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Functional organisation of the cell nucleus in the fission yeast, *Schizosaccharomyces pombe*

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Abstract
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In eukaryotes the genome adopts a non-random spatial organisation, which is important for gene regulation. However, very little is known about the driving forces behind nuclear organisation. In the simple model eukaryote fission yeast, *Schizosaccharomyces pombe*, it has been known for a long time that transcriptionally repressed heterochromatin localise to the nuclear membrane (NM); the centromeres attaches to spindle pole body (SPB), while the telomeres are positioned at the NM on the opposite side of the nucleus compared to the SPB. Studies presented in this thesis aimed at advancing our knowledge of nuclear organisation in *Schizosaccharomyces pombe*.

We show that the heterochromatic mating-type region localises to the NM in the vicinity of the SPB. This positioning was completely dependent on Clr4, a histone methyl transferase crucial for the formation of heterochromatin. Additional factors important for localisation were also identified: the chromo domain protein Swi6, and the two boundary elements *IR-L* and *IR-R* surrounding this locus. We further identify two other chromo domain proteins; Chp1 and Chp2, as crucial factors for correct subnuclear localisation of this region. From these results we suggest that the boundary elements together with chromodomain proteins in balanced dosage and composition cooperate in organising the mating-type chromatin.

Gene regulation can affect the subnuclear localisation of genes. Using nitrogen starvation in *S. pombe* as a model for gene induction we determined the subnuclear localisation of two gene clusters repressed by nitrogen: Chr1 and Tel1. When repressed these loci localise to the NM, and this positioning is dependent on the histone deacetylase Clr3. During induction the gene clusters moved towards the nuclear interior in a transcription dependent manner.

The knowledge gained from work presented in this thesis, regarding nuclear organisation in the *S. pombe* model system, can hopefully aid to a better understanding of human nuclear organisation.

Keywords: fission yeast, heterochromatin, subnuclear organisation, chromo domain proteins, boundary elements, transcriptional regulation, epigenetics

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Tillägnad min Mamma och Pappa

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Alfredsson-Timmins J.**, Henningson F., Bjerling P. (2007)
The Clr4 methyltransferase determines the subnuclear localization of the mating-type region in fission yeast.
Journal of Cell Science 120; 1935.

II **Alfredsson-Timmins J.**, Ishida M., Nakayama J. and Bjerling P. (2009)
Chromo domain proteins in balanced dosage together with boundary elements cooperate in organising the mating-type chromatin in fission yeast.
Manuscript

III **Alfredsson-Timmins J.**, Kristell C., Henningson F., Lyckman S., and Bjerling P. (2009)
Reorganization of chromatin is an early response to nitrogen starvation in *Schizosaccharomyces pombe.*
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Contents

Abbreviations

4C	genomewide Chromatin Conformational Capture
BE	Boundary Elements
cc	central core
CD	chromo domain
CFP	Cyan Fluorescent Protein
ChIP	Chromatin Immunoprecipitation
CSD	chromoshadow domain
CT	Chromosome Territory
CT-IC	Chromosome Territory-Interchromatin space
DCC	Dosage Compensation Complex
DNA	Deoxyribonucleic acid
EB	Enchancer-Blocking insulator
FISH	Fluorescence *in situ* hybridisation
GFP	Green Fluorescent Protein
H3K9	histone H3 lysine 9
H3K9Ac	histone H3 acetylated at lysine 9
H3K4Me	histone H3 methylated at lysine 4
H3K9Me	histone H3 methylated at lysine 9
H3K27Me	histone H3 methylated at lysine 27
HATs	Histone Acetyl Transferases
HDACs	Histone Deacetylases
HDMs	Histone Demethylases
HMTs	Histone Methyltransferases
HP1	Heterochromatin Protein 1
ICN	Interchromosome Network
IF	immunofluorescence
INM	Inner Nuclear Membrane
imr	innermost repeats
LADs	Lamin Associated Domains
LCR	Locus Control Region
MHC	Major Histocompatability Complex
NAD	Nicotinamide Adenine Dinucleotide
NM	Nuclear Membrane
NPC	Nuclear Pore Complex
otr	outermost repeats
PCR	Polymerase Chain Reaction

PEV	Position Effects Variegation
qRT-PCR	Quantitative Real Time- Polymerase Chain Reaction
RITS	RNA Induced Transcriptional Silencing
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
siRNA	small interfering RNA
SPB	Spindle Pole Body

General introduction

Gregor Mendel showed in the mid 19th century through simple genetic crossing experiments using peas that certain traits are inherited and transferred to the next generation in a non-random fashion. Later these heritable traits were given the term genes. Although the mechanism of inheritance of genetic material was known then, what the genes were made of was not. It took until the mid 20th century to unravel this. Little more than 50 years ago now it was discovered that the genetic material of all living organisms is made up of deoxyribonucleic acid (DNA) (Avery et al., 1944; Watson and Crick, 1953).

All the DNA of a living organism is known as the genome. In eukaryotes the genome is split up onto a number of chromosomes that in turn contain the genes. The number of chromosomes and genes varies between different species. Eukaryotic cells also have a membrane partitioning the genome from the rest of the cell forming a nucleus.

The cellular structure known as the nucleus was first described in 1831 by the Scottish botanist Robert Brown, and in the late 19th century it was first hypothesised that each specific chromosome occupies a distinct area inside the cell nucleus (Lamond and Earnshaw, 1998; Cremer and Cremer, 2006a; Cremer and Cremer, 2006b).

With rapidly improving imaging and molecular techniques in the second half of the 20th century the area of research investigating subnuclear organisation has seen major advances. Despite these many advances, there is still a large ongoing debate on what factors help establish and maintain the very tightly regulated organisation of the cell nucleus, and as a consequence several independent mutually exclusive models have been proposed (Branco and Pombo, 2007).

How specific genes are up- or down-regulated in different cells at any given time can change in response to signals from the surrounding environment. If i.e. the availability of nutrients becomes restricted, the temperature changes, or if the cells are subjected to different types of stress such as infections from microorganisms, the gene expression is altered so the cells are able to respond to these changes and adapt to them. In recent years there has also been growing evidence that the actual subnuclear position of genes, or clusters of genes, also influences the level of gene expression. In addition, the subnuclear localisation of these genes changes if they become activated or turned off (Lanctot et al., 2007). Adding further to the complexity of gene

regulation is the vast array of epigenetic mechanisms such as DNA methylation and histone modifications. Apart from being involved in regulating transcription, these modifications also play important roles in structurally shaping the chromatin. As a consequence, studying the interplay between gene regulation and subnuclear organisation is proving a very complex task indeed.

So far much of the research has focused on investigating the transcription of individual genes at the promoter level to see how this effects the organisation of the local chromatin structure (McPherson et al., 2001; Venter et al., 2001). Even though studying the expression of individual genes may provide some initial clues on the influence transcription has on subnuclear localisation, it has become evident that merely investigating single genes does not hold all the answers. As a result, several new techniques have been developed employing genomewide approaches for studying transcription and DNA:protein interactions at the genome level, as well as inter- and intra-chromosome interactions. As more such detailed genomewide studies emerge, these may hold some important clues to how subnuclear organisation is established and maintained.

By investigating the impact subnuclear position has on individual genes, entire chromosomes or the complete genome in normal cells, one hopes to find some answers regarding what happens to this organisation in for example cancer cells. Evidence from several studies on cancer cell lines have indicated that the subnuclear organisation becomes distorted in the tumour cells. Whether this observed distortion is a cause or a consequence of the disease state of theses cells remains to be elucidated. However, if the underlying mechanisms to what causes the disease state is known the goal is to develop more specialised drugs, as the use of more specialised drugs would mean that the disease could be targeted more efficiently and treated with less detrimental side effects. Even though the understanding of how subnuclear organisation may influence gene expression has advanced rapidly over the last few years there are still many questions left unanswered. Then again, with rapidly increasing advances in molecular and microscopic techniques, alongside increasingly powerful computer hardware and more sophisticated software packages in addition to comparative studies across the species, many of the questions will most surely be answered in a not too distant future.

Fission yeast as a model organism

In order to understand some of the underlying mechanisms of several disciplines, including genetics, the use of model organisms has proven invaluable research tools. By studying molecular mechanisms in a simple model organism it is possible to draw some conclusions on how these mechanisms might work in higher organisms. This is possible due to evolutionary conservation of important molecular pathways and processes. Advances in technologies and availability of more genome sequences and protein:protein interaction data sets from increasing number of organisms have also enabled more detailed comparisons between species. Different model organisms have their own advantages and disadvantages, and depending on what molecular processes you want to study the choice of model organism is important when stating your hypothesis in order to address it correctly.

The fission yeast, *Schizosaccharomyces pombe*, is a simple unicellular eukaryote that shared a common ancestor with humans approximately 1.5 million years ago (Heckman et al., 2001; Hedges, 2002). Despite this length of time, many of the essential molecular pathways in this simple yeast are very well conserved through evolution all the way to humans. As a consequence, it has become a popular model organism for basic research for a number of different reasons. First and foremost, it is cheap and easy to maintain in both liquid culture or on solid media. It has a conventional cell cycle with a relatively short generation time of 2-4 hours, and during normal growth conditions the cells alternate between growth and mitotic division. In *S. pombe* like in other fungi the nuclear envelope stays more or less intact throughout the cell cycle, unlike in higher eukaryotes where the nuclear envelope breaks down at the end of mitosis.

The *S. pombe* genome is completely sequenced and contains around 5000 protein-coding genes. Like in all eukaryotes the genome is contained inside the cell nucleus. The genome is 13.8 Mb in size, divided onto three chromosomes; 3.5, 4.6 and 5.7 Mb in size respectively (Wood et al., 2002).

During normal growth conditions the *S. pombe* genome is haploid. This is a great advantage as the effects of gene deletions, or expression from reporter genes, can easily be monitored since there is no effect by the allele on the other chromosome like in mammals. *S. pombe* also undergoes natural homologous recombination during mitotic growth that makes it possible to easily knock out genes or to introduce markers at specific sites in the ge-

nome (Bahler et al., 1998). Furthermore, as all laboratory strains originate from a single isolate it makes it possible to simply cross two strains and study genetic traits in the progeny as the strains share the same genetic background (Egel, 2004).

As a model organism, *S. pombe* has turned out very informative for studying cell-cycle regulation employing both classical and molecular genetic techniques. In addition, in recent years, fission yeast has also become a very popular tool for epigenetic studies. The main reason being that fission yeast shares many conserved features of higher eukaryotes such as the ribonucleicacid interference (RNAi) machinery and histone modifications, both of which are important for the assembly and regulation of transcriptionally repressed chromatin, heterochromatin. In *S. pombe,* like in higher eukaryotes, the formation of heterochromatin is mediated via the binding of chromo domain proteins to di- and tri-methylated histone H3 Lysine 9 (H3K9Me), a feature not present in the budding yeast, *Saccharomyces cerevisiae,* another popular model organism for studying gene regulating and genetics (Martienssen et al., 2005). The fact that *S. pombe* shares this conserved pathway of heterochromatin structure formation and maintenance via Swi6, a Heterochromatin Protein 1 (HP1) homologue, with higher eukaryotes, has made fission yeast an excellent model organism for both chromatin and epigenetic studies. More in depth mechanisms of the chromatin structure formation and maintenance in *S. pombe* is discussed in the subchapter 'Heterochromatin in *S. pombe*' of the chapter 'The structure of DNA and chromatin'.

The structure of DNA and chromatin

The naked DNA molecule is made up of a sugar-phosphate backbone to which the four bases; adenine, thymine, cytosine and guanine are attached making up a single strand. Two complementary single strands basepair through hydrogen-bonds making up the double helix. Van der Waals- interactions make the helix bend in a left-handed turn also known as an alpha helix. The DNA molecule itself is negatively charged and associates through electrostatic interaction with positively charged structural globular proteins called histones (Kornberg, 1977). The DNA bound to these histone proteins forms the chromatin, the basic structural unit of which is the core nucleosome consisting of a 146 bp stretch of DNA wrapped 1.65 times around a histone octamer. Wrapping the DNA around these histone proteins help to package the DNA to make it fit inside the nucleus. The histone octamer itself is made up of two copies each of the core histones; H2A, H2B, H3 and H4. Two H3-H4 dimers form a tetramer at the centre of the octamer flanked on either side by H2A-H2B dimers. The nucleosome also contains the linker DNA, and in higher eukaryotes also the linker histone H1, connecting the octamers to each other. Histone H1 binds the inside of the helix, and this not only helps to stabilise the molecule, but also causes it to bend and twist resulting in further compaction of the DNA molecule (Luger et al., 1997; Richmond and Davey, 2003; Schalch et al., 2005).

Apart from the four core histones, there are a number of histone variants present in all eukaryotes. These histone variants differ slightly in sequence to the core histones and replace these at specific sites in the genome where they perform highly specialised functions. For example, H3 is replaced at the central core of the centromeres by CENP-A, a variant essential for kinetochore assembly. Another variant is H2A.X, which replaces H2A at sites of DNA damage (Bjerling and Ekwall, 2002; Henikoff and Ahmad, 2005).

In addition, the N-terminal histone tails of the core histones are unstructured and protrude out from the core and can be modified. Together with the linker histone these modifications are then a platform for recruiting factors necessary for organising the DNA into higher order structures (Bednar et al., 1998; Schalch et al., 2005; Robinson et al., 2006).

Covalent histone modifications

Several amino acids on the N-terminal tails of the core histones can be subjected to a range of different post-translational covalent modifications. Some of these; methylation, acetylation or phosphorylation, involves the addition of a chemical group, while others such as ubiquitination and sumolyation result in the addition of a polypeptide (Lee et al., 2005; Groth et al., 2007; Kouzarides, 2007). These histone modifications are important for several processes including; regulating transcription, heterochromatin formation, imprinting and recruiting the DNA damage response machinery (Kouzarides, 2007).

Additionally, histone modifications are also important in regulating the condensation of chromatin into higher order structures such as the 30 nm fibre or into metaphase chromosomes where the linker histone H1 also has an important role. In contrast, it is hypothesised that large modifications such as ubiquitination aid in physically opening up the chromatin to create access for the transcriptional machinery to the underlying DNA sequence (Horn and Peterson, 2002; Kouzarides, 2007; Misteli, 2007). It has also been proposed that the different modifications set up a specific 'histone-code' involving cross-talk between the different modifications. This code is then read and interpreted with great accuracy, which is crucial for correct regulation of gene expression. Recent studies involving genome-wide approaches have supported this hypothesis (Jenuwein and Allis, 2001; Suganuma and Workman, 2008).

Histone modifications can be inherited through mitosis and sometimes meiosis in the same fashion as the DNA sequence itself. This inheritance of specific modifications is known as epigenetic inheritance. These modifications are important not only for regulating gene expression but also for genetic memory through performing imprinting functions. However, most epigenetic marks are erased as part of a major epigenetic reprogramming in the nucleus after fertilisation. Epigenetic reprogramming can also occur in disease cells, although it is not clear in these cases if this reprogramming is a cause or a consequence of the disease (Morgan et al., 2005; Martin and Zhang, 2007; Probst et al., 2009).

Methylation and acetylation

The most widely studied and best understood of the histone modifications are acetylation and methylation. Both these modifications are reversible and act on specific lysine or arginine residues of histones H3 and H4. They are particularly important in regulating transcription and in heterochromatic gene silencing.

Methyl modifications on specific lysine residues of the N-terminal histone tails creates precise binding surfaces for specific proteins such as activators

or repressors, resulting in either switching transcription on or off in that part of the genome. Some proteins have specificity for a particular modification whilst others have a broader specificity. These methyl-binding proteins contain i.e. chromo domains, WD40 domains or bromo domains. Binding of these types of proteins to methylated lysines is necessary for the recruitment of specific transcriptional regulators such as the SAGA or Mediator complexes or in recruiting the heterochromatin regulating SHREC complex (Kelleher et al., 1990; Millar and Grunstein, 2006; Daniel and Grant, 2007; Kouzarides, 2007; Sugiyama et al., 2007).

Acetylation of lysine residues involves the addition of an acetyl group onto the ε-amino acid of lysines and is thought to play a central role in altering the folding properties of chromatin. Because of its positive charge, the lysine residue normally binds strongly to the negatively charged DNA. However, acetylation negates the positive charge of the lysine leading to a weakened binding between the histones and the DNA, which in turn can aid in opening up of the chromatin during processes like transcription (Millar and Grunstein, 2006; Jiang and Pugh, 2009).

The implications of specific modifications in terms of setting up different types of chromatin are discussed further below in the subchapter 'Euchromatin and Heterochromatin'.

Histone modifying enzymes

The process of modifying the histones by adding or removing different residues is carried out by specific histone modifying enzymes. The mode of action of these enzymes is not only tightly regulated, but also very well conserved across species. Furthermore, some of these modifying enzymes have specificity for a particular residue of a particular histone or even for a specific modification, whereas others have much broader specificity (Kurdistani and Grunstein, 2003; Lee et al., 2005; Kouzarides, 2007).

Histone acetyltransferases and deacetylases

The transfer of an acetyl group onto a lysine residue is known as acetylation, a process carried out by specific enzymes called histone acetyl transferases [HATs or KATs (Allis et al., 2007)]. The opposing reaction to acetylation, the removal of acetyl groups from lysine residues, is deacetylation and is carried out by enzymes called histone deacetylases (HDACs). Some HATs and HDACs have broad enzymatic specificity while others are more specific. For example, the mammalian HAT PCAF [KAT2B (Allis et al., 2007)], or its homologue in budding yeast Gcn5 [KAT2 (Allis et al., 2007)], specifically acetylates K9, K14 and K18 of histone H3. It is thought that acetylation of histone H3 lysine 9 (H3K9Ac) prevents methylation of that same residue

thereby blocking the spread of repressive heterochromatin into regions of active transcription (Fig. 1) (Litt et al., 2001).

The HDACs can be divided into three main classes; class I, class II and class III. Class I and class II HDACs are phylogenetically related and share a common enzymatic domain. Class III HDACs differ from class I and class II HDACs as they are nicotinamide adenine dinucleotide (NAD) dependent.

In *S. pombe*, deacetylation is carried out by six different HDACs. Clr6 and Hos2 are class I, Clr3 is a class II, and Sir2, Hst2 and Hst4 are class III HDACs. The class I HDAC Clr6 is an essential gene in *S. pombe* and has a broad specificity (Bjerling et al., 2002). In addition, Clr6 is the main enzyme for deacetylation of promoter elements (Wiren et al., 2005). The class III enzyme Sir2 specifically deacetylates H3K9 and H3K14, while the class II HDAC Clr3 on the other hand shows substrate specificity for H3K14. Furthermore, Sir2 is shown to cooperate with Clr3 across the genome (Bjerling et al., 2002; Shankaranarayana et al., 2003; Wiren et al., 2005). Clr3 is also required for the recruitment of the histone methyltransferase [HMT or KMT (Allis et al., 2007)] Clr4 [KMT1 (Allis et al., 2007)] to heterochromatic regions through two redundant pathways. One is RNAi-dependent while the other is dependent on the recruitment of Aft1/Pcr1. The recruitment of Clr4 in turn results in H3K9Me followed by heterochromatin assembly and nucleation (Nakayama et al., 2001; Jia et al., 2004; Yamada et al., 2005).

Histone methyltransferases and demethylases

In contrast to HATs and HDACs, HMTs and histone demethylases [HDMs or KDMs (Allis et al., 2007)] have a narrow specificity for a specific lysine residue on a specific histone. HMTs transfer a methyl group onto a deacetylated lysine residue. As the lysine residues can be either mono-, di- or trimethylated this adds another layer to the complexity of gene regulation and chromatin formation. In mammalian cells different enzymes carry out the different states of methylation and the different methylation marks correspond to different degrees of gene silencing (Peters et al., 2003; Rice et al., 2003). Methylation of lysine residues is linked both to repression and activation of genes. One example of repression is the tri-methylation of H3K9 that creates a binding site for the chromo domain protein HP1. Binding of HP1 then initiates the formation of heterochromatin thus causing transcriptional repression (Fig.1) (James and Elgin, 1986). For a long time it was thought that methylation of histones were non-reversible modifications. However, that changed when it was discovery that LSD1 [KDM1 (Allis et al., 2007)] possess demethylation activity (Shi et al., 2004). LSD1 is a HDM with specificity for H3K4, and since the discovery of LSD1 several other HDMs have been characterised in many species including both the budding and fission yeasts (Clissold and Ponting, 2001). The largest family of HDMs is the Ju-

monjii domain family of HDMs (Balciunas and Ronne, 2000; Takeuchi et al., 2006).

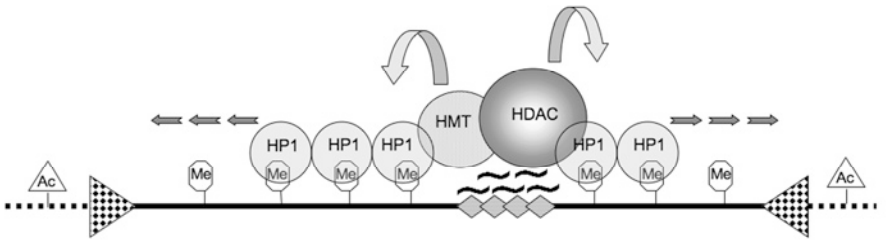

Figure 1. Heterochromatin formation

The RNAi machinery produces si-RNAs (small black curved lines) from repetitive elements (diamond shapes) across the genome. These si-RNAs help recruit histone-modifying factors such as histone deacetylaces (HDAC) and histone methyl transferases (HMT) important for the formation of structural heterochromatin. In *S. pombe* they include the HDAC Clr3 and the HMT Clr4. Once the action of the HDACs and HMTs is carried out, resulting in methylation of histone H3, members of the HP1 family of proteins can bind to the methylated histones. Structural heterochromatin then nucleates *in trans* through HP1 binding to another HP1 molecule via its chromoshadow domain preceded by the simultaneous actions of HDACs/HMTs. In fission yeast the HP1 family protein Swi6 is responsible for this nucleation. Transcriptionally silenced heterochromatin is characterised by the epigenetic mark of methylated histone H3 (Me) and transcriptionally active euchromatin by acetylated histones (Ac). Insulators present in the genome (inverted chequered triangles) act as barriers between heterochromatin (solid black line) and euchromatin (dashed line) thus preventing spreading of chromatin and shielding nearby regions from position effect variegation. [Modelled on Fig. 1 (Grewal and Jia, 2007)].

Euchromatin and heterochromatin

Actively transcribed regions of the genome are traditionally known as euchromatin. These euchromatic regions, share common characteristics with actively transcribed genes including hyperacetylated histones and histone H3 methylated at lysine 4 (H3K4Me) (Litt et al., 2001; Noma et al., 2001). Heterochromatic regions, also known as silent chromatin are areas in the genome with very low levels of transcription. These regions also share common features such as low acetylation levels of histones, H3K9Me and binding of chromo domain protein HP1 (Fig. 1) (Bannister et al., 2001; Litt et al., 2001).

The hallmarks of transcriptionally active or inactive chromatin are highly conserved between different species. For example, both fission yeast and the fruitfly, *Drosophila melanogaster*, share some of the same specific histone modifications and structural components of chromatin as are found in mammals. Although, *S. pombe* does not have histone H3 methylated at lysine 27

(H3K27Me) and Polycomb binding, which is important for silencing in both mammals and the fruitfly. The budding yeast is also different in some aspects compared to mammals but not in others in terms of chromatin formation. Most but not all the modifications that regulate the formation of chromatin are the same in budding yeast as in mammals, but the structural components making up the chromatin differs. For example, budding yeast does not have H3K9Me or HP1 homologues but does have HDACs, giving *S. pombe* an advantage and making it a more suitable model organism for chromatin studies (Lomberk et al., 2006).

The different covalent histone modifications are not only important for structurally organising the chromatin or for regulating transcription at the single gene level. H3K4Me and H3K9Me are also important for barrier functions across the genome, setting up boundaries between transcriptionally active euchromatin and inactive heterochromatin regions (Fig. 1). These barriers ensure there is no spread of heterochromatin into regions that needs to be transcriptionally active as well as keeping silenced parts of the genome switched off. This is extremely important as faulty gene expression could lead to disease due to either lack of or over expression of proteins (Kurdistani and Grunstein, 2003; Sinha et al., 2006; Bhaumik et al., 2007).

Chromo domain proteins

The formation of transcriptionally repressed heterochromatin is facilitated by the binding and subsequent nucleation of chromo domain proteins. These chromo domain proteins were first discovered in the fruitfly. In the fruitfly certain rearrangements of the DNA result in an unstable expression of the gene encoding the eye-colour with a clonal inheritance (James and Elgin, 1986; Eissenberg et al., 1990). This type of variegated expression of the genes encoding the eye-colour in flies is called position effect variegation (PEV) (Reuter and Spierer, 1992). Certain mutations either enhance, *E(var)*, or suppress, *Su(var)*, the variegated phenotype. For example the *Su(var)2-5* mutation causes a suppression of PEV, resulting in an eye-colour that resembles the wild type red eye. This is due to a disruption of the dominant repression by the protein that is a major structural component of heterochromatin.

The gene *Su(var)2-5* in the fruitfly was found to encode a protein, HP1, that was to become the founding member of the HP1 family of proteins. This family of non-histone chromosomal proteins is highly phylogenetically conserved and is important for the formation and maintenance of heterochromatin as well as genome integrity. Structural homologues have been identified in most eukaryotes, and HP1 proteins in these associate with heterochromatic loci; centromeres, telomeres and in addition with rDNA. PEV also occur in other systems at these heterochromatic loci if there are mutations in

the HP1 gene (Eissenberg et al., 1990; Ekwall et al., 1995; Eissenberg, 2001; Cam et al., 2005; Fanti and Pimpinelli, 2008). The association of HP1 protein with single silenced genes have also been detected (Greil et al., 2003; Cam and Grewal, 2004; Kouzarides, 2007).

In many organisms there are more than one gene encoding different isoforms of the HP1 proteins that perform slightly different functions (Lomberk et al., 2006; Fanti and Pimpinelli, 2008). HP1 proteins were commonly thought to be repressors of transcription, but this notion changed as isoforms of HP1, i.e. in the fruitfly, were found to associate with actively transcribed regions of the genome (de Wit et al., 2007). However, in the budding yeast there have been no proteins belonging to the HP1 family detected. In this organism the regulation of heterochromatin is instead facilitated by a group of proteins called silent information regulatory (SIR) proteins.

The formation of structural heterochromatin is not just necessary for transcriptional silencing of genes or larger regions in the genome, but also create further binding sites for other proteins or protein complexes that help form higher order chromatin structures. One example is the recruitment of cohesin to HP1/Swi6 that facilitates the condensation of the metaphase chromosome (Reuter and Spierer, 1992; Nonaka et al., 2002).

Proteins belonging to the HP1 family of proteins all have a chromo domain (CD) in the N-terminal part of the protein (Fig. 2) (Aasland and Stewart, 1995). They sometimes also have a second domain at the C-terminal end known as the chromoshadow domain (CSD) (Fig. 2). The CSD shows a weak although significant sequence homology to the CD (Paro and Hogness, 1991). In addition, there are also many proteins outside the HP1 family that contain a CD (Koonin et al., 1995).

The chromo domain

The CD is made up of a three-stranded antiparallel β-sheet against an α-helix in the secondary structure of the CD. Binding of HP1 proteins to H3K9Me is reliant on the CD (Figs. 1 and 2) (Ball et al., 1997; Jacobs et al., 2001). More specifically, the binding depends on the actual hydrophobic binding pocket of the CD. This pocket has a net positive charge that makes it fit and basepair perfectly through electrostatic interaction with the negatively charged DNA molecule. This binding is completely lost in a V26M mutation in the CD of HP1 as the DNA molecule will no longer ‘fit into’ the pocket (Jacobs and Khorasanizadeh, 2002). This binding-pocket is conserved across HP1 family of proteins and recognises a consensus heptamer motif (Smothers and Henikoff, 2000).

The chromoshadow domain

The structure of the CSD resembles that of the CD in that it also consists of a three-stranded antiparallel β-sheet against an α-helix in the secondary structure. In addition, the CSD contains a second α-helix. Once the HP1 protein has bound the DNA via its CD, the nucleation of constitutive heterochromatin is mediated through the interaction between the additional α-helix of the CSD of one HP1 molecule to another, resulting in the nucleation and thereby spreading of heterochromatin (Figs. 1 and 2) (Cowieson et al., 2000).

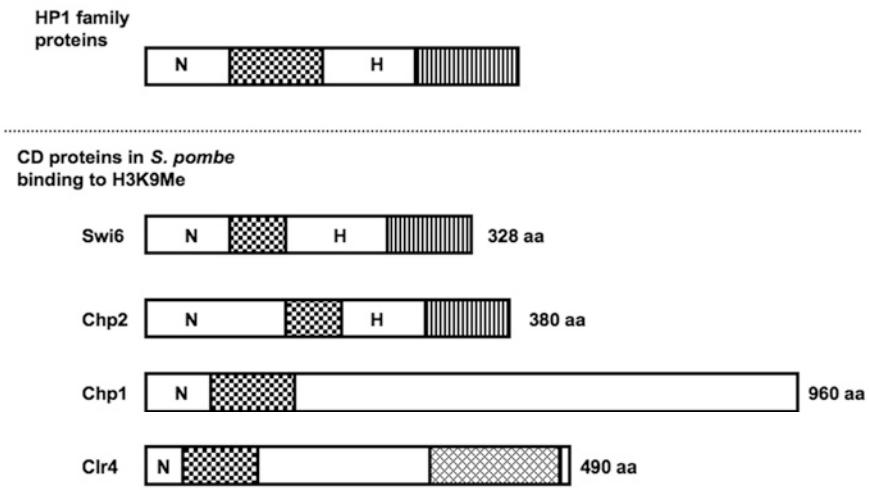

Figure 2. Chromo domain proteins

Top; The HP1 family of chromo domain proteins contain the four characteristic domains: the N-terminal domain (N), the chromo domain (CD) (chequered box), the hinge region (H) and a C-terminal chromoshadow domain (CSD) (striped box).
Bottom; In *S. pombe* four proteins belonging to the chromo domain super family of proteins all contain a CD with specificity for H3K9Me. Swi6 is a HP1 homologue in *S. pombe*, and Chp2 is an iso-form of Swi6. These two HP1 family proteins both also contain the characteristic CSD. Chp1 is a component of the RNA-induced transcriptional silencing (RITS) complex in *S. pombe*. Clr4 is the sole H3K9Me histone methyl transferase in fission yeast. This enzyme also contains a SET domain (diamond shaped box) responsible for the enzymatic activity of Clr4.

The interaction between one CSD and another occur through a conserved consensus binding motif, namely the PXVXL motif. This motif is sufficient for the dimerisation of the CSD and is also present in several other HP1 interacting proteins. This PXVXL motif together with H3K9Me is necessary to recruit HP1 proteins to heterochromatin (Cowieson et al., 2000; Smothers and Henikoff, 2000; Thiru et al., 2004).

The CSD is not just important in the function of binding to other chromo domain proteins. Interactions between the CSD of HP1 proteins to other sets of proteins and nuclear structures such as transcriptional co-repressors, chromatin remodelling factors and the lamin B receptor, have also been implicated through interactions via its additional α-helix (Brehm et al., 2004; Lomberk et al., 2006). In higher eukaryotes the interaction of HP1 proteins with nuclear receptors, such as the lamin B and emerin receptors, is necessary for attaching silent chromatin at the nuclear periphery (Somech et al., 2005).

The hinge region

The region separating the CD from the CSD is called the linker region but is also commonly known as the hinge region (Fig. 2). In the folded protein the hinge region is thought to be flexible and exposed to the surface. Unlike the CD and the CSD that are very highly conserved between HP1 family proteins, the hinge region has the most variable amino acid sequence. This variability in sequence is not just seen between HP1 proteins from different species but also in subsets within the same species (Singh and Georgatos, 2002). The hinge region can be subjected to several different post-translational modifications. It is thought that the principal regulation of HP1 family proteins could be mediated through these modifications, especially through phosphorylation. Evidence shows that this and other modifications do have an effect on both the function as well as on interaction with other proteins and localisation (Lomberk et al., 2006; Shimada et al., 2009). Perhaps the observed variability in the hinge region is a consequence of the structure of the HP1 proteins where this region is exposed to the surface. By altering the sequence of the hinge region, the subsets of HP1 protein could easily change their functions in order to adopt more specialised functions (Lomberk et al., 2006).

Chromo domain proteins in *S. pombe*

In fission yeast there are three members belonging to the HP1 family of proteins; Chp1, Swi6 and Chp2 (Fig. 2). All three proteins play important functions at the heterochromatic regions of the *S. pombe* genome although the interdependencies at the different loci vary (Thon and Verhein-Hansen, 2000; Sadaie et al., 2004; Sadaie et al., 2008; Alfredsson-Timmins et al., 2009a).

Chp1 is a main component of the RNA Induced Transcriptional Silencing (RITS) Complex and is important in establishing heterochromatin at all of the major heterochromatic regions: the centromeres, the telomeres and at the mating-type locus (Sadaie et al., 2004; Verdel et al., 2004). In addition, deleting

chp1$^+$ causes centromere specific silencing defects whereas the mating-type region and the telomeres are unaffected in this mutant (Sadaie et al., 2004).

Swi6 is a functional homologue of human HP1 and is the main component of structural heterochromatin in fission yeast (Fig. 2) (Lorentz et al., 1994; Ekwall et al., 1995). Chp2 is an iso-form of Swi6 (Fig. 2), and just like Swi6, Chp2 is involved in the formation and maintenance of heterochromatin at all heterochromatic loci in *S. pombe* (Thon and Verhein-Hansen, 2000; Sadaie et al., 2004). Swi6 and Chp2 act cooperatively at these loci although their respective roles at these sites are distinct (Sadaie et al., 2004; Sadaie et al., 2008). Localisation of Chp2 to the mating-type locus and the telomeres is dependent on Swi6, whereas Swi6 localisation to theses loci is only slightly impaired in a *chp2*Δ strain (Sadaie et al., 2008; Alfredsson-Timmins et al., 2009a). In addition, a proper balance between Swi6 and Chp2 is critical for establishing and organising heterochromatin at the heterochromatic loci (Sadaie et al., 2008). A proper balance between all three of the HP1 family proteins in *S. pombe*: Chp1, Swi6 and Chp2 is also crucial for correct positioning of the mating-type region at the NM in the vicinity of the spndle pole body (SPB) (Alfredsson-Timmins et al., 2009a).

Both Swi6 and Chp2 are also important in recruiting the HDAC Clr3 to heterochromatic regions, although the way in which they facilitate the recruitment differs and still remains to be properly dissected (Sadaie et al., 2008). Interestingly, a *chp2*Δ*clr3*Δ double mutant showed additive silencing defects implicating Chp2 in other specific roles in heterochromatin formation apart from recruiting Clr3 (Sadaie et al., 2008). The distinct roles of Swi6 and Chp2 are further supported by chromatin fractionation assays where Swi6 was found to localise to both the soluble and the nuclear fractions. Chp2 on the other hand was found to be tightly associated with the pellet fraction further showing their different functions in chromatin maintenance (Sadaie et al., 2008). Live-cell analysis of the subnuclear localisation of the mating-type locus also supports a structural role of Chp2 in the formation of higher-order heterochromatin structures. In a strain where *chp2*$^+$ is deleted the mating-type locus is more severely delocalised than in a *swi6*Δ strain (Alfredsson-Timmins et al., 2007; Alfredsson-Timmins et al., 2009a).

Another important CD protein in fission yeast is the HMT Clr4. Like all CD proteins it contains a CD in the N-terminal part of the protein. In the C-terminal end Clr4 has a SET domain responsible for the HMT activity of Clr4 (Fig. 2). So far Clr4 is the sole H3K9 HMT identified in *S. pombe* (Ivanova et al., 1998; Rea et al., 2000). The association of HP1 family CD proteins in *S. pombe* to the heterochromatic regions is dependent on binding to H3K9Me mediated via Clr4 at these loci (Fig. 1) (Rea et al., 2000; Bannister et al., 2001).

Heterochromatin in *S. pombe*

In the *S. pombe* genome there are three main regions of heterochromatin on the chromosomes; the pericentric regions of the centromeres, at the chromosome ends where the telomeres are found, and the silent mating-type locus. All these heterochromatic regions share features of heterochromatin with higher eukaryotes such as; low acetylation levels of the histones, H3K9Me and binding of CD proteins (Fig. 1). In *S. pombe* the HMT Clr4, a homologue of the HMT SUV39H1 [KMT1A (Allis et al., 2007)] first characterised in the fruitfly, is the sole HMT in fission yeast identified so far. Clr4 carries out the mono-, di- and tri-methylation of H3K9 in *S. pombe* (Rea et al., 2000). This creates binding sites for the *S. pombe* CD proteins Swi6, Chp1 and Chp2 (Fig. 1) (Rea et al., 2000; Bannister et al., 2001). The mechanism of establishing heterochromatin at the pericentric regions is dependent on the RNAi machinery (Fig. 1) (Volpe et al., 2002; Verdel et al., 2004). At the mating-type locus and at the telomeres redundant pathways are also important in establishing heterochromatin (Jia et al., 2004; Kanoh et al., 2005). Apart form being regulated by redundant pathways, the maintenance of heterochromatin once established within these regions also differs. One thing these three regions have in common is that insertion of reporter genes into any of these heterochromatic loci result in them becoming transcriptionally repressed due to this specialised chromatin environment (Allshire et al., 1995; Martienssen et al., 2005; Horn and Peterson, 2006; Grewal and Jia, 2007; White and Allshire, 2008).

Centromeres

Centromeric silencing has been extensively studied in fission yeast, and the centromeres in *S. pombe* structurally resemble those of higher eukaryotes. They are relatively large, ranging from 35-110 kb in size (Takahashi et al., 1992; Steiner et al., 1993). The main function of the centromeres is their involvement in pairing-up of the two sister-chromatids and to ensure their even segregation. The centromeres are the attachment sites for the kinetochores, and the establishment of the kinetochores is dependent on heterochromatin. In a recent study it was found that cohesin recruited to the centromere via heterochromatin is responsible for setting up the geometry that gives the kinetochore its bi-orientation. This is an important feature to ensure correct segregation of the sister-chromatids at mitosis (Sakuno et al., 2009).

Just like in higher eukaryotes the centromeres contain large repetitive elements that are in heterochromatin structure. In *S. pombe* theses repetitive sequences are known as the innermost (*imr*) and outermost (*otr*) repeats and surrounds the central cores (*cc*) of the centromeres (White and Allshire, 2008). The *cc* and part of the *imr* is bound by CENP-A, and most of the *imr* and the *otr* are in heterochromatin structures (Partridge et al., 2000). The border between heterochromatin and euchromatin at the centromeres are

found within the *otr* and coincide with the presence of tRNA genes acting as boundary elements at five of the six chromosome arms (Partridge et al., 2000; Cam and Grewal, 2004).

The repeats themselves were thought to be transcriptionally inactive, but accumulation of anti-sense transcripts from the repeats was later detected in mutants where the degradation of transcripts was inhibited. It turns out that these repeats are actively transcribed and processed in the process known as RNAi (Volpe et al., 2002; Verdel et al., 2004). In fission yeast it is now well established that the nucleation of heterochromatin is initiated by the formation of small interfering RNA (siRNA) by the RNAi machinery, and that the recruitment of Clr4 by the RNAi machinery is necessary for the formation of heterochromatin (Fig. 1). Since there is only a single copy of each gene encoding the components of the RNAi machinery in *S. pombe* makes it an excellent model for studying RNAi. Mutants in either of the *S. pombe* RNAi genes; *ago1*, *dcr1* or *rdp1* causes transcriptional derepression of reporter genes at the centromeres whereas the mating-type locus is unaffected (Hall et al., 2002; Provost et al., 2002; Volpe et al., 2002; Martienssen et al., 2005).

The processing of anti-sense transcripts is necessary for directing the RITS complex to the centromeric repeats. The recruitment of RITS to the repeats is necessary in establishing the formation of heterochromatin at the centromeres and the repeats themselves are the main nucleation site for centromeric heterochromatin in *S. pombe* (Fig. 1) (Amor et al., 2004; Vos et al., 2006). Interestingly in a recently published study, it was found that the production of transcripts from the *otr,* and the RNAi machinery itself are dispensable for the formation of centromeric heterochromatin. When Clr4 was artificially tethered to chromatin in the *S. pombe* genome, this so called synthetic heterochromatin could still be formed at these sites both in the presence and absence of the RNAi machinery. Tethering of Clr4 also promoted *de novo* CENP-A incorporation and kinetochore assembly (Kagansky et al., 2009).

Telomeres

Unlike for the centromeres, the mechanistics of how telomeric heterochromatin is established and maintained is not as well understood. The telomeric heterochromatic areas are found in the subtelomeric regions spanning approximately 40 kb from the telomere ends. At the telomeres the border between heterochromatin and euchromatin are not as defined as at the centromere and at the mating-type locus. Rather than sequence specific barriers, the balance between the opposing effects of histone modifications and/or histone variants is thought to regulate these borders (Cam et al., 2005; Gordon et al., 2007).

Just like at the centromeres the presence of repetitive elements help to direct the RITS complex to establish the formation of heterochromatin at these repeats. However, the RNAi machinery is dispensable for heterochromatin formation at the telomeres, as in RNAi mutants Swi6 is still localised to the telomeres and transcriptional silencing is retained. This is achieved via a redundant pathway mediated via the DNA binding telomere repeat protein Taz1. Taz1 can establish heterochromatin at the telomeres independently of the RNAi machinery via independent recruitment of Clr4 and Swi6 to telomeric repeats. Interestingly, deletion of Taz1 results in both elongation of telomeric repeats and loss of silencing despite the fact that Swi6 remains bound to the region (Nimmo et al., 1994; Cooper et al., 1997; Kanoh et al., 2005).

The importance of heterochromatin at the telomeres is not as clear as at the centromeres. However, the heterochromatin structures at the subtelomeric regions are thought to prevent end-to-end fusion and homologous recombination between the different telomere ends (Tham and Zakian, 2000). At interphase the *S. pombe* telomeres are anchored to the nuclear periphery at two to five distinct foci. At the fission yeast specific event at beginning of meiosis known as the 'horse-tail stage' the telomeres and centromeres change positions resulting in the telomeres being attached to the SPB (Funabiki et al., 1993). Unlike in the budding yeast *S. cerevisiae,* where anchoring of the telomeres to the NM is facilitated by Ku70, Esc1, members of the Sir protein family and SUN domain protein Mps3, how this attachment is achieved in *S. pombe* is still largely unknown (Gotta et al., 1996; Hediger et al., 2002; Bupp et al., 2007). However, in fission yeast RNAi mutants the clustering of the telomeres is lost although they still remain bound to the NM (Hall et al., 2002).

The mating-type region

The mating-type region in *S. pombe* is situated on the right arm of chromosome II in no close physical proximity to either the centromere or the telomere (Fig. 3). This locus consist of *mat1* that is expressed and two silent storage cassettes; *mat2-P* and *mat3-M*, containing the mating-type information. The genetic information present at *mat1*, determines the mating-type of the cell, either P or M. *mat1* is separated from *mat2-P* by the 15 kb long L-region, and *mat2-P* is separated from *mat-3M* by the 11 kb K-region (Fig. 3) (Grewal and Klar, 1997; Arcangioli, 2004). In a wild type strain, homothallic h^{90}, cells switch mating-types every second cell division through a gene conversion event where information is moved from one of the silent storage cassettes to the expressed *mat1* locus. This interconversion is aided by an imprint in the form of a protected single-stranded break and possibly by the incorporation of a ribonucleotide during DNA replication (Arcangioli, 2004; Egel, 2005; Vengrova and Dalgaard, 2006).

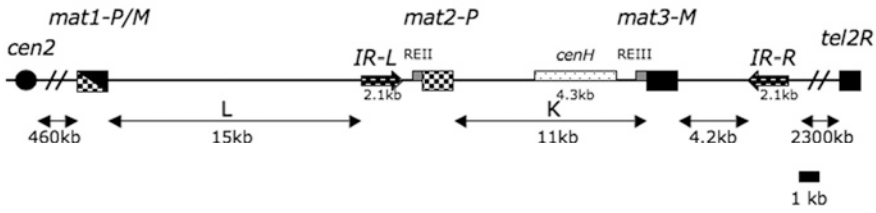

Figure 3. Schematic representation of the mating-type region in *S. pombe*

In *S. pombe* the mating-type region is located on the right arm of chromosome II. The mating-type region consists of three linked loci: *mat1* (chequered/black box), *mat2-P* (chequered box) and *mat3-M* (black box). *mat1* is expressed and determines the mating-type of the cell. *mat2-P* and *mat2/3 mat3-M* are two silent storage cassettes and are surrounded by two inverted repeats, *IR-L* and *IR-R* (block arrows). These boundary elements have perfect sequence identity. In the K-region separating *mat2-P* and *mat3-M* there is a 4.3 kb sequence, denoted *cenH* (white box), with 96% homology to the repeats at *cen2*. This element is the main nucleation site for Clr4 mediated Swi6 heterochromatin formation. Additionally there are two elements in the *mat2/3* region where heterochromatin nucleates via a redundant pathway targeted by Aft1/Pcr1 via Clr3; REII centomere proximal to *mat2-P*, and REIII centromere proximal to *mat3-M* (grey boxes).

Despite a physical distance of 11 kb between *mat2-P* and *mat-3M*, the *mat2/3* interval is completely devoid of meiotic recombination (Fig. 3). This is due to tight repression of this region caused by the heterochromatin structure. Two redundant pathways are involved in establishing heterochromatin in the mating-type region. One pathway is mediated by the RNAi machinery, and acts via the *cenH* element in the K-region (Fig. 3). The *cenH* element is a 4.3 kb sequence that shares 96% homology with the *dg* repeats surrounding centromere II (Fig. 3) (Grewal and Klar, 1997). The RITS complex is directed to *cenH* via siRNA transcribed from this element. This results in H3K9Me by the HMT Clr4 and the binding and nucleation of Swi6 and establishment of heterochromatin causing transcriptional repression of this region (Grewal and Klar, 1997; Hall et al., 2002). In mutant strains where the K-region has been deleted an epigenetic switch occurs resulting in the cells being able to switch with low frequencies between a transcriptionally repressed and derepressed state (Grewal and Klar, 1996; Thon and Friis, 1997). The other pathway is dependent on two members of the ATF/CREB family of DNA-binding proteins; Pcr1 and Atf1, binding to the *REIII* element located just centromere-proximal to the *mat3-M* cassette (Fig. 3) (Thon et al., 1999; Jia et al., 2004; Kim et al., 2004). This results in the recruitment of the SHREC complex that in turn recruits Swi6 and heterochromatin can thus be established in the mating-type region (Yamada et al., 2005; Sugiyama et al., 2007).

Genome organisation

How the chromatin is organised at the nucleosome level has been shown and is now widely accepted (Felsenfeld and Groudine, 2003). Although the actual mechanistics of how the chromosomes condense and organise into the 30 nm fibre, and how the chromosomes adopts higher order structures, as they align on the metaphase plate preceding cell division, is only characterised to some degree (Tremethick, 2007).

Due to the physical sizes of the highly condensed metaphase chromosomes they were early on visible in very primitive light microscopes (Cremer and Cremer, 2006a). How the individual chromosomes are arranged inside the cell nucleus at interphase is still largely unknown, but by investigating how the genome is organised at interphase is of great significance in order to eventually fully understand the functional implications of nuclear architecture. Evidence is emerging indicating that the nuclear architecture is of great importance in transcriptional regulation, not just at the single gene level but also at the genome level controlling large chromatin domains or even whole chromosomes. Through investigating functional implications of subnuclear organisation the goal is to increase the understanding on how the genome is regulated as this may help in for example early diagnosis of disease.

Close proximity of two chromosomes in the nucleus can also explain high frequency of translocations between them. The implications of such events can be changes in the transcriptional programme leading to faulty gene expression. The best characterised translocation is the 9;22 translocation that results in fusion of the *BCR* and *ABL* genes causing Chronic Myeloid Leukaemia (Guasconi et al., 2005). However, if the reorganisation of the genome observed in tumour cells is a cause or a consequence of the disease state still needs to be dissected (Meaburn et al., 2007). Understanding the functions of intergenic, intra- and interchromosomal interactions will also help in advancing biotechnological applications such as stem cell differentiation and somatic cloning as these techniques are highly reliant in completely understanding the regulatory network responsible for patterning the cell upon differentiation involving both local and global chromatin organisation (Misteli, 2007).

Transcription and genome reorganisation

There are several documented events where activation of genes, or a subset of genes, results in a change in the subnuclear localisation (Sexton et al., 2007). For example, the immunoglobulin loci *IgH* and *Igk* relocate from the nuclear periphery to the interior when they are activated, while repression of the *CD2* gene by Ikaros results in a relocation of *CD2* to the pericentromeric heterochromatin (Brown et al., 1997; Kosak et al., 2002). The human CFTR gene also displays a change in subnuclear position upon activation. In this case the change is shown to be transcription dependent (Zink et al., 2004). In contrast, when the *Hoxb1* transgene is transposed into *Hoxd* of the *Hox* gene cluster it causes the chromatin to open up and the region becomes delocalised. However, this occurs without transcriptional activation indicating that perhaps delocalisation is an upstream event of transcription (Morey et al., 2008). In *S. pombe*, two gene clusters on the left arm of chromosome 1; Chr1 and Tel1, are induced during nitrogen starvation (Mata et al., 2002). When induced by nitrogen starvation these loci change their subnuclear position, moving away from the nuclear periphery to a more interior location. An event that at least for Tel1 was transcriptionally dependent (Alfredsson-Timmins et al., 2009b).

When genes are activated the local chromatin environment also changes. Gene activation is generally accompanied by changes in histone modifications, as discussed in the previous chapter 'Histone modifications', but also by a change in nucleosome positioning in the activated region (Jiang and Pugh, 2009). Genome-wide studies in the budding yeast have shown that nucleosomes are evicted from promoters of activated genes, and in some cases also from the coding region of highly activated genes (Lee et al., 2004; Shivaswamy et al., 2008).

Interestingly, transcriptional activation by nitrogen starvation in *S. pombe* causes a reduction of nucleosome density of the promoters as well as the coding regions of all the 118 genes upregulated 20 minutes after induction. Moreover, the strongly upregulated genes in the Chr1 cluster displayed a drastic nucleosome loss with only 20% of the nucleosomes remaining in the coding regions. This nucleosome loss was accompanied by an increase in H3K9Ac, an epigenetic mark of active transcription (Kristell et al., 2009).

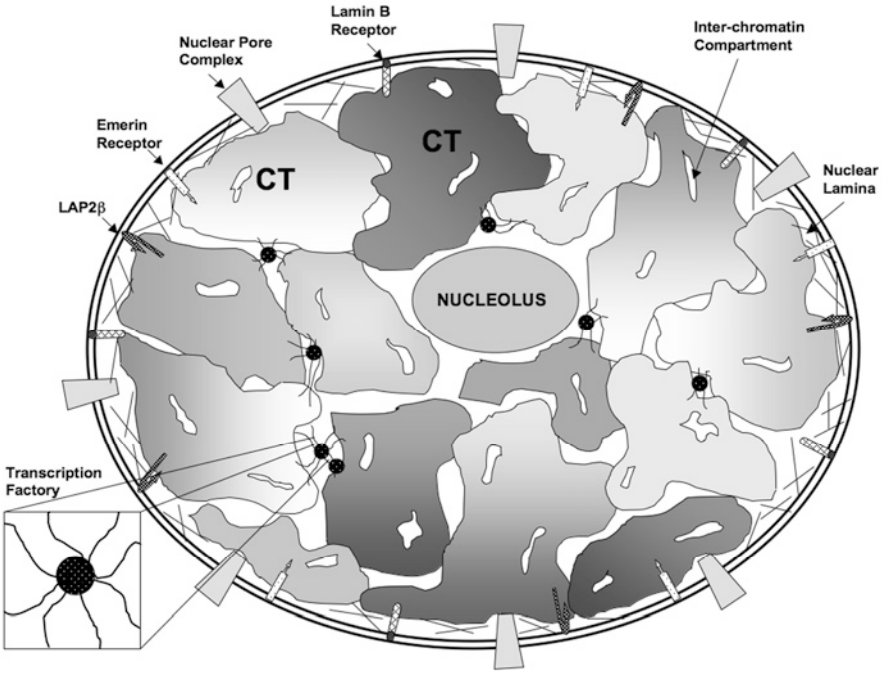

Figure 4. Proposed mammalian nuclear organisation

It is widely accepted that in mammalian cells the chromosomes occupy distinct areas inside the nucleus. These areas are known as chromosome territories (CT). At the edges of the CTs there is a certain degree of intermingling of chromosomes. The chromosomes are thought to attach to the nuclear membrane through interactions between different DNA binding protein:protein interactions, membrane receptors or nuclear lamina at the nuclear periphery. The chromosomes also adopt a radial positioning where gene-rich chromosomes localise to the nuclear interior and gene-poor to the nuclear periphery. Actively transcribed genes tend to localise on the outside of the CTs, as well as to the nuclear interior. Upon activation, genes loop out of their respective CT into the interchromatin space where they are transcribed in so called Transcription Factories. These Transcription Factories are shared by loci from different chromosomes. It has also been shown for some genes that they associate with nuclear pore structures when they are activated, a process thought to maximise mRNA export. [Modelled on Fig. 1 (Lanctot et al., 2007) and Fig. 1 (Fraser, 2006)].

Chromosome territories

The individual chromosomes occupy distinct regions inside the cell nucleus and this has been known for a long time. The areas occupied by each chromosome are known as chromosome territories (CTs), a term that was first used by Rabl and Bovery in the late 19th century (Fig. 4). However, it was not until 1977 it was first shown experimentally, using the CHO cell line from Chinese hamster in G1 by solvent fixation, that this was indeed the case.

Visualising the individual chromosomes in interphase cell nuclei was later possible using a technique called fluorescence *in situ* hybridisation (FISH) (Stack et al., 1977; Cremer et al., 1982; Cremer and Cremer, 2006a; Cremer and Cremer, 2006b). This technique involves the use of chromosome specific probes conjugated with different fluorescent conjugates. Entire individual chromosomes can then be painted by these probes and consequently be visualised in fixed cells (Cremer et al., 1988; Lichter et al., 1988).

With the use of different *in vitro* and *in vivo* labelling techniques, individual chromosomes have been visualised as almost globular domains inside the interphase nucleus (Fig. 4). Using these techniques it is also clear that each chromosome has its distinct position inside the nucleus, and that specific chromosomes tend to have the same neighbouring chromosomes. Gene-rich chromosomes are found to localise together in the nuclear interior, whereas gene-poor chromosomes localise to the nuclear periphery (Fig. 4). In addition, there also seem to be a correlation between gene-density and chromatin structure as gene-rich regions tend to be in the more open euchromatin structure while gene-poor regions are in a more compact heterochromatin structure. Furthermore, the subnuclear positioning of individual chromosomes has also been shown to be cell type as well as tissue specific (Croft et al., 1999). Interestingly, the organisation of certain chromosomes inside the nucleus is evolutionary conserved across some species. For example, in human lymphocytes chromosomes 18 and 19 occupy a peripheral and an internal position respectively, and this organisation is conserved as far back in the evolutionary tree as the Old World monkeys (Tanabe et al., 2002). It is hypothesised that maintaining this conserved chromosome organisation is a result of optimising evolutionary conserved intracellular signalling pathways, adding to the importance in studying subnuclear organisation in order to fully understand the functional implications of this organisation (Croft et al., 1999).

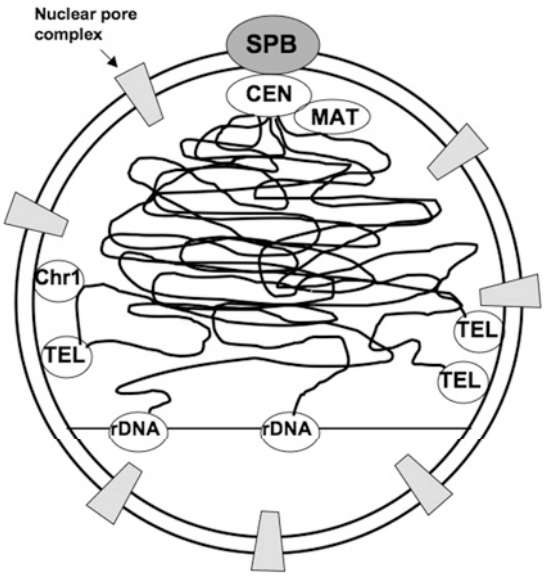

Figure 5. Subnuclear organisation in *S. pombe*

At interphase the fission yeast chromosomes adopts the so called Rabl formation inside the cell nucleus. The centromeres (CEN) localises to the nuclear periphery by attaching to the spindle pole body (SPB). The SPB is the mammalian counter part of the centriole structures and is always embedded into the nuclear membrane. The mating-type region (MAT) localises to the nuclear membrane in the vicinity of the SPB. The chromosome ends; the telomeres (TEL) and the rDNA (rDNA), localise to the nuclear membrane and the nucleolus in two to five distinct foci. A gene cluster regulated by nitrogen and positioned in the middle of the left arm of chromosome 1 (Chr1), also localise to the NM when repressed. The Chr1 locus relocates to a more interior position when induced by nitrogen

Yeast chromosomes adopt the so called 'Rabl formation' inside the cell nucleus. The centromeres cluster together by attaching to the SPB, and the telomeres and rDNA form two to five foci at the opposite side of the cell nucleus as compared to the SPB attaching to the NM or the rDNA forming the nucleolus (Fig. 5). How the rest of the genome is organised is not well understood for fission yeast. Although some evidence is emerging on how changes in gene expressions are influencing organisational changes to individual genes, gene clusters or chromosomal regions in both budding and fission yeast, in addition to factors important for organising the chromatin into higher order structures (Fig. 5) (Taddei et al., 2006; Alfredsson-Timmins et al., 2007; Alfredsson-Timmins et al., 2009b).

Organisation within chromosome territories

It is not just the nucleus that is highly organised. Each CT also has its own topology and complex organisation inside the nuclear space. Within their respective territories the chromosomes adopt a polarised organisation. Active genes tend to be found on the outside of their respective CT whilst inactive genes are found inside the CT (Ferreira et al., 1997; Zink et al., 1998; Sadoni et al., 1999; Lanctot et al., 2007). Speculations as to why this polarised organisation has been adopted results from the hypothesis that by actively transcribed genes being in physical proximity inside the cell nucleus would make them more accessible to the transcriptional machinery and other regulatory factors (Fig. 4). This closeness to components necessary for transcription would then enable them to share these components, as well as rapidly switch on transcription when necessary thereby optimising transcription. This hypothesis is supported by that fact that many regulatory components are found in specialised nuclear compartments such as nuclear speckles and PML bodies, themselves distinct in their composition and abundance, as well as the occurrence of so called transcription factories dispersed throughout the nuclear space where transcription takes place (Fig. 4) (Cremer et al., 2006; Fraser, 2006).

The influence of chromatin loops on organisation and gene regulation

How the chromatin is organised within the CTs at the local level is still under much debate. As several studies from experiments in cells from different tissues and species have provided very different results regarding the repositioning of genes or gene clusters upon activation or repression, a few independent theories have emerged (Branco and Pombo, 2007). Although these different theories on nuclear organisation differ, they all have a common denominator which is that the chromatin forms specialised loop structures as part of organisation and reorganisation when the transcriptional status is changed. Evidence has shown that giant chromatin loops, several megabases in size, extending beyond its CT upon activation play important roles in genome regulation. These loops contain one or several genes that are activated and the formation of these giant loops is thought to help optimise the transcription of these genes by actively moving them to sites where factors of the transcriptional machinery are found (Fig. 4). Giant loops of this kind are observed both in the human Major Histocompatability Complex (MHC) II as well as the mouse Hox gene cluster (Volpi et al., 2000; Chambeyron and Bickmore, 2004). Another example is the β-gene locus where upon activation an enhancer element physically interact with the locus control region (LCR) in the main body of the gene situated approximately 50 kb downstream of the enhancer element (Wijgerde et al., 1995). Perhaps the regulated expression of the genes coding for the olfactory receptor in neurons is

the most fascinating in terms of long-range chromosomal interactions. Here an enhancer, the so-called H-element, interacts with only one of thirteen hundred olfactory family genes in a particular neuron resulting in a very specific receptor expression (Kumaran et al., 2008).

The theory of forming loop structures in order to optimise transcription of a single gene or a subset of genes is in concordance with the transcription factory theory proposed by Fraser. According to this, genes loop into transcription factories when transcribed (Fig. 4) (Osborne et al., 2004; Fraser, 2006). This looping out of their respective CT was first shown in studies of the MHC in B-lympoblastoid cells. In these cells it was observed that only a subset of interferon-activated MHC class genes looped out upon activation as a response to the activation (Volpi et al., 2000). For the β-globin locus loop-formation is also observed in erythroid cells where activation involving the LCR is thought to result in the reorganisation of this locus (Ragoczy et al., 2003).

At the *Hoxb* locus the expression of the individual genes of the locus follows a strict sequential order. This sequential activation of the genes in this cluster correlates with a decondensation of the chromatin in a 3' to 5' direction resulting in a more 'open' chromatin structure when genes in this cluster are activated. Activation also coincides with a temporal looping out the individual genes out of the CT as they are activated (Chambeyron and Bickmore, 2004; Chambeyron et al., 2005).

Insulators/boundary elements in organisation

The formation of chromatin loops may also serve as gene silencers, and may be facilitated by regulatory DNA elements called insulators. There are two different types of insulators that are characterised according to their different functional properties (Gaszner and Felsenfeld, 2006). Insulators can be either enhancer-blocking insulators (EB) or boundary elements (BE). The functional role of EBs is to prevent communication between distal sequences such as enhancers and promoters thereby preventing transcriptional activation (Wallace and Felsenfeld, 2007). EBs were first discovered in the fruitfly, where the *scs* and *scs'* elements surrounding the *hsp70A* locus displayed insulator activity (Udvardy et al., 1985). Later these two elements were found to be in close proximity and possibly forming a loop structure (Blanton et al., 2003).

The main function of BEs is to act as shields protecting genes from PEV of nearby heterochromatin, and also to prevent the spread of euchromatin into heterochromatin regions disrupting these important structures (Fig. 1). Preventing the spread of heterochromatin into transcriptionally active regions is important as incorrect silencing of genes could have detrimental effects as well as incorrect activation if euchromatin spreads. The E and I elements surrounding the HMR mating-type locus in budding yeast act as such borders between silent and active chromatin (Dorman et al., 2007).

Although there are two subclasses of insulators some types can possess both EB as well as BE activity. This is the case of the 5'HS4 element in the β-globin locus in vertebrates. This element has binding sites for both CTCF and the transcription factors USF1 and USF2. When CTCF binds the 5'HS4 element is acts as a EB and as a BE if USF1 and USF2 is bound to this element (Dorman et al., 2007).

The IR-L and IR-R boundary elements in S. pombe

In the mating-type region of fission yeast, the boundaries between heterochromatin and the surrounding euchromatin region on either side of this region are very sharp. These borders are set up by two BEs, *IR-L* and *IR-R*. *IR-L* is centromere distal and *IR-R* centromere proximal to the *mat2/3* interval (Fig. 3). These BEs are inverted repeats and share 100% sequence identity over a 2.1 kb region. When these elements are deleted it causes the spread of euchromatin into the normally silent region. As a consequence, reporter genes inserted in this area become expressed (Thon et al., 2002). In contrast to the findings by Thon et al, experiments using strains containing the *swi6-333* allele, expressing three times the normal amount of the heterochromatin protein Swi6, the heterochromatin spread out of the silent region when the BEs *IR-L* and *IR-R* are deleted (Noma et al., 2001). In addition, the euchromatin spreads into the normally silent region in a *swi6-333* strain where the BE are deleted (Alfredsson-Timmins et al., 2009a). The evidence from these investigations demonstrates the importance of these BEs in separating the euchromatin from heterochromatin in this region. When these BEs are deleted it seems to cause spreading of both euchromatin and heterochromatin resulting in a mixed chromatin structure at the edges of the *mat2/3* region.

Evidently, heterochromatin does play an important role in transcriptional regulation as well as organising the chromatin into higher order structures. *IR-L* and *IR-R* are thought to play a role in organising the *mat2/3* chromatin. As there is evidence from several independent studies on the importance of chromatin loops in regulating gene expression it is intriguing to speculate that perhaps a loop structure is formed possibly through the physical interaction between these two BEs, *IR-L* and *IR-R*, found in the mating-type region. This is supported by the fact that these BE do contain so called B-box sequences where the factor TFIIIC binds. Besides, the maintenance of their boundary activity is reliant on having TFIIIC bound to these B-box sequences. TFIIIC in turn has been shown to cluster together at foci at the NM (Noma et al., 2006). In addition, binding of chromo domain proteins in balanced dosage to the mating-type region is important in organising the local chromatin structure (Sadaie et al., 2008; Alfredsson-Timmins et al., 2009a). Although more detailed studies are needed in order to fully elucidate the specific roles of the BEs and/or chromodomain proteins in organising the mating-type chromatin into higher order structures.

The nuclear envelope and transcription

It is now widely accepted that the nucleus is a highly structured and organised organelle inside the cell. Several independent studies have shown that this organisation correlates directly with gene expression. Inside the cell nucleus there are specialised compartments where there is active transcription and others known to be transcriptionally silent (Fig. 4). Highly compacted dense heterochromatin where there is no, or low levels of transcription, frequently localises to the inner nuclear membrane (INM) at the nuclear periphery (Brown and Silver, 2007; Fraser and Bickmore, 2007).

Localisation of genes to the nuclear periphery has generally been regarded as a means of silencing transcription. In the budding yeast it was shown that tethering a reporter gene under the control of a crippled silencer to the nuclear periphery resulted in transcriptional silencing (Andrulis et al., 1998). In the budding yeast it was later shown that this peripheral silencing is mediated via Esc1 and a pool of SIR proteins. The SIR proteins are found in distinct areas between the nuclear pores at the nuclear periphery (Hediger et al., 2002).

In human cells it has also been shown that genes localise to the nuclear periphery when silenced. This localisation often coincides with an association with lamin A (Fig. 4). Lamin A in turn is shown to bind to several LEM-domain containing membrane receptors in mammalian cells (Fig. 4) (Wagner and Krohne, 2007). This lamin A:LEM-domain receptor association is important in gene silencing in human cells as many genetic disorders harbouring mutations in the lamina genes result in genomic instability causing translocations and distorted nuclear organisation. This is for example the case in the premature ageing syndrome, Hutchinson-Gilford progeria and in muscular dystrophy (Akhtar and Gasser, 2007; Schirmer, 2008).

In vivo interactions of genes with lamina were identified in a genome-wide study in the fruitfly using a DamID technique combined with microarray profiling. Here they found a clear correlation of lamin-association and transcriptionally silent chromatin. These transcriptionally silent regions also coincided with previously identified regions of possible nuclear envelope association (Paddy et al., 1990; Pickersgill et al., 2006). DamID in human cells have also identified Lamin Associated Domains (LADs). These LADs are characterised as having low expression levels of associated genes, by containing CpG islands and binding of the repressor protein CFTC to insulator elements at the borders of the LADs (Guelen et al., 2008).

Three recent studies have also shown that nuclear membrane association is also an important function in influencing transcription in human cells (Finlan et al., 2008; Kumaran and Spector, 2008; Reddy et al., 2008). Although they were conducted independently they all made use of the same technique where the LacI is fused to a protein of the nuclear periphery and *lacO* arrays are integrated at a locus of interest together with a marker. One study used the LacI fused directly to Lamin B1 resulting in tethering the loci

to the NM. They found that the RNA transcript from the transgene next to *lacO* did accumulate (Kumaran and Spector, 2008). The other two studies used LacI fused to either Emerin or Lap2β, both nuclear receptors of the INM. Where the loci was tethered to the Emerin receptor the expression from the reporter gene next to the tethered loci reduced significantly (Reddy et al., 2008). Fusion of the LacI with Lap2β also showed a reduction of transcription in a fraction of the genes of the tagged loci in the third study. Interestingly this reduction was shown to be HDAC-dependent (Finlan et al., 2008).

Although the nuclear envelope has long been considered as a nuclear compartment associated with transcriptional silencing, the 'Gene-gating' hypothesis was proposed in the 1980's. According to this hypothesis, genes are moved to the nuclear pores in the nuclear envelope where they associate with specialised nuclear pore complexes when activated in order to maximise mRNA export (Fig. 4) (Blobel, 1985). This theory has later been supported by several studies in different model systems, but it was first shown in the budding yeast, *S. cerevisiae*. The first published study showing that actively transcribed genes localises to the nuclear periphery used a genome-wide localisation approach. They found that a large proportion of activated genes localised to the NM in a process that is highly dynamic and occur at the global level (Casolari et al., 2004). Two further investigations have supported the interaction with members of the nuclear transport machinery with the NPC. One study found the NPC to be involved in boundary functions through the NPCs component Nup2 (Ishii et al., 2002), while the second study showed interactions between Sus1 and the SAGA and Sac3-Thp1 complexes both which are important in activating transcription (Rodriguez-Navarro et al., 2004).

Peripheral activation of genes have now also been shown in a few other eukaryotes. In *D. melanogaster* transcription of the Dosage Compensation Complex (DCC) goes down if NPC genes are knocked-down. An indication that peripheral localisation of the DCC in the vicinity of the NPC could be important for activation and for mRNA export (Mendjan et al., 2006). Studies in higher eukaryotes have also implicated localisation to the nuclear periphery. During T-helper cell differentiation the IFN-γ locus shows a peripheral localisation that coincides with high levels of expression (Hewitt et al., 2004). Moreover, in immature murine erythroid cells the β-globin genes are highly active and are found at the nuclear periphery. The locus then relocates to a more interior position at a later stage when cells become differentiated (Ragoczy et al., 2006).

As the evidence shows, the nuclear membrane is a highly dynamic cellular compartment. It also suggests that the common notion where the periphery is

seen as a transcriptionally silent region does not hold for all species or cell types. It merely indicate the importance of cross-species comparative studies as well as studying several different cell types within a species in order to fully understand the complex correlation between gene regulation and genome organisation.

Models on genome organisation

There is an ongoing debate on what mechanisms are involved in setting up and maintaining subnuclear organisation. Evidence from several studies have provided some clues how this may be achieved and how regulatory elements, nuclear landmarks and transcription all play roles in these functions. To date the collective evidence have converged into several mutually exclusive models on how subnuclear organisation is set up and maintained (Branco and Pombo, 2007).

Chromosome territory-interchromatin space model

The oldest and most widely accepted model is known as the chromosome territory-interchromatin space (CT-IC) model. This model stipulates that actively transcribed genes are found at the periphery of the CT while inactive genes are found in the interior of the CT. According to the CT-IC model, active transcription occurs in specialised interchromosome compartments (Fig. 4). As further evidence has emerged the CT-IC model has later been extended to include intrachromosomal domains within the CTs, as lately it has been shown that transcription can also occur within these (Cremer and Cremer, 2001; Cremer et al., 2006). Moreover, this model supports minimal intermingling of separate chromosomes and chromatin domains, and does not incorporate potential long-range intrachromosomal interactions that actually has been observed by some experiments (Branco and Pombo, 2006; Branco and Pombo, 2007).

The lattice model

The lattice model dispels any form of higher order structures above the 30 nm fibre of the interphase chromatin. This model proposes that within the nuclear space, the chromatin fibres are organised in varying local concentrations of 10 and 30 nm fibres. This loose and open chromatin structure would allow macromolecules to move freely by diffusion across the whole nuclear space thereby easily gain access to the chromatin (Verschure et al., 2003; Dehghani et al., 2005; Branco and Pombo, 2007).

Advances in molecular techniques have revealed several intra- and interchromosomal interactions. Techniques such as the genomewide Chromo-

some Conformational Capture (4C) and cryo-FISH have provided evidence for a greater degree of intermingling of chromosomes than was previously thought (Branco and Pombo, 2006). This is in support of the lattice model. Furthermore, this model also supports the transcription factory theory as the sites of intermingling coincide with foci of active RNA polII transcription (Fig. 4), and this pattern changes when transcription is inhibited (Branco and Pombo, 2006; Zhao et al., 2006; Fraser and Bickmore, 2007).

The interchromosome network model

Evidence of intermingling of chromosomes, and the concurrence of these intermingling-sites with active transcription, brought about a new model termed the interchromosome network model (ICN). The ICN model suggests that intra- and interchromosomal interactions together with tethering of chromatin regions to nuclear landmarks are important for setting up the sunuclear network in order to establish a particular functional relationship (Branco and Pombo, 2007). Besides, observations that chromosomal translocations, a process that requires physical proximity and interchromosomal interactions, occur more frequently between specific chromosomes or genomic regions known to be in close proximity inside the nucleus could be explained by this model (Guasconi et al., 2005; Meaburn et al., 2007).

As there is still much uncertainty on how the chromatin is organised at interphase both at the local and global level, all the proposed models on subnuclear organisation are plausible and hold on their own. One possible explanation to the contradictory results obtained by independent studies regarding subnuclear organisation may merely be representative of the organisation in a particular tissue or cell type, or of a specific transcriptional status. The use of different microscope techniques could also explain the differing results (Dehghani et al., 2005). Although there are some indications on the mechanistics that help to set up and maintain subnuclear organisation, many important questions are still left unanswered. The exact role of heterochromatin, boundary elements, specific protein:protein interactions, as well as higher order structure in subnuclear genome organisation still remains to be elucidated. However, as both molecular and biochemical techniques as well as sophisticated imaging techniques are continually advancing, any uncertainties regarding genome organisation will hopefully be explained and the true organisation of the genome inside the nucleus will be resolved.

Aims

The main aims of this thesis were to investigate how the genome in *S. pombe* is organised, as well as trying to identifying what factor/s are necessary for setting up and maintaining this organisation. Additionally, to investigate how gene regulation is influenced by this organisation. More detailed aims are stated below.

Paper I

The main aim of the study in paper I was to determine the subnuclear localisation of the silent mating-type locus in interphase nuclei of cells in a wild type *S. pombe* strain. In addition, to identify what factor/s are responsible for correct localisation by studying mutant strains harbouring described *cis*- and *trans*-acting silencing mutations resulting in derepression of this normally transcriptionally silent region.

Paper II

In paper II, a follow-on study to paper I, the main aim was to determine which of the two models on the organisation of the mating-type chromatin proposed in paper I is correct. Furthermore, to investigate more in depth what drives nuclear organisation, specifically what additional factors contribute to the subnuclear localisation of the mating-type region and organisation of the local chromatin structure.

Paper III

The aim of the study in paper III was to investigate the relationship between subnuclear localisation and gene induction. The subnuclear localisation of two gene clusters; Chr1 and Tel1, both containing genes repressed by nitrogen via the histone deacetylase Clr3, as well as the gene *ssm4*$^{+}$, covered by a local heterochromatin island, were investigated; first during normal growth conditions, then when cells were starved for nitrogen.

Paper I

The Clr4 methyltransferase determines the subnuclear localization of the mating-type region in fission yeast.

In *S. pombe*, both the centromeres and the telomeres are in a heterochromatin structure. At interphase, the centomeres localise to the nuclear periphery by attaching to the SPB, while the telomeres are localised at the nuclear membrane in two to five distinct foci on the opposite side of the cell nucleus as compared to the SPB (Funabiki et al., 1993).

The mating-type region, that determines the mating-type of the fission yeast, consists of three linked loci and is found on the middle of the right arm of chromosome II (Fig. 3). *mat1* is expressed and the genetic information present here determines the mating-type of the cell. The *mat2/3* region works as a silent storage cassette and is in a heterochromatin structure. Reporter genes inserted into the *mat2/3* region become transcriptionally repressed as a consequence of the specialised chromatin structure at this locus.

The mating-type region was visualised in live fission yeast cells via the *lacO*/Lac-R system (Robinett et al., 1996). In our strains Lac-R fused to Green Fluorescent Protein (GFP) was expressed from the *his7*$^{+}$ locus and the array of *lacO* repeats were inserted at the *his2* locus approximately 25 kb downstream of *mat3*-M (Shimada et al., 2003). The *lacO* containing strains were then crossed to a strain where the nuclear membrane protein Pom152 had been labelled with the fluorochrome DsRed. In the same strain the SPB component Cut12 were labelled with the fluorochrome Cyan Fluorescent Protein (CFP). The resulting strains allowed for live cell microscopy of the *lacO* labelled loci in relation to the nuclear periphery and to the SPB. The SPB provides a fixed nuclear landmark when measuring subnuclear distances in three dimensions, which is important when using two-dimensional images for measuring three-dimensional distances.

Applying live-cell confocal microscopy the subnuclear localisation of this region could then be analysed. Firstly we investigated the subnuclear localisation of the mating-type region in a wild type strain by measuring the distance in µm between the SPB, and the mating-type region. In addition, the distance between the mating-type region and the NM was also measured. Secondly, strains harbouring known silencing mutations defective in heterochromatin formation were studied to see if the localisation of this region was different in these mutants.

We found that in interphase cells of a wild type strain the mating-type region localise together with the centromeres in the vicinity of the SPB (Fig. 5). This is the area of the *S. pombe* nucleus where the pericentromeric heterochromatin is also found (Ekwall et al., 1995). Furthermore, in a mutant

strain where the gene encoding the HMT Clr4, crucial for the formation of heterochromatin, had been deleted, the mating-type region had a random localisation in the nucleus. A similar random localisation was observed in a strain tagged at the euchromatic *cut3*$^{+}$ locus. Thus without heterochromatin formation by Clr4 the mating-type region could move freely in the nucleus like a euchromatic locus like *cut3*$^{+}$.

Furthermore, the BEs *IR-L* and *IR-R* surrounding the mating-type region were also shown to play a role in positioning the region (Fig. 3). In a strain where these BEs had been deleted the mating-type region was also displaced from its position at the proximity of the SPB, although it remained in the vicinity of the NM.

In addition, we found a correlation between transcriptional derepression with displacement of the region as in all strains that were investigated that contain mutations resulting in defective silencing, the distance between the mating-type region and the SPB was increased as compared to the wild type. Transcriptional derepression was monitored from a reporter gene inserted into the mating-type region by reverse transcriptase polymerase chain reaction (RT-PCR).

Most importantly, out of all the mutants investigated, the displacement was most severe in the Clr4 mutant, resulting in two mutually exclusive models of how the mating-type chromatin is organised in the cell nucleus.

The first model proposes that it is solely the amount of heterochromatin in the mating-type region that determines the proximity to the SPB, and all effects on chromatin organisation is a result of reduced amounts of heterochromatin in this region.
The second model postulates that the amount of Clr4 formed heterochromatin together with additional factors acting via the boundary elements *IR-L* and *IR-R* cooperate in anchoring the mating-type region to the NM in the vicinity of the SPB.

The data presented in paper I is the first published paper showing the precise localisation of the mating-type region at the SPB in living fission yeast cells, although a previous publication had indicated a localisation to the nuclear periphery in fixed cells (Noma et al., 2006). The results in this paper where we show a correlation between nuclear positioning and transcriptional states has provided some important clues as to what factors are involved in nuclear positioning of repressed chromatin. The finding that the establishment of heterochromatin is necessary for this positioning in fission yeast is in agreement with studies in higher eukaryotes. This proves the integrity of a simple model organism for studying complex molecular pathways for providing evidence for how these might work in higher eukaryotes.

Paper II

Chromo domain proteins in balanced dosage together with boundary elements cooperate in organising the mating-type chromatin in fission yeast.

This study involves some more in depth investigations on the driving forces behind nuclear organisation. Most importantly, the two models proposed in (Alfredsson-Timmins et al., 2007) on what determines the localisation of the mating-type chromatin are tested. The data presented in paper II clearly disproves the first model proposed in paper I, as well as identifies additional factors necessary for correct localisation of the *mat2/3* region.

To investigate if the delocalisation phenotype observed in the strain where the BEs *IR-L* and *IR-R* had been deleted, the *swi6-333* allele was crossed into this strain. The *swi6-333* allele results in expression of tripled amounts of the CD protein Swi6. This meant testing if the theory whereby increasing the amount of Swi6 bound to the mating-type region through over-expression could rescue the subtle delocalisation phenotype observed in the strain with deleted BEs. In the strain with tripled amounts of Swi6 and lacking the BE, the mating-type region was still delocalised from the proximity of the SPB. As a consequence, we can dismiss the first model stipulating that it is solely the amount of heterochromatin that determines the subnuclear localisation of the mating-type region. Moreover, in a wild type strain with tripled amount of Swi6 the mating-type region was also delocalised

In a wild type strain Swi6 has a distinct localisation pattern, forming two to five spots in the nucleus (Ekwall et al., 1995). Interestingly, using antibodies against Swi6 a more diffuse staining pattern of Swi6 is observed in the strain expressing tripled amounts of this protein compared to a wild type strain (Alfredsson-Timmins and Bjerling, data not shown). The diffusion of Swi6 protein in this strain could explain the enhanced silencing that is observed although the mating-type region is displaced from its normal position in the vicinity of the SPB at the NM. This is reminiscent of a study in the budding yeast where dispersed pools of Sir proteins can silence a reporter gene under a strong silencer element in the nuclear interior (Taddei et al., 2009).

Analysis of a strain where the CD protein Chp1, had been deleted also had a delocalisation phenotype. This was unexpected, as Chp1 is not needed for silencing at the mating-type locus, but Chp1 is important in initiating heterochromatin formation at the pericentromeric regions through recruiting the RNAi machinery. This is in contrast to findings in paper I where we found that deleting the gene encoding the RNAi component Dcr1 had no effect on localisation of the mating-type region.

In addition, a strain where another CD protein Chp2, a Swi6 homologue had been deleted, also had a delocalisation phenotype. In fact, this mutant had the most severe delocalisation phenotype of all the mutants. ChIP analysis of the mating-type region confirms reduced amounts of Swi6 binding to the *mat2/3* region in the *chp2Δ* strain. A previous study showed that Chp2 was associated with the insoluble fraction of the cell nucleus indicating a possible membrane interaction for Chp2.

Furthermore, a strain deleted of the HDAC *clr3*$^{+}$ also had a delocalised mating-type locus, but the delocalisation was not as severe as in the CD protein deleted strains. Interestingly, a strain with a point-mutation in *clr3*$^{+}$, the *clr3-735* allele, producing a structurally normal but enzymatically inactive Clr3 protein in normal amounts, showed an intermediate phenotype between the wild type and Clr3 deletion strain. This raises the possibility that Clr3 may have dual functions: gene repression by histone deacetylation and attachment of chromatin to the NM and/or proteins at the nuclear periphery (Sadaie et al., 2008). Interestingly, Clr3 is quite concentrated at the nuclear periphery (Bjerling et al., 2002). Moreover, Chp2 has also been shown to be part of the Clr3 containing SHREC complex (Motamedi et al., 2008).

The results from this study so far suggest that the boundary elements together with CD proteins Swi6, Chp1 and Chp2 in balanced dosage and composition cooperate in organising the mating-type chromatin. This would be in agreement with model II proposed in (Alfredsson-Timmins et al., 2007).

Paper III

Reorganization of chromatin is an early response to nitrogen starvation in *Schizosaccharomyces pombe*.

There are several documented events where genes change their subnuclear localisation, away from the nuclear periphery to a more internal localisation of the nuclear interior when induced, or where they relocalise to a more pericentric locations when transcriptionally repressed (Brown et al., 1997; Kosak et al., 2002).

In *S. pombe*, two gene clusters on the left arm of chromosome I were identified in a gene expression microarray experiment where they were found to be upregulated one hour after nitrogen starvation (Mata et al., 2002). One cluster, by us named Tel1, is located in the subtelomeric region. Tel1 is an 11 kb region containing 6 genes induced after one hour of nitrogen starvation (Alfredsson-Timmins et al., 2009b). The other cluster, named Chr1, is located on the middle of the left chromosome arm and is in no physical proximity to either the telomere or the centromere. This cluster is

22 kb in length and contains seven known or predicted genes. Out of these, six were induced one hour after nitrogen starvation in the study by Mata et al. In addition, these two regions have been identified in other studies to be under the control of the class II HDAC Clr3 (Hansen et al., 2005; Wiren et al., 2005).

Furthermore, in (Mata et al., 2002) the gene *ssm4*$^{+}$ situated in the middle of the right arm of chromosome I was also found to be upregulated by nitrogen starvation. This was interesting as this locus is covered by a small heterochromatin island containing the known heterochromatin hallmarks: H3K9Me and bound Swi6. Additionally, the binding of Swi6 to *ssm4*$^{+}$ is dependent on the HMT Clr4 (Cam et al., 2005)

Tel1, Chr1 as well as the gene *ssm4*$^{+}$ were labelled chromosomally with an array of *lacO* repeats. In the strains where the *lacO* repeats had been successfully integrated the Lac-R fused to Green Fluorescent Protein (GFP) was expressed from the *his7*$^{+}$ locus to allow visualisation of the loci in live cells (Robinett et al., 1996; Shimada et al., 2003; Alfredsson-Timmins et al., 2007).

Two genes from each gene cluster; one at the end and one in the middle of each cluster, and *ssm4*$^{+,}$ was monitored for mRNA expression by RT-PCR, first during normal growth conditions with nitrogen, then upon nitrogen starvation for 20 minutes. The expression from *act1* was used as a reference, since this is a gene not upregulated by nitrogen starvation. The gene clusters were indeed upregulated 20 minutes after nitrogen starvation in our strain background.

The cells were then applied to live-cell imaging and the clusters, Chr1 and Tel1, were found to localise at the NM at the opposite side of the cell nucleus as compared to the SPB during normal growth conditions (Fig. 5). When induced by nitrogen starvation, this upregulation coincides with a change in subnuclear positioning of both the Chr1 and the Tel1 clusters, locating away from the periphery to position more into the nuclear interior and closer to the SPB.

With a documented relocalisation for both Chr1 and Tel1 upon induction, the question was then if this change was a cause or a consequence of transcriptional activation. To try and dissect this we used 1,10-phenantroline, a transcriptional inhibitor. The cells were treated with this agent both during growth with nitrogen and during nitrogen starvation. Using this transcriptional inhibitor we showed that at least for one of the two clusters, Tel1 that the movement observed during nitrogen starvation was transcription dependent. However, for the Chr1 cluster the drug itself caused a slight effect on localisation even in the presence of nitrogen during uninduced conditions and could thereby not be confirmed as a cause of transcription.

In addition, in a strain lacking Clr3 the two gene clusters were delocalised from the NM. In fact, in a *clr3Δ* strain both the Chr1 and Tel1 loci had the same localisation in the presence and absence of nitrogen. Moreover, using a

strain with a point mutation in *clr3*$^{+}$ resulting in a structurally normal but enzymatically inactive Clr3 protein the gene clusters were partially delocalised. The strain with the *clr3-735* allele had a localisation phenotype between wild type and *clr3Δ*.

The data presented in this paper is the first documented relocalisation event of activated genes in *S. pombe.* This is the reverse to what mainly have been reported in the budding yeast, *S. cerevisiae,* where genes have been shown to move to the pores in the nuclear membrane when activated. The fact that activation coincides with a relocalisation away from the nuclear periphery towards the nuclear interior is in agreement with findings in higher eukaryotes where genes are shown to relocate to a more interior localisation of the nucleus when they become activated.

Concluding remarks

Over the past centuries, curiosity and an urge for knowledge has driven Scientists forward in their research. Finding the vital clues that would show how living organisms set up the exact right subnuclear environment in order to regulate their gene expression would be a great achievement. The research area of functional subnuclear organisation has seen many advances over the past years, much owing to simultaneous advances in molecular techniques, microscopic and image analysis as well as handling of large data sets. For a long time it was thought that the genome did not adopt a particular organisation inside the cell nucleus. The chromosomes were thought to be like 'spaghetti in a bowl', tangled up and disordered. This notion changed as experimental advances meant subnuclear structures could be studied more closely. Interestingly, as results from independent studies indicate contrasting findings it has become clear that the areas comprising subnuclear organisation and transcription are very complex indeed (Guasconi et al., 2005; Ruault et al., 2008). In a study by (Croft et al., 1999) they showed that in human cells chromosomes 18 and 19, that are approximately equal in size, adopt radial positioning inside the nucleus. The more gene dense chromosome 19 has a more internal localisation while the gene poor chromosome 18 has a more peripheral position. This was also the finding by (Cremer et al., 2003). Additionally, they also found that this localisation pattern of chromosomes 18 and 19 was altered in a high proportion of nuclei of tumour cells. In these tumour cells they found that chromosome 18 was more interiorly positioned than chromosome 19 (Cremer et al., 2003). A structured organisation where chromosomes localise in a certain CTs, where gene rich chromosomes tend to localise to the nuclear interior and gene poor to the periphery is now a common view (Fig. 4).

Chromosomes also localise in the proximity to a particular set of chromosomes, which in turn can explain why there appears to be a higher proportion of translocations between certain chromosomes in tumour cells (Parada et al., 2002; Cremer et al., 2003). However, it is worth bearing in mind that many of these studies are conducted in cell cultures and might only reflect the situation in that particular system. Though, a recently published study applying multi-colour FISH on tissue sections from different cancer tissues found that the chromatin pattern in certain cancer types were abnormal as compare to tissue from normal cells. This abnormal chromatin conformation could explain the altered expression observed in these disease cells (Murata

et al., 2007). Another recent study combining FISH and a newly developed computational analysis investigated the localisation pattern of six chromosomes in two normal cell lines as well as in cancerous cells (Marella et al., 2009). They generated a model on how these chromosomes were organised in proximity to one another in normal cells and could thereby show that the observed chromosome rearrangements in the cancerous cells were statistically different (Marella et al., 2009).

There is also the question of 'How is the chromatin attached and positioned inside the nucleus?'. Several reports have indicated nuclear lamina, lining the INM, as well as different membrane receptors of the INM to have roles in organising the chromatin (Fig. 4). Lamina and the receptors are proposed to interact with the chromatin both *in cis*, via specialised sequences spread across the genome, or *in trans* through indirect binding of proteins and protien-complexes. For example the lamin B receptor interacts with HP1, both the Emerin receptor and Lap2β interact with HDAC3 as well as Lap2β interacting with the DNA binding protein BAF at the nuclear periphery (Fig. 4). All these receptors belong to the LEM-domain family of receptors (Wagner and Krohne, 2007).

Although nuclear lamina does not exist in yeast, it was proposed through sequence analysis that the central domain of Sir4 in budding yeast shows significant homology to lamins A and C and my thus be involved in transcriptional silencing (Diffley and Stillman, 1989). Moreover, a lamin B receptor homologue has been identified in the budding yeast (Georgatos et al., 1989), and two homologues have been identified by sequence homology in *S. pombe*; *sts1* and *erg24* and theses both contain a LEM-domain (Wood et al., 2002). Interestingly, when chicken lamin B2 cDNA is expressed in fission yeast it assembles into a structure that associates with the nuclear membrane (Enoch et al., 1991).

In addition, in fission yeast, Swi6 localises to the NM where a large portion of Clr3 is also found (Ekwall et al., 1995; Bjerling et al., 2002). There these proteins could possibly interact with specific receptors of the INM thereby assisting in shaping the chromatin. Furthermore, mutants in Chp2 a Swi6 homologue in *S. pombe* caused a more severe delocalisation phenotype of the mating-type region than a Swi6 mutant (Alfredsson-Timmins et al., 2009a). Chp2 is also tightly associated with the pellet fraction indicating a possible membrane association (Sadaie et al., 2008). Chp2 is also genetically linked to Clr3 (Sadaie et al., 2008), as well as associates with the Clr3 containing SHREC complex (Motamedi et al., 2008). A point mutation in Clr3 had an intermediate delocalisation phenotype compared to the wild type and deletion of Clr3, indicating structural roles of Clr3 in organising the chromatin (Alfredsson-Timmins et al., 2009a; Alfredsson-Timmins et al., 2009b). Albeit speculations it is intriguing to implement possible roles in nuclear

organisation of HP1 family proteins and HDACs and lamin- and/or lamin receptor precursors in yeast.

How the organisation of the genome relates to its function has also proven to be important. Although as the evidence discussed in this thesis suggests, there is still some controversy regarding how this is achieved. What has become evident is that the nucleus is a complex subcellular structure where there are specialised compartments responsible for different processes. Many questions remain regarding how this complex organisation is achieved and maintained. For example, is has been shown that some genes loop out of their respective CT or move to a more interior position in the nucleus when they are activated (Fig. 4) (Chambeyron and Bickmore, 2004). However, does this relocalisation happens before activation or is it a consequence of it, and how is this movement achieved?

Blockage of transcriptional activation showed that the relocalisation of genes when induced in Murine erythroleukemia (MEL) cells is indeed a consequence of transcription (Francastel et al., 2001). Studies of nitrogen induced genes in fission yeast also indicates relocalisation of induced genes as a cause of transcription. When the transcriptional inhibitor 1,10-phenantroline was used there was no observed relocalisation of induced loci upon nitrogen starvation as was the case when no inhibitor was used (Alfredsson-Timmins et al., 2009b).

Several reports have indicated possible roles of actin and myosin I in the nucleus, perhaps indicative of active movement involving these in shaping the subnuclear landscape thus aiding in transcriptional activation or repression (de Lanerolle et al., 2005). Actin has been shown to co-purify with RNA polymerase II (Egly et al., 1984), as well as having a direct role in transcription (Rungger et al., 1979; Scheer et al., 1984). In addition to this, in a study by (Zhao et al., 1998) they showed that the SWI/SNF chromatin remodelling complex BAF associates with β-actin as well as the actin-related protein BAF53, indicating a regulatory role of actin in chromatin structure. With the discovery of a nuclear form of myosin I, Nuclear Myosin I (NMI), it provides further evidence of possible active movement inside the nucleus involving actin and NMI (Pestic-Dragovich et al., 2000).

The nucleus is a complex organelle whose functional organisation is of great importance in regulating gene expression. Through integrating molecular, imaging and computational approaches many clues to how this is achieved have increased and still is. The use of cultured cells and *in vivo* systems, including simple model organisms like fission yeast, has all contributed to this. Surely, investigations across species and disciplines will eventually lead to unravelling the many questions on how genome regulation and maintenance is achieved.

Sammanfattning på svenska

Alla levande organismers arvsmassa är uppbyggd av DNA, och allt DNA i en organism kallas för ett genom. I eukaryota organismer är genomet i sin tur fördelat på ett antal kromosomer och på kromosomerna finns generna. Eukaryoter har celler med en cellkärna, d.v.s. ett membran som skiljer genomet från den övriga cellen.

Generna kan bli upp- eller nedreglerade, d.v.s. sättas på eller stängas av, och detta kallas för genreglering. När och hur specifika gener slås av eller sätts på är väldigt viktigt. Denna reglering är vad som i sin tur gör varje art och individ unik, och är viktig för att transformera stamceller till alla de olika celltyper som finns i en multicellulär organism.

Hur generna är uttryckta i en viss cell vid ett visst tillfälle kan även ändras i respons till specifika signaler från den omliggande miljön. Om t.ex. tillgången till näring är restriktiv, temperaturen ändras eller vid olika typer av stress som vid infektioner då immunförsvaret aktiveras för att effektivt bekämpa dessa.

Om allt DNA inuti en cell sträcktes ut så skulle det bli en ca 2 m lång sträng. För att kunna få plats inuti en cellkärna så lindas DNA:t runt så kallade histonproteiner. DNA:t tillsammans med dessa histonproteiner kallas för kromatin. Histonproteinerna kan modifieras kemiskt, d.v.s. små molekyler eller proteiner kan hakas på eller av. Modifieringarna påverkar inte den underliggande DNA sekvensen, men kan i vissa fall nedärvas precis som DNA sekvenser och kallas för eipgenetiska modifieringar.

Studier i bl.a. människoceller har visat att när genomet packas så har det en viss organisation men hur denna organisation skapas och bibehållas är fortfarande till stor del inte känt. Det har visat sig att denna specifika organisation av genomet även har stor betydelse för genregleringen. Det är även känt att olika typer av modifieringar av kromatinet påverkar hur gener regleras.

I denna avhandling så har organisationen av genomet i en eukaryot modellorgaism, fissionsjästen *Schizosaccharomyces pombe,* undersökts. Denna encelliga jäst är välbeskriven och har en kromatin struktur som är väldigt likt den i högre eukaryoter som t.ex. människan. Genom att studera en enkel modellorganism där man lätt kan mutera olika faktorer som påverkar genuttryck och bildandet av kromatin, så kan man sedan utforma modeller om hur

det kan fungera i högre organismer. Målet med arbetet i denna avhandling var att undersöka hur genomet är organiserat i fissionsjästen *S. pombe* och att identifiera faktorer som är viktiga för att skapa och bibehålla denna organisation. Vidare har vi även undersökt hur denna organisation är viktig för genreglering.

Metoderna som använts för att studera detta har till stor del varit mikroskopisk analys av levande jästceller. För att kunna göra detta så har det nukleära membranet, spindelpolkroppen, samt den region av genomet vi undersökt märkts in med olika fluorokromer. Om man belyser jästcellerna med fluorocerande ljus så exciteras dessa fluorokromer och emitterar sedan ljus i olika våglängder vilka är visuella som olika färger. Detta gör att man då i levande jästceller kan se exakt vart de olika regionerna finns i cellen då de är synliga med olika färger. Bildserier av vildtyps jäststammar, vilka har normal kromatin struktur eller genuttryck, samt olika mutanter där bildandet av kromatin och/eller där genuttrycket är defekt har analyserats. Vi har även jämfört samma stam vid två olika tillväxt förhållanden. Bilderna vi tagit har analyserats genom att mäta avstånden i mikrometer (µm) från de olika referenspunkterna: nukleära membranet och spindelpolkroppen, till den delen av genomet som vi undersökt. Mätningarna har sedan testats med olika statistiska analyser för att se om organisationen påverkas när kromatinet inte bildas som det ska eller om genuttrycket är felaktigt. Då mätningarna gjorts i två dimensioner så har spindelpolkroppen, vilken är en fast struktur som är inbäddad i det nukleära membranet, fungerat som referenspunkt för att göra det möjligt med statistiska analyser av två-dimensionella mätningar.

Att bibehålla en korrekt organisation av genomet är viktigt för att genuttrycket ska vara normalt. Studier av kromatinet vid olika genetiska sjukdomar och cancer har visat att i många av dessa sjukdomsfall så är det just organiseringen av genomet som är felaktigt. Hur denna felaktiga organisering påverkar genutryck och i sin tur leder till olika sjukdomstillstånd är fortfarande i hög grad okänt och det råder oenighet i fältet. Det är också oklart om det är fel i organisationen som leder till sjukdomen eller om det är sjukdomen som leder till fel i organisationen. Genom att studera nukleär organisering i en enkel encellig eukaryot hoppas vi kunna hitta svaren på några av de många frågor angående hur det kan fungera i högre organismer.

I artikel I så har vi undersökt vad som driver nukleär organisation genom att undersöka var i *S. pombes* cellkärna som parningstypsregionen finns samt vilka faktorer som påverkar denna lokalisering. Denna region är väl undersökt i avseende på sin kromatin struktur och består till stor del av s.k. heterokromatin. Heterokromatin är hårt packat kromatin som är transkriptionellt nedtystat, d.v.s. generna i heterokromatiska regioner av genomet är avstängda. Var i cellkärnan som parningstypsregionen finns var tidigare inte helt

utrett. I denna studie så visar vi att denna region är positionerad vid spindelpolkroppen vid den nukleära periferin (Alfredsson-Timmins et al., 2007). Detta är den plats där centromererna, som även de är i heterokromatin struktur, finns (Funabiki et al., 1993). Vidare fann vi att bildandet av heterokromatin är nödvändigt för denna lokalisering då en jäststam som saknar proteinet Swi6, som är en strukturell komponent av heterokromatin, hade en delokaliserad parningstypsregion. I en annan stam som saknade ett annat protein, Clr4 vilket är nödvändigt för att Swi6 ska kunna binda in till denna region så var denna region ännu mer delokaliserad. Stammar med mutationer där den mer lokala kromatinstrukturen ändras hade även de lokaliserings defekter. Detta ledde till två olika modeller om hur parningstypsregionen blir korrekt positionerad vid SPB i den nukleära periferin. Modell 1 stipulerar att enbart mängden heterokromatin i parningstypsregionen är viktig för att korrekt positionera denna intill det nukleära membranet vid spindelpolkroppen. Modell 2 däremot föreskriver att mängden heterokromatin tillsammans med andra faktorer som verkar via de två barriärelementen som omger parningstypsregionen är viktiga for korrekt lokalisering.

I artikel nummer II så undersöker vi mer detaljerat vad som driver nukleär organisering samt testar de två modellerna i artikel I.

De s.k. barriärelementen, vilka omger parningstypsregionen är viktiga för att förhindra att heterokromatinet sprids ut ur den nedtystade regionen eller att aktivt kromatin sprids in i den normalt tysta regionen. En stam som saknar dessa är något defekt i bildandet av heterokromatin, och resulterade i att region blev aningen delokaliserad. Genom att öka den totala mängden av Swi6 proteinet inne i cellen så kan vi se om det är möjligt att kompensera för minskad mängd i parningstypsregionen i denna mutant, och se om detta resulterar i att parningstypsregionen blir lokaliserad som i en vildtyps stam. Detta gjordes genom att överuttrycka Swi6 så att det finns tre gånger mer av detta än normalt inne i cellerna. Överuttryck av Swi6 resulterade i att regionen fortfarande var delokaliserad och betyder att en av modellerna i artikel I om vad som bestämmer bildandet kunde förkastas.

Vidare fann vi att jäststammar som saknade antingen Chp1 eller Chp2, två andra proteiner som konkurrerar med Swi6 om inbindningsställen, hade även de en delokaliserad parningstypsregion. Vad det gäller Chp1 så var det ett överraskande resultat då Chp1 inte är nödvändigt för bildandet av heterokromatin i parningstypsregionen. Chp2 å andra sidan hade en ännu mer delokaliserad region än någon av de andra heterokromatin mutanterna.

Clr3 är en annan faktor viktig för bildandet av heterokromatin, då det är ett enzym som kemiskt modifierar histoner. Clr3 visade sig även det vara viktig för korrekt lokalisering av parningstypsregionen. En stam som har en punktmutation i Clr3, vilket resulterar i ett protein som saknar sin enzymatiska aktivitet, så var regionen inte lika delokaliserad som i en stam som

saknar Clr3 helt. Vilket indikerar att Clr3 kan ha fler funktioner i genomorganisation, dels en enzymatisk men kanske även en strukturell.

Hittills indikerar resultaten från denna studie att en balanserad mängd av de tre olika proteinerna Swi6, Chp1 och Chp2 är viktig för korrekt organisering och lokalisering av regionen

Hur genomet är organiserat är som nämnt viktigt för genreglering. I bagerijästen så har man dokumenterat en viss nukleär organisation och i vissa fall visat att gener som induceras, d.v.s. slås på, flyttas till porer i det nukleära membranet. I människoceller har man däremot visat i ett flertal exempel att gener flyttas från den nukleära periferin mot cellkärnans mitt när de slås på. I artikel III så undersöker vi sambandet mellan geninduktion och subnukleär lokalisering. Detta görs genom att undersöka två olika genkluster, Tel1 och Chr1. Dessa kluster blev identifierade i en studie som undersökte hur uttrycket av alla gener i *S. pombe* genomet förändrades i respons till kvävesvält. Under normal tillväxt med kväve så är dessa genkluster repressade, d.v.s. avstängda, men klustrena induceras vid kvävesvält. M.h.a. mikroskopanalys av levande jästceller så kunde vi påvisa att båda dessa kluster finns vid det nukleära membranet under normala tillväxtförhållanden. Däremot när cellerna utsätts för kvävesvält så sker en förflyttning av dessa kluster från periferin till en mer central position i cellkärnan. Vi kunde även visa att denna förflyttning är transkriptionsberoende. En s.k. transkriptionsinhibitor förhindrar att generna slås på och, i närvaro av en sådan skedde ingen förflyttning för åtminstone ett av klustrena.

Vidare så fann vi att klustrenas lokalisering till det nukleära membranet var beroende av både Clr3:s enzymatiska aktivitet samt att Clr3 även har en mer strukturell roll. Detta är intressant då man i humana celler har visat att HDAC3, som är en homolog till Clr3 behövs för att stänga av gener genom att fästa dem via HDAC3 till en receptor i det nukleära membranet.
Flytt av gener har tidigare inte påvisats i jästen *S. pombe*, och det faktum att det sker en förflyttning ifrån periferin till cellkärnans mitt precis som tidigare visats i människoceller, indikerar att kanske reorganisation av genomet när gener induceras i denna jäst liknar det som sker i människoceller.

Sammanfattningsvis så kan man säga att resultaten av studierna i denna avhandling har lett till att öka förståelsen om hur organisering och genreglering är viktig för normal genfunktion. Resultaten hittills indikerar att fissions jästen *S. pombe* påvisar ett system som är relativt likt människans. Både i avseende av organisationen av den globala samt lokala kromatinstrukturen, samt hur genreglering fungerar och hur dessa förändras när gener induceras. Ytterligare studier som undersöker fler mutanter samt andra regioner och/eller gener under induktion/repression är trots detta nödvändiga för att kunna dra mer generella slutsatser.

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