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CHROMATIN REMODELING AND DNA TOPOLOGY IN TRANSCRIPTION AND GENOME STABILITY

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ABSTRACT

Eukaryotic DNA is wrapped around histone proteins to form nucleosomes, the fundamental repeating unit of chromatin. DNA packaging into chromatin both solves and creates problems. It allows the centimeters, or even meters, of DNA that constitute a eukaryotic genome to fit inside a micrometer-scale cell nucleus. Nucleosomes also block access to the DNA, necessitating complex rearrangements to allow for transcription, replication, recombination, or repair, while also providing a way to regulate these processes. ATP-dependent chromatin remodelers slide, assemble, disassemble, and alter nucleosomes to enable and regulate DNA-dependent processes. In parallel, topoisomerases relieve the tangles, torsional stress, and supercoils generated when DNA is exposed and unwound. Topoisomerases also enable efficient nucleosome remodeling. In this thesis, we use genomewide and single-locus techniques to study the interplay between DNA topoisomerases, Snf2 family chromatin remodelers, and transcription in the fission yeast *Schizosaccharomyces pombe.* We find that topoisomerase activity is essential for transcription elongation and for proper chromatin structure at genes, which in turn are required for efficient transcription initiation and termination. This is partially mediated by cooperation with chromatin remodelers. We also find that the fission yeast Chd1 subfamily remodelers maintain correct gene body nucleosome positioning, which inhibits cryptic transcription initiation. Finally, we show that the Fun30 subfamily chromatin remodeler Fft2 is involved in centromere function and heterochromatic silencing, as well as the full transcription of highly transcribed genes. Fft2 and its paralog Fft3 also regulate the transcriptional response to stress. As a part of this function, Fft2 and Fft3 repress retrotransposons by a novel mechanism, in which they enforce the use of an alternative transcription start site.

LIST OF PUBLICATIONS

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

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**Equal contribution*

RELATED PUBLICATIONS

Hogan CJ, Aligianni S, Durand-Dubief M, **Persson J**, Will WR, Webster J, Wheeler L, Mathews CK, Elderkin S, Oxley D, Ekwall K, Varga-Weisz PD (2010). Fission yeast Iec1-ino80-mediated nucleosome eviction regulates nucleotide and phosphate metabolism. *Mol Cell Biol* **30**: 657-674

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LIST OF ABBREVIATIONS

Ac	Acetylation
Ago1	Argonaute 1
ALC1	Amplified in Liver Cancer 1
ATP	Adenosine TriPhosphate
bp	Base Pair
CHD	Chromodomain, Helicase domain, DNA-binding
ChIP	Chromatin ImmunoPrecipitation
CTD	C-Terminal Domain
Dcr1	Dicer 1
DNA	DeoxyriboNucleic Acid
DSB	Double Stranded Break
Etl1	Enhancer Trap Locus 1
FISH	Fluorescence *In Situ* Hybridization
Fft1	Fission yeast Fun Thirty 1
Fft2	Fission yeast Fun Thirty 2
Fft3	Fission yeast Fun Thirty 3
Fun30	Function UNknown 30
HDAC	Histone DeACetylase
H2A/2B/3/4	Histone H2A/2B/3/4
Iswi	Imitation SWI/snf
K	Lysine
KAT	Lysine AcetylTransferase
KDM	Lysine DeMethylase
KMT	Lysine MethylTransferase
lncRNA	Long NonCoding RNA
Lsh	Lymphoid Specific Helicase
LTR	Long Terminal Repeat
me	Methylation
MNase	Micrococcal Nuclease
mRNA	Messenger RNA
NDR	Nucleosome Depleted Region
NHEJ	NonHomologous End Joining

PIC	Preinitiation Complex
piRNA	Piwi-Interacting RNA
Piwi	P-element Induced Wimpy Testes
ph	Phosphorylation
RdDM	RNA Directed DNA Methylation
Rdp1	RNA Directed RNA polymerase 1
RdRP	RNA Directed RNA Polymerase
RNA	RiboNucleic Acid
RNAPII	RNA Polymerase II
rRNA	Ribosomal RNA
S	Serine
siRNA	small interfering RNA
SMARCAD1	SWI/SNF-related, Matrix.associated, Actin-dependent Regulator of Chromatin, subfamily A, containing DEAD/H box 1
SRCAP	Swi/snf Related CBP Activating Protein
sRNA	Small RNA
Swr1	SWi/snf-Related 1
Swi/Snf	SWItch/Sucrose NonFermentable
TGS	Transcriptional Gene Silencing
Top/Topo	Topoisomerase
tRNA	Transfer RNA
TSS	Transcription Start Site
TTS	Transcription Termination Site
ub	Ubiquitination
WT	Wild Type

1 INTRODUCTION

1.1 AN INTRODUCTION TO CHROMATIN

All of the information required to build an organism is encoded in its genome. The DNA of the human genome is approximately two meters long (Lander *et al*, 2001; Venter *et al*, 2001), which is more than 300,000 times the diameter of the average human cell nucleus. This problem is common to all eukaryotes, with even the unicellular baker's yeast having to compact half a centimeter of DNA (Goffeau *et al*, 1996) into a microscopic nucleus. Furthermore, to avoid wasting energy yet be able to respond to a changing environment, the thousands of genes encoded by that genome have to be under fine regulatory control. A sophisticated packaging system has evolved to meet these two challenges. Eukaryotic DNA is wrapped around basic proteins called histones, providing both compaction and regulatory control. Together, the DNA and histone proteins are called chromatin. The fundamental unit of chromatin, 147 base pairs of DNA wrapped 1.7 times around an octamer of histones, is called a nucleosome. Two each of the canonical histones H2A, H2B, H3, and H4 build the nucleosome core, arranged into an H3/H4 tetramer and two outer H2A/H2B dimers (Figure 1A-C; Kornberg, 1974; Luger *et al*, 1997; Richmond & Davey, 2003). The length of DNA between nucleosomes, the linker DNA, varies, and in some species is also bound by the linker histone H1. Known variants exist for all histones, and the incorporation of these histone variants can have important functional effects on the chromatin (Ahmad & Henikoff, 2002; Kamakaka & Biggins, 2005; Probst *et al*, 2009; Talbert & Henikoff, 2010; Millar, 2013; Skene & Henikoff, 2013). In addition, both the C- and N-terminal tails of histones can be covalently modified; they can for example be methylated, acetylated, monoubiquitinated, ADP-ribosylated or sumoylated. By altering the histone charge and/or recruiting additional chromatin modifying enzymes and transcription factors, histone tail modifications enhance the regulatory versatility of chromatin (Kouzarides, 2007; Campos & Reinberg, 2009; Tropberger & Schneider, 2010; Zentner & Henikoff, 2013).

Nucleosome positioning is also important for regulation, as DNA that is within a nucleosome is far less accessible than unbound DNA (Liu *et al*, 2006). The precise position of a nucleosome can, for example, determine whether transcription factors can bind and whether a given gene will be transcribed (Struhl & Segal, 2013). Beyond transcriptional regulation, nucleosome positioning and repositioning are important for DNA replication, recombination, and repair, as well as for constructing structural components of chromosomes like centromeres. Nucleosome spacing also contributes to higher-order chromatin compaction. The Snf2 family of ATP-dependent chromatin remodelers controls nucleosome positioning and composition, assembling, disassembling and sliding nucleosomes as well as performing dimer exchange (Clapier & Cairns, 2009). DNA topology, including supercoiling, is an important factor in nucleosomal stability and the thermodynamics of ATP-dependent chromatin remodeling. Processes like DNA replication and transcription affect local DNA topology. The DNA topoisomerases that relieve torsional strain are essential to allow these processes and the chromatin fiber to coexist (Vos *et al*, 2011).

In addition, noncoding RNAs can target specific genomic sequences for regulation (Sabin *et al*, 2013). The DNA itself can be covalently modified, with DNA methylation being the most studied mark (Yoder *et al*, 1997; Selker *et al*, 2003; Law & Jacobsen, 2010; Smith & Meissner, 2013). All of these processes are engaged in a complex cross-talk, wherein one histone modification or stretch of methylated DNA recruits a given modifying enzyme or ATP-dependent remodeler, which may exist in a complex with other modifiers. The result is a many-layered and sometimes redundant regulatory network that plays a vital role in all DNA-based processes.

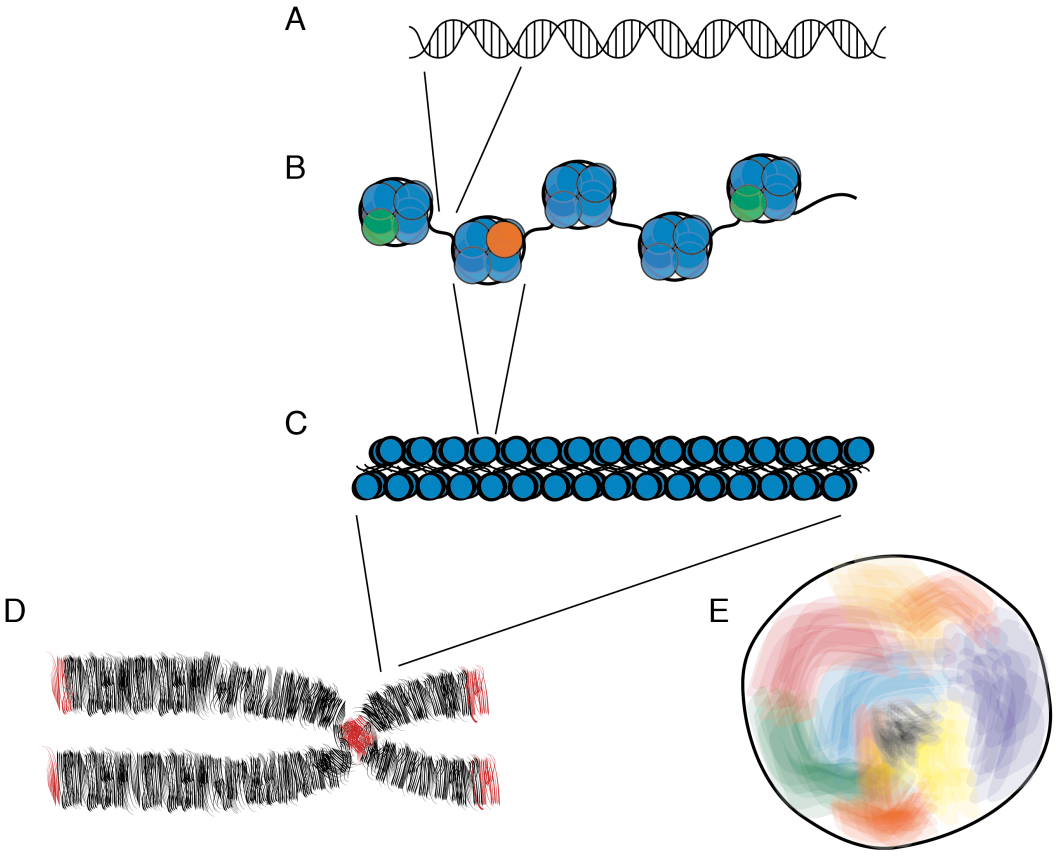

Figure 1. Chromatin. A) The DNA molecule, which can be covalently modified with e.g. methyl groups, is B) compacted into nucleosomes by wrapping around octamers of histones (blue, green, orange circles). Nucleosomes differ due to covalent histone modifications and histone variant (green, orange circles) incorporation, which contribute to C) higher order chromatin structure. D) Centromeric, telomeric, and other silent chromatin domains are characterized by repressive heterochromatin (red). Euchromatin characterizes active regions of the chromosome (black). In preparation for cell division, chromosomes become highly compacted, and sister chromatids are joined at the centromere. E) In contrast, interphase chromosomes are more diffuse, but still occupy distinct territories in the nucleus.

In very general terms, chromatin can be divided into two types: euchromatin and heterochromatin (Figure 1D). Named for their differential staining and appearance by microscopy (Heitz, 1928; Brown, 1966), euchromatin is a broad term for active chromatin, while heterochromatin is generally inactive (Strålfors & Ekwall, 2011). Euchromatin promotes and is shaped by transcription, having a less compact structure than heterochromatin and being characterized by ‘active’ histone modifications and nucleosome remodeling. Heterochromatin, in contrast, is more compact and generally refractory to transcription. Heterochromatin can be further subdivided into constitutive and facultative heterochromatin. Structural elements of chromosomes, such as

centromeres and telomeres, tend to be composed of repeat elements and are compacted into constitutive heterochromatin. Other regions may contain coding genes but be transcriptionally repressed under a given developmental program. A distinct facultative form of heterochromatin forms at such regions (Trojer & Reinberg, 2007; Wutz, 2011). Barrier elements prevent the spreading and intermingling of these distinct forms of chromatin (Strålfors & Ekwall, 2011; Barkess & West, 2012). Finally, chromosomes occupy distinct territories within the nucleus, displaying numerous nonlinear intra- and interchromosomal interactions (Figure 1E; Cavalli & Misteli, 2013).

1.2 THE NUCLEOSOME

1.2.1 Introduction

In the eukaryotic nucleus, DNA is wrapped around an octamer of histone proteins to form nucleosomes, the repeating unit of chromatin (Kornberg, 1974; Luger *et al*, 1997; Richmond & Davey, 2003). The DNA wrap around the octamer is typically left-handed, absorbing negative supercoils (Luger *et al*, 1997). The properties of histone proteins vary, but all have a histone fold domain that mediates di- and tetramerization (H2A with H2B; H3 with H4) (Arents & Moudrianakis, 1995) and unstructured tails that protrude from the nucleosome. Histones can be post-translationally modified, and canonical histones can be exchanged for histone variants, lending considerable variability to the chromatin fiber (Talbert & Henikoff, 2010; Zentner & Henikoff, 2013). The most fundamental way in which nucleosomes affect DNA-based processes, however, is by their simple presence, or positioning.

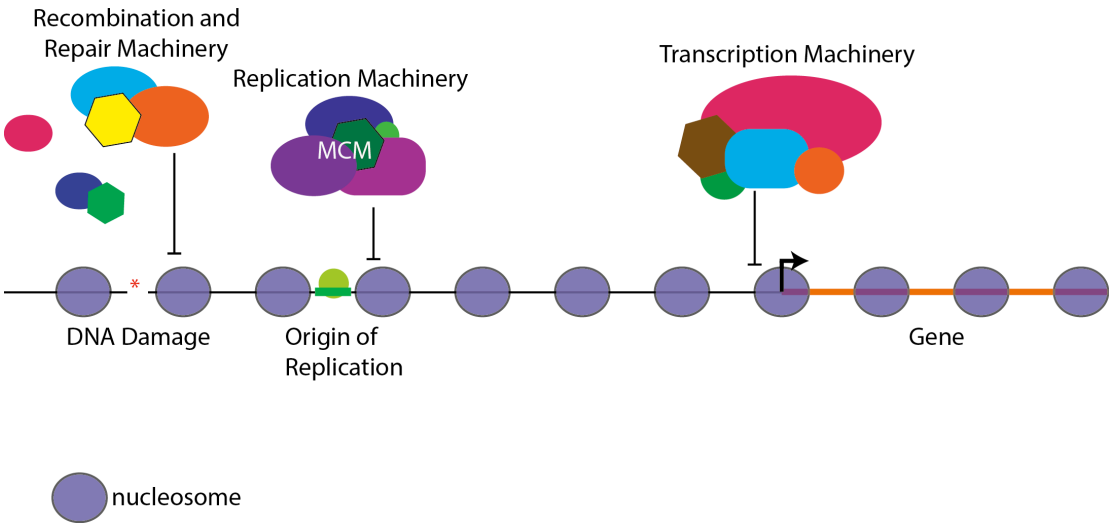

Figure 2. Nucleosomes restrict access to the DNA. Nucleosome positioning regulates, and must be altered to allow, DNA-based processes. Recombination, in both meiosis and DNA repair, replication fork progression, and transcription must all overcome the barrier presented by nucleosomes.

1.2.2 Nucleosome positioning

Nucleosome positioning has important implications for DNA accessibility and as such is subject to regulation (Struhl & Segal, 2013). The machinery of transcription, replication, recombination, and repair, as well as the myriad DNA-binding proteins that

regulate them, must all access the DNA molecule through its nucleosomal structure (Figure 2). As a result, whether or not a binding site is covered by a nucleosome can have physiological consequences.

Nucleosome positioning has now been mapped in many organisms (Yuan *et al*, 2005; Lee *et al*, 2007; Mavrich *et al*, 2008b; Schones *et al*, 2008; Shivaswamy *et al*, 2008; Valouev *et al*, 2008; Lantermann *et al*, 2010), though most biochemical studies and genetic analyses have been conducted in yeast. These studies have shed considerable light on where nucleosomes tend to be positioned, how this is altered in response to transcriptional changes, and which factors affect positioning. Nucleosome positioning refers to the precise 147 bp wrapped around a single histone octamer. What is actually measured, however, is generally nucleosome occupancy; that is, the fraction of an assayed population of cells that have a nucleosome at a given 147 bp window.

Certain principles of nucleosome occupancy appear to be universal: the transcription start and termination sites (TSS and TTS) of transcribed genes tend to be depleted of nucleosomes (nucleosome depleted region, NDR), as do enhancer elements (Struhl & Segal, 2013). Other aspects are more species-dependent. In budding yeast (*Saccharomyces cerevisiae*), both the -1 nucleosome directly upstream of the TSS and the +1 nucleosome directly downstream of the TSS are highly positioned (Mavrich *et al*, 2008a; Shivaswamy *et al*, 2008). Arrays of regularly spaced nucleosomes then extend in both directions from the TSS NDR. In contrast, the fission yeast (*Schizosaccharomyces pombe*) +1 nucleosomes are highly positioned, while positioned -1 nucleosomes are only present at some genes. Neither does fission yeast have a regular nucleosomal array upstream of the TSS (Figure 3; Lantermann *et al*, 2010). In both cases, TTS nucleosome depletion appears to be more dependent on RNAPII elongation and distance to the next promoter than TSS depletion (Fan *et al*, 2010). Gene bodies are generally covered by regularly spaced nucleosomal arrays, with linker DNA length (the distance between one nucleosome and the next) varying by species (Mavrich *et al*, 2008b; Shivaswamy *et al*, 2008; Lantermann *et al*, 2010; Givens *et al*, 2012).

Nucleosome positioning is determined by the combination of sequence favorability, the action of nucleosome remodelers, and the binding of transcription factors, the transcription machinery, and other DNA-binding proteins (Struhl & Segal, 2013). Nucleosome formation favorability ranges over three orders of magnitude based on DNA sequence alone (Thastrom *et al*, 1999). The difference in favorability arises from the differing flexibility of DNA sequences, with AT/TA pairs being the most flexible and homopolymeric stretches being the least. However, most of the yeast genome exhibits the favorable ten-bp periodicity that puts AT/TA pairs on the face of the DNA helix that is in contact with the histone octamer. Gene body nucleosomal arrays and the strongly positioned +/-1 nucleosomes are not replicated *in vitro* (Zhang *et al*, 2009). Furthermore, while AT-rich promoter regions can partially account for TSS nucleosome depletion, such promoters are uncommon in fission yeast (*Schizosaccharomyces pombe*), in more complex species, and at inducible genes (Lantermann *et al*, 2010; Tsankov *et al*, 2011). While DNA sequence factors provide local energy minima and may help chromatin remodelers to get positioning exactly ‘right,’ they cannot generate *in vivo* patterns of nucleosome spacing alone.

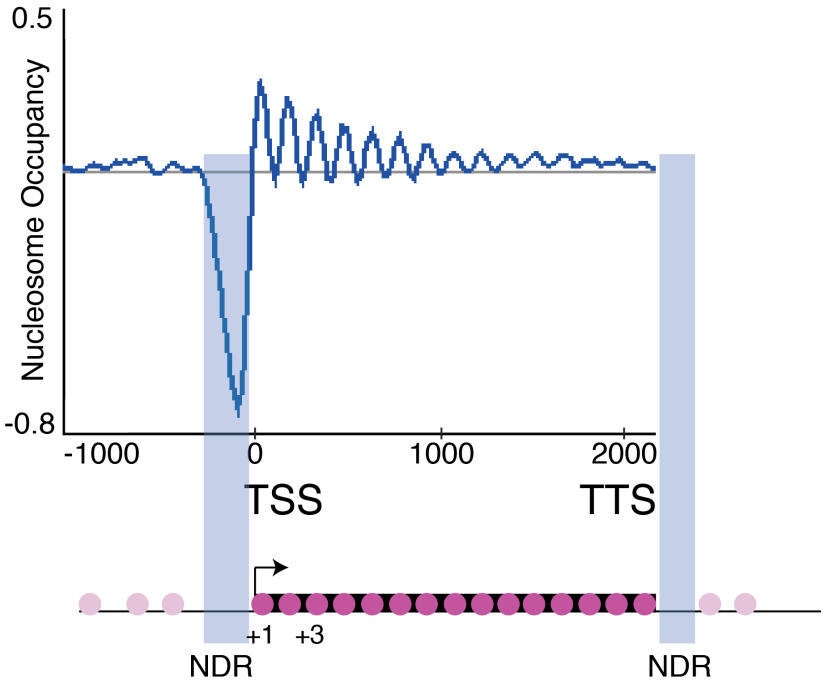

Figure 3. Nucleosome positioning over an average fission yeast gene. Nucleosome occupancy averaged over 4013 genes aligned at the transcription start site. A prominent nucleosome depleted region (NDR) is visible directly upstream of the TSS. An array of evenly spaced nucleosomes covers gene body. Fission yeast does not have a prominent nucleosomal array upstream of the NDR. Nucleosome occupancy signal is relative to genomic DNA signal. Dark pink circles represent positioned nucleosomes; light pink circles represent nonpositioned nucleosomes.

There are numerous examples of genetic and biochemical studies describing the importance of Snf2-family chromatin remodelers for nucleosome positioning (Alen *et al*, 2002; Walfridsson *et al*, 2007). Assembling, disassembling and sliding nucleosomes along DNA is, after all, a primary function of this diverse group of enzymes. Remodeling enzymes are needed for the maintenance of TSS and TTS nucleosome depleted regions (Alen *et al*, 2002; Walfridsson *et al*, 2007; Whitehouse *et al*, 2007; Badis *et al*, 2008) and for linking gene body nucleosomal arrays to the +1 nucleosome (Gkikopoulos *et al*, 2011; Hennig *et al*, 2012; Pointner *et al*, 2012; Shim *et al*, 2012). The loss of individual remodelers or combinations of partially redundant remodelers has physiological consequences. In addition to affecting the processes of transcription, replication and repair (Seeber *et al*, 2013), nucleosome positioning by chromatin remodelers is essential in maintaining boundary elements, e.g. between heterochromatin and euchromatin (Strålfors *et al*, 2011), and can even affect the flexibility and motility of the entire chromatin fiber (Neumann *et al*, 2012). Maintaining a NDR at the TSS of transcribed genes allows the transcription machinery to assemble; in cases where the TSS NDR is compromised, highly transcribed genes are downregulated (Whitehouse & Tsukiyama, 2006; Whitehouse *et al*, 2007; Yen *et al*, 2012). In parallel, when the TTS NDR is not maintained transcription termination can be compromised, resulting in readthrough transcription (Alen *et al*, 2002). Disruption of the regularly spaced genic array appears to allow for cryptic transcription

to initiate in the gene body (Hennig *et al*, 2012; Pointner *et al*, 2012; Shim *et al*, 2012; Smolle *et al*, 2012).

Finally, other DNA-binding proteins can compete with nucleosomes for access. Transcription factors that can bind DNA despite its being incorporated into a nucleosome are called 'pioneer' transcription factors (Zaret & Carroll, 2011). Once bound, pioneer factors can recruit ATP-dependent chromatin remodelers, which can further open the chromatin and allow other transcription factors to access the DNA. The strong positioning of the +1 nucleosome immediately after the TSS also suggests the influence of transcription machinery binding on nucleosome positioning (Zhang *et al*, 2009; Struhl & Segal, 2013).

1.2.3 Histone variants

The canonical histones H2A, H2B, H3 and H4 are synthesized and incorporated at the time of DNA replication (Heintz *et al*, 1983; Harris *et al*, 1991). In addition, histone variants exist and are incorporated into nucleosomes in place of the canonical histones in specific situations, loci or tissues. Histone variants are involved in processes from transcriptional regulation to centromere function to sex chromosome condensation. Many variants emerged early in eukaryotic evolution and are well-conserved, while others appear only in specific lineages. Histone variants add versatility to the chromatin fiber by affecting nucleosomal structure, bearing unique post-transcriptional modifications, or recruiting effector proteins (Talbert & Henikoff, 2010; Millar, 2013; Skene & Henikoff, 2013). Histone incorporation into chromatin, both generally and in histone variant exchange, is accomplished by the combined effort of histone chaperones and ATP-dependent nucleosome remodelers (Park & Luger, 2008; Burgess & Zhang, 2013; Skene & Henikoff, 2013; Torigoe *et al*, 2013).

A centromeric variant of H3, known as CenH3 (CENP-A in human, Cse4 in budding yeast, Cnp1 in fission yeast), is nearly universal among eukaryotes. CenH3 marks the site of kinetochore construction and is essential for centromeric identity and function (Earnshaw & Migeon, 1985; Stoler *et al*, 1995; Takahashi *et al*, 2000; Padeganeh *et al*, 2013a). CenH3 nucleosomes may actually be hemisomes, or DNA wrapped in a right-handed turn around a histone tetramer, rather than typical left-handed octameric nucleosomes (Dalal *et al*, 2007; Furuyama & Henikoff, 2009) although this is still controversial (Padeganeh *et al*, 2013b). Arguably the most dramatic example of a histone variant altering chromatin properties, this hemisomal chromatin may facilitate kinetochore assembly during mitosis (Furuyama & Henikoff, 2009). In contrast with CenH3, the H3.3 variant differs from canonical H3 (H3.1) by only a few amino acids. Unlike H3.1, which is only incorporated into chromatin during DNA replication or repair, H3.3 can be incorporated into chromatin both during and independent of replication (Ahmad & Henikoff, 2002). Nucleosome incorporation independent of chromatin replication is important for many processes, including chromatinization of the male pronucleus after fertilization (Loppin *et al*, 2005; Konev *et al*, 2007). Many species, including yeasts, have only a single non-centromeric H3 variant, like H3.3 incorporated at any point in the cell cycle (Talbert & Henikoff, 2010). Despite its similarity to H3.1, H3.3 appears to reduce nucleosome stability, with important regulatory consequences (Jin & Felsenfeld, 2007; Henikoff, 2009; Kurumizaka *et al*,

2013).

H2A has two highly conserved variants, H2A.Z and H2A.X, as well as numerous more lineage-restricted variants (West & Bonner, 1980; Millar, 2013). The former diverged early in eukaryotic evolution, while the latter is the result of repeated convergent evolution (Malik & Henikoff, 2003). H2A.Z is found in nucleosomes flanking the TSS NDR of both active and inactive genes (Raisner *et al*, 2005), and has been proposed to play a role in gene regulation (Halley *et al*, 2010). The relationship between H2A.Z occupancy and gene expression is a complex one, however, varying by developmental stage, tissue, and species (Millar, 2013). Reported roles in the full transcription of active genes, the induction of repressed genes, and even occasionally gene repression, may in part be reconciled by varying post-transcriptional modifications (Bruce *et al*, 2005; Millar *et al*, 2006; Gevry *et al*, 2007; Halley *et al*, 2010; Gallant-Behm *et al*, 2012; Millar, 2013). The effect of H2A.Z incorporation on the nucleosome could also stem from its structure, which may render a nucleosome containing both H2A and H2A.Z unstable (Li *et al*, 1993; Suto *et al*, 2000; Park *et al*, 2004; Zhang *et al*, 2005; Thambirajah *et al*, 2006; Hoch *et al*, 2007; Higashi *et al*, 2007; Bonisch & Hake, 2012). Incorporated by the Swr1 nucleosome remodeling complex SWR-C (Krogan *et al*, 2003) and removed by the Ino80 remodeling complex (Papamichos-Chronakis *et al*, 2011), H2A.Z also plays important roles in development, DNA damage repair, heterochromatin and chromatin boundary formation (Millar, 2013). H2A.X, an S139 phosphorylatable H2A variant, is absent in yeasts and fruit fly (*Drosophila melanogaster*). In yeasts, however, canonical H2A can be phosphorylated and appears to have the same functions as H2A.Xph (Pinto & Flaus, 2010; Rozenzhak *et al*, 2010; Szilard *et al*, 2010; Kitada *et al*, 2011). H2A.Xph, or γ-H2A.X, marks sites of DNA damage, where it recruits repair factors including SWR-C and INO80. SWR-C in turn transiently incorporates H2A.Z, which may ease nucleosome eviction proximal to the damage site (Papamichos-Chronakis & Peterson, 2013). γ-H2A.X is also associated with replication fork collapse and heterochromatin (Rozenzhak *et al*, 2010; Szilard *et al*, 2010; Kitada *et al*, 2011). Although H2A.X can be found genomewide (Seo *et al*, 2012), its functions beyond damage repair are poorly understood.

While H4 and H2B variants do exist, they are far less common than variants of H3 and H2A (Ota *et al*, 2004; Bonenfant *et al*, 2006; Wu *et al*, 2009; Gonzalez-Romero *et al*, 2010; Jufvas *et al*, 2011; Talbert *et al*, 2012). This is likely due to evolutionary constraints on allowing both histones in the core dimers to vary (Gonzalez-Romero *et al*, 2010). Finally, a fifth histone, H1, has been identified in species from ciliates to human. Known as the linker histone, H1 binds the DNA where it leaves the core nucleosome. Reflecting the diversity of somatic- and gamete-specific H1 variants, the linker histone can influence nucleosome spacing, higher order chromatin structure, gene expression, and development (Kowalski & Palyga, 2012; Öberg *et al*, 2012).

1.2.4 Histone modifications

In addition to serving as the scaffold that allows DNA to be compacted, histones play a much-studied regulatory role. Histones can be post-translationally modified, most commonly on their protruding tails but also on the globular core domain (Cosgrove *et al*, 2004), with a variety of chemical and protein groups. These include methylation of

arginine and lysine residues, acetylation of lysine residues, phosphorylation of serine and tyrosine residues, and ubiquitination/sumoylation of lysine residues (Kouzarides, 2007; Campos & Reinberg, 2009; Tropberger & Schneider, 2010; Zentner & Henikoff, 2013). It was once expected that, in combination, these different modifications would form a 'histone code,' with specific combinations giving rise to specific outcomes (Strahl & Allis, 2000). It has since become apparent, however, that histone modifications are often redundant (Martin *et al*, 2004; Dion *et al*, 2005). Rather than modifications forming myriad meaningful combinations, there appears to be a more limited number of common combinations associated with different chromatin states (Wang *et al*, 2008; Filion *et al*, 2010). Though these histone modification patterns could still be considered a code, it is a highly redundant, and thus highly resilient, one (discussed in Sims & Reinberg, 2008; Campos & Reinberg, 2009).

Histones are modified by a plethora of enzymes (Kouzarides, 2007). Lysine methyl transferases (KMTs), lysine acetyl transferases (KATs), kinases and E3 ubiquitin ligases are key enzymes involved in modifying histones. Modifications can also be removed, through the action of lysine demethylases (KDMs), histone deacetylases (HDACs), deubiquitinases and phosphatases, among others. These enzymes have crucial regulatory and signaling roles in all processes that have chromatin as their substrate, e.g. transcription and DNA repair. They are also crucial for the maintenance of heterochromatin and euchromatin (Dalla Rosa *et al*, 2009; Zentner & Henikoff, 2013).

There are two principal ways in which histone modifications can affect nuclear events. In the first, modifications can have a general effect on chromatin structure. Acetylation and phosphorylation reduce the positive charge of the histone, weakening the interaction with the negatively charged DNA backbone and increasing DNA accessibility (Hyland *et al*, 2005; Masumoto *et al*, 2005; Xu *et al*, 2005; Rufiange *et al*, 2007; Dawson *et al*, 2009; Manohar *et al*, 2009; Neumann *et al*, 2009). Histone tail modifications have been shown to alter higher order chromatin structures, which are held together by interactions between histones in different nucleosomes (Simpson, 1978; Tse *et al*, 1998; Garcia-Ramirez *et al*, 1995). For example, H4K16Ac antagonizes chromatin compaction (Shogren-Knaak *et al*, 2006; Robinson *et al*, 2008), as does H2Bub (Fierz *et al*, 2011). In contrast, histone tail modifications may have only minor effects on the stability of individual nucleosomes (Widlund *et al*, 2000).

Histone modifications can also specifically recruit effector proteins and/or influence the activity of those proteins (Tamkun *et al*, 1992; Bannister *et al*, 2001; Hon *et al*, 2009; Erdel *et al*, 2011; Musselman *et al*, 2012; Zentner & Henikoff, 2013). These proteins, alone or accompanied by large complexes, can then modify other histones, remodel nucleosomes, alter the chromatin compaction or otherwise influence chromatin-based processes. This recruitment is mediated by domains in the recruited proteins that recognize different histone modifications. Chromodomains, for example, are able to bind methylated lysine residues (Bannister *et al*, 2001; Eissenberg, 2012), while bromodomains bind acetylated lysines (Zeng & Zhou, 2002). Many modifications both recruit specific effectors and influence general chromatin properties. The complex crosstalk between chromatin modifiers generates a regulatory network, resilient in its

redundancy, that influences processes including transcription, DNA repair, recombination and replication.

1.3 ATP-DEPENDENT CHROMATIN REMODELERS

1.3.1 Introduction

The specific positioning of a nucleosome along the DNA can expose or block transcription factor binding sites, as well as contribute to overall DNA topology. Furthermore, DNA-based processes like transcription, recombination, DNA repair and DNA replication must contend with nucleosomes for access to the DNA molecule. The SNF2 family of ATP-dependent helicase-like nucleosome remodelers is responsible for assembling and disassembling nucleosomes, exchanging H2A-H2B dimers, histone variant incorporation and nucleosome sliding (Clapier & Cairns, 2009; Hargreaves & Crabtree, 2011). In addition to ATPase and helicase-like DNA translocase domains (Fyodorov & Kadonaga, 2002; Saha *et al*, 2002; Whitehouse *et al*, 2003; Singleton *et al*, 2007), chromatin remodelers often have domains by which they can be recruited to specific chromatin landscapes (Erdel *et al*, 2011). SWI/SNF (SNF2), the founding member of the family, and its homologs have a bromodomain, which binds to acetylated lysine and thus recruits the remodeler to open, active chromatin (Tamkun *et al*, 1992; Zeng & Zhou, 2002). Chromatin remodelers are often active in eponymous complexes, the subunits of which can vary by tissue and developmental stage in more complex organisms. These other subunits lend further specificity by modulating remodeler activity and targeting (Clapier & Cairns, 2009; Hargreaves & Crabtree, 2011).

Given the importance of Snf2 family chromatin remodelers to so many nuclear processes, it is unsurprising that the mutation or loss of these enzymes is associated with an array of diseases. In addition to numerous ties to cancer, (Bagchi *et al*, 2007; Wilson & Roberts, 2011), chromatin remodeling defects are associated with neural and other developmental abnormalities (Bultman *et al*, 2000; Boerkoel *et al*, 2002; Martin, 2010), some of them lethal.

The Snf2 family chromatin remodelers can be subdivided in a variety of ways, ranging from the historical to the structural. Table 1 reflects a mixed classification, retaining the common group names while still clustering subfamilies according to the sequence and structure of the helicase-like domain, a division that appears to parallel functional differentiation (Flaus *et al*, 2006). The Snf2 ATPases have been reviewed extensively elsewhere (Clapier & Cairns, 2009; Flaus & Owen-Hughes, 2011; Hargreaves & Crabtree, 2011; Ryan & Owen-Hughes, 2011). Importantly, there are many more members of the Snf2 family than are outlined in Table 1. Many of these enzymes do not have nucleosomes as their primary substrate, however, and translocate DNA for other purposes (Flaus *et al*, 2006; San Filippo *et al*, 2008; Viswanathan & Auble, 2011)

Table 1. ATP-dependent Snf2 Family Chromatin Remodelers. A subset of the Snf2 family ATPases, encompassing the better characterized chromatin remodeling groups, and their homologs in humans, *Arabidopsis thaliana*, and two yeasts. The first three groups (blue), Swi/Snf, Iswi, and Chd, can be further clustered as distinct from the more distantly related Ino80-like remodeling subfamilies (purple). (Flaus *et al*, 2006)

Group	Subfamily	Name in Fission Yeast	Name in Budding Yeast	Name in *Arabidopsos thaliana* (Thale Cress)	Name in Human
Swi/Snf	Swi/Snf	Snf22 Snf21	SNF2 (SWI/SNF) STH1 (RSC)	CHR12; CHR23; CHR3/SPLAYED ; CHR2/BRM	SMARCA4/BRG1 SMARCA2/BRM
	Lsh	-	IRC5	DDM1	SMARCA6/ HELLS
Iswi	Iswi	-	ISW1; ISW2	CHR11; CHR17	SMARCA1/SNF2L SMARCA5/SNF2H
	ALC1	-	-	CHR10/ASG3	CHD1L/ALC1
Chd	Chd1	Hrp1; Hrp3	CHD1	CHR5	CHD1; CHD2
	Mi-2	Mit1	-	CHR4/PKR1; CHR6/CHD3/ PICKLE; CHR7/PKR2	CHD3/Mi2-α; CHD4/Mi2-β; CHD5
	Chd7	-	-	-	CHD6; CHD7; CHD8/HELSNF1; CHD9
Ino80-like	Fun30	Fft1; Fft2; Fft3	FUN30	CHR19/ETL1	SMARCAD1
	Ino80	Ino80	INO80	INO80	INO80
	Swr1	Swr1	SWR1	PIE1	SRCAP
	EP400	-	-	-	EP400

The exact mechanism by which remodelers translocate DNA around nucleosomes is still under discussion, and is likely to differ between different subfamilies (Flaus & Owen-Hughes, 2011). Any manipulation of the nucleosome that requires breaking the interactions between the positive histones and negative DNA requires ATP-dependent remodeling activity. This includes nucleosome sliding, assembly, disassembly, and histone dimer exchange (Figure 4). Chromatin is assembled by the combined effort of histone chaperones and Snf2-family chromatin remodelers. First, chaperones deposit histones on the DNA in an ATP-independent manner (Das *et al*, 2010; Burgess & Zhang, 2013). The result is a histone-DNA complex that resembles a nucleosome, but does not supercoil DNA like a mature nucleosome and is more vulnerable to digestion by micrococcal nuclease (Torigoe *et al*, 2013). Next, the Snf2-family remodeler assembles the histone-DNA complex into a canonical nucleosome in a process that utilizes ATP (Torigoe *et al*, 2013). This nucleosome assembly, or maturation, is distinct from the remodeler's other functions such as spacing the nucleosomes (Torigoe *et al*, 2013).

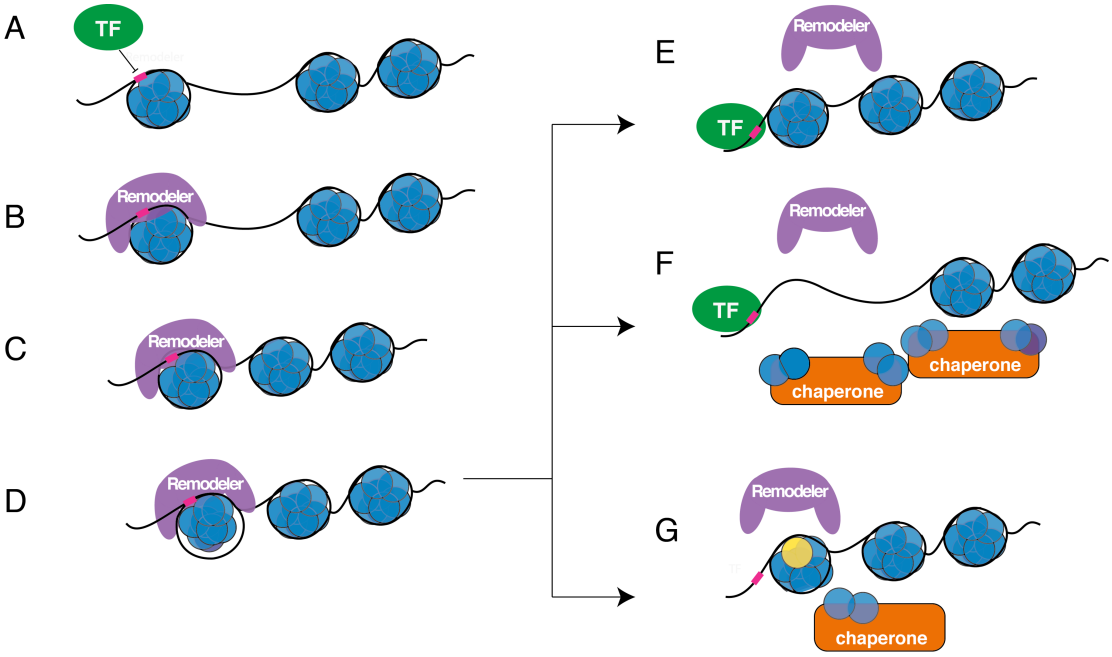

Figure 4. DNA translocation by a Snf2 family chromatin remodeler. A) An ATP-dependent chromatin remodeler can expose DNA that is obscured by a nucleosome. B-D) This may happen in a series of enzymatic cycles, in which the remodeler pulls/pushes DNA into the nucleosome, disrupting histone-DNA interactions. This would allow E) nucleosome sliding, F) eviction, or G) dimer exchange. Histones, blue/yellow; Remodeler, purple; Transcription factor, green; Chaperone, orange.

ATP-dependent chromatin remodeling is involved throughout the process DNA damage repair. There are many kinds of DNA damage, repaired by a wide variety of mechanisms (Branzei & Foiani, 2008), of which double stranded break (DSB) repair involves the most interaction with chromatin (Papamichos-Chronakis & Peterson, 2013; Price & D'Andrea, 2013). The role of chromatin remodeling is perhaps most critical in DSB repair via homologous recombination, although nonhomologous end joining repair also involves Snf2 family chromatin remodelers (Ogiwara *et al*, 2011). The role of ATP-dependent chromatin remodeling in DNA damage repair likely follows the classic Access-Repair-Restore model (Smerdon, 1991): after DSBs are detected, repair proteins need assistance to access the lesion. Remodeling is further required to allow resectioning, the creation of a single-stranded DNA (ssDNA) intermediate capable of homologous recombination, and to restore normal chromatin after repair is completed.

Upon detection of a DSB, the RSC complex (catalytic subunit Snf21) is immediately recruited to the site, where it slides or ejects immediately proximal nucleosomes (Chai *et al*, 2005; Shim *et al*, 2005; 2007; Kent *et al*, 2007). Ino80 and Fun30 are recruited more slowly, and while remodeling by Fun30 is redundant with the activity of Ino80 and RSC proximal to the DSB, it is required for extensive resection (Papamichos-Chronakis *et al*, 2006; Tsukuda *et al*, 2009; Chen *et al*, 2012; Costelloe *et al*, 2012; Eapen *et al*, 2012; Neumann *et al*, 2012). Swr1c is also recruited to DSBs, where it transiently stimulates the incorporation of H2A.Z, possibly easing nucleosome ejection (van Attikum *et al*, 2007; Kalocsay *et al*, 2009; Rosa *et al*, 2013). How essential these

remodeling activities are for efficiency of DSB repair by homologous recombination is still unclear. In addition to apparent interspecies variation, studies disagree on the amount of nucleosome loss and the importance of remodeling for the kinetics of recombination DSB repair (discussed in Papamichos-Chronakis & Peterson, 2013). Once the ssDNA intermediate has been formed it seeks homologous sequence with which to recombine, requiring increased chromosome flexibility and mobility (Kruhlak *et al*, 2006; Soutoglou *et al*, 2007; Torres-Rosell *et al*, 2007; Chiolo *et al*, 2011; Dion *et al*, 2012; Mine-Hattab & Rothstein, 2012). This may also be enhanced by ATP-dependent chromatin remodelers, as Ino80c has been demonstrated to enhance chromosomal flexibility and locus mobility, perhaps by disrupting a chromosomal anchor (Neumann *et al*, 2012).

Meiotic homologous recombination, which involves many of the same principles as DSB repair recombination, also involves Snf2 ATPases (Colas *et al*, 2008; Kim *et al*, 2012; Rosa *et al*, 2013). Chromatin remodeling plays crucial roles in DNA replication, a process in which nucleosomes must be not only circumvented but also reassembled in such a way as to preserve the chromatin state on both daughter chromatids. In budding yeast, INO80 travels with the replication fork and evicts H2A.Z (Papamichos-Chronakis *et al*, 2011). In the absence of INO80, stalled replication forks are more prone to collapse, perhaps because of excess H2A.Z (Papantonis *et al*, 2008; Shimada *et al*, 2008). In humans and budding yeast, SNF2H/ISW2 facilitates replication through heterochromatin (Collins *et al*, 2002; Vincent *et al*, 2008), while human SMARCAD1 recruits HDACs to the replication fork (Rowbotham *et al*, 2011). This is important because histones are acetylated before incorporation, and without the action of HDACs chromatin can become hyperacetlyated, leading to a loss of heterochromatin. Chromatin remodeling is also essential for transcription. Remodelers give RNA polymerases access to the DNA template, reassemble chromatin behind the polymerases, and prevent inappropriate (‘cryptic’) transcription initiation. This is discussed further in section 1.6.2.

Unsurprisingly, chromatin remodeling is heavily involved in developmental regulation. The diversity of chromatin remodeling enzymes and complexes that supports situation-appropriate transcriptional programs in unicellular organisms is also essential to multicellular development. Snf2 enzymes are active from the earliest stages of egg fertilization (Konev *et al*, 2007) to the determination of differentiated cell fate (Aichinger *et al*, 2009; Yoo & Crabtree, 2009; Ronan *et al*, 2013). Chromatin remodelers are also important for the maintenance of an undifferentiated state (Aichinger *et al*, 2009; Gaspar-Maia *et al*, 2009). The molecular and developmental functions of two remodeler subfamilies, Chd1 and Fun30, are discussed in detail in the next sections.

1.3.2 The Chd1 subfamily of chromatin remodelers

Mouse Chd1 was the first member of the Chd group of remodelers to be discovered (Delmas *et al*, 1993). Named for their Chromodomains, Helicase domains, and DNA binding, early characterization suggested that Chd1 is a euchromatic factor, binding the active interbands and puffs of fruit fly polytene chromosomes (Stokes *et al*, 1996). Furthermore, the remodeler was observed to be expressed at different levels in different

human tissues, with expression high in placenta and skeletal muscle but almost absent from brain tissue (Woodage *et al*, 1997).

Since their initial characterization, the Chd1 remodelers have been studied extensively both *in vivo* and *in vitro.* Unlike many other Snf2 family remodeling enzymes, Chd1 is not found in stable complexes, but rather functions as a monomer or perhaps homodimer (Tran *et al*, 2000; Lusser *et al*, 2005). Chd1 is capable of assembling and sliding nucleosomes (Lusser *et al*, 2005), and cooperates with the Nap1 histone chaperone both *in vivo* (Walfridsson *et al*, 2007) and *in vitro* (Lusser *et al*, 2005). Chd1 slides end-positioned nucleosomes toward the middle of DNA fragments *in vitro* (Stockdale *et al*, 2006), a process that is dependent on the DNA binding domain (McKnight *et al*, 2011; Ryan & Owen-Hughes, 2011). Chd1 may also slide nucleosomes toward its own position on longer extranucleosomal DNA, making the DNA binding domain important for sliding directionality (McKnight *et al*, 2011; Patel *et al*, 2013).

The function of the tandem Chd1 chromodomains has been more difficult to determine. Chromodomains are found in all studied eukaryotes and mediate protein-protein or protein-nucleic acid interactions (Eissenberg, 2012). Human CHD1 has a strong affinity for H3K4 di- and trimethylation *in vitro* (Sims *et al*, 2005) and *in vivo* (Sims *et al*, 2007), where the chromodomains are important for Chd1 interaction with chromatin (Kelley *et al*, 1999). In contrast, fruit fly chromodomains appear to be dispensable for Chd1 recruitment to active chromatin *in vivo* and for H3K4me2 affinity *in vitro* (Morettini *et al*, 2011), while budding yeast CHD1 shows no *in vitro* affinity for methylated H3K4 (Sims *et al*, 2005). Rather than being important for recruitment of Chd1 to chromatin, fruit fly Chd1 chromodomains appear to be essential for full enzymatic function (Morettini *et al*, 2011). This makes sense in light of mechanistic studies that revealed the regulatory control of chromodomains over Chd1 ATPase activity (Hauk *et al*, 2010). This study shows that in addition to perhaps helping to target Chd1 in some species, in the absence of nucleosomes the chromodomains block DNA from accessing and activating the ATPase motor, allowing Chd1 to restrict its activity to nucleosomal DNA. Similarly, a NegC domain between the DNA binding domain and the ATPase/DNA translocase domains appears to inhibit remodeling in the absence of extranucleosomal DNA (Patel *et al*, 2011; Sharma *et al*, 2011).

In vivo, Chd1 has diverse, context-dependent functions. The remodeler is implicated in all stages of transcription, from initiation to termination. At fission yeast gene promoters, Chd1 remodelers are associated with maintaining the NDR, as H3 occupancy increases in their absence (Walfridsson *et al*, 2007). Budding yeast CHD1 is important for clearing the promoter of nucleosomes during *PHO5* gene activation, particularly in the absence of ISW1 (Ehrensberger & Kornberg, 2011). Both budding yeast and fruit fly Chd1 remodelers increase the turnover of histone H3 at the 5' end of genes (Radman-Livaja *et al*, 2012). The Mediator complex, which triggers assembly of the RNAPII PIC, interacts physically with both fission yeast Hrp1 and human Chd1, coordinating remodeler recruitment with PIC assembly (Khorosjutina *et al*, 2010; Lin *et al*, 2011). However, the relationship between Chd1 promoter remodeling and transcription level is ambiguous. Although Chd1 is required for the proper expression of some genes under certain conditions (Belden *et al*, 2011; Ehrensberger & Kornberg,

2011; Lin *et al*, 2011; Raduwan *et al*, 2013), genomewide correlations are weak (Tran *et al*, 2000; Walfridsson *et al*, 2007; Khorosjutina *et al*, 2010). The NDRs of gene promoters may also provide a convenient base of operations for Chd1 activity in the gene body. Because Chd1 remodeling requires a longer stretch of naked DNA than the standard internucleosomal linker, it may first bind the stable promoter NDR and then hop to the more transient nucleosome depleted regions that form in the wake of the elongating RNAPII (Zentner *et al*, 2013).

Over gene bodies Chd1 remodelers are involved in linking the regularly spaced chromatin array to the highly positioned +1 nucleosome (Gkikopoulos *et al*, 2011; Hennig *et al*, 2012; Pointner *et al*, 2012; Shim *et al*, 2012). The placement of nucleosomes over coding regions appears to be important for preventing the initiation of transcription from cryptic promoters in the gene body (Hennig *et al*, 2012; Pointner *et al*, 2012; Shim *et al*, 2012; Smolle *et al*, 2012), rather than correlating with changes in sense transcription. In budding yeast, CHD1 is partially redundant with ISWI remodelers in maintaining these genic nucleosomal arrays. At the same time CHD1, with ISW1 and ISW2, prevents the exchange of gene body histones with newly synthesized histones during transcription in budding yeast (Smolle *et al*, 2012). This is important to maintain H3K36me and hypoacetylation at gene bodies, which also prevents cryptic transcription. In agreement with this finding, in fruit fly and budding yeast CHD1 represses H3 turnover at the 3' end of gene bodies, particularly in long genes. The particular vulnerability of long genes to elevated H3 turnover may have to do with DNA topology (Durand-Dubief *et al*, 2010) and is further discussed in section 1.5. Indeed, Chd1 association with the long genes of fruit fly polytene chromosomes was noted quite early in its characterization (Kelley *et al*, 1999). Chd1 recruitment to active gene bodies is likely mediated by its physical association with elongation factors like PAF1, SPT4-SPT5 and FACT (Kelley *et al*, 1999; Simic *et al*, 2003). Finally, human CHD1 has been implicated in recruiting the spliceosome for pre-mRNA processing (Sims *et al*, 2007), while budding yeast CHD1 remodels the TTS with Isw1 and Isw2 for proper transcription termination (Alen *et al*, 2002).

Beyond transcription, Chd1 remodelers have a role in the heterochromatin/euchromatin balance in the genome. Chd1 opposes excessive heterochromatin formation in fruit fly (Bugga *et al*, 2013), while fission yeast Chd1 remodelers Hrp1 and Hrp3 are required for proper silencing of pericentromeric and silent mating type region heterochromatin (Yoo *et al*, 2000; Walfridsson *et al*, 2007). Chd1 mutants have chromosome segregation defects (Yoo *et al*, 2000), and in fission yeast, chicken and human Chd1 remodeling is required for WT levels of the centromeric H3 variant CENP-A.

Finally, Chd1 is essential in the early stages of both mammalian and insect development. Experiments in flies uncovered a requirement for Chd1, in cooperation with the HIRA histone chaperone, in unpacking the male pronucleus upon egg fertilization (Konev *et al*, 2007). Chd1 incorporates H3.3 into the paternally contributed chromosomes, which is essential if these chromosomes are to participate in mitosis (Konev *et al*, 2007). In silk worm (*Bombyx mori*), Chd1 remodels nucleosomes at the promoters of developmental genes, leading to transcription factor binding and transcription (Papantonis *et al*, 2008). Chd1 is also essential for mouse ESC pluripotence. Without Chd1, heterochromatic foci form and the stem cells are pushed

toward the neural lineage, while becoming incapable of endodermal differentiation (Gaspar-Maia *et al*, 2009).

It is surprising that, although studies have observed either increased promoter H3 occupancy or decreased promoter H3 turnover in Chd1 mutants (Walfridsson *et al*, 2007; Ehrensberger & Kornberg, 2011; Radman-Livaja *et al*, 2012), MNase digestion in the same mutants does not indicate any decrease in NDR depth (Gkikopoulos *et al*, 2011; Hennig *et al*, 2012; Pointner *et al*, 2012; Shim *et al*, 2012). This discrepancy may in part be explained by observations that *in vitro*, Chd1 both slides nucleosomes and 'matures' the disorganized histone-DNA complex that is the result of histone deposition by chaperones (Torigoe *et al*, 2013). Such products of chaperone deposition are more vulnerable to MNase digestion than are mature nucleosomes, and could explain the differences observed between H3-ChIP and MNase digestion experiments (Torigoe *et al*, 2013). It is likely that maintaining the promoter NDR is a highly redundant process involving many remodelers, any of which could mature randomly deposited histones into nucleosomes and slide them away or expel them from the NDR. Loss of Chd1 remodeling reduces the efficiency of this process such that 'immature' nucleosome occupancy is elevated in the NDR, but this is not detected by MNase digestion. Furthermore, if these histones do not efficiently block access to the DNA then they may be of little functional importance at constitutively active genes, explaining the puzzling lack of genomewide correlation between elevated promoter H3 occupancy and transcriptional changes in the absence of Chd1 (Walfridsson *et al*, 2007).

1.3.3 The Fun30 subfamily of chromatin remodelers

The Fun30 (Function Unknown 30) subfamily of chromatin remodelers are relatively poorly characterized. The single budding yeast homolog, Fun30, was the first member of the subfamily discovered (Clark *et al*, 1992), and mutants were observed to be resistant to UV radiation (Barton & Kaback, 1994). The mouse Fun30 homolog, Etl1, is expressed from the 2-cell stage of embryonic development and then throughout embryogenesis, with especially high levels in the ICM, throughout the embryo during gastrulation, and in the fetal CNS, epithelia and thymus (Soininen *et al*, 1992; Schoor *et al*, 1993). Despite this and continued expression throughout the adult mouse, Etl1 knockout mice are viable and Etl1 is not required for normal proliferation in mESC. Knockout mice do, however, exhibit growth retardation, skeletal dysplasias and reduced fertility (Schoor *et al*, 1999). Although not yet linked to more serious conditions, a splicing mutation of the human homolog SMARCAD1 can cause adermatoglyphia, or absence of fingerprints. Adermatoglyphia is a rare condition causing inconvenience to sufferers when they pass through immigration controls (Nousbeck *et al*, 2011).

More recent purification and *in vitro* characterization of the budding yeast Fun30 reveals that the remodeler exists primarily as a homodimer and that it performs both histone dimer exchange and whole nucleosome sliding (Awad *et al*, 2010; Byeon *et al*, 2013). In addition to ATPase and helicase-like domains, the Fun30 family has a conserved CUE motif (Neves-Costa *et al*, 2009). CUE motifs are known to interact with ubiquitin, but budding yeast FUN30 did not show a preference for ubiquitinated

HeLa histones *in vitro* (Awad *et al*, 2010).

In vivo studies in budding yeast show that deletion of FUN30, or inactivation of its ATPase domain, leads to loss of silencing at telomeres, rDNA repeats and the HMR mating type locus. In support of a direct effect, Fun30 binds both the HMR locus and the HMR boundary element, at which it alters chromatin structure (Neves-Costa *et al*, 2009). Fission yeast has three Fun30 subfamily remodelers, Fft1, Fft2 and Fft3 (Fission yeast FunThirty 1-3). Of these, Fft3 is the best characterized. Fft3 binds insulator elements and has a role in maintaining the boundaries between euchromatin and heterochromatin at centromeres and subtelomeres. In the absence of Fft3, euchromatin invades subtelomeres and the centromeric central core, alleviating gene silencing and causing mitotic defects (Strålfors *et al*, 2011). Budding yeast Fun30 is also important for centromeric function, maintaining proper chromatin at and around the centromeric nucleosome and preventing mitotic segregation defects (Durand-Dubief *et al*, 2012). In human, SMARCAD1 physically interacts with heterochromatin proteins and HDACs, localizes to DNA replication forks, and is required to deacetylate histones incorporated during replication (Rowbotham *et al*, 2011). SMARCAD1-depleted cells have elevated histone acetylation, reduced centromeric H3K9 methylation, and mild mitotic defects (Rowbotham *et al*, 2011).

Members of the Fun30 subfamily may also play a more direct role in gene regulation. Budding yeast Fun30 remodels nucleosomes at gene TSSs, with a generally repressive result (Byeon *et al*, 2013). This may be conserved in humans, as SMARCAD1 has also been observed to bind the TSS of several genes (Okazaki *et al*, 2008). Finally, Fun30 and SMARCAD1 have recently been found to have corresponding functions in the repair of double stranded DNA breaks (DSBs). DSBs can be repaired by homologous recombination, which requires 'resectioning,' or the production of a single stranded DNA intermediate from DNA that is embedded in chromatin. Although Fun30 remodeling is redundant with that of INO80 and RSC immediately adjacent to the DSB, it is required for extensive resectioning, and without it cells become sensitive to DSB-inducing agents (Chen *et al*, 2012; Costelloe *et al*, 2012; Eapen *et al*, 2012).

1.4 NONCODING RNA

The days of thinking of RNA molecules as coming in three principal flavors, mRNA, tRNA, and rRNA, are over. Instead, it has become clear that an astonishing variety of non-protein-coding RNAs regulate gene expression at all levels. This is hardly surprising in light of the 'RNA World' hypothesis, which suggests that the DNA-RNA-protein life system arose from a system in which RNA performed both data storage and catalytic functions (Gilbert, 1986). There are over 100 potentially reversible modifications for RNA nucleotides, contributing an additional layer of complexity and regulation to the transcriptome (Kellner *et al*, 2010; Squires *et al*, 2012). Functional RNAs can be divided into two categories based on their length: long noncoding RNAs (lncRNAs; Mercer & Mattick, 2013) and small RNAs (sRNAs; Sabin *et al*, 2013). Of the two, various species of sRNA have been the objects of far more study.

The arrival of deeper sequencing techniques has revealed that the majority of the human and mouse genomes is transcribed at some level (Okazaki *et al*, 2002; Carninci

et al, 2005; Kapranov *et al*, 2007). Surprisingly, the non-protein-coding transcription appears to be more cell-type specific than that of coding genes (Cabili *et al*, 2011; Derrien *et al*, 2012). lncRNAs are typically regulated and transcribed like mRNAs, by RNAPII, with active gene marks at their TSSs and polyadenylation of their transcripts (Guttman *et al*, 2009). Indeed, many lncRNAs are also coding mRNAs, with dual roles in the cell. lncRNAs can interact with other molecules either by base pairing or via their secondary structure, in the manner of a folded protein (Plath *et al*, 2003; Lescoute & Westhof, 2006; Cruz & Westhof, 2009). This versatility opens several routes to influence gene transcription and cellular processes in general. lncRNAs can regulate histone modifications, for example Xist recruitment of the Eed-Ezh2 Polycomb complex to methylate H3K27 on the inactive X chromosome (Plath *et al*, 2003). They can also prevent transcription factors from entering the nucleus by binding directly to specific importin proteins (Willingham *et al*, 2005). After transcription, lncRNAs can base pair with mRNAs and influence their fates, targeting them for amplification (Carrieri *et al*, 2012) or destruction (Gong & Maquat, 2011). lncRNAs are also involved in chromosomal interactions (Shevtsov & Dundr, 2011; Wang *et al*, 2011) and in telomere maintenance (Azzalin *et al*, 2007; Schoeftner & Blasco, 2008).

In contrast with lncRNAs, most sRNAs function through a limited number of effector protein complexes. sRNAs are typically cleaved from longer RNA molecules by a Dicer class protein, after which they target Argonaute class proteins to complementary sequences. This process is often amplified by an RNA-directed RNA polymerase (RdRP), and can silence targets at either the chromatin or mRNA level (Sabin *et al*, 2013). The precursor RNA molecule can be of either endogenous or exogenous origin, with the origin of the pathway hypothesized to be as a defense against transposable elements and other genomic parasites (Wong & Choo, 2004; Werren, 2011). In post-transcriptional gene silencing (PTGS), sRNAs guide Argonaute complexes to complementary mRNAs in the cytoplasm, which are then either cleaved or translationally repressed. Here the focus will be on transcriptional gene silencing (TGS), in which sRNAs guide effector complexes to alter chromatin and repress transcription.

Transcriptional silencing at fission yeast pericentromeric heterochromatin (Volpe *et al*, 2002; Motamedi *et al*, 2004) and other genomic loci (Djupedal & Ekwall, 2009; Yamanaka *et al*, 2013) is mediated by small interfering RNAs (siRNAs: ~20-23 nt). These siRNAs are produced from target locus primary transcripts, which are first made double stranded by the fission yeast RdRP (Rdp1) and then cleaved by the fission yeast Dicer protein (Dcr1). The siRNAs are finally loaded into the fission yeast Argonaute protein (Ago1), targeting it to the target locus nascent transcripts. Ago1 is part of the RNA induced initiation of transcriptional silencing (RITS) complex, which contains other heterochromatic proteins that contribute to TGS. RITS binding also leads to the recruitment of Clr4, the fission yeast H3K9 methyltransferase (Zhang & Pommier, 2008). A similar system operates in the germ lines of several species, including flies and humans, to defend against transposon activation (O'Donnell & Boeke, 2007). The Piwi (P-element induced wimpy testes) clade of Argonaute proteins, which tend to be essential for gamete production and fertility, are targeted to transposons by Piwi-interacting RNAs (piRNAs: ~23-29 nt). Piwi argonautes guided by piRNAs are able to cleave transposon transcripts (Brennecke *et al*, 2007; Gunawardane *et al*, 2007), and

also support TGS via H3K9 methyltransferase recruitment (Shpiz *et al*, 2011; Wang *et al*, 2011; Sienski *et al*, 2012; Le Thomas *et al*, 2013; Rozhkov *et al*, 2013).

Noncoding RNAs are also capable of directing DNA methylation. Although some species, including fission yeast and fruit fly, have lost the ability to methylate DNA, this dynamic modification has key regulatory functions in many species. DNA is methylated by *de novo* and maintenance DNA methyltransferases, and often leads to gene silencing (Law & Jacobsen, 2010; Smith & Meissner, 2013). RNA directed DNA methylation (RdDM) was first discovered in tobacco (Wassenegger *et al*, 1994; Pelissier *et al*, 1999), though the mechanism has been elucidated in *A. thaliana* and mouse (Zhang & Zhu, 2011; Sabin *et al*, 2013). In *A. thaliana,* transposon-rich repetitive regions near centromeres are transcribed by a specialized polymerase, RNA Pol IV. The long resultant transcripts are made double stranded by the RdRP RDR2, diced into siRNA by DCL3, and used by Argonaute AGO4 to target the transposons and repeats. AGO4 in turn recruits DRM2, a *de novo* DNA methyltransferase, leading to transcriptional silencing (Mette *et al*, 2000; Aufsatz *et al*, 2002; Onodera *et al*, 2005; Castel & Martienssen, 2013; Sabin *et al*, 2013). In mice, the piRNA guided system targets transposable elements for DNA methylation and TGS in male germ cells (Carmell *et al*, 2007; Kuramochi-Miyagawa *et al*, 2008).

1.5 TOPOISOMERASES

1.5.1 Introduction

The nature of the DNA double helix is such that, while allowing for duplication and transmission of the genetic material, local changes in the number of twists in the DNA will result in topological changes such as supercoiling. This happens whenever the DNA is unwound or the strands are separated by a tracking protein like RNAPII. The supercoils will remain in a chromosome until relieved, as even linear eukaryotic chromosomes are too encumbered by chromatin, DNA-binding proteins, and interactions with the nuclear membrane and other chromosomes to twist freely. The polymerase is also often restricted from rotating around the DNA as it progresses, for example due to drag from the nascent mRNA and associated pre-mRNA processing complexes. In the case of a progressive polymerase, positive supercoils will accumulate ahead of the polymerase while negative supercoils will form behind, in what are known as Twin Supercoiled Domains (Liu & Wang, 1987; Wu *et al*, 1988). Local supercoil accumulation has functional consequences for chromatin composition and gene expression. Because the formation of a nucleosome absorbs one negative supercoil (Worcel *et al*, 1981), negatively supercoiled chromatin favors nucleosome formation while positively supercoiled chromatin disfavors nucleosome formation. The accumulation of supercoils can also impede the progress of RNA or DNA polymerases (Kim & Wang, 1989; Gartenberg & Wang, 1992; Hiasa & Marians, 1994; Postow *et al*, 2001; Peter *et al*, 2004; Durand-Dubief *et al*, 2010). DNA molecules can become tangled and interlocked (catenanes) during processes such as replication, repair, and meiotic recombination. If this is not relieved before chromosome condensation and cell division, genome instability and cell death will follow (Vos *et al*, 2011).

1.5.2 Classification

The solution to all of these topological problems, from supercoiling to catenation, lies in the activity of topoisomerases. Topoisomerases are essential to all DNA-based life, and are even common in DNA viruses (Forterre *et al*, 2007). Topoisomerases are generally divided into four groups, Types IA, IB, IIA, and IIB (Forterre *et al*, 2007; Vos *et al*, 2011). Although all topoisomerases sever one or both DNA strands by nucleophilic attack, the topoisomerase subfamilies do not appear to have a common origin. It has been hypothesized that they developed independently at the transition from RNA- to DNA-based genomes, in the common ancestor of bacteria, archaea and eukaryotes (Forterre *et al*, 2007). Type I topoisomerases sever a single strand of DNA and, with one exception (Kikuchi & Asai, 1984; Perugino *et al*, 2009), do not utilize ATP. These topoisomerases relax superhelical tension and resolve single stranded catenanes. Type I is further subdivided into Type IA enzymes capable of relaxing only negative supercoils and Type IB enzymes, which can relax both positive and negative supercoils. Type IA topoisomerases actively pass the intact strand through the severed one (Brown & Cozzarelli, 1981), while Type IB topoisomerases allow the severed strand to rotate freely until tension is released (Champoux & Dulbecco, 1972; Koster *et al*, 2005). The Type IA topoisomerase TopIII is also important for resolving single strand catenanes, such as are formed during homologous recombination (Wallis *et al*, 1989; Hiasa & Marians, 1994).

Type II topoisomerases, in contrast, sever both strands of their target DNA molecule (Mizuuchi *et al*, 1980; Collins *et al*, 2009). This ability is essential for untangling the interlocked knots and catenanes formed during DNA replication and recombination. Type II topoisomerases, which function as ATP-dependent multimers, are also capable of relaxing positive and negative supercoils (Wigley *et al*, 1991; Berger *et al*, 1996; McClendon *et al*, 2005). Type IIA topoisomerases are ubiquitous and essential throughout the tree of life. In contrast, Type IIB topoisomerases have so far only been found in archaea, which lack Type IIA, in plants, and in some bacteria and algae (Bergerat *et al*, 1994; Malik *et al*, 2007). The plant TopVI is important for endoreduplication (Sugimoto-Shirasu *et al*, 2005), the process by which polyploid tissues are formed. Two less common topoisomerases are actually able to induce supercoils. The bacterial Type IIA topoisomerase DNA gyrase induces negative supercoils (Gellert *et al*, 1976), while thermophilic archaea and some bacteria have a Type IA topoisomerase capable of increasing positive supercoiling, reverse gyrase (Kikuchi & Asai, 1984; Forterre *et al*, 1985). Although there is considerable redundancy between topoisomerases, there is also evidence of specialization. For example, Type IB enzymes can detect and preferentially bind supercoiled substrates (Patel *et al*, 2010). Some Type IB enzymes show a preference for binding and relieving positive supercoils (Frohlich *et al*, 2007), while some Type IIA topoisomerases recognize and preferentially resolve DNA crossovers and positively supercoiled DNA (Dong & Berger, 2007; Baxter *et al*, 2011).

Table 2. Topoisomerase classification, cellular function, and distribution. (Forterre *et al*, 2007)

Topoisomerase Type	Role	Selected Members
IA	1. ATP-independent 2. Cut one DNA strand 3. relax negative supercoils 4. single strand decatenation	Eukaryotic TopIII (α and β) Bacterial TopoI and TopoIII Archaeal TopoI Archaeal/Bacterial Reverse Gyrase
IB	1. ATP-independent 2. Cut one DNA strand 3. relax supercoils ('swivelase')	Eukaryotic TopoI Mitochondrial TopoI Bacterial TopoIB Poxvirus TopoIB
IIA	1. ATP-dependent 2. Cut both DNA strands 3. Relax supercoils 4. resolve knots 5. decatenation	Eukaryotic TopoII (α and β) Bacterial gyrase Bacterial TopoIV Bacteriophage T4 TopoIIA Archaeal gyrase
IIB	1. ATP-dependent 2. Cut both DNA strands 3. Relax supercoils 4. resolve knots 5. decatenation	plant TopoVI archaeal TopoVI

1.5.3 Function

The cellular roles of topoisomerases are diverse and are discussed in many reviews (Forterre *et al*, 2007; Vos *et al*, 2011). As this thesis is mainly concerned with the role of topoisomerases in eukaryotic transcription, only a brief overview of topoisomerase function in DNA replication, condensation, recombination, and repair is presented here. Topoisomerases are needed for the initiation of DNA replication, with TopoIB and TopoIIα binding replication origins and assisting with their firing. During DNA polymerase elongation, the polymerase must either rotate around the DNA axis, resulting in interlocked sister chromatids, or it must push DNA twist ahead of it, resulting in the buildup of positive supercoils. In either of these scenarios, topoisomerase activity is required to allow for progressive replication. Both type I and II topoisomerases relieve the positive supercoiling (Kim & Wang, 1989; McClendon *et al*, 2005; Bermejo *et al*, 2007), and without them replication of longer chromosomes is delayed (Kegel *et al*, 2011). Top2 (TopoII), with its ability to pass one DNA strand through another, is crucial to disentangling the sister chromatids (Adams *et al*, 1992; Baxter & Diffley, 2008). Sister chromatids that are left interlinked will tear during mitosis. The eukaryotic host-encoded Type IB mitochondrial topoisomerase is essential for replication of the mitochondrial genome (Zhang & Pommier, 2008; Dalla Rosa *et al*, 2009). TopoIII also plays a role, with its ability to resolve single stranded catenanes possibly unlinking the hemicatenanes that form when two replication forks meet (Nurse *et al*, 2003). Finally, at least budding yeast TopoII (TOP2) is involved in replication fork termination at genomic pausing elements (Fachinetti *et al*, 2010).

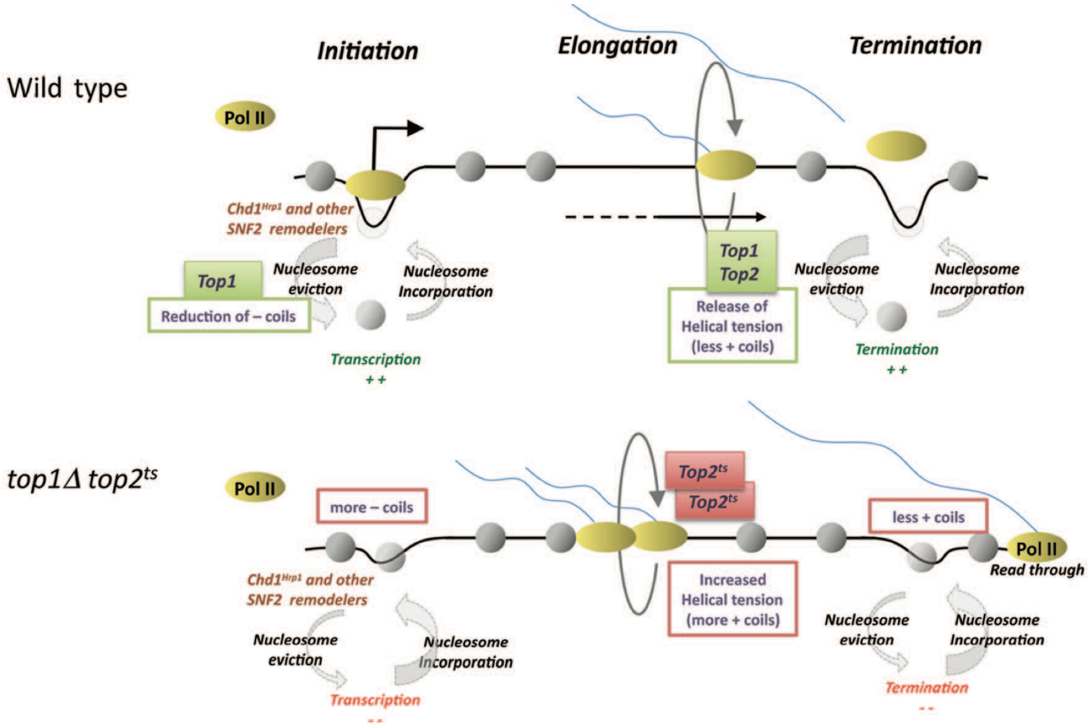

Figure 5. Model of DNA topology and topoisomerases in transcription. Top: In WT fission yeast cells, topoisomerases 5' of the gene relieve the negative supercoiling generated upstream of the elongating RNAPII. Snf2 family remodelers are then able to keep the promoter NDR clear, resulting in normal transcription levels. At the 3' end of the gene body, Type IIA topoisomerase Top2 relieves positive supercoiling, allowing the RNAPII to progress to the end of the gene. This results in positive supercoiling at the TTS, destabilizing nucleosomes and contributing to the maintenance of an NDR there. **Bottom**: In the absence of topoisomerase activity, negative supercoils accumulate at the TSS. This shifts the chromatin balance toward nucleosome assembly, and highly transcribed genes are downregulated. Positive supercoils accumulate ahead of elongating RNAPII molecules, reducing their ability to reach the end of genes, particularly long genes. Because fewer RNAPII reach the TTS, there are fewer positive supercoils to support nucleosome depletion. With the TTS NDR compromised, transcription termination is affected and readthrough transcription increases. *Reprinted with permission from Durand-Dubief* et al *(2011)*

In addition to decatenating sister chromatids (Gasser *et al*, 1986; Rose *et al*, 1990; Charron & Hancock, 1991), topoisomerases contribute to cell division at the level of chromatin condensation and centromere structure. There is evidence that TopoII promotes mitotic chromatin condensation by clamping strands together (Hizume *et al*, 2007), while the fission yeast Top3 maintains proper topology at centromeres. In the absence of Top3, levels of centromeric H3 variant CENP-A rise, possibly contributing to mitotic defects (Norman-Axelsson *et al*, 2013). Topoisomerases, particularly TopoIII, are also involved in resolving the double Holliday junction tangles that result from homologous recombination. As decatenation by TopoIII avoids the exchange of longer stretches of DNA between homologous chromosomes, this may be the preferred method during DNA repair in mitotic cells, rather than dominating meiotic homologous recombination (Wu & Hickson, 2003; Chang *et al*, 2005; Plank *et al*, 2006).

Transcription, like DNA replication, involves a progressive polymerase passing between the DNA strands, pushing positive supercoils ahead and leaving negative supercoils behind (Liu & Wang, 1987; Wu *et al*, 1988). There are functional consequences of these 'Twin Supercoiled Domains' at both the DNA and chromatin levels (Figure 5; Vos *et al*, 2011). First, if the positive supercoils ahead of the elongating RNA polymerase are not relieved, the polymerase may fail to reach the end of the gene (Gartenberg & Wang, 1992; Merino *et al*, 1993; Mondal *et al*, 2003; Durand-Dubief *et al*, 2010). This effect is stronger on long genes, comparable to the increased difficulty of replicating longer chromosomes in the absence of TopoI (Kegel *et al*, 2011). Both TopoI and TopoII are involved in relieving positive supercoils during transcriptional elongation, although TopoII may have a special role at long genes (Durand-Dubief *et al*, 2010). Underwound DNA behind the polymerase can hybridize more easily with RNA molecules (Drolet *et al*, 1994), which can lead to DNA damage (Tuduri *et al*, 2009). Supercoiling can also affect nucleosome stability, which is enhanced by negative supercoils and reduced by positive supercoils (Worcel *et al*, 1981; Clark & Felsenfeld, 1991; Negri *et al*, 1994; Hizume *et al*, 2004). ATP-dependent chromatin remodelers regulate gene body and gene-proximal nucleosome occupancy in sometimes redundant concert (Petty & Pillus, 2013). However, there is considerable evidence that DNA topology can interfere with the kinetics of the remodeling reaction (Almouzni & Mechali, 1988). When topoisomerase activity, particularly that of TopoI, is removed or reduced, negative supercoils accumulate upstream of the TSS. Excess negative supercoiling in a region that is normally depleted of nucleosomes can reduce the ability of chromatin remodelers to remove nucleosomes, which in turn reduces the transcription of highly transcribed genes (Durand-Dubief *et al*, 2010). Interestingly, some transcriptionally generated supercoiling may have a normal physiological role. The NDR at the TTS of many genes seems to depend on positive supercoils. When, under conditions of reduced topoisomerase activity, the RNAPII reaches the end of the gene less frequently, the TTS NDR becomes less defined and polymerases that do reach the gene end are more prone to readthrough transcription (Durand-Dubief *et al*, 2011). The diversity of topoisomerases in multicellular eukaryotes may contribute to the developmental program by fine-tuning gene activation and repression (Thakurela *et al*, 2013).

Because Topoisomerases are so essential to dividing cells, they are attractive therapeutic targets in the fights against cancer and bacterial infection. Poisoned topoisomerases, unable to religate the strands they have cleaved, induce DNA damage while failing to resolve supercoils and catenanes. Inhibiting topoisomerase activity will also inhibit cell replication. Several of the most effective anti-cancer therapies, including camptothecin and etoposide, target topoisomerase activity (Vos *et al*, 2011; Kathiravan *et al*, 2013).

1.6 TRANSCRIPTION

1.6.1 General mechanisms

Transcription is the process of producing an RNA molecule from a DNA template, and is highly conserved from yeast to humans. RNA molecules play a wide variety of roles, from influencing chromatin structure to regulating other RNA molecules to composing

much of the protein-constructing ribosome. Perhaps the best understood RNA function, however, is that of messenger RNA (mRNA). mRNA molecules are transcripts of protein coding genes, and after their production by RNA polymerase II (RNAPII) they exit the nucleus and are translated into protein by the ribosomal machinery. Only a fraction of possible gene products are needed at a given time in a given cell, and so the process of transcription is tightly regulated (Zhou *et al*, 2012; Liu *et al*, 2013; Luse, 2013; Mischo & Proudfoot, 2013).

In general, transcription of protein coding genes (Thomas & Chiang, 2006; Hahn & Young, 2011) is initiated when activator proteins bind upstream of the promoter. The transcriptional machinery and many transcription factors are unable to bind nucleosomal DNA, so early 'pioneer' factors that can bind nucleosomal DNA are essential to begin the activation of a gene. These activators then recruit the mediator complex and factors that modify histones and remodel nucleosomes, preparing the promoter DNA for the general transcription machinery. The general transcription machinery consists of RNAPII itself and several transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) (Liu *et al*, 2013). Once these have assembled into the Pre-Initiation Complex (PIC), Rad25 (in humans, the XBP of TFIIH) unwinds the transcription start site so that the active site of RNAPII can bind a single stranded template. At the same time, Ser5 of the C-terminal domain (CTD) of Rpb1, the largest subunit of RNAPII, is phosphorylated. This allows for some early elongation, but subsequent phosphorylation of Ser2 leads to the recruitment of several additional factors and efficient elongation (Luse, 2013). These factors aid in elongation, termination, and mRNA processing, and include histone modifiers and nucleosome remodelers (Zhou *et al*, 2012; Mischo & Proudfoot, 2013). Newly formed RNA molecules are generally stabilized by either polyadenylation or binding to specific proteins. This stabilization is linked to RNAPII termination (Millevoi & Vagner, 2010; Kuehner *et al*, 2011).

1.6.2 Interaction with chromatin

As is touched upon in the previous section, chromatinization of the DNA template complicates every stage of transcription (Figure 6; Clapier & Cairns, 2009; Smolle & Workman, 2013). Assembling the components of the PIC and granting them access to the DNA are prerequisites for transcription initiation. This process involves nucleosome remodeling and histone modification by a wide range of enzymes. KAT coactivator complexes like SAGA and NuA3 acetylate promoter-region histones (Grant *et al*, 1999), stimulating the recruitment of other proteins by their bromodomains. The Snf2 ATPases at the heart of the SWI/SNF and RSC remodeling complexes both have bromodomains, and remodel promoter-region nucleosomes to both repress and promote transcription in different contexts (Whitehouse *et al*, 1999; Bruno *et al*, 2003; Vicent *et al*, 2004; Monahan *et al*, 2008; Parnell *et al*, 2008). However, inactivation of Snf21, the catalytic subunit of the RSC complex, did not affect NDR depth in fission yeast (Pointner *et al*, 2012). It is unclear whether this is due to redundancy with other remodelers or insufficiently harsh inactivation.

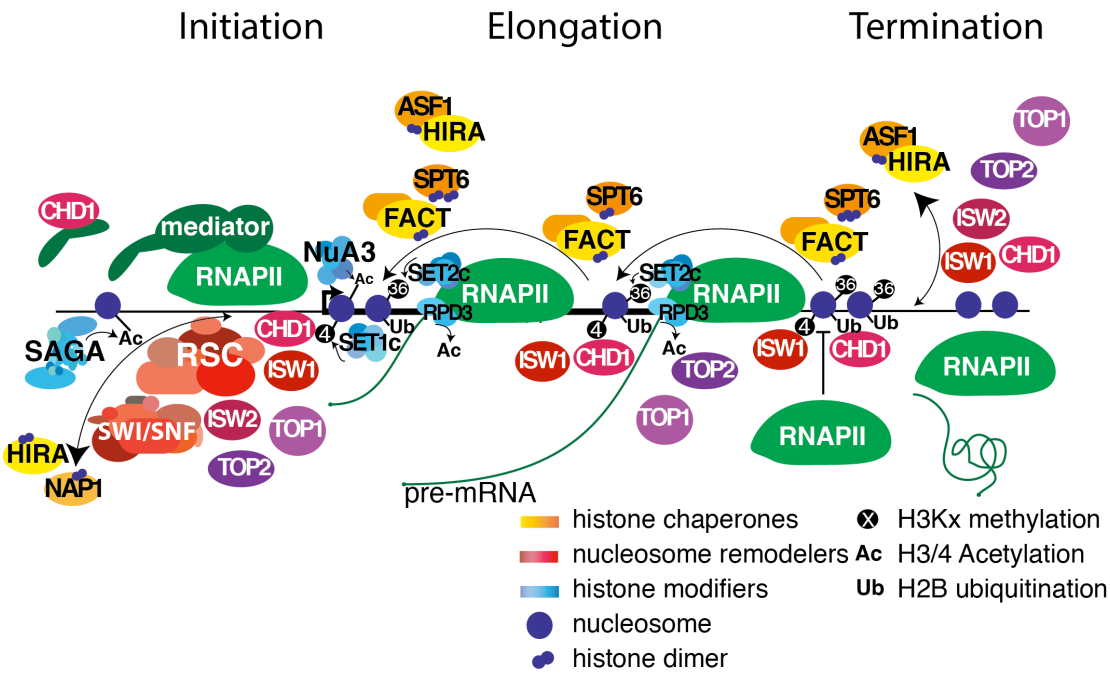

Figure 6. Transcription in the Context of Chromatin. A non-comprehensive illustration of some of the chromatin-related factors involved in transcription.

Trimethylation of H3K4 by Set1 is also characteristic of active promoters, while di- and monomethylation are found over gene bodies. H3K4me, which is dependent on a complex cascade of prior events including H2B ubiquitination, in turn recruits other chromatin modifiers (Smolle & Workman, 2013). Human Chd1 and NURF (an Iswi subfamily remodeling complex), as well as the budding yeast SAGA and NuA3 KATs, are recruited to promoters by H3K4me3 (Flanagan *et al*, 2005; Sims *et al*, 2005; Li *et al*, 2006; Taverna *et al*, 2006; Vermeulen *et al*, 2010). Chd1 is also recruited to promoters by interactions with Mediator (Khorosjutina *et al*, 2010; Lin *et al*, 2011). Chd1 is known to be important for gene activation and maintenance of open chromatin (Gaspar-Maia *et al*, 2009; Morettini *et al*, 2011), moving the +1 nucleosome away from budding yeast promoters in concert with ISW1b (Yen *et al*, 2012). Conversely, budding yeast ISW1a and ISW2 move the +1 nucleosome into the promoter and are associated with gene repression (Whitehouse & Tsukiyama, 2006; Whitehouse *et al*, 2007; Yen *et al*, 2012). Histone chaperones like Nap1 are also involved in regulating promoter-region chromatin (Walfridsson *et al*, 2007).

In contrast to promoter regions, where nucleosomes are depleted to allow the assembly of the transcription machinery, gene body nucleosomes are dense and regularly spaced. Here, the need to accommodate passage of the elongating RNAPII must be balanced with the need to prevent cryptic mid-gene transcription initiation. Either irregularly spaced nucleosomes or the loss of appropriate histone modifications appears to allow cryptic transcription initiation (Hennig *et al*, 2012; Pointner *et al*, 2012; Shim *et al*, 2012; Smolle *et al*, 2012; Smolle & Workman, 2013). Although RNAPII cannot transcribe through a full histone octamer, it is able to pass through hexamers *in vitro* (Kireeva *et al*, 2002; Belotserkovskaya *et al*, 2003). Dismantling the entire octamer appears only to happen at highly transcribed genes (Thiriet & Hayes, 2005; Dion *et al*,

2007), while at more weakly transcribed genes H2A-H2B dimer removal is sufficient. Removal of histone dimers or entire octamers ahead of the elongating polymerase, and their replacement behind, is mediated by histone chaperones like FACT, Asf1, Nap1, and Spt6 (Belotserkovskaya *et al*, 2003; Robinson & Schultz, 2003; Adkins & Tyler, 2004; Schwabish & Struhl, 2006; Walfridsson *et al*, 2007). Chromatin remodelers of the Iswi and Chd1 subfamilies then remodel the nucleosomes so that they are regularly spaced and linked to the +1 nucleosome (Gkikopoulos *et al*, 2011; Hennig *et al*, 2012; Pointner *et al*, 2012; Shim *et al*, 2012). The combined activity of chaperones and remodelers is important to ensure that histones are recycled rather than exchanged for new ones, as this helps maintain histone modification patterns (Hennig *et al*, 2012; Shim *et al*, 2012; Smolle *et al*, 2012). Set2 interacts directly with the elongating polymerase (Krogan *et al*, 2003; Li *et al*, 2003; Kizer *et al*, 2005) and methylates H3K36 in the gene body. H3K36me is needed for the deacetylation activity of the budding yeast RPD3S (fission yeast Clr6c) (Carrozza *et al*, 2005; Li *et al*, 2007; Venkatesh *et al*, 2012), without which chromatin becomes hyperacetylated and permissive of transcription initiation (Carrozza *et al*, 2005; Li *et al*, 2007). Budding yeast ISW1b and CHD1 also have an affinity for H3K36me (Smolle *et al*, 2012). There is some evidence that Isw1 and Chd1 have differing affinities or importance for genes of different length or transcriptional strength, though there is considerable overlap in their functions (Radman-Livaja *et al*, 2012; Shim *et al*, 2012; Smolle *et al*, 2012). Finally, gene body H2Bub stabilizes nucleosomes and is linked to high expression levels (Robzyk *et al*, 2000; Henry *et al*, 2003; Wood *et al*, 2005; Pavri *et al*, 2006; Minsky *et al*, 2008; Kim *et al*, 2009). Loss of Chd1 decreases the level of H2Bub (Lee *et al*, 2012), and loss of either Chd1 or H2Bub results in lower gene body nucleosome occupancy (Batta *et al*, 2011; Lee *et al*, 2012).

Isw1, Isw2, and sometimes Chd1, remodel nucleosomes to ensure correct transcription termination in budding and sometimes in fission yeast (Alen *et al*, 2002; Yen *et al*, 2012). Finally, this complex balance of nucleosome occupancy, modification, and recycling is supported at all times by the activity of topoisomerases, which relieve the supercoiling generated by transcription and nucleosome remodeling (see section 1.5). The numerous redundancies and highly interconnected crosstalk of transcriptional regulation by chromatin indicates the importance of this level of control.

1.7 TRANSPOSONS

1.7.1 Introduction

Genomes are made up of more than an organism's own coding genes. In addition to structural and regulatory elements, parasitic DNA elements can make up a significant fraction of a cell's genetic material (Kidwell & Lisch, 2000). These elements, transposons, use a wide variety of techniques to replicate and spread within the genomes of their hosts, driving the hosts to develop equally varied regulatory arsenals to keep them in check (Wong & Choo, 2004; Werren, 2011). This struggle, as well as successful transposition events and recombination between homologous transposon sequences in nonhomologous loci, are powerful drivers of evolution (Kidwell & Lisch, 2000; Houle & Nuzhdin, 2004; Hedges & Batzer, 2005; Wang & Kirkness, 2005; Gentles *et al*, 2007; Cordaux & Batzer, 2009; Hollister & Gaut, 2009). Transposons

can be divided into two major types: retrotransposons and DNA transposons (Wicker *et al*, 2007). DNA transposons spread by being cut out of the genome and pasted into a new location by their own transposase or that of another transposon. Retrotransposons, in contrast, spread via an RNA intermediate. They either encode a reverse transcriptase and endonuclease or, in the case of nonautonomous retrotransposons like the human SINE elements, utilize the enzymes of autonomous retrotransposons. LTR retrotransposons, which are flanked by Long Terminal Repeats (LTRs), are believed to be the ancestors of retroviruses (Malik *et al*, 2000). In addition to reverse transcriptase and endonuclease, these retrotransposons encode a capsid protein and form virus-like-particles as a step in their transposition process. LTR retrotransposons appear to be present in all eukaryotic genomes (Huang *et al*, 2012).

Table 3. Transposable element content, type and activity in six fungal, plant and animal species. (Goffeau *et al*, 1996; The Arabidopsis Genome Initiative, 2000; Lander *et al*, 2001; Venter *et al*, 2001; Waterston *et al*, 2002; Wood *et al*, 2002; Schnable *et al*, 2009; Beck *et al*, 2010)

Species	DNA transposons	LTR retrotransposons	Non-LTR retrotransposons	Total
Saccharomyces cerevisiae **(budding yeast)**	0	3% active	0	3%
Schizosaccharomyces pombe **(fission yeast)**	0	0.35% active	0	0.35%
Arabidopsis thaliana **(thale cress)**	6.7% active	3.1% active	1.2% quiescent	11%
Zea mays **(maize)**	8.6% active	74.6% active	1%	84%
Mus musculus **(mouse)**	0.9% (defective copies)	9.9% active	27.4% active	38%
Homo sapiens **(human)**	3% (defective copies)	8.6% Recently active	34.6% active	46%

1.7.2 Host-transposon interaction

The potential disadvantages to the host of allowing free transposition are obvious. Transposons can insert into exons, affecting protein activity or introducing a premature stop codon, as exemplified by a human hemophilia allele (Sukarova *et al*, 2001). Transposition can also affect splicing or disrupt promoters and enhancers, altering transcript composition and quantity (Rebollo *et al*, 2011; Huang *et al*, 2012), while rearrangements during transposition or meiotic recombination can dramatically alter the genome (McClintock, 1951). The results of somatic cell transposition events can have results as harmless as kernel color variation in maize (McClintock, 1951; Wessler, 1988) or as detrimental as cancer (Miki *et al*, 1992; Iskow *et al*, 2010). Finally, the genomes of many species contain a high fraction of transposon sequence (Table 3). The simple cost of replicating so much excess DNA is nontrivial. This being the case, it is unsurprising that host species have evolved layers of overlapping, even redundant,

strategies to regulate their transposons. These strategies frequently differ between somatic and germline cells, and even at different stages of development.

DNA methylation and targeting by small ncRNAs are two of the most common mechanisms of transposon silencing. Regulatory methylation of cytosine residues in DNA to form 5-methyl-cytosine is common in flowering plants, vertebrates and some fungi. DNA methylation often silences transcription, and transposable elements are major, in some species the primary, targets of this silencing (Yoder *et al*, 1997; Selker *et al*, 2003). DNA methylation has been known for decades to silence transposable elements in maize (Chandler & Walbot, 1986; Fedoroff *et al*, 1995). Transposon promoters are hypermethylated in normal human cells (Thayer *et al*, 1993; Lees-Murdock *et al*, 2003), and in the fungus *Neorospora crassa* DNA methylation is involved in TE silencing in both somatic and germ cells. In many species, loss of DNA methylation allows TE transcription and, in mice, is associated with derepression of TEs and meiotic failure in male germ cells (Bourc'his & Bestor, 2004). The genome-wide hypomethylation associated with many cancers appears to derepress transposable elements, allowing for *de novo* transposition in, e.g., non-small-cell lung tumors (Iskow *et al*, 2010) and breast carcinomas (Belancio *et al*, 2010). Although the mechanisms by which mammalian TEs are targeted for DNA methylation are poorly understood, targeting in flowering plants is better characterized (Law & Jacobsen, 2010). In both cases, targeting by noncoding RNAs plays an essential role (see section 1.4). Chromatin remodeling by the LSH/HELLS/DDM1 subfamily is also known to promote DNA methylation (Huang *et al*, 2004). Histone deacetylation, nuclear organization, and antisense transcription are among the multiple additional silencing mechanisms that target transposable elements (Durand-Dubief *et al*, 2007; Cam *et al*, 2008; Matsuda & Garfinkel, 2009).

Despite the dangers of allowing uncontrolled transposition, it has long been suspected that host cells could harness these processes to adapt to stressful situations (McClintock, 1984). Active transposition has, for example, been observed in response to DNA damage (McClintock, 1950), compromised telomeric stability (Morrish *et al*, 2007), and oxygen or nutrient starvation (Dai *et al*, 2007; Sehgal *et al*, 2007). Furthermore, in some cases this increase in transposition is demonstrably under host control (Dai *et al*, 2007), suggesting that host cells may leverage transposition for rapid genetic change and stress adaptation. Indeed, *S. pombe* LTR retrotransposons are upregulated by stress (Chen *et al*, 2003; Sehgal *et al*, 2007) and tend to integrate into the promoters of stress-response genes (Guo & Levin, 2010). Similarly, heavy metals stimulate human LINE-1 retrotransposition (Kale *et al*, 2006). In addition to being a stress response, somatic transposition in the brain is suggested to enhance neural plasticity (Singer *et al*, 2010; Baillie *et al*, 2011). Transposable elements can also be coopted by the host genome for regulatory purposes. Melon species use the chromatin silencing associated with a transposon insertion for sex determination (Martin *et al*, 2009). In other species, the transposon-derived CENP-B proteins have become essential regulators of genome stability (Casola *et al*, 2008) and even cluster transposon sequences for regulatory purposes (Cam *et al*, 2008; Lorenz *et al*, 2012).

Transposable elements form essential chromosomal structural components in several species. Fruit fly telomeres are composed of TART, HeT-A, and TAHRE non-LTR

retrotransposons, which integrate near chromosome ends when they replicate (Biessmann *et al*, 1992; Levis *et al*, 1993; George *et al*, 2010). This process, also seen in the silk worm *Bombyx mori,* appears to function in place of telomerase in maintaining chromosome ends (Fujiwara *et al*, 2005). The *Penelope* clade of non-LTR retrotransposons, which includes the human LINE elements, are also present at telomeres (Morrish *et al*, 2002; 2007). Finally, the centromeres of many species are also derived from transposable elements (Wong & Choo, 2004).

1.7.3 The retrotransposons of budding yeast

Budding yeast has five families of LTR retrotransposons, *Ty1-5* (Goffeau *et al*, 1996). Several of these families have mechanisms of insertion that avoid major detriment to the host cell. Both *Ty1* and *Ty3* integrate upstream of PolIII transcribed sequences (Devine & Boeke, 1996; Sandmeyer, 2003). In contrast, *Ty5* integrates into heterochromatin under normal circumstances (Zou *et al*, 1996; Zhu *et al*, 2003). The *Ty5* integrase (IN) protein bears a Targeting Domain that tethers the integrase to Sir4p (Gai & Voytas, 1998; Xie *et al*, 2001), an element of budding yeast heterochromatin. The interaction with Sir4p is dependent on phosphorylation of the IN targeting domain, however, and under stress conditions this phosphorylation is reduced, transforming *Ty5* into a powerful mutagen (Dai *et al*, 2007). Budding yeast lacks both DNA methylation and RNAi, both of which are involved in controlling TEs in other species. However, antisense transcripts from Ty1 elements are capable of post-translationally reducing the quantity of Ty1-derived reverse transcriptase and integrase (Matsuda & Garfinkel, 2009).

1.7.4 The retrotransposons of fission yeast

The relatively compact fission yeast genome is only 0.35% composed of intact transposable elements (Wood *et al*, 2002). Two families of transposons, both LTR retrotransposons belonging to the *gypsy* family, have been identified in the fission yeast genome (Levin *et al*, 1990). Called *Tf1* and *Tf2* (transposon of fission yeast 1 and 2), *Tf2* is the only family present in the sequenced *972* strain (Wood *et al*, 2002). The *tf* retrotransposons of fission yeast were the first to be identified as being able to self-prime the reverse transcription step of their life cycle (Figure 7; Levin, 1995). Both *Tf1* and *Tf2* are known to target the promoters of PolII transcribed genes (Guo & Levin, 2010), and *Tf1* targeting involves the transcription factor Atf1 (Activating transcription factor 1) (Majumdar *et al*, 2011). Next generation sequencing used to track thousands of new integration events has confirmed that *Tf1* integrates upstream of ORFs, particularly those of stress-induced genes (Guo & Levin, 2010). Furthermore, *Tf1* elements are able to stimulate the transcription of adjacent genes (Leem *et al*, 2008) and environmental stress causes upregulation of *Tf2* elements (Chen *et al*, 2003; Sehgal *et al*, 2007). Taken together, it seems likely that fission yeast, like budding yeast, activates transposition to improve fitness in the face of stress (Levin & Moran, 2011).

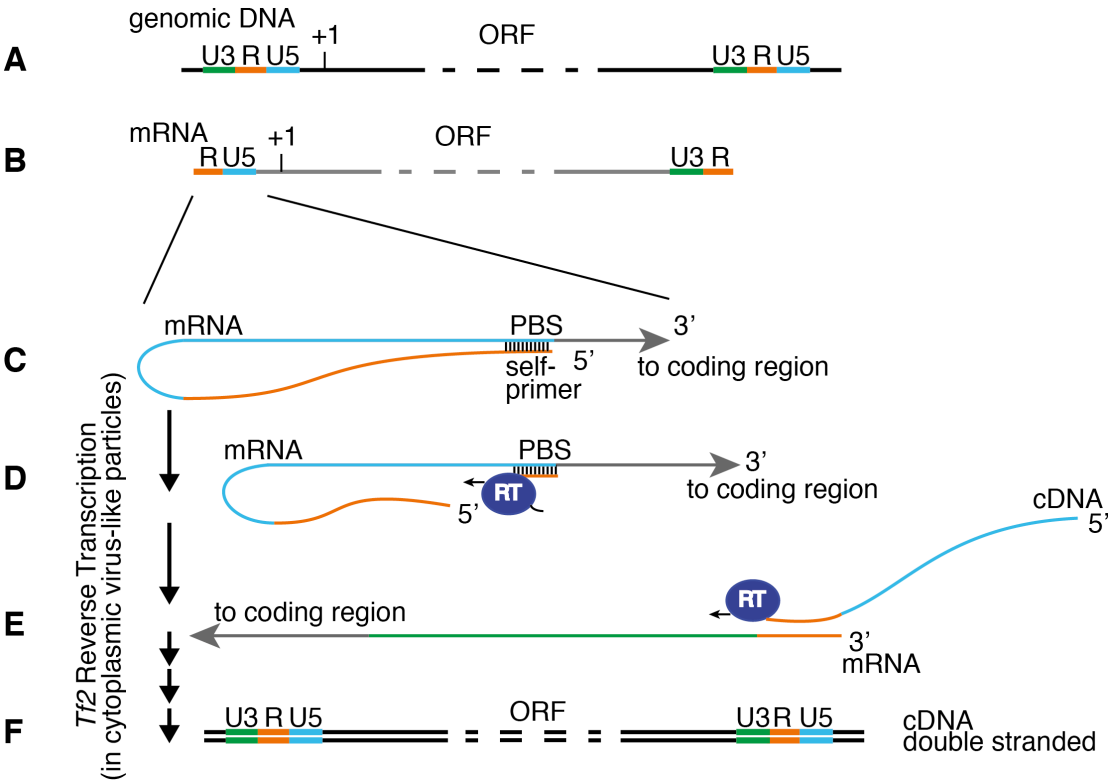

Figure 7. The lifecycle of fission yeast *tf* retrotransposons. A) Genomic DNA: *Tf2* retrotransposons are flanked by LTRs, which are composed of U3, R, and U5 sequences. B) mRNA is transcribed from within the LTR. C) A self-primer in the R region hybridizes to the Primer Binding Site (PBS) in the U5. D) The *tf2*-encoding reverse transcriptase (RT) cleaves the self-primer from the rest of the mRNA and generates cDNA complementary to the U5 and R sequences while digesting the mRNA template. E) The RT and short cDNA transcript are able to hybridize at the 3' end of the mRNA, continuing reverse transcription and eventually producing a double stranded cDNA capable of reintegration into the genome.

Fission yeast, like other species, employ a variety of overlapping and partially redundant mechanisms to control *Tf2* transcription. *Tf2* transcripts are generally targeted for degradation by the exosome. When the exosome is inactivated, however, the RNAi machinery causes heterochromatinization of the retrotransposon genes (Yamanaka *et al*, 2013). Furthermore, the fission yeast CENP-B proteins Abp1, Cbh1 and Cbh2 have been shown to bind and repress *Tf2* elements. Abp1 in particular is a crucial recruiter of HDACs Clr3 and Clr6, and via its dimerization domain is also important for clustering the retrotransposons into 'Tf2 bodies' (Cam *et al*, 2008). Other histone modifying enzymes, including the HDACs Hst2 and Hst4 and the H3K4 methyltransferase Set1 have also been implicated in maintaining *Tf2* clustering and transcriptional repression (Durand-Dubief *et al*, 2007; Lorenz *et al*, 2012). Deletion of the Abp1 dimerization domain does not cause significant transcriptional upregulation (Cam *et al*, 2008), however, and loss of chromatin modifiers can cause declustering (Lorenz *et al*, 2012), highlighting the importance of chromatin context.

2 METHODS

2.1 FISSION YEAST

Schizosaccharomyces pombe, the fission yeast species most commonly used as a model organism, is named after the African millet beer from which it was first isolated (Egel, 2004). *S. pombe*, called fission yeast in this thesis and in the scientific literature, has a 12.57 Mbp sequenced genome divided between 3 chromosomes and containing 5123 protein coding genes (Wood *et al*, 2002). The fission yeast lineage diverged from that of *Saccharomyces cerevisiae*, the more familiar budding (brewer/baker's) yeast, approximately 1,140 million years ago (Hedges, 2002). Thus, although both *S. pombe* and *S. cerevisiae* are unicellular fungi capable of converting sugar to alcohol (Lin & Li, 2011), they also differ in many ways. Like budding yeast, fission yeast has an easily manipulable genome and short (~2.5 hours) generation time. However, fission yeast has large centromeres surrounded by pericentromeric heterochromatin, similar to mammals and totally unlike the single nucleotide point centromere of budding yeast (Fishel *et al*, 1988; Clarke, 1990). Fission yeast also has 'typical' heterochromatin and the full RNAi apparatus (Aravind *et al*, 2000; Volpe *et al*, 2002), which budding yeast lacks. Finally, the fission yeast cell cycle differs from that of budding yeast or most mammalian cell types in that roughly 70% of the cell cycle is spent in G2 (Figure 8; Egel, 2004).

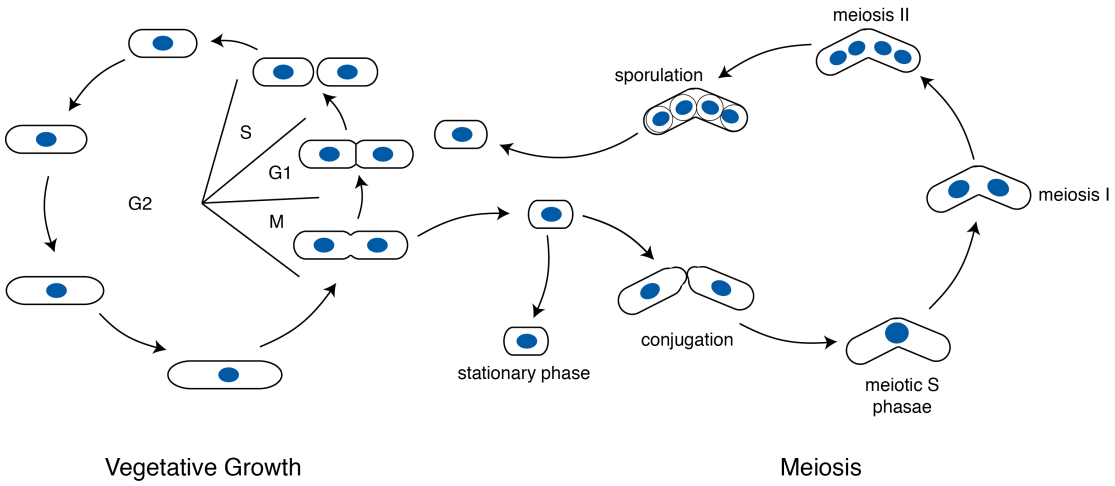

Figure 8. The fission yeast cell cycle.

2.2 CHROMATIN IMMUNOPRECIPITATION

Chromatin Immunoprecipitation (ChIP), is a method for determining where in the genome a protein of interest binds. A method for using ChIP in fission yeast is described in Durand-Dubief & Ekwall (2009). Briefly, cells are crosslinked by a gentle formaldehyde fixation (1% w/v final concentration). After the fixation has been quenched with glycine, cells are lysed with glass beads in the presence of protease inhibitors and chromatin is fragmented by sonication to approximately 400-500 bp fragments. This chromatin extract can then be incubated with an antibody against the

protein of interest and the antibody-chromatin complex captured by protein A-coated beads. After washing, elution from the beads, and protease digestion, the captured DNA can be quantified by PCR, microarray hybridization, or sequencing. Other ChIP methods can use different methods of fixation and chromatin fragmentation, e.g., enzymatic digestion.

It is important to compare ChIPed DNA with relevant controls. In the papers in this thesis, up to four types of control comparisons have been made. First, an antibody-free quality control (protein A beads only) is always run in parallel with ChIP samples and assayed by PCR. If ChIP signal is not sufficiently elevated over this background then the samples are discarded. Second, whenever possible we perform a 'no-antigen control' ChIP to determine the level of background and cross-reactivity of our antibody. Third, unprecipitated chromatin extract (input) is useful to control for differences in fragmentation and chromatin concentration. Finally, mutants are always compared to the relevant WT strain.

2.3 NUCLEOSOME POSITION MAPPING

DNA that is incorporated into nucleosomes is protected from cleavage. This allows the mapping of *in vivo* nucleosome positions by enzymatic or chemical digestion of chromatin by agents that will efficiently cleave linker DNA. One of the most common choices for nucleosome mapping is digestion by micrococcal nuclease (MNase), which is used in Paper III of this thesis. The method is described in Lantermann *et al* (2009). Briefly, cells are gently fixed with formaldehyde (0.5%), after which the cell wall is digested with zymolyase to produce spheroplasts. The chromatin is then partially digested with MNase, which can enter the spheroplasts. The purified DNA can be probed by southern blot, or mononucleosomes can be hybridized to microarrays or sequenced. The digestion product can also simply be visualized by agarose gel to screen for bulk nucleosome spacing alterations. In Paper III we use DNaseI-fragmented genomic DNA as a control for normalization.

2.4 MICROARRAYS

Microarrays are a densely spotted collection of probes, generally corresponding to genomic loci, to which fragmented DNA or cDNA can be hybridized. For example, an array can have one probe for each unique cDNA for a given organism. In tiling microarrays, short, slightly overlapping probes cover an entire region or even genome, allowing for high resolution mapping of RNA or, commonly, ChIPed DNA. The microarray Affymetrix GeneChip S. pombe Tiling 1.0FR Array was used in all papers in this thesis. This array covers the entire *S. pombe* genome with 25 bp probes. These probes overlap by 5 bp, providing an approximately 20 bp resolution. By labeling the ChIP or cDNA sample, it is possible to measure the hybridization signal strength for each probe. The raw data can then be normalized, visualized, and further analyzed using a combination of Tiling Analysis Software (TAS) and IGB from Affymetrix, R/Bioconductor, and Podbat (Sadeghi *et al*, 2011).

2.5 FLUORESCENCE *IN SITU* HYBRIDIZATION

Fluorescence *in situ* hybridization (FISH) allows the visualization of a DNA locus of interest in the nucleus. This method has been described in (Ekwall *et al*, 1996). Briefly, cells are fixed with paraformaldehyde solution. Chromosomal DNA is then denatured by incubations with saline sodium citrate (SSC) buffer and formamide and probed. The probe can be a large PCR product or cosmid, digested to an average length of approximately 200 bp and labeled with, in this thesis, Digoxigenin-11-dUTP. Here we used anti-digoxigenin-fluorescein Fab fragments to highlight the probe, and confocal microscopy to visualize the cells. FISH can be combined with standard immunofluorescence.

2.6 PAPER II

In Paper II of this thesis, microarray data from Paper I was normalized in TAS (Affymetrix) as described in Paper I and analyzed in Podbat (Sadeghi *et al*, 2011). Total RNA was prepared by hot acid phenol extraction. Briefly, logarithmically growing cells were harvested by centrifugation, immediately resuspended in TES buffer (10mM TrisHCl, pH 7.5, 10mM EDTA, 0.5% SDS), and vortexed with 65°C acid phenol for 45 minutes. RNA was then purified by phenol/chloroform extraction and precipitated. Reverse transcription was performed with gene and strand specific primers and SuperScript II Reverse Transcriptase (Invitrogen) before quantification of normal and readthrough transcript by qPCR.

3 RESULTS AND DISCUSSION

3.1 PAPER I: TOPOISOMERASE I REGULATES OPEN CHROMATIN AND CONTROLS GENE EXPRESSION *IN VIVO*

Processes that involve exposing or unwinding the DNA molecule, like transcription, DNA replication, repair, and recombination, generate torsional stress. DNA topoisomerases are essential to these processes, relieving torsional stress and supercoiling, as well as decatenating tangled strands of DNA (Almouzni & Mechali, 1988; Mondal *et al*, 2003). Unrelieved, torsional stress affects chromatin structure. The assembly of one nucleosome absorbs one negative supercoil, so while negative supercoils ease nucleosome formation, positive supercoils promote nucleosome disassembly (Clark & Felsenfeld, 1991; Negri *et al*, 1994; Hizume *et al*, 2004). Several studies have shown the importance of topoisomerase activity to transcription and chromatin structure both *in vitro* and *in vivo* (Stewart & Schutz, 1987; Schultz *et al*, 1992; Kretzschmar *et al*, 1993; Collins *et al*, 2001).

In papers I and II, we sought to understand the genomewide interplay between transcription, chromatin structure, and relief of torsional stress by topoisomerases. We studied the loss of topoisomerase function in a fission yeast double mutant with the Type IB topoisomerase *top1* gene deleted and with a temperature sensitive allele of the Type IIA topoisomerase *top2* gene (*top2-191*). Top1 and Top2 are both able to relieve both positive and negative supercoils and are able to substitute for one another (Uemura & Yanagida, 1984). Nevertheless, we found evidence of specialization *in vivo*.

Using ChIP-chip, we found that both Top1 and Top2 bind to intergenic regions (IGRs). However, only Top1 IGR binding strength showed a positive correlation with gene transcription level. Furthermore, we noted that intergenic histone H3 levels are elevated in *top1Δ top2-191*, which is associated with the downregulation of the most highly transcribed genes. Using the drug camptothecin, we were able to trap active Top1 and demonstrate that Top1 is active at the promoters of affected genes. We hypothesized that topoisomerases, particularly Top1, relieve promoter-region supercoiling to ease the kinetics of the nucleosome remodeling reactions that keep these regions nucleosome depleted. Without topoisomerase activity, H3 levels increase and transcription initiation is inhibited. There is a strong correlation between the biding patterns of Top1 and Top2 and that of the Chd1 subfamily chromatin remodeler Hrp1. This, in combination with the similar effects on IGR H3 levels in *top1Δ top2-191* and in *hrp1Δ*, led us to hypothesize that Hrp1 is one of the Snf2 chromatin remodelers to depend on topoisomerase activity for its full activity.

We also observe a special role for Top2 in RNAPII elongation over long genes. Top2, but not Top1, is enriched in the gene body of genes over 2000 bp long. This appears support the RNAPII in progressing to the end of the gene, as the level of RNAPII at the 3' end of long genes is depleted in *top1Δ top2-191* relative to in WT. We propose that, although both TypeIB and Type IIA topoisomerases are capable of relieving either positive or negative supercoils, they have preferential specialties *in vivo*. Although they

can substitute for each other, Top1 specializes in removing the negative supercoils generated upstream of RNAPII, particularly at highly transcribed genes. This supports open promoters and efficient transcription initiation. Top2, in contrast, specializes in relieving the positive supercoils that accumulate ahead of the elongating polymerase. This allows the polymerase to reach the end of even the longest genes. We also propose that Hrp1 is a major beneficiary of topoisomerase activity. Chd1, the mouse homolog of Hrp1, is required to maintain open chromatin and pluripotency in embryonic stem cells (Gaspar-Maia *et al*, 2009). Top1 is also required for early multicellular development. Our findings suggest a role for topoisomerases in supporting developmental transcriptional programs, perhaps in cooperation with Chd1.

3.2 PAPER II: TOPOISOMERASES, CHROMATIN AND TRANSCRIPTION TERMINATION

In this paper, we continued our study of the role of topoisomerases on nucleosome occupancy and transcription genomewide, using the newly available Podbat analysis tool (Sadeghi *et al*, 2011). Nucleosomes are known to be depleted at gene transcription termination sites in a manner that is dependent on transcription (Fan *et al*, 2010; Lantermann *et al*, 2010). In Paper I, we observed enriched topoisomerase occupancy at the 3' NDR of RNAPII transcribed genes. This enrichment was particularly strong 3' of highly transcribed genes. We wondered whether topoisomerases could also be involved in maintaining the 3' NDR.

We found that in the *top1Δ top2-191* mutant, nucleosome occupancy in the 3' NDR is increased. This effect was particularly strong for genes that end at least 1000 bp from the next known transcript. The loss of the 3' NDR has physiological consequences. Transcription termination is reduced, such that RNAPII occupancy and RNA production beyond the transcription termination site (TTS) are increased in *top1Δ top2-191*. This effect was particularly visible at convergent genes; we were able to measure a two-to-four-fold increase in readthrough transcription at two target pairs by PCR.

Altered chromatin structure at the TTS NDR are known to cause a termination defect in budding yeast. In that system, Iswi subfamily members regulate TTS nucleosome depletion, which promotes pausing of the polymerase and its separation from the mRNA (Alen *et al*, 2002; Ehrensberger & Kornberg, 2011). Hrp1 has been implicated in transcriptional termination of a reporter gene in fission yeast, although that role may not be widespread (Alen *et al*, 2002). We propose that at the TTS, the positive supercoils generated ahead of the elongating RNAPII support nucleosome eviction by Snf2 family chromatin remodelers. In the absence of topoisomerase activity, however, the fraction of elongating polymerases that reach the end of the gene is reduced. The result is that, while positive supercoiling ahead of the polymerase is increased without topoisomerase activity, the supercoiling accumulates in the gene body rather than at the TTS. This in turn shifts the nucleosome incorporation/eviction balance at the TTS NDR in the direction of incorporation, reducing the NDR depth and permitting readthrough transcription.

3.3 PAPER III: CHD1 REMODELERS REGULATE NUCLEOSOME SPACING *IN VITRO* AND ALIGN NUCLEOSOMAL ARRAYS OVER

GENE CODING REGIONS IN *S. POMBE*

Nucleosome positioning, which affects access to the underlying DNA molecule, has now been mapped in several species. The mechanisms that establish nucleosome positioning are still under active study, however. DNA sequence, while influencing the favorability of nucleosome incorporation, cannot alone establish *in vivo* positioning patterns. Rather, competition from DNA-binding proteins and remodeling by Snf2 family enzymes contributes much of the specificity of *in vivo* nucleosome positioning. In paper III, we try to understand the contribution of different Snf2 family chromatin remodelers to nucleosome positioning in fission yeast. We use a combined approach, comparing nucleosome positioning mapped by MNase digestion with transcriptomic data and *in vitro* characterization.

The highly positioned nucleosomes following or flanking the TSS NDR tend to be enriched for H2A.Z, a histone variant incorporated by the Swr1 remodeler (Mizuguchi *et al*, 2004; Raisner *et al*, 2005; Buchanan *et al*, 2009). In budding yeast, H2A.Z deposition is dependent on NDR formation, but the reverse is not true (Hartley & Madhani, 2009). More important for NDR formation is the activity of the RSC remodeling complex, catalyzed by the Snf2 protein STH1 (Badis *et al*, 2008; Parnell *et al*, 2008; Hartley & Madhani, 2009). We found that in fission yeast, as in budding yeast, H2A.Z is unimportant for positioning the typically H2A.Z-enriched +1 nucleosome. This was true even at genes where H2A.Z is normally enriched, and at genes with altered transcription in the absence of H2A.Z. In contrast to budding yeast, however, we did not observe any role for the STH1 homolog Snf21 in positioning nucleosomes at fission yeast genes. Snf21, like STH1, is essential, and so its function was studied in a conditional loss of function system. It is possible that our conditions did not completely eliminate Snf21 remodeling function, but our results may indicate that other remodelers can support promoter-region nucleosome depletion in fission yeast.

We also looked at the importance of chromatin remodeling for regularly spaced gene body nucleosomal arrays. In budding yeast, these arrays are dependent on the combined remodeling activity of Isw1, Isw2, and Chd1. Fission yeast lacks the Iswi subfamily but has three Chd-type remodelers: Mit1, of the Mi-2 subfamily, and Hrp1 and Hrp3 of the Chd1 subfamily. Although our groups had previously published that Mit1 is important for gene body nucleosomal arrays (Lantermann *et al*, 2010), with improved methodology we found little effect in a *mit1Δ* mutant. Rather, Hrp1 and Hrp3 have an additive role in nucleosome spacing and in linking genic arrays to the +1 nucleosome. Regular genic arrays appear to be important to prevent cryptic transcription initiation, as antisense transcripts were elevated in the single and double Hrp deletion mutants. We also show for the first time that Hrp1 and Hrp3 have ATP-dependent nucleosome spacing activity *in vitro*. Our study demonstrates considerable evolutionary divergence between budding yeast and fission yeast, with the same key functions being performed by different combinations of chromatin remodelers in the two species. We also provide a partial explanation for how fission yeast survives without Iswi subfamily remodelers Our findings highlight the importance of maintaining regular genic nucleosomal arrays, as both yeast species have redundant remodelers performing the task.

3.4 PAPER IV: CONTROLLING RETROTRANSPOSONS AND MAINTAINING GENOME INTEGRITY: FUN30 REMODELER COOPERATION

The Fun30 chromatin remodelers have, until recently, been extremely poorly characterized. Although they are now implicated in heterochromatin maintenance, centromere function, chromatin boundaries, DNA damage repair, and transcriptional regulation (Rowbotham *et al*, 2011; Strålfors *et al*, 2011; Chen *et al*, 2012; Costelloe *et al*, 2012; Durand-Dubief *et al*, 2012; Eapen *et al*, 2012; Byeon *et al*, 2013), Fun30 enzymes are still not as well understood as most chromatin remodelers. Interestingly, fission yeast has three Fun30 subfamily members, while most species have only one (Flaus *et al*, 2006). This expansion in fission yeast has paralleled the disappearance of the Iswi subfamily (Rhind *et al*, 2011). In paper IV, we characterize the novel chromatin remodeler Fft2 (Fission yeast Fun Thirty 2) and demonstrate its role in a new mechanism of retrotransposon regulation.

Fft2 has a generally repressive role genomewide. Like budding yeast Fun30, it is required for proper transcriptional silencing at telomeric, rDNA repeat, and silent mating type heterochromatic loci (Neves-Costa *et al*, 2009). Fft2 also cooperates with Fft3 to repress several categories of stress and nutrient deprivation response genes under optimal growth conditions. In contrast to Fft3, however, Fft2 can also support gene activity: Fft2 is required for the full transcription of the most strongly transcribed genes.

One of the most interesting features of Fft2 and Fft3 binding patterns genomewide is the way that one can compensate for the absence of the other. As a result, the most dramatic phenotypes are exposed in the double mutant. Both Fft2 and Fft3 bind the centromere and associated heterochromatin. The remodelers redistribute in single *fft2Δ* or *fft3Δ* mutants and can at least partially replace each other. The double *fft2Δ fft3Δ* mutant, in contrast, has a severe mitotic defect. This compensatory redistribution is visible at other genomic loci as well. LTRs and *tf2* retrotransposons are a striking example, with loss of both Fft2 and Fft3 leading to dramatic *tf2* upregulation. We found that not only are the retrotransposons upregulated in *fft2Δ fft3Δ*, but the transcripts are longer. Like most LTR retrotransposons, the *tf2* lifecycle is dependent on reverse transcription primed from a site in the LTR sequence (Levin, 1995). In WT cells, however, *tf2* transcription initiates 5' of the LTR, producing reverse transcriptionally incompetent RNA that will never be able to transpose. In *fft2Δ fft3Δ* cells, however, *tf2* transcripts include this crucial primer binding site, as well as a self-primer. Transposon activation is a common stress response in many species, and is often under host cell control (Levin & Moran, 2011). Given the role of Fft2 and Fft3 in repressing other stress response genes, and the fact that these remodelers are downregulated in response to stress (Chen *et al*, 2003), we propose that *tf2* retrotransposition is induced as part of the fission yeast stress response. This mechanism of regulation, with Fun30 remodelers enforcing transcription from an alternative transcription start site, would allow for rapid retrotransposon activation. Finally, we speculate that the ability of LTRs to function as remodeler-dependent boundary elements (Strålfors *et al*, 2011) may have evolved as a result of remodeler affinity for and repression of retrotransposons.

4 CONCLUSION

This thesis explores the ways in which nucleosome positioning and occupancy, as regulated by DNA topoisomerases and chromatin remodelers, affect transcription. We show that topoisomerases, in addition to relieving supercoils ahead of elongating RNAPII, are important for nucleosome depletion 5' and 3' of genes. At the 5' NDR, elevated nucleosome occupancy limits the rate at which transcription can initiate, leading to the downregulation of highly transcribed genes. At the 3' NDR, elevated nucleosome occupancy increases the level of readthrough transcription, perhaps by interfering with the RNAPII pausing that is associated with termination. We hypothesize that relief of supercoils by topoisomerases is needed for efficient nucleosome eviction by chromatin remodelers at the 5' NDR, and suggest that Hrp1 is key to this disassembly. In contrast, the positive supercoils generated ahead of an elongating RNA polymerase normally destabilize nucleosomes in the 3' NDR, supporting their eviction by remodelers. In the absence of topoisomerase activity, however, fewer RNAPII reach the end of the gene and positive supercoils are mislocalized to the gene body rather than the termination site.

While nucleosome depletion at the transcription start and termination sites support transcription initiation and termination, gene body nucleosomal arrays are required to prevent cryptic transcription initiation. In the absence of the Chd1 subfamily remodelers Hrp1 and Hrp3, gene body nucleosome positioning becomes disordered and permissive of cryptic antisense transcription. Finally, the Fun30 chromatin remodelers Fft2 and Fft3 regulate the transcription of stress response genes. In particular, Fft2 and Fft3 control retrotransposons by a novel regulatory mechanism in which they enforce retrotransposon transcription from an alternative, nonproductive transcription start site. Taken together, these studies expand our understanding of the interplay between transcription, nucleosome positioning, and the chromatin remodelers and DNA topoisomerases that regulate them.

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