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DNA TOPOISOMERASES AND NUCLEOSOME DYNAMICS IN FISSION YEAST

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“Real science is a revision in progress, always.
It proceeds in fits and starts of ignorance.”

Stuart Firestein

ABSTRACT

The organization of DNA and associated proteins into chromatin allows for compaction and protection of large eukaryotic genomes. However, it also poses a challenge to fundamental cellular processes such as DNA replication, recombination, repair and transcription. In order to orchestrate and regulate these processes chromatin structure needs to be both variable and dynamic. The basic unit of chromatin is the nucleosome. ATP dependent chromatin remodelers and DNA topoisomerases have emerged as important factors in regulating nucleosome transactions. The studies presented in this thesis further our understanding of the roles of such enzymes in nucleosome organization using high-resolution genome-wide techniques in the model organism *Schizosaccharomyces pombe*.

We find that the DNA topoisomerases Top1 and Top2 play overlapping, yet distinct, roles in relieving supercoiling during transcription. Top1 removes negative supercoiling behind the RNA polymerase, helping to maintain the nucleosome depleted region (NDR). This is particularly important for sustaining successive rounds of initiation and elongation at highly transcribed genes. Both Top1 and Top2 also relieve positive supercoiling ahead of the RNA polymerase, thereby preventing stalling of the polymerase during elongation, with Top2 being particularly important at long genes. We also identify a new role for Top3 in maintaining normal levels of the centromere specific histone H3 variant CENP-A. This is largely independent of the role of Top3 in homologous recombination and we suggest that it reflects a role for Top3 in regulating supercoiling at centromeres, thereby affecting CENP-A nucleosome dynamics and perhaps structure.

Furthermore, we find that the fission yeast CHD1-type chromatin remodelers Hrp1 and Hrp3 have an important role in maintaining the characteristic pattern of nucleosome positioning at transcribed genes. We demonstrate that Hrp1 and Hrp3 have nucleosome assembly and spacing activity *in vitro*, and are required for linking regular nucleosomal arrays to the 5' end of genes, thereby preventing cryptic transcription.

Last, we present the first genome-wide map of replication-independent nucleosome turnover in fission yeast, and show that successive mono-, di-, and trimethylation of H4K20 can be used as a proxy marker for nucleosome age. We find that transcription at low and intermediate levels promotes conservation of old nucleosomes in gene bodies and suggest that this reflect efficient recycling of histones behind the RNA polymerase. Moreover, we show that transcription promotes incorporation of newly synthesized nucleosomes at the borders of genes.

Overall, these studies support a model in which nucleosome dynamics are dependent on a large number of factors, including the cooperation between DNA topoisomerases, chromatin remodelers and histone chaperones, and DNA-dependent processes, such as transcription.

LIST OF PUBLICATIONS

I. Durand Dubief M, Person J, **Norman U**, Hartsuiker E, Ekwall K (2010). Topoisomerase I regulates open chromatin and controls gene expression *in vivo*. *EMBO J.* **29**: 2126-3214.

II. Pointner J*, Person J*, Prasad P*, **Norman-Axelsson U**, Strålfors A, Khorosjutina O, Krietenstein N, Svensson JP, Ekwall K, Korber P (2012). CHD1 remodelers regulate nucleosome spacing *in vitro* and align nucleosomal arrays over gene coding regions in *S. pombe*. *EMBO J.* **31**: 4388-4403.

III. **Norman-Axelsson U**, Durand Dubief M, Prasad P, Ekwall K (2013). DNA topoisomerase III localizes to centromeres and affects centromeric CENP-A levels in fission yeast. *PLoS Genet.* **9**: e1003371.

IV. Svensson JP, Shukla M, Menendez-Benito V, **Norman-Axelsson U**, Audergon P, Sinha I, Tanny JC, Allshire RC, Ekwall K (2015). A nucleosome turnover map reveals that the stability of histone H4 Lys20 methylation depends on histone recycling in transcribed chromatin. *Genome Res.* Mar 16. pii: gr.188870.114. [Epub ahead of print]

**Equal contribution*

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LIST OF ABBREVIATIONS

A	adenosine
ACF	ATP-utilizing chromatin remodeling and assembly factor
actD	actinomycin D
ASF1	Anti-silencing function 1
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
bp	base pair
BLM	Bloom syndrome
C	cytosine
CATCH-IT	covalent attachment of tags to capture histones and identify turnover
CATD	CENP-A targeting domain
CCAN	constitutive centromere-associated network
CD	chromatin domain
CDK	Cyclin-dependent kinase
cDNA	complementary DNA
CDS	coding sequence
CENP-A	Centromere protein A
CHD1	Chromo domain helicase DNA binding protein 1
ChIP	chromatin immunoprecipitation
ChIP-chip	ChIP and DNA microarray
ChIP-exo	ChIP and lambda exonuclease digestion
ChIP-qPCR	ChIP and qPCR
ChIP-seq	ChIP and high-throughput sequencing
CPT	camptothecin
CTD	C-terminal domain
dHJ	double Holliday junction
DNA	deoxyribonucleic acid
DSB	DNA double-stranded break
EBD	estrogen-binding domain
FACT	Facilitates chromatin transcription
G	guanine
GTF	general transcription factor
GTPase	guanosine triphospathase
HA	hemagglutinin
HAT	histone acetyl transferase
HDAC	histone deacetylase
HFD	histone fold domain
HIRA	Histone cell cycle regulation defective homolog A
HJURP	Holliday junction recognition protein
HKDM	histone lysine demethylase
HKMT	histone lysine methyl transferase
HR	homologous recombination
Hrp1/3	Heterogeneous nuclear ribonucleoprotein 1/3
IGR	intergenic region
INO80	Inositol requiring mutant 80
ISWI	Imitation Switch
K	lysine
kb	kilo base
LAD	lamina-associated domain
Lk	linking number

Lys	lysine
M	molar
me	methylation
me1	monomethylation
me2	dimethylation
me3	trimethylation
MIS18	Minichromosome instability 18
MIS18BP1	MIS18 binding protein 1
Mit1	Muc1 expressed independent of TEC1
MNase	micrococcal nuclease
MNase-chip	MNase digestion and DNA microarray
MNase-seq	MNase digestion and high-throughput sequencing
NAP1	Nucleosome assembly protein 1
NDR	nucleosome depleted region
NL	nuclear lamina
nm	nanometer
NRL	nucleosome repeat length
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PIC	pre-initiation complex
PLK1	Polo-like kinase 1
PTM	posttranslational modification
qPCR	quantitative PCR
R	arginine
RbAp48	Retinoblastoma-associated protein 48
RC	replication-coupled
REQL4	RecQ protein-like 4
RI	replication-independent
RITE	recombination-induced tag exchange
RMI	RecQ-mediated genome instability
RNA	ribonucleic acid
rRNA	ribosomal RNA
RSC	Remodels the structure of chromatin
RSF	Remodeling and spacing factor
RTR	RecQ-Top3-RMI
S	serine
SANT	Swi3, Ada2, N-Cor, and TFIIIB
SLIDE	SANT-like ISWI domain
S/MAR	scaffold/matrix-associated region
SNF	Sucrose non-fermentable
SPT6	Suppressor of Ty 6
SWI	Switch
SWR1	SWI/SNF-related 1
T	thymine (in ribonucleic acids) tyrosine (in proteins)
TAD	topologically associated domain
TF	transcription factor
Top1	DNA topoisomerase I
Top2	DNA topoisomerase II
Top3	DNA topoisomerase III
tRNA	transfer RNA
TSS	transcription start site

TTS	transcription termination site
Tw	twist
ub	ubiquitination
UTR	untranslated region
Wr	writhe
WRN	Werner syndrome
Å	angstrom

1 INTRODUCTION

1.1 BASIC CHROMATIN ORGANIZATION

The development and function of all the species populating earth today is a tremendous task that has taken endless years of evolution to master. The key to this complexity and diversity lies in the detailed instruction manual for each species, which is encoded in almost every cell and inherited through generations. The manual is written as genetic information, the full set of which makes up the genome of an organism.

1.1.1 DNA and chromatin

For eukaryotes, the elegant solution to faithful storage, use and transmission of genetic information revolves around molecules of deoxyribonucleic acid (DNA) (Watson and Crick 1953). DNA is a polymer of nucleotides, each consisting of a deoxyribose unit, a phosphate group and one of the nitrogenous bases adenine (A), thymine (T), guanine (G) and cytosine (C). Covalent binding between the deoxyribose moiety of one nucleotide and the phosphate group of the next results in formation of a macromolecule with an alternating sugar-phosphate backbone. Genetic information is encoded in the sequence of nucleotides and the key to its transmission lies in the fact that two complementary polynucleotide strands come together to form an anti-parallel helix. The strands of the DNA double helix are intertwined by right-handed wrapping around a central axis and held together by non-covalent interactions between complementary base pairs (bp), where A pairs with T and G pairs with C. This organization allows semi-conservative replication of the genome.

Eukaryotic genomes are mostly comprised of linear DNA molecules, termed chromosomes, which are harbored within the cell nucleus. In most species, some genetic information can also be found on circular DNA molecules in mitochondria and in chloroplasts. The number of chromosomes, as well as their individual sizes, varies between species, with humans having 46 chromosomes, including 22 autosomal chromosome pairs and one pair of sex chromosomes (X and Y). In total, the human genome contains approximately 3 billion bp, and remarkably, it must be compacted approximately 100 000 times to fit within the cell nucleus (Lander, Linton et al. 2001; Venter, Adams et al. 2001). This is achieved by elaborate packaging and organization of DNA together with a plethora of proteins into a complex known as chromatin (figure 1). Chromatin serves to compact and protect the genome, but is also the template upon which all DNA-dependent cellular processes act, and thereby it plays a major role in its functional use and offers many opportunities for coordination and regulation of genome functions.

1.1.2 The nucleosome

The primary level of chromatin organization is the formation of nucleosomes along DNA (figure 2) (Kornberg 1974; Olins and Olins 1974; Oudet, Gross-Bellard et al. 1975). Nucleosomes form basic repeating units distributed like beads-on-a-string along the DNA molecules. Each nucleosome consists of approximately 147 bp of DNA wrapped 1.65 times in a left-handed direction around an octamere of histone proteins (Simpson 1978;

Richmond, Finch et al. 1984; Arents, Burlingame et al. 1991; Luger, Mader et al. 1997; Richmond and Davey 2003). A canonical nucleosome contains two copies each of the highly conserved core histones H2A, H2B, H3 and H4. Histones are basic proteins that are characterized by the presence of a histone fold domain (HFD), and N terminal tails that are protruding from the nucleosomal structure (Arents and Moudrianakis 1995; Luger and Richmond 1998). Histone H2A and H2B also have short C terminal tails. The HFD mediates pair wise anti-parallel associations of H2A with H2B and of H3 with H4.

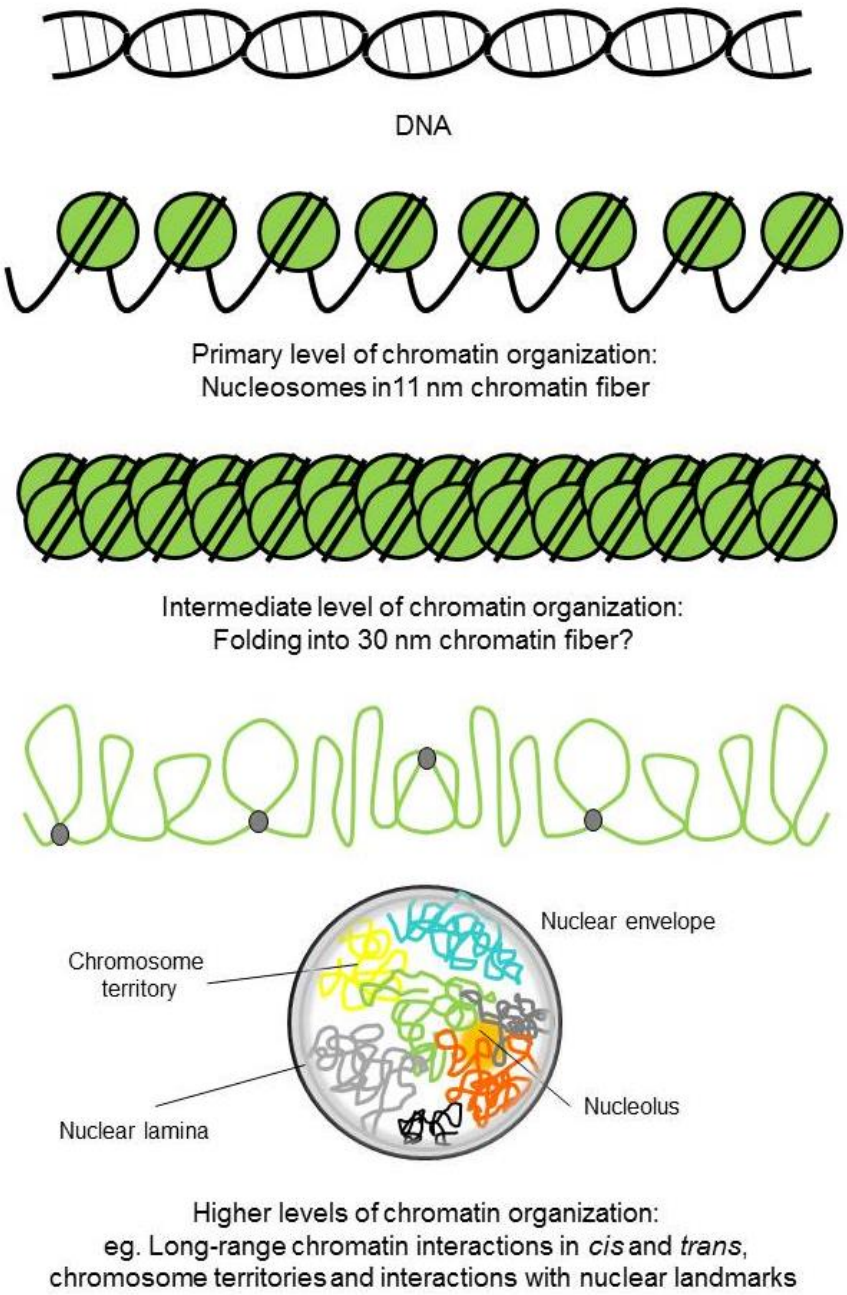

Figure 1. Schematic view of basic chromatin organization. Chromatin is organized at several hierarchical levels, starting with the formation of nucleosomes along DNA. Further compaction is achieved by interactions between neighboring nucleosomes. Three-dimensional organization of chromatin in the nucleus is governed by long-range interactions between more distant chromatin regions and interactions with nuclear landmarks.

In the nucleosome, two H3-H4 heterodimers become tightly associated through interactions between the H3 histones, and mainly organizes the central ~80 bp of DNA. The octameric structure is completed by binding of two H2A-H2B heterodimers on opposite sides by weaker interactions between H4 and H2B, which are organizing the peripheral ~40 bp of DNA on each side (Eickbush and Moudrianakis 1978). Neighboring nucleosomes are separated by linker DNA, the length of which varies between species, ranging between 20-50 bp (Woodcock, Skoultchi et al. 2006). In many species, the linker histone H1 binds to the surface of the nucleosome core particle and contacts DNA at the entry and exit points, with profound effects on higher-order chromatin organization.

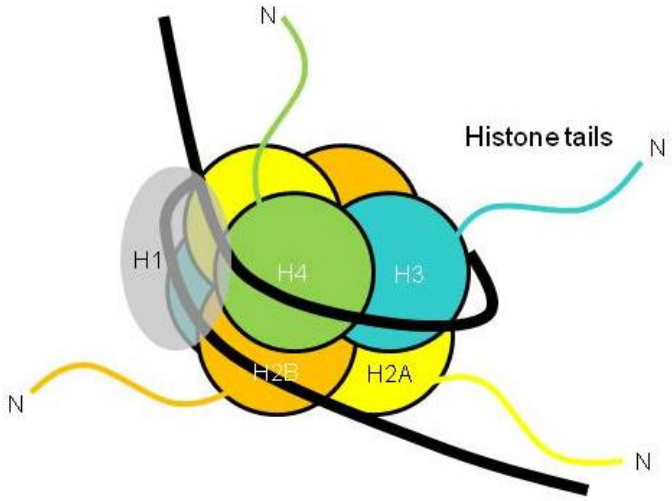

Figure 2. The nucleosome. 147 bp of DNA is wrapped in a left-handed direction around a core consisting of two copies each of histone H2A, H2B, H3 and H4. The histones have protruding N terminal tails that can be modified by posttranslational modifications.

1.1.3 Chromatin fibers

The formation of nucleosomes along DNA results in six fold compaction of DNA and gives chromatin fibers with a diameter of 11 nanometers (nm). Much less is known about the intermediate level of chromatin organization, where neighboring nucleosomes interact with each other to provide further compaction (figure 1). Salt-dependent folding of reconstituted nucleosomal arrays as well as studies of chromatin isolated from cells points towards the organization of chromatin into 30 nm fibers (van Steensel 2011; Bian and Belmont 2012; Grigoryev and Woodcock 2012). Several repetitive structures have been suggested for the potential folding of nucleosomal DNA into thicker fibers, including a solenoid structure with bent linkers, a zigzag structure with straight linkers, or a combination of these. Some observations of 30 nm fibers have also been made *in vivo*, but are limited to a few specific loci and cell types. Rather, when chromatin is studied in cells it seems that the intermediate organization of chromatin occurs by irregular aggregation of nucleosomes (van Steensel 2011; Bian and Belmont 2012; Grigoryev and Woodcock 2012). One possibility is that while nucleosomal DNA can form fibers, the extreme compaction in the cell nucleus results in a high degree of interdigitation, where interactions between nucleosomes of different fibers my start to compete with intra-fiber integrity.

1.1.4 Higher-order chromatin organization

Higher-order chromatin organization describes the large-scale three dimensional organization of chromatin in the nucleus (figure 1) (Meldi and Brickner 2011; van Steensel 2011; Bian and Belmont 2012; Bickmore and van Steensel 2013; Cavalli and Misteli 2013; Dekker, Marti-Renom et al. 2013; Gibcus and Dekker 2013). One major aspect of this is the formation of long- and short-range interactions between relatively widely spaced loci. These interactions may depend on specific protein-DNA interactions, but are also often dependent on stochastic encounters. Several studies have demonstrated that intra-chromosomal interactions are much more frequent than inter-chromosomal interactions (Lieberman-Aiden, van Berkum et al. 2009; Kalhor, Tjong et al. 2012; Sexton, Yaffe et al. 2012; Zhang, McCord et al. 2012). Studies of chromatin interactions in human, mouse and *D. melanogaster* have revealed the existence of discrete linear stretches of loci that preferentially interact with one another, referred to as topologically associated domains (TADs), usually a few hundreds of kilo bases (kb) in size (Dixon, Selvaraj et al. 2012; Hou, Li et al. 2012; Nora, Lajoie et al. 2012; Sexton, Yaffe et al. 2012). TADs that are overall gene rich and highly expressed tend to associate with one another, while TADs that are overall gene poor and lowly expressed tend to cluster together. Overall, there is a strong tendency for gene-rich, highly transcribed and open chromatin regions to interact, both in *cis* and in *trans* (Lieberman-Aiden, van Berkum et al. 2009; Hou, Li et al. 2012; Kalhor, Tjong et al. 2012; Sexton, Yaffe et al. 2012; Zhang, McCord et al. 2012).

Another aspect of higher-order chromatin organization is the anchoring of particular chromatin regions to different nuclear landmarks and the formation of nuclear bodies, which are characterized by specific protein and DNA content (Meldi and Brickner 2011; van Steensel 2011; Bickmore and van Steensel 2013; Cavalli and Misteli 2013; de Graaf and van Steensel 2013; Gibcus and Dekker 2013). For example, associations between the nuclear matrix and non-coding regions termed scaffold/matrix-associated regions (S/MARs) have been suggested to act as a general organizing feature within the cell nucleus. There are also many regions that associate with the nuclear lamina (NL). Such lamina-associated domains (LADs) tend to have low transcriptional activity. One example of a nuclear body is the organization of genes encoding ribosomal ribonucleic acid (rRNA) together with RNA polymerase I and III, as well as ribosomal proteins, in the nucleolus.

Lastly, higher-order chromatin organization involves several global chromosomal configurations (Meldi and Brickner 2011; Cavalli and Misteli 2013; Gibcus and Dekker 2013). One example is the polarization of chromosomes within the nucleus of some organisms and cell types. In yeast, centromeres and the mating type region cluster together near the spindle pole body at the nuclear periphery, while telomeres and rRNA-coding genes form peripheral foci at the opposite end of the nucleus, in what is known as a Rabl conformation (Funabiki, Hagan et al. 1993). Another example is the folding of the bulk of each chromosome in a discrete spatial region in the nucleus, resulting in formation of chromosome territories in many species (Cremer and Cremer 2010). Chromosome territories also tend to be radially spaced, with gene rich chromosomes having a tendency to be located at the nuclear interior in many cells (Bickmore 2013). A similar tendency is also seen for gene rich chromatin regions within chromosomes.

1.2 VARIATIONS AND DYNAMICS OF CHROMATIN

It has become increasingly clear that chromatin structure and organization is highly variable and dynamic, and that these variations strongly influence the readout of the underlying DNA sequence. A lot of the variation and dynamics occur at the level of nucleosomes, including posttranslational modifications (PTMs) of histones, incorporation of histone variants, and chromatin remodelling to control nucleosome positioning, occupancy and turnover. These mechanisms play essential roles in all DNA-dependent cellular processes and are tightly controlled by an intricate network of modifiers, which display multiple layers of cross-talk and interplay, giving a complex toolbox for genome regulation. This is essential to development and differentiation, as well as to various responses to environmental cues. A multitude of intrinsic and extrinsic signals converges on chromatin, resulting in dynamic changes in global and local chromatin organization and dynamics, thereby modulating genome functions.

1.2.1 Histone modifications

Histones can be modified in a large number of ways and the list of modifications is continuously growing, now comprising more than 100 different histone PTMs (Kouzarides 2007; Campos and Reinberg 2009; Bannister and Kouzarides 2011; Rothbart and Strahl 2014). Modifications are added to specific amino acid residues, which are often located in the protruding histone tails, but occasionally found inside the histone core (Tropberger and Schneider 2010). Methylation (me), acetylation (ac) and ubiquitinylation (ub) of lysine (K) residues, methylation of arginine (R) residues, as well as phosphorylation (p) of tyrosine (T) and serine (S) residues are among the most well studied histone modifications. Methyl groups are added consecutively, resulting in mono-, di- or trimethylated lysine, and mono- or dimethylated arginine, making it an extremely versatile modification.

For most of the known histone modifications, enzymes that catalyze their addition and removal, respectively, have been identified (Kouzarides 2007; Campos and Reinberg 2009; Bannister and Kouzarides 2011; Zentner and Henikoff 2013; Rothbart and Strahl 2014). For example, methylation of lysines is mediated by histone lysine methyl transferases (HKMTs) and reversed by histone lysine demethylases (HKDMs), acetyl groups are added to lysine by histone acetyl transferases (HATs) and removed by histone deacetylases (HDACs), and phosphorylation is catalyzed by kinases and dephosphorylation by phosphatases. Some histone modifying enzymes have relatively broad specificities, acting on several different amino acid residues, while others are more specific. Particularly HKMTs and HKDMs tend to have a narrow specificity for both the amino acid residue that they target and the degree of methylation that they act on. Histone modifying enzymes are often components of large multi protein complexes, acting in different genomic locations or cellular functions, in which interactions with other factors may further direct and modify their activities. The counteracting activities of enzymes that add and remove histone modifications creates a highly dynamic situation, where turnover can be rapid and the steady-state level of each histone modification is the result of an enzymatic equilibrium that can be rapidly shifted in response to various cellular signals and processes.

Histone modifications may either have direct effects on chromatin structure or regulate the recruitment and/or activity of effector proteins (Kouzarides 2007; Campos and Reinberg 2009; Bannister and Kouzarides 2011; Zentner and Henikoff 2013; Rothbart and Strahl 2014). Some histone modifications have a direct effect on chromatin organization by altering the intrinsic properties of histones, whereby they can affect histone-DNA contacts within nucleosomes, nucleosome stability and/or associations with other nucleosomes. For example, acetylation neutralizes the positive charge of lysine residues, thereby affecting nucleosome conformation and stability (Manohar, Mooney et al. 2009; Neumann, Hancock et al. 2009), histone-DNA interactions (Hong, Schroth et al. 1993) and chromatin compaction *in vitro* (Shogren-Knaak, Ishii et al. 2006; Robinson, An et al. 2008). The importance of general charge neutralization has also been supported *in vivo* (Martin, Pouchnik et al. 2004; Dion, Altschuler et al. 2005). Effector proteins generally contain one or several conserved domains that mediate direct interactions with modified histones, such as chromo domains that bind certain methylated lysine residues (Eissenberg 2001) or bromo domains that bind acetylated lysine residues (Zeng and Zhou 2002). Effector proteins often mediate chromatin remodeling or further modifications of histones, resulting in sequential cascades events that change the structure and function of chromatin.

The genome-wide average enrichment of various histone modifications has been mapped in many different organisms and cell types (Kouzarides 2007; Campos and Reinberg 2009; Bannister and Kouzarides 2011). Generally, histone modifications are non-randomly distributed in relation to different genomic loci, such as gene regions, and their relative enrichment often correlates with specific genomic functions. This way, histone modifications have been implied both in local the orchestration of various DNA-dependent cellular functions and in the more global establishment of different chromatin environments across large regions (Kouzarides 2007; Campos and Reinberg 2009; Bannister and Kouzarides 2011; Rothbart and Strahl 2014). The latter reaches from the classical classification of chromatin into euchromatin and heterochromatin, to the more recent attempts at identifying a larger but yet limited number of preferred chromatin states, characterized by different combinations of histone modifications and protein compositions (Filion, van Bemmel et al. 2010; Gerstein, Lu et al. 2010; Ernst, Kheradpour et al. 2011; Kharchenko, Alekseyenko et al. 2011; Roudier, Ahmed et al. 2011). This way, chromatin can be divided into large chromatin domains (CDs), which are separated by insulators and boundary elements, and associated with different levels of gene expression, (van Steensel 2011; Bickmore and van Steensel 2013; Cavalli and Misteli 2013; de Graaf and van Steensel 2013; Gibcus and Dekker 2013). However, all such correlations between histone modifications and genomic functions does not imply a casual relationships, as they are often just consequences of different chromatin functions (Henikoff and Shilatifard 2011). Much effort has been put into deciphering a 'histone code', where histone modifications would act singly or in a specific combination to dictate a specific and predictable functional outcome (Strahl and Allis 2000; Turner 2000; Jenuwein and Allis 2001). However, the 'histone code' hypothesis is still debated and has proven difficult to validate experimentally, possible because of the complexity of chromatin organization. A plethora of modifications can be present even in a small chromatin region, making it likely that functional outcomes depend on a large number of histone marks (Fischle, Wang et al.

2003; Lee, Smith et al. 2010; Suganuma and Workman 2011; Rothbart and Strahl 2014). Furthermore, functional outcomes may be dependent on availability of effector proteins, chromatin context, genomic location, cell cycle stage, timing and cell type. Moreover, there is an extensive degree of crosstalk between different modifications as well as with other mechanisms of chromatin regulation, such as chromatin remodeling. Lately, histones have been suggested to be "patterning", rather than encoding (Rothbart and Strahl 2014). They are indeed one of many players in a complex network of dynamic regulatory pathways responding to numerous cellular and environmental stimuli, in order to coordinate various DNA dependent processes.

1.2.2 Histone variants

In many eukaryotes, variants of the canonical core histones H2A, H2B and H3 have evolved and can be incorporated into nucleosomes in their place (Talbert and Henikoff 2010; Millar 2013; Skene and Henikoff 2013; Yuan and Zhu 2013). Canonical histones are expressed from tandem gene arrays at high levels during a short period in S phase and subsequently incorporated into chromatin by replication-coupled (RC) nucleosome assembly. In addition, they can be incorporated by replication-independent (RI) pathways in other parts of the cell cycle. Histones variants are constitutively expressed at low levels from single or low copy number genes and incorporated into chromatin by distinct RI pathways. Incorporation of a histone variant is usually limited to specific chromatin regions, specific time points or specific cell types. Histone variants differ to varying degrees in amino acid sequence relative to the canonical histones and contribute to diversification of chromatin by alternating chromatin structure and stability, either directly or indirectly by recruitment of other factors.

While canonical histones function primarily in genome compaction and regulation of gene expression, histone variants have additional roles in DNA repair, chromosome segregation, recombination and heterochromatin formation. While some histone variants are highly conserved between eukaryotic species and ubiquitously expressed, others are restricted to specific lineages or cell types. For histone H2A there are two variants that are almost universal in eukaryotes (Talbert and Henikoff 2010; Millar 2013; Skene and Henikoff 2013; Yuan and Zhu 2013). Histone H2A.Z is highly enriched at nucleosomes flanking transcription start sites (TSSs), and has been implicated in both activation and repression of transcription. These seemingly opposing functions may depend on differences in nucleosome composition, the underlying DNA sequence, histone modifications and available effectors. While homotypic H2A.Z containing nucleosomes seems to confer nucleosome stability, nucleosomes containing both H2A.Z and H3.3, which are enriched at promoters and the 5′end of transcribed genes, confers nucleosome instability (Ishibashi, Dryhurst et al. 2009; Jin, Zang et al. 2009). H2A.Z is also involved in heterochromatin and boundary formation, DNA repair, suppression of antisense RNA, and chromosome segregation. Histone H2A.X is found all over the genome and has a critical role in DNA repair, as well as functions in meiotic silencing and heterochromatin formation. In yeast and *D. melanogaster*, the function of H2A.X is performed by H2A and H2A.Z, respectively.

Histone H3 has two variants that are present in essentially all eukaryotes (Talbert and Henikoff 2010; Millar 2013; Skene and Henikoff 2013; Yuan and Zhu 2013). The centromere specific histone H3 variant Centromere Protein A (CENP-A) has a universal and essential role in centromere specification and function. Mammals and *D. melanogaster* display ubiquitous expression of the non-centromeric H3 variant H3.3, which is the major H3 molecule available for RI nucleosome deposition. Histone H3.3 is enriched at promoters and gene bodies of active genes but is also found at promoters of inactive genes, transcription factor binding sites, boundary elements, telomeres and pericentromeric regions. Some studies suggest that histone H3.3 plays a role in the regulation of transcription by affecting nucleosome stability and dynamics. In addition, histone H3.3 has been implicated in chromatinization of the male pronucleus following fertilization, silencing of telomeric repeats, and heterochromatin formation. Ascomycetes, including yeast, and some unicellular organisms have only one non-centromeric histone H3, which is most similar to H3.3 in other species.

Most eukaryotes encode only canonical histone H2B and H4, although some lineages have variants also of these histones. The lack of histone H2B and H4 variants may be due to evolutionary constraints restricting both histones in each the dimer to vary (Gonzalez-Romero, Rivera-Casas et al. 2010). Moreover, histone H4 makes extensive contacts with the other three histones and is extensively modified, resulting in higher evolutionary constraints compared to other histones.

1.2.3 Histone chaperones and chromatin remodelers

The assembly and disassembly of nucleosomes are crucial for maintaining chromatin organization in DNA-dependent processes such as DNA replication, transcription, repair and recombination. Moreover, it is highly important for regulation of these processes. Although the nucleosome is an energetically favourable confirmation, mixtures of DNA and histones tend to form disordered aggregates under physiological conditions. Therefore, nucleosome transactions generally rely on the cooperated action of histone chaperones and chromatin remodelers.

Histone chaperones are negatively charged proteins that can shield the positively charged histones from aggregation with negatively charged DNA until they are properly assembled into chromatin (Park and Luger 2008; Das, Tyler et al. 2010; Burgess and Zhang 2013; Li, Burgess et al. 2013). Histone chaperones present a structurally diverse family of proteins. Most histone chaperones preferentially bind either histone H2A-H2B or histone H3-H4 heterodimers, and the variant histones usually have their own dedicated chaperones. Newly synthesized histones are rapidly bound by histone chaperones in the cytoplasm and actively imported into the nucleus for assembly. In recent years histone PTMs have been shown to play important roles in coordination of the assembly process and in nuclear import (Keck and Pemberton 2012). The assembly pathways are often complex and may include consecutive interactions with several different chaperones as well as other factors. In agreement, histone chaperones often act in large multimeric complexes together with both chromatin remodelers and histone modifiers.

Group	Family	Subfamily	Human	*S. cerevisiae*	*S. pombe*
Snf2-like	SWI/SNF (SNF2)	Swi/Snf	SMARCA2/BRM SMARCA4/BRG1	STH1 (RSC) SWI2/SNF2	Snf21 Snf22
	ISWI	Iswi	SMARCA5/SNF2H SRACA1/SNF2L	ISWI1, ISWI2	-
	CHD1	Chd1	CHD1, CHD2	CHD1	Hrp1, Hrp3
		Mi-2	CHD3/Mi2-α, CHD4/Mi2-β, CHD5	-	Mit1
		Chd7	CHD6, CHD7, CHD8/HELSNF1, CHD9	-	-
Swr1-like	INO80/SWR1	Ino80	INO80	INO80	Ino80
		Swr1	SRCAP	SWR1	Swr1
		Fun30/Etl1	SMARCAD1	FUN30	Fft1, Fft2, Fft3
		EP400	EP400	-	-

Table 1. Classification of a subset of Snf2 family chromatin remodeling ATPases.

Chromatin remodelers are a large family of enzymes that use the energy from adenosine triphosphate (ATP) hydrolysis to alter histone-DNA contacts (Clapier and Cairns 2009; Flaus and Owen-Hughes 2011; Hargreaves and Crabtree 2011; Hota and Bartholomew 2011; Ryan and Owen-Hughes 2011; Bartholomew 2014). All chromatin remodelers are characterized by an adenosine triphosphatase (ATPase) domain and a Sucrose non-fermentable 2 (SNF2) helicase-like translocase domain. The major families of chromatin remodelers are the Switch (SWI)/SNF, Immitation Switch (ISWI), Helicase DNA-binding (CHD), and Inositol requiring mutant 80 (INO80)/SWI/SNF-related 1 (SWR1), which are then sub-classified based on sequence homology of their conserved ATPase subunits and the presence of other domains (table 1) (Hota and Bartholomew 2011; Bartholomew 2014). Such additional domains often mediate recognition of nucleosomes, either by recognizing histones or DNA. Histone binding domains can also promote recruitment to specific chromatin regions by binding to specific modified histone tails, such as the tandem chromo domains of the remodeler Chromo domain (CHD) 1 binding to H3K4me2/3 and to a lesser extent me1 (Flanagan, Mi et al. 2005; Sims, Chen et al. 2005), although not in yeast (Sims, Chen et al. 2005; Flanagan, Blus et al. 2007). Interestingly, the chromo domains of CHD1 have also been suggested to regulate the activity of the enzyme (Hauk, McKnight et al. 2010; Morettini, Tribus et al. 2011). DNA binding domains contribute to the affinity and stability of the remodeling complex with nucleosomes, but may also have additional roles. For example, CHD-type remodelers contain two DNA binding domains in form of a Swi3, Ada2, N-Cor, and TFIIIB (SANT) domain and a SANT-Like ISWI domain (SLIDE) domain (Ryan, Sundaramoorthy et al. 2011). The SLIDE domain is involved in targeting as well as in the directionality of nucleosome sliding (McKnight, Jenkins et al. 2011). Similar to histone modifying enzymes and histone chaperones, chromatin remodelers are often present in large effector complexes with other components that contribute to targeting, activity and remodelling outcome.

The mechanisms behind chromatin remodelling are not entirely clear and may differ between enzymes. In general, the energy from ATP hydrolysis is used to modify the interaction between histones and DNA, the outcome of which may be nucleosome assembly, disassembly, exchange, or sliding with potential spacing (figure 3) (Clapier and Cairns 2009; Bowman 2010; Hargreaves and Crabtree 2011; Hota and Bartholomew 2011; Bartholomew 2014). The outcome of remodelling varies between different types of remodelers, partially due to enzymatic differences and partially related to their association

with other factors. For example, SWI/SNF-type remodelers characteristically promote nucleosome disassembly (Boeger, Griesenbeck et al. 2004; Schwabish and Struhl 2007; Gkikopoulos, Havas et al. 2009; Dechassa, Sabri et al. 2010), while CHD-type remodelers mediate assembly and spacing of nucleosomes (Brehm, Langst et al. 2000; Lusser, Urwin et al. 2005; Stockdale, Flaus et al. 2006). However, a fission yeast CHD1 homolog has also been suggested to be able to evict nucleosomes *in vivo* (Walfridsson, Khorosjutina et al. 2007), and *S. cerevisiae* CHD1 can move nucleosomes of the edges ends of DNA when targeted to histones instead of DNA *in vitro*, making it behave much similar to SWI/SNF (Patel, Chakravarthy et al. 2012).

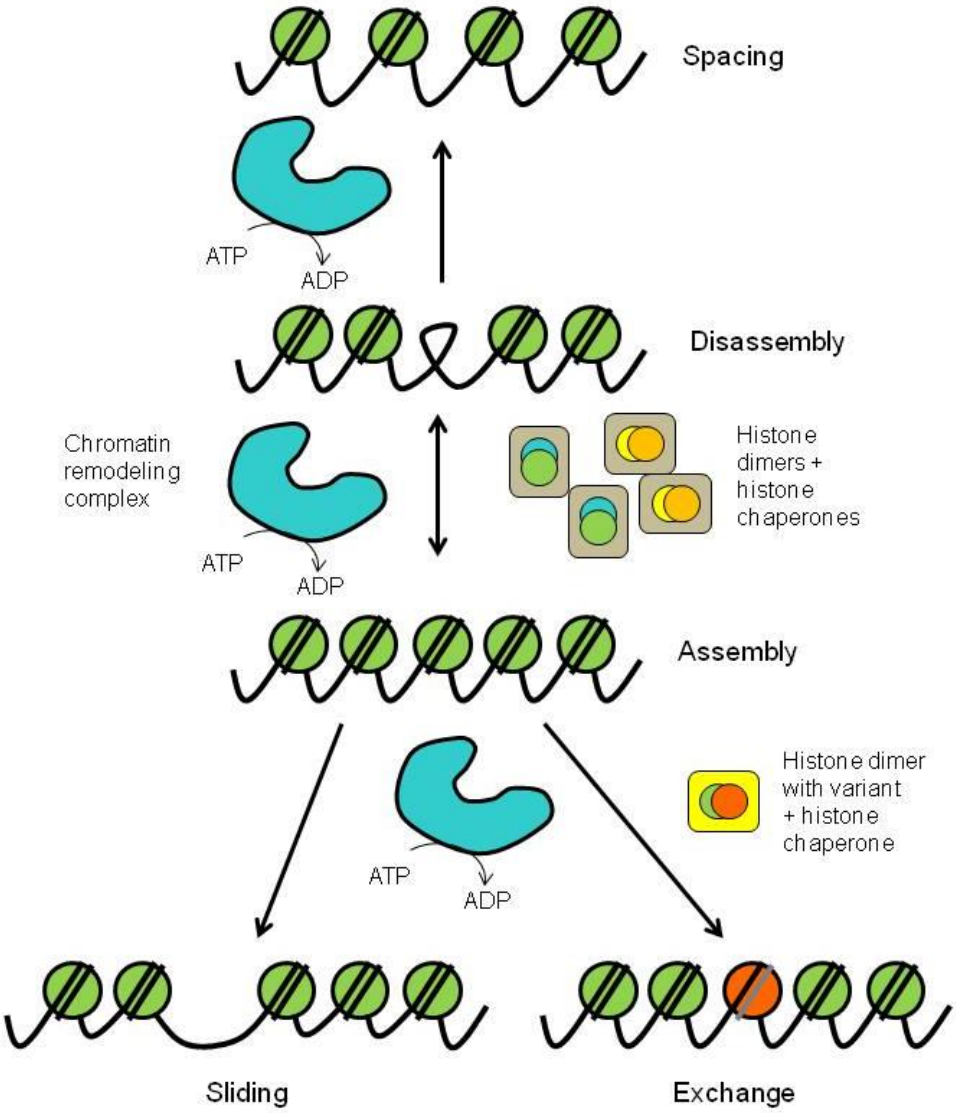

Figure 3. Outcomes of chromatin remodelling. Chromatin remodelers use the energy from ATP hydrolysis to disrupt histone-DNA contacts and are important players in assembly, disassembly, exchange, sliding and spacing of nucleosomes.

Nucleosome assembly occurs in a stepwise fashion, which is to a large extent guided by histone chaperones, but which may also be assisted by chromatin remodelers (Haushalter and Kadonaga 2003; Park and Luger 2008; Das, Tyler et al. 2010; Keck and Pemberton 2012; Burgess and Zhang 2013). The first step is the assembly of a tetrasome by deposition of two histone H3-H4 dimers onto DNA. The histone H3-H4 tetramer may form already on

a histone chaperone, before deposition onto DNA, or by sequential deposition of two dimers on DNA. This is followed by rapid incorporation of two histone H2A-H2B dimers on opposite sides, possibly via an intermediate represented by a hexasome. This sequence of events can be attributed to the fact that histone H3-H4 dimers have high affinity for DNA, while histone H2A-H2B dimers have high affinity for histone H3-H4 bound to DNA (Smith and Stillman 1991; Nakagawa, Bulger et al. 2001). Nucleosome assembly can occur by several parallel pathways, each involving multiple steps of shuffling between different chaperones, ultimately guiding the folding process down the free-energy gradient. The role of chromatin remodelers in nucleosome assembly may be to disrupt non-specific associations between histones and DNA along the assembly process and to remove kinetically trapped intermediates (Haushalter and Kadonaga 2003; Das, Tyler et al. 2010).

Disassembly of nucleosomes also occurs in a stepwise fashion that involves histone chaperones, and importantly, it always requires ATP-dependent chromatin remodelers to break the contacts between histones and DNA. The first step is the opening of the interaction between a H2A-H2B dimer and the H3-H4 tetramer, followed by release of both H2A-H2B dimers from the DNA (Gansen, Valeri et al. 2009; Bohm, Hieb et al. 2011). Histone chaperones now act as acceptors of histones and are important to prevent re-assembly. Nucleosome disassembly may be complete or partial, in the latter case resulting in eviction of dimers or just local unwrapping of nucleosomal DNA. Histone exchange is basically the combined processes of nucleosome disassembly and assembly with replacement of one or more histones, usually with a histone variant. As an example, the chromatin remodeler SWR1 acts in a large complex that catalyzes the replacement of an H2A-H2B dimer with an H2A.Z-H2B dimer (Morrison and Shen 2009).

Nucleosome sliding is basically the repositioning of nucleosomes along DNA. The dominating mechanistic models for nucleosome sliding are the DNA twist model and the bulge propagation model, respectively. The DNA twist model proposes that a 1 bp twist propagates through the entire nucleosome, thus moving it 1 bp along DNA. The bulge propagation model suggests that a larger segment of DNA is displaced from the nucleosome and replaced by another segment, creating a bulge that moves through the nucleosome until it exits. Some chromatin remodelers, like ISWI and CHD-type remodelers, slide nucleosomes until there is a minimal length of linked DNA left to the adjacent nucleosome, resulting in regular spacing of nucleosomes. At least for some remodelers, such as SWI/SNF, nucleosome disassembly actually involves sliding of one nucleosome towards a second nucleosome, displacing the DNA and eventually releasing the histones (Batholomey 2014). Moreover, sliding may contribute to nucleosome assembly by exposing more DNA for deposition of nucleosomes. Thus, nucleosome assembly, disassembly and sliding are likely highly intertwined *in vivo*.

1.2.4 Nucleosome positioning and occupancy

The compact and elaborate organization of chromatin makes it possible to fit large eukaryotic genomes into the limited space of a cell nucleus and to protect it from damage. However, it also limits the accessibility for DNA-dependent processes and DNA-binding regulatory factors. Nucleosomal DNA is generally less accessible than naked DNA and

accessibility to DNA varies throughout the nucleosome (Bell, Tiwari et al. 2011). Therefore, nucleosome positioning and occupancy have profound effects on the orchestration and regulation of genomic functions, including transcription, replication, recombination and DNA repair (Arya, Maitra et al. 2010; Iyer 2012; Struhl and Segal 2013; Hughes and Rando 2014; Lieleg, Krietenstein et al. 2014). In agreement, distribution of nucleosomes throughout the genome is non-random, and governed by both intrinsic properties of the DNA sequence and by extrinsic factors in the cell.

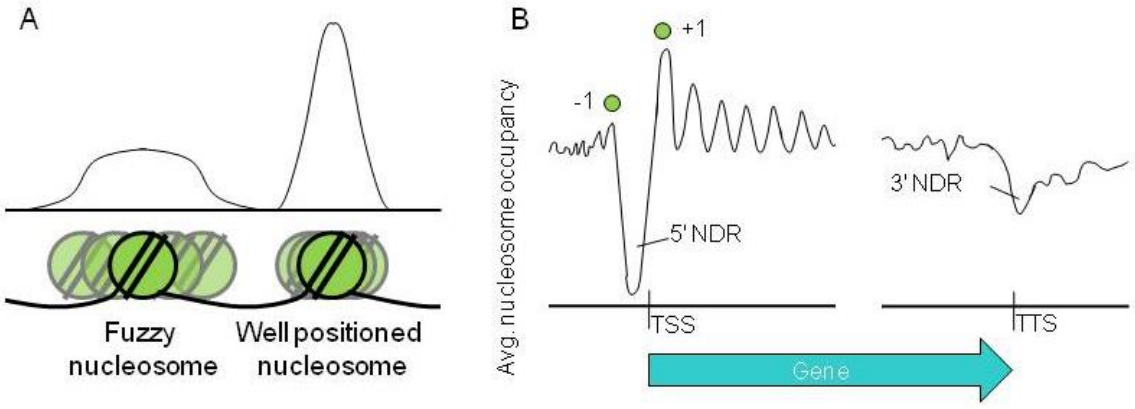

Figure 4. Nucleosome positioning and occupancy. A. Schematic view of well positioned nucleosomes, resulting for little variation within the cell population, and more fuzzy nucleosomes, resulting from more variation within the cell population. B. Average nucleosome organization at transcribed genes typically reveals a 5′ NDR at the promoter region, followed by a regular array of highly positioned nucleosomes in the gene body, and a less pronounced 3′ NDR at the terminator when genes are aligned at the TSS and TTS.

Nucleosome occupancy describes the probability that a given base pair will be present in a nucleosome, while nucleosome positioning describes the probability that a given base pair will be at the central dyad position of a nucleosome. Genome-wide maps of nucleosome positioning and occupancy, measured as the average steady-state situation in a large population of cells, have been generated for most major model organisms (Arya, Maitra et al. 2010; Iyer 2012; Struhl and Segal 2013; Hughes and Rando 2014; Lieleg, Krietenstein et al. 2014). This reveals a landscape of well positioned nucleosomes, resulting from little variation within the cell population, and more fuzzy nucleosomes (figure 4a). The degree of nucleosome occupancy varies from being complete to more or less undetectable. Partial nucleosome occupancy reflects that a position is occupied by a nucleosome in some genomes and unoccupied in others. Thus nucleosome maps give a snapshot of a variable and likely also very dynamic situation. The spacing between nucleosomes is determined by the length of linker DNA and is usually determined from the overlay of many nucleosomal arrays. Nucleosomes are described as regularly spaced if consecutive nucleosomes have the same linker length. The average spacing between nucleosomes in the whole genome, referred to as the nucleosome repeat length (NRL), varies between species, but is usually around 170-210 bp.

Nucleosome occupancy and positioning varies between different functional regions (Arya, Maitra et al. 2010; Iyer 2012; Struhl and Segal 2013; Hughes and Rando 2014; Lieleg, Krietenstein et al. 2014). Transcription units have a very distinct pattern of nucleosome

organization that accounts for many of the well-positioned nucleosomes found across genomes (figure 4B). A common feature is the strong depletion of nucleosomes from a region of 100-200 bp just upstream the TSS. Such regions are generally referred to as nucleosome depleted regions (NDRs). The 5′ NDRs plays an important role in transcription initiation and is enriched for transcription factor (TF) binding sites, TATA boxes and TATA-like elements. The extent of depletion at the 5′ NDR is highly variable between genes and correlates with transcription rate. Downstream of this region is an array of regularly spaced and well positioned nucleosomes, with the TSS generally placed a few base pairs inside the +1 nucleosome border. For most genes, these nucleosomes have the same positions relative to the 5′ end of the gene, resulting in clear nucleosomal arrays in composite plots after alignment of genes at the TSS or the +1 nucleosome. Nucleosomal arrays may also present upstream of the NDR, but are generally less pronounced than downstream arrays, especially in *S. pombe* (Lantermann, Straub et al. 2010). The precision of nucleosome arrays depends on transcription rate and organization decreases with increased distance from the TSS. In yeast, both the 5′ NDR and the downstream nucleosomal array are particularly pronounced at genes with constitutive expression, while inducible genes that depends on cofactors for expression tend to have non-stereotypical organization of nucleosomes (Lieleg, Krietenstein et al. 2014). In human cells, the characteristic nucleosome positioning pattern is found at active genes and genes with paused RNA polymerase, but not at inactive genes (Schones, Cui et al. 2008).

Nucleosome occupancy and positioning are largely determined by the combined effects of DNA sequence, chromatin remodelling, protein-binding and DNA-dependent processes, such as transcription (Arya, Maitra et al. 2010; Iyer 2012; Struhl and Segal 2013; Hughes and Rando 2014; Lieleg, Krietenstein et al. 2014). Intrinsic structural and thermodynamical properties of DNA sequences have long been known to be more or less strong determinants for nucleosome occupancy and positioning (Segal, Fondufe-Mittendorf et al. 2006; Kaplan, Moore et al. 2009; Segal and Widom 2009; Kaplan, Moore et al. 2010). This can at least partially be attributed to the fact that the path of DNA in nucleosomes is curved so that flexible DNA sequences are energetically favourable for nucleosome formation. For example, poly(d:A/dT) stretches confer the lowest flexibility and resist nucleosome assembly *in* vitro (Segal and Widom 2009). Such sequences are enriched at *S. cerevisiae* NDRs (Yuan, Liu et al. 2005; Lee, Tillo et al. 2007) and can even direct NDR formation and affect transcription (Raveh-Sadka, Levo et al. 2012; Small, Xi et al. 2014). Surprisingly, poly(dA:dT) sequences does not seem to promote nucleosome depletion in *S. pombe* (Lantermann, Straub et al. 2010; Moyle-Heyrman, Zaichuk et al. 2013). Nucleosome formation is also affected by dinucleotide periodicity. Bending of DNA around the histone octamer necessitates widening and compression of the helix, which is facilitated by the positioning of certain dinucleotides, like dA:dA, dT:dA, and dT:dT in minor grooves facing toward and dG:dC in minor grooves facing away from the histone octamer. This effect is evident as a 10 bp nucleotide periodicity in nucleosome sequences *in vivo* and can dictate co called ‘rotational positioning’ of nucleosomes (Brogaard, Xi et al. 2012; Moyle-Heyrman, Zaichuk et al. 2013). Computer models that take thermodynamical properties of the DNA sequence into account and *in vitro* reconstitution of chromatin partially mimics the situation *in vivo* (Ioshikhes, Albert et al. 2006; Field,

Kaplan et al. 2008; Yuan and Liu 2008; Kaplan, Moore et al. 2009; Segal and Widom 2009; Tillo and Hughes 2009; Zhang, Moqtaderi et al. 2009). However, the DNA sequence alone fails to direct all NDRs and as well as the strong positioning of +/-1 nucleosomes and flanking nucleosomal arrays. Thus, it is clear that *trans*-acting factors have a significant influence on the chromatin landscape and can override sequence preferences for nucleosome positioning *in vivo*.

The most prominent *trans*-acting factors involved in nucleosome occupancy and positioning are ATP-dependent chromatin remodelers in cooperation with histone chaperones (Arya, Maitra et al. 2010; Iyer 2012; Struhl and Segal 2013; Hughes and Rando 2014; Lieleg, Krietenstein et al. 2014). Their presence in a cell free-extract supplemented with ATP clearly enhances nucleosome depletion at promoter regions, improves the formation of well positioned -1/+1 nucleosomes, and generates nucleosomal arrays (Korber and Horz 2004; Wippo, Israel et al. 2011; Zhang, Wippo et al. 2011). Mutating a single chromatin remodeler generally has mild effects, while double or triple mutations result in severe disruptions of chromatin organization, indicating a high degree of redundancy among these enzymes. At transcription units, yeast SWI/SNF (Hirschhorn, Brown et al. 1992; Gregory, Schmid et al. 1999; Chandy, Gutierrez et al. 2006; Gutierrez, Paredes et al. 2007; Schwabish and Struhl 2007; Brown, Mao et al. 2011; Tolkunov, Zawadzki et al. 2011), ISW1a (Morillon, Karabetsou et al. 2003), and CHD1 (Walfridsson, Khorosjutina et al. 2007; Ehrensberger and Kornberg 2011; Siggens, Cordeddu et al. 2015) have been implicated in formation and maintenance of nucleosome depletion at the 5′ NDR. Nucleosome positioning at the regions immediately flanking the NDR depend on the Remodels structure of chromatin (RSC) complex (Badis, Chan et al. 2008; Parnell, Huff et al. 2008; Hartley and Madhani 2009; Wippo, Israel et al. 2011), ISW2 (Whitehouse, Rando et al. 2007), and CHD1 (Skene, Hernandez et al. 2014). Histone chaperones acting at the promoter region include Histone cell cycle regulation defective homolog A (HIRA) (Kim, Seol et al. 2007) and Anti-silencing function 1 (ASF1) (Adkins, Howar et al. 2004; Korber, Barbaric et al. 2006; Kim, Seol et al. 2007). Nucleosome positioning in gene bodies and formation of nucleosomal arrays seems to involve the chromatin remodelers ISW1b and CHD1 (Morillon, Karabetsou et al. 2003; Xella, Goding et al. 2006; Tirosh, Sigal et al. 2010; Gkikopoulos, Schofield et al. 2011; Hennig, Bendrin et al. 2012; Pointner, Persson et al. 2012; Shim, Choi et al. 2012), RSC (Parnell, Huff et al. 2008) and SWI/SNF (Schwabish and Struhl 2007). In yeast, ISW1 and CHD1 seem to have a prominent role in re-assembly and positioning of nucleosomes behind the polymerase (Smolle 2012). Histone chaperones acting in gene bodies include the Facilitates chromatin transcription (FACT) complex (Belotserkovskaya, Oh et al. 2003; Kaplan, Laprade et al. 2003; Schwabish and Struhl 2004; Jamai, Puglisi et al. 2009), Suppressor of Ty 6 (Spt6) (Kaplan, Laprade et al. 2003; Adkins, Howar et al. 2004), ASF1 (Schwabish and Struhl 2006; Kim, Seol et al. 2007), and HIRA (Kim, Seol et al. 2007). FACT, in cooperation with H2Bub1, has an important role in re-deposition of parental histone H3-H4, while ASF1 is involved in deposition of newly synthesized histone H3-H4, in the wake of elongating RNA polymerase II (Kaplan, Laprade et al. 2003; Schwabish and Struhl 2004; Fleming, Kao et al. 2008; Jamai, Puglisi et al. 2009). Last, chromatin structure at the TTS depends on

CHD1 and ISW1a (Alen, Kent et al. 2002; Morillon, Karabetsou et al. 2003; Whitehouse, Rando et al. 2007).

The process of transcription has a strong influence on, and is also strongly dependent on, nucleosome occupancy and positioning (Arya, Maitra et al. 2010; Iyer 2012; Petesch and Lis 2012; Struhl and Segal 2013; Hughes and Rando 2014; Lieleg, Krietenstein et al. 2014; Venkatesh and Workman 2015). *In vitro*, chromatin presents a strong physical barrier to RNA polymerase II, both at the stages of initiation and elongation (Knezetic and Luse 1986; Lorch, LaPointe et al. 1987; Izban and Luse 1991). Since elongation rates measured *in vivo* are close to rates on naked DNA *in vitro*, there are efficient ways of overcoming this barrier in cells (Ardehali and Lis 2009). First, initiation is associated with nucleosome depletion form the promoter region, which is required for assembly of general transcription factors (GTFs) and the RNA polymerase II holoenzyme into a pre-initiation complex (PIC) (Knezetic and Luse 1986; Lorch, LaPointe et al. 1987; Han and Grunstein 1988; Knezetic, Jacob et al. 1988; Boeger, Griesenbeck et al. 2003; Boeger, Griesenbeck et al. 2004; Korber, Luckenbach et al. 2004; Lee, Shibata et al. 2004; Zhang and Reese 2007). So called pioneering transcription factors induce nucleosome depletion, either by directly competing with histones for binding to DNA, and/or by recruiting co-activators, such as chromatin remodelers, histone chaperones and histone deacetylases (Tsukiyama, Becker et al. 1994; Yu and Morse 1999; Bernstein, Liu et al. 2004; Yarragudi, Miyake et al. 2004; Bai, Ondracka et al. 2011; Ganapathi, Palumbo et al. 2011; Ozonov and van Nimwegen 2013). All these factors cooperate to establish and maintain nucleosome depletion at active promoters (Cosma, Tanaka et al. 1999; Natarajan, Jackson et al. 1999; Deckert and Struhl 2001; Hassan, Neely et al. 2001; Neely, Hassan et al. 2002; Ng, Robert et al. 2002; Mitra, Parnell et al. 2006). Then, elongation begins with the release of RNA polymerase II from the GTFs at the promoter regions and the recruitment of elongation factors. During elongation, nucleosomes are temporarily disassembled or displaced ahead of and reassembled behind the transcription machinery, using both old and new histones (Kristjuhan and Svejstrup 2004; Schwabish and Struhl 2004; Workman 2006; Bintu, Kopaczynska et al. 2011). There are some indications that RNA polymerase II can enter the first 30 bp of nucleosomal DNA and that this initial transversal can facilitate the release of one H2A/H2B dimer (Kireeva, Walter et al. 2002; Hodges, Bintu et al. 2009; Bintu, Kopaczynska et al. 2011). Moreover, elongation involves a number of histone chaperones, including HIRA (Kim, Seol et al. 2007), ASF1 (Schwabish and Struhl 2006; Kim, Seol et al. 2007), spt6 (Ardehali, Yao et al. 2009; Ivanovska, Jacques et al. 2011), Nap1 (Del Rosario and Pemberton 2008) and FACT (Belotserkovskaya, Oh et al. 2003; Kaplan, Laprade et al. 2003; Mason and Struhl 2003; Schwabish and Struhl 2004; Jamai, Puglisi et al. 2009), and chromatin remodelers, such as SWI/SNF (Treand, du Chene et al. 2006; Schwabish and Struhl 2007; Shivaswamy and Iyer 2008), ISW1b (Morillon, Karabetsou et al. 2003; Smolle, Venkatesh et al. 2012) and CHD1 (Smolle, Venkatesh et al. 2012; Skene, Hernandez et al. 2014). Interestingly, elongation seems to shift parental nucleosomes upstream, perhaps contributing to stacking of nucleosomes against a fixed barrier around the TSS and thereby the formation of nucleosomal arrays in gene bodies (Weiner, Hughes et al. 2010; Radman-Livaja, Verzijlbergen et al. 2011; Zhang, Wippo et al. 2011). The restoration of nucleosome structure in the wake of elongation, including the strong

positioning of the +1 nucleosome and the formation of linked regular nucleosomal arrays in gene bodies, is highly important to prevent cryptic transcription from within genes (Kaplan, Laprade et al. 2003; Mason and Struhl 2003; Schwabish and Struhl 2004; Schwabish and Struhl 2006; Gkikopoulos, Schofield et al. 2011; Hennig, Bendrin et al. 2012; Pointner, Persson et al. 2012; Shim, Choi et al. 2012). Whether it also facilitates elongation remains to be discovered. Formation and maintenance of the NDR at the TTS is also dependent on elongation, and it may have an important role in transcription termination (Alen, Kent et al. 2002; Morillon, Karabetsou et al. 2003; Fan, Moqtaderi et al. 2010; Durand-Dubief, Svensson et al. 2011).

1.2.5 Replication-independent nucleosome turnover

In recent years it has become clear that nucleosomes are highly dynamic. Every round of replication brings about the genome-wide turnover of half the histones associated with DNA. In addition, there is a substantial degree of RI turnover of both canonical and variant histones outside of S phase (Kimura and Cook 2001; Ahmad and Henikoff 2002; Choi, Shin et al. 2005; Thiriet and Hayes 2005; Linger and Tyler 2006). While histone H2A-H2B turns over rapidly across the genome, turnover of histone H3-H4 varies between different regions, and seems to be largely dependent on transcription.

The first studies on genome-wide RI histone turnover relied on inducible expression of tagged histones from ectopic loci in *S. cerevisiae* arrested in the G1 phase of the cell cycle (Dion, Kaplan et al. 2007; Jamai, Imoberdorf et al. 2007; Rufiange, Jacques et al. 2007). In *S. cerevisiae*, promoters of genes are characterized by high levels of histone H3 turnover, while gene bodies display much lower levels. In both regions, turnover correlates with transcription rate. Interestingly, high turnover of histone H3 at promoters also coincides with low steady-state histone H3 occupancy, suggesting that nucleosome depletion at promoters may reflect a highly dynamic equilibrium between nucleosome disassembly and assembly. In support, activation of inducible genes has been shown to trigger both a decrease in histone occupancy and an increase in turnover of histone H3 (Kim, Seol et al. 2007). Low levels of histone H3 turnover are also seen at many inactive promoters, particularly at the nucleosomes surrounding the NDR (Dion, Kaplan et al. 2007; Jamai, Imoberdorf et al. 2007; Rufiange, Jacques et al. 2007). This indicates that nucleosome turnover at promoters is not absolutely dependent on transcription and that some inactive genes display a basal level of nucleosome turnover, possibly to allow for rapid induction. More recently, genome wide histone turnover in *S. cerevisiae* was assessed using the recombination-induced tag exchange (RITE) method to map differentially tagged old and new histone H3 expressed from an endogenous locus (Verzijlbergen, Menendez-Benito et al. 2010). Intriguingly, RI turnover could replace half of the old histones associated with chromatin after just 5 hours of arrest.

In *D. melanogaster*, newly synthesized native histones have been mapped using a method referred to as covalent attachment of tags to capture histones and identify turnover (CATCH-IT) (Deal, Henikoff et al. 2010). This method relies on metabolic pulse labelling of histone H3-H4. In fruit fly, gene bodies display high levels of histone H3-H4 turnover, correlating with transcription, while promoters display much lower levels of histone H3-

H4 turnover, independently of transcription. The difference in the pattern of turnover as compared to yeast may be related to experimental differences, or reflect biological differences between species. Interestingly, when CATCH-IT was applied to mammalian cells, the pattern of histone H3-H4 turnover at gene regions more resembled that of *S. cerevisiae* (Skene, Hernandez et al. 2014). This study also revealed that turnover is particularly pronounced at the regions immediately flanking promoter NDRs.

Several studies have identified factors that influence nucleosome turnover, and as expected, they overlap with factors that determine nucleosome occupancy and positioning, and that have known roles in transcription. The histone chaperones HIRA and FACT, both of which are involved in re-deposition of old histone H3-H4 in the wake of transcription, have been shown to inhibit histone H3 turnover over coding regions (Kim, Seol et al. 2007; Jamai, Puglisi et al. 2009). Conversely, ASF1, which is involved in deposition of new histone H3-H4 in the wake of transcription, has been shown to promote transcription-dependent turnover of histone H3 over coding regions (Rufiange, Jacques et al. 2007). Similarly, the chromatin remodelers CHD1 and ISW1b, both of which are important for nucleosome reassembly and positioning in gene bodies, inhibits nucleosome turnover in coding regions (Radman-Livaja, Quan et al. 2012; Smolle, Venkatesh et al. 2012; Skene, Hernandez et al. 2014). Interestingly, CHD1 also promotes turnover of histone H3 at promoters and the 5' ends of gene bodies, which seems to be important for promoter escape by RNA polymerase II (Skene, Hernandez et al. 2014). A similar role for CHD1 has also been identified at boundaries between introns and exons (Park, Shivram et al. 2014). Last, a screen for genes that promote histone H3 turnover in *S. cerevisiae* have identified a potential role for HAT1, the histone chaperone Nucleosome assembly protein 1 (NAP1) as well as the chromatin remodelers RSC, SWI/SNF and SWR1, in nucleosome turnover (Verzijlbergen, van Welsem et al. 2011).

1.3 CENTROMERIC CHROMATIN AND CENP-A

The ability to accurately transmit genetic information at mitosis and meiosis is a fundamental cellular function. Following genome replication, sister chromatids must be equally distributed to the daughter cells. Chromosome segregation is dependent on the mitotic spindle, and the attachment of microtubuli originating from opposite poles of the spindle to each sister chromatid. The attachment occurs at the kinetochore, which is a large protein structure in most species formed at a chromosomal region that is referred to as the centromere (figure 5).

1.3.1 Centromeric chromatin

Centromeric DNA often contains both unique and repetitive elements, but there is a remarkable variation in both size and sequence between centromeres of different organisms and different chromosomes (Henikoff, Ahmad et al. 2001; Buscaino, Allshire et al. 2010). In most eukaryotes, centromeric DNA sequences are neither necessary nor sufficient for centromere function and evidence suggests that centromere identity is epigenetically specified (Allshire and Karpen 2008; Bernad, Sanchez et al. 2009; Buscaino, Allshire et al. 2010; Henikoff and Furuyama 2010; Stimpson and Sullivan 2010; Maddox,

Corbett et al. 2012; Sekulic and Black 2012). At the heart of epigenetic specification of centromeres is the centromere-specific histone H3 variant CENP-A, which is both required and sufficient for centromere formation and function when tethered to an ectopic loci (Howman, Fowler et al. 2000; Foltz, Jansen et al. 2006; Liu, Rattner et al. 2006; Guse, Carroll et al. 2011; Mendiburo, Padeken et al. 2011). However, some studies indicate that CENP-A is not sufficient for establishment of functional kinetochores at native centromeres, suggesting that there may be additional factors at work (Van Hooser, Ouspenski et al. 2001; Gascoigne, Takeuchi et al. 2011).

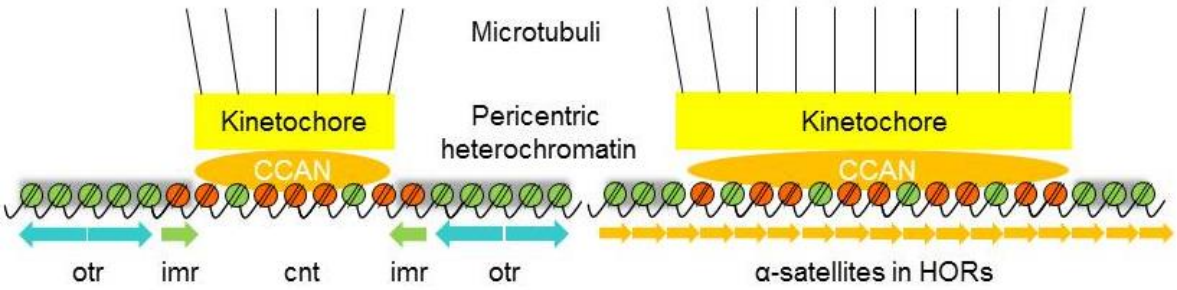

Figure 5. Schematic view of *S. pombe* and human centromeres. Centromeric chromatin containing CENP-A associates proteins of the CCAN throughout the cell cycle. The CCAN then forms the foundation for kinetochore assembly at mitosis. CENP-A assembles on the central core (cnt) and innermost repeat (imr) regions of fission yeast centromeric DNA and is flanked by pericentric heterochromatin formed on the outermost repeat (otr) regions. Human centromeric DNA contains different α-satellite repeats organized in higher order repeats (HORs). CENP-A nucleosomes form on a subset of the repeats and is flanked by centromeric heterochromatin.

1.3.2 Structure of CENP-A nucleosomes and chromatin

Chromatin containing CENP-A has an important role in the physical and functional specification of centromeres. The constitutive centromere-associated network (CCAN), which is present at CENP-A containing chromatin throughout the cell cycle, mediates assembly of functional kinetochores at mitosis (figure 5).

CENP-A displays more than ~60% sequence similarity to canonical histone H3 over the C-terminal domains and the HFD, but differs from all other known histones in the N terminal domain (Palmer, O'Day et al. 1987). The key to kinetochore formation lies in the N and C terminal tails of CENP-A (Chen, Baker et al. 2000; Ravi, Kwong et al. 2010; Fachinetti, Folco et al. 2013). The CCAN component CENP-C binds to the C terminal of CENP-A, which is sufficient to recruit CENP-C (Carroll, Milks et al. 2010; Guse, Carroll et al. 2011). However, this interaction it is not absolutely required, indicating that there are other mechanisms of CCAN recruitment to centromeres (Black, Jansen et al. 2007). The key to centromere targeting lies in the presence of a unique CENP-A targeting domain (CATD) in the HFD of CENP-A (Black, Jansen et al. 2007). This region also binds to the CCAN component CENP-N (Carroll, Silva et al. 2009). Studies of crystal structures have also revealed several unique features of nucleosomes containing CENP-A, including unique surface-accessible bulge that is required for stable incorporation of CENP-A (Sekulic, Bassett et al. 2010; Tachiwana, Kagawa et al. 2011; Tachiwana, Kagawa et al. 2012), a more rigid and compact structure mediated by the CATD (Black, Jansen et al. 2007;

Sekulic, Bassett et al. 2010; Cho and Harrison 2011), and yet more loose terminal DNA contacts (Conde e Silva, Black et al. 2007; Dechassa, Wyns et al. 2011; Kingston, Yung et al. 2011; Panchenko, Sorensen et al. 2011; Tachiwana, Kagawa et al. 2011; Hasson, Panchenko et al. 2013). Comparisons of CENP-A- and H3-containing nucleosomal arrays also suggest a compaction at the chromatin level (Panchenko, Sorensen et al. 2011; Geiss, Keramisanou et al. 2014).

Interestingly, there is also an ongoing debate about the molecular architecture of CENP-A nucleosomes (Black and Cleveland 2011; Henikoff and Furuyama 2012; Maddox, Corbett et al. 2012; Bui, Walkiewicz et al. 2013). This discussion mostly revolves around formation of CENP-A octasomes versus formation of CENP-A hemisomes. First, *in vitro* assembly of CENP-A with histone H4, H2A and H2B on DNA has been shown to produce conventional octameric nucleosomes with left-handed wrapping of DNA (Yoda, Ando et al. 2000; Camahort, Shivaraju et al. 2009; Sekulic, Bassett et al. 2010; Dechassa, Wyns et al. 2011; Kingston, Yung et al. 2011; Tachiwana, Kagawa et al. 2011) or tetrameric hemisomes with right-handed wrapping of DNA (figure 6) (Furuyama and Henikoff 2009; Furuyama, Codomo et al. 2013). Similarly, there is seemingly contradicting *in vivo* evidence for CENP-A-containing octasomes (Shelby, Vafa et al. 1997; Erhardt, Mellone et al. 2008; Camahort, Shivaraju et al. 2009; Tachiwana, Kagawa et al. 2011; Bassett, DeNizio et al. 2012; Tachiwana, Kagawa et al. 2012; Zhang, Colmenares et al. 2012; Aravamudhan, Felzer-Kim et al. 2013; Hasson, Panchenko et al. 2013; Padeganeh, Ryan et al. 2013) and hemisomes (Dalal, Wang et al. 2007; Furuyama and Henikoff 2009; Dimitriadis, Weber et al. 2010; Huang, Hajra et al. 2011; Krassovsky, Henikoff et al. 2012). Some recent studies have attempted to reconcile these findings by presenting evidence for cell cycle-dependent transitions in the structure of CENP-A nucleosomes (Bui, Dimitriadis et al. 2012; Shivaraju, Unruh et al. 2012). One possibility is that CENP-A nucleosomes are hemisomes following replication, and that these mature into octamers when newly synthesized CENP-A is incorporated. If CENP-A nucleosomes are hemisomes at some point, this unique feature may well contribute to the epigenetic inheritance as well as structural specification of centromeric chromatin (Henikoff and Furuyama 2010).

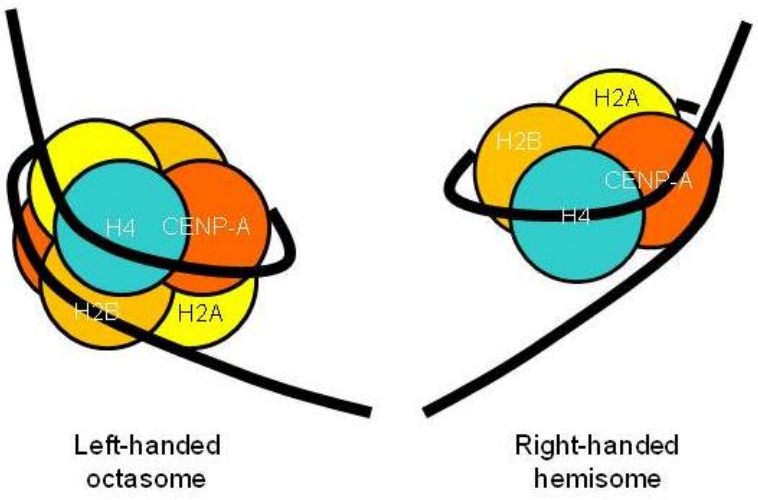

Figure 6. Alternative models for the composition and structure of CENP-A nucleosomes. Different studies have found seemingly contradicting evidence for formation of conventional octasomes with left-handed wrapping of DNA and hemisomes with right handed-wrapping of DNA.

In human cells and *D. melanogaster*, nucleosomes containing histone H3.1 and H3.3 are interspersed with nucleosomes containing CENP-A at the central domains of centromeres (figure 5), and histone H3.3 seem to act as a placeholder after replication, which is subsequently removed upon assembly of newly synthesized CENP-A (Blower, Sullivan et al. 2002; Dunleavy, Almouzni et al. 2011). However, recent studies indicate that centromeric chromatin is folded so that both nucleosomes containing CENP-A and H3 are available and thereby both species could be involved in kinetochore formation (Ribeiro, Vagnarelli et al. 2010). Intriguingly, centromeric histone H3 carries a unique pattern of histone modifications, including some euchromatic marks, which have been implied in CENP-A deposition (Sullivan and Karpen 2004; Nakano, Cardinale et al. 2008; Cardinale, Bergmann et al. 2009; Bergmann, Rodriguez et al. 2011; Ohzeki, Bergmann et al. 2012). It has also been suggested that H3 nucleosomes are required for centromeric association of the CENP-T/W/S/X complex with centromeres (Hori, Amano et al. 2008; Prendergast, van Vuuren et al. 2011; Nishino, Takeuchi et al. 2012). Interestingly, the CENP-T/W/S/X complex forms a nucleosome-like structure that induces positive DNA supercoiling, similar to hemisomes.

1.3.3 Incorporation and maintenance of CENP-A

CENP-A localizes almost exclusively to active centromeres, although over expression leads to promiscuous localization. Upon DNA replication, parental CENP-A histones are equally partitioned to sister centromeres (Shelby, Monier et al. 2000; Jansen, Black et al. 2007; Dunleavy, Almouzni et al. 2011; Mellone, Grive et al. 2011). This is followed by RI incorporation of newly synthesized CENP-A at pre-existing centromeres, the timing of which varies between species (Pearson, Yeh et al. 2004; Jansen, Black et al. 2007; Hemmerich, Weidtkamp-Peters et al. 2008; Takayama, Sato et al. 2008; Boyarchuk, Montes de Oca et al. 2011; Mellone, Grive et al. 2011). In several species, including humans, CENP-A is not replenished until G1, meaning that centromeric chromatin contains half the complement of CENP-A at mitosis. Temporal restriction of CENP-A deposition is central to centromere inheritance and function, and is at least partially achieved by cell cycle regulated recruitment of CENP-A assembly factors. Deposition of newly synthesized CENP-A is achieved by the coordinated action of an intricate network of molecular players (De Rop, Padeganeh et al. 2012; Falk and Black 2012; Maddox, Corbett et al. 2012; Stellfox, Bailey et al. 2013; Muller and Almouzni 2014). Regional restriction of CENP-A deposition is in part achieved by a feed-forward mechanism between pre-existing centromeric chromatin and kinetochore components, and the CENP-A assembly machinery (Fachinetti, Folco et al. 2013; Hori, Shang et al. 2013).

Incorporation of newly synthesized CENP-A can be divided into initiation, deposition and maintenance. A central player in the initiation, or priming, process is the Minichromosome instability 18 (MIS18) complex, consisting of MIS18α, MIS18β and MIS18 binding protein 1 (MIS18BP1), which are all required for CENP-A deposition (Hayashi, Fujita et al. 2004; Fujita, Hayashi et al. 2007; Maddox, Hyndman et al. 2007). Centromere specific recruitment of the Mis18 complex is in part mediated by a direct interaction between MIS18BP1 and CENP-C, thus coupling CENP-A assembly to pre-existing centromeric chromatin (Moree, Meyer et al. 2011; Dambacher, Deng et al. 2012). Moreover, assembly

of the human MIS18 complex is regulated by cell-cycle dependent phosphorylation of MIS18BP1 by Cyclin-dependent kinase (CDK) 1 and 2 (Silva, Bodor et al. 2012), while centromeric localization of the complex is licensed by phosphorylation of MIS18BP1 by Polo-like kinase 1 (PLK1) (McKinley and Cheeseman 2014), thus tightly coupling CENP-A assembly to cell cycle progression. The mechanism behind centromere priming by the MIS18 complex remains elusive, but is hypothesized to involve changes in histone acetylation and DNA methylation (Hayashi, Fujita et al. 2004; Fujita, Hayashi et al. 2007; Kim, Lee et al. 2012; Ohzeki, Bergmann et al. 2012).

Assembly of newly synthesized CENP-A is mediated by the CENP-A-specific chaperone Holliday junction recognition protein (HJURP) (Mizuguchi, Xiao et al. 2007; Stoler, Rogers et al. 2007; Dunleavy, Roche et al. 2009; Foltz, Jansen et al. 2009; Pidoux, Choi et al. 2009; Williams, Hayashi et al. 2009; Barnhart, Kuich et al. 2011; Bernad, Sanchez et al. 2011; Dechassa, Wyns et al. 2011). HJURP interacts with the CENP-A-H4 heterodimer with the major binding surface contacting the CATD of CENP-A, thereby stabilizing the pre-nucleosomal complex CENP-A-H4 and preventing DNA binding until deposition at centromeres (Shuaib, Ouararhni et al. 2010; Cho and Harrison 2011; Hu, Liu et al. 2011; Zhou, Feng et al. 2011; Bassett, DeNizio et al. 2012). Moreover, HJURP was recently shown to have an active role in the actual loading mechanism of CENP-A (Muller, Montes de Oca et al. 2014). In yeast, the HJURP homolog Scm3 may have additional help from the chaperone Sim3 in escorting CENP-A (Dunleavy, Pidoux et al. 2007). In both yeast and mammalian cells, the MIS18 complex is required for targeting of HJURP to centromeres, but it is only in fission yeast that a direct interaction between these factors have been found (Dunleavy, Roche et al. 2009; Pidoux, Choi et al. 2009; Barnhart, Kuich et al. 2011; Wang, Liu et al. 2014). In human cells, recruitment of HJURP by MIS18β is regulated by CDK1-dependent phosphorylation of HJURP (Muller, Montes de Oca et al. 2014; Wang, Liu et al. 2014). CDK1 also phosphorylates CENP-A, thereby preventing binding to HJURP, further strengthening cell cycle-coupled control of CENP-A assembly (Yu, Zhou et al. 2015). In human cells, recruitment of HJURP is also affected by H3K9 methylation (Ohzeki, Bergmann et al. 2012), H3K4 methylation and H3 acetylation (Bergmann, Rodriguez et al. 2011). In recent years it has become clear that transcription of centromeric chromatin plays a role in deposition and/or maintenance of CENP-A (Wong, Brettingham-Moore et al. 2007; Choi, Stralfors et al. 2011; Ohkuni and Kitagawa 2011; Chan, Marshall et al. 2012). A recent study in fission yeast suggest that initiation of RNA polymerase II transcription combined with frequent stalling during subsequent elongation, as dictated by centromeric DNA sequences, promotes CENP-A incorporation (Catania, Pidoux et al. 2015). One suggestion is that transcription-coupled remodeling enables replacement of histone H3 with CENP-A. Alternatively or additionally, transcription may be important for generating a chromatin environment that is appropriate for CENP-A assembly. Moreover, centromeric non-coding RNA has been shown to stabilize CENP-C (Wong, Brettingham-Moore et al. 2007; Du, Topp et al. 2010) and to play a role in kinetochore regulation (Ferri, Bouzinba-Segard et al. 2009). The need for transcription may explain why the non-specific histone chaperone Retinoblastoma-associated protein 48 (RbAp48) (Hayashi, Fujita et al. 2004; Fujita, Hayashi et al. 2007), CHD1 and components of the FACT complex (Walfridsson, Bjerling et al. 2005; Okada, Okawa et al. 2009; Chan, Marshall et al. 2012), and H2Bub

mediated by RNF20 (Sadeghi, Siggens et al. 2014) have been shown to affect the levels of centromeric CENP-A.

Some evidence suggests that newly incorporated CENP-A nucleosomes needs to go through an active maturation process before becoming fully stable. This may involve the Remodeling and spacing factor (RSF) complex (Perpelescu, Nozaki et al. 2009) and small Guanosine triphospathase (GTPase) activity (Lagana, Dorn et al. 2010; Prendergast and Sullivan 2010). Moreover, non-centromeric CENP-A is actively degraded via the ubiquitin pathway (Collins, Furuyama et al. 2004; Moreno-Moreno, Torras-Llort et al. 2006; Hewawasam, Shivaraju et al. 2010; Ranjitkar, Press et al. 2010). Interestingly, this is somehow also dependent on the SWI/SNF complex in *S. cerevisiae* (Gkikopoulos, Singh et al. 2011).

The CENP-A-containing central cores of regional centromeres are generally flanked by pericentric heterochromatin, usually assembled on repetitive DNA sequences (figure 5). At least in fission yeast, this produces RNA polymerase II-dependent siRNAs that mediate heterochromatin formation via the RNAi-machinery (Reinhart and Bartel 2002; Volpe, Kidner et al. 2002; Djupedal, Portoso et al. 2005; Kato, Goto et al. 2005). Fission yeast pericentric heterochromatin has an essential role at least in *de novo* assembly CENP-A (Folco, Pidoux et al. 2008; Ishii, Ogiyama et al. 2008) and may also prevent spreading of CENP-A. In support, factors required to maintain the boundary between heterochromatin and CENP-A chromatin, such as the fission yeast chromatin remodeler Fft3 (Stralfors, Walfridsson et al. 2011), are also required for maintaining normal levels of CENP-A. In other species, the role of heterochromatin remains less well understood (Chan and Wong 2012)

1.4 DNA TOPOLOGY AND TOPOISOMERASES

The intertwining of two complementary strands in the DNA double helix is ideal for faithful propagation of genetic information, but imposes important constraints on DNA structure. DNA topology is a fundamental property that depicts the three-dimensional shape and path of the DNA double helix in terms of supercoiling, catenation and knotting (figure 7).

1.4.1 Supercoiling

Supercoiling describes one type of topological strain in the DNA double helix using the parameters of twist (Tw) and writhe (Wr) (Barbi, Mozziconacci et al. 2012). Under physiological conditions, each helical turn about the central axis of relaxed DNA consists of approximately 10 bp. Twist describes an under- or over-winding of the DNA strands in comparison to this state, while writhe describes the plectonemic or solenoid wrapping of the helix around itself. The total number of times that the DNA double helix rotates around the axis is referred to as the linking number (Lk) and is determined by the sum of twist and writhe (Lk = Tw + Wr). Twist and writhe are interchangeable, and as the amount of twist that can be accommodated in DNA molecules is limited, supercoiling is mostly presented as writhe.

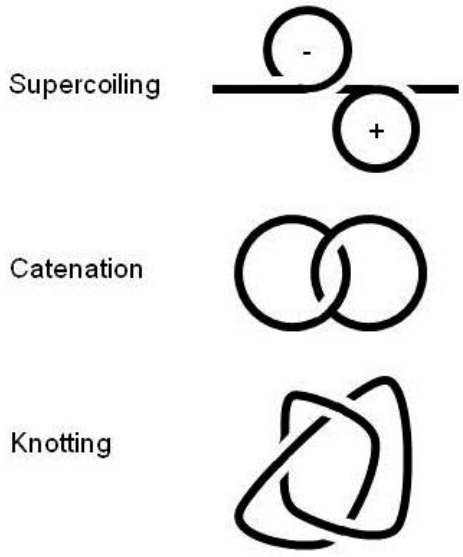

Figure 7. DNA topology. DNA topology describes the three-dimensional shape and path of the double helix in terms of supercoiling, catenation and knotting.

There is an intimate interplay between supercoiling and DNA-dependent cellular processes (Wang 1985; Wang 1996; Champoux 2001; Wang 2002; Vos, Tretter et al. 2011; Chen, Chan et al. 2013). First, since the topological state affects the energy state, structure and stiffness of the template, DNA-dependent transactions and DNA-binding factors are affected by the current topological state of DNA. Second, many DNA-dependent processes themselves impose topological strains in DNA by causing unwinding, bending or writhing of the double helix. If a DNA molecule is free to rotate, supercoiling can dissipate along the molecule and be released from the ends. However, because of the size of eukaryotic chromosomes and their organization into large domains with fixed ends, such rotation is limited (Joshi, Pina et al. 2010; Kegel, Betts-Lindroos et al. 2011). This allows for accumulation of topological stress. In fact, recent studies of eukaryotic genomes have revealed a landscape of domains with different degrees of supercoiling, as well as local accumulation of supercoiling in particular regions, such as promoters of genes (Bermudez, Garcia-Martinez et al. 2010; Naughton, Avlonitis et al. 2013).

1.4.2 DNA topoisomerases

As excessive accumulation of supercoiling may deregulate and inhibit DNA dependent cellular processes, careful regulation of DNA topology is an important cellular function, which also offers an opportunity for regulation of a number of cellular processes. DNA topoisomerases are a family of ubiquitous enzymes that mediates topological transformations in DNA by cutting, shuffling and re-ligating DNA strands (Wang 1985; Wang 1996; Champoux 2001; Wang 2002; Corbett and Berger 2004; Vos, Tretter et al. 2011; Chen, Chan et al. 2013). DNA topoisomerases catalyzes a reversible trans-esterification reaction between a tyrosine residue at the active site and a phosphate group in the DNA backbone. The first reaction creates a covalent adduct between the enzyme and DNA and opens a transient nick in the DNA backbone. This allows for topological transformations. The second reaction, which is the reverse of the previous, re-ligates the DNA backbone and resets the enzyme.

	Class	Human	Yeast	Molecular activities	Major cellular functions
DNA topoisomerase I	Type 1B	Top1 Top1mt	Top1	Relaxation of positive and negative supercoiling	Replication Transcription Chromosome condensation Mitochondrial replication and transcription
DNA topoisomerase II	Type 2A	Top2α Top2β	Top2	Relaxation of positive and negative supercoiling Decatenation	Decatenation of sister chromatids at mitosis Replication Transcription Chromosome condensation
DNA topoisomerase III	Type 1A	Top3α Top3β	Top3	Relaxation of negative supercoiling Decatenation	Homologous recombination Replication S-phase checkpoint modulation Translation Mitochrondrial replication and transcription

Table 2. Eukaryotic DNA topoisomerases.

Based on differences in structure and in the enzymatic reaction, DNA topoisomerases are divided into class I and II, which are further divided into subfamilies A, B and C (table 2) (Champoux 2001; Wang 2002; Corbett and Berger 2004; Vos, Tretter et al. 2011; Chen, Chan et al. 2013). Type I DNA topoisomerases create a single-stranded gap in the DNA backbone. Members of the type IB family includes eukaryotic topoisomerase I (Top1) and mitochondrial Top1 (mtTop1) in mammalian cells. These enzymes uses a mechanism based on free rotation of the cut strand to relax supercoiling (Stewart, Redinbo et al. 1998), and are key enzymes for relieving supercoiling associated with replication and transcription (Leppard and Champoux 2005). Members of the type 1A family include eukaryotic Topoisomerase III (Top3), reverse gyrase in thermophilic bacteria and archaea, and bacterial and archaeal Top1 and Top3. These enzymes use a gate mechanism for passage of an intact strand through the cut strand (Viard and de la Tour 2007), and have a preference for relaxing negatively supercoiled DNA *in vitro* (Kim and Wang 1992; Hanai, Caron et al. 1996; Seki, Seki et al. 1998; Goulaouic, Roulon et al. 1999; Wilson, Chen et al. 2000). Eukaryotic Top3 is essential for genome stability and is mostly recognized for its important function in homologous recombination (HR), thus affecting DNA repair, meiosis and recombination (Plank, Wu et al. 2006; Mankouri and Hickson 2007; Bizard and Hickson 2014; Swuec and Costa 2014). The type 1C subfamily is represented by archaeal Top5, which is similar but evolutionary distinct from members of the IB subfamily, with an additional unique role in DNA repair. Type II DNA topoisomerases act as multimers and passes a segment of intact duplex DNA through a double-stranded gap in the DNA backbone in an ATP-dependent manner, to relax supercoiling and to perform catenation or decatenation (Wang 1998). The type IIA family includes eukaryotic topoisomerase II (Top2), bacterial DNA gyrase and Top4, and some viral enzymes. Eukaryotic Top2 which

plays critical roles in disentanglement of catenated sister chromatids at mitosis, but is also implicated in other aspects of replication, mitotic chromatin condensation, and transcription. The type IIB family is represented by Top6, found in archaea, plants, and some bacteria, protists and algae. Top6 uses a similar mechanism as type IIA DNA topoisomerases, but is structurally distinct. Interestingly, eukaryotic Spo11 shows clear homology with Top6 and is essential for generating double stranded breaks to initiate meiotic recombination.

The functions of different DNA topoisomerases are both distinct and redundant. Their roles in different cellular processes depend on the nature of the topological problem to be solved, the chromatin location and environment, posttranslational modifications of the enzymes and the presence of other factors. DNA topoisomerases are often parts of larger complexes that act in specific pathways and on specific DNA structures.

1.4.3 DNA topology and nucleosome dynamics

Chromatin is constantly changing its structure in order to accommodate and control DNA dependent cellular processes. Nucleosomes are constantly being assembled, disassembled and remodeled as a part of various cellular processes. Since DNA is wrapped around the nucleosome core by left-handed solenoid wrapping, chromatin assembly and remodeling involves changes in the path of the DNA double helix. In agreement, chromatin remodeling is associated with changes in DNA topology (Havas, Flaus et al. 2000; Gavin, Horn et al. 2001). Nucleosome assembly traps negative writhe in the DNA template (Simpson, Thoma et al. 1985; Pfaffle and Jackson 1990), which is compensated by positive supercoiling in free regions of DNA in order to maintain a constant linking number (Clark and Felsenfeld 1991; Gupta, Zlatanova et al. 2009). Conversely, nucleosome disassembly releases unconstrained negative supercoiling in unconstructed regions of DNA (Clark and Felsenfeld 1991). In agreement, nucleosome assembly is facilitated by negative supercoiling (Pfaffle and Jackson 1990; Clark and Felsenfeld 1991; Patterton and von Holt 1993; Hizume, Yoshimura et al. 2004), while positive supercoiling prevents nucleosome assembly and promotes histone H2A-H2B dimer loss and nucleosome disruption (figure 8) (Pfaffle, Gerlach et al. 1990; Levchenko, Jackson et al. 2005; Sheinin, Li et al. 2013).

Studies on nucleosome assembly using cell-free extracts from *Xenopus* and *Drosophila* indicate that relaxation of supercoiling during this process is performed mainly by Top1 (Almouzni and Mechali 1988; Becker and Wu 1992). Similar studies, using *Xenopus* and yeast extracts have shown that relaxation is mediated redundantly by both Top1 and Top2 (Sekiguchi and Kmiec 1988; Garinther and Schultz 1997). Inhibition or mutation of DNA topoisomerases results in rather subtle changes in nucleosome occupancy *in vivo* (Cavalli, Bachmann et al. 1996; Teves and Henikoff 2014).

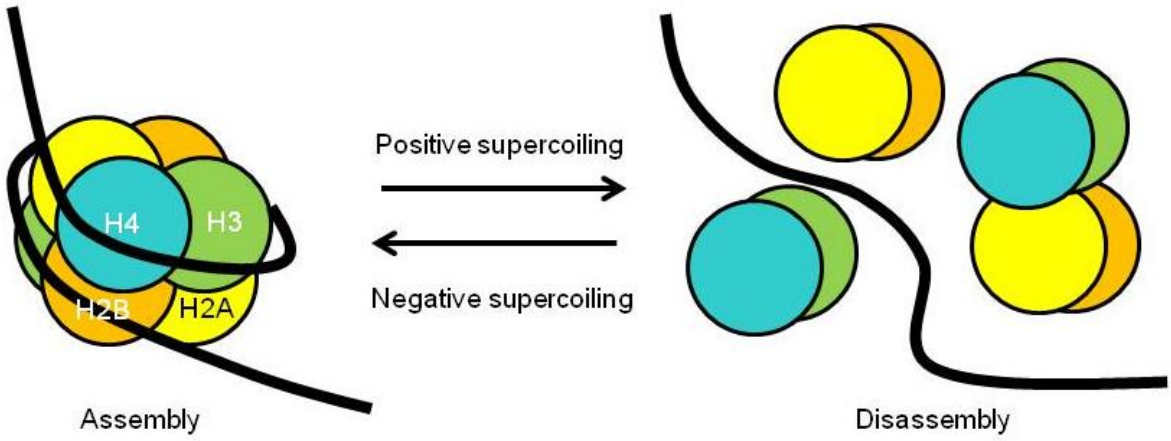

Figure 8. DNA topology and nucleosome dynamics. Negative supercoiling promotes nucleosome assembly while positive supercoiling promotes nucleosome disassembly.

However, recent studies in mammalian cells indicate that there is a profound effect on turnover of nucleosomes in these mutants (Teves and Henikoff 2014). In gene bodies, where inhibition of DNA topoisomerases results in increased positive supercoiling, nucleosomes display decreased stability and increased turn-over. At promoters and terminators, inhibition of DNA topoisomerases results in increased negative supercoiling and subsequently, nucleosomes display increased stability and decreased turn-over.

1.4.4 DNA topology and transcription

Transcription is highly dependent on the topological state of the DNA template and transcription itself is a strong generator of topological strains (Roca 2011; Baranello, Kouzine et al. 2013; Ma and Wang 2014). Upon initiation, binding of transcription factors and assembly of the transcriptional machinery are dependent on the topological state of the underlying DNA, and may in turn impose changes in this parameter (Mizutani, Ohta et al. 1991; Mizutani, Ura et al. 1991; Diffley and Stillman 1992; Bazett-Jones, Leblanc et al. 1994; Giese, Pagel et al. 1997; Kahn 2000; Kouzine, Sanford et al. 2008). Moreover, interactions between the promoter and distant regulatory elements, such as enhancers, can be affected by supercoiling (Liu, Bondarenko et al. 2001). Elongation involves tracking of the transcription machinery along the DNA double helix with limited rotation (Nelson 1999), thereby building up positive supercoiling ahead while leaving negative supercoiling behind, in what is referred to as the 'twin supercoiled domain' model of transcription (figure 9) (Liu and Wang 1987). In agreement, transcriptional elongation is a powerful generator of supercoiling *in vitro* and *in vivo* (Wu, Shyy et al. 1988; Tsao, Wu et al. 1989; Yang, Jessee et al. 1989; Rahmouni and Wells 1992; Kouzine, Sanford et al. 2008; Kouzine, Gupta et al. 2013). Accumulation of negative supercoiling at behind the polymerase inhibits further elongation by promoting formation of RNA:DNA hybrids between the nascent transcript and the DNA template strand, known as R loops (Drolet, Broccoli et al. 2003; Tous and Aguilera 2007). Negative supercoiling at promoters affects chromatin structure and protein binding at the promoter, ultimately affecting subsequent rounds of initiation (Schultz, Brill et al. 1992). Accumulation of positive supercoiling ahead of the polymerase also inhibits elongation by promoting stalling of the RNA

polymerase (Gartenberg and Wang 1992; Schultz, Brill et al. 1992; Yin, Wang et al. 1995; Mondal, Zhang et al. 2003; Kouzine, Sanford et al. 2008; Joshi, Pina et al. 2010).

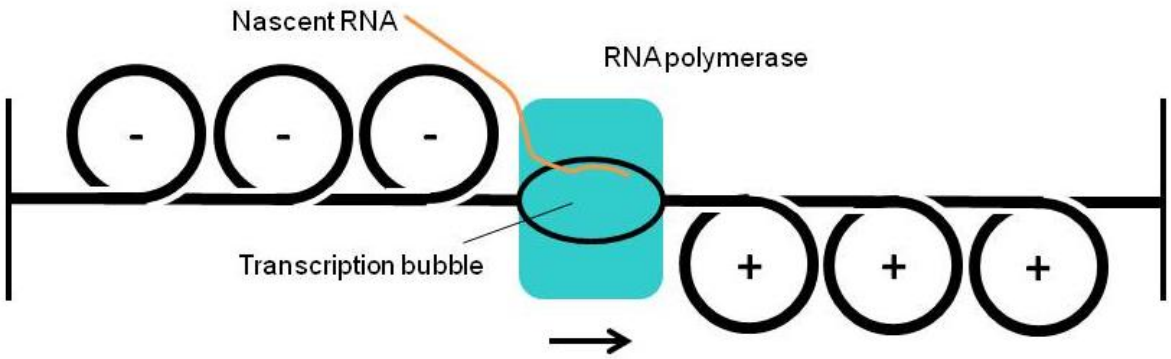

Figure 9. The 'twin supercoiled domain' model of transcription. Translocation od RNA polymerases along the double helix with limited rotation results in formation of positive supercoiling ahead and negative supercoiling behind the enzyme.

It has become increasingly clear that both eukaryotic Top1 and Top2 play important, and at least partially overlapping, roles at all stages of transcription (Mondal and Parvin 2001; Mondal, Zhang et al. 2003). Overall, binding of Top1 is preferentially seen at transcribed regions of chromatin (Fleischmann, Pflugfelder et al. 1984; Gilmour, Pflugfelder et al. 1986; Zhang, Wang et al. 1988; Stewart, Herrera et al. 1990). Similarly, Top1 is preferentially enriched at under-wound topological domains, which are characterized by open chromatin structure, high gene density and higher levels of transcription (Naughton, Avlonitis et al. 2013). Top1 is considered the major DNA topoisomerase for relieving torsional stress generated during transcriptional elongation. It is hypothesized to act as a DNA swivel for removal of positive writhe just ahead of the polymerase and to control supercoiling at promoters of moderately expressed genes. In agreement, it has been shown that Top1 is required to ensure progressivity of elongation, to avoid stalling of the RNA polymerase and to prevent R loop formation (Brill and Sternglanz 1988; Zhang, Wang et al. 1988; Schultz, Brill et al. 1992; Ljungman and Hanawalt 1996; Mondal, Zhang et al. 2003; El Hage, French et al. 2010; Teves and Henikoff 2014). In fact, Top1 seems particularly suitable for acting right in front of the elongating polymerase, where helical tension is displayed as positive twist and nucleosomes are disassembled. This is because Top1 is sensitive to DNA torque (Koster, Croquette et al. 2005) and has a preference for nucleosome-free templates (Salceda, Fernandez et al. 2006) *in vitro*. The interaction between Top1 and the phosphorylated C-terminal domain (CTD) of elongating RNA polymerase II may help to recruit and position the enzyme (Wu, Phatnani et al. 2010). In addition, Top1 activity is required to keep negative supercoiling at an appropriate level to support initiation and maintenance of the NDR (French, Sikes et al. 2011; Kouzine, Gupta et al. 2013; Teves and Henikoff 2014). Top1 has been implicated in both activation and repression of transcription (Kretzschmar, Meisterernst et al. 1993; Merino, Madden et al. 1993) and can affect transcription factor binding (Palecek, Vlk et al. 1997; Shykind, Kim et al. 1997; Jagelska, Brazda et al. 2008), RNA polymerase recruitment (Sperling, Jeong et al. 2011), and enhancer activation (Puc, Kozbial et al. 2015), and promoter-proximal pausing (Ma, Bai et al. 2013). However, although inhibition of Top1 clearly alters the kinetics of both initiating and elongating DNA polymerase II, the over-all effects on

transcription and transcribed chromatin *in vivo* are rather small, mainly affecting rDNA (Brill, DiNardo et al. 1987; Zhang, Wang et al. 1988; Collins, Weber et al. 2001; Teves and Henikoff 2014). This may be due to redundancy with Top2 in transcription.

In contrast to Top1, Top2 is preferentially enriched at over-wound topological domains, which tend to have a closed chromatin structure, fewer genes and overall lower levels of transcription (Naughton, Avlonitis et al. 2013). Indeed, Top2 is most efficient on highly over-wound chromatin templates *in vitro*, probably because over-wound chromatin is associated with increased writhe and the formation of DNA crossovers, which juxtaposes DNA segments in a way that promotes Top2 activity (Salceda, Fernandez et al. 2006; Fernandez, Diaz-Ingelmo et al. 2014). Similar to Top1, Top2 is involved in release of positive supercoiling during elongation, but seems to play a particular role at long genes (French, Sikes et al. 2011; Joshi, Pina et al. 2012). Indeed, Top2α is part of the RNA polymerase II holo-enzyme in mammalian cells (Mondal and Parvin 2001). Top2 is also found at promoters of very highly transcribed genes, specifically at the negatively supercoiled NDR close to the TSS (Sperling, Jeong et al. 2011; Kouzine, Gupta et al. 2013; Thakurela, Garding et al. 2013). Impairment of Top2 activity at these promoters reduces recruitment of RNA polymerase and also reduces its conversion from the initiating to the elongating form. Although Top2 is less efficient in relaxing negative supercoiling in chromatin (Fernandez, Diaz-Ingelmo et al. 2014), the extreme helical tension at very highly transcribed promoters also results in a high degree of writhe, thereby providing a good template for Top2 (Roca and Wang 1996; Salceda, Fernandez et al. 2006). Similar to Top1, Top2 may either act as an activator or repressor of transcription. At some promoters, activation involves cleavage by human Top2β and subsequent recruitment of DNA repair proteins (Ju, Lunyak et al. 2006; Lyu, Lin et al. 2006). Overall, the recruitment and activity of Top1 and Top2 at transcribed regions may be highly overlapping and the differences in their effect on different genes may to a large extent be context dependent.

Both Top1 and Top2 may also be involved in termination of transcription. In cells with reduced levels of topoisomerase activity, the NDR at the TSS becomes less defined and levels of read through transcription increases (Durand-Dubief, Svensson et al. 2011). One hypothesis is that the NDR at the TTS depends on positive supercoiling. Thus, when elongation is inhibited and RNA polymerase II reaches the end of the gene less frequently in the topoisomerase mutants, chromatin structure at the 3' end is perturbed, preventing proper termination for polymerases that manage to transcribe through the whole gene.

1.4.5 The known roles of Top3

Eukaryotic Top3 is less well studied compared to Top1 and Top2. While a single Top3 enzyme is present in yeast, both *D. melanogaster*, mouse and human cells harbour the two isoforms Top3α and Top3β (Kim and Wang 1992; Hanai, Caron et al. 1996; Seki, Seki et al. 1998; Seki, Seki et al. 1998; Goodwin, Wang et al. 1999; Maftahi, Han et al. 1999; Ng, Liu et al. 1999; Wilson, Chen et al. 2000). Top3 was initially considered a rather weak enzyme because it is limited to partial relaxation of negative supercoiling *in vitro* (Kim and Wang 1992; Hanai, Caron et al. 1996; Seki, Seki et al. 1998; Goulaouic, Roulon et al. 1999; Wilson, Chen et al. 2000). This selectivity is hypothesized to reflect the need for a

single stranded region in the DNA substrate, the formation of which is facilitated by the partial unwinding of the strands in negatively supercoiled DNA. However, Top3 is essential in many organisms and impairment result in severe phenotypes, including genome instability and chromosome segregation defects (Wallis, Chrebet et al. 1989; Li and Wang 1998; Goodwin, Wang et al. 1999; Maftahi, Han et al. 1999; Kwan and Wang 2001; Oh, Choi et al. 2002; Kwan, Moens et al. 2003). Highly under-wound substrates are created during the cellular processes of replication, transcription and recombination, and may also be specifically induced by the action of a helicase, creating hotspots for Top3 activity. Moreover, additional factors present *in vivo* have been shown to enhance and modulate the activity of Top3.

It is now well known that yeast Top3 and mammalian Top3α have important roles in HR (Plank, Wu et al. 2006; Mankouri and Hickson 2007; Bizard and Hickson 2014; Swuec and Costa 2014), which has implications for various cellular processes, including DNA repair (Chakraverty, Kearsey et al. 2001; Oh, Choi et al. 2002; Laursen, Ampatzidou et al. 2003; Mankouri and Hickson 2006), meiosis (Gangloff, de Massy et al. 1999; Kwan, Moens et al. 2003), and telomere maintenance (Kim, Caron et al. 1995; Tsai, Huang et al. 2006). Top3 acts in close physical and mechanistic collaboration with the RecQ family of DNA helicases and RecQ-mediated genome instability (RMI) proteins, forming what is known as the RecQ-Top3-RMI (RTR) complex (Gangloff, McDonald et al. 1994; Goodwin, Wang et al. 1999; Maftahi, Han et al. 1999; Bennett, Noirot-Gros et al. 2000; Wu, Davies et al. 2000; Chakraverty, Kearsey et al. 2001; Hu, Beresten et al. 2001; Laursen, Ampatzidou et al. 2003; Ahmad and Stewart 2005; Mullen, Nallaseth et al. 2005; Wu, Bachrati et al. 2006; Raynard, Zhao et al. 2008; Singh, Ali et al. 2008; Xu, Guo et al. 2008; Tang, Wu et al. 2015). The components of RTR complex mutually stimulate and modulate the activities of each other *in vitro* (Harmon, DiGate et al. 1999; Wu and Hickson 2002; Harmon, Brockman et al. 2003; Wu, Bachrati et al. 2006; Chen and Brill 2007; Raynard, Zhao et al. 2008; Cejka, Plank et al. 2010; Yang, Bachrati et al. 2010; Cejka, Plank et al. 2012). The RTR complex acts in a pathway of HR known as dissolution, which results in gene conversion without crossing over (Ira, Malkova et al. 2003; Wu and Hickson 2003; Hope, Cruzata et al. 2007). The RTR complex catalyzes resolution of double Holliday junctions (dHJs) by coupling DNA unwinding by the RecQ helicase to decatenation of intertwined strands by Top3. The 'convergent branch migration' model and the 'unravel and unlink' model describe potential mechanisms for dissolution (Plank and Hsieh 2009). A similar mechanism can be used to resolve single-stranded DNA entanglements that arise when replication forks converge (Chan, North et al. 2007). Recent studies also indicate that Top3 and Rmi1 also have unique functions in early and late HR, that are independent of RecQ helicases, such as the dissolution of D loops created upon strand invasion (Fasching, Cejka et al. 2015; Kaur, De Muyt et al. 2015).

The function of Top3β is less well studied, but it was recently shown to reduce formation of R loops during transcription, thereby promoting elongation (Wilson-Sali and Hsieh 2002; Yang, McBride et al. 2014). In addition, Top3β processes RNA topoisomerase activity and has been implied in translation (Stoll, Pietilainen et al. 2013; Xu, Shen et al. 2013).

2 AIM AND SIGNIFICANCE

The studies presented in this thesis has aimed at further characterizing the *in vivo* roles of DNA topoisomerases and CHD1-type chromatin remodelers in chromatin organization, nucleosome dynamics and transcription, using the fission yeast *Schizosaccharomyces pombe* as a model organism.

In the post genomic era, genome-wide studies of the chromatin landscape, the mechanisms shaping this and its effect on cellular functions, play important roles in expanding the knowledge about the orchestration and regulation of genome functions. A major part in regulation of DNA-dependent processes revolves around the question if a particular stretch of DNA is accessible or occluded by nucleosomes. Therefore, the identification of factors and mechanisms that govern nucleosome occupancy, positioning and turnover is fundamental. Much remains to be explored about the roles of DNA topoisomerases in the organization of chromatin and transcription. While it has been known for several decades that DNA topology and topoisomerases influences nucleosome dynamics *in vitro*, there have been rather few studies exploring this function *in vivo*. Questions remain regarding the redundant and specific functions of different DNA topoisomerases, their roles in different genomic regions, their targeting and regulation, and their interplay with other cellular factors. There is also a need for further characterizing the complex and often redundant roles of different ATP-dependent chromatin remodelers in chromatin organization. Pinpointing their roles in different cellular processes and regions, their mechanisms of action, their recruitment and regulation and their associations with other factors is an important task that needs to be fulfilled.

Apart from furthering basic knowledge of chromatin organization and the implications of DNA-dependent processes, these studies are of medical significance. Dysregulation of factors that govern chromatin structure and dynamics is a hallmark of several human diseases. Mutations and altered expression of Top1 and Top2 is seen in many types of cancer and is in many cases of prognostic value (Chen, Chan et al. 2013). Mutations in the genes encoding Top3 have not been implicated in any disease, but polymorphisms in Top3α, RMI1 and BLM have been associated with increased risk of cancer (Broberg, Huynh et al. 2009). Moreover, mutations in the human RecQ helicases Werner syndrome (WRN), Bloom syndrome (BLM) and RecQ protein-like 4 (RECQL4) results in rare genetic disorders characterized by cancer predisposition, congenital defects and premature ageing (Hanada and Hickson 2007). Similarly, chromatin remodelers are targets of cancer (Neely and Workman 2002; Nair and Kumar 2012), as well as congenital and developmental disorders (Boerkoel, Takashima et al. 2002; Martin 2010). Studies that aim at exploring the cellular roles of DNA topoisomerases and chromatin remodelers provide a foundation for new and improved treatment of these diseases.

Intriguingly, while DNA mutations are permanent, chromatin structure is dynamic and perturbations are potentially reversible. Drugs that target enzymes regulating chromatin structure have proven effective in treatment of various diseases. Inhibitors of DNA Top1, Top2α and Top2β are among the most effective and widely prescribed anti-cancer drugs

for solid tumors and hematological malignancies, and inhibitors of bacterial DNA topoisomerases are used as antibiotics (Pommier 2013). However, these treatmenst are associated with rather severe side-effects (Pommier 2013; Pendleton, Lindsey et al. 2014) and with development of drug resistance (Pilati, Nitti et al. 2012; Beretta, Gatti et al. 2013). Moreover, there is not yet any clinically useful drug that targets Top3, although its involvement in DNA repair suggests that such compounds can be used for sensitizing tumor cells to chemotherapeutic drugs. Exploring the cellular roles of DNA topoisomerases is a prerequisite for understanding and minimizing the potential side effects, and improving the desired effects, for example by combination treatments.

3 METHODS

3.1 FISSION YEAST

The fission yeast *Schizosaccharomyces pombe* is a free living unicellular fungus that was first isolated from East African millet beer. The rod shaped fission yeast cells grow exclusively from the ends and divide by medial fission (figure 10). The cells usually reside in a haploid life cycle, but cells of opposite mating types fuse and enters meiosis under certain conditions.

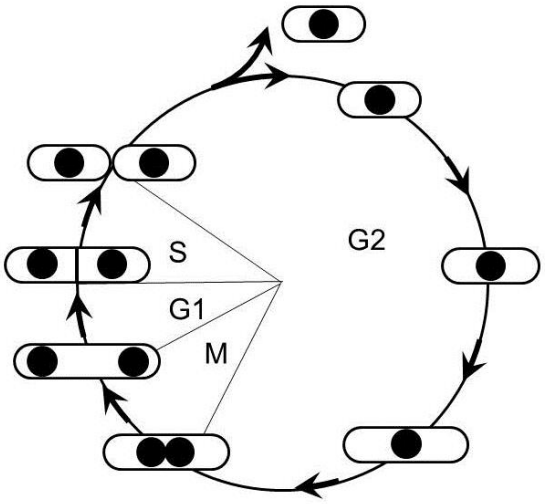

Figure 10. The haploid (mitotic) cell cycle of *S. pombe*. The rod-shaped cells grow from the tips and divide by medial fission. The G2 phase is long and takes about 70-80% of the division time. Cytokinesis is separate from nuclear division and is not completed until the beginning of S phase.

S. pombe was introduced as a model organism for studying genetics and cell cycle regulation in the 1950s. It is now a notable model organism for studies of basic principles in molecular and cell biology (Forsburg 1999; Wixon 2002). *S. pombe* is easily grown with a short generation time of 2-4 hours, and can be studied using a large set of molecular tools (Forsburg 1999; Wixon 2002; Forsburg and Rhind 2006). It has a small 13.8 Mb genome in which approximately 4800 protein coding genes have been predicted (Wood, Gwilliam et al. 2002). Fission yeast is easily subjected to genetic manipulation and there is a close to complete mutant collection. Furthermore, fission yeast is separated from the budding yeast model organism *Saccharomyces cerevisiae* by an estimated evolutional divergence of 1,140 million years (Hedges 2002). Thus, cross-species comparisons between these yeasts can give clues to general biological mechanisms for eukaryotic cells as well as alternative solutions to a biological problem. Some major differences between these yeast species at the molecular level also makes them complement each other as model organisms (Forsburg 1999; Wood, Gwilliam et al. 2002; Forsburg 2005). Similar to metazoans, fission yeast has large and degenerate chromosomes, with long regulatory regions (Wood, Gwilliam et al. 2002). Fission yeast also harbors a complete machinery for RNA interference (Aravind, Watanabe et al. 2000) and heterochromatin organization is more similar to vertebrates. Moreover, similar to mammalian cells, fission yeast has epigenetically defined regional centromeres surrounded by heterochromatic repeat regions (Clarke 1990). These features make *S. pombe* particularly useful as a model organism in chromatin research.

3.2 CHROMATIN IMMUNOPRECIPITATION

Insights to the distribution of particular proteins on chromosomal DNA sequences are important for understanding the mechanisms that govern DNA-dependent cellular processes. Chromatin immunoprecipitation (ChIP) was introduced as a method for mapping the *in vivo* associations between a protein of interest and DNA. In our time, ChIP provides a versatile method for generating a snapshot of the chromatin landscape in cells (Kuo 1999, Orlando 2000, Das 2004, Kim 2006, Collas 2010).

The basic steps of ChIP include *in vivo* cross-linking of chromatin, fragmentation of crosslinked chromatin, selective immunoprecipitation of protein-DNA complexes, reversal of cross-links, and identification of the recovered DNA fragments (figure 11) (Kuo and Allis 1999; Orlando 2000; Das, Ramachandran et al. 2004; Collas 2010).

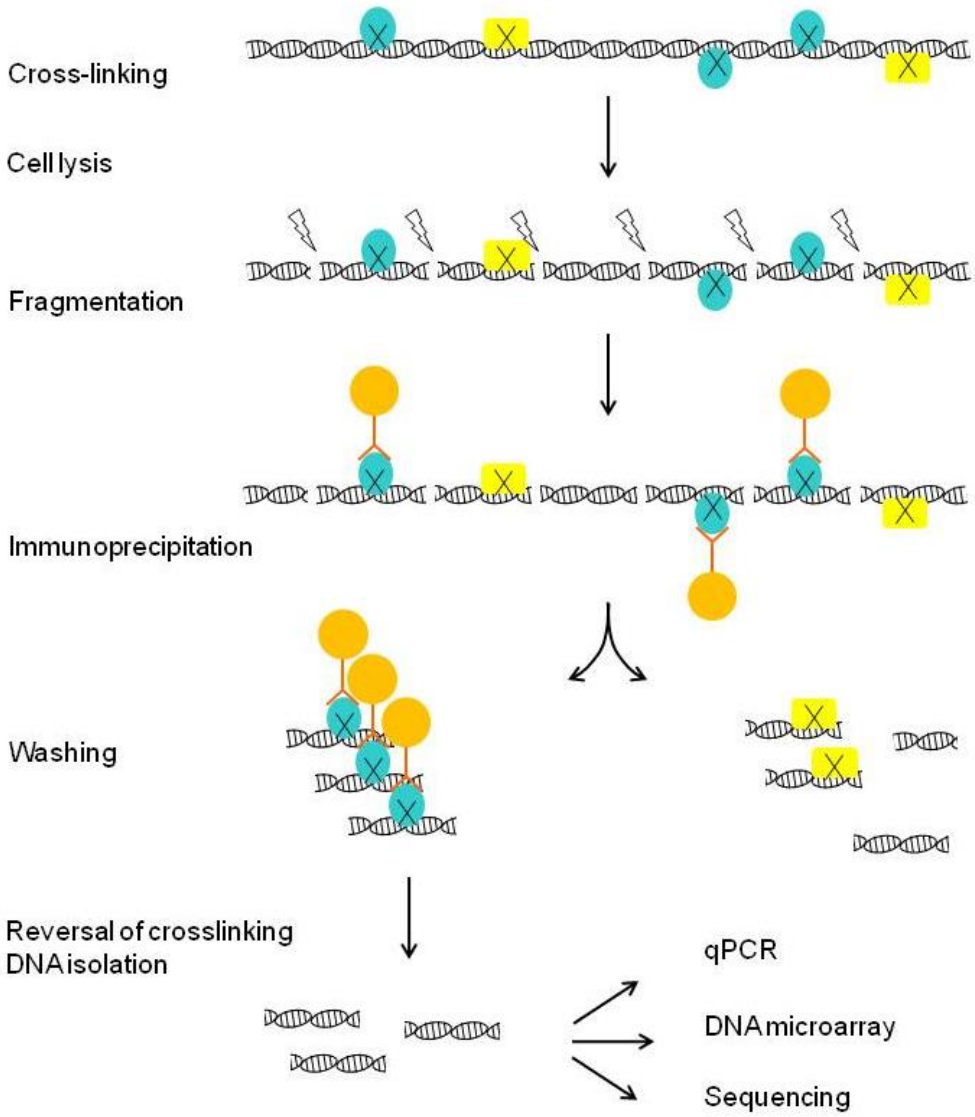

Figure 11. The basic steps of chromatin immunoprecipitation. Chromatin immunoprecipitation usually involves cross-linking proteins and DNA, fragmentation of chromatin, immunoprecipitation of fragments bound to the protein of interest and washing to remove unbound fragments. This is followed by reversal of cross-linking, isolation of DNA and analysis to identity regions bound by the protein of interest.

Covalent, but reversible, cross-links are commonly produced by the addition of formaldehyde, which creates protein-protein, protein-DNA and protein-RNA cross-links over a distance of 2 angstrom (Å). Thus, cross-linking may also occur with proteins that are indirectly bound to DNA. The cells are lysed and chromatin randomly fragmented by sonication or nuclease treatment. After clearing the lysate, agarose or magnetic beads and highly specific antibodies are used to select for fragments cross-linked to the protein of interest, either in its native form or tagged with a non-disruptive epitope tag. Unspecifically bound protein-DNA complexes are removed by stringent washes. Cross-links are then reversed by heat treatment, and proteins removed by digestion with proteinase K. The recovered DNA fragments are purified, followed by identification and quantification, aiming to determine the relative enrichment of the protein of interest over background at different chromosomal regions.

ChIP combined with quantitative polymerase chain reaction (ChIP-qPCR) allows for targeted analysis of candidate regions, while microarrays (ChIP-chip) and high-throughput sequencing (ChIP-seq) allows for genome-wide analysis (Collas and Dahl 2008; Aleksic and Russell 2009; Collas 2010). The choice between these methods is largely dependent on the aim of the study and prior knowledge. A disadvantage of the genome-wide methods is the higher cost and more time required, but much more information can be deduced from such experiments. In either case, analysis calls for the use of appropriate controls. The background can be deduced from a parallel control sample using extract from an untagged isogenic strain, a control antibody or a mock purification, thereby controlling for cross-reactivity of the antibody and/or unspecific binding of DNA to the beads. A un-immunoprecipitated input control sample controls for differences in chromatin concentration and fragmentation between samples. Replicate samples are made to determine which parts of the signal attributable to technical variation and which are biologically relevant. Moreover, mutants are always compared to relevant wild type strains. A large number of variations to the basic ChIP procedure have been developed to better suit small cell numbers and more specific applications (Collas and Dahl 2008; Collas 2010).

3.3 DNA MICROARRAYS

DNA microarray technology was introduced as a method for identification and quantification of a large number of DNA sequences in a complex mixture (Schena, Shalon et al. 1995). Since then, the power of DNA microarrays have revolved to provide coverage of whole genomes at high resolution and analysis of the results have improved to become more streamlined, making it a commonly used technique (Mockler, Chan et al. 2005).

A DNA microarray is basically a small chip containing a large number of DNA probes immobilized as spots in an ordered two-dimensional pattern on substrate, such as a nylon membrane or a glass slide. The probes are designed to be complementary to the sequences they aim to detect. Modern microarrays usually consist of oligonucleotides that are synthesized *in situ* at high density, thereby enabling high coverage and resolution. For example, tiling microarrays are built from small overlapping probes contiguously covering

a particular part of or a whole genome, thereby generating unbiased data at very high resolution. After applying an unknown mixture of DNA fragments, which are usually radioactively or fluorescently labeled, hybridization signals for each probe spot are recorded by a detector. The signals are then mapped to the corresponding positions in the genome.

A large number of streamlined analysis tools for data extraction, including spot finding and signal quantification, and data processing, including normalization, genome alignment, data smoothing and statistical tests, are available (Aleksic and Russell 2009; Gottardo 2009). As the amplification of immunoprecipitated DNA may generate bias, comparison with a non-immunoprecipitated input sample is useful. Spiking controls, consisting of control DNA of known composition that is added before amplification, can be utilized for normalization between samples and experiments. Normalization aims at removing systematic biases, to ease comparisons within and between arrays, and to ease the recognition of true signal from noise. Hybridization bias can occur due to cross hybridization between homologous regions on arrays, non-specific cross hybridization and hybridization biases due to dependence of signal intensity on base composition. Cross hybridization can be corrected for by the normalizing the signal to mismatch probes on the array or by deducing the signal from several neighboring probes, for example by applying a sliding window. The processed data can be visualized using a variety of genome browsers.

The availability of DNA microarrays together with reference genome sequences for various organisms has clearly increased the power of ChIP experiments (Ren, Robert et al. 2000). The resolution of ChIP-chip depends on the length of the sheared DNA fragments and the array probes, and the former usually remains the limiting factor. Moreover, ChIP-chip cannot probe for repeats or highly homologous regions. Another drawback is that all possible variations to the processing and normalization of data makes detailed comparisons, especially of absolute numbers, between ChIP-chip experiments performed in different laboratories difficult.

3.4 HIGH-THROUGHPUT SEQUENCING

The development of second generation massively parallel high-throughput DNA sequencing has revolutionized the identification and quantification of DNA in complex mixtures, such as ChIP material (Aleksic and Russell 2009; Collas 2010). DNA sequencing is now powerful, quick and straightforward.

There are several solutions to high-throughput sequencing, including Solexa, SOLiD, and 454-sequencing. In general, the DNA fragments to be analyzed are arrayed across a surface and sequenced upon amplification on site. Sequencing is often achieved by successive cycles of single-base extension and identification of the incorporated nucleotide using differential fluorescence. Short reads, sequenced from one or both ends of the DNA fragments, are used to create a map for frequencies of sequence reads across the genome. The number of reads generated determines sequencing depth, which must be enough to

give full genome coverage and resolution. The first step in the analysis of sequencing data involves base-calling of the sequence reads from the image profiles produced by the sequencer. The sequence reads are then mapped to the genome using one of several available alignment software tools.

ChIP-seq has the advantage of providing complete coverage of the whole genome, including repetitive regions, as well as increased sensitivity and higher resolution compared to ChIP-chip. However, fragment size of sheared DNA still remains a limited factor. To determine the relative enrichment of different genomic regions a background level must be deduced either using empirically determined model from standard control samples or using a computationally generated model. Several normalization and peak-finding algorithms are then available. Similar to ChIP-chip, there are many ways of processing and presenting the data. In the end, both ChIP-chip and ChIP-seq provides a set of statistically-enriched high-occupancy binding regions, but it can rarely give a complete and precise set of bound regions. False positives are generated from unspecific binding of DNA to the beads and from cross-reactivity of the antibody, while false negatives are generated by data processing, filtering and thresholding.

3.5 CHROMATIN IMMUNOPRECIPITATION AND EXONUCLEASE DIGESTION

ChIP and lambda exonuclease digestion (ChIP-exo) followed by high-throughput sequencing is an adaptation of ChIP that allows for high resolution mapping of protein-DNA interactions with high sensitivity and little background (Rhee and Pugh 2011; Rhee and Pugh 2012).

In ChIP-exo, the immunoprecipitated DNA fragments are treated with lambda exonuclease and $RecJ_f$ (Rhee and Pugh 2012). Lambda exonuclease removes mononucleotides from one strand of double stranded DNA in the 5′ to 3′ direction to within a few base pairs from the cross-linking point. The part of each DNA strand that resides 3′ to the cross-linking point remains intact. $RecJ_f$ degrades single stranded DNA in the 5′ to 3′ direction, thereby degrading the product from lambda nuclease digestion as well as DNA that is unspecifically bound to the beads. The remaining fragments are sequenced from the 5′ end to identify both boundaries of the cross-linking point for the immunoprecipitated protein. Upon analysis, bound regions are thus identified as peak-pairs, with one peak on the forward strand and one peak on the reverse strand. Chip-exo enables up to single nucleotide resolution of protein binding with very little background.

3.6 MICROCOCCAL NUCLEASE DIGESTION FOR NUCEOSOME MAPPING

A common method for genome-wide mapping of nucleosome positions relies of treatment of whole genome chromatin with micrococcal nuclease (MNase) (Lieleg, Krietenstein et al. 2014). This enzyme preferentially cuts DNA in linker regions and NDRs, leaving intact fragments of 140-150 bp of DNA that are protected by presence of a nucleosome.

For MNase mapping, cells are usually fixed with a low concentration of formaldehyde in permeabilized cells or in isolated nuclei. Chromatin is digested with MNase at a concentration that has been carefully titrated to generate mostly mononucleosomal DNA. DNA fragments of the correct size are purified and can either be probed by Southern blot, or mapped to genomic locations using high-resolution tiling microarrays (MNase-chip) (Yuan, Liu et al. 2005; Lee, Tillo et al. 2007) or high-throughput sequencing (MNase-seq) (Albert, Mavrich et al. 2007; Mavrich, Jiang et al. 2008). If arrays are used, the DNA must be further fragmented since hybridization of full-length mononucleosomal DNA generates an artificial shift in nucleosome positioning. The results are usually displayed by plotting hybridization signals across the genome, thus rather giving a display of average nucleosome occupancy (Zhang and Pugh 2011). Deep sequencing of the complete fragments or paired-end sequencing of both ends of the fragments enables plotting of the central dyads of nucleosomes across the genome, thus more readily giving a display of nucleosome positioning (Kent, Adams et al. 2011; Zhang, Wippo et al. 2011). Alternatively, if single-end sequencing is used, the positions of dyad axes can be estimated from and estimation of fragment length.

The major key to nucleosome mapping is the optimization of MNase digestion. As "breathing" of nucleosomal DNA off of the histone surface especially near the nucleosome edges allows for MNase to cut the DNA inside nucleosomes, over-digestion results in subnucleosomal fragments. Over-digestion also increases the effect of MNase sequence bias, which is generated from the fact that dA:dT base pairs are cut with higher probability than at dG:dC base pairs. Further refinement of MNase-seq can be achieved by introducing a ChIP step with antibodies recognizing histone H3 after MNase digestion (MNase-ChIP-seq) (Albert, Mavrich et al. 2007; Mavrich, Jiang et al. 2008).

3.7 RECOMBINATION-INDUCED TAG EXCHANGE

The recombination-induced tag exchange (RITE) method was developed for studying the dynamic behavior of proteins (Verzijlbergen, Menendez-Benito et al. 2010). It is a genetic method that allows for a hormone-induced permanent epitope-tag switch at the endogenous locus for the gene encoding the protein of interest. This enables parallel detection and tracking of proteins synthesized before and after the switch, using various biochemical methods.

The RITE method relies on integration of a RITE cassette immediately downstream of the target gene of interest by homologous recombination (figure 12) (Verzijlbergen, Menendez-Benito et al. 2010). This cassette begins with a transcribed short flexible spacer, shown to be required for viability of strains expressing the tagged proteins. The spacer is followed by a LoxP site, which is also part of the transcribed sequence, and a C-terminal epitope tag with a stop codon. Downstream of the first tag is a selectable marker and a second LoxP site, immediately followed by an orphan sequence that encodes a second tag. Recombination between the LoxP sequences is mediated by the Cre recombinase, and results in a switch that replaces the first tag with the second tag as part of the transcribed

region. The Cre recombinase is stably integrated at an ectopic locus and constitutively expressed as a fusion protein with the estrogen-binding domain (EBD) of the human estrogen receptor. The fusion protein remains inactive and is sequestered by heat-shock proteins until addition of β-estradiol to the cells, thereby inducing the tag switch. The switch can be monitored by the elimination of the selectable marker upon recombination between the LoxP sites.

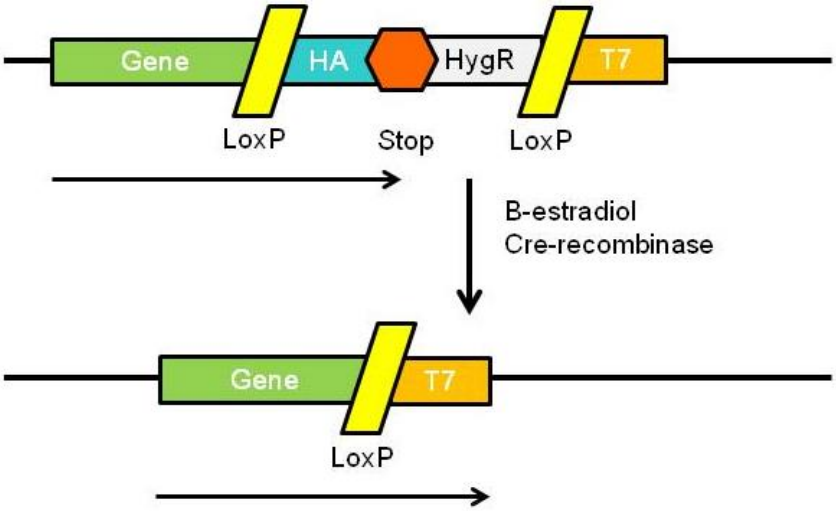

Figure 12. The RITE cassette. Homologous recombination is used for integrating the RITE cassette in frame with a gene of interest at the endogenous locus. A construct encoding the Cre recombinase fused to the EBD of the estrogen receptor is also integrated in the genome. Activation of Cre recombinase by addition of β-estradiol induces a genetic switch from the HA to the T7 epitope tag.

The study of nucleosome turnover using the RITE system provides several advantages over the use of inducible expression of tagged histones from an ectopic locus. In the RITE approach, gene expression is under normal control and maintained at the endogenous level, eliminating the risk of bias due to over-expression of histones. The switch is also introduced without changing the conditions in the cells and enables parallel analysis of both old and new proteins, even over generations. If turnover is measured by the enrichment of newly synthesized tagged histones over endogenous histones, ongoing synthesis of the endogenous gene copy means that the endogenous histones represent old as well as new proteins. As a consequence, the induced and endogenous proteins quickly reach a new steady state. This problem can be eliminated when using the RITE system.

3.8 TRANSCRIPTOME ANALYSIS

The repertoire of transcripts, the transcriptome, of a cell is a key link between information encoded in DNA and phenotype. Analysis of genome-wide transcription, or rather RNA levels, was introduced with the use of gene expression microarrays (Schena, Shalon et al. 1995). RNA is extracted from cells with the method of choice and reverse transcribed into cDNA using random primers, oligo-dT primers or gene specific primers. The cDNA is then labelled and hybridized to microarrays, with probes designed for known ORFs or covering the whole genome. Strand specific labelling of cDNA can be used to allow for separation of signals originating from the forward and reverse strands, respectively, thereby enabling detection

of both sense and anti-sense transcription from known genomic elements. To reduce potential bias from the secondary DNA-dependent DNA polymerase activity of reverse transcriptase, leading to a artificial correlation between sense and antisense transcription, the drug actinomycin D (actD) can included in the reaction (Perocchi, Xu et al. 2007). In recent years, transcriptome profiling has been revolutionized by the use of RNA sequencing (RNA-seq) (Wang, Gerstein et al. 2009; Costa, Angelini et al. 2010; Ozsolak and Milos 2011). Purified RNA, enriched for the required RNA species, is reverse transcribed into cDNA and fragmented, before applying high-throughput sequencing. This provides direct access to the actual sequence of transcripts, allowing for much more detailed analysis, including analysis of alternative splicing, RNA editing, and straightforward identification of new transcripts, even when genome sequence information is lacking. The reads can be mapped to a reference genome, or assembled *de novo*, using different algorithms. Since conversion of RNA into cDNA may introduce biases and artifacts that can interfere with the characterization and quantification of transcripts, attempts have been made at developing methods for direct RNA sequencing (DRS), but it is not yet commercially available (Ozsolak and Milos 2011).

3.9 IN VITRO RECONSTITUTION OF CHROMATIN AND REMODELLING ASSAYS

Studying the molecular mechanisms of DNA-dependent processes *in vitro* generally involves reconstitution of DNA and histones into a suitable chromatin template, ranging from mononucleosomal core particles to more complete nucleosomal arrays (Lusser and Kadonaga 2004). Histones may either be purified directly from the experimental organism, or isolated as recombinant histones after over-expression in bacteria, thus lacking posttranslational modification but also being devoid of contaminating eukaryotic factors. Similarly, the DNA template may be from an endogenous source, or be produced by enzymatic digestion or PCR from a plasmid that has been amplified in bacteria. The choice of assembly method and components depend on the downstream assay to be used, and on the native properties of chromatin in the experimental organism.

Random deposition of histones onto DNA can be achieved by salt dialysis or by assembly in the presence of histone chaperones, such as NAP1. In the salt dialysis method, DNA and histones are first mixed in the presence of 2 molar (M) sodium chloride (NaCl). This results in octamere formation and prevents aggregation. Salt concentration is then slowly decreased by step-wise dilution or dialysis of the mixture, resulting in deposition of histone H3-H4 tetramers and then histone H2A-H2B dimers onto DNA. For assembly using NAP1, histones are first incubated with an equal mass of purified recombinant NAP1 and then combined with DNA under physiological salt conditions. In both of these assays, the resulting chromatin is purified by sucrose gradient sedimentation. Periodic nucleosomal arrays, reminiscent of those of bulk native chromatin, can be assembled in an ATP-dependent manner using crude extracts or purified chromatin remodelers, such as the ATP-utilizing Chromatin remodeling and assembly Factor (ACF) complex or the RSF complex. Furthermore, local positioning of nucleosomes can be achieved by the use of sequence-

specific DNA binding factors, or by salt dialysis using a template with a single or tandemly repeated nucleosome positioning sequences.

There are a number of assays available for studying nucleosome remodelling *in vitro* (Becker and Horz 2002). One assay that monitors both nucleosome assembly and spacing (Tsukiyama, Palmer et al. 1999; Lusser, Urwin et al. 2005) relies on *in vitro* assembly of chromatin in the presence of NAP1 at physiological salt conditions. NAP1 alone generates chromatin that is of low quality and therefore rather susceptible to MNase digestion (Ito, Tyler et al. 1996). Generation of more extensive and regular MNase ladders in the presence of ATP and the chromatin remodeler to be studied is an indication of increased efficiency of assembly and nucleosome periodicity. This may be due to an ATP-dependent direct assembly of regular nucleosomal array by NAP1 and the chromatin remodeler, or result from ATP-dependent spacing of nucleosomes by the remodeler after their random deposition onto DNA by NAP1. To distinguish between these mechanisms, nucleosome assembly and nucleosome positioning can be studied separately. An assay than specifically detects nucleosome spacing can be performed using salt-gradient dialysis to assemble recombinant histones on DNA (Tsukiyama, Palmer et al. 1999; Lusser, Urwin et al. 2005). This generates high-quality chromatin, containing canonical nucleosomes, but lacking regular spacing of nucleosomes. Spacing activity can be detected by monitoring an ATP-dependent increase in regularity of the MNase cleavage pattern after addition of the chromatin-remodeling factor.

4 RESULTS AND DISCUSSION

4.1 PAPER I: TOPOISOMERSE I REGULATES OPEN CHROMAIN AND CONTROLS GENE EXPRESSION *IN VIVO*

In this study, we investigated the genome-wide roles of *Schizosaccharomyces pombe* DNA topoisomerase I and II, Top1 and Top2, in transcription and nucleosome organization *in vivo*, mainly using ChIP-chip with high-resolution tiling microarrays. First, we examined the RI genome-wide associations of Top1 and Top2 with chromatin, and found that both are strongly enriched at intergenic regions (IGRs) compared to open reading frames (ORFs) of genes. However, particularly the relative enrichment of Top1 at the 5' IGR displayed a positive correlation with transcription. Moreover, the levels of Top1 increased upon transcription an inducible gene. This suggests that Top1 may have a particular role at the promoter of genes that is related to transcription initiation.

Because fission yeast Top2 is essential and can substitute for Top1 (Uemura and Yanagida 1984), we used a *top1Δ top2ts* double mutant to characterize the roles of Top1 and Top2 in transcription and chromatin organization at gene regions. Although the *top1Δ top2ts* mutant displayed relatively little changes in global transcription levels, there was a clear down-regulation of genes that are highly transcribed in wild type cells. This is in agreement with previous studies (Brill, DiNardo et al. 1987; Zhang, Wang et al. 1988; Collins, Weber et al. 2001; Teves and Henikoff 2014) and may well be due to complete or partial redundancy between Top1 and Top2 in transcription. Also, reduced topoisomerase activity may well sustain transcription at lowly transcribed genes, but the importance of topoisomerases in transcription becomes evident at highly transcribed genes. At these genes there are simultaneous events of both initiation and elongation, allowing for pronounced accumulation of negative supercoiling towards the 5' end of genes and positive supercoiling towards the 3' end of genes.

Moreover, the *top1Δtop2ts* mutant displayed increased nucleosome occupancy at 5'IGRs and reduced levels of RNA polymerase II at ORFs at highly transcribed genes. This suggests that topoisomerase activity is required for high levels of transcription, possibly through maintaining nucleosome depletion at promoter regions, thereby supporting initiation. Indeed, increased levels of H3 at 5' IGRs correlated with reduced transcription. At individual Top1 binding target genes, we confirmed that reduced H3 promoter occupancy correlated with reduced transcription, as well as reduced levels of H3K9 acetylation, which is generally a mark of active transcription. In addition, we showed that Top1 is catalytically active at these genes by ChIP of Top1 cleavage-intermediates trapped on DNA after treatment of cells with the drug camptothecin (CPT). This suggests that Top1 is required for efficient transcription by a direct role in maintaining low nucleosome occupancy at promoters. In agreement, more recent studies show that Top1 is important for relieving negative supercoiling behind the polymerase (French, Sikes et al. 2011) and to prevent accumulation of excessive negative supercoiling at promoters of highly transcribed genes, which otherwise prevents maintenance of the NDR and inhibits initiation (Teves and Henikoff 2014). A role for Top1 at promoters is also in agreement with previous studies in which Top1 has been implicated in promoter functions, such as initiation

(Kretzschmar, Meisterernst et al. 1993; Merino, Madden et al. 1993), TF binding (Palecek, Vlk et al. 1997; Shykind, Kim et al. 1997; Jagelska, Brazda et al. 2008), RNA polymerase II recruitment (Sperling, Jeong et al. 2011), promoter pausing (Ma, Bai et al. 2013), and enhancer looping (Puc, Kozbial et al. 2015). Furthermore, we show that Top1 enrichment at IGRs correlated with enrichment of the *S. pombe* CHD1-type remodeler Heterogeneous nuclear ribonucleoprotein 1 (Hrp1). Hrp1 has previously been implicated in maintaining low nucleosome occupancy at promoters (Walfridsson, Khorosjutina et al. 2007). Therefore, we propose a model in which Top1 cooperates with Hrp1 in nucleosome disassembly at promoters by catalyzing removal of negative supercoils in this region.

The twin-supercoiled domain model of transcription predicts that long genes will be more affected by accumulation of positive supercoiling ahead of the polymerase, inhibiting further elongation and resulting in stalled transcription if not removed. In agreement, we found that the levels of RNA polymerase II increased in the 5' ends and decreased in 3' ends of long genes in the *top1Δtop2ts* mutant. This implies that topoisomerases are important for transcriptional elongation. This is in agreement with studies showing that Top1 also plays a major role in relieve of positive supercoiling ahead of the RNA polymerase (Brill and Sternglanz 1988; Zhang, Wang et al. 1988; Schultz, Brill et al. 1992; Ljungman and Hanawalt 1996; Mondal, Zhang et al. 2003; El Hage, French et al. 2010; Teves and Henikoff 2014). Moreover, we show that binding of Top2 to ORFs correlated with gene length, suggesting that Top2 could be the main enzyme responsible for removal of positive supercoils ahead of elongating RNA polymerase II at the extreme situation found at long genes. This correlation may also be explained by the fact that the average transcription level of long genes is lower than of short genes, and that Top2 tends to associate with more lowly transcribed regions (Naughton, Avlonitis et al. 2013). However, more recent studies support the idea that Top2 is required for elongation at long genes (French, Sikes et al. 2011; Joshi, Pina et al. 2012), where extreme over-winding of the template is associated with increased writhe and the formation of DNA crossovers, which juxtaposes DNA segments in a way that promotes Top2 activity (Salceda, Fernandez et al. 2006; Fernandez, Diaz-Ingelmo et al. 2014).

4.2 PAPER II: CHD1 REMODELERS REGULATE NUCLEOSOME SPACING *IN VITRO* AND ALIGN NUCLEOSOMAL ARRAYS OVER GENE BODIES IN *S. POMBE*

In this study, we investigated the roles of several *S. pombe* chromatin remodelers in genome-wide nucleosome positioning using MNase-chip, transcriptome mapping and *in vitro* remodeling assays. First, we examined the role of the SWR1 remodeling complex, which is known to be required for deposition of the histone H2A variant H2A.Z at the highly positioned +1 and -1 nucleosomes of euchromatic genes (Mizuguchi, Shen et al. 2004; Raisner, Hartley et al. 2005; Buchanan, Durand-Dubief et al. 2009). In budding yeast, deposition of H2A.Z is dependent on the NDR, but the NDR is established independently of SWR1 (Hartley and Madhani 2009). In agreement, we found that deletion of the gene encoding H2A.Z or the gene encoding *S. pombe* Swr1, only mildly influenced the average nucleosome occupancy at the +1 position. Next, we examined the role of the

RSC remodeling complex, which has a prominent role in nucleosome positioning in budding yeast. However, impairment of the ATPase subunit of the fission yeast RSC complex, Snf21, resulted in only a slight reduction of average nucleosome occupancy at -1 and +1 positions.

While *S. pombe* lacks ISWI-type chromatin remodelers, which play prominent roles in nucleosome positioning in budding yeast, it has three CHD-type chromatin remodelers. Among these, we found that Muc1 expressed independent of TEC1 (Mit1) of the MI-2 subfamily had no effect on average nucleosome positioning in euchromatic gene regions, but that Hrp1 and Hrp3 of the CHD1-subfamily play a prominent role in the generation of nucleosomal arrays downstream of the TSS. While the effect in the *hrp1Δ* single mutant was rather mild, the *hrp3Δ* single mutant displayed clearly reduced amplitude of the nucleosomal array downstream of the TSS. Furthermore, the *hrp1Δ hrp3Δ* double mutant completely lacked highly positioned nucleosomes downstream of the +2 position. This effect was even more pronounced for Hrp3 binding target genes, arguing for a direct effect of at least Hrp3. Thus, there has been an evolutionary shift in the use of remodelers for the generation of TSS-aligned nucleosomal arrays, from a combination of ISWI- and CHD1-type remodelers in *S. cerevisiae* to an expanded repertoire of CHD1-type remodelers in *S. pombe*.

Next, we looked at transcription levels in the *hrp1Δ*, *hrp3Δ* and *hrp1Δ hrp3Δ* mutants. We observed both up- and down regulation of sense transcription of genes, and few genes displayed a >1.5-fold change. Moreover, there was no correlation between altered chromatin structure and changes in sense transcription, arguing against a direct casual relationship between impaired nucleosomal arrays and altered sense transcription in these mutants. On the other hand, perturbed chromatin organization in gene bodies have been shown to result in increased cryptic transcription from within genes in budding yeast, leading us to also investigate anti-sense transcription in the mutants (Kaplan, Laprade et al. 2003; Mason and Struhl 2003; Quan and Hartzog 2010; Gkikopoulos, Schofield et al. 2011). There was a clear preference for up-regulation of cryptic antisense transcription in the *hrp1Δ*, *hrp3Δ* and especially the *hrp1Δ hrp3Δ* mutant. However, the effects on nucleosome positioning were similar for genes that showed unaltered cryptic antisense transcription, indicating that impaired nucleosomal arrays downstream of the TSS can, but are not always sufficient to, cause increased antisense transcription.

Subsequently, we investigated bulk nucleosomal spacing in the mutant strains by limited MNase digestion. Surprisingly, the MNase ladders generated from chromatin isolated from the mutants were similar to wild type, demonstrating that Hrp1 and Hrp3 are not required for regular spacing of bulk nucleosomes. Therefore, we suggest that the role of Hrp1 and Hrp3 is in linking the regularly spaced nucleosomes to a focal point at the TSS. This is required for appearance of nucleosomal arrays upon alignment of genes at the TSS, as it results in little variation in the positioning of individual nucleosomes between genes and within a population of cells.

D. melanogaster and *S. cerevisiae* CHD1 have been shown to have nucleosome spacing activity *in vitro* (Lusser, Urwin et al. 2005; Stockdale, Flaus et al. 2006). To test the activities of the *S. pombe* CHD1-type chromatin remodelers *in vitro*, we purified catalytically active endogenous Hrp1 and Hrp3. We also prepared *in vitro* reconstituted chromatin by purifying recombinant *S. pombe* histones and assembling them on lambda DNA, using either the NAP1 chaperone or the salt dialysis method. First, the presence of Hrp1 or Hrp3 increased the formation of MNase-resistant regular nucleosomal arrays in an ATP-dependent manner in a NAP1-assembly assay. This assay monitors both nucleosome assembly and spacing. Furthermore, the addition of Hrp1 or Hrp3, together with ATP, to low-quality chromatin that had already been assembled by salt dialysis generated more extensive nucleosomal arrays, demonstrating that that Hrp1 and Hrp3 possess ATP-dependent nucleosome spacing activity *in vitro*. This is in agreement with a role in formation of nucleosomal arrays in gene bodies.

Two additional studies on the role of Hrp1 and Hrp3 in nucleosome positioning and cryptic transcription were published simultaneously with our study, with similar results (Hennig, Bendrin et al. 2012; Shim, Choi et al. 2012). Intriguingly, later studies have demonstrated that nucleosome turnover is reduced at promoter regions, but increased over coding regions in the absence of CHD1 in *S. cerevisiae* (Radman-Livaja, Quan et al. 2012; Smolle, Venkatesh et al. 2012) and in mammalian cells (Skene, Hernandez et al. 2014). Thus, CHD1 seems to promote replacement of old nucleosomes with newly synthesized nucleosomes bat promoters, and specifically at nucleosomes immediately flanking NDRs (Skene, Hernandez et al. 2014). This may either be due to an active role for CHD1 in disassembly of nucleosomes in these regions, or it may reflect assembly and directional sliding of nucleosomes by CHD1 from the edges of gene regions. Similar to our study, lack of CHD1-type remodelling activity resulted in no change or slightly reduced nucleosome occupancy at promoters, including NDRs in most studies using MNase-ChIP, opposing a role for CHD1 in nucleosome disassembly (Hennig, Bendrin et al. 2012; Shim, Choi et al. 2012; Skene, Hernandez et al. 2014). This is in contrast to the preferential increase in nucleosome occupancy at promoters of Hrp1- and Hrp3 binding target in these mutants found by histone H3 ChIP-chip (Walfridsson, Khorosjutina et al. 2007). This discrepancy may reflect formation of MNase-sensitive non-canonical nucleosomes at promoters in the mutants. However, an increase in MNase-ChIP signal was in fact observed at promoters in the *S. cerevisiae* CHD1 mutant (Gkikopoulos, Schofield et al. 2011). Thus, it is unclear wheatear CHD1 maintains high turnover at promoters by actively participating in the assembly and sliding of histones or in disassembly of histones, or perhaps both.

4.3 PAPER III: DNA TOPOISOMERASE III LOCALIZES TO CENTROMERES AND AFFECTS CENTROMERIC CENP-A LEVELS IN FISSION YEAST

In this study, we present a new role for fission yeast Top3 in maintaining centromeric chromatin structure and in controlling the levels of centromeric CENP-A. The fission yeast RTR complex consists of Top3, Rmi1 and the RecQ helicase Rqh1 (Laursen, Ampatzidou et al. 2003). We show that a thermo-sensitive *top3* (*top3ts*) mutant displays growth and chromosome segregation defects. This has previously been shown to at least partially

depend on accumulation of RecQ-mediated recombination intermediates downstream of Rad51 (Oakley, Goodwin et al. 2002; Shor, Gangloff et al. 2002; Laursen, Ampatzidou et al. 2003; Mankouri, Ashton et al. 2011). However, we find that the chromosome segregation defect can only be partially rescued by deletion of *rqh1* or *rad51*, and that both the *rqh1Δ* mutant (Win, Mankouri et al. 2005) and the *top3ts rqh1Δ* double mutant still display defects in chromosome segregation. This indicates that there is an additional role for the fission yeast RTR complex in chromosome segregation.

When we investigated the genome-wide *in vivo* associations of Top3 with chromatin using ChIP and high-resolution tiling microarrays, we observed a consistently high relative enrichment of Top3 at the central domains of all three centromeres, where the enrichment of Top2 and Top1 is low. Moreover, we found a unique positive correlation between the relative enrichment of Top3 and CENP-A in this region, leading us to investigate the levels of CENP-A at centromeres in the *top3ts*, *rqh1* and *top3ts rqh1Δ* mutants. Using ChIP-chip and chip-qPCR we demonstrated that impairment of Top3 and/or Rqh1 results in a clear increase in the levels of CENP-A at the centromeric central domains. Thus, Top3 and Rqh1 affect centromeric chromatin in a way that normally limits the levels of CENP-A in the central domains. Furthermore, we found that the increased levels of CENP-A are associated with reduced levels of HJURP at centromeres. Last, we demonstrated that the increase in centromeric CENP-A in the *top3ts* and *rqh1Δ* mutants is independent of homologous recombination downstream of Rhp51.

Similar to the assembly and disassembly of canonical nucleosomes, transactions involving CENP-A nucleosomes are associated with changes in DNA topology. We suggest that Top3 has a role in controlling DNA topology at centromeres, including the relaxation of topological strains created during assembly or disassembly of CENP-A nucleosomes. Interestingly, recent studies have shown that Rmi1 modulates the outcome of Top3 activity by promoting decatenation and inhibiting relaxation of supercoiling (Cejka, Plank et al. 2012; Bocquet, Bizard et al. 2014). It is possible that there are cellular processes or locations, such as centromeres, where Top3 may acts in the absence of Rmi1, thus favoring its relaxation activity. Moreover, the residue mutated in the *top3ts* mutant protein lies in the region where Rmi1 interacts with Top3, and may thus affect the modulation of Top3 activity by Rmi1. The role of Rmi1 at centromeres remains to be explored.

If CENP-A nucleosomes are octasomes, removal only of negative supercoiling by Top3 should limit their assembly and/or promote their disassembly at centromeres. *In vitro*, efficient relaxation of negative supercoils by Top3 is dependent on RecQ helicases (Wu and Hickson 2002; Harmon, Brockman et al. 2003), providing an explanation for the similar effect of Rqh1. Reduced levels of chromatin-bound HJURP in these mutants may reflect facilitated loading of CENP-A-H4 from the pre-nucleosomal complex onto DNA, resulting in more rapid turnover of the chaperone. Intriguingly, if CENP-A-containing nucleosomes are hemisomes with right-handed wrapping of DNA, removal of negative supercoiling by Top3 should have the opposite effect, promoting assembly of right-handed hemisomes over left-handed octameres. In the *top3* and *rqh1* mutants, lack of this activity may result in formation of octameric CENP-A-containing nucleosomes instead, thereby

leading to increased levels CENP-A. In this case, the presence of Top3 and relative absence of the other DNA topoisomerases at centromeric central domains may specify the assembly of hemisomes, which may in turn contribute to the functional and structural specification of centromeric chromatin.

A recent study also demonstrated that incorporation of newly synthesized CENP-A is promoted by RNA polymerase II-dependent transcription with a high degree of polymerase stalling, imposed by the centromeric DNA sequence (Catania, Pidoux et al. 2015). Lack of Top3 activity, shifting supercoiling towards a more negative state, may promote initiation of transcription by RNA of polymerase II, and at the same time increase R-loop formation and thereby stalling during elongation. This could be an additional or alternative way in which impairment of the Top3-Rqh1 complex leads to increased levels of CENP-A. In either case, the effect of the Top3-Rqh1 complex on CENP-A levels likely contributes to the observed chromosome segregation defects in *top3* and *rqh1* mutants.

4.4 PAPER IV: A NUCLEOSOME TURNOVER MAP REVEALS THAT THE STABILITY OF HISTONE H4 LYS20 METHYLATION DEPENDS ON HISTONE RECYCLING IN TRANSCRIBED CHROMATIN

In this paper, we studied RI turnover of histone H3 by adapting the RITE method and ChIP-exo to *S. pombe*. The RITE method enabled us to monitor nucleosome turnover as the preservation of old histone H3 tagged with hemagglutinin (HA) and the incorporation of new histone H3 tagged with T7. ChIP-exo and deep sequencing then allowed us to map the genome-wide high-resolution relative enrichment of H3-HA and H3-T7 in G2-arrested cells at 0 h and at 2 h after inducing the switch.

In fission yeast, histone H3 incorporated into nucleosomes in heterochromatin is highly stable. In euchromatic gene regions, the average turnover of histone H3 is lowest in the middle of genes and higher at the 3'- and particularly at the 5' untranslated regions (UTRs) at the borders of genes. This is in agreement with previous studies in *S. cerevisiae* and mammalian cells (Dion, Altschuler et al. 2005; Jamai, Imoberdorf et al. 2007; Rufiange, Jacques et al. 2007; Verzijlbergen, Menendez-Benito et al. 2010; Skene, Hernandez et al. 2014). Similar to mammalian cells (Skene, Hernandez et al. 2014), nucleosome turnover is particularly high in regions flanking the 5' NDR, and to lesser extent also the 3' NDR. This demonstrates that that newly synthesized histones are preferentially incorporated at the edges of transcribed regions, while histone recycling dominates in the middle part of genes in fission yeast. There may also be a directional movement of recycled nucleosomes towards the central parts of genes, so that potential gaps that need to be filled by incorporation of new histones are preferentially created at the borders of transcribed regions. In some previous studies, high turnover at promoters was shown to be correlated with low steady state nucleosome occupancy, suggesting that nucleosome depletion at NDRs may reflect a highly dynamic equilibrium (Dion, Kaplan et al. 2007; Jamai, Imoberdorf et al. 2007; Rufiange, Jacques et al. 2007). However, in our hands, histone H3 turnover is particularly low at the actual NDRs, at least at the 5' NDR, suggesting that this region is truly depleted of nucleosomes.

Surprisingly, when looking at nucleosome turnover relative to transcription levels of genes in G2, we found that the average turnover of histone H3 is anti-correlated with transcription both at the 5' UTR and at the CDS, and positively correlated with transcription only at 3' UTRs of lowly and moderately transcribed genes. This is partially in contrast with previous studies. It may reflect very efficient recycling of old histones in the wake of transcription, resulting in a conservatory role for transcription at these genes. However, at highly transcribed genes, there is a positive correlation between turnover and transcription at all regions, suggesting that the molecular machinery for recycling of old histones eventually becomes saturated, reaching a plateau level for this activity, above which transcription becomes disruptive and results in incorporation of newly synthesized histones in also in gene bodies. Similarly, in *S. cerevisiae* the positive correlation between histone H3 turnover in gene bodies and transcription is more pronounced for highly transcribed genes (Dion, Altschuler et al. 2005; Jamai, Imoberdorf et al. 2007; Rufiange, Jacques et al. 2007).

Next, we looked at the genome-wide distribution of H4K20me1, H4K20me2 and H4K20me3. In *S. pombe*, methylation of H4K20 is mediated by Set9 in a consecutive manner, but there is no known demethylase these modifications. Thus, H4K20 methylation should be removed by nucleosome turnover and thus may act as a mark of nucleosome stability, similar to what has been observed in *D. melanogaster* (Scharf, Meier et al. 2009) and for H3K79 methylation in other organisms (De Vos, Frederiks et al. 2011). In agreement with a previous study, we found that *S. pombe* heterochromatin is devoid of H4K20 methylation (Carneiro, Khair et al. 2010). In euchromatic gene regions, methylation of H4K20 is preferentially seen in the transcribed region of genes. High average relative enrichment of H4K20me1 is seen just downstream of the TSS and at regions flanking the TTS of genes. H4K20me2 and H4K20me3 are preferentially seen further into gene bodies. Thus, on a genome-wide scale, H4K20me1 is found in regions of higher histone H3 (and presumably histone H4) turnover, while H4K20me2 and me3 are found in regions of lower turnover. Interestingly, at very highly transcribed genes, where transcription results in much higher nucleosome turnover also in the CDS, H4K20me1 is found at the middle part of the transcribed region, while the levels of H4K20me2 and H4K20me3 remain low. Furthermore, H4K20me2 and me3 shows a slight positive correlation with H3-HA, and an anti-correlation with H3-T7. This supports a model in which the sequential methylation of H4K20 correlates with nucleosome age in euchromatic gene regions, with H4K20me2 and H4K20me3 being marks of old nucleosomes. In support, we show that the levels of H4K20me1 and me3 at genes are reduced upon mutation of *spt16*, encoding the *S. pombe* homolog of FACT. Thus, maintenance of H4K20 methylation seems to depend on FACT-mediated recycling of histones following transcription (Kaplan, Laprade et al. 2003; Schwabish and Struhl 2004; Fleming, Kao et al. 2008; Jamai, Puglisi et al. 2009). This further supports the role of histone recycling in maintaining low nucleosome turnover in gene bodies.

5 CONCLUSIONS

This thesis explores how DNA topoisomerases and nucleosome remodelers affect chromatin structure and dynamics in fission yeast. We show that the three fission yeast DNA topoisomerases have different effects on chromatin organization throughout the genome. We shed new light on the roles of Top1 and Top2 in transcription, in which they act to promote nucleosome depletion at promoter regions and to facilitate transcriptional elongation by preventing stalling of RNA polymerase II. Top1 and Top2 are at least partially redundant in these functions, but Top1 seems to the enzyme that is preferentially recruited to promoter regions in correlation with transcription at the majority of genes, and Top2 seems to be preferentially recruited to gene bodies of long genes. This specification likely reflects the different propensities for Top1 and Top2 to act on different types of topological strains and in different chromatin environments. Their recruitment to gene regions may be both an active process dependent on interactions with the transcription machinery, and to some degree an effect of random encounters with different topological substrates. Interestingly, Top3 displays very similar binding patterns to Top1 and Top2 at euchromatic gene regions, but its role at such regions is unknown. We identify a new role for Top3 at centromeres, where the activity of Top3 limits the levels of CENP-A. Since this is largely independent of HR, we suggest that this reflects a role for Top3 in relaxing negative supercoiling at central domains, thereby controlling the topological state and topology-dependent nucleosome transaction at centromeres. Preferential relaxation of negative supercoiling should inhibit assembly and/or promote disassembly of conventional CENP-A octasomes, or it may specify the assembly of non-conventional CENP-A hemisomes. In the latter case, Top3 would contribute to the structural and functional specification of centromeric chromatin.

This thesis also explores the role of the fission yeast CHD1-type chromatin remodelers Hrp1 and Hrp3 in nucleosome occupancy and positioning. We demonstrate that Hrp1 and Hrp3 have nucleosome spacing and assembly activity *in vitro*, and they are important for formation of nucleosomal arrays in gene bodies, and particularly for their alignment at the TSS. We show that, similar to *S. cerevisiae*, the characteristic pattern of nucleosome positioning in gene bodies is important to prevent cryptic transcription. In our studies using MNase-chip, Hrp1 and Hrp3 does not affect average nucleosome occupancy at the NDR. However, it seems likely that they could have an effect on nucleosome turnover both in gene bodies and around the NDR, similar to CHD1-type chromatin remodelers in budding yeast and mammalian cells. The last study in this thesis provides a genome-wide map of nucleosome turnover in fission yeast, and shed new light on how nucleosome turnover correlates with transcription and certain histone modifications.

Overall, this thesis supports a view in which nucleosome organization and dynamics are governed by an intricate interplay between different factors, including DNA topoisomerases, chromatin remodelers and the transcription machinery. Heterogeneity across the genome is likely produced by the different distributions and activities of these *trans*-acting factors in different regions, in concert with the intrinsic properties of the underlying DNA template.

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