones and DNA became visually clear with the electron micrograph obtained almost at the same time by Don and Ada Olins and Chris Woodcock (Olins and Olins 1974, Woodcock et al., 1976). They called 'nu bodies' the beads that soon after, trough Electron microscopy of in vitro reconstituted histone-DNA complexes, were identified as nucleosomes (Finch et al., 1975). The coined term beads-on-a-string refers to the linear array of nucleosomes or 10 nm chromatin fiber and today its structure and organization in higher order complex is still subject to many studies.
One year later Finch and Klug found that purified 10 nm fiber in presence of H1 or high concentration of Mg^{2+} ions were folded into higher order structures with a diameter of 30 nm, the so

called ‘solenoids’ or ‘30 nm fibers’ (Finch and Klug, 1976) (Figure 3a).

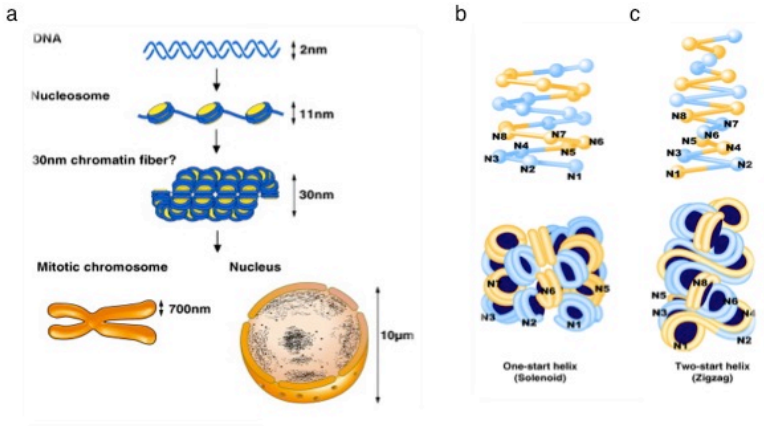


Figure 3. Classical view of chromatin compaction. (a). The long DNA molecule of 2 nm is wrapped around a core histone octamer and forms the nucleosome fiber with a diameter of 11 nm, which then fold into a 30 nm structure and subsequently into the higher order of interphase chromatin or mitotic chromosomes. In (b) and (c) are shown the two proposed models for the 30 nm chromatin structure: one-start helix or solenoid in (b) and the two-start helix (zigzag) in (c). Adapted from Maeshima et al., 2010.

Since then many studies using different techniques including biochemistry, biophysics, X-ray crystallography, conventional EM, cryo-EM and small angle X-ray scattering (SAXS) (Finch and Klug 1976; Woodcock et al. 1984; Widom and Klug 1985; Dorigo et al. 2004; Schalch et al. 2005; Robinson et al. 2006; Bordas et al. 1986; Langmore and Paulson 1983; Hansen 2002; Gilbert et al. 2004; Bystricky et al. 2004; Kruithof et al. 2009), have been used to identify the arrangement of single nucleosomes inside the 30 nm structure. All of them can be

recollected into two mainly proposed models: a. “One-start helix” where consecutive nucleosomes are located adjacent to one another in the fiber making the ‘solenoid’ (Finch and Klug, 1976) (Figure 3a); b. the “Two-start helix” assuming that nucleosomes arrange in a zigzag manner, where a nucleosome in the fiber is bound to the second neighbor (Woodcock et al., 1984) (Figure 3b).

Although a unique structure remains undetermined it has been assumed that the 10 nm fiber folds into 30 nm fibers that subsequently compact with the help of condensins (Woodcock and Dimitrov, 2001) in hierarchical higher-order structures up to the mitotic chromosomes (Widom and Klug, 1985). (For a recent Reviews see Grigoryev and Woodcock, 2012).

3.1 DOES THE 30-NM CHROMATIN FIBER EXIST *IN VIVO?*

The first evidences against a 30 nm structure in 'native' mitotic chromosome were published in 1986 when Dubochet group observed under cryo-EM microscopy vitrified sections of mammalian mitotic cells. They observed that chromosomes had a homogeneous, grainy texture, which on optical diffraction gave rise to reflections corresponding to ~11 nm spacing. They concluded that the basic structure of the chromosome was a liquid-like compact aggregation of 10-nm fibers (McDowall et al., 1986; Dubochet et al., 1988).

To resolve the controversy, Maeshima group found that the ~30-nm peak previously observed in mitotic HeLa chromosomes using previous synchrotron X-ray scattering (SAXS) experiments, was due to ribosome contamination on chromosome surface (Figure 4a). The ribosomes were stacked regularly at ~30-nm intervals. After the removal of the ribosomes from the surface of the mitotic chromosomes only the 11- and 6-nm peaks were detected by SAXS (Nishino et al., 2012). The group had the same result by examining interphase nuclei after washing out the ribosomes (Joti et al., 2012). No other larger regular structures, between ~30- and 1000-nm, was detected when they investigated an entire chromosomal region by ultra small X-ray scattering (USAXS). For a recent Reviews see Maeshima et al., 2014).

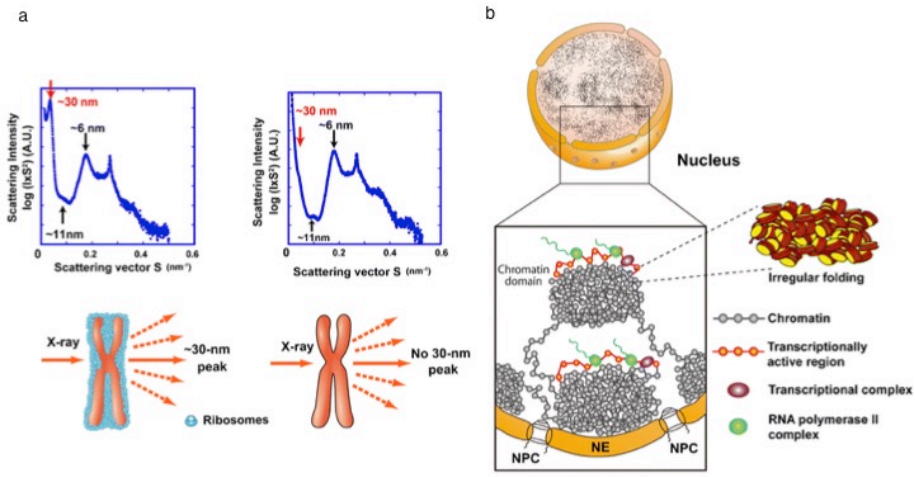


Figure 4 New model of chromatin organization. (a) (Upper left) Typical SAXS patterns of purified mitotic HeLa chromosome fractions. Three peaks at ~6, ~11 (weak), and ~30 nm were detected (arrows). (Upper right) After the removal of ribosome aggregates, the 30- nm peak disappeared, whereas the other peaks remained. (Bottom) A model whereby the 30-nm peak in SAXS results from regularly spaced ribosome aggregates and not from the chromosomes (Nishino et al., 2012). (b) Active chromatin regions are transcribed on the surfaces of chromatin domains with transcriptional complexes (purple spheres) and RNA polymerase II (green spheres). NPC nuclear pore complex, NE nuclear envelope. b (Left) Condensed chromatin is more resistant to radiation damage or chemical attack. (Right) Reactive radicals arising from the radiolysis of water molecules by irradiation can damage decondensed chromatin; decondensed chromatin is also more accessible to chemicals (labeled "Ch"). Adapted by Maeshima et al., 2014.

Maeshima and co-workers concluded that both mitotic and interphase chromosomes were constituted by irregularly folded nucleosome fibers, resembling 'chromatin liquid drops' in correspondence of the closed chromatin domains whereas the active chromatin regions were transcribed on the surface of the drop (Figure 4b). They introduced also the 'polymer melt' concept implying that nucleosome fibers may be constantly moving and rearranging (Maeshima et al., 2010).

In support of these new theories which were against the 30-nm fiber, Dekker by combining chromosome conformation capture (3C) data and polymer modeling, provided a map of chromatin interactions in yeast and suggested that in actively transcribed domains, chromatin did not form 30-nm structures but instead it was extended in a loose arrangement of nucleosomes. Also in the neighboring domains, where chromatin is more compact, he found the mass density still below that of a canonical 30-nm fiber (Dekker J., 2008).

4. NUCLEOSOME POSITIONING AND GENE REGULATION

The sharp bending of DNA on the nucleosomes occurs when the major groove of the DNA faces inwards, towards the histone octamer, every ~10 bp and then again ~5 bp away when it faces outward in the opposite direction (Segal et al., 2006).

Octamers' affinity to wrap the DNA is known to be dependent on the specific DNA sequence that also influences nucleosome stability and dynamics (Satchwell et al.,1986; Widom J., 2001; van Holde, 1989).

Neighbouring nucleosomes are spaced by a naked linker DNA whose length can vary from a minimum of 20 bp in budding yeast, to ~75 bp in echinoderm sperm (van Holde, 1989). In the 1992 Widom presented an extensive work where by measuring nucleosome repeat lengths (NRLs) of different cell types and species, he demonstrated that NRLs vary within a single nucleus. Moreover Routh and his group reveled a direct effect of

the NRLs and the histone linker stoichiometry on the genome folding (Routh et al., 2008). The accessibility to DNA binding sites for particular factors such as polymerases, repair or regulatory complexes, can be affected by nucleosome position. Indeed sites in the naked linker DNA are likely easier to be accessed than those wrapped by nucleosomes. Thus the location of the nucleosomes along the DNA can have important role in gene expression regulation (Kornberg and Lorch, 1999; Wyrick, 1999).

More recently the development of genome-wide nucleosome mapping methods allowed the description of the preferential positioning of nucleosomes at many genomic loci.

Segal and co-workers in 2006, by combining experimental and computational approaches, showed the existence of a genomic code for nucleosome positioning in regions that are critical for gene regulation and specific chromosome function. Investigating nucleosome occupancy across different types of chromosomal regions, for example, they found the highest predicted occupancy over the centromeres, as opposed to a low nucleosome occupancy at functional binding sites and at transcription start sites (Segal et al., 2006).

The genome map inside coding sequences revealed that at the upstream of the transcription start site (TSS), there are precisely located nucleosomes (named +1, -1) flanking a nucleosome free region (NFR). Both -1 and +1 nucleosomes are likely to be evicted during transcription and to contain histone variants or histone tail modifications, but it seems they return immediately after Pol II has passed to their original position (Venkatesh and Workman, 2015).

Discovery of the NFR demonstrated that open promoters are stable and present also at genes that are almost turned off.

Nucleosome positioning is known to be determined by the combination of DNA sequence, nucleosome remodelers and transcription factors, including the pre-initiation complex, activator and the elongating Pol II that impose ordered nucleosome arrays along the transcribing coding region (Struhl and Segal, 2013).

In human cells the data are controversial. Genome-wide deep sequence experiments have shown that the majority of human genome has substantial flexibility of nucleosome positioning and the cellular environment often can drive nucleosomes to occupy what would be unfavorable DNA elements, or evict nucleosomes from favorable sites (Valouvel et al. 2011).
Conversely, a high-resolution map of nucleosome occupancy obtained by paired-end sequencing of micrococcal nuclease-digested chromatin (MNase-seq) data, revealed that most nucleosomes have more consistent positioning then expected by chance and the 8.7% of nucleosomes have moderate to strong positioning. This work concluded also that almost half of the genome contains nucleosome arrays enriched in active insulators, promoters and enhancers, but depleted within actively elongating genes (Gaffney et al., 2012).

Related to the nucleosome positioning is the nucleosome occupancy concept. It reflects the percentage of genomic DNA occupied by nucleosomes.

Segal et al., in 2008 extended their model published in the former work to make a prediction of the whole yeast genome occupancy, and they found around 68% of genome is occupied by histone octamers, thus many DNA regions are nucleosome depleted.

Through which mechanisms nucleosome positioning can be modulated to regulate DNA accessibility?

Jiang and Pugh reviewed three possible ways: 1) DNA accessibility without catalysis where DNA regulatory sites reside near the entry and exit site of nucleosomes, inducing only a partially disassembled state; 2) DNA accessibility through ATP-dependent remodeling complexes that can drive nucleosome dynamics in different ways among which nucleosome sliding might be important to regulate DNA access to sites close to the nucleosome borders; 3) the nucleosome eviction mechanism that can occurs as response to environmental stresses or signals, leading to transcriptional reprogramming (Jiang and Pugh, 2009).

5. MODELLING NUCLEOSOME POSITIONING

Computer modeling can help in understanding many properties of the genome structure. DNA (with or without nucleosomes) can be modeled at many different levels. At the atomic resolution, DNA can be modeled using molecular dynamics simulations. However, due to the very long computational times required for

modeling a large biological structure, models often incorporate coarse-graining: collections of atoms are considered as one unit that behaves as a rigid object. The model can then incorporate varying levels of details and interactions, such as bending and rotation of the adjacent segments and long range interactions between non-adjacent segments (Langowski, 2006). These interactions may be due to DNA-DNA interactions, DNA-nucleosome interactions and/or nucleosome-nucleosome interactions. In the most simple scenario, the Gaussian Chain Model assumes that the links between the individual segments are freely jointed and each segment can assume any orientation. In the case of a Worm Like Chain model on the other hand the successive segments are correlated and point in a similar direction as long as they are smaller than the persistence length of the polymer (Langowski and Heermann, 2007) .

A group of physicists presented a Monte Carlo model for

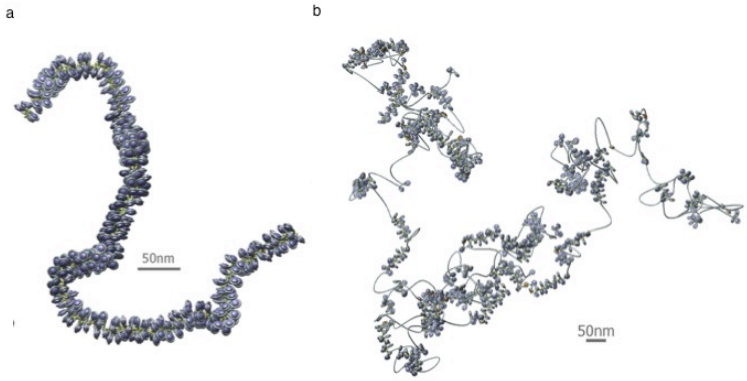


Figure 5. Examples of chromatin conformation with or without depletion effects. The light blue tubes represent the DNA, the histone octamers are modeled as purple cylinders, and the linker histone are marked in light yellow. In (a) the conformation of a chromatin strength of length 40 Kbp without depletion effect and a diametr of ~34 nm. In (b) conformation of a chromatin fiber with depletion effects: linker histone skip rate of 6% and nucleosome skip rate of 8% is shown. The fiber has a total length of 394 kbp. Adapted from Diesinger and Heermann, 2009).

genome folding into the 30-nm scale to investigate the impact of the linker length and nucleosome depletion on the chromatin compaction and on the flexibility of the chromatin fiber (Diesinger and Heermann, 2009). Using experimental data from Widom's work on NRLs distribution and the prediction on nucleosome occupancy of Segal et al. in 2008, they found that parameter distributions do not lead to one specific chromatin fiber but rather to a wide distribution of structures.

They showed that depletion effects lead not only to a much more flexible fiber but also to a more coiled conformation then one would expect from existing chromatin models of higher regular order compaction (Figure 4).

The concept of a regular 30-nm fiber no longer holds, since it needs to be completly saturated with nucleosomes, a situation that seems unlikely also from a thermodynamic point of view. A linker histone skip rate of 6% and a nucleosome skip rate of 8% (the average depletion rate in yeast genome) give a very flexible coil like structures of compact regions separated by naked DNA.

6. NUCLEAR ARCHITECTURE

6.1 HETEROCHROMATIN AND EUCHROMATIN

The work of Heitz around 1982 leads to the distinction of two different chromatin structures inside an interphase nucleus. Based on differences visible through a new in situ method suitable for chromosomal stains, he suggested the term heterochromatin for the regions of chromosomes that remain

condensed through interphase and the term euchromatin for the regions of chromosomes that become invisible at late telophase (Heitz, 1928).

Electron micrograph of many interphase nuclei also have shown clear electron dense patch of chromatin along the nuclear envelope and around the nucleolus, thought to be the higher order structured chromatin. The functional genome distinction between heterochromatin, which corresponds to the more compact one and it is associated with inactive genes, and euchromatin, which is more open and associated with actively transcribed genes, nowadays remains used and has been characterized by biophysical chromatin fractionation or chromatin immunoprecipitation (ChiP) methods (Rodriguez and Bjerling, 2013).

Within euchromatin histone's tails are enriched in acetylation and in di/tri-methyl group on H3 Lysine 4 (H3K4me2/3), instead heterochromatin is characterized by low transcriptional activity, low abundance of genes, many repetitive sequences, hypoacetylation and high amounts of di/tri-methyl groups on H3 Lysine 9 (H3K9me2/3). This last modification is also important for the recruitment of the heterochromatin protein 1 (HP1) through its chromodomain (Fischle, Tseng et al. 2005).

Heterochromatin is further divided into constitutive heterochromatin that is always compact, found mainly at the telomers and the centromeres of chromosomes where it assembles on repetitive regions and in facultative heterochromatin that can reversibly undergo transition from a

compact and silent state to a more open and transcriptionally active state, as for example it happens in differentiated cells during their reprogramming to pluripotency (Fussner et al., 2011).

6.2 ATTACHMENT TO NUCLEAR LANDMARKS

The interactions of the genome with the nuclear 'landmarks' also seem to give an important contribution to the folding of chromosomes (Figure 6).

The nuclear lamina (NL), composed by lamin polymers, coats the inner nuclear membrane providing a big surface area for potential contacts with the genome. Several studies have shown that also in human the genome interacts with the NL via the lamina-associated domains (LADs) which have a median size of 0.5 Mb and cover about 35-40% of the genome (Guelen et al., 2008). Using DNA adenine methyltransferase identification technique (DamID) it has been found that most genes in LADs are transcriptionally silent indicating that the NL contributes to gene repression (Guelen et al., 2008). Furthermore the contact of LADs to NL is dependent on the activity of the methyl H3K9 methyltransferase G9a and high H3K9me2 (Kind et al., 2013). In flies lacking one of the lamins a derepression of NL-associated genes was detected (Sheveloyev et al., 2009). In accordance with this theory,

during differentiation, a reorganization of the LADs was identified and several genes were observed to move from the NL (Peric-Hupkes et al., 2010).

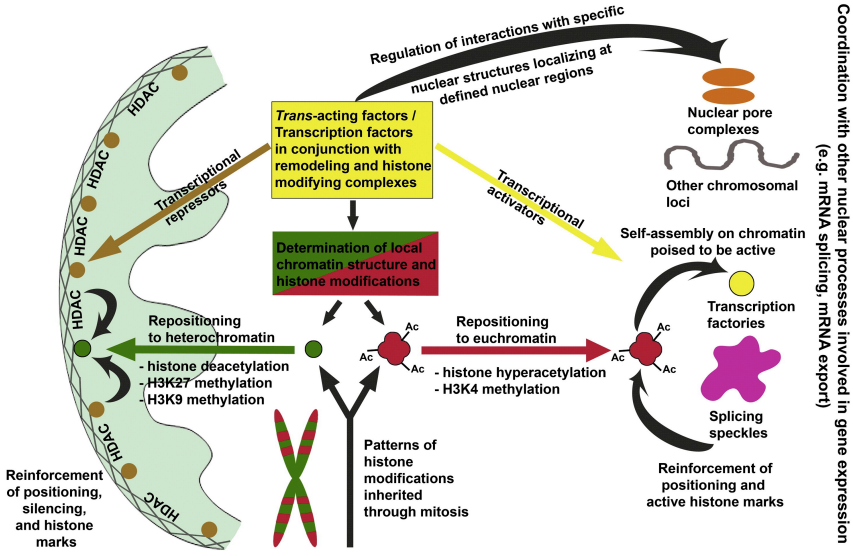


Figure 6. Regulation of chromatin positioning and of transcriptional activity at specific nuclear regions. Sequence-specific transcription factors in conjunction with chromatin remodeling and histone modifying complexes determine local chromatin structure and histone modifications (dark-green: condensed, silenced, hypoacetylated and other "inactive" histone marks; red with Ac: decondensed, active, hyperacetylated and other "active" histone marks). Radial chromatin positioning, interactions with the lamina, and differential associations with heterochromatic and euchromatic nuclear areas might be determined by local chromatin structure and histone modifications and in particular by the patterns of histone acetylation (red and green arrows pointing to the different nuclear regions and sub-structures). The patterns of histone acetylation of the corresponding different chromosomal domains are inherited through mitosis (red and green banding pattern on mitotic chromosome) and this might provide a pathway for the re-establishment of chromatin arrangements and radial nuclear order after mitosis (bottom, black arrows). Transcription factors are not evenly distributed in the nuclear space and transcriptional repressors (brown dots) are enriched at the nuclear periphery (brown arrow), while transcriptional activators are enriched in areas containing transcription factories (yellow arrow). At the nuclear periphery the

enrichment in transcriptional repressors and histone deacetylase (HDAC)3-interacting with the nuclear lamina and associated INM proteins (grey Xs) – might contribute to a reinforcement (left, black arrows) of histone marks, positioning, and silencing of chromatin residing in this area (silenced locus: dark-green dot, perinuclear heterochromatin: light-green).

At active nuclear regions (right) trans-acting and transcription factors associated with chromatin modifying complexes regulate interactions with other structures involved in gene expression (top, black arrow), such as transcription factories (yellow) and nuclear pores (orange) . In addition, gene loci (grey) also interact with each other and such interactions can take place while loci are associated with other nuclear structures. Interaction with other components like nuclear pores and splicing speckles (pink) might also help to coordinate transcription with pre-mRNA splicing and RNA export. The formation of transcription factories and of splicing speckles in the nuclear interior might be driven by self-assembly on active chromatin and chromatin poised to be active harboring corresponding histone marks and localizing in the area (black arrow, right, middle). Self-assembly on corresponding chromatin domains and other interactions with active chromatin/chromatin poised to be active might also confine these domains to the active nuclear interior. Conversely, interactions with transcription factories and splicing speckles and other domains might stabilize and refine chromatin positioning in this nuclear area and reinforce and refine local chromatin structure and histone modifications (black arrow, right, bottom). Fedorova and Zink, 2008.

Another landmark is the nuclear pore complexes (NPCs) that are located in gaps in the NL and interact with specific genomic loci. However, it has become clear that most of NPC proteins freely diffuse over the nucleoplasm and often interact with the target genes in the interior of the nucleus rather than at the pore complex (Capelson et al., 2010; Kalverda et al., 2010).

Last landmark that may provide an anchoring platform for the genome is the nucleolus. Recent mapping studies have identified many nucleolus-associated domains (NADs), which tend to harbor specific chromatin domains from most human chromosome (Nemeth et al., 2010; van Koningsbruggen et al.,

2010). Some NADs seems to overlap with LADs, suggesting that there are genomic regions contacting both NL and nucleoli (van Steensel, 2011).

6.3 CHROMOSOME TERRITORIES

The question on how whole chromosomes are spatially organized within the nucleus interested scientists since the 19th century. Carl Rabl in 1885 studying epithelial cells from Salamandra, formulated a first hypothesis regarding a territorial chromosome arrangement in the interphase nucleus, later on Theodor Boveri introduced the term chromosome territory (CT) in his studies on blastomere stages. He argued that CT order is maintained during interphase and that the chromosome neighborhood pattern change from prophase to metaphase, when new arrangements happen, then conserved through anaphase and telophase and resulting in a symmetrical arrangement of the CTs in the two daughter nuclei (Boveri, 1909). With the advent of electron microscopy however, high-resolution images of interphase nuclei showed intermingle of chromatin fibers of about 10-30 nm structures with no evidence of individual chromosomes. Thus the CT theory was replaced by the 'spaghetti' theory with the nucleus filled of intermingling chromatin fibers, until the development of fluorescence in situ hybridization (FISH) and confocal microscopy technicques provided evidence for a nonrandom radial arrangement of CTs. A multicolor 3D FISH, using labeled chromosome painting probes, allowed the colorful discrimination of the 22 pairs of

autosomal CTs and the two sex chromosome CTs in a diploid human fibroblast (46,XY) (Bolzer et al., 2005).

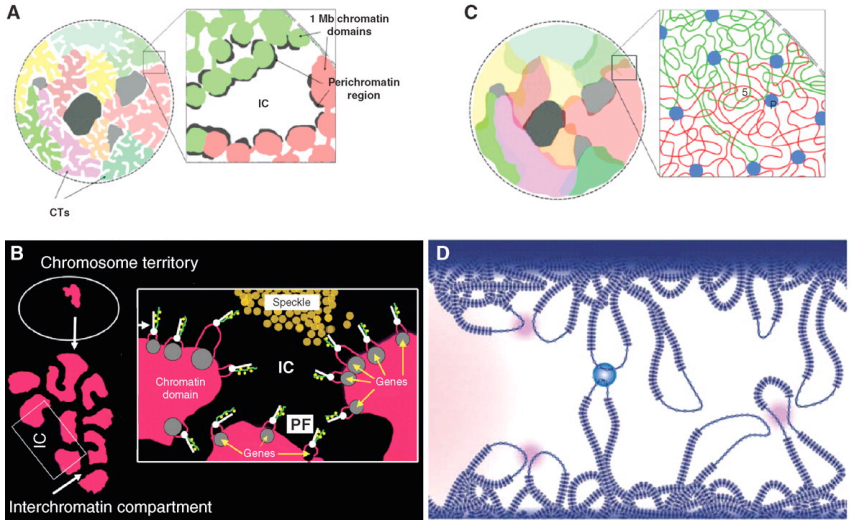


Figure 7. Different models of nuclear architecture. (A) Chromosome territory-interchromatin compartment (CT-IC) model (for description, see text). (B) Hypothetical view of the functional nuclear architecture according to the CT-IC model. Chromatin domains are considered the major constituents of a CT. The IC expands between these domains as a rather DNA free nuclear compartment carrying splicing speckles and nuclear bodies. The width of the IC space is highly variable depending on Brownian movements of chromatin domains and allowing transient contacts of domain surfaces in cis and trans. During ongoing transcription, genes are at least partially decondensed at any given time into the perichromatin region (PR) located at the domain periphery. Perichromatin fibrils (PF) are generated there. Each PF carries a nascent transcript (green) from a different gene. White dots with a line symbolize RNA Pol II molecules with their CTD domain, which may play a role in the structural organization of splicing events. Splicing speckles located in the IC provide the splicing factors to PFs, which also represent the structures in which cotranscriptional splicing occurs. (C) According to the interchromatin network (ICN) model (Branco and Pombo 2006), intermingling chromatin fibers/loops from the same CT, as well as from neighboring CTs, can make contact in cis and trans. Blue dots represent sites of intrachromosomal and interchromosomal contacts with unknown composition. Although there is extensive space between chromatin fibers/loops, this space should not be confused with the functional relationship of the IC and PR

predicted by the CT-IC model. (D) Model suggested by Fraser and Bickmore (Fraser and Bickmore 2007, figure reprinted with permission from Macmillan Publishers Ltd). These authors review evidence arguing for the colocalization of genes in the nucleus for expression or coregulation. Transcription factories (dark pink) can recruit genes in cis and trans located on decondensed chromatin loops that extend outside chromosome territories. The pale pink area on the left represents a splicing-factor enriched speckle. The blue circle exemplifies an interaction for coregulation in trans, which can occur between regulatory elements and/or gene loci. From Cremer and Cremer, 2010.

Although many studies tried to find general determinants of CT spatial distribution in the interphase nucleus, it seems to be more an interplay of factors playing a role.
Active genes for example colocalize to shared sites of ongoing transcription (Osborne et al., 2004), CTs architecture seems to change during differentiation (Stadler et al., 2004), in a given cell the relative position of a gene into a CT is conserved through interphase and between daughter cells (Strickfaden et al., 2010).

Despite the organization of the CTs is an accepted principle further supported by the DNA interaction maps obtained by 3C experiments, discussion is still open on the nuclear architecture and there are two are the proposed models (Figure7):

- The chromosome territory-interchromatin compartment (CT-IC) model arguing that nuclei are built up from two principal components, chromosome territories CT and interchromatin compartment (IC) (Cremer et al., 1995), asserting a DNA free space in which there are splicing speckles and non-chromatin nuclear bodies.
- The interchromatin network (ICN) model predicts that chromatin fibers and loops intermingle in a rather uniform way both in the

interior of individual CTs and between differentially labeled neighboring CTs, making any distinction between the interior or periphery of distinct chromatin domains functionally meaningless. In this model loops can extend from one CT to meet loops from another CT (Branco and Pombo, 2006).

What is clear though, is that chromatin is dynamic and its active nature directly influences genome activity and nuclear functions (Gasser, 2002; Misteli, 2001)

6.4 NUCLEAR ARCHITECTURE IN EMBRYONIC STEM CELLS AND IN DIFFERENTIATED CELLS

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of a blastocyst before implantation in the uterus and are characterized by two main features: the capacity to self-renew indefinitely in culture and the potential to differentiate into all derivatives of the three germ layers (ectoderm, endoderm, mesoderm).
The ability of self-renewal requires the ES cell genome to divide maintaining the pluripotent potential, on the other hand differentiation requires complex changes in gene expression to commit the ES genome towards any specific lineage. Key events during the process of differentiation are the selective silencing and activation of a specific subset of genes, however it is becoming well demonstrated that chromatin plays an important

role for pluripotency, stem cell identity, regulation of differentiation and cellular fate (Mattout and Meshorer, 2010).
Starting from the functional compartmentalization of the ES cell nucleus, the first big difference with a differentiated nucleus, is the absence of the inner nuclear lamina. As described in the previous section 6.2, nuclear lamina is composed by a meshwork of filaments interspersed with lamina-associated proteins and interacts through the LADs with chromatin causing down-regulation of gene expression and accumulation of facultative heterochromatin. Both human and mouse ESCs only express the subtypes B1 and B2, lacking the lamina subtype A/C that appears just during differentiation. Absence of lamin A has been related to contribute to the plasticity and chromatin mobility typical of an ESC nucleus (Pajerowski et al., 2007).

The spatial distribution of CT has been found to be similar in ESCs and differentiated cells, meaning that the functional positioning of chromosomes is already established at the blastocyst stage. Looking at the location of specific genes within the CT, a relocation from a more interior part to a more peripheral position has been observed, as demonstrated for the pluripotent *Oct4* locus that is decondensed and on the outside of its CT in human ESCs but within the interior of the CT in lymphoblastoid cells (LCLs) (Wiblin et al., 2005). In addition, CT containing pluripotency genes, such as the region containing the human chromosome 12 that harbors the locus for the stem cell marker *Nanog,* is embedded in a region surrounded by other pluripotency genes and in a more central position in ESCs compared to LCLs (Wiblin et al., 2005).

Other higher-order features of ESC chromatin structure is the location of the centromere clusters that localize more towards the nuclear interior or around the nucleoli in ESCs than in differentiated cells where centromeric markers such as α-satellite/CENP-A, have been found on the nuclear periphery (Bartova et al., 2008).

A functionally important hallmark of pluripotency is also the higher mobility of architectural chromatin. Using fluorescence recovery after photobleaching (FRAP) it was demonstrated that the heterochromatin protein 1 (HP1α) and the linker histone H1 exist in a hyperdinamic, loosely bound or soluble fraction in ESCs with a reduction of protein mobility during differentiation (Meshorer et al., 2006). The same authors of this work suggested that the dynamic nature of chromatin-associated proteins might also help the regulatory factors to gain access to regulatory regions and activate for example lineage specific gene expression (Meshorer and Misteli 2006).

More in general, comparison of heterochromatin domains marking HP1α protein or using DNA probe against the major satellite repeat, revealed many distinct heterochromatin foci in differentiated neuronal precursor cells (NPCs), whereas in ESCs only diffusely labeled structures were observed.
Looking more close to epigenetic marks, a unique feature of the ESC genome is the presence of the so-called 'bivalent domains' consisting of large regions of the repressive histone modification H3K27me3 harboring small regions of the active mark H3K4me3. These regions overlay with developmental specific genes that in ESCs are expressed just at very low levels but they

are kept poised for activation or silencing upon differentiation towards a specific cell lineage (Bernstein et al., 2006). Furthermore histones in ESCs are enriched in specific post-translational modifications that have been correlated with a more transcriptionally active chromatin, such as acetylation (histone H3 and H4 acetylation) and H3K4me3. Upon differentiation a reduction of these marks and an increase of several repressive epigenetic signals has been found, like the mono, di-, tri-, methylation of the Lys9 on histone H3 (H3K9me1/-me2/-me3) (Meshorer et al., 2006; Wen B. et al., 2009; Hawkins RD et al., 2010; Krejcí J. et al., 2009).

The general idea of open chromatin in pluripotent stem cells implies an overall less condensed structure with a ratio between euchromatin and heterochromatin that is higher with respect to differentiated cells (Morris, Chotalia and Pombo, 2010; Mattou and Meshorer, 2010).

6.5 CHROMATIN REORGANIZATION LEADS TO SOMATIC CELL REPROGRAMMING

Somatic cell reprogramming can be referred to as the transition from one cell type into another. There are two major types of reprogramming: reprogramming of differentiated cells into pluripotent cells; and lineage reprogramming of differentiated cells into different somatic cells (transdifferentiation). Different techniques have been used to induce reprograming: nuclear

transfer (Gurdon et al., 1958), when a somatic nucleus is transferred into an enucleated oocyte, to produce a cloned animal; cell fusion between ESCs and somatic cells, which results in fully reprogrammed hybrids that can be selected as heterokaryons with two separated nuclei, or as synkaryons with one tetraploid nucleus (Tada et al., 1997; Silva et al., 2006; Lluis et al., 2008; Pereira et al., 2008; Bhutani et al., 2010); and direct reprogramming, via transduction of specific transcription factors, such as Oct4, Klf4, Sox2 and c-Myc, to generate induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006, Stadtfeld and Hochedlinger, 2010).

The potential and efficiency of the reprogramming process varies between cell types and very little is known about the molecular mechanisms that promote it. However, several experiments have shown that the cascade of events in somatic cell reprogramming to a pluripotent state involves genome-wide changes in the euchromatic histone modifications followed by large-scale epigenetic remodeling to reactivate genes that are essential for pluripotency and to silence somatic genes (Gaspar-Maia, Alajem et al., 2011).

Luis et al., 2008 demonstrated that the activation of the Wnt/β-catenin pathway, enhances reprogramming of a variety of somatic cells, furthermore the deletion of Tcf3, a transcriptional repressor factor of β-catenin target genes in ESCs greatly enhances the efficiency of cell-fusion-mediated reprogramming (Lluis F et al., 2011). Tcf3 deletion increases AcH3 and decreases the number of H3K9me3 heterochromatin foci, thus reprogramming by TCF3 deleted mESC is due to massive

epigenome modifications in the somatic genome that occur very early and before re- expression of the endogenous stem-cell genes. Therefore overcoming the epigenetic barriers is highly important for enhancing efficient reprogramming and favouring pluripotent ground state.

Fussner and co-workers characterized the physical structure of heterochromatin domains in iPS cells by correlative electron spectroscopic imaging (LM/ESI) with indirect labeling of H3K9me3 to delineate chromocentres.

Phosphorus density analysis demonstrates that the chromocenters of ESCs and in fully reprogrammed iPS cells, with high Nanog level, are difficult to delineate from the surrounding chromatin and are characterized by dispersed open domains comprised of 10 nm chromatin fibers. In contrast chromocenters in differentiated MEFs and partial iPS cells, with low Nanog level, were densely packed. The conversion of the partial iPS cells to a pluripotent state, by culturing the cells in a media containing MEK/GSK3 2i inhibitor (Silva et al., 2008), has been shown to be accompanied by the dispersion of the densely packed heterochromatin fibers (Fussner et al., 2011).

These results imply that heterochromatin reorganization is dependent on the establishment of the pluripotent network, moreover they are in accordance with the previous observation that chromatin in ES cells is mainly composed of dispersed chromatin fiber (Efroni et al., 2008) and suggests that the transition between ‘open’ and ‘close’ domains means in part a transition from dispersed to densely packed 10 nm fibers.

7. IMAGING THE CHROMATIN

7.1 ELECTRON MICROSCOPY BASED TECHNIQUES

Microscopy techniques have been essential to study chromatin organization.

EM gave important clues in understanding chromatin structure. First it suggested the sub-compartimentalization of the mammalian nucleus in hetero- and euchromatin, revealed by electron dense regions along the nuclear envelope and around the nucleoli (Heitz et al., 1928). Then electron micrographs showing the 'nu bodies' led to the discovery of the fundamental repeating unit of the chromatin arranged into the 'beads-on-a-string' fiber (Olins and Olins 1974; Woodcock et al., 1976). EM, Cryo-EM, or AFM have been used to study the compaction of the 10 nm fiber in presence of the linker histone H1, high salt concentration or different linker lengths (Thoma et al., 1979; Woodcock et al., 1984; Routh et al., 2008; Clauselle et al., 2009).

Although the optical resolution achievable with electron microscopy is in the order of nanometers, the heavy atom contrast reagents required to coat biological structures obscure the high-resolution details of the chromatin fibers. Moreover different biochemical structures in a nucleus have different affinities for contrast agents creating the illusion of electron-dense and mass-depleted regions without a molecular specificity. If cryo-EM in part overcame limitations due to the

harsh sample preparation because it retains chromatin in a hydrated environment, on the other hand it works only with extracted chromatin fibers or metaphase chromosomes but not in intact interphase nuclei, mainly for the low contrast of chromatin *in situ* in interphase nuclei. Step forward was the advent of Electron spectroscopic imaging (ESI), a specialized form of transmission electron microscopy based on electron energy loss spectroscopy. It enables the visualization of phosphate and nitrogen in formaldehyde- fixed cells, without any other heavy contrast agents, thus chromatin serves as the best cellular structures readily visualized by ESI. Nucleolus, nuclear pore complex, compact chromatin domains at the nuclear periphery or surrounding the nucleolus and highly dispersed fibers of open chromatin domains could be easily distinguished in interphase nuclei of many cell types (Bazett-Jones et al., 2008; Efroni et al., 2008; Ahmed et al., 2010; Fussner et al., 2011). ESI gained resolution with respect to EM but remains restricted to the phosphorus and nitrogen context.

7.2 LIGHT MICROSCOPY TECHNIQUES

Light microscopy allows intact samples and living cells to be studied in their natural environment and to image almost any structure inside the cell with high molecular specificity. The vast toolbox of fluorescence probes and the increasing number of available fluorescent proteins, make fluorescence light microscopy the method of choice in the majority of applications in life sciences.

Chromatin structure however has been hard to visualize by this powerful technique which is limited by the intrinsic wave-like nature of light, the so called 'diffraction limit' of spatial resolution (Figure 8).

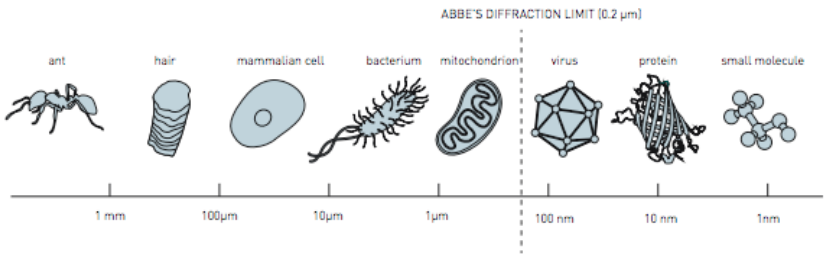


Figure 8. Lenght scales in life biology. At the end of the 19th century, Ernst Abbe defined the limit for optical microscope resolution to roughly half the wavelength of light, about 0.2 micrometer. Adapted from 'How the optical microscope became a nanoscope' (http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2014/popular-chemistryprize2014.pdf)

Even a point source, when observed with a light microscope, results in a broad image. This phenomenon has been studied by Ernest Abbe, it is due to the diffraction of light and depends on the wavelength and the finite size of the objective lens of the microscope. Critically for fluorescent microscopy this limit means also that it is impossible to resolve two elements of a structure which are closer to each other than about half the wavelength (λ) in the lateral (x,y) plane and even further apart in the longitudinal plane (z), thus two objects within a distance of between 400/2=200 nm (far blue) and 700/2=350 nm (far red) cannot be resolved.

Although conventional fluorescence microscopy allowed large-scale nuclear organization studies, showing the existence of CT for example, and the further derived novel tools, such as fluorescence recovery after photobleaching (FRAP) (Belmont, 2001; Lippincott-Schartz and Patterson, 2003; Misteli, 2001), fluorescence loss in photobleaching (FLIP) (Dundr and Misteli, 2003), fluorescence correlation spectroscopy (FCS) (Weidemann et al., 2003) and fluorescence resonance energy transfer (FRET) (Rao et al., 2007), were useful to follow nuclear proteins dynamics and protein-chromatin interactions, it has not possible to access the nanoscale structure of the chromatin fiber.

7.3 SUPER-RESOLUTION MICROSCOPY

The diffraction limit has been recently overcome by the development of new 'super resolved fluorescent microscopes', cutting edge optical techniques that receive the Nobel Prize in Chemistry in 2014.

The first to be implemented was the stimulated emission depletion (STED) of fluorescence from all molecules in a sample except those in a small region of the studied object, which can be made arbitrarily small then the diffraction-limited size (Klar and Hell 1999). Based on a similar principle of "structuring the illumination light", in 2005 the saturated structured-illumination microscopy (SSIM) was developed (Gustafsson 2005).

The second principle is based on the a priori knowledge that all the photons coming from individual fluorophores that are separated from each other by distances larger than the Abbe's limit can be detected, and the position of their emitting point sources can be estimated with precision much higher than that allowed by the diffraction limit. Thus, based on this principle, in 2006 the stochastic optical reconstruction microscopy (STORM) (Rust, Bates, and Zhuang 2006), photoactivated localization microscopy (PALM) (Betzig et al., 2006), and fluorescence photoactivation localization microscopy (fPALM) (Hess, Girirajan, and Mason, 2006) were demonstrated. All these techniques made it possible to improve the nanometer-scale resolution with far field fluorescence microscopy by one order of magnitude (~20 nm in the lateral and ~50 nm in the axial dimensions).

7.3.1 STORM

As anticipated in the previous section localization microscopy relies on the possibility to identify the precise position of individual fluorescence emitters when their images separated by distances larger than the diffraction limit,($\lambda/2NA$) and thus not overlapping.

STORM combines the single molecule localization concept and the fluorophore photoswitching concept (Patterson andLippincott-Scwartz, 2002, Bates, Blosser, and Zhuang, 2005, Hailemann et al., 2005). Photoswitching makes it possible to "turn off" most fluorophores into a dark state and "turn on" only a

small subset of them at a time (Figure 9). As a result, the images of the "active" fluorophores are isolated in space and their positions can be localized with high precision. Once all the fluorophores are imaged and their positions are localized, a high-resolution image can be reconstructed from these localizations.

Photoswitchable fluorophores can be small organic dyes or fluorescent proteins. Organic dyes can switch many times between a dark and a bright state. In STORM, A647, Cy5.5 and Cy7 are often combined with a second fluorophore such as Alexa Fluor 405 (A405), Cy2, A488, or Cy3 in an activator-reporter pair configuration to increase the photoswitching efficiency and to facilitate multi-color imaging (Bates et al. 2007, Bates et al. 2012). With the pair configuration, the fluorescent state of the reporter (normally the red or near infrared dye) can be recovered upon illumination of the activator dye with the corresponding wavelength laser. These fluorophores are typically linked to antibodies or nanobodies, which are used to immunostain the sample.

Fluorescent proteins can be photoactivatable (from a dark to a bright state) or photoconvertible (from one state to another state with different spectral properties) or photoswitchable fluorescent proteins (reversibly photoactivable). Examples of photoactivable proteins are PA-GFP and PA-mCherry (Patterson andLippincott-Scwartz, 2002, Subach et al., 2009); Dendra2 and mEOS2 are examples of typically used green to red photocovertible proteins upon UV light illumination (Wiedenmann et al., 2004, McKinney et al., 2009, Gurkaya et al., 2006, Zhang et al., 2012) (See Oddone A. et al., 2014).

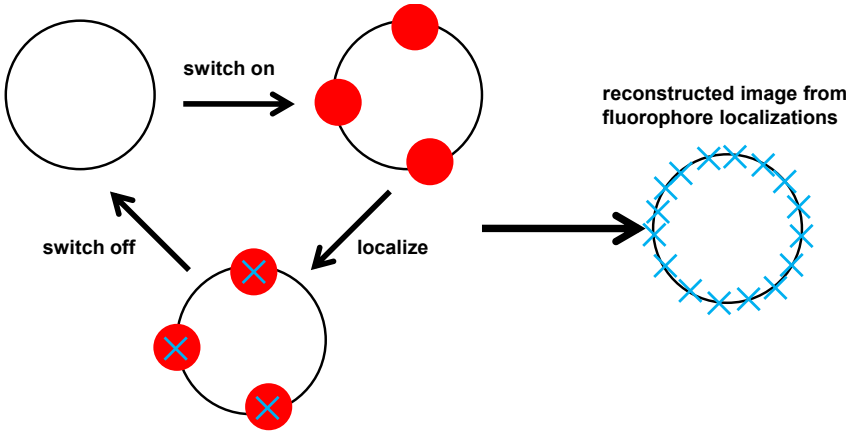


Figure 9. Single molecule detection and localization. Initially the fluorophores labelling the sample are in a dark state. Using light excitation, a small subset of these fluorophores is "switched on" and their positions are precisely localized before they switch off. Through repeated cycles of activation, localization and de-activation, a super resolution image of the underlying structure (here given by a small circle) can be reconstructed from fluorophore positions. **Adapted from Lakadamyali, 2012.**

Super-resolution imaging has revealed the organization of microtubule cytoskeleton (Huang et al., 2008a), actin cytoskeleton (Xu et al., 2013), and nuclear pore complexes (Szymborska et al., 2013) with an unprecedented level of detail. It has also been used to visualize chromatin in interphase (Benke and Manley, 2012; Bohn et al., 2010; Markaki et al., 2010; Wombacher et al., 2010; Zessin et al., 2012) and dividing nuclei (Matsuda et al., 2010).

Up to date, however, the super-resolution studies of DNA and histones have not addressed questions regarding the organization of single or groups of nucleosomes, the overall nucleosome occupancy level of DNA, whether these parameters are consistent with the 30 nm fiber model of chromatin and the

link between chromatin organization and the pluripotency state of a cell.

Aims

This project has been developed in collaboration with Dr. Prof. Melike Lakadamyali at ICFO (The Institute of Photonic Sciences, Castelldefels, Barcelona).
The main objectives of the project were:

- To dissect the structure of chromatin fibers at high-resolution level and in single cells.

- To identify chromatin organization in embryonic pluripotent cells and it's changes during differentiation and reprogramming.

- To understand whether chromatin fiber structure might be used as marker for pluripotency or differentiation.

PART II

RESULTS

Chromatin Fibers Are Formed by Heterogeneous Groups of Nucleosomes In Vivo

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Chromatin fibers are formed by heterogeneous groups of nucleosomes in vivo

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Keywords: STORM; Chromatin; histones; iPSC; induced Pluripotent Cells; ESCs; Embryonic Stem Cells; pluripotency

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PART III

DISCUSSION AND CONCLUSIONS

Discussion

Understanding chromatin structure, the complex of DNA with all its associated proteins, has always been of big interest for scientists since the time it became clear that chromatin has not only the structural function to package the DNA but play key roles in many cellular processes like transcription, development, differentiation and reprogramming (Gasser, 2002; Misteli, 2001; Wolffe AP and Gushin D, 2000).

The use of electron microscopy techniques gave important clues for the identification of nucleosomes and the compaction of the DNA fiber from the primary structure "beads-on-string" (Olins and Olins, 1974) into highly ordered arrangement, such as the one-start solenoid or the two-start helix structure (Woodcock and Ghosh, 2010). The advent of fluorescence microscopy then, was fundamental to highlight and characterize the histone's tail modifications, the compartmentation into hetero- and euchromatin and the chromatin-transcriptional factor interactions, among other features, in different cell states and many cell types (Boveri, 1909; Cremer and Cremer, 2006; Meshorer et al., 2006). Moreover light microscopy was essential to dissect chromatin dynamics (Miyanari et al., 2013).
Light microscopy remains a powerful method to study chromatin structure and function at the single cell level. The advent of super resolution microscopy, moreover, overcomes the diffraction limit allowing imaging at very high spatial resolution.

The breakthrough of our work is the direct visualization of heterogeneous groups of nucleosomes forming the chromatin fibers. The absence of regularly folded chromatin fiber in both heterochromatin and euchromatin compartment has been

recently demonstrated in different cell types (Efroni et al., 2010; Fussner et al., 2011) however the procedures used in those studies lacked molecular specificity and required harsh sample preparation. Here super resolution microscopy allowed us to study nucleosomes organization with high molecular specificity and at nanoscale resolution in single live cells.

STORM images revealed that nucleosomes do not form a highly ordered organization but rather arrange into discrete groups, the clutches, of various sizes and densities, which are interspaced by nucleosome-depleted regions.

Within a single nucleus clutch size is highly heterogeneous but strikingly we found that the median clutch size and clutch density is closely related to the cell type. Quantitative analyses allowed us to count the number of nucleosomes per clutch showing that ground-state pluripotent stem cells have low-density clutches containing on average only a few nucleosomes with respect to differentiated cells.

The link between nuclear architecture and pluripotency has already been demonstrated (Efroni et al., 2008; Fussner et al., 2011), however here we can go further inside the concept of 'open' and 'closed' chromatin domains, characterizing them as low- and high-density nucleosome clutches respectively, and relate clutch size to cellular state.

mESCs cultured in Serum plus the cytokine leukemia inhibitory factor (sLif condition) constitute a heterogeneous population in which some cells are committed to differentiation whereas others maintain the 'ground state' of pluripotency. We were able not only to catch this heterogeneity but also relate the bigger clutch size of some cells to the lower level of the pluripotency marker

Nanog and so to a loss of the naïve state of their transcriptional profile (Marks et al., 2012).

Actually in 2iLif culturing media and in presence of the TCF3 factor deletion, mESCs have all very few nucleosomes per clutches with a low clutch density, data in accordance with the maintainance of the 'ground state' of pluripotency already demonstrated in these conditions (Cole et al., 2008; Tam et al., 2008; Yi et al., 2008).

The organization of nucleosomes in small, low-density clutches in stem cells is a new feature of ESCs and it was so robust that it also highly correlated with and was predictive of the pluripotency grade of hiPSCs at the single cell level. The identification of high-grade pluripotent hiPSCs is time consuming, requiring the generation of teratomas in mice and several additional pluripotency tests. The correlation between the clutch size and the pluripotency grade opens a novel way for the identification of stemness as well as of diversity between cell lines. Indeed, we showed that changes in the assembly of nucleosomes resemble changes in cellular phenotype when passing from pluripotent to somatic state and *vice-versa*. It would be interesting study, for example, nucleosome clutches organization and dynamics during cellular transformation, which mechanism may have big similarities with the reprogramming and differentiations processes.

Our data show also that large clutches with higher nucleosome compaction include more H1, moreover centromeric regions, where constitutive heterochromatin is located, contain ~2 fold higher number of nucleosomes in their clutches compared to the rest of the nuclei. Whereas the small clutches with lower

nucleosome compaction likely correspond to active chromatin regions since they are associated to RNA Polymerase II.
Nuclear periphery contains heterochromatin associated to silenced regions of the genome, here in fact we found an enhanced density of clutches compared to the nuclear interior. In a recent study, A. Olins and D. Olins investigated the organization of the Nuclear envelope-limited chromatin sheets by three different methods and interestingly they found that the fiber thickness was variable but primarily ~10 nm, with occasional thicker fibers (Eltsov et al., 2014).
These data are in agreement with latest evidence against the hierarchical model of chromatin compaction and suggest that interphase chromatin forms numerous condensed chromatin domains consisting of irregularly folded 10-nm nucleosome fibers (Maeshima et al., 2010, Joti et al., 2012).

It cannot be excluded that within certain clutches, nucleosomes arrange in a regular structure with ~30 nm diameter, since this is beyond our resolution limit. Nevertheless our in-silico model could recapitulate the experimental results including as variables only nucleosome removal or linker-DNA length modifications and without constrains for specific arrangement such as the 30 nm fiber. Our findings are overall consistent with a 'mixed' chromatin fiber model likely including areas of 10 nm which allow RNA Polymerase II accessibility and areas of larger size which are the heterochromatin regions, which are enriched in H1.

Nucleosome arrangement on the genome can modulate accessibility of regulatory proteins on DNA, further nucleosome

positioning and nucleosome occupancy are subject of high interest. Considering an average linker-DNA length of around 50 bp between subsequent nucleosomes (Kornberg, 1977; Valouev et al., 2011; Widom, 1992) a fully occupied fiber by nucleosomes would correspond to a DNA occupancy of around 75%.

The comparison of STORM images of nucleosome clutches to the nucleosome removal and linker-DNA length modification models also allowed us to calculate the nucleosome occupancy probability in human fibroblast cells, which we estimated to be around 60%, corresponding to an average linker-DNA length of around 100 bp. Although nucleosome occupancy in human cells is not known, our result determined from single cells, provide experimental evidence for previous modeling studies, which predicted that a heterogeneous fiber with nucleosome or histone linker depleted regions, corresponding to a nucleosome occupancy in yeast of around 65%, is more flexible and can fit more optimally inside the nuclear space (Diesinger and Heermann, 2009). Furthermore our result are consistent with genome wide population studies that found nucleosomes depleted regions at many enhancers, promoters, transcription starting sites and terminator region in human cells (Valouvel et al., 2011). Indeed, we found that after TSA treatment, which is known to lead to chromatin decondensation and transcriptional activation, occupancy decreased to around 45%.

In the future would be really interesting the visualization and the clutches organization analysis at specific gene loci during reprogramming or differentiation. DNA fiber staining by FISH, coupled with super resolution microscopy is possible but sample preparation may alter chromatin structure and is not compatible with live imaging. The recently developed CRISPR-based

(clustered regularly interspaced short palindromic repeats) techniques have already been used for sequence-specific visualization of genomic elements in living human cells, with robust results for the imaging of repetitive elements, such as in telomeres regions (Chen B. et al., 2013). Likely CRISPR technology coupled with a new versatile platform called 'Sun-tag', which allows the amplification of single protein signal in living cells (Tanenbaum et al., 2014), will make possible the gene locus imaging also in non-repetitive regions.

Conclusions

1. Nucleosomes do not form a highly ordered organization but rather arrange into discrete groups, the clutches, of various sizes and densities, which are interspaced by nucleosome-depleted regions;

2. There is a striking correlation between spatial distribution, size and compaction of nucleosome clutches and cell pluripotency;

3. Ground-state stem cells have low-density clutches containing on average only a few nucleosomes;

4. Large clutches with higher nucleosome compaction corresponds to heterochromatin and include more H1,

5. Small clutches with lower nucleosome compaction correspond to active chromatin regions since they are associated to RNA Polymerase .

ANNEX 1

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ANNEX 2

Abbreviations

AFM	Atomic force microscopy
CHIP	Chromatin immunoprecipitation
CRISPR	Clustered regularly interspaced short palindromic repeats
CT	Chromosome territories
CT-IC	Chromosome territory-interchromatin compartment
DamID	DNA adenine methyltransferase identification
EM	Electron microscopy
ESCs	Embryonic stem cells
ESI	Electron spectroscopic imaging
FCS	Fluorescence correlation spectroscopy
FISH	Fluorescence in situ hybridization
FLIP	Fluorescence loss in photobleaching
FRAP	Fluorescence recovery after photobleaching
FRET	Fluorescence resonance energy transfer
HFbs	Human fibroblast cells
HP1	Heterochromatin protein 1
ICM	Inner cell membrane
ICN	Interchromatin network
INM	Inner nuclear membrane
iPS	Induced pluripotent stem
LAD	Lamina associated domain
LCLs	Lymphoblastoid cells
LL	Linker length model
LM/ESI	Light microscopy and electron spectroscopic imaging

MEFs	Mouse embryonic fibroblast cells
MNase	Micrococcal Nuclease
NAD	Nucleolus-associated domains
NCP	Nucleosome core particle
NE	Nuclear envelope
NFR	Nucleosome free region
NL	Nuclear lamina
NND	Nearest neighbor distance
NPC	Nuclear pore complex
NPCs	Neuronal progenitors cells
NR	Nucleosome removal model
NRL	Nucleosome repeat lenght
PALM	Photoactivated localization microscopy
PF	Perichromatin fibrils
PTM	Post-translational modification
Pol II	RNA Polymerase II
PR	Perichromatin region
SAXS	Small-angle X-ray scattering
sLif	Serum plus cytokine leukemia inhibitory factor
SSIM	Structured-illumination microscopy
STED	Stimulated emission depletion
STORM	Stochastic optical reconstruction microscopy
TSA	Trichostatin A
TSS	Transcription start site
WLC	Worm like chain model
2iLif	Mek and Gsk3 kinases inhibitors plus cytokine leukemia inhibitory factor
3C	Chromosome conformation capture

ANNEX 3

Publications

- Chromatin fibers are formed by heterogeneous groups of nucleosomes in vivo
Maria Aurelia Ricci, Carlo Manzo, María Filomena García-Parajo, Melike Lakadamyali, and Maria Pia Cosma
Cell, 2015 March 12,
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Marucci L, Barton DA, Cantone I, **Ricci MA**, Cosma MP, Santini S, di Bernardo D, di Bernardo M.
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Cantone I, Marucci L, Iorio F, **Ricci MA**, Belcastro V, Bansal M, Santini S, di Bernardo M, di Bernardo D, Cosma MP. Cell. 2009 Apr 3;137(1):172-81.