THE DYNAMIC CHROMATIN LANDSCAPE IN SACCHAROMYCES CEREVISIAE

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The accurate and faithful segregation of chromosomes during mitosis is essential for cellular survival. Current paradigms of chromosome segregation focus on the mechanical contributions of the mitotic spindle without considering the biomechanical properties of the chromatin itself. In order to further our understanding of how the inherent physical properties of chromatin contribute to cellular processes like chromosome segregation, we have examined both histone and chromatin dynamics in the budding yeast *Saccharomyces cerevisiae*. During mitosis, the mitotic spindle exerts force on the pericentromeric chromatin, which adjusts structurally to accommodate this force. By measuring the fluorescence recovery after photobleaching (FRAP), we found that histones are more dynamic in the pericentromeric region as compared to the chromosome arm, and these increased recovery rates are dependent on spindle-based tension. The tension-dependent histone dynamics in the pericentromere are dependent on the chromatin remodeling activities of the Remodels the Structure of Chromatin (RSC) and Imitation Switch (ISWI) ATP-dependent chromatin remodeling complexes. Thus, balanced histone removal and reincorporation in the pericentromere provide a mechanism for accommodation of spindle-based tension and the maintenance of chromatin packaging. Having measured the dynamic nature of histone turnover within the chromatin polymer in response to spindle-based tension, we subsequently examined the spatio-temporal fluctuations of the chromatin polymer. We combined *in vivo* chromatin motion analysis and mathematical modeling to elucidate the physical properties of the chromatin polymer underlying dynamic
fluctuations. The range of chromatin motion and its effective spring constants are found to vary along the length of the chromosome, in a manner dependent on tethering at the centromere. These polymer properties of the chromatin are dependent on both histone occupancy and cohesin packaging. As a whole, the work detailed in this dissertation contributes valuable insights into the importance of dynamic histone occupancy and chromatin motion in defining and maintaining the biomechanical polymer properties of chromosomes in vivo.
ACKNOWLEDGEMENTS

It is bittersweet to finally reach this point in my life. I’ve spent much time and effort to get here and am excited for the next challenge, but am sad that this chapter is almost over. My graduate experience at UNC Chapel Hill has been filled with both endless excitement and frustrations, but I would not change any single moment.

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express how tremendously helpful it has been for me as a student and scientist to have had so much encouragement in lab.

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“The most exciting phrase to hear in science, the one that heralds new discoveries, is not ‘Eureka!’ but ‘That’s funny...” – Issac Asimov

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Cr</td>
<td>compaction ratio</td>
</tr>
<tr>
<td>CEN</td>
<td>centromere</td>
</tr>
<tr>
<td>Gal</td>
<td>galactose</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>Glu</td>
<td>glucose</td>
</tr>
<tr>
<td>$k_s$</td>
<td>spring constant</td>
</tr>
<tr>
<td>$L_c$</td>
<td>contour length</td>
</tr>
<tr>
<td>$L_p$</td>
<td>persistence length</td>
</tr>
<tr>
<td>MSD</td>
<td>mean square displacement</td>
</tr>
<tr>
<td>Noc</td>
<td>nocodazole</td>
</tr>
<tr>
<td>paGFP</td>
<td>photoactivatable GFP</td>
</tr>
<tr>
<td>Rc</td>
<td>radius of confinement</td>
</tr>
<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
</tr>
<tr>
<td>SPB</td>
<td>spindle pole body</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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CHAPTER 1: INTRODUCTION

The integrity of the genome must be maintained throughout fundamental cellular processes including chromosome segregation. The canonical paradigms for investigating mitosis are centered on developing a “parts list” or examining the motion of the spindle. While these provide important insights into the molecular basis for faithful chromosome segregation, in this view the chromosomes are generally considered passive components of the segregation machinery. It is clear, however, that chromosomes are complex polymers whose behaviors and properties are essential components to the larger mitotic chromosome segregation apparatus (Bloom, 2008; Stephens et al., 2011; Stephens et al., 2013). My graduate work has focused on expanding our understanding of the importance of dynamics, both of histones and chromatin, in the study of cellular processes such as chromosome segregation and DNA repair.

Current and previous work from the Bloom lab described the geometric organization of the pericentromere and found it to be under tension (Bouck and Bloom, 2007; Haase et al., 2012; Stephens et al., 2011; Yeh et al., 2008). Building on that framework, chapter 2 starts with a review of mitosis and the composition of the centromere, moves into a discussion of the organization of the pericentromeric chromatin flanking the centromere, and discuss how the polymer properties of the chromatin contribute to segregation (Verdaasdonk and Bloom, 2011). Histone proteins are the fundamental level of chromatin compaction, and thus likely contribute in large part to the polymer properties of the chromosome. Chapter 3 explores the nature of histone dynamics in the pericentromere using Fluorescence Recovery After Photobleaching (FRAP) of histones H2B and H4.
We find that histones are more dynamic in the pericentromere as compared to the chromosome arm, and the increased removal and reincorporation is due to spindle-based tension. In pioneering work measuring photoactivation kinetics performed by Ryan Gardner, an undergraduate student I mentored, we show that the increased dynamics in the pericentromere are the result of increased histone removal. Further experiments reveal that the observed dynamics are, in part, the result of ATP-dependent chromatin remodeling activities of Sth1p/Nps1p (RSC complex) and Isw2p (ISWI complex). We propose that these dynamics are essential for the chromatin to accommodate spindle-based tension while maintaining proper packaging.

The second half of my graduate work shifted from histone dynamics to the dynamic motion of the chromatin polymer as a whole. As described in chapter 4, we utilized Gaussian fitting to obtain sub-pixel precision (Verdaasdonk et al., 2013a). By examining the motion of lacO/lacI-GFP labeled chromatin spots at various distances from the centromere, and comparing these to literature values, we find that the radius of confinement varies along the length of the chromosome (Verdaasdonk et al., 2013b) Chromatin close to the centromere tether point is more confined, and this confinement is dependent on tethering at the centromere. Utilizing mathematical modeling, we show that the motion of a confined and tethered bead-spring chain can capture the observed experimental dynamics. In addition, we provide novel mathematical basis for calculation of an effective spring constant from the in vivo motion of chromatin. In order to examine the molecular basis for chromatin confinement, we measured motion upon depletion of nucleosome occupancy or cohesin. Nucleosome depletion results in increased confinement and relative stiffening of the chromatin fiber. Cohesin depletion results in reduced confinement, likely the result of reduced looping activity upon depletion. Although further work is needed to better understand the polymer
properties of chromatin and how these contribute to chromosome segregation, the work here presents a basis for understanding the dynamic chromatin landscape in *Saccharomyces cerevisiae*. 
CHAPTER 2: CENTROMERES: UNIQUE CHROMATIN STRUCTURES THAT DRIVE CHROMOSOME SEGREGATION

INTRODUCTION

The canonical cell cycle is divided into interphase and mitosis (Figure 2.1). During interphase, cells undergo growth (G1 phase) and DNA replication (S phase). After S phase, cells undergo another phase of growth (G2) and prepare to enter mitosis (M phase). During mitosis the sister chromatids need to be accurately segregated to each daughter cell, thereby ensuring survival from one generation to the next. This is facilitated by a complex array of proteins that regulate the timing and accuracy of chromosome segregation. Chromosome segregation is directed by the centromere, a chromosomal locus that is required for mitosis and acts as the site of kinetochore formation. The proteinaceous kinetochore (Appendix 2.1) ensures proper segregation by linking the chromosome to the dynamic microtubules (composed of tubulin dimers), thereby forming the mitotic spindle. Because the centromere mediates chromosome segregation, it is essential that the cell form only one centromere and associated kinetochore attachment per chromosome to prevent breakage, although organisms with holocentric chromosomes, such as Caenorhabditis elegans, have attachment sites spread throughout the length of the chromosome.

Upon entry into mitosis, chromosomes condense, and the primary constriction forms at the centromere, the region of the chromosome defined by the incorporation of a histone H3 variant,

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CENPA (Moore and Roth, 2001; Sullivan and Karpen, 2004). The kinetochore is recruited to the centromere, and signalling proteins, such as cyclin-dependent kinases (CDKs), signal to microtubules originating at the centrosomes to form a bipolar spindle and attach to the kinetochores. This links the microtubules and chromosomes mechanically and through signalling processes that sense attachment and force to ensure that all chromosomes are bioriented and bound before anaphase onset (for a more detailed review of mitosis, see (Bouck et al., 2008; Cheeseman and Desai, 2008; Fukagawa and De Wulf, 2009; Santaguida and Musacchio, 2009)).

Although much is known about the organization and structure of the kinetochore, the physical structures of centromeric chromatin and pericentromeric chromatin, and their contribution to fidelity during chromosome segregation, are not as clear. Recent studies have shed new light on a range of topics, including the composition of the centromeric nucleosome, the histone modifications and variants that are unique to the centromeric chromatin, and the physical organization of this region during mitosis. These studies have expanded our understanding of how the centromere functions to ensure segregation fidelity and prevent errors that lead to aneuploidy, which can ultimately lead to cancer and diseases such as Down’s syndrome, Edwards’ syndrome and Patau’s syndrome.

In this Review we describe the distinct properties that define centromeric chromatin, including associated proteins, epigenetics and histone variants. We also examine the physical architecture of the chromatin and its spatial geometry, and discuss the mechanical and physical properties of the pericentric chromatin and the importance of maintaining the balances of forces during mitosis. From this work, it is evident that the centromere does not only serve passively as a site (or “landing pad”) for kinetochore formation, but that the underlying chromatin itself dictates, in part, the geometry and function of the kinetochore and mitotic spindle and therefore has an
THE CENTROMERE

The centromere is a unique region on the chromosome that is required for attachment to the mitotic spindle and chromosome segregation. Simple eukaryotic organisms, such as budding yeast, have point centromeres, defined by a specific DNA sequence found on all chromosomes (see below), whereas more complex eukaryotes have larger regional centromeres that are defined by hierarchical arrays of satellite repeats. Many similarities between centromeres in different species have been observed that highlight the unique nature and essential functions of the centromere.

DNA AT THE CENTROMERE

The *Saccharomyces cerevisiae* centromere DNA was the first eukaryotic centromere to be isolated (Clarke and Carbon, 1980) and is now known to have three conserved DNA sequences common to all chromosomes, a feature not seen in higher eukaryotes (Figure 2.2) (Clarke and Carbon, 1983). These are termed centromere DNA element I (CDE I) CDE II, and CDE III, and together form a 116-120 bp sequence that is sufficient to confer mitotic stability when introduced into plasmids (Clarke and Carbon, 1980; Fitzgerald-Hayes et al., 1982; Hieter et al., 1985). CDE I is 8 bp and is required for high fidelity chromosome segregation; CDE II is a 78-86 bp AT-rich region that is required for chromosome segregation; and CDE III is 26 bp and contains seven invariant nucleotides conserved across all chromosomes, the mutation of which abolishes centromere function (McGrew et al., 1986). Because they are small, not complex and have a single microtubule
attachment per chromosome, the centromeres of budding yeast and of the yeast *Kluyveromyces lactis* have been termed point centromeres.

By contrast, the regional centromeres of the fission yeast *Schizosaccharomyces pombe* are longer (between 40-100 kb), have multiple microtubule attachment sites per centromere and do not contain a conserved DNA sequence common to all chromosomes (Baum et al., 1994; Chikashige et al., 1989; Clarke et al., 1986; Joglekar et al., 2008; Sullivan, 2009). Instead, these centromeres are composed of core region 1 (cnt1), cnt2 and cnt3 bordered on either side by inverted repeat sequence L (imrL) and imrR, which are flanked by outer repeat L (otrL) and otrR (Pidoux and Allshire, 2004). These regions do not show DNA sequence homology to the centromere of budding yeast.

Centromeres have been identified in *Candida albicans* (Sanyal et al., 2004), *Neurospora crassa* (Centola and Carbon, 1994), *Arabidopsis thaliana* (Copenhaver et al., 1999), *Drosophila melanogaster* (Sun et al., 2003) and *Homo sapiens* (Schueler et al., 2001). The regional centromeres of higher eukaryotes are more difficult to study because they are large and contain arrays of tandem repeats. Indeed, human centromeres can be up to 5 Mb and contain 1-4 Mb of 171 bp α-satellite repeats (Choo, 2001; Maio, 1971; Pidoux and Allshire, 2004).

**CENTROMERE-ASSOCIATED PROTEINS**

Despite large divergence in centromere DNA sequences across organisms, CENPs are highly conserved (see (Malik and Henikoff, 2009) for a discussion of the evolutionary characteristics of centromeres). CENPs are not the only centromere-associated proteins; indeed, many others, for example the budding yeast proteins chromosome transmission fidelity 3 (Ctf3) and Mif2, were discovered and named prior to identifying their centromeric associations. However, we focus on CENPA and CENPC, as they have been extensively studied and shown to be required to form centromeres and the associated kinetochores; other constitutively centromere-associated network
(CCAN) proteins are briefly described (TABLE 1). The CCAN proteins are generally classified as part of either the CENPA-containing nucleosome-associated complex (NAC) or the CENPA-containing nucleosome distal (CAD) proteins (Foltz et al., 2006; Panchenko and Black, 2009).

CENPA is a centromere-associated protein that acts as a histone variant and is required to build a fully functioning kinetochore (Blower and Karpen, 2001; Howman et al., 2000; Oegema et al., 2001; Sullivan et al., 1994). CENPA was shown to co-purify with nucleosome core particles, which indicated that it probably forms a complex with the core histones (Palmer et al., 1987). Homologues of CENPA have been identified in species from yeast to mammals. Complete loss of CENPA has been found to be lethal in every organism studied to date, although mammalian cells can tolerate a 90% reduction in the level of CENPA.

CENPC is a DNA-binding protein that associates with the inner-kinetochore plate (Saitoh et al., 1992). CENPC homologues have been identified in many model organisms, including yeasts, flies, plants and mammals, and it has been shown to be essential for proper progression through mitosis and chromosome segregation (Dawe et al., 1999; Erhardt et al., 2008; Fukagawa et al., 2001; Moore and Roth, 2001; Ogura et al., 2004; Schuh et al., 2007; Tomkiel et al., 1994). Indeed, loss of CENPC at the centromere has been shown in human cells to result in small or absent kinetochores (Tomkiel et al., 1994). CENPC localizes to CENPA-containing chromatin, and this requires CENPA; reciprocally, CENPA localization requires CENPC (Erhardt et al., 2008; Oegema et al., 2001). CENPC binds two different groups of proteins that serve distinct functions: to the Mis12 complex, which is part of the KMN network (which comprises the Knl1 complex, Mis12 complex, and Ndc80 complex) of the outer kinetochore that is also needed for recruitment of checkpoint proteins (Przewloka et al., 2011; Screpanti et al., 2011); and other CCAN components such as CENPH, CENPI, CENPK and CENPT (Carroll et al., 2010). Furthermore, CENPC, together with CENPN, binds distinct domains in CENPA to direct the assembly of other centromere and kinetochore proteins (Carroll et al., 2010).
The CENPT-CENPW subcomplex is recruited to H3-containing centromeric chromatin and has been proposed to be an alternative to CENPC for the connection between the centromere and the kinetochore. The CENPT-CENPW subcomplex functions upstream of the CENPH subcomplex (Hori et al., 2008a), which comprises CENPH, CENPI and CENPK. These proteins are recruited to the centromere by CENPC (Carroll et al., 2010). They are essential for kinetochore function in vertebrates and have been found to play a part in CENPA loading and the recruitment of other, more distal centromere complexes (Okada et al., 2006). The CENPO subcomplex (made up of CENPO, CENPP, CENPQ, and CENPR) and the similar CENP-U protein (also called CENP50) are needed to prevent premature separation upon spindle damage (Hori et al., 2008b). The CENPS subcomplex (comprising CENPS and CENPX) is required for proper and stable formation of the outer kinetochore, and localization of these proteins to the centromere requires CENPT or CENPK (Amano et al., 2009).

**HISTONES AT THE CENTROMERE**

Histone modifications and histone variants serve to demarcate unique regions of the chromosome, including the centromere. The centromeric DNA sequence is rapidly evolving and, as such, centromere function does not depend solely on primary DNA sequence, but also on the presence of essential proteins, such as histone variants (including CENPA and H2A.Z) and the local chromatin context as defined by distinct histone modifications.

**PATTERNS OF HISTONE MODIFICATIONS AT THE CENTROMERE**

The epigenetic specification of centromeres was first postulated by Earnshaw and Migeon, with the observation that the two centromeres placed on a dicentric chromosome differed in their ability to direct chromatid segregation by the presence or absence of centromeric proteins.
(Earnshaw and Migeon, 1985). This indicated that additional proteins at the centromere are required to facilitate segregation. Position effect variegation studies have shown that genes placed adjacent to centromeres lead to stochastic inheritance of gene expression (Ekwall, 2007). Subsequent studies have identified epigenetic factors required for centromere function in S. cerevisiae (Mythreye and Bloom, 2003), C. albicans (Mishra et al., 2007), S. pombe (Folco et al., 2008) and H. sapiens (Morris and Moazed, 2007).

Chromatin can be flagged up for transcriptional activation (euchromatin) or repression (heterochromatin) through the modification of the amino-terminal tails of canonical histones by methylation, acetylation and phosphorylation (Glynn et al., 2010; Sullivan, 2009). Heterochromatin surrounding the centromere is known to contribute to sister chromatid cohesion and condensation (Bernard et al., 2001; Giet and Glover, 2001; Hagstrom et al., 2002; Hendzel et al., 1997; Jager et al., 2005; Maddox et al., 2007). At regional centromeres in organisms such as humans, mice, flies and fission yeast, the nucleosomes containing the canonical H3 histone (as opposed to the variant CENPA, discussed below) are dimethylated at Lys4 (H3K4me2) (Figure 2.2), a modification associated with euchromatin. This modification is thought to be important for the physical organization of the centromere (Dunleavy et al., 2005); indeed, depletion of H3K4me2 has been shown to result in a lack of recruitment of Holliday junction recognition protein (HJURP; see below), leading to failed incorporation of CENPA (Bergmann et al., 2011).

The pericentric chromatin can also be defined by methylation (Greaves et al., 2007; Guenatri et al., 2004). For example, H3 and H4 in heterochromatic regions surrounding the centromere can be methylated at Lys9 (H3K9me2 and H3K9me3) (Guenatri et al., 2004; Sullivan and Karpen, 2004) and Lys20 (H4K20me3) (Martens et al., 2005; Peters et al., 2003), and these modifications serve to recruit proteins such as cohesin (Nonaka et al., 2002) and to maintain the structure of pericentric chromatin. The formation and maintenance of heterochromatin in pericentric chromatin has
recently been shown to require the nucleolar remodelling complex (NoRC) in mice (Guetg et al., 2010). Interestingly, loss of the heterochromatic modification H4K20me3 is thought to result in aberrant centromere function, as in humans it has been associated with the presence of cancer cells, which are characterized by a high degree of aneuploidy (Fraga et al., 2005).

In addition to methylation (correlating with silenced chromatin), centromeric histones lack acetylation, which denotes actively transcribed chromatin. The hypoacetylation of the histones and the methylation of the DNA at the centromere alter the chemical interactions of the histones and DNA and define a region distinct from traditional euchromatin and heterochromatin (Dunleavy et al., 2005; Sullivan and Karpen, 2004; Zhang et al., 2008). These modifications maintain the heterochromatic nature of centromeric chromatin, which is thought to contribute to the physical structure of the centromere as well as to sister chromatid cohesion and condensation (Bernard et al., 2001; Giet and Glover, 2001; Hagstrom et al., 2002; Hendzel et al., 1997; Jager et al., 2005; Maddox et al., 2007). Histone modifications are also essential for defining and maintaining the centromeric region. Recent evidence has shown that it is possible to engineer a human artificial chromosome (HAC) to assess the contribution of chromatin state adjacent to the centromere without perturbing all the centromeres of the cell (Cardinale et al., 2009; Nakano et al., 2008). These experiments reveal that altering the chromatin to a more closed state results in loss of essential centromere proteins as well as depletion of the H3K4me2 modification and accumulation of the H3K9me3 (found in heterochromatin adjacent to the centromere) (Nakano et al., 2008). The loss of centromere function is not solely due to loss of CENPA; instead the authors observed a hierarchical loss of various components of the inner centromere (including CENPC and CENPH) (Cardinale et al., 2009).
CENPA, AN H3 VARIANT UNIQUE TO THE CENTROMERE

CENPA is a variant of histone H3, and the homology is found mainly at the \(\alpha\)-helical carboxy-terminal histone-fold domain (Luger et al., 1997). The amino-terminal tail of CENPA is highly variable between species and is required to recruit kinetochore proteins to the centromere (Henikoff and Dalal, 2005; Van Hooser et al., 2001). The CENPA centromere targeting domain (CATD) within the histone fold domain is known to be required for centromere targeting and function (Black et al., 2004; Black et al., 2007). Work in *S. cerevisiae* has shown that specific residues within the CATD are required for interaction of CENPA with suppressor of chromosome missegregation 3 (Scm3) (Zhou et al., 2011). At point centromeres, such as those of budding yeast, a single CENPA-containing nucleosome forms the basis for kinetochore formation and microtubule attachment (Furuyama and Biggins, 2007). Larger regional centromeres have multiple CENPA-containing nucleosomes interspersed between canonical H3-containing nucleosomes (Blower et al., 2002). Tetrasomes of CENPA and H4 have been found to be more rigid than the H3–H4 tetramer, a feature inherent in the structure of the histones (Black et al., 2004). Furthermore, CENPA-containing nucleosomes are more prone to unwrapping and releasing the H2A–H2B dimer (Conde e Silva et al., 2007; Sekulic et al., 2010), which suggests that CENPA nucleosomes may be pliable, possibly to allow for easier removal from non-centromeric chromatin. These findings indicate that CENPA-containing nucleosomes are structurally distinct from canonical H3-H4-containing nucleosomes, and that they may facilitate the exposure of CENPA-containing nucleosomes on the outer surface at the centromere, as opposed to becoming buried within the bulk chromatin.

Since the structure of the canonical nucleosome octamer was published (Luger et al., 1997), much work has been done to determine the structure of the centromeric nucleosome. A wide range of possibilities for the structure of the centromeric protein *in vivo* have been proposed, including an octamer in which histone H3 is replaced by the variant CENPA (Blower et al., 2002; Camahort et al.,
2009; Chen et al., 2000; Foltz et al., 2006; Sekulic et al., 2010; Shelby et al., 1997), a nucleosome with only four histones (tetrasome (Williams et al., 2009) or hemisomes (Dalal et al., 2007a; Dalal et al., 2007b; Furuyama and Henikoff, 2009)) or an alternative structure containing the non-histone protein Scm3 (Furuyama and Henikoff, 2009; Mizuguchi et al., 2007) (Figure 2.3A). These different theories have been proposed in various model organisms, and more work will be needed to further understand differences in centromeric structure and to develop a cohesive model to reconcile these observations. For example, human centromeric nucleosomes are thought to be composed of eight histones, and most are homotypic octamers containing two copies of CENPA (Shelby et al., 1997). More recent work, however, has shown that about 10% of human centromeric nucleosomes form a heterotypic octamer containing one CENPA histone and one canonical H3 histone (Foltz et al., 2006). Furthermore, work in D. melanogaster has found that different compositions of the centromeric nucleosome are present: a nucleosome containing at least two copies of Centromere identifier (CID; the D. melanogaster homologue of CENPA) (Erhardt et al., 2008) and a hemisome structure composed of one copy each of H2A, H2B, H4 and CID (Dalal et al., 2007a; Dalal et al., 2007b). These experiments found that purified cross-linked centromeric nucleosomes had a molecular mass equal to half of an octamer, and were calculated by atomic force microscopy to be half as high as would be expected for an octamer (Dalal et al., 2007a; Dalal et al., 2007b). It is possible that these represent different stages of assembly or variations found at different stages of the cell cycle.

More controversial is the recent work in S. cerevisiae that has revealed a new hexameric nucleosome structure at the centromere that lacks H2A and H2B and instead contains Scm3 (Mizuguchi et al., 2007). This protein was first identified in a screen for suppressors of chromosome segregation 4 (Cse4; the S. cerevisiae homologue of CENPA, which is known as Cnp1 in S. pombe) mutants and found to interact with the histone-fold domain of Cse4, but not the essential amino-terminal domain (Chen et al., 2000). Various recent studies in both budding and fission yeast have
shown that Scm3 is a kinetochore protein that is needed to target Cse4 to the centromeric DNA and to ensure kinetochore function (Camahort et al., 2007; Camahort et al., 2009; Pidoux et al., 2009; Stoler et al., 2007; Williams et al., 2009). As such, it seems likely that the observed hexameric nucleosome represents perhaps a transitional state during assembly or that Scm3 is associated with, but not part of, the centromeric nucleosome, especially in light of recent work in budding yeast suggesting that binding of DNA to a Cse4-containing nucleosome is incompatible with Scm3 binding (Zhou et al., 2011). A homologue of Scm3 has not been identified in species other than S. cerevisiae and S. pombe, but functional and sequence analysis has shown that the mammalian HJURP and yeast Scm3 are orthologues that share the same functional domain and are required for proper deposition of CENPA (Aravind et al., 2007; Dunleavy et al., 2009; Foltz et al., 2009; Sanchez-Pulido et al., 2009; Shuaib et al., 2010). Interestingly, Scm3 was shown to prevent ubiquitylation of CENPA by Psh1 (POB3 and SPT16 histone-associated), an E3 ubiquitin ligase that is thought to control the level of CENPA and remove misincorporated protein (Hewawasam et al., 2010; Ranjitkar et al., 2010).

The physical properties of centromeric nucleosomes are starting to emerge. Intriguing findings were obtained by the crystallization of a protein fragment of CENPA (Sekulic et al., 2010). These experiments found that CENPA nucleosomes formed canonical octameric nucleosomes that wrap DNA in a left-handed manner (Figure 2.3A). However, the region of CENPA cleaved prior to crystallization is the flexible, and highly variable, amino-terminal region, and it is unclear what effect the removal of this portion of the protein has on the subsequent structure of the nucleosome (Sekulic et al., 2010). Furthermore, these experiments were carried out using a plasmid lacking centromeric DNA sequence and without any histone chaperones. Histone chaperones, such as RbAp48 (also known as RBBP4) or Scm3, are needed to assemble CENPA-containing nucleosomes in vitro and that this assembly is known to induce positive supercoiling (Dalal et al., 2007a; Furuyama et al., 2006; Furuyama and Henikoff, 2009; Lavelle et al., 2009). Thus, it is likely that the structure of
the centromeric nucleosome is not exclusively dictated by the histone particles themselves, but by the underlying DNA and histone chaperones as well.

By contrast, other studies have indicated that CENPA-containing nucleosomes induce positive supercoils, which result from right-handed wrapping of the DNA around the nucleosome (Dalal et al., 2007a; Furuyama and Henikoff, 2009). Positive supercoiling would serve to differentiate the centromere from the negatively supercoiled bulk chromatin. The experiments examining the effects of CENPA-containing nucleosomes on chromatin were carried out by in vitro reconstitution of Drosophila proteins using circular minichromosomes. DNA wrapping around nucleosomes can be detected by changes in plasmid supercoiling following protein removal. The supercoiling is topologically defined by linking number, which is the sum of twist and writhe. In this study, the linking number was changed in the presence of CENPA, which could be explained by altered supercoiling; however, this could also be explained by loss of a nucleosome. If the centromeric nucleosome is positively supercoiled, under tension the centromere nucleosome may split into two hemisomes or become more tightly wrapped in response to the applied force (Furuyama and Henikoff, 2009; Lavelle et al., 2009). The positive supercoiling of this nucleosome would focus the spindle tension on the centromere, and could serve as a place for the checkpoint to monitor attachment (Figure 2.3B).

THE H2A.Z VARIANT AT THE CENTROMERE

CENPA-containing nucleosomes contain canonical H2A, whereas the variant H2A.Z is associated with nucleosomes containing H3K4me2 (and to a smaller extent H3K9me3) in the centromere of human and mouse cells (although the H2A.Z variant is not unique to the centromere). H2A.Z is one of the most studied histone variants, and the structure of the H2A.Z-containing nucleosome has been solved by X-ray crystallography and found to be similar to the canonical H2A-
containing nucleosome (Greaves et al., 2007; Luger et al., 1997; Suto et al., 2000). Interestingly, however, the interaction between H2A.Z and the H3-H4 tetramer is destabilized owing to the differences in amino acid sequence between H2A and H2A.Z. Furthermore, an acidic region on the surface of the H2A.Z-containing nucleosomes allows interactions with non-histone proteins and serves as a signpost to direct chromatin-remodelling factors (Suto et al., 2000). H2A.Z also functions as a boundary between heterochromatin and euchromatin by antagonizing silencing. The H2A.Z-containing nucleosomes are more resistant to condensation and form a boundary between heterochromatin and euchromatin (Abbott et al., 2001; Bruce et al., 2005; Fan et al., 2002; Meneghini et al., 2003). Indeed, loss of the H2A.Z homologue in yeast (Htz1) leads to spreading of the silencing factors silent information regulator (Sir2), Sir3, and Sir4, which affects centromere function by perturbing the structural organization of the chromatin and aberrant gene expression throughout the genome.

ASSEMBLING THE CENTROMERE

Various hypotheses have been proposed to explain how the cell identifies and maintains the centromeric region, including the presence of CENPA on the parental chromatid (Shelby et al., 1997), chromatin tension and conformation (Mellone and Allshire, 2003), and heterochromatin modifications in the pericentromere (Folco et al., 2008; Henikoff et al., 2000).

CENPA LOADING

The timing of CENPA loading varies across different species. In animals such as humans and D. melanogaster, CENPA is loaded between anaphase and G1 (Ahmad and Henikoff, 2001; Jansen et al., 2007; Schuh et al., 2007; Shelby et al., 2000), plants load CENPA in late G2 (Lermontova et al.,
2007; Lermontova et al., 2006), and fission and budding yeast load CENPA in S-G2 (Pearson et al., 2004; Takahashi et al., 2005). Much work recently has centered on identifying the proteins responsible for loading and maintaining CENPA at the centromere (Mellone et al., 2009).

In addition to the CENPA targeting domain, various proteins have known roles in CENPA loading, including the human proteins RbAp46 (also known as RBBP7) and RbAp48 (which is similar to S. pombe Mis16 (Hayashi et al., 2004)), CENPH and CENPI (Fujita et al., 2007; Okada et al., 2006). Furthermore, Scm3 and its mammalian orthologue, HJURP (which is part of the CENPA prenucleosomal complex (Foltz et al., 2006; Kato et al., 2007)), are also thought to have a role in CENPA loading. Work in S. pombe has shown that Scm3 localization to the centromere requires Mis16 and Mis18, and it is thought that Scm3, Mis16 and Mis18 act as assembly factors to bring and load CENPA at the centromere (Pidoux et al., 2009; Williams et al., 2009). These data have led to a multistep model for centromere histone loading, distinguishing between licensing and loading (Mellone et al., 2009; Perpelescu et al., 2009). Licensing is proposed to occur in humans by the recruitment of MIS18 (Mis16-Mis18 in S. pombe), CENPH, CENPI, and RbAp46 and RbAp48 (Foltz et al., 2009; Fujita et al., 2007), followed by the recruitment of loading factors, such as KNL2 (Maddox et al., 2007) and HJURP (Scm3 in yeast) (Dunleavy et al., 2009; Foltz et al., 2009) to load new CENPA into centromeric chromatin, and proper spacing by the remodeling and spacing factor (RSF) complex (Perpelescu et al., 2009). Further work into the targeting, loading and proper incorporation of CENPA will continue to develop our understanding in this field.

Histone chaperones serve many functions, including recruiting, loading and removing histone proteins from chromatin, but do not form part of the chromatin itself. The histone chaperone complexes chromatin assembly factor 1 (CAF1) and histone regulator (HIR) are known to be required for histone H3 and H4 deposition and to affect chromatin structure at silent and centromeric loci in budding yeast (Kaufman et al., 1998; Sharp et al., 2002). Recently, CAF1 and HIR
have been implicated in preventing extra-centromeric incorporation of CENPA by regulating histone eviction (removal of inappropriate incorporation of histones) (Lopes da Rosa et al., 2010). Another histone chaperone, anti-silencing function (ASF1), works together with CAF1 and HIR to deposit histone H3 variants in human cells, although it is not clear whether this interaction is needed for CENPA loading (Galvani et al., 2008). Interestingly, Sir1 has been found to be present at budding yeast centromeres to and bind to a component of CAF1, helping retain it there (Sharp et al., 2003). This role for Sir1 in budding yeast indicates that, although point and regional centromeres have different mechanisms for forming and maintaining heterochromatin, both form unique chromatin architecture to maintain fidelity in chromosome segregation.

**CHROMATIN REMODELLING AT THE CENTROMERE**

Various chromosome-remodelling complexes function at the centromere to maintain the unique chromatin architecture that underlies the proper functioning of the centromere. Topoisomerase II is thought to function at the centromere in an ATP-dependent manner to remove topological linkages (decatenation), to create these linkages to ensure sister chromatids stay together (catenation) and to maintain chromatin packaging when the pericentric chromatin is under tension to provide balancing inwards force (discussed below) (Lee and Bachant, 2009). The CCAN (TABLE 1) has been shown to affect chromatin structure in the pericentromere independently of CENPA (Hori et al., 2008a). The RSC (remodels the structure of chromatin) complex in budding yeast is known to localize to the pericentromere and to maintain the chromatin structure flanking the centromeric nucleosomes (Hsu et al., 2003). The loss of this remodelling activity results in loss of fidelity in chromosome segregation and suggests that the architecture found at the centromere is needed to facilitate chromosome segregation. This activity is also found in humans: the switch-sucrose nonfermentable (SWI/SNFB) remodeling complex is related to the RSC complex of budding
yeast and also localizes to kinetochores during mitosis (Xue et al., 2000). Further research into the roles of remodelling and chromatin-modifying complexes at the centromere will advance our understanding of the structure of the centromeric chromatin and how this is maintained.

**ARCHITECTURE OF CENTROMERIC CHROMATIN**

The physical organization of the chromatin plays an important part in preventing aneuploidy during mitosis. The cohesin and condensin complexes, which are enriched in the pericentric region (Glynn et al., 2004), have been identified as major contributors to the physical organization and packaging of the mitotic chromosome.

**TENSION AND BI-ORIENTATION OF CENTROMERIC CHROMATIN**

Packaging proteins, including cohesin and condensin, serve to organize the large amounts of DNA inside the nucleus into manageable and discrete units that can be successfully segregated. In addition, these proteins allow the cell to sense bi-orientation of the chromosomes and generate tension across the centromeric DNA. Cohesin is composed of structural maintenance of chromosomes 1 (SMC1), SMC3, SCC1 and SCC3, and is needed to maintain sister chromatid cohesion. Condensin is composed of SMC2, SMC4, and other non-SMC proteins, and is needed to compact the chromosome and prevent tangles of duplex DNA, termed catenations, which can impede accurate chromosome segregation. Cohesin and condensin have been extensively reviewed recently, and we will focus on the contributions of these proteins to organizing the centromeric chromatin (Hirano, 2006; Nasmyth and Haering, 2009; Wood et al., 2010).

Cohesin and condensin are enriched at the pericentric region, a fact that confounded researchers for years, as sister centromeres are separated during metaphase when visualized *in vivo*
How could proteins that specifically bind DNA together be enriched in a region where they appear to be separated and transiently together? Two models have been proposed to reconcile these observations: transient disassociation of cohesin from pericentric chromatin, and the formation of intramolecular loops in the pericentromere, as shown in *S. cerevisiae* (Ocampo-Hafalla et al., 2007; Yeh et al., 2008).

Bi-orientation of sister chromatids during mitosis and tension generation by the mitotic spindle are both thought to require cohesin. It is essential that the chromatin not break when under tension from the mitotic spindle, this tension is accommodated by cohesin, together with the chromatin itself (Dewar et al., 2004; Tanaka et al., 2000). In addition to balancing tension, cohesin is important to organize the geometry of the centromere, ensuring that sister centromeres (and associated kinetochores) face opposite spindle poles and bi-orient (Sakuno et al., 2009). Proper bi-orientation is essential for survival to ensure that the sister chromatids are equally segregated to daughter cells. Two probably overlapping methods have been proposed to promote amphitelic attachment: error correction and geometric bias (Indjeian and Murray, 2007; Stumpff and Asbury, 2008). However, the relative contributions of these mechanisms remain unclear.

Error correction promotes detachment in the absence of tension, and this is accomplished by Aurora B (increase-in-ploidy (Ipl1) in *S. cerevisiae*). It is thought that Aurora B is spatially confined and that phosphorylation by Aurora B facilitates detachment of incorrect attachments, whereas the tension generated across correct attachments physically separates them from Aurora B activity (Maresca and Salmon, 2009, 2010; Santaguida and Musacchio, 2009).

The underlying geometry of the centromere (see below) is thought to facilitate segregation by exposing the centromere on the outer surface of the chromosome. Given the high level of conservation of proteins across different species, it is likely that this geometry displays similar conservation.
GEOMETRIC ORGANIZATION OF THE CENTROMERE

Various models have been proposed for the geometric organization of the eukaryotic centromere, including the looping model (Dalal et al., 2007a; Yeh et al., 2008; Zinkowski et al., 1991), the solenoid model (Birchler et al., 2009; Blower et al., 2002; Sullivan and Karpen, 2004) and the sinusoidal patch model (Ribeiro et al., 2010) (Figure 2.4A). The looping model proposes that the pericentric chromatin is looped out from the bulk chromatin towards the spindle pole, whereas the solenoid model proposes that the pericentric chromatin forms a coil with the CENPA-containing nucleosomes facing the spindle pole. The sinusoidal patch model attempts to explain the observed location of various CCAN proteins and the unfolding of the vertebrate kinetochore (Ribeiro et al., 2010).

These models all propose an organization that would favour CENPA-containing nucleosomes (and therefore kinetochore formation) facing the spindle pole to facilitate attachment to the kinetochore at the centromere. The sinusoidal patch model also allows for H3-containing nucleosomes to be present on the surface of the centromeric chromatin, where they have been shown to interact with the CENPT-CENPW subcomplex (Hori et al., 2008a). The heterochromatin would face inwards towards the sister chromatid, which would provide the physical basis for generating tension across chromosomes and serve as a geometric bias for biorientation.

Although geometric orientation increases the likelihood of correct amphitelic attachment, it is not essential and is dispensable for bi-orientation (Dewar et al., 2004; Indjeian and Murray, 2007). By contrast, tension is needed to correct erroneous microtubule-kinetochore attachments (Dewar et al., 2004). Tension is generated across the centromeres of sister chromatids when they are attached to opposite sides of the mitotic spindle, and this tension allows the cell to sense and correct erroneous attachments. Several lines of evidence show that CENPA is interspersed with canonical
H3 and heterochromatin, and that CENPA must be aligned outwards towards the spindle pole bodies to allow kinetochore formation and microtubule attachment, which supports the three models mentioned above.

We propose that the whole budding yeast mitotic spindle serves as a model for a single regional centromere with multiple microtubule attachments per chromosome, and the cruciform structure found at budding yeast centromeres is analogous to the looping model for more complex centromeres (Figure 2.4B) (Zinkowski et al., 1991). The cruciform structure of the pericentromere places the centromeres at the apex of the intramolecular loop loaded with cohesin, maximizing the distance between sister centromeres and thus reconciling increased cohesin and maximal spot separation during mitosis (Yeh et al., 2008). Further work has revealed that the formation of this structure is promoted by the DNA-binding components of the kinetochore (Anderson et al., 2009). This function is likely inherent in the structure of the proteins. For example, the S. cerevisiae protein Ndc10 (also known as Cbf3a) is needed to form the looping cruciform structure; it is thought to bind as a dimer, and it is possible that these dimers serve to bring two regions of chromatin together to form a loop. Given the high level of conservation in composition between yeast and higher eukaryotic kinetochores, one view is that multiple binding site kinetochores of regional centromeres are repeats of the basic kinetochore of budding yeast, as proposed by the repeated subunit hypothesis (Joglekar et al., 2009; Joglekar et al., 2008; Joglekar et al., 2006; Johnston et al., 2010; Wan et al., 2009; Zinkowski et al., 1991). However, electron microscopy work has suggested that the mammalian kinetochore is disorganized and lacks the recurring subunits proposed by the repeated subunit hypothesis (Dong et al., 2007; McEwen and Dong, 2010). A view that reconciles these perspectives is the inner-kinetochore-centromere interface resembles a woven fabric, rather than two separate fixed structures.
In addition to the effects of the kinetochore, histone variants at the centromere also contribute to the geometry of the centromere. Work in human cell lines has shown that the incorporation of H2A.Z and epigenetic modifications contribute to the spatial organization of the centromere, although the authors do not distinguish between a solenoid and a looping model (Greaves et al., 2007). Furuyama and Henikoff showed that reconstituted CENPA-containing nucleosomes from D. melanogaster wrap DNA in a right-handed manner, opposite of canonical H3-containing nucleosomes (Figure 2.3A) (Furuyama and Henikoff, 2009). The reverse-wrap nucleosome and the proposed hemisome structure combine to suggest that centromeric chromatin is packaged fundamentally differently from underlying heterochromatin, promoting the idea that these regions are excluded from the normal packaging of the chromosome (Birchler et al., 2009; Dalal et al., 2007a; Dalal et al., 2007b). This exclusion would serve to separate the centromere from the bulk chromatin to allow the formation of the kinetochore and increase the likelihood of microtubule capture. In addition, the relative enrichment of cohesin and condensin at the centromere suggests a unique physical architecture that allows stable attachment to the mitotic spindle and movement to maintain force balance during metaphase. As the microtubules exhibit dynamic instability and are always in a state of lengthening or shortening, the pericentric chromatin must allow for this movement while remaining attached. The geometry of the centromere would promote interactions between the centromere and kinetochore, beyond serving simply as a fixed site of attachment.

Genome-wide assays for chromatin looping have uncovered a high level of nuclear organization and have shown that centromeric regions tended to not interact over longer ranges and formed a cluster of centromeres lasting throughout, and facilitating progression of, the cell cycle (Duan et al., 2010). Visualization experiments correspond to these results and have shown that centromeres cluster together throughout the cell cycle. These data all suggest that centromeric
chromatin forms a geometry that promotes clustering of the centromeres at the surface of the chromosome, which reduces the region that the microtubules must ‘search’ to attach to the centromeres.

POLYMER PHYSICS AT THE CENTROMERE

The molecular architecture of the centromere – the ‘parts list’ – and the in vivo interactions between the many protein complexes that function to load and remodel the centromere, as well as aid in chromosome segregation, continue to be comprehensively studied. In addition to the valuable data these studies provide, polymer physics can be applied to the mitotic spindle to understand chromosome segregation (Bloom, 2007). Often these concepts seem counterintuitive, and it is essential to remember that the scale and forces at work inside the cell are not immediately obvious to us in our everyday lives.

CHROMOSOME SEGREGATION BY ENTROPY

Entropy (S) is a measure of the distribution of energy in a system, and can be defined by the following equation:

\[ S = k_B \ln(W), \]

in which \( k_B \) is Boltzmann’s constant (4.1 pN x nm at room temperature), \( \ln \) is the natural logarithm and \( W \) is the number of possible ways the molecule or polymer can occupy the space (Grosberg and Khokhlov, 1997). From this equation, we see that, as the number of possible conformations for a polymer increases, the entropy will also increase, which is energetically favourable and pushes the system in that direction.
Entropy has been proposed to drive segregation of molecules like chromatin (for example, in bacteria (Finan et al., 2010; Jun and Wright, 2010)). This may seem counterintuitive. However, if one considers two long connected chains (such as chromatin) in a confined space (such as the nucleus), the mixing of these chains will reduce the number of possible entropic states, whereas chain segregation will increase the number of possible conformations for each chain, making this an entropically favourable process. It is possible that the mitotic spindle serves to direct chromosome segregation during mitosis, ensuring that the proper complement of chromosomes goes to each daughter cell, but that entropic forces are responsible for segregation of the bulk chromatin (Figure 2.5A).

Furthermore, entropy is thought to have a role in removing entanglements between larger eukaryotic chromosomes before anaphase onset (Koszul and Kleckner, 2009; Marko, 2009). Even though sister chromatids are proposed to form two distinct structures, cohesin continues to hold these together until anaphase, so entropy resolves topological entanglements between chromosomes to ensure that chromosomes can segregate faithfully. Modelling of larger eukaryotic chromosomes has also shown that a force, termed depletion-attraction, results in looping of chromosomes (Marenduzzo et al., 2006a; Marenduzzo et al., 2006b) (for example, during transcription or in a proposed centromeric structure (Yeh et al., 2008)), which in turn increases the entropic segregation of chromosomes owing to the increased repulsive forces between them (Bohn and Heermann, 2011). Depletion-attraction force is generated when two large particles (in this case, separated chromosomal regions) are brought together and interact, which results in an increase in the space available for other smaller particles to occupy.

At the size scale inside the nucleus, forces such as viscosity and thermal motion dominate, whereas weight and inertia are less relevant. The maximal forces generated by microtubules on chromosomes have been measured to be 47 pN of force per microtubule (Jannink et al., 1996;
Nicklas, 1983). This number represents the stall force, and the force required to segregate the chromosome is thought to be much lower (~5 pN). Assuming that the mitotic spindle directs chromosome segregation and serves to harness thermal motion to drive segregation, the forces generated by the mitotic spindle on the chromatin must exceed the forces generated by thermal motion. To explain this concept, chromosome movement in thermal motion can be compared to pulling a boat in the ocean. A person (the mitotic spindle) must generate enough force on the boat (chromosomes) to exceed the forces of the waves (thermal motion) to pull the boat in a certain direction, and the waves will continue to push the boat in that direction.

**MAINTAINING CHROMATIN-SPINDLE FORCE BALANCE**

Although much work has been done to examine the roles of packaging complexes, such as cohesin, to maintain chromosome packaging under tension from the mitotic spindle, the contribution of the chromatin itself in maintaining the balance of forces during mitosis is not well studied (Figure 2.5B) (Chai et al., 2010; Tanaka et al., 2005; Zhang et al., 2006). Chromatin must be able to resist the forces applied by the microtubules by stretching instead of breaking, and it has been shown that reduction of the chromatin packaging (by depleting histones) is needed to maintain spindle length in response to spindle tension in budding yeast (Bouck and Bloom, 2007). The effects of pulling or pushing a material are quantified in its Young’s modulus (E), which represents the relationship of stress to strain and is measured in pascals (1 Pa = 1 N/m² = 1 kg/(m x s²)). DNA and microtubules have a similar Young’s modulus, in the order of 1-2 GPa, which is similar to hard plastics. Although it has been more challenging to determine the Young’s modulus of the whole chromosome because it is not readily reconstituted outside of the cell, experiments have measured it to be between 40 and 400 Pa (Marshall et al., 2001; Nicklas, 1983).
Thus, it seems that the relative flexibility of chromatin could be acting as a buffer or ‘shock absorber’ to temper the forces generated by the spindle on the chromatin to prevent DNA breakage but still allow for tension-sensing mechanisms to correctly establish bi-orientation. Further work into the geometry and packaging of the pericentric chromatin will elucidate the role of the chromatin spring in balancing the outward forces of the microtubules.

**THE FUTURE**

The centromere is an essential site on every eukaryotic chromosome, and errors can lead to a wide range of diseases including cancer, aneuploidy-related disorders such as Down’s syndrome, and death. Although the level of complexity and underlying chromatin sequence varies across model organisms, a fundamental group of proteins, including the histone variant CENPA, is needed for attachment and function of the centromere. The structure, deposition and regulation of the centromeric nucleosome continue to be studied in great detail. CENPA variants have been identified in a wide range of eukaryotic organisms, but the structure of the nucleosome seems to vary by organism, and it will be important to understand the effects and implications of these differences. The geometry and physical properties of the pericentric chromatin are essential to our understanding of how chromosome segregation occurs, and we must continue to expand our understanding of the forces involved. Future work will incorporate the contributions of various fields to develop a comprehensive model of centromere–kinetochore attachment and the nature of the centromeric and pericentromeric chromatin. Approaching chromosome segregation from an interdisciplinary viewpoint - by combining the molecular and the mechanical properties of the centromere and kinetochore - will allow a better understanding of how fidelity in segregation is maintained.
FIGURE 2.1 - Chromosome segregation in the cell cycle

(A) The various stages of the cell cycle are depicted. During interphase the cell undergoes growth and replication of the DNA. Upon replication of the spindle pole body and DNA, the cell undergoes a second round of growth, and subsequently enters mitosis. Mitosis is divided into prophase (when the chromatin is condensed), prometaphase (kinetochore microtubules start to interact with kinetochores), metaphase (chromosomes bi-oriented), anaphase (the sister chromatids segregate to opposite spindle poles), and telophase (chromosomes decondense). In most eukaryotes, the nuclear membrane degrades during mitosis and reforms during telophase, but this does not occur in budding yeast. (B) The microscopy images (Canman et al., 2003; Ohta et al., 2010) show chromosomes in red and microtubules forming the mitotic spindle in green. The proteinacious kinetochore forms at the centromere and mediates attachment to the spindle. Shown are images of metaphase and anaphase cells.
Bioriented chromosomes (red) attached the spindle by kinetochore microtubules at their centromeres (blue). Spindle pole bodies are shown in green and interpolar microtubules are thicker black lines.
FIGURE 2.2 - Characteristics of point and regional centromeres

The point centromeres of budding yeast form a single microtubule attachment per chromosome, whereas larger regional centromeres will form multiple attachments. The budding yeast centromere DNA is composed of the conserved centromere DNA element I (CDEI), CDEII and CDEIII. Larger regional centromeres do not contain DNA sequences, but the presence of a centromeric protein A (CENPA)-containing nucleosome is conserved. H3K4me2, histone H3 dimethylated at Lys4; H3K9me, histone H3 methylated at Lys9; H3K20me3, histone H3 trimethylated at Lys20; imr, inverted repeat sequence; otr, outer repeat.
(A) Work in different organisms on the composition and physical properties (direction of DNA wrapping and supercoiling induction) of the centromeric nucleosome has given rise to a range of different possibilities: homotypic octamer (*Saccharomyces cerevisiae* (Camahort et al., 2009; Chen et al., 2000), *Drosophila melanogaster* (Blower et al., 2002) and human cells (Blower et al., 2002; Foltz et al., 2006; Sekulic et al., 2010; Shelby et al., 1997)), heterotypic octamer (human cells (Foltz et al., 2006)), reverse octamer (molecular dynamics (Bancaud et al., 2007; Lavelle et al., 2009)), homotypic tetramer or tetrasome (*Schizosaccharomyces pombe* (Williams et al., 2009)) and heterotypic tetramer or hemisome (*S. cerevisiae* (Furuyama and Henikoff, 2009) and *D. melanogaster* (Dalal et al., 2007a; Furuyama and Henikoff, 2009)). Furthermore, two alternative structures that contain the non-histone protein Scm3 have been proposed: hexasomes (*S. cerevisiae* (Mizuguchi et al., 2007)) and trisomes (*S. cerevisiae* (Furuyama and Henikoff, 2009) and *D. melanogaster* (Furuyama and Henikoff, 2009)). (Adapted from (Black and Cleveland, 2011)).

(B) Tension on the right-handed reverse-wrapped centromeric protein A (CENPA)-containing nucleosome may cause it to split into two hemisomes (histone H2A, H2B, CENPA, and H4), and could serve as a site for the spindle assembly checkpoint to monitor attachment. HJURP, Holliday junction recognition protein.
### A.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Homotypic octamer</th>
<th>Heterotypic octamer</th>
<th>Reverse octamer (&quot;reverseome&quot;)</th>
<th>Homotypic tetramer (&quot;tetrasome&quot;)</th>
<th>Heterotypic tetramer (&quot;hemisome&quot;)</th>
<th>Scm3/HJURP-containing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 copies each of H2A, H2B, CENP-A, and H4</td>
<td>1 copy each of CENP-A and H3; 2 copies each of H2A, H2B, and H4</td>
<td>2 copies each of H2A, H2B, CENP-A, and H4</td>
<td>2 copies each of CENP-A and H4</td>
<td>1 copy each of CENP-A, H4, and Scm3/HJURP</td>
<td>2 copies each of CENP-A, H4, and Scm3/HJURP</td>
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<tr>
<td>DNA wrapping</td>
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<td>Left handed</td>
<td>Right handed</td>
<td>Left handed</td>
<td>Right handed</td>
<td>Left handed</td>
</tr>
<tr>
<td>Supercoiling</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
</tr>
</tbody>
</table>

- **Modelling**
  - *S. cerevisiae*: Chen et al., 2000; Camahort et al., 2009
  - *S. pombe*: Williams et al., 2009
  - *Drosophila*: Blower et al., 2002
  - Human/HeLa: Shelby et al., 1997; Blower et al., 2002; Foltz et al., 2006; Sekulic et al., 2010
  - Lavelle et al., 2009
  - Furuyama & Henikoff, 2009
  - Mizuguchi et al., 2007
  - Furuyama & Henikoff, 2009

### B.

- **Canonical H3-containing octamer**
- **Reverse wrapped CENP-A-containing octamer**
- Under tension, right-handed octamer (reverseome) is split into two hemisomes
- Two reverse wrapped hemisomes are formed
FIGURE 2.4 - Chromatin geometry at the centromere

(A) Three models for the organization of the regional centromere have been proposed: the looping model (Dalal et al., 2007a; Yeh et al., 2008; Zinkowski et al., 1991), the solenoid model (Birchler et al., 2009; Blower et al., 2002; Sullivan and Karpen, 2004) and the sinusoidal patch model (Ribeiro et al., 2010). The looping model proposes that the pericentric chromatin is looped out from the bulk chromatin towards the spindle pole. The solenoid model proposes that the pericentric chromatin forms a coil with centromeric protein A (CENPA)-containing nucleosomes facing the spindle pole. The sinusoidal patch model attempts to explain the observed location of various constitutively centromere-associated network (CCAN) proteins and the unfolding of the vertebrate kinetochore.

(B) The budding yeast pericentromere adopts a cruciform (C-loop) structure, which serves to place the centromere (and therefore the kinetochore) on the poleward-facing side of the chromosomes (Yeh et al., 2008). We equate the multiple loops of the looping model in part A to the whole mitotic spindle of budding yeast.
A. Regional centromere

B. Point centromere
FIGURE 2.5 - Applying the principles of polymer physics to chromosome segregation

(A) Entropic forces drive the segregation of bulk chromatin. This is because it is energetically favourable for the polymers to segregate, as this allows them to adopt higher entropic states. The mitotic apparatus provides directionality for this segregation and ensures that sister chromatids are equally segregated to daughter cells. (B) It is important that the forces present at the mitotic spindle remain balanced to prevent breakage of the chromatin while maintaining tension along the chromatin (to sense bi-orientation). The microtubules exert an outwards force (towards the spindle pole), whereas the chromatin maintains an inwards force and is flexible enough to accommodate microtubule-based tension.
TABLE 2.1 – Centromere-associated proteins

Proteins and protein complexes associated with the centromere in various model organisms (Amano et al., 2009; Foltz et al., 2006; Fukagawa and De Wulf, 2009; Ohta et al., 2010; Panchenko and Black, 2009; Przewloka and Glover, 2009). Protein names in red have been shown to be recruited directly by CENPA–containing nucleosomes (Foltz et al., 2006). Overall, proteins and complexes are organized with respect to distance from CENPA–containing nucleosomes, and names highlighted in green are proposed to be part of the CENPA–nucleosome associated complex (NAC) and names in yellow are part of the CENPA–nucleosome distal proteins (CAD) (Foltz et al., 2006; Panchenko and Black, 2009). (-) indicates protein is unknown or unidentified.

<table>
<thead>
<tr>
<th>Human</th>
<th>D. melanogaster</th>
<th>C. elegans</th>
<th>S. pombe</th>
<th>S. cerevisiae</th>
</tr>
</thead>
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<tr>
<td>CENP-A</td>
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<td>HCP-3</td>
<td>Cnp1</td>
<td>Cen4</td>
</tr>
<tr>
<td>CENP-B</td>
<td>-</td>
<td>-</td>
<td>Abpl, Cbh1, Cnh2</td>
<td>-</td>
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<tr>
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<td>CENP-C</td>
<td>HCP-1</td>
<td>Cnp3</td>
<td>Mif2</td>
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<tr>
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<td>-</td>
<td>Vis17</td>
<td>Iml3</td>
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<td>-</td>
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<td>Vis15</td>
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<td>-</td>
<td>Fta1</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
<td>SPB800</td>
<td>-</td>
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<td>CENP-W</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
<td>Fta3</td>
<td>Mcm16</td>
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<td>-</td>
<td>Mis6</td>
<td>Ctf3</td>
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<td>-</td>
<td>Sm4</td>
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<td>-</td>
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<tr>
<td>CENP-G</td>
<td>-</td>
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</tr>
</tbody>
</table>
APPENDIX 2.1

BOX – The Kinetochore

The kinetochore is a large, multiprotein complex needed to link the sister chromatids to the mitotic spindle during chromosome segregation. The physical organization of the kinetochore into a trilaminar structure (Brinkley and Stubblefield, 1966; Jokelainen, 1967) is visible in electron microscopy images of vertebrate kinetochores, and the molecular composition follows this three-layer organization (Cheeseman and Desai, 2008; Joglekar et al., 2009; Joglekar et al., 2008; Joglekar et al., 2006; Santaguida and Musacchio, 2009; Welburn and Cheeseman, 2008). The kinetochore forms on the microtubule plus end as a ‘basket’ of elongated molecules (namely Ndc80), that recruit the outer-kinetochore components of the KMN network (which comprises the Knl1 complex, the Mis12 complex, and the Ndc80 complex) (Figure 2.6). These outer-kinetochore proteins dangle from the expanded basket surface generated by Ndc80 and can interact with the chromatin and proteins of the constitutively centromere-associated network (CCAN). The purpose of the basket is to allow the outer-kinetochore components to move over a greater distance and increase the likelihood of encountering an unattached centromere. The geometry of the centromeric protein A (CENPA)-containing chromatin predisposes CENPA to be at the surface (see main text) and recruit the CENPA-containing nucleosome-associated complex (NAC) (Table 2.1). The two halves of the kinetochore can then interact and form a stable attachment, connecting the chromosome to the microtubule. The kinetochore serves several important roles during chromosome segregation: it links chromosome movement to microtubule dynamics, monitors chromosome bi-orientation and serves as a site of catalysis for synchronizing chromosome segregation with cell cycle events.
Appendix 2.2

GLOSSARY TERMS

KINETOCHORE: A multiprotein complex that assembles on centromeric DNA and mediates the attachment and movement of chromosomes along the microtubules of the mitotic spindle.

HOLOCENTRIC CHROMOSOMES: Chromosomes lacking a localized centromere and primary constriction site. In holocentric chromosomes, kinetochores are diffuse and kinetochore microtubules attach along the length of the chromosome.

CENTROSOME: Specialized organelles that duplicate during interphase and that constitutes the centre of the mitotic spindle.

CENTROMERIC CHROMATIN: The chromatin where centromeric protein A (CENPA) is incorporated, underlying the kinetochore.

PERICENTRIC CHROMATIN: The chromatin flanking the centromeric chromatin.

NUCLEOSOME: The basic structural subunit of chromatin, which consists of ~147 base pairs of DNA wrapped ~1.7 times around an octamer of histones (2 copies each of H2A, H2B, H3 and H4).

SATELLITE REPEATS: Specific DNA sequences that are repeated many times in long tandem arrays.

DICENTRIC CHROMOSOME: A chromosome that carries two centromeres, which arise from the aberrant fusion of 'naked' telomeres or interstitial double-strand breaks. These can also be experimentally generated by inserting a second conditional centromere into a chromosome.

POSITION EFFECT VARIEGATION: Variable expression of DNA sequence based on temporal or quantitative effects from adjacent chromatin; for example, if an active gene is relocated to a heterochromatic region, it can randomly be silenced.
**HEMISOME**: (Heterotypic tetramer) A proposed nucleosome structure found at the centromere and composed of one copy each of H2A, H2B, CENPA, and H4.

**TETRASOMES**: (Homotypic tetramers) Proposed nucleosome structures found at the centromere and composed of two copies each of centromeric protein A (CENPA) and H4.

**HEMISOME**: (Heterotypic tetramer) A proposed nucleosome structure found at the centromere and composed of one copy each of H2A, H2B, centromeric protein A (CENPA) and H4.

**HOMOTYPIC OCTAMERS**: In the context of nucleosome composition, octamers in which both copies of H3 have been replaced by centromeric protein A (CENPA).

**HETEROTYPIC OCTAMER**: In the context of nucleosome composition, an octamer in which only one copy of H3 has been replaced by centromeric protein A (CENPA).

**AMPHITELIC ATTACHMENT**: Connection of sister kinetochores to microtubules that emanate from opposite spindle pole bodies.

**INTRAMOLECULAR LOOP**: A loop of chromatin formed by bringing distant regions of the same sister chromatid together, as opposed to intermolecular interactions between sister chromatid pairs. It is the proposed structure of the pericentromeric chromatin in budding yeast.

**ENTROPY**: A thermodynamic property related to the state of disorder of a system.

**THERMAL MOTION**: The random motion and collision of particles owing to temperature.

**YOUNG’S MODULUS**: (Also known as elastic modulus.) A measure of the stiffness of a polymer, measured as stress divided by strain.

**STRESS**: In the context of polymer physics, stress is defined as the force per unit area and measures how a material responds to external force.

**STRAIN**: In the context of polymer physics, strain measures the deformation of the material, measured as change in length over length (ΔL/L).
CHAPTER 3: TENSION-DEPENDENT NUCLEOSOME REMODELING AT THE PERICENTROMERE IN YEAST

INTRODUCTION

Nucleosomes form the basis for packaging of DNA into chromatin. Two copies each of histones H2A, H2B, H3 and H4, are wrapped by 145-147 bp of DNA (Luger et al., 1997). Histone protein levels are tightly regulated, as both overexpression and depletion have deleterious effects, including disruption of nucleosome organization surrounding the centromere (Saunders et al., 1990). Histone genes are transcribed and the protein incorporated during DNA replication. Histone deposition is thought to occur in a step-wise manner, with H3-H4 tetramers bound first, followed by two H2A-H2B dimers (Akey and Luger, 2003; Verreault, 2000). Histone eviction has been proposed to occur in reverse, with H2A/H2B being more mobile than H3/H4 (Jamai et al., 2007; Kimura and Cook, 2001). Outside of replication-dependent histone incorporation, histones are known to be dynamic during transcription in a manner dependent on RNA polymerase II (Chen et al., 2005; Deal et al., 2010; Dion et al., 2007; Kim et al., 2007; Kimura, 2005; Lee et al., 2004; Lopes da Rosa et al., 2011; Thiriet and Hayes, 2005; Widmer et al., 1984).

Individual histones display different dynamic properties within actively transcribed or silent regions. Histone H2B is dynamic at both active and inactive loci, whereas histone H3 is dynamic

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1 This chapter previously appeared as an article in Molecular Biology of the Cell. The original citation is as follows: Verdaasdonk, J.S., Gardner, R., Stephens, A.D., Yeh, E., and Bloom, K. (2012). Tension-dependent nucleosome remodeling at the pericentromere in yeast. Mol Biol Cell 23, 2560-2570.
predominantly at active loci (Jamai et al., 2007; Pusarla and Bhargava, 2005). Histone H3 displays rapid exchange at highly transcribed regions, such as rRNA gene loci, associated with the incorporation of an H3 variant (Ahmad and Henikoff, 2002; Lopes da Rosa et al., 2011; Thiriet and Hayes, 2005). Histones are sTable during metaphase in HeLa cells when transcription is silenced, and when transcription resumes upon anaphase onset histones are found to be more dynamic (Chen et al., 2005). Similarly, histones are exchanged during a single cell cycle in yeast (Cho et al., 1998; Schwabish and Struhl, 2004).

Histone dynamics are regulated by ATP-dependent chromatin remodeling complexes, which in budding yeast include the ISWI and SWI2/SNF2 (SWI/SNF and RSC) families of chromatin remodelers (Clapier and Cairns, 2009). The ISW2 Switch (ISWI) family of is known to remodel nucleosomes as well as function as a chromatin assembly factor (Corona et al., 1999). Isw2p is the nucleosome-stimulated ATPase of the ISW2 complex in the ISWI family of chromatin remodelers that exhibits nucleosome spacing activities resulting in increased nucleosome occupancy (Flaus and Owen-Hughes, 2003; Tsukiyama et al., 1999; Whitehouse et al., 2007; Whitehouse et al., 2003). Isw2p is located throughout the nucleus in budding yeast (Figure 3.S1). Antagonistic activities of ISW2 and SWI/SNF control gene expression; ISW2 increases nucleosome occupancy to exclude SWI/SNF and silence gene expression (Tomar et al., 2009). The yeast SWI/SNF (switching defective/sucrose nonfermenting) and RSC (remodels the structure of chromatin) remodeling complexes contain the conserved homologous ATPase subunits Swi2p/Snf2p and Sth1p/Nps1p, respectively (Cairns et al., 1996; Du et al., 1998; Tsuchiya et al., 1992; Vignali et al., 2000). Sth1p/Nps1p demonstrates DNA-dependent ATPase activity resulting in the eviction of nucleosomes (Chaban et al., 2008; Erkina et al., 2010; Lorch et al., 2006; Parnell et al., 2008), is essential for mitotic growth (Cairns et al., 1996; Cao et al., 1997; Saha et al., 2002; Tsuchiya et al., 1998; Xue et al., 2000) and is located throughout the nucleus (Figure 3.S1).
RSC is required for chromatin organization in the pericentromere and kinetochore structure in budding yeast (Hsu et al., 2003; Tsuchiya et al., 1998). Loss of Sth1p/Nps1p function results in reduced histone occupancy around the centromere by nucleosome-scanning assay and ChIP (Desai et al., 2009) and leads to cell cycle arrest at G2/M. Nucleosomes flanking the centromere are subject to disruptive tension from the spindle which would require removal of mislocalized nucleosomes and reloading them in the proper position. In the absence of nucleosome removal (ie., loss of RSC), the nucleosomes flanking the centromere cannot be efficiently repositioned, become disorganized and the net effect is reduced overall occupancy (Desai et al., 2009).

The pericentromere is approximately 50kb of chromatin flanking the centromere forming a C-loop (Yeh et al., 2008). This chromatin is located in a defined region in metaphase cells (~800 by ~300 nm cylinder between the spindle pole bodies, Figure 3.1A), corresponding to the region enriched for cohesin and condensin (Stephens et al., 2011; Yeh et al., 2008). The pericentromeric chromatin acts as an elastic spring to balance the outward forces exerted by the mitotic spindle in metaphase (Bouck and Bloom, 2007; Stephens et al., 2011). To determine the contribution of histone dynamics in packaging and maintaining the metaphase chromatin spring we measured histone turnover within the pericentromere and its regulation by ATP-dependent chromatin remodeling factors. We show that regulation of histone dynamics by chromatin remodelers is important for kinetochore structure, pericentromeric chromatin organization and metaphase spindle length.
RESULTS

HISTONE DYNAMICS DIFFER IN THE PERICENTROMERE AND CHROMOSOME ARM DURING METAPHASE

We have determined the dynamics of histones H2B and H4 in the pericentromere and the chromosome arm during metaphase in *Saccharomyces cerevisiae* by measuring the half life ($t_{1/2}$) of fluorescence recovery after photobleaching (FRAP). Strains containing histone tagged with GFP and spindle pole bodies tagged with RFP (Materials and Methods, Table 3.1) allowed us to demarcate the pericentromere from chromosomal arms in a living cell. The pericentromere lies between the spindle pole bodies (Yeh et al., 2008) and the chromosome arms are distal to the spindle (Figure 3.1).

Histones within the pericentromere exhibit faster turnover rates than in the chromosome arms. Histone H2B has a half life ($t_{1/2}$) of 62 seconds in the pericentromere during metaphase compared to 87 seconds in the arm (Figures 3.1B and 3.2A, and Table 3.S1). Histone H4 has a half life of 76 seconds in the pericentromere compared to 121 seconds in the chromosome arm (Figures 3.1C and 3.2A, and Table 3.S2). H2B is more dynamic than H4 in both regions of the chromosome. For both H2B and H4, the half life values of the chromosome arm are significantly slower compared to the pericentromere (Student’s t-test, p<0.05). The final percent recoveries of histone protein were found to be similar for both regions and histones measured indicating similar levels of mobile protein (Figure 3.2B and Tables 3.S1 and 3.S2). The individual dynamics of H2B and H4 both within the pericentromere and the arms is consistent with the observations that each histone pair, H2A/H2B and H3/H4 are independently regulated (Akey and Luger, 2003; Jackson, 1987; Jin et al., 2005; Ladoux et al., 2000; Smith and Stillman, 1991; Thiriet and Hayes, 2005).
**HISTONE DYNAMICS IN THE PERICENTROMERE ARE REDUCED UPON LOSS OF SPINDLE-BASED TENSION**

To determine whether histone dynamics in the pericentromere were influenced by spindle-based tension, we treated cells with the microtubule depolymerizing drug nocodazole (noc.) and examined histone half-life. In nocodazole arrested cells, the spindle pole bodies collapse into a single diffraction-limited spot and the pericentromeric chromatin remains adjacent to the spindle pole bodies. Upon photobleaching, we observed two populations of histone recovery. There was a significant reduction in the number of cells with measurable H4 recovery dynamics in the pericentromere, with the chromosome arm largely unaffected (pericentromere: 92% untreated WT vs. 55% noc. treated; arm: 97% untreated WT vs. 85% noc. treated; Fisher’s exact test, p<0.05, Figure 3.3A). H2B also showed a decrease in cells exhibiting measurable dynamics (pericentromere: 92% untreated WT vs. 74% noc. treated; arm: 100% untreated WT vs. 83% noc. treated), but these were not found to be statistically significant (Fisher’s exact test, p<0.05, Figure 3.3A).

When histone recovery was measurable, H2B half life was significantly slowed in the pericentromere (62 seconds untreated WT vs. 121 seconds noc. treated; Student’s t-test, p<0.05, Figure 3.3B and Table 3.S1). There was no significant change in H2B recovery in the chromosome arm upon nocodazole treatment, or any significant changes in final percent recovery (Figures 3.3B and 3.3C). Therefore, upon reduction of spindle tension by nocodazole treatment, the dynamics of pericentromeric H2B and H4 are reduced.

An alternative method to reduce pericentric tension was employed by introducing a conditional allele of the kinetochore protein Nuf2 (Gal-NUF2). On galactose media the cells express NUF2 and are able to assemble the kinetochore, whereas on glucose media NUF2 expression is repressed compromising kinetochore function. Loss of Nuf2p resulted in reduced histone dynamics in the pericentromere but not the chromosome arm for both H2B and H4 (H2B: pericentromere: 62
seconds WT vs. 94 seconds Gal-NUF2 on glu; arm: 87 seconds WT vs. 86 seconds Gal-NUF2 on glu; H4: pericentromere: 76 seconds WT vs. 97 seconds Gal-NUF2 on glu; arm: 121 seconds WT vs. 135 seconds Gal-NUF2 on glu; Student’s t-test, p<0.05, Figure 3.3B and Tables 3.S1 and 3.S2). The final percent recovery of H2B was not significantly affected, whereas the final percent recovery of H4 in the pericentromere was significantly reduced (64% WT vs. 30% Gal-NUF2 on glu; Figure 3.3C), indicating a reduced level of mobile histones. Thus, like nocodazole treatment, the loss of spindle tension via reduction of kinetochore function results in significantly reduced histone dynamics in the pericentromere and not in the chromosome arm.

**INCREASED HISTONE DYNAMICS ARE THE RESULT OF INCREASED HISTONE REMOVAL**

At least two properties of histone dynamics can contribute to the observed behavior in the pericentromere: either the histones are removed from DNA more frequently in the pericentromere, or histones are replaced more quickly leaving binding sites in the arm unbound longer. In order to address these possible explanations, we examined the dynamics of H2B tagged with a photoactivatable GFP (paGFP) fluorophore (Vorvis et al., 2008).

The highly dynamic nature of proteins can be visualized using photoactivation (Figure 3.4A). As a control, we examined the dispersion characteristics of photoactivated Erg6p, a membrane protein involved in ergosterol biosynthesis (Gaber et al., 1989; Vorvis et al., 2008). Erg6p exhibited dispersion in all of the examined cells, indicative of a high level of dynamics (Figure 3.4B and Table 3.S3). Dispersion was measured by quantifying the loss of signal intensity in a 2.6 μm x 2.6 μm area over time (20 x 20 pixels, Materials and Methods). Photoactivation of H2B in the pericentromere and chromosome arm reveals that the percentage of cells showing histone dispersion is not significantly different in the pericentromere and the chromosome arm (79% vs. 83%, respectively; Fisher’s exact test, p<0.05, Figure 3.4B). The removal dynamics of histone protein are not different
in the pericentromere and chromosome arm. Therefore the increased histone dynamics observed by FRAP in the pericentromere under tension are likely the result of active processes replacing lost histones more rapidly.

Loss of spindle tension leads to a significant decrease in the percentage of cells displaying dispersion of photoactivated H2B in the pericentromere but not the chromosome arm (pericentromere: 79% untreated WT vs. 33% noc. treated; arm: 83% untreated WT vs. 46% noc. treated; Fisher’s exact test, p<0.05, Figure 3.4B). The reduced histone dispersion in the pericentromere in collapsed spindles points to reduced histone removal from the DNA in the absence of tension. The fluorescence recovery after photobleaching and photoactivation data indicate an active histone replacement time under tension, and an increased histone dwell time (slower off rate) in the pericentromere upon loss of spindle tension.

LOSS OF STH1p/NPS1p OR ISW1p LEADS TO REDUCED HISTONE TURNOVER IN THE PERICENTROMERE

To address whether chromatin remodelers are involved in nucleosome exchange at the pericentromere, we measured histone dynamics in mutations in RSC (Sth1p/Nps1p) and Isw2p. In the absence of RSC activity (nps1-105 ts allele), cells arrest in metaphase with defects in kinetochore assembly and segregation (Hsu et al., 2003; Tsuchiya et al., 1998). In the nps1-105 mutant at permissive temperature (24°C), there is a significant decrease in the percentage of cells exhibiting measurable histone-GFP recovery in the pericentromere (H2B: 92% WT vs. 60% nps1-105; H4: 92% WT vs. 58% nps1-105; Fisher’s exact test, p<0.05, Figure 3.3A and Tables 3.S1 and 3.S2). Of the cells with measurable histone recovery, the half life (t½) of H2B is significantly slowed as compared to wild-type (pericentromere: 62 seconds WT vs. 116 seconds nps1-105; arm: 87 seconds WT vs. 125 seconds nps1-105; Student’s t-test, p<0.05, Figure 3.3B). The half life of H4 is also significantly
altered in both the pericentromere and chromosome arm in nps1-105 cells as compared to wild-type (pericentromere: 76 seconds WT vs. 119 seconds nps1-105; arm: 121 seconds WT vs. 75 seconds nps1-105; Student’s t-test, p<0.05, Figure 3.3B). The final percent recovery of histone H4 in nps1-105 cells is significantly reduced from wild-type in the pericentromere but unaffected in the chromosome arm (pericentromere: 64% WT vs. 33% nps1-105; arm: 52% WT vs. 60% nps1-105; Student’s t-test, p<0.05, Figure 3.3C). Histone exchange in the pericentromere is dependent upon a fully functional RSC complex. Using the photoactivatable H2B, the fraction of cells exhibiting dispersion is unchanged (Fisher’s exact test, p<0.05, Figure 3.4B). Thus, histones are evicted in nps1-105, but the mechanisms replacing lost histones are diminished (Figures 3.3A and 3.3B).

RSC chromatin remodeling during metaphase primarily affects the histone dynamics in the pericentromere, with histone dynamics in the chromosome arm affected to a lesser degree. The nucleus-wide alteration of histone dynamics is consistent with the essential nature of STH1/NPS1. However, histones in the pericentromere more often display no measurable recovery (Figure 3.3A), indicating a regional specificity for RSC chromatin remodeling activity.

The requirement for antagonistic chromatin remodeling has been demonstrated at promoter regions to control expression levels (Erkina et al., 2010; Tomar et al., 2009). We reasoned that histone occupancy at the pericentromere may also reflect balanced chromatin remodeling. ISW2 has been found to counter the histone removal activity of SWI/SNF chromatin remodeling (Tomar et al., 2009).

In the absence of ISW2 activity (isw2Δ), the half life (t½) of both histones H2B and H4 is significantly slower in the pericentromere but not the chromosome arm as compared to wild-type cells. H2B half life slows from 62 seconds in wild-type to 103 in isw2Δ cells, and H4 half life slows from 76 seconds in wild-type cells to 119 seconds in isw2Δ cells (Student’s t-test, p<0.05, Figure 3.3B and Tables 3.51 and 3.52). Similarly, the final percent recovery is significantly lower in the
pericentromere but not the chromosome arm for both histones H2B and H4 (Student’s t-test, p<0.05, Figure 3.3C). Wild-type H2B percent recovery in the pericentromere is 58% and is reduced to 40% in isw2Δ cells. H4 percent recovery in the pericentromere is 64% in wild-type cells and is reduced to 37% in isw2Δ cells. Consistent with the non-essential nature of ISW2, there is no significant difference in the percent of cells showing measurable histone recovery between wild-type and isw2Δ cells (Fisher’s exact test, p<0.05, Figure 3.3A). As in the nps1-105 cells, there was no significant change in percent of cells exhibiting dispersion after photoactivation in isw2Δ cells as compared to wild-type (Fisher’s exact test, p<0.05, Figure 3.4B). This data suggests that the primary role for ISW2 is maintenance of nucleosome occupancy under tension by reloading histones rather than eviction, as there is no decrease in percent of cells exhibiting measurable recovery (Figure 3.3A).

CHROMATIN PACKAGING CONTRIBUTES TO KINETOCHORE ORGANIZATION

In yeast, the 16 kinetochores are clustered into a close-to-diffraction limited spot. To address whether histone occupancy is important for this organization, we examined the structure of the inner (Ame1p-GFP or Mif2p-GFP) and outer (Spc24p-GFP or Nuf2p-GFP) kinetochore (Figures 3.5A and 3.5B). From this analysis, we observed significant disruption of the kinetochore in conditions that perturb chromatin packaging.

We first examined kinetochore structure upon the depletion of histone H3, and found that the inner, but not the outer (as in Bouck and Bloom, 2007), kinetochore is disrupted (Figure 3.5C and Table 3.S4). Cells expressing the sole copy of H3 under the galactose promoter exhibit disruption of the inner kinetochore (Mif2p-GFP) in 6% of cells. Upon reduction of histone H3, 30% of the cells show disruption of the inner kinetochore, a significant increase (Fisher’s exact test, p<0.05, Figure 3.5C). Decreasing histone density specifically affects the inner kinetochore
organization leaving the microtubule binding components (Nuf2p-GFP) structurally intact. The significant disruption of the inner kinetochores observed in H3-repressed cells is not simply the disaggregation of the 16 individual kinetochores because the outer kinetochore components remains properly organized. Thus, the underlying pericentromeric chromatin contributes to the structure of the inner kinetochore and the correct linkage with the microtubule binding outer kinetochore.

Loss of RSC function (nps1-105 at restrictive temperature, 37°C) results in significant disruption of both the inner and outer kinetochores. The inner (Ame1p-GFP) and outer (Spc24p-GFP) kinetochores of nps1-105 cells are disrupted 37% and 24%, respectively, as compared to 6% and 3% in wild-type cells (Fisher’s exact test, p<0.05, Figure 3.5C and Table 3.54). The increase in disruption is more dramatic in the inner kinetochore (6% WT vs. 37% nps1-105) supporting the hypothesis that disruption of the underlying chromatin results in disrupted kinetochore organization. The disruption of the outer kinetochore (Spc24p-GFP) in nps1-105 cells may suggest a role for RSC in kinetochore organization or stability. We did not observe increased kinetochore disruption in isw2Δ cells (Fisher’s exact test, p<0.05, Figure 3.5C). Thus, nucleosome density and mobility within pericentromeric chromatin is essential in maintaining kinetochore structure.

DISCUSSION

PATTERNS OF HISTONE DYNAMICS IN METAPHASE

The proper organization of the pericentromere is essential for balancing spindle forces in metaphase as well as the attachment and alignment of sister chromatids. The work presented here provides a model for maintenance of histone occupancy in the pericentromere under tension though the balanced remodeling activities of RSC and ISW2 (Figure 3.6). The chromatin remodeling
activities of RSC and ISW2 are needed to maintain a balance of on and off rates of histones in the pericentromere. Loss of RSC activity (Figure 3.6D) results in reduced off rates (increased dwell time, Figure 3.3B) and slowed reloading of histones that are displaced (Figure 3.3B), as well as disrupted kinetochore organization (Figure 3.5C). These data are consistent with roles for RSC in both histone removal and reloading. The loss of Isw2p (Figure 3.6E) also results in slower histone dynamics (Figure 3.3B), likely due to disrupted reloading of histones. Given that ISW2 is non-essential, other remodeling complexes may contribute to reloading histones at the pericentromere. ISW2 is known to interact genetically with various components of both the INO80 chromatin remodeling complex and Chromatin Assembly Complex (CAF-1) (Collins et al., 2007; Costanzo et al., 2010; Hannum et al., 2009; Vincent et al., 2008), suggesting possible roles for these remodelers in the maintenance of histone occupancy in the pericentromere. Balanced remodeling at gene promoters has been shown to be required for maintenance of proper gene expression (Tomar et al., 2009). These experiments demonstrated synthetic lethality between Isw2 and Snf2 of the SWI/SNF chromatin remodeling complex (Nps1/Sth1 is a Snf2 homolog). The remodeling activities of RSC and ISW2 are critical for nucleosome occupancy in the pericentromere while accommodating physical tension.

During chromosome segregation, the mitotic spindle exerts an outward force on the chromosomes that exceeds the amount of force required for nucleosome eviction (Mihardja et al., 2006; Nicklas, 1983, 1988; Yan et al., 2007). We hypothesize that the eviction of nucleosomes under tension serves to equalize the tension across the pericentromeric chromatin. The cell must maintain a balance between nucleosome eviction and reloading to maintain kinetochore organization. Here we provide evidence coupling the imposition of mechanical force (spindle tension) to a distinct chemical reaction to remodel chromatin. Tension sensing is an important component of the spindle-assembly checkpoint, required for preventing aneuploidy and chromosome missegregation (Biggins and Murray, 2001; Luo et al., 2010; Musacchio and Salmon, 2007; Nicklas, 1997; Nicklas et
In order to ensure consistent tension sensing, the chromatin spring must accommodate the fluctuating forces exerted by growing and shortening microtubules without DNA breaks. This consistent tension sensing is accomplished by the balanced off and on rates dictated, at least in part, by RSC and ISW2 chromatin remodeling.

In addition to examining the dynamics of nucleosome turnover in response to tension, this work suggests an ordered sequence of histone removal and deposition (Akey and Luger, 2003; Jamai et al., 2007; Kimura and Cook, 2001; Verreault, 2000). We find that in wild-type cells H2B dynamics are more rapid than H4 (Figure 3.2A). In the absence of tension due to nocodazole treatment, H2B turnover is significantly slower (Figure 3.3B) and fewer cells exhibit H4 recovery (Figure 3.3A) in the pericentromere. Upon repression of an essential kinetochore protein (Gal-Nuf2), both H2B and H4 dynamics are slowed and H4 exhibits a lower final percent recovery, which indicates a lower mobile fraction (Figures 3.3B and 3.3C). From these data, we hypothesize that the H2A-H2B dimer must be removed to allow for H3-H4 tetramer mobility. This normal sequence of eviction and deposition seems to be abolished in the RSC mutant (nps1-105), as H4 turnover is more rapid than that of H2B in the chromosome arm (Figure 3.3B) and overall dynamics are suppressed in the pericentromere (Figure 3.3A). The observed differences in histone dynamics point to an ordered remodeling which is accomplished in large part by RSC remodeling throughout the nucleus. From these data, we can hypothesize that histone eviction as a result of physical tension occurs in a two-step manner, with H2B being more mobile than H4.

REDEFINING THE PERICENTROMERE

This research shows that the pericentromere surrounding the point centromere of budding yeast is functionally distinct from the bulk chromosome arms during mitosis. Traditionally the pericentromere is delineated by histone modifications and variants that result in the unique state of
chromatin at the centromere, which has been termed centrochromatin (Sullivan and Karpen, 2004). In addition to histone modifications and variants, the physical state of the chromatin serves to define the pericentromere. Chromatin under tension exhibits a distinct pattern of nucleosome dynamics that might functionally distinguish pericentromeric chromatin during mitosis in budding yeast.

The underlying pericentromeric chromatin is required to form kinetochore-microtubule attachments and maintain kinetochore clustering when under spindle-based tension. The platform on which the kinetochore is built depends on the sequence-specific centromere DNA as well as the flanking pericentromeric chromatin. Unlike promoters and repressors that serve as sign posts for starting or stopping transcription, the centromere DNA locus forms a node within a larger chromatin network upon which the kinetochore is built. The finding that 30% of the inner kinetochores are disrupted without disruption of the outer kinetochore in H3 repressed cells reveals that the inner kinetochore relies upon an intact chromatin foundation. In contrast, that the outer kinetochore is less dependent on the underlying chromatin. We propose that the pericentromeric chromatin surrounding a point centromere contributes to the maintenance of kinetochore organization. The structure of the underlying foundation is likely to consist of chromatin meshwork under tension, organized around nodes of Cse4p.

We have previously shown that the force produced by the mitotic spindle is not exerted in a linear fashion between the sister centromeres (Stephens et al., 2011; Yeh et al., 2008). Rather, the chromatin loops, which are radially dispersed relative to the microtubule spindle axis, provide a vector perpendicular to the site of kinetochore-microtubule attachment and microtubule lengthening and shortening axis. The consequence of this organization is that the chromatin platform occupies a larger area than the sites of microtubule or kinetochore attachment. The organization of the pericentromeric chromatin into a surface platform containing both Cse4p- and
H3-containing nucleosomes may be analogous to the organization of chromatin in larger regional centromeres where a large surface of centromere chromatin is exposed on the surface of the chromosome. It is known that pericentromeric chromatin of regional centromeres is organized into higher order structure in which inner kinetochore components (such as CENP-T/W) are known to interact with H3-containing nucleosomes (Marshall et al., 2008; Ribeiro et al., 2010; Santaguida and Musacchio, 2009). The filamentous NDC80 complex connects the inner kinetochore plate to the outer microtubule-binding side of the kinetochore by associating with CENP-T/W via the Mis12 and Knl1 complexes. By building the inner kinetochore on a larger chromatin platform instead of foci of Cse4p, the linkage from the microtubule can be distributed across a larger area of chromatin.

MATERIALS AND METHODS

YEAST STRAINS AND IMAGING

All strains in this study were constructed in the YEF473A background (Bi and Pringle, 1996) unless otherwise noted. Most proteins were tagged with fluorescent proteins through homologous recombination at the C-terminus using PCR-amplified fragments (Joglekar et al., 2009). NUF2-GFP and Gal-H3 strains were constructed as previously described (Bouck and Bloom, 2007). Tagging with photoactivatable GFP was performed as previously described (Vorvis et al., 2008).

Yeast strains were grown in YPD (2% glucose, 2% peptone, and 1% yeast extract) prior to imaging at 32°C for untreated wild-type and nocodazole treated cells and 24°C for nps1-105 and isw2Δ strains (Table 3.1, strain list). Overnight cultures were diluted into fresh YPD media several hours prior to imaging and grown to early-mid logarithmic phase. Cells were washed and imaged on 2% glucose slab media. Nocodazole treatment to depolymerize microtubules was done by adding nocodazole to a final concentration of 20 μg/mL one hour prior to imaging. Gal-H3 cells were
depleted of H3 as described in (Bouck and Bloom, 2007). Gal-Nuf2 strains were grown overnight in YPG (2% galactose, 2% peptone, and 1% yeast extract) at 24°C to early-mid logarithmic phase. Approximately one hour prior to imaging, cells were washed with water and resuspended in YPD at 24°C to repress Nuf2 expression.

Cells were imaged on a wide field Nikon Eclipse TE2000-U or Nikon FN600 (Nikon, East Rutherford, NJ, USA) microscope stand with a 100X Plan Apo 1.4 NA digital interference contrast (DIC) oil immersion lens and an Orca ER Camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan). MetaMorph 6.1 (Molecular Devices, Downington, PA, USA) was used to acquire 2x2 binned 5 plane z-series stacks every 500 nm for FRAP experiments. For characterization of kinetochore organization, unbinned 5plane z-series stacks were acquired and the single brightest (most in focus) plane was analyzed.

**FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING**

FRAP experiments were performed by acquiring a z-series in both the GFP and RFP channels prior to photobleaching. Throughout, a neutral density (ND4) filter was used to reduce photobleaching during photoacquisition. Proteins were photobleached with a single 50 ms pulse of 488nm laser light focused on the image plane in a diffraction limited spot. A 5 plane z-series of the GFP channel was acquired immediately post-bleach and every 20 seconds for 6 minutes. After the end of the GFP timelapse, a z-series of the RFP channel was taken to ensure that the cell did not enter anaphase.

The RFP spindle pole bodies were used to determine if the cells were in metaphase (spindle length between 1.4-1.8 micron) and the position of the photobleached region. For nocodazole treated cells we examined medium budded cells with fully collapsed spindle pole bodies. To ensure that our analysis in the nps1-105 mutant is also of metaphase cells, we examined spindle pole
(Spc29p-RFP) to kinetochore (Ame1p-GFP) distances in both wild-type and nps1-105 cells. Upon anaphase onset, the distance between the spindle pole and kinetochore is reduced. In wild-type cells this occurred when the spindle was approximately 1.9-2 microns, and similar values were observed in nps1-105 cells at permissive temperature (Figure 3.S2). Therefore, we can use the same spindle length criteria (between 1.4-1.8 micron) to examine metaphase histone dynamics. Gal-Nuf2 cells exhibited a metaphase arrest so we examined only cells with medium buds, spindles less than 1.8 microns, and wild-type roughly circular histone signal (corresponding to pre-anaphase nuclear shape).

Post-acquisition, maximum projection images were compiled from each z-series at each timepoint. A 5 x 5 pixel square was drawn over the photobleached region and copied to the RFP image of the spindle pole bodies. The pericentromere was defined by a rectangle corresponding to the region of cohesin enrichment, approximately 800 nm by 300 nm between the spindle pole bodies (Yeh et al., 2008). The 5x5 photobleached region was defined as pericentromeric if more than half of the pixels fell within the cohesin cylinder rectangle dimension (Figure 3.1).

To calculate half life ($t_{1/2}$) and percent protein recovery, integrated intensity values were measured at each time point for a 5 x 5 region over the photobleached spot, an unbleached region in the same nucleus, the cell background, and an unbleached cell in the same field of view. These values were further analyzed using Microsoft Excel (Microsoft, Richmond, Washington). We corrected for photobleaching during acquisition by measuring the unbleached intensity over time. The FRAP rate constant ($k$) was calculated as follows: $[F_{inf} - F(t)]/[F_{inf} - F(0)] = e^{-kt}$, where $F_{inf}$ is the average fluorescence intensity after maximum recovery, $F(t)$ is the fluorescence intensity at each time point, $F(0)$ is the fluorescence intensity at t=0 sec immediately post-bleach, $k$ is the rate constant for exponential decay, and $t$ is time. $t_{1/2}$ was calculated as $\ln(2)/k$, and percent recovery equals $[F_{inf} - F(0)]/[F_{pre} - F(0)]$. 

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Samples with a negative percent recovery were classified as displaying no measurable recovery. The number of samples exhibiting measurable recovery or not was compared to wild-type values by Fisher’s exact test of statistical significance. Fisher’s exact test is a statistical significance test used to analyze contingency tables, similar to a chi-squared test but allows for smaller sample sizes. Of the cells displaying recovery, further statistical analysis was done using Microsoft Excel. The upper and lower ranges were calculated as quartile (Q)1 – 1.5 x interquartile range (IQR) and Q3 + 1.5 x IQR. Values below or above these limits were defined as outliers. Mean averages and standard deviations of t½ and percent recovery were calculated (summarized in Tables 3.S1 and 3.S2). To determine if data sets to be compared had statistically significantly different variances, we first performed an F-test (p<0.05). Subsequently, two-tailed Student’s t-tests (type 2 or 3, depending on F-test result) were performed to determine if the values were statistically significant (p<0.05).

PHOTOACTIVATION

Similar to the FRAP technique described above, photoactivation experiments were performed by acquiring a z-series in both the GFP and RFP channels prior to photoactivation. A neutral density (ND4) filter was used to reduce photoactivation during photoacquisition. Proteins were photoactivated with 1-3 800ms pulses of 458/10nm laser light. We have also imaged these strains using 405nm excitation light exciting the full field of view as a control. A z-series of the GFP channel was acquired immediately post-bleach and every 15 seconds for 6 minutes. After the end of the GFP timelapse, a z-series of the RFP channel was taken to ensure that the cell did not enter anaphase. The RFP spindle pole bodies were used to determine if the cells were in metaphase (spindle length between 1.4-1.8 micron) and the position of the photoactivated region.
For analysis, z-series stacks were compiled into maximum projection images for each
timepoint. The background intensity value was obtained from the pre-photoactivation images.
These are defined as the average intensity of a 20x20 pixel region plus three standard deviations.
For each post-photoactivation frame, we determined the number of pixels in the region that were
brighter than the background value. We then corrected for photoactivation during acquisition by
subtracting the average intensity of a 20x20 pixel region in an unphotoactivated cell in the same
field of view. The corrected number of pixels brighter than background were plotted over time.
Proteins were characterized as dispersive or not dispersive based on the slope – dispersive samples
(such as the Erg6p control) exhibited negative slopes suggesting the spot was getting dimmer over
time. Non-dispersive samples exhibited a positive slope. The number of samples exhibiting
dispersion or not was compared to wild-type values by Fisher’s exact test of statistical significance.

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Figure 3.1 - *In vivo* photobleaching of the pericentromere and chromosome arm using spindle pole bodies as fiduciary markers.

**(A)** Diagram showing the organization of the pericentromeric chromatin in budding yeast (Yeh et al., 2008). The pericentromere is defined by the region of cohesin enrichment between the spindle pole bodies. **(B and C)** Representative images of fluorescence recovery after photobleaching (FRAP) experiments in the pericentromere (B) and chromosome arm (C). Shown is the histone-GFP signal before photobleaching, post photobleaching (with bleached area outlined by black square), 3 minutes post photobleaching, and 6 minutes post photobleaching. The color align image shows the spindle pole bodies (Spc29p-RFP) relative to the post photobleaching H2B-GFP with location of bleaching denoted by the 5x5 pixel white square. The spindle axis (solid black line) and the pericentromere (dotted black line) are shown in relation to the photobleached spot. Bar, 1 μm.
Figure 3.2 - Histones in the pericentromere are more dynamic than those of the chromosome arm.

(A) Graph of average half life in seconds measured by FRAP for histones H2B and H4 in the pericentromere and chromosome arm. Asterisks indicate statistically significant differences (Student’s t-test, p<0.05) between arm and pericentromere regions for each histone. All data are summarized in Tables 3.51 (H2B) and 3.52 (H4). (B) Graph of final percent recovery of histone fluorescence signal after photobleaching. These values are not statistically significantly different (Student’s t-test, p<0.05). Graph, mean ± standard deviation.
Figure 3.3 - Loss of spindle tension or chromatin remodeling activity results in reduced histone dynamics primarily at the pericentromere.

(A) Graph showing percent of samples showing measurable recovery after photobleaching. Asterisks indicate statistically significant differences between sample and corresponding wild-type value (Fisher’s exact test, p<0.05). (B) Graph of average histone half life (seconds). *nps1-105* at permissive temperature (24°C). (C) Graph of final histone fluorescence percent recovery. For both (A) and (B), asterisks indicate statistically significant differences between sample and corresponding wild-type value (Student’s t-test, p<0.05). All data are summarized in Tables 3.S1 (H2B) and 3.S2 (H4). Graph, mean ± standard deviation.
Figure 3.4 - Loss of spindle tension results in reduced dispersal of photoactivated histone H2B.

(A) Representative images showing nuclear region prior to photoactivation (pre activation), post photoactivation, halfway through timelapse (+3 min) and at end of timelapse (+6min). The top row shows the dispersion of the control strain containing Erg6p-paGFP. The second and third rows show representative images of dispersive (row 2) and not dispersive (row 3) H2B-paGFP. Bar, 1 μm. (B) Percentage of cells showing dispersion of photoactivated Erg6p or H2B in the arm and pericentromere (wild-type = untreated, nps1-105 at permissive temperature (24°C)). Dispersion is defined by the loss of fluorescence intensity over the course of the timelapse (Materials and Methods). Asterisks indicate statistically significant differences between sample and corresponding wild-type value (Fisher’s exact test, p<0.05, Table 3.S3).
Figure 3.5 - Disruption of the underlying chromatin platform results in disruption of the kinetochore.

(A) Diagram of kinetochore location in relation to pericentromeric chromatin, as denoted by green dotted line. (B) Representative images of both normal and disrupted kinetochores. Either inner kinetochore (Ame1p-GFP) or outer kinetochore (Spc24p-GFP) is shown in green and spindle pole bodies (Spc29p-RFP) are shown in red. Bar, 1 μm. (C) Graph showing percent of kinetochores disrupted in single plane images. Asterisks indicate statistically significant differences between sample and corresponding wild-type value (Fisher’s exact test, p<0.05, Table 3.S4).
Figure 3.6 - Model diagram of histone occupancy in the pericentromere and arm under various experimental conditions.

(A) Diagram of replicated bioriented chromosome indicating the locations of the arm and the pericentromere in relation to the centromere and kinetochore. (B) In wild-type cells, histones are more dynamic in the pericentromere than the arm (illustrated by larger arrows at the pericentromere), which is the result of being replaced more rapidly in the pericentromere. Histone on and off rates are balanced (equal sized arrows) to maintain proper histone occupancy. (C) Upon loss of spindle tension, histones are not removed as frequently from pericentromeric chromatin and the arm is unaffected. (D) Loss of RSC function results in reduced histone dynamics (slower reloading) at the pericentromere and slower histone dynamics throughout the nucleus. Kinetochore appears disrupted due to disturbance of the underlying chromatin structure required for kinetochore organization. (E) Loss of ISW2 results in slower histone dynamics at the pericentromere, likely due to disrupted histone reloading, whereas the chromosome arm is unaffected.
A
Chromosome Arm
Kinetochore
Pericentromere
Centromere

B
WILD-TYPE
Pericentromeric histones are more dynamic than the arm because they are replaced more rapidly

C
LOSS OF SPINDLE TENSION
Pericentromeric histones are less dynamic, slowed because they are removed less;
the arm is unaffected

D
LOSS OF RSC (nps1-105)
Pericentromeric histones are less mobile and less dynamic because they are replaced more slowly;
arm dynamics are slowed; kinetochores are disrupted

E
LOSS OF ISW2 (isw2Δ)
Pericentromeric histones are replaced more slowly; the arm is unaffected
Table 3.1 - *Saccharomyces cerevisiae* strains.

<table>
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<th>Stain Number</th>
<th>Genotype</th>
<th>Source</th>
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<td>Bloom lab This study This study This study This study This study This study This study This study This study This study This study This study This study This study This study This study This study This study (Vorvis et al., 2008)</td>
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* Parent nps1-105 strain from Tsuchiya et al., 1998
Figure 3.51 - RSC and ISW2 both localize throughout the budding yeast nucleus.

Representative images of NPS1-GFP and ISW2-GFP in relation to spindle pole bodies (SPC29-RFP).

No obvious consistent region of enrichment is observed for either chromatin remodeler. Bar, 1 μm.
Figure 3.52 - Loss of RSC function (*nps1-105*) does not alter metaphase spindle length at permissive (24°C) temperature.

Graph of spindle pole body (Spc29p-RFP) to kinetochore (Ame1p-GFP) distance binned by spindle length. Upon anaphase onset, this distance decreases. In wild-type cells this occurs at a spindle length of approximately 1.9 microns, and in *nps1-105* cells a significant decrease is observed at 2 microns.
Table 3.S1 - Summary of all data for FRAP of histone H2B.

Supplemental Table 1

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Table 3.S3 - Summary of all data for photoactivation of H2B.

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Table 3.S4 - Summary of all data for kinetochore organization.

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INTRODUCTION

As scientists who use microscopes to address biological questions, we strive to acquire ever better images and push the limits of resolution. The image formed of a specimen by a light microscope occurs as the combination of light diffracted by the specimen, the interference of these light rays, and the collection of these light rays by the objective lens. The ability to detect objects—the image contrast—depends on the signal-to-noise ratio. In order to clearly identify an object, its signal intensity must be high enough over the background noise. The level of detail in an image, or alternatively the ability to distinguish two objects as separate, is its resolution limit. The limits of resolution (the ability to distinguish as separate two points of light) were first described by Ernst Abbe, and are dependent on both the sample and the microscope hardware (Abbe, 1873). As a result of the wavelength of the light and its diffraction by the microscope optics, a single point of light will not appear as a spot but instead as a hazy circle surrounded by diffraction rings in the focal plane. In three dimensions, this blurring is termed the point spread function (PSF) and defines the limits of resolution (Figure 4.1A). The lateral dimensions of this hazy blurring can be described mathematically as an Airy pattern (Airy, 1835), and the bright central region (Airy disk) has a radius determined by the wavelength of light (\(\lambda\)) and numerical aperture (NA) of the objective lens (Inoué, 2006; Waters, 2009).
The Airy disk can be approximated by a Gaussian distribution (Figure 4.1B). By the Rayleigh criterion, two point objects are said to resolvable when they are separated by a distance equal to or greater than the radius of the Airy disk (Rayleigh, 1896). For example, in order to be able to distinguish two separated sister kinetochores in the budding yeast mitotic spindle, these must physically be separated by at least 222 nm, assuming they are labeled with GFP and imaged with a 1.4 NA objective lens. Otherwise, they are said to be within a single diffraction limited spot (Figure 4.1C and D). As we shall see, even this fundamental limit is being pushed by advancements in methods of image acquisition and mathematical image processing.

So how can we image two objects that are close together or more precisely find the position of a single point of light? Newer imaging technologies have emerged that use a range of methods to improve the resolution of a light microscopy image (Inoué, 2006; Weisshart et al., 2013). Modern confocal microscopy is based on the principles first described in 1957 by Marvin Minsky and use a combination of pinholes to illuminate a smaller area of the total sample and eliminate the collection of out of focus (scattered) light rays (Minsky, 1988). Multi-photon microscopy (MPM) utilizes the simultaneous absorption of two (or more) photons to excite a single fluorophore as an alternative method to confine excitation to a smaller area of the sample and allowing for deeper penetration into thick samples. In Structured Illumination Microscopy (SIM), a sample is illuminated with highly structured light that has passed through a grating. This process effectively allows the microscope to capture finer details in the sample and can double resolution of a typical widefield microscope. PALM (photoactivated localization microscopy), STORM (stochastic optical reconstruction microscopy), and a wide range of similar techniques utilize specialized fluorophores or modulate fluorophore behavior (e.g., photoactivation and photoswitching) to sequentially image and precisely
localize subsets of fluorophores (Betzig et al., 2006; Rust et al., 2006; Schermelleh et al., 2010; Sengupta et al., 2012).

However, these techniques are not always optimal for imaging in vivo dynamic events in budding yeast. Imaging live cells requires trade-offs to optimize imaging parameters for a given experiment. These include total time imaged (timelapsing), z axis resolution, image acquisition speed, and signal to noise. Confocal and multi-photon microscopy require greater excitation intensity to collect a similar number of photons per second as compared to widefield microscopy. This increased intensity can lead to cell damage and death, hampering the ability to collect data for longer periods of time. SIM requires the collection of multiple images with different grating patterns to reconstruct a single plane, drastically increasing total imaging time (preventing rapid acquisition) and this increased photobleaching reduces total images that can be acquired over time. The extensive rounds of imaging required for PALM/STORM image reconstruction is not feasible in live and dynamic cells. Thus, for imaging relatively thin, highly dynamic and rapidly photobleaching samples like budding yeast, we prefer widefield microscopy.

In this review, we will outline three post-acquisition methods to improve the spatial resolution of a widefield microscopy image, and their applications to yeast cell biology. Deconvolution utilizes mathematical algorithms to remove or reverse image blurring and improve contrast. Image analysis by model convolution allows the user to examine how computer generated geometrical structures are blurred by the microscope and compare these to experimentally obtained images. Gaussian fitting of single point objects allows for sub-pixel localization of structures of interest and can be used to precisely track motion over time. In order to maximize signal to noise and reach spatial resolution limited by the physics and optics of the microscope, it is essential to properly prepare samples. Discussions of optimizing sample preparation and imaging
parameters, however, are beyond the scope of this article (Cannell et al., 2006; Dailey et al., 2006; Goldman et al., 2010; Rines et al., 2002; Waters, 2009).

DECONVOLUTION – PUTTING LIGHT (BACK) IN ITS PROPER PLACE

The observed image generated by the microscope is generated from the actual image convolved by the PSF and contains noise (Biggs, 2010) (Figure 4.2A, left). Thus, although the image is degraded, it occurs in a mathematically predictable manner. Image restoration is achieved either by removal (deblurring) or reassignment (deconvolution) of blurred light, based on knowledge of how the image was degraded during acquisition. In this section, we discuss measurement of the PSF, briefly outline the various techniques used in image deconvolution, and highlight some applications of image deconvolution to the study of mitosis in budding yeast.

The PSF forms the basis for image deconvolution and depends on the microscope (objective lens properties) and imaging conditions. The PSF can be estimated mathematically or measured under experimental conditions (Cannell et al., 2006; Gibson and Lanni, 1991; Swedlow, 2007; Wallace et al., 2001). Measurement of the PSF is most commonly done using sub-resolution (100-200nm) fluorescent beads under identical conditions as the experiment by imaging through the axial (z) direction (Biggs, 2010). Adding these fluorescent beads to the experimental slide to further ensure PSF measurements are consistent (Cannell et al., 2006). It is important to image multiple beads throughout the field of view to ensure that the measured PSF is consistent throughout and detect any possible aberrations in the imaging set up (e.g., coma, astigmatism, spherical and chromatic) that may alter the PSF. The shape of the PSF is especially sensitive to spherical aberrations of the objective lens, as these cause oblique light rays to be focused at a different focal plane than the central light rays. The PSF can be translated to frequency, or Fourier, space to ease
mathematical analysis, as convolution functions translate to multiplication in the frequency domain (Biggs, 2010). The Fourier transform of the PSF is termed the optical transfer function (OTF) (Sibarita, 2005).

Deblurring algorithms – including nearest neighbor, no neighbor, multi-neighbor – improve image appearance by removing or reducing blur but the resulting image cannot be used for subsequent quantification (Agard, 1984; Agard et al., 1989; Biggs, 2010; Sibarita, 2005; Swedlow, 2007; Wallace et al., 2001) (Figure 4.2A, center). These analyses have the benefit of being computationally simple and fast, but since these do not use all out of focus planes their application is limited.

In contrast, fully three dimensional deconvolution algorithms reassign blurred light preserving signal intensity and include linear (Weiner) filters and non-linear (iterative) filters (Biggs, 2010; Wallace et al., 2001) (Figure 4.2A, right). Wiener and other linear filters are a direct method of image reconstruction and are very sensitive to PSF measurements. Linear filters are limited by noise and errors in reconstruction (Cannell et al., 2006; Sibarita, 2005). The application of constraints or limitations during reconstructions can limit noise (Wallace et al., 2001). More recently, nonlinear iterative deconvolution has become standard and linear deconvolution can be used as a first approximation for these iterative processes (Sibarita, 2005).

Nonlinear iterative deconvolution algorithms are based on repeatedly generating a reconstructed image until the reconstruction reaches a level acceptable to the user or software chosen by the user (Swedlow, 2007). Classical iterative methods are based on the Jansson Van-Cittert and Gold algorithms (Agard et al., 1989; Gold et al., 1964; Jansson, 2009; Shaw, 2006). Reconstruction can be slow and unsatisfactory if the raw data is too noisy (Sibarita, 2005). Thus, deconvolution becomes a balance between removing noise by filtering and a less sharp final image due to increased smoothing. Statistical iterative deconvolution is similar to classical algorithms and
improves image quality by reducing noise. The metric to be applied depends on the source of the noise and its distribution (Gaussian or Poisson) (Biggs, 2010; Lucy, 1974; Richardson, 1972; Sibarita, 2005). Blind deconvolution, also called adaptive PSF deconvolution, is iterative reconstruction of both the image and the PSF from an estimated starting point with constraints (Biggs, 2010; Holmes, 1992; Holmes et al., 2006; Krishnamurthi et al., 1995). This method has the advantage of not requiring measurement of the PSF but tends to be slower than non-blind iterative methods (Holmes et al., 2006).

Image restoration by deconvolution is a powerful tool to improve image quality but does have inherent limitations. Deconvolved images provide a high resolution, high contrast image that can provide additional information about biological structures. If done with certain algorithms, the resulting images can be quantitatively analyzed (Wallace et al., 2001). Advances in computing power will continue to increase speed and ease of deconvolution, making it an attractive option for improving image quality. Imaging followed by deconvolution is a good option for live cell imaging where cell viability must be balanced against exposure time and light intensity. However, it is essential that deconvolved images be compared to raw data, and if a structure is present in the deconvolved image, it should also be visible in the raw data (Sibarita, 2005). One must exercise caution to avoid artifacts or aberrations in the reconstructed image (Murray, 2005; Wallace et al., 2001) (Figure 4.2B and C).

**MODEL CONVOLUTION – BLURRING THE LINES BETWEEN REALITY AND SIMULATION**

Model convolution is an approach used to understand the possible distributions of fluorophores that give rise to an experimentally acquired image. Model convolution takes the opposite approach of deconvolution. Instead of trying to deduce fluorophore positions/distribution
from a noise-filled experimental image, model convolution uses computer simulated fluorophore positions/distributions then generates a noise-filled simulated image. This is accomplished by attaching the experimentally determined PSF of the microscope to each of the fluorophores to recapitulate the spread of fluorescence through the microscope objective (Figure 4.3A). The convolution of the entire fluorophore geometry or distribution is the summarization of the contributions of the fluorescence from each simulated fluorophore position in x, y, and z to the image plane (Agard et al., 1989; Sprague et al., 2003). Image(x,y) = Σ Fluorophore matrix (x,y,x) * PSF(x,y,z). Signal to noise ratios can be recapitulated by adding in the experimentally measured background, brightest intensity, and noise variation. Simulated images from model convolution can then be compared directly to experimental microscopy images in a statistically rigorous fashion to determine a best fit.

Model convolution has various positives and negatives as compared to deconvolution. Deconvolution methods are centered on producing a clearer image of a fluorescently labeled structure. However, when trying to use deconvolution algorithms to determine correct fluorophore positions in a structure, poor signal to noise ratios can impede deconvolution or introduce artifacts (Gardner et al., 2010). Alternatively, model convolution can be used to determine the distribution of fluorophores in a structure through running possible iterations and finding the best match. This approach relies on knowing how many fluorescent molecules are in the structure. Modeling can provide information about gradients, clustering, and/or random dispersion of the fluorophores within the structure. The limitation of model convolution is that you are still subject to the resolution of your microscope - the ability to discriminate two objects or fluorophores. However, modeling intensity changes over a structure along with the ability to alter fluorophore positions with sub-pixel accuracy makes model convolution a useful tool to analyze experimental data at the resolution limit.
Model convolution has been instrumental in determining the shape, size, and distribution of molecules in the pericentric cohesin and condensin structures that are two major components of the mitotic chromatin spring. While deconvolution was effective in determining shape and size of the cohesin barrel (Figure 4.2B), model convolution allowed insight into the distribution of fluorophores (Figure 4.3B and C). Using the measured number of fluorophores for cohesin we simulated different gradients from a major loading site, the centromere, and major site of function, the sister cohesion axis (Figure 4.3C). Interestingly, the simulated sister cohesin axis gradient accurately recapitulated experimental size measurements but failed to match the intensity distributions throughout the structure. Instead, cohesin randomly distributed throughout the structure recapitulated fluorescence intensity along the cohesin barrel. This approach of matching changes in intensities is the cornerstone of model convolution. Model convolution allows one to determine how fluorophores are distributed within a structure and assess the validity of mathematical models of biological processes by comparing simulated and experimental images.

As scientists and researchers we love to see an image, because seeing is believing. However, when you consider the PSF and Abbe diffraction limit, what you see might not be what you get. This is where model convolution excels. The output of a microscope image may not align with your preconceived notion of the underlying structure. However, with this technique we can explore possible structures and distributions and analyze how they are changed by the spreading of light through the microscope. One example of failed intuition is that two smaller hollow cylinders will look like one line, while one larger hollow cylinder will look like two lines. This occurs because the two smaller cylinders are too close to differentiate, while for the larger cylinder we only see the top and the bottom of the cylinder in focus. Model convolution goes beyond measurements by allowing us to understand how a defined geometry or distribution of fluorophores results in the blurred image we see in the microscope.
The other place that model convolution excels is in computer modeling. In the quest to understand the biology of life computer models are being used to investigate complex cellular process. Microscopy holds a wealth of data that is necessary to incorporate into modeling approaches. Through the use of programs such as Microscope Simulator 2.0 we can produce fluorescence simulated images from computational models to compare to experimental data (Quammen et al., 2008; Stephens et al., 2013).

GAUSSIAN FITTING – CUTTING THROUGH THE HAZE

As described above, point sources of light are blurred in a characteristic manner by the widefield light microscope, termed the point spread function (PSF). In the image plane, the point of light appears as a bright maximum cone of light surrounded by concentric diffraction rings. The shape of this maximum cone, the Airy disk, can be approximated by a Gaussian fit (Figure 4.1B). The fitting of a single molecule’s Airy disk with a Gaussian function to identify its centroid with nanometer resolution forms the basis of PALM/STORM microscopy, which relies on various techniques (e.g., photoactivation and photoswitching) to sequentially image subsets of fluorophores (Betzig et al., 2006; Rust et al., 2006; Schermelleh et al., 2010; Sengupta et al., 2012). Here we outline sub-diffraction particle localization methods, and the application of Gaussian fitting to track motion or localize proteins within a complex structure in vivo.

The shape of the theoretical PSF can be approximated by either a Gaussian function or center of mass calculation in order to localize the sub-resolution position of the fluorophore (Cheezum et al., 2001; Deschout et al., 2012; Levi and Gratton, 2007; Thompson et al., 2002). Approximation of the PSF by a Gaussian function is relatively simple, quick computationally, and more robust under conditions of low signal to noise than the center of mass method (Cheezum et
al., 2001; Churchman and Spudich, 2012; Levi and Gratton, 2007; Santos and Young, 2000; Stallinga and Rieger, 2010; Thomann et al., 2002). The approximation of position by center of mass does not rely on a normal distribution of fluorescence intensity, making it a better choice for larger or asymmetric particles, and has been shown to more accurately estimate lateral motion (Deschout et al., 2012; Levi and Gratton, 2007). It is also important to note that computationally a Gaussian fitting can be done by either least squares fitting or maximum likelihood estimation. Maximum likelihood estimation is a more accurate method, but more complex than least squares fitting (Larson et al., 2010; Laurence and Chromy, 2010; Mortensen et al., 2010; Smith et al., 2010). Close proximity of fluorophores or structures can confound the precision localization of basic Gaussian fitting methods. Advancements in mathematical processing of PALM/STORM datasets has allowed for localization analysis of fluorophores in close proximity (Babcock et al., 2012; Cox et al., 2012; Holden et al., 2011; Huang et al., 2011). With the advancement of computational power, the development of more robust algorithms for particle localization will continue to be developed and more precise localization may be possible.

Precise tracking of motion by Gaussian fitting has been used to address a wide range of biological questions including nucleocytoplasmic transport, myosin V motor motion, organelle dynamics and chromatin motion (Churchman et al., 2005; Goryaynov et al., 2012; Jaqaman et al., 2008; Levi and Gratton, 2007). We have developed a methodology for tracking chromatin motion to quantify confinement and physical properties such as spring constant. As mentioned previously, imaging setup is essential to obtain images with good signal to noise for sufficiently long timelapses. Once images are acquired, we perform a 2D Gaussian fitting by least squares using a custom MatLab (The Mathworks, Natick, MA, USA) script (Wan et al., 2009; Wan et al., 2008) (Figure 4.4A). We assume that the labeled chromatin array forms a close to diffraction limited spot, and the program
is able to account for small asymmetries by the incorporation of a rotation transformation (Wan et al., 2008).

In addition to tracking particles over time, Gaussian fitting has been used to characterize the architecture of the kinetochore and measure the number of proteins within this complex. The kinetochore is a large multi-protein complex that mediates the attachment of the centromere to the microtubule during chromosome division (Bloom and Joglekar, 2010). Essential to further understanding the role of the kinetochore in establishing and maintaining proper attachment is the development of a precise map of protein numbers and organization. Measurement of signal intensity by Gaussian fitting to determine number of proteins in a single focus within the kinetochore has been performed in a range of yeast species (Joglekar et al., 2008a; Joglekar et al., 2006; Joglekar et al., 2008b). These have revealed a conserved fundamental building block present in both point and regional centromeres. Protein numbers are reported as ratio values to a known standard, as a wide range of factors can influence signal intensity including inherent properties of the fluorophore and imaging conditions. Due to the stereotypic nature of the budding yeast point centromere, the fluorescence intensity of Cse4p (HsCENP-A) has been used as a standard. Recent studies using a variety of imaging and molecular techniques have shed new light on the number of Cse4p molecules in the single focus and further work will be needed to clarify the use of Cse4p as an imaging standard for counting by fluorescence intensity (Aravamudhan et al., 2013; Coffman and Wu, 2012; Lawrimore et al., 2011; Padeganeh et al., 2013a; Padeganeh et al., 2013b; Shivaraju et al., 2012).

Precision localization by Gaussian fitting can also be used to determine distances between kinetochore components. The SHREC (single molecule high-resolution colocalization) method serves as a basis for this methodology, and is based on imaging two points of interest within the same protein labeled with two different fluorophores and precisely localizing these using least squares fit
to a 2D Gaussian curve (Churchman et al., 2005). This approach has been expanded to incorporate distance measurements throughout the human kinetochore, which suggested an intra-kinetochore tension-sensing mechanism important for cell cycle regulation (Wan et al., 2009). Work examining the distances between different kinetochore proteins using 2 color imaging and fitting to a Gaussian function by maximum likelihood estimation has revealed that distances within the budding yeast kinetochore have also been shown to change upon loss of tension, perhaps serving a similar role in cell cycle checkpoint signaling during mitosis (Joglekar et al., 2009).

The architecture of the budding yeast kinetochore has further been characterized by Gaussian fitting to describe kinetochore spot shapes and possible positions within the stereotypical mitotic spindle structure. By measuring kinetochore spot sizes during metaphase along defined axes during metaphase, we have shown that the proteins of the inner kinetochore (adjacent to the chromatin) are anisotropic and elongated along the axis perpendicular to the spindle (Haase et al., 2012) (Figure 4.4B and C). These measurements were done by rotating the fluorescent images using the spindle pole body markers to bring the spindle axis to a horizontal level (y-axis). After rotation, we measured the full width-full maximum of the Gaussian fit to the kinetochore signal along the spindle axis (y-axis) and along the x-axis. In addition to characterizing the shape of individual kinetochore foci, the precise measurement of coordinates by Gaussian fitting can be used to examine the distribution of possible positions occupied by the protein of interest. In order to normalize the data across many individual cells, the spindle pole body coordinates are set to (0,0) and positions of kinetochore proteins or labeled chromatin measured in many cells are plotted relative to the normalized spindle pole body (Haase et al., 2012). The resulting heat map illustrates the average position as well as the spread of possible positions.
CONCLUSION

In this review, we have highlighted three methods that can be used to extract greater detail from wide-field images – deconvolution, model convolution, and precision localization by Gaussian fitting. These methods can be used independently or together in order to best address the scientific questions of interest. Acquiring a good image (i.e., good signal to noise, maintaining cell viability, fully capturing processes of interest) is only part of the battle; for the image to be of value it must also be analyzed in a quantitative and rigorous manner. We would encourage any researcher to experiment with various analysis techniques and software packages to determine which is most suitable for the problem at hand.

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(A) Experimental images of a sub-resolution bead illustrating the characteristic blurring of a point source of light by a wide-field microscope. In X and Y (left), the bead appears as a bright central spot surrounded by more faint rings, termed the Airy disk and Airy rings. In X and Z (right), the light is flared along the Z-axis. (B) The blurring of light by the microscope, the point spread function (PSF), can be approximated by a Gaussian distribution. (C) Examples of two point sources of light less that ~250nm apart (left) and more than ~250nm apart (right). At smaller separating distances, the two spots appear as one. (D) A linescan illustrating the intensity profile of the images in (C). The spots in close proximity appear as a single, higher peak, whereas the two more separated spots appear as two distinct peaks.
**Figure 4.2 –**

(A) Point sources of light are significantly blurred by both the point spread function (PSF) of the microscope and noise. Two distinct methods exist for post-acquisition processing of a blurred image. Deblurring algorithms remove out of focus planes, producing a sharper image but reducing the total information available and thus rendering the image unquantifiable. Deconvolution algorithms preserve the information, and restore the blurred fluorescence to the original point source using a range of methods as described in the text. (B) and (C) Cohesin forms a barrel-like structure during metaphase in budding yeast, which appears as a doughnut when viewed end-on and bilobed when viewed side-on (Stephens et al., 2011; Yeh et al., 2008). Original and deconvolved images of cohesin (SMC3-GFP) illustrating how both input image quality and deconvolution algorithm parameters can lead to very different output images and potentially introduce artifacts.
(A) Model convolution requires knowledge of the point spread function (PSF) and a simulated geometry to produce an image that can then be compared to experimentally acquired data. (B) Experimental image of cohesin (SMC3-GFP) viewed side-on appears bilobed. (C) In order to further understand the distribution and organization of cohesin during metaphase, we compared the experimental distribution to various modeled geometries. Introducing a gradient from either the centromere or the center (sister chromatid cohesion axis), resulted in intensity profiles that did not match the experimental data in (B). When cohesin distribution was modeled as distributed throughout the pericentromere region, the intensity profile matched experimental data, showing that cohesin distribution is not characterized by a concentration gradient.
A  

PSF + Simulated geometry = Model convolution

B  

Experimental

Intensity vs. Position

C  

Gradient from CEN

Gradient from sister cohesion axis

Throughout pericentromere
Figure 4.4 –

(A) The precise localization of a blurred point source of light can be achieved by fitting the fluorescent signal with a Gaussian distribution, as illustrated by the blue arrow at the apex. An example of precise localization of a labeled chromatin spot performed over time is shown, and these data can be used to perform mean square displacement (MSD) analysis or examine viscoelastic properties of the polymer. (B) In addition to measuring precise localization (blue arrows), fitting with Gaussian distributions can be used to measure spot size (black arrows). This method reveals differences in kinetochore spot shape between the inner (Cse4) and outer (Nuf2) kinetochores (Haase et al., 2012). (C) Various individual images illustrating the increased spot size along the vertical axis of the inner kinetochore (mean = 655nm) not present when examining outer kinetochore spots (mean = 582nm) (Haase et al., 2012).
A

Movement of Chromatin Spot Over Time

Y position (micron)

X position (micron)

Time elapsed (seconds)

LacO/LacI-GFP at 240kb from CEN II, Spc29-RFP
scale bar = 2 micron

B

Inner Kinetochore  Outer Kinetochore

C

Inner Kinetochore “Height” (Cse4 GFP), 655nm

Outer Kinetochore “Height” (Nuf2 GFP), 582nm

96
CHAPTER 5: CENTROMERE TETHERING CONFINES CHROMOSOME DOMAINS

INTRODUCTION

The foundations for our understanding of the physical organization of chromosomes originated in the work of Rabl and Boveri who articulated a characteristic conformation in which centromeres and telomeres are located at opposite sides of the nucleus and this organization is maintained throughout the cell cycle (Boveri, 1909; Cremer and Cremer, 2010; Rabl, 1885; Spector, 2003). Chromosomes in budding yeast display a Rabl-like configuration in interphase (reviewed in (Albert et al., 2012; Taddei and Gasser, 2012; Taddei et al., 2010; Zimmer and Fabre, 2011)).

Centromeres are clustered and attached by microtubules to an unduplicated spindle pole body (SPB) (Dekker et al., 2002; Jin et al., 2000; O'Toole et al., 1999). Telomeres are located at the nuclear periphery in five to eight clusters in a manner dictated, at least in part, by chromosome arm length with telomeres on arms of similar lengths clustering together (Bystricky et al., 2005; Dekker et al., 2002; Hediger et al., 2002; Jin et al., 2000; Schober et al., 2008). More recently, the characterization of the physical organization of chromatin within the nucleus has been described using 3C (chromosome conformation capture) and high-throughput variants of this technique (de Wit and de Laat, 2012; Dekker et al., 2002; Dixon et al., 2012; Sanyal et al., 2011). Using a 4C (circular chromosome conformation capture) followed by deep sequencing protocol, Duan et al. (Duan et al., 2010) showed that budding yeast chromosomes occupy discrete areas of the nucleus around the

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tethered centromeres. Population imaging of yeast nuclei has furthermore established the existence of chromosome territories (Berger et al., 2008) that are now perceived as a fundamental organizational feature of the nucleus (Austin and Bellini, 2010; Bickmore and van Steensel, 2013; Cremer and Cremer, 2010; Dixon et al., 2012; Hubner and Spector, 2010; Spector, 2003).

Various computational models have examined the formation of chromosomal territories and have shown that this organization can be explained by the inherent properties of a fluctuating polymer (Rosa and Everaers, 2008; Tjong et al., 2012; Wong et al., 2012). These models identify tethering, by simulating attachment at the centromere and telomere, and confinement, either by nuclear membrane or crowded polymer effects, as essential in modeling chromosome behavior and validate the starting point of our polymer model. By simulating the positioning of self-avoiding polymers, it has been suggested that entropic forces are sufficient to recapitulate the observed chromosomal territories (Cook and Marenduzzo, 2009; Finan et al., 2011). However, both the 3C variants and imaging to date have primarily examined the organization of nuclei in a whole population and lack information about the dynamics of chromatin organization within the cell nucleus.

We have quantified dynamic fluctuations along the length of the chromosome. The radius of confinement ($R_c$) is smaller at positions closer to the site of centromere attachment. We have examined the position dependent fluctuations using a bead-spring polymer model of chromatin together with the biological constraints of nuclear confinement, crowding, and tethering. In vivo chromatin tethering and fluctuations underlie chromosome organization and dynamics. Thus, the organization of chromatin within the nucleus of interphase yeast cells is dictated by its confinement and proximity to an attachment point and the dynamics can be approximated by the motion of an entropic spring.
RESULTS

**CHROMATIN CONFINEMENT VARIES ALONG THE LENGTH OF THE CHROMOSOME**

We examined the *in vivo* dynamics of chromatin during interphase to determine the wild type (WT) radius of confinement ($R_c$) at a discrete number of sites along the length of the chromosome. To do this, we tracked both a GFP-labeled chromatin array (lacO/lacI-GFP) at 6.8 kb (ChrXV), 8.8 kb (ChrIII), 24 kb (ChrIII), and 240 kb (ChrII) from the centromere (CEN) and relative to the unduplicated SPB (Spc29-RFP) over 10 minutes at 30 second intervals and measured sub-pixel localization by Gaussian fitting over time. The 240 kb lacO array is positioned roughly midway between the centromere and telomere. The distribution of spot positions show that arrays integrated further from the centromere can explore a larger space as compared to those more proximal to the point of attachment (Figure 5.1A). In order to quantify the sub-nuclear confinement observed, we use two methods to calculate $R_c$. From the plateau of the mean square displacement (MSD) curve (Figure 5.1B), 2D MSD gives (Neumann et al., 2012) (Experimental Procedures)

$$R_c = \frac{5}{4} \cdot \sqrt{\text{MSD}_{\text{plateau}}}.$$  
(1)

From the standard deviation of spot positions, $\sigma$, and the average squared deviation from the mean position, $\langle \Delta r_0^2 \rangle$, we applied the equipartition theorem to calculate $R_c$ from random chromatin motion (Scheffold et al., 2010; Uhlenbeck and Ornstein, 1930) (Experimental Procedures),

$$R_c = \frac{5}{4} \cdot \sqrt{2\sigma^2 + \langle \Delta r_0^2 \rangle}.$$  
(2)

Equations 1 and 2 are not statistically different for positions close to the tether (Student’s t-test, $p<0.05$, Table S1, Figure 5.S1A). The plateau value from MSD is more variable as it is based on long lag times between spot measurements and uses a fraction of the total data. Therefore, we use
Equation 2 and the standard deviation obtained with the entire data set to calculate $R_e$ throughout this work.

The $R_e$ is largest for the chromatin spot furthest from the centromere at 240 kb (705 nm, 43 cells) and smallest for spots proximal to the centromere at 8.8 kb (274 nm, 40 cells) and 6.8 kb (396 nm, 54 cells), suggesting that the attachment at the centromere functions to constrain chromatin movement (Figure 5.1C, Table 5.1). Statistical comparison revealed that $R_e$ values of chromatin spots at these four distances from the centromere are all statistically different from each other (Levene’s test, $p<0.05$, Figure 5.52A) (Levene, 1960). This pattern is a generalized feature of chromosomes in yeast (Figure 5.1D) (Bystricky et al., 2005; Dion et al., 2012; Hediger et al., 2002; Heun et al., 2001; Mine-Hattab and Rothstein, 2012; Neumann et al., 2012).

**CHROMATIN DYNAMICS IN INTERPHASE ARE DICTATED BY TETHERING**

In order to determine whether the $R_e$ observed adjacent to the centromere is dictated by microtubule attachment or an inherent property of the pericentric chromatin, we tracked chromatin motion in cells in which the centromere has been detached through its conditional inactivation (Hill and Bloom, 1987) (Figure 5.2A). The insertion of the GAL promoter adjacent to the centromere allows the centromere to function normally when grown on glucose and inactivated when on galactose. Detachment upon centromere inactivation results in a dramatic increase in the $R_e$ at 8.8 kb from the CEN from 274 nm (40 cells) to 745 nm (23 cells) (Figure 5.2B, Table 5.1), demonstrating that this chromatin region can explore a larger space when no longer attached to the SPB. This increased motion is unlikely the result of transcription induced by the GAL promoter as these loci have previously been shown to be confined at the periphery (Brickner et al., 2007; Drubin et al., 2006). Chromatin confinement at maximal distance from attachment (lacO at 240 kb) and detached upon centromere inactivation (Gal-CEN at 8.8 kb on galactose) is not statistically different (Levene’s
test, p<0.05). LacO at 8.8 kb is statistically different from both lacO at 240 kb and Gal-CEN at 8.8 kb (Levene’s test, p<0.05, Figure 5.S2A). This indicates that the confinement of the 8.8 kb chromatin spot is due to attachment at the centromere and not an inherent property of this region of chromatin. The tethering of chromatin is a universal organizational feature and has important implications for the temporal and spatial fluctuations of chromosomes.

**THE CHROMATIN POLYMER BEHAVES LIKE AN ELASTIC FILAMENT DURING INTERPHASE**

Throughout our *in vivo* time lapse movies, we observed transient spot expansion of lacO/lacI-GFP labeled chromatin arrays in G1 (Figure 5.3A-D). Chromatin arrays stretch during mitosis, presumably as a consequence of microtubule pulling force (Stephens et al., 2011; Stephens et al., 2013). Spot expansion during G1 could be the result of microtubule dynamics (as chromosomes remain attached at their centromeres) and/or the inherent spring properties of the polymer. We defined a change in the lacO arrays by measuring the ratio of the axes of a 2D Gaussian fit to the GFP signal. A spot was defined as expanded when the long axis was at least 1.5 times larger than the smaller axis. The lacO/lacI-GFP array at 240 kb from the CEN was found to exhibit expansion in 16% of time points imaged (141/879 planes) and the centromere-proximal (6.8 kb from the CEN) chromatin spot exhibited expansion in 10% of time points imaged (109/1105 planes) (Figure 5.3A and C). Examples of lacO/lacI-GFP dynamics in live cells are plotted in Figure 5.3B (240 kb) and D (6.8 kb). These transient extensions are of relatively short duration, lasting less than a few minutes. On average, the chromatin spot at 240 kb from the CEN had higher mean and median ratios than the 6.8 kb chromatin spot (Figure 5.S3A). We observe recoiling of these chromatin spots, indicating that the chromatin is behaving like an elastic filament. The spot expansion and contraction is variable and often deformed (see variability of expansion shapes, Figure 5.3A and C), thus chromatin motion in interphase shows no coherent properties. The random trajectory of
energy-dependent processes acting on the chromatin validates the rationale for using the
equipartition theorem to estimate confinement (Equation 2). In addition to aspect ratio, we
examined variance in distance between two loci (lacO/lacI-GFP and tetO/tetR-CFP) adjacent to the
centromere of chromosome XI to assess chromatin polymer elasticity (Figure S3B). We find that
both aspect ratio and variance in spot distances reveal the elastic nature of the chromatin.

**MODELING THE CHROMATIN SPRING AS A DOUBLY TETHERED, CONFINED BEAD-SPRING CHAIN**

**WITH EXCLUDED VOLUME INTERACTIONS CAN RECAPITULATE EXPERIMENTAL DYNAMICS**

In order to model the dynamic behavior and gain insight into chromosome organization, we
construct a 2D bead-spring model of a doubly tethered polymer chain, using Brownian beads
connected by linear springs (Doi and Edwards, 1986) (Experimental Procedures, Figure 5.4A). The
chain is tethered at both ends to simulate the centromere and telomere attachments, confined
within a 1 μm circle (the nucleus), and subject to excluded volume interactions. The bead-spring
chain has a persistence length ($L_p$) of 50 nm, corresponding to the known value for DNA. The $L_p$ is
defined as the distance over which the correlation of the direction of the two ends is lost, and longer
$L_p$ implies stiffer polymer chains (Bloom, 2008). We model one arm of a chromosome as 100 beads
connected by 99 springs with a packing density in between that of the 11 nm and 30 nm fibers
(Experimental Procedures, “Defining model variables”). $R_c$ values along the chain compare to
experimental values and are smaller at positions closer to the tether point (Figure 5.1C, black
squares). The varying radii of confinement observed in vivo can be recapitulated by a doubly
tethered bead-spring model. We note that in the absence of tethering, all positions within the chain
will have the same $R_c$ (i.e., the radius of the circle) (Rosa and Everaers, 2008). Thus tethering results
in variations of $R_c$ with respect to the distance from the centromere. We found that the **qualitative**
behavior of $R_c$ along the chain remains unchanged when the radius of the nucleus is changed, however the magnitude of $R_c$ decreases with the radius.

While the bead-spring chain consists of identical springs between each pair of beads, the effects of tethering, geometric confinement, and excluded volume interactions result in distinct statistical fluctuations of each bead (lacO/lacI-GFP position experimentally) along the chain. This leads to a position-dependent effective spring constant ($k_s$), as seen by a particular bead relative to the tether points, and measured based on that bead's fluctuations as described below. The tendency of the spring to adopt a random coil can be represented in terms of a spring constant that reflects the spring stiffness (Bloom, 2008). The effective spring constant ($k_s$) for bead $i$ in our model is given by

$$k_{s,i} = 3k_B T \left( \frac{C_r}{2L_p \times L_c} \right) \left( \frac{1}{p(1-p)} \right).$$

where $k_B$ is the Boltzmann constant, $T$ is temperature (Kelvin), $L_p$ is persistence length, $L_c$ is contour length, $C_r$ is ratio of compaction, and $p$ is the percentage of the chain from the centromere (Experimental Procedures). From the displacement of the beads in our model, we estimate $k_s$ as a function of position along the chain (Figure 5.4B). This observation reveals the limitation in deducing a spring constant from measurements of a single spot along the chain. The $k_s$ is smaller for beads that “explore” a larger space, and so the $k_s$ will be highest (most stiff) for positions close to the tether point and softest in the middle of the chain. Thus, tethering of an otherwise homogeneous bead-spring chain results in a gradient of $k_s$ along the chain and introduces variations in local mobility.
THE EFFECTIVE SPRING CONSTANT ALONG THE ENTROPIC CHROMATINSpring CAN BE MEASURED IN VIVO

We calculate an effective $k_s$ from our in vivo time lapse data using two methods (Experimental Procedures). Using the MSD plateau value and the average squared deviation of each step from the mean position $\langle \Delta r^2 \rangle$, we calculated (Bruno et al., 2011; Kamiti and van de Ven, 1996),

$$k_s = \frac{2k_BT}{(\text{MSD}_{\text{plateau}}) - \langle \Delta r^2 \rangle}.$$  

(4)

Using the equipartition theorem, we measured the standard deviation ($\sigma$) of each step from the mean position to calculate (Scheffold et al., 2010),

$$k_s = \frac{k_BT}{\sigma^2}.$$  

(5)

Similar to $R_c$, $k_s$ values were calculated using both methods for lacO at 6.8 kb, 8.8 kb, 24 kb, and 240 kb from the CEN (Figure 5.S1B, Table 5.S1). We found significant agreement between the two methods; a Student’s t-test comparing $k_s$ values calculated using Equations 4 and 5 for individual cells showed no statistical differences between the two methods (Student’s t-test, p<0.05). As previously described for $R_c$ calculations, the plateau method uses a fraction of the total data set. Thus we use the equipartition method (Equation 5) and the standard deviation obtained from the entire data set for remaining calculations.

From Equation 5, we see that in general stiffness varies inversely with position variance, meaning that for loci exhibiting smaller variance, the chromatin will have a higher effective spring constant (Figure 5.1A). As predicted by our model, $k_s$ was found to vary with distance from the tether point, and appeared stiffer at points closer to the site of attachment and regions within the pericentromere domain exhibit a variation in stiffness (lacO at 6.8 kb - $1.6 \times 10^{-4}$ pN/nm; 8.8 kb - $3.4 \times 10^{-4}$ pN/nm; 24 kb - $1.3 \times 10^{-4}$ pN/nm; 240 kb - $5.1 \times 10^{-5}$ pN/nm) (Figure 5.4D, Table 5.1). Statistical comparison of population variances showed these to all be significantly different from each other (Levene’s test, p<0.05, Figure 5.52A). Application of equipartition methods to our data represents a
starting point to quantify these dynamics, and further work will refine the applied mathematics to more closely match \textit{in vivo} conditions.

Upon detachment from the centromere (Gal-CEN), the $k_s$ for a chromatin spot 8.8 kb from the CEN is reduced as compared to WT at 8.8 kb and appears softer and approaches the value of the chromatin arm at 240 kb (Gal-CEN at 8.8 kb - $4.6 \times 10^5$ pN/nm, Figure 5.4D, Table 5.1). Statistical comparison found Gal-CEN at 8.8 kb to be significantly different from WT at 8.8 kb but not significantly different from WT at 240 kb (Levené’s test, $p<0.05$, Figure 5.52A). This confirms the prediction that the apparent properties of the chromatin polymer are dictated by the attachment to a tether point like the centromere. The gradient of $k_s$ is the result of tethering and provides a mechanism to build variations in local mobility along the chromatin chain. This implies a role for tethering in the differential regulation of various regions of chromatin by altering polymer properties such as dynamics and stiffness. By attaching or detaching chromatin from a tether, the cell can efficiently alter the stiffness, as well as the range of motion of the chromatin.

\textit{COHESIN CONTRIBUTES TO LOCAL CLAMPING OF CHROMATIN}

A major source of chromatin organization is the structural maintenance of chromosomes (SMC) protein complex cohesin. While the role of cohesin in holding sister chromatids together in mitosis is well-established, it is becoming increasingly evident that cohesin also serves a vital role in interphase chromatin gene regulation through looping (as reviewed in (Haering and Jessberger, 2012; Seitan and Merkenschlager, 2012; Sofueva and Hadjur, 2012)). Given the regulatory role for cohesin looping, we predict a role for cohesin in the organization of chromatin into territories and maintaining chromatin dynamics during interphase.

We examined $R_c$ and effective $k_s$ of the lacO/lacI-GFP array at 240 kb from the CEN in WT and \textit{mcd1-1} cells at permissive (24°C) and restrictive (37°C) temperature (Figure 5.5A). The $R_c$ is
increased in \( \text{mcd1-1} \) cells at restrictive temperature as compared to \( \text{mcd1-1} \) at permissive or WT (WT at 24°C- 705 nm, 43 cells; WT at 37°C- 691 nm, 16 cells; \( \text{mcd1-1} \) at 24°C- 892 nm, 25 cells; \( \text{mcd1-1} \) at 37°C- 958 nm, 33 cells; Figure 5.5B, Table 5.1). This does not result from increased temperature, as WT cells at 37°C do not show a similar increase in \( R_c \). In addition, \( k_s \) is decreased in \( \text{mcd1-1} \) cells at restrictive temperature as compared to WT at 37°C, suggesting that the chromatin spring is softer upon the loss of cohesin (WT at 24°C- 5.1 \( \times 10^{-5} \) pN/nm; WT at 37°C- 5.4 \( \times 10^{-5} \) pN/ nm; \( \text{mcd1-1} \) at 24°C- 3.2 \( \times 10^{-5} \) pN/ nm; \( \text{mcd1-1} \) at 37°C- 2.8 \( \times 10^{-5} \) pN/ nm; Figure 5.5C, Table 5.1). Statistical comparison of population variances found no statistical difference between WT at 24°C and WT at 37°C, whereas \( \text{mcd1-1} \) at 24°C and \( \text{mcd1-1} \) at 37°C were both statistically different from WT and from each other (Levene’s test, \( p<0.05 \), Figure 5.52B).

To model the reduction of cohesin and predict its physiological role, we have examined the dynamics of chains composed of more beads (from \( N=100 \) to \( N=150 \) at a constant \( L_p = 50 \) nm) (Experimental Procedures, Figure 5.5D). We reason that chromatin in loops will not contribute to the effective chromatin length, and upon loss of cohesin and release of loops, additional chromatin will lengthen the chain. In the model this reduces the compaction ratio, and at the same \( L_p \), which increases the number of beads (# of beads = \((L_c/C)_r/2L_p))\). The model predicts that upon an increase in effective chromatin length (i.e., more beads in the chain), the \( R_c \) for any given point should increase and the \( k_s \) will decrease (Figure 5.5D), consistent with the experimental results.

**NUCLEOSOME DEPLETION RESULTS IN A STIFFER CHROMATIN FIBER**

We hypothesize that changes to nucleosome density would have important effects on chromatin fluctuations. We tested the effects of changing chromatin packaging on dynamics by measuring \( R_c \) and effective \( k_s \) in cells with reduced nucleosome occupancy. We measured the MSD dynamics of a chromatin spot 6.8 kb from the CEN in a stain depleted of histone H3 that results in a
2-fold reduction of nucleosome density (Bouck and Bloom, 2007) (Figure 5.5E). The $R_c$ was statistically significantly reduced from 396 nm (54 cells) to 319 nm (55 cells) (Levene’s test, p<0.05, Figure 5.5F, Table 5.1, Figure 5.52C). The $k_s$ was statistically significantly increased, becoming slightly stiffer from $1.6 \times 10^{-4}$ pN/nm to $2.5 \times 10^{-4}$ pN/nm (Levene’s test, p<0.05, Figure 5.5G, Table 5.1). These effects were not due to $\alpha$ factor arrest, which was not found to significantly alter $R_c$ or $k_s$ (Figures 5E-G, Table 5.1, Figure 5.52C). From these data, we conclude that nucleosomal packaging is an important factor in dictating the spatio-temporal organization and fluctuations of the chromatin polymer.

The reduced nucleosomal density in H3 depleted cells will increase the fraction of naked DNA from ~20 bp/nucleosome (based on linker length) to ~160 bp/2 nucleosomes, or ~80 bp/nucleosome (based on loss of $\frac{1}{2}$ the number of nucleosomes). This will disproportionally alter the physical properties of the chromatin. Unlike cohesin depletion in which the fraction of naked DNA is constant, we model the increase in naked DNA as a decrease in persistence length ($L_p$). Bystricky et al. have reported that DNA has a substantially shorter $L_p$ than chromatin (Bystricky et al., 2004). The model predicts that by decreasing $L_p$, $R_c$ for any given bead decreases (data not shown) and its $k_s$ increases (Figure 5.5H).

The lacO/lacI-GFP spot morphology provides a quantitative assay for chromatin elasticity. The centromere-proximal (6.8 kb) chromatin spot exhibited expansion in 10% of time points imaged (WT, 109/1105 time points) and reduced to 3% upon histone H3 depletion (36/1148 time points) (Figure 5.3E). The stiffer $k_s$ at 6.8 kb from the CEN observed in nucleosome depleted cells would predict that fewer cells would exhibit expansion (aspect ratio > 1.5) of the centromere-proximal lacO/lacI-GFP array since it would take more energy to extend the entropic spring. Reduced nucleosome density would result in unwrapping of the DNA from nucleosomes and a general increase in spot size. We examined the morphology of the centromere-proximal lacO/lacI-GFP
labeled chromatin spot and observed an average increase in spot size for both compacted (aspect ratio < 1.5) and decompacted (aspect ratio >1.5) spots (Figure 5.54). We have directly assessed the dynamic physical consequences of changing the histone compaction and measured a higher effective $k_s$ for a chromatin spot 6.8 kb from the CEN in nucleosome depleted cells. Nucleosomal density and therefore $L_p$ and linker length are important factors in determining the physical properties of the entopic chromatin spring.

**DYNAMIC FLUCTUATIONS UNDERLIE CHROMOSOME TERRITORIES**

Chromosome territories within a population of cells can be visualized in chromosome interaction maps. In order to examine if our tethered bead-spring model describes the formation of chromosome territories within the dynamic nucleus, we generate interaction maps of four tethered chains within a circle. We examined the bead position distributions in our polymer model (Figures 7A-B) and plotted the average normalized bead separation (Figures 7C-D). Telomeric attachment in budding yeast is known to occur in five to eight foci and in Rabl-like configuration (Bystricky et al., 2005), and therefore we consider either five attachment points analogous to one centromere position and four discrete telomere attachment points (Figure 5.7A), or three attachment points (one centromere position and two telomere points) (Figure 5.7B). The combination of excluded volume interactions and shorter distance between tether points results in a more uniform heat map distribution (Figures 7A and C - chains 1 and 4, Figures 7B and D - chains 3 and 4). When the tethers are further apart and the chain is able to explore a larger space, beads along the middle of the chain rarely come into contact (blue on the heat maps) and excluded volume interactions dominate over confinement. The chromosome interaction maps reveal a potential regulatory mechanism in the position of the telomere attachment site. The tethered regions of a single chain, while sampled infrequently, provide the ability for disparate chains to physically interact with one another. This
provides a mechanism for distinct chains within the nucleus to share genetic information, while satisfying the territories of individual chains.

DISCUSSION

IN VIVO MEASUREMENTS OF FUNDAMENTAL CHROMOSOME PROPERTIES – $R_c$ AND EFFECTIVE $k_s$

Through a combination of experimental observation and mathematical modeling we have shown that interphase chromatin fluctuations are mainly governed by attachment at the centromere and telomere. Loss of attachment allowed centromere-proximal chromatin to soften (lower effective $k_s$) and explore more space (higher $R_c$), behaving like a region out on the chromosome arm. By formulating a 2D bead-spring model based on simple polymer physics, we capture the observed $k_s$ along the length of the chromosome. To capture relevant experimental features the model requires: (1) tethering at both ends, (2) confinement within a domain, and (3) excluded volume interactions between beads.

In order to determine the effect of polymer length on chromosome fluctuations, we examined dynamics in cohesin-depleted cells. Loss of cohesin effectively lengthens the chromosome since less of the total length is confined in loops. This led to softening of the chromatin fiber at 240 kb from the CEN and allowed this region to explore a larger space. Thus, the overall length of the chromosome is an important factor in determining its spatio-temporal fluctuations, consistent with polymer theory (Equation 6) in which the spring constant is inversely related to number of segments (N). In addition to chromosome length, the persistence length ($L_p$) of the polymer is predicted to play an important role in chromosome fluctuations. As $L_p$ is decreased, the spring constant will increase by virtue of the increase in number of entropic states, and the polymer will occupy a smaller radius of gyration (Bloom, 2008). In order to test this, we hypothesized that reduced
nucleosome occupancy results in a lower average persistence length (DNA $L_p = 50$ nm vs. chromatin $L_p = 170-220$ nm (Bystricky et al., 2004)). Consistent with the theory, we observed stiffening of the centromere-proximal chromatin (higher $k_s$) and smaller $R_c$ in nucleosome depleted cells.

**EXAMINING CHROMOSOME TERRITORIES TO UNDERSTAND CELLULAR BEHAVIORS LIKE REPAIR**

Our *in vivo* observations of chromatin fluctuations highlight dynamics and predict significant interactions to allow for a variety of cellular processes (Figure 5.7). We hypothesize that the organization of chromosomes within the nucleus into territories dictates chromosome interactions. Altering the location of tethering or detaching one chromosome end would allow chromatin to explore a larger volume. These genome-wide changes could be quickly reversed by subsequent reattachment of the chromosomes. The radius of confinement is determined by multiple factors including tethering, compaction by cohesin, and nucleosomal wrapping (Figures 1D, 2B, 5B and 6B).

Various model systems have shown that the total radius of confinement is similar across species (Chubb and Bickmore, 2003; Gasser, 2002; Soutoglou and Misteli, 2007), suggesting that this may be at least in part dictated by an inherent property of the polymer. Attenuation of these properties could contribute to facilitating chromosome interactions upon damage, and form the basis for mechanism of action for a wide range of pathways that serve to increase or decrease chromatin motion such as DNA repair or gene gating. Based on the increased confinement and stiffening of the chromosome upon reduction of nucleosome occupancy, we hypothesize that attenuation of nucleosomal wrapping could play an important role in dictating the dynamics of DNA repair by reducing persistence length. We have previously shown that dynamic exchange of nucleosomes is important for maintenance of the pericentromeric chromatin under tension (Verdaasdonk et al., 2012). The work presented here provides a basic framework for an integrated understanding of
physical organization and dynamic interactions dictated by chromatin modifications to explain complex cellular behaviors such as DNA repair.

Changes in the radius of confinement have been observed upon DNA damage, both for damaged and undamaged chromosomes (Dion et al., 2012; Haber and Leung, 1996; Mine-Hattab and Rothstein, 2012). The increased range of motion of a DSB is thought to allow the damaged site to explore a much larger area within the nucleus to promote homology search for repair. The increased motion of damaged chromatin is known to require the recombination proteins Rad51 and Rad54, and the DNA damage response pathway components Mec1 and Rad9 (Dion et al., 2012; Mine-Hattab and Rothstein, 2012). Interestingly, Mec1 checkpoint activity is important to maintain replication fork integrity by detaching tethered and highly transcribed regions from the nuclear pore (Bermejo et al., 2011). This mechanism of detachment cannot explain the increased motion observed upon DNA damage (Ira and Hastings, 2012), but could suggest an alternative role for the DNA damage response pathway in maintaining chromatin tethering. These data highlight the importance of tethering to regulate chromatin motion, and further work should examine which points of tethering are altered upon DNA damage.

**EXPERIMENTAL PROCEDURES**

For detailed growth and imaging conditions, see supplemental experimental procedures. Strains used are listed in Table 5.S2.

**IMAGE ANALYSIS**

For MSD analysis, the images were identically analyzed using MetaMorph (Molecular Devices, Sunnyvale, CA, USA) and MATLAB (The Mathworks, Natick, MA, USA). The GFP and RFP foci of the
brightest planes per time point were tracked using a custom MATLAB program (Speckle Tracker) as previously described (Wan, 2008; Wan et al., 2012; Wan et al., 2009). These coordinates were further analyzed using MATLAB and Excel (Microsoft, Redmond, WA, USA) software to determine mean square displacement. The RFP coordinates were subtracted from the GFP coordinates to eliminate cell and nuclear motion. We then examined the 2D change in position of the lacO/lacI-GFP chromatin spot over increasing time lags using a classical MSD approach at each time interval,

\[ MSD_\tau = \tau_1^2 + \tau_2^2, \quad (6) \]

for all time lags \( \tau \). Only cells whose MSD curves exhibited a linear slope within the (1.5*IQR) range were included in subsequent analysis. Figures were made using SigmaPlot (Systat Software, San Jose, CA, USA).

To measure spot size, we used a custom GUI written in MATLAB as described previously (Haase et al., 2012). The spot intensity distribution is fit with a two-dimensional Gaussian function and full width-half maximum values are used to determine spot size.

**CALCULATING \( R_c \) FROM EXPERIMENTAL DATA**

We calculate \( R_c \) from the 2D MSD plateau value as (Neumann et al., 2012)

\[ R_c = \frac{5}{4} \times \sqrt{MSD_{\text{plateau}}}, \quad (1) \]

where the plateau is measured from the 330–390 s region of the timelapse, as most cells have reached confinement at this point while minimizing potential errors introduced by MSD analysis at longer time lags.

We calculate the variance of the distribution of spot positions as \( \sigma^2 = \text{mean} (\sigma_x^2, \sigma_y^2) \) where these are measured using MATLAB to fit the spot positions as \([\mu_x, \sigma_x] = \text{normfit}(x - x_{\text{mean}})\) and \([\mu_y, \sigma_y] = \text{normfit}(y - y_{\text{mean}})\). We then use \( \sigma^2 \) to calculate \( R_c \) as
where the average squared deviation from the mean position is \( \langle \Delta r_0^2 \rangle = \langle \Delta x_0^2 \rangle + \langle \Delta y_0^2 \rangle \).

### Entropic Bead-Spring Chain Model

We model chromosomes as chains composed of \( N+1 \) beads connected by \( N \) linear springs. The equation of motion for a bead \( i \) at position \( X_i \) is given by (Doi and Edwards, 1986),

\[
\frac{d\tilde{X}_i}{dt} = \frac{1}{\zeta} \left( \tilde{F}_B^i(t) + \tilde{F}_S^i(t) + \tilde{F}_{EV}^i(t) + \tilde{F}_W^i(t) \right),
\]

for \( i = 0, 1, \ldots, N + 1 \). Here \( \zeta \) is the bead drag coefficient, \( F_B^i \) is the Brownian force, \( F_S^i \) is the spring force, \( F_{EV}^i \) is the excluded volume force, and \( F_W^i \) captures the interaction of the bead with the cell walls. In this work we use the following dimensionless variables,

\[
X_i = \tilde{X}_i \frac{k_s^0}{k_B T}, \quad t = \tilde{t} \frac{2k_s^0}{\zeta}, \quad F = \tilde{F} \frac{1}{\sqrt{k_s^0 k_B T}}, \quad \text{where} \quad k_s^0 = \frac{3 k_B T}{(2L_p)^2}.
\]

- **Brownian force.** The Brownian force is \( \tilde{F}_B^i = \sqrt{2k_s^0 \zeta} \mathbf{W}_i \), or in dimensionless form,

\[
F_B^i = \mathbf{W}_i,
\]

where \( \mathbf{W}_i \) is a Wiener process:

\[
\langle \mathbf{W}_i(t) \rangle = 0, \\
\langle \mathbf{W}_i(t) \mathbf{W}_j(t') \rangle = \delta_{ij} \min(t, t').
\]

- **Spring force.** We consider linear springs so that the spring force acting on bead \( i \) is,

\[
F_S^i = 2X_i - X_{i-1} - X_{i+1},
\]

for \( i = 1, 2, \ldots, N \).

- **Excluded volume force.** The excluded volume interaction is modeled using a soft potential as in (Jendrejack, 2002),
\[ F^E_i = \frac{Z}{4d^5} \left( \sum_{j=0,j \neq i}^{N} (X_i - X_j) \exp \left[ -\frac{(X_i - X_j)^2}{2d^2} \right] \right), \]  

(11)

Here the dimensionless parameters \( z \) and \( d \) are, respectively, measures of the strength and range of the interaction.

- **Wall interaction.** To model the bead-wall interaction, whenever a bead moves outside the confining circle, it is moved to the nearest point on the boundary before the next time step (Jones et al., 2011).

**EFFECTIVE SPRING CONSTANT IN A DOUBLE-TETHERED ROUSE CHAIN**

If consider that the only forces are \( F^B_i \) and \( F^S_i \), the model becomes a double-tethered Rouse chain. In this case the effective spring constant for bead \( i \) can be found explicitly as,

\[ k_{s,i} = 3k_BT \left( \frac{c_r}{2L_p \times L_c} \right) \left( \frac{1}{p(1-p)} \right), \]  

(3)

where \( k_B \) is the Boltzmann constant, \( T \) is temperature (Kelvin), \( L_p \) is persistence length, \( L_c \) is contour length, \( C_r \) is ratio of compaction, and \( p \) is the percentage of the chain from the centromere (i.e., for centromere \( p=0 \) and for the telomere \( p=1 \)). The first term, \( 3k_BT \), is the thermal contribution, the second, \( \left( \frac{c_r}{2L_p \times L_c} \right) \), captures the properties of the chromatin, and the last, \( \left( \frac{1}{p(1-p)} \right) \), measures relative location within the chromatin.

**DEFINING MODEL VARIABLES**

The model requires two parameters: \( N \) and \( L_p \). To determine \( N \), we first estimate chromosome length in the cell by dividing the chromosome length in bp (contour length, \( L_c \)) by the packing density (ratio of compaction, \( C_r \)). For an average chromosome \( L_c \sim 800,000 \) bp and a packing density is \( 80 \) bp/nm (\( \sim 4X \) nucleosomal chromatin), then its length in the nucleus is \( 10,000 \) nm (\( L_c/C_r \)). The
packing density is less than the 30 nm fiber (~ 6X the 11 nm fiber) due to the lack of evidence for the 30 nm fiber and the reduced compaction of yeast chromatin relative to chromosomes in larger cells. The number of beads is derived from the nuclear length divided by the persistence length ($L_p$), 10,000 nm/50 nm = 200 beads. Since we model the polymer from the centromere to the telomere, we use $N = 100$ beads. $L_p$ and packing density vary proportionally and cannot be independently deduced from the model. Simulations of 100 beads with $L_p = 50$ nm or 25 beads with $L_p = 100$ nm predict motion plots comparable to that shown in Figure 5.1A. The modeled $R_c$ using 100 beads and $L_p = 50$ nm compares closely with literature values (dashed line, Figure 5.1D). These assigned values are not unique parameters, and it is likely that in vivo conditions include a wide range of values.

**CALCULATING EFFECTIVE $k_s$ FROM EXPERIMENTAL DATA**

Although the motion in vivo is ATP-dependent (Figure 5.55) (Weber et al., 2012), it is still random in nature with step sizes following a Gaussian distribution, supporting the assumption that the beads move in a harmonic potential well at some effective temperature (Tokuda et al., 2012). In this manner, it is possible to group the different components of energy-dependent motion in the single non-directional temperature parameter ($T$).

To calculate effective $k_s$ from the plateau value of the 2D MSD plot, we consider the equation (Uhlenbeck and Ornstein, 1930)

$$MSD = 2\frac{k_b T}{k_s} (1 - e^{-\zeta t/(2k_s)}) + 2\langle\Delta r_0^2\rangle (1 - e^{-\zeta t/(k_s)})^2 .$$  \hspace{1cm} (12)

This means that when $t$ is very large, the plateau value is

$$MSD_{plateau} = 2\frac{k_b T}{k_s} + \langle\Delta r_0^2\rangle ,$$  \hspace{1cm} (13)

where the average squared deviation from the mean position is

$$\langle\Delta x_0^2\rangle = \langle\Delta x_0^2\rangle + \langle\Delta y_0^2\rangle .$$  \hspace{1cm} (14)

Solving Equation 13 gives the spring constant as,
To illustrate how to calculate effective $k_s$ from variance of lacO spot position, we look at the simplest case: a bead moving by Brownian motion and attached to a fixed point by a linear spring. The bead position obeys the following Langevin Equation,

$$\zeta d\mathbf{X} = -k_s \mathbf{X} dt + \sqrt{2k_BT\zeta} d\mathbf{W}$$

(15)

where, as before, $\mathbf{W}$ is a Wiener process.

Equation 15 has a corresponding Fokker-Planck Equation with solution,

$$P(X) \sim \exp \left( -\frac{k_s X^2}{k_BT} \right).$$

(16)

This means that a histogram the distribution of bead positions has a Gaussian form with variance $\sigma^2 = k_BT/k_s$. Then from the variance of the distribution, one can obtain the spring constant as,

$$k_s = \frac{k_BT}{\sigma^2}.$$  

(5)

STATISTICAL ANALYSIS

We used the equipartition Equations 2 and 5 and the standard deviation from the whole population of cells for remaining comparisons. This results in a single value from the whole population standard deviation (with no associated error bars) and for statistical comparison we compare the homogeneity of population variances by Levene’s test (Figure 5.S2) (Levene, 1960). For additional details, see Supplemental Information and Figure 5.S1.

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Figure 5.1 – \( R_c \) of chromatin varies along the length of the chromosome.

**A** Scatter plots illustrating variance in WT lacO/lacl-GFP spot position relative to spindle pole pole body. **B** MSD curves of WT chromatin spot motion at various distances from the centromere. **C** \( R_c \) values calculated using Equation 2 and whole population standard deviation values (Table 5.1). \( R_c \) values are all significantly different from each other (Levene’s test, \( p<0.05 \), Figure 5.S2A). For comparison of \( R_c \) calculations using Equations 1 and 2, see Table 5.S1 and Figure 5.S1A. **D** Experimental and literature \( R_c \) values plotted by percent distance from centromere (0%) and telomere (100%). The dashed line illustrates the general trend of reduced confinement at increasing distances from the attachment point. Red – this work using individual cell variance, mean ± standard deviation (Table 5.S1); orange – (Bystricky et al., 2005); yellow – (Dion et al., 2012); light blue – (Hediger et al., 2002); dark blue – (Heun et al., 2001); green – (Mine-Hattab and Rothstein, 2012); purple – (Neumann et al., 2012); black squares – our tethered bead-spring model (see text).
**Figure 5.2 –** \( R_c \) is dictated by the attachment at the centromere.

(A) MSD analysis of cells in which the centromere has been detached through its conditional inactivation (Gal-CEN on GAL) revealed reduced confinement of a lacO/lacI-GFP labeled chromatin spot 8.8 kb from the CEN as compared to WT at 8.8 kb. (B) Bar graph of \( R_c \) values (Equation 2). Statistical comparison show WT at 8.8 kb and Inactive CEN at 8.8 kb to be statistically significantly different from each other, whereas WT at 240 kb and Inactive CEN at 8.8 kb are not statistically significantly different from each other (Levene’s test, \( p<0.05 \), Figure 5.2A).
Figure 5.3 – Interphase chromatin is dynamic.

(A) Example images of lacO/lacI-GFP at 240 kb from the CEN with aspect ratios less than and greater than 1.5 (compacted and decompacted). Measured aspect ratios are shown. (B) Graph of aspect ratio change over time for two cells, colors corresponding to outlined images in (A). (C) Example images of lacO/lacI-GFP at 6.8 kb and (D) graphs of aspect ratio changes for two examples. WT cells exhibit transient expansion and recompaction of chromatin arrays along random and occasionally non-linear trajectories. (E) Example images of lacO/lacI-GFP at 6.8 kb in Gal-H3 cells in which nucleosomes have been depleted (see text), and (F) graphs of aspect ratio changes over time. Aspect ratio values of lacO/lacI-GFP signal = large axis/small axis. Scale bar = 1 μm. Time lapse images were taken every 30 s. Color images: lacO/lacI-GFP – green, and spindle pole body (Spc29-RFP) – red. Black and white images show lacO/lacI-GFP with corresponding aspect ratios alongside.
Figure 5.4 – Modeling interphase chromatin dynamics as a doubly tethered bead-spring polymer chain.

(A) The chromatin polymer (dark blue) can be modeled as a bead-spring polymer chain (dotted blue line indicates original chromatin polymer chain). Diagram of a bead-spring chain composed of 100 beads tethered at both ends and confined within a circle of radius 1 μm. (B) The effective spring constant \(k_s\) (Equation 3) is highest (most stiff) for beads closest to the tether points (beads 0 and 100) and softest in the middle of the chain (at bead 50). (C) Measurement of \(k_s\) from in vivo dynamic MSD data at various positions from the centromere. We compared \(k_s\) calculated using the MSD plateau value (gray, Equation 4) or the variance of the distribution of spot positions (light blue, Equation 5) and found no significant difference in WT cells at 240 kb from the CEN (Student’s t-test, \(p>0.05, \text{Table 5.S1}\)). Black bars indicate median, red bars indicate mean. Black dots above and below indicate 95%/5% percentiles of the data distribution. (For comparisons at 24 kb, 8.8 kb, and 6.8 kb from the CEN, see Figure 5.S1B). (D) \(k_s\) values calculated using the variance of the distribution of spot positions of the whole population (Equation 5, Table 5.1). Consistent with the doubly tethered bead-spring polymer chain model, the chromatin exhibited a higher \(k_s\) (stiffer spring) closer to the tether point (at 6.8 kb and 8.8 kb from the CEN), and lower \(k_s\) (softer spring) in the middle of the chromosome (at 240 kb from the CEN). The high \(k_s\) observed close to the centromere is due to attachment at the centromere and is not an inherent property of this region of chromatin. When the centromere is detached through its conditional inactivation (Inactive CEN), the \(k_s\) at 8.8 kb from the CEN is reduced. Statistical comparisons at four WT positions are statistically significantly different from each other. Upon conditional centromere inactivation (Inactive CEN), the measured \(k_s\) at 8.8 kb is significantly softer from the measured \(k_s\) for WT at 8.8 kb (Levene’s test, \(p<0.05, \text{Figure 5.S2A}\)).
Figure 5.5 – Loss of cohesin or nucleosome depletion during interphase result in altered confinement and chromatin stiffness.

(A) Upon depletion of cohesin (mcd1-1 at 37 °C, light blue diamonds) we observed a decrease in confinement of chromatin at 240 kb from the CEN that is not due to the increased temperature (compare to WT at 37 °C, dark blue triangles). (B) Bar graph of $R_c$ values (Equation 2). Statistical comparisons reveal significant increased $R_c$ upon depletion of cohesin in mcd1-1 cells (Levene’s test, $p<0.05$, Figure 5.S2B). (C) $K_s$ (Equation 5) is reduced (softened) upon depletion of cohesin as compared to WT at 24 °C and 37 °C. (D) We can simulate the effects of depleting cohesin and losing chromatin looping as an increase in the number of beads in our model (inset). This predicts that increasing the number of beads in the chain will result in reduced confinement (688 nm) as compared to the same bead position (middle, red circle) on a shorter chain (577 nm), consistent with experimentally observed data. (E) MSD curves of lacO/lacI-GFP at 6.8 kb from the CEN in WT, histone depleted (Gal-H3) and α factor treated cells. (F) $R_c$ is reduced (more confined, Equation 2) and (G) effective $k_s$ is increased (stiffer, Equation 5) upon depletion of histone H3. Statistical comparisons show a significant difference in both $R_c$ and $k_s$ upon histone depletion ($p<0.05$) and no significant difference upon α factor treatment ($p>0.05$) (Levene’s test, $p<0.05$, Figure 5.S2C). (H) We hypothesize that reducing nucleosome density by depleting histone H3 will result in reduced $L_p$ (from chromatin to DNA, (Bloom, 2008)). Our doubly tethered bead-spring model predicts an increase in $k_s$ upon reduction of $L_p$ (from $L_p=50$ to $L_p=25$ nm), consistent with experimentally observed increase in $k_s$ at 6.8 kb from the CEN upon nucleosome depletion.
Figure 5.6 – Modeling the chromatin spring as a doubly tethered, confined bead-spring chain with excluded volume interactions can recapitulate chromosome territory formation as observed by chromosome interaction heat maps.

We have tracked bead positions over time for a single run for chains in which one end (centromere-SPB attachment) is at the top of the circle (at 0 degrees) and the other end (telomere attachment) is tethered at (A) four discrete positions (equally distributed between 90 and 298 degrees) or (B) tethered at two discrete positions (90 and 298 degrees). Heat map representation for the average distance during a run between all beads for (C) four discrete attachment points and (D) two attachment points. Heat map values have been normalized to 1 to account for different maximum distances between model runs. For a value of 0, the beads are separated by a small distance and thus highly likely to come into physical contact, whereas a value of 1 represents a large spatial separation and low probability of contact. Chain 1 – red; chain 2 – blue; chain 3 – black; chain 4 – green.
Table 5.1 – Summary of $R_c$ and $k_s$ measurements from population variance

<table>
<thead>
<tr>
<th>LacO array distance from centromere</th>
<th>Relevant genotype/conditions</th>
<th>Sample size</th>
<th>Radius of confinement ($R_c$) (nm)</th>
<th>Effective spring constant ($k_s$) (pN/nm)</th>
<th>Fold over entropic spring</th>
</tr>
</thead>
<tbody>
<tr>
<td>240 kb</td>
<td>WT (Chr II)</td>
<td>43</td>
<td>705</td>
<td>$5.1 \times 10^{-5}$</td>
<td>33x</td>
</tr>
<tr>
<td>24 kb</td>
<td>WT (Chr III)</td>
<td>25</td>
<td>441</td>
<td>$1.3 \times 10^{-4}$</td>
<td>9x</td>
</tr>
<tr>
<td>8.8 kb</td>
<td>WT (Chr III)</td>
<td>40</td>
<td>274</td>
<td>$3.4 \times 10^{-4}$</td>
<td>8x</td>
</tr>
<tr>
<td>6.8 kb</td>
<td>WT (Chr XV)</td>
<td>54</td>
<td>396</td>
<td>$1.6 \times 10^{-4}$</td>
<td>3x</td>
</tr>
<tr>
<td>8.8 kb</td>
<td>Gal-CEN on galactose</td>
<td>23</td>
<td>745</td>
<td>$4.6 \times 10^{-5}$</td>
<td>1x</td>
</tr>
<tr>
<td>240 kb</td>
<td>WT, 37°C</td>
<td>16</td>
<td>691</td>
<td>$5.4 \times 10^{-5}$</td>
<td>35x</td>
</tr>
<tr>
<td>240 kb</td>
<td>mcd1-1</td>
<td>25</td>
<td>892</td>
<td>$3.2 \times 10^{-5}$</td>
<td>21x</td>
</tr>
<tr>
<td>240 kb</td>
<td>mcd1-1, 37°C</td>
<td>33</td>
<td>958</td>
<td>$2.8 \times 10^{-5}$</td>
<td>18x</td>
</tr>
<tr>
<td>6.8 kb</td>
<td>Gal-H3 on glucose</td>
<td>55</td>
<td>319</td>
<td>$2.5 \times 10^{-4}$</td>
<td>5x</td>
</tr>
<tr>
<td>6.8 kb</td>
<td>WT, α factor</td>
<td>43</td>
<td>380</td>
<td>$1.8 \times 10^{-4}$</td>
<td>3x</td>
</tr>
</tbody>
</table>
APPENDIX 5.1

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

YEAST STRAINS AND CELL PREPARATIONS
For WT, sodium azide treatment, and mcd1-1 strains, cells were grown to logarithmic phase at 24°C in rich YPD media. To deplete cellular ATP, cells were incubated for 10 minutes prior to imaging on a gelatin slab containing 0.02% sodium azide and 1 μM 2-deoxyglucose as the only carbon source. To deplete cohesin, logarithmically growing mcd1-1 cells were shifted from 24°C to 37°C for approximately 3 hours prior to imaging (as described in (Stephens et al., 2011)). Histone depletion of GAL-H3 strain was carried out as described in (Bouck and Bloom, 2007). Cells were grown overnight in YPGal media, arrested in G1 with 10 μg/mL α factor for 2 hours, and resuspended in YPD media at 24°C for approximately 3 hours to deplete histone H3. In order to detach the chromosome from the centromere tether point, we employed a conditional centromere as described in (Hill and Bloom, 1987). Cells were grown overnight in YPD (transcriptional repression of the GAL1 promoter allowing for centromere function), overnight in YPL, and resuspended in YPG (transcriptional activation of the GAL1 promoter on galactose containing media silencing the centromere) approximately 5 hours prior to imaging. For imaging, cells were washed and resuspended in YC complete media with 2% glucose for WT, mcd1-1, and histone depleted (Gal-H3) cells, YC complete media with 2% galactose for centromere inactivation (Gal-CEN) cells, or sterile water for azide imaging.

MICROSCOPY
Images were acquired on a Nikon Eclipse Ti wide-field inverted microscope with a 100x Apo TIRF 1.49 NA objective (Nikon, Melville, New York, USA) and Andor Clara CCD camera (Andor, South
Windsor, Connecticut, USA). Images were acquired at room temperature with Nikon NIS Elements imaging software (Nikon, Melville, New York, USA). Seven plane Z sections at 200 nm steps were acquired every 30 seconds for a total of 10 minutes for both GFP and RFP.

**STATISTICAL ANALYSIS**

To allow for direct comparisons of the plateau and equipartition analysis methods for $R_c$ and effective $k_{eq}$, we calculated these for each cell and performed Student’s $t$-tests for statistical analysis. We found no statistical difference between these methods for WT cells at positions close to the tether point at $p<0.05$, and $R_c$ values at 240 kb were not different at $p<0.01$ (Student’s $t$-test, Table 5.1, Figure 5.S1). Since the results using the plateau value showed greater variability and used a fraction of the total data set, we used the equipartition Equations 2 and 5 and the standard deviation from the whole population of cells for remaining comparisons. This results in a single value from the whole population standard deviation (with no associated error bars) and for statistical comparison we compare the homogeneity of population variances by Levene’s test (Figure 5.S2) (Levene, 1960).
Figure 5.51 – Statistical comparison of equations used to calculate \( R_c \) and effective \( k_s \), Related to Figures 5.1 and 5.4.

Box plots of (A) \( R_c \) from Equations 1 (plateau, gray) and 2 (variance, light blue) and (B) \( k_s \) from Equations 4 (plateau, gray) and 5 (variance, light blue). Median values are indicated by a black line, mean values are indicated by a red line, and 95%/5% percentiles are shown as black dots. Data is summarized in Table 5.51. Statistical comparison by Student’s t-tests (p<0.05) did not show any significant differences for positions close to the centromere (WT at 6.8 kb, WT at 8.8 kb, and WT at 24 kb). WT at 240 kb is not significantly different at p<0.01 (Student’s t-test). P values are given.
Figure 5.52 – Statistical comparisons of data sets, Related to Figures 5.1, 5.2, 5.4, and 5.5.

Box plots comparing distribution of \([x - x_{\text{mean}}]\) and \([y - y_{\text{mean}}]\) values in nm used to calculate \(\sigma\) (see Equations 2 and 5, Experimental Procedures). Tables show p-values for Levene’s test for homogeneity of variance between populations, values highlighted in red at \(p<0.05\). **(A)** Comparison of WT at 240 kb, 24 kb, 8.8 kb, and 6.8 kb from the centromere and Inactive CEN (conditional inactivation of the centromere) at 8.8 kb. **(B)** Comparison of WT at 240 kb at 24 °C and 37 °C and \(mcd1-1\) at 240 kb at 24 °C and 37 °C. **(C)** Comparison of WT at 6.8 kb, Gal-H3 at 6.8 kb (conditional depletion of nucleosomes), and WT + \(\alpha\) factor at 6.8 kb from the centromere.

![Box plots](image-url)

<table>
<thead>
<tr>
<th>Levene’s test ((p=0.05))</th>
<th>WT LαO at 8.8 kb</th>
<th>WT LαO at 240 kb</th>
<th>WT LαO at 240 kb</th>
</tr>
</thead>
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<tr>
<td>WT LαO at 6.8 kb</td>
<td>2.5E-22</td>
<td>1.3E-03</td>
<td>3.9E-01</td>
</tr>
<tr>
<td>WT LαO at 8.8 kb</td>
<td>4.2E-40</td>
<td>6.7E-37</td>
<td>9.3E-30</td>
</tr>
<tr>
<td>WT LαO at 240 kb</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Levene’s test ((p=0.05))</th>
<th>WT LαO at 8.8 kb</th>
<th>Gal-CEN LαO at 8.8 kb</th>
<th>Inactive LαO at 8.8 kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT LαO at 8.8 kb</td>
<td>6.9E-29</td>
<td>3.5E-49</td>
<td>1.3E-01</td>
</tr>
<tr>
<td>WT LαO at 240 kb</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Levene’s test ((p=0.05))</th>
<th>mcd1-1 240 C1</th>
<th>mcd1-1 37°C C1</th>
<th>LαO at 240 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT LαO at 240 kb</td>
<td>2.6E-10</td>
<td>2.6E-20</td>
<td>6.4E-01</td>
</tr>
<tr>
<td>mcd1-1 240 C1</td>
<td>2.5E-02</td>
<td>6.2E-05</td>
<td>1.8E-03</td>
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<tr>
<td>mcd1-1 37°C C1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LαO at 240 37°C</td>
<td></td>
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<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Levene’s test ((p=0.05))</th>
<th>Gal-H3 at 6 kb</th>
<th>Alpha Factor LαO at 6.8 kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT LαO at 6.8 kb</td>
<td>2.1E-13</td>
<td>1.8E-01</td>
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<tr>
<td>Gal-H3 at 6.8 kb</td>
<td>2.8E-09</td>
<td></td>
</tr>
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</table>
Figure 5.53 – Additional information regarding chromatin elasticity measurements, Related to Figure 5.3.

The chromatin polymer behaves like an elastic filament. (A) Chromatin spots at 240 kb from the centromere exhibit greater variability in spot decompaction as compared to spots 6.8 kb from the centromere. Box plot showing distribution of measured aspect ratios (=large axis length/small axis length). Black bars indicate median, red bars indicate mean. Black dots above and below indicate 95%/5% percentiles of the data distribution. (B) Scatter plot showing the variance in distance between two chromosomal loci on the same chromosome (red, lacO/lacI-GFP and tetO/tetR-CFP adjacent to the centromere of chromosome XI) compared to variance in WT at 6.8 kb spot position relative to the SPB (black). The variance in spot position (either relative to another chromosomal locus or the SPB) reveals the elastic properties of the chromatin filament.
Nucleosome depletion results in chromatin spot decompaction. (A) Full width-full maximum Gaussian fit measurements of lacO/lacI-GFP spot size show that both compacted and decompacted chromatin spots are statistically significantly larger upon nucleosome depletion (Student’s t-test, p<0.05). Data are represented as mean ± standard deviation. (B) The observed spot shape changes are not the result of changed lacO/lacI-GFP binding. Background corrected lacI-GFP signal intensity is not significantly different between WT and Gal-H3 at 6.8 kb. Data are represented as mean ± standard deviation.
ATP-depletion by sodium azide and deoxyglucose leads to reduced chromatin motion (increased confinement). MSD curves of chromatin spot motion over time of lacO/lacI-GFP integrated at 240 kb or 8.8 kb from the centromere with and without sodium azide and deoxyglucose. $R_c$ values are calculated using Equation 2 and population variance.
Table 5.51 - Summary of WT results from equations used to calculate $R_c$ and effective $k_s$, Related to Figures 5.1 and 5.4.

<table>
<thead>
<tr>
<th>LacO array distance from centromere</th>
<th>240kb</th>
<th>24kb</th>
<th>8.8kb</th>
<th>6.8kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>43</td>
<td>25</td>
<td>40</td>
<td>54</td>
</tr>
<tr>
<td>Radius of confinement $(R_c)$ (nm)</td>
<td>$802 \pm 328$</td>
<td>$494 \pm 137$</td>
<td>$278 \pm 119$</td>
<td>$420 \pm 173$</td>
</tr>
<tr>
<td>$\sigma$ (individual cell)</td>
<td>$682 \pm 209$</td>
<td>$437 \pm 94$</td>
<td>$255 \pm 97$</td>
<td>$381 \pm 126$</td>
</tr>
<tr>
<td>$\sigma$ (entire data set)</td>
<td>705</td>
<td>441</td>
<td>274</td>
<td>396</td>
</tr>
<tr>
<td>Effective spring constant $(k_s)$ (pN/nm)</td>
<td>$6.6 \times 10^{-5}$</td>
<td>$1.6 \times 10^{-4}$</td>
<td>$6.9 \times 10^{-4}$</td>
<td>$2.3 \times 10^{-4}$</td>
</tr>
<tr>
<td>$\sigma$ (individual cell)</td>
<td>$7.8 \times 10^{-5}$</td>
<td>$1.6 \times 10^{-4}$</td>
<td>$8.6 \times 10^{-4}$</td>
<td>$2.4 \times 10^{-4}$</td>
</tr>
<tr>
<td>$\sigma$ (entire data set)</td>
<td>$5.1 \times 10^{-5}$</td>
<td>$1.3 \times 10^{-4}$</td>
<td>$3.4 \times 10^{-4}$</td>
<td>$1.6 \times 10^{-4}$</td>
</tr>
</tbody>
</table>
Table 5.52 - Saccharomyces cerevisiae strains used in this paper, Related to Experimental Procedures.

<table>
<thead>
<tr>
<th>Strain Number</th>
<th>LacO array distance from centromere (centroid)</th>
<th>Relevant genotype</th>
<th>Full genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS406</td>
<td>240 kb from CEN II</td>
<td>WT</td>
<td>MAT α CEN2(240kb)-GFP[10kb] ade5-1, trp1-289, ura3Δ, leu2-3, 112, lys2::insl-Sce1, lacO array next to RAD16 promoter, tetO array next to LYS2 promoter, arg4::hisG Gal1/10 I-Sce1, thr1::HispLacGFP::Nat, ade1::URAptetR-CFP::Hyg, Spc29-RFP::Bsd</td>
</tr>
<tr>
<td>KBY8815</td>
<td>24 kb from CEN III</td>
<td>WT</td>
<td>Mata CEN3(24)-GFP[10kb] ade2-1, his3-11,15, trp1-1, ura3-1, leu2-3, can1-100, LacInLSGFP::HIS3, lacO::LEU2, Spc29-RFP::Hyg</td>
</tr>
<tr>
<td>KBY8812</td>
<td>8.8 kb from CEN III</td>
<td>WT</td>
<td>Mata CEN3(3.8)-GFP[10kb] W303 ade2, ura3, leu2, trp1, his3, can1-100 Cen linked LacO is URA3 tagged (at 3.8kb from CEN3, 10kb array); Spc29-RFP::Hyg</td>
</tr>
<tr>
<td>KBY8065</td>
<td>6.8 kb from CEN XV</td>
<td>WT</td>
<td>Mata CEN15(1.8)-GFP[10kb] ade2-1, his3-11, trp1-1, ura3-1, leu2-3, 112, can1-100, LacInLSGFP::HIS3, lacO::URA3, Spc29RFP::Hyg</td>
</tr>
<tr>
<td>SGD10.2</td>
<td>8.8 kb from CEN III</td>
<td>Gal-CEN</td>
<td>Mata CEN3(3.8)-GFP[10kb] ade2-1, his3-11,15, trp1-1, ura3-1, leu2-3, can1-100, LacInLSGFP::HIS3, lacO::URA3, UraΔNat, GalCEN3::URA, Spc29-RFP::Hyg</td>
</tr>
<tr>
<td>KBY407</td>
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<td>mcd1-1</td>
<td>MAT α CEN2(240kb)-GFP[10kb] ade5-1, trp1-289, ura3Δ, leu2-3, 112, lys2::insl-Sce1, lacO array next to RAD16 promoter, tetO array next to LYS2 promoter, arg4::hisG Gal1/10 I-Sce1, thr1::HispLacGFP::Nat, ade1::URAptetR-CFP::Hyg, Spc29-RFP::Bsd, mcd1-1</td>
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<tr>
<td>KBY8062</td>
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<td>Gal-H3</td>
<td>Mata CEN15(1.8)-GFP[10kb] ade2-1, his3-11, trp1-1, ura3-1, leu2-3, 112, can1-100, LacInLSGFP::HIS3, lacO::URA3, Spc29RFP::Hyg, HHT1::TRP, KAN-GAL-HHT2</td>
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<tr>
<td>KBY9424</td>
<td>LacO/LacI-GFP at 4.5 kb from CEN XI</td>
<td>2-color</td>
<td>Mata ade1 met14 ura3-52 leu2-3, 112 his3-11,15 lys2::lact-GFP-NLS::Nat, met14::lactO-Hyg, trp::[Ura, pSR8(tetO-his3) at Met14, Hyg::[Trp1, pDB49 (tetR-CFP-Hyg), Hyg::Leu, Spc29-RFP-Hyg, Nat::Kan</td>
</tr>
</tbody>
</table>
APPENDIX 5.2

FORMULAE

Starting calculations

\[
\text{plateau} = \text{mean of MSD from } 330 - 390 \text{ s} \quad (\text{nm}^2)
\]

\[
\sigma^2 = \text{mean} \left( \sigma_x^2, \sigma_y^2 \right) \quad (\text{nm}^2)
\]

\[
[\mu_x, \sigma_x] = \text{normfit}(x_t - x_{\text{mean}})
\]

\[
[\mu_y, \sigma_y] = \text{normfit}(y_t - y_{\text{mean}})
\]

\[
\text{Pop } \sigma^2 = \text{mean} \left( \text{pop } \sigma_x^2, \text{pop } \sigma_y^2 \right) \quad (\text{nm}^2)
\]

\[
\text{pop } [\mu_x, \sigma_x] = \text{normfit}(x_{t,\text{cell}} - x_{\text{mean,cell}})
\]

\[
\text{pop } [\mu_y, \sigma_y] = \text{normfit}(y_{t,\text{cell}} - y_{\text{mean,cell}})
\]

\[
\langle \Delta r_0^2 \rangle = \langle x_0^2 \rangle + \langle y_0^2 \rangle \quad (\text{nm}^2)
\]

\[
\langle x_0^2 \rangle = \langle (x_t - x_{\text{mean}})^2 \rangle
\]

\[
\langle y_0^2 \rangle = \langle (y_t - y_{\text{mean}})^2 \rangle
\]

Radius of confinement equations

From plateau

\[
R_c = \frac{5}{2d} \sqrt{\text{plateau}} \quad (\text{nm})
\]

From variance

\[
R_c = \frac{5}{2d} \sqrt{d \sigma^2 + \langle \Delta r_0^2 \rangle}
\]

Effective spring constant equations

From plateau

\[
\text{eff } k_s = \frac{dk_{\beta \Gamma}}{\text{plateau} - \langle \Delta r_0^2 \rangle} \quad (\text{pN per nm})
\]

From variance

\[
\text{eff } k_s = \frac{k_{\beta \Gamma}}{\sigma^2}
\]
REFERENCES


Lee, M.T., and Bachant, J. (2009). SUMO modification of DNA topoisomerase II: trying to get a CENse of it all. DNA Repair (Amst) 8, 557-568.


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