Cell cycle regulation of centromere assembly in *Drosophila* male meiosis

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List of figures

List of tables

Abbreviations

ActD	Actinomycin D
APC:	Anaphase Promoting Complex
BDSC	Bloomington Stock Center
BLAST	Basic Local Alignment Search tool
BSA	Bovine Serum Albumin
C. *elegans*	*Caenorhabditis elegans*
CAL1	Chromosome Alignment Defect 1
CCAN	Constitutive Centromere Associated Network
Cdk	Cyclin dependent kinase
cDNA	complementary DNA
CENP	Centromere Protein
CID	Centromere Identifier
Cnp1	Centromeric Protein 1
Cse4	Chromosome Segregation Protein 4
CYCA	Cyclin A
CYCB	Cyclin B
DAPI	4',6-Diamidino-2-Phenylindole
DGRC	*Drosophila* Genomics Resource Centre
Dm	*Drosophila melanogaster*
DMSO	Dimethyl Sulfoxide

DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotides
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
EU	5-Ethynyl Uridine F
FBS	Foetal Bovine Serum
Fig	Figure
FISH	Fluorescence In Situ Hybridisation
G1/2	Growth phase
GAL4	Galactose Induced Protein 4
H2Av	Histone 2A Variant
HJURP	Holliday Junction Recognition Protein
HRP	Horse Radish Peroxidase
hs	heat shock
IF	Immunofluorescence
LacI	Lac-Inducer
LacO	Lac operator
LB	Luria broth
LDS	Lithium Dodecyl Sulfate

MEI-S332	meiotic from via Salaria 332
mg	milligram
µM	micromolar
µg	microgram
ml	milliliters
mM	millimolar
mRNA	messenger RNA
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PMSF	Phenylmethanesulfonyl fluoride
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid intereference
RT	Room Temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium Dodecyl Sulfate
Ser	Serine
S phase	Synthesis phase
S. pombe	*Schizosaccharomyces pombe*
Scm3	Suppressor of Chromosome Missegregation 3

UAS	Upstream Activating Sequence
UV	Ultraviolet
VDRC	Vienna *Drosophila* Resource Center
LAP	Localisation and Affinity Purification
*****	$p \leq 0.05$
******	$p \leq 0.01$
*******	$p \leq 0.001$
*******	$p \leq 0.0001$
ns	$p > 0.05$

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This thesis is to the memory of my father

Abstract

Centromeres are chromosomal sites required for faithful chromosome segregation. CENP-A is the histone H3 variant that demarcates centromeres from bulk chromatin. Errors in centromere assembly result in segregation defects that can lead to genome instability, sterility and genetic disorders. In *Drosophila*, the CENP-A assembly partners CENP-C and CAL1 assist in constructing a functional centromere that provides a platform for kinetochore assembly.

In this study, we exploit *Cenp-C*$^{Z3\text{-}4375}$ and *cal1*^{2k32} mutants to explore the role of CENP-C and CAL1 in CENP-A assembly in male meiosis (Chapter 3). We find that levels of CENP-A are reduced in *Cenp-C*$^{Z3\text{-}4375}$ and *cal1*^{2k32} mutants. We report for the first time that CENP-A is sequestered in the nucleolus in wild type spermatocytes. We further show a correlation between nucleolar integrity and CENP-A levels. Nucleolar disruption and reduced global RNA transcription correlates to reduced centromeric CENP-A in *Cenp-C*$^{Z3\text{-}4375}$ spermatocytes and segregation defects including lagging chromosomes and decondensed chromatin in spermatids.

In Chapter 4 and 5 we characterise cell cycle regulation of meiotic CENP-A assembly given its temporal disconnect from mitosis. Meiotic CENP-A assembly is coupled to high Cdk activity, which in mitosis, inhibits assembly. We report that Cdk1/2 inhibition in early prophase I spermatocytes stimulates CENP-A incorporation to centromeres. We find that Cdk1/2 binding partners CYCA and CYCB localise to meiotic centromeres and that overexpression of stable CYCA or CYCB compromises CENP-A assembly. Testis-specific RNAi and site directed mutagenesis studies implicate CAL1 as the potential substrate through which CYCA or CYCB associated kinase activity mediates this inhibitory regulation. In Chapter 6 we uncover a single phosphorylation site on CAL1 that is important for Cdk mediated regulation of meiotic CENP-A assembly.

We conclude that perturbation of CENP-C or CAL1 has adverse effects on centromere assembly and ultimately chromosome segregation and fertility.

Chapter 1. Introduction

1.1. The centromere

The centromere is a primary constriction site on chromosomes (Flemming 1882), required for microtubule attachments and thus accurate mitotic and meiotic cell divisions. It is now appreciated that centromeres are regions of highly specialised chromatin where the kinetochore assembles (Brinkley & Stubblefield, 1966; Robbins & Gonatas, 1964), for the equipartition of genes, (figure 1.1). Errors in centromere assembly can result in aneuploidy. In somatic cells such failures can drive cancer (Amato et al., 2009; Tomonaga et al., 2003) while in the germ line defective segregation has profound effects on fertility (Hassold & Hunt, 2001).

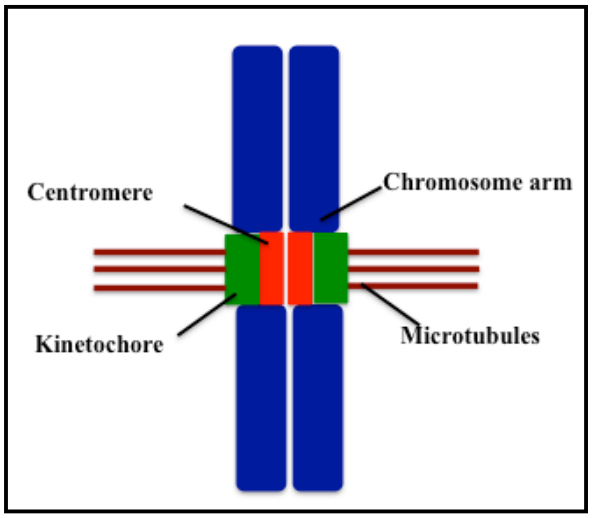


Figure 1.1. Schematic of a chromosome. The centromere is shown in red, the kinetochore in green and microtubules are shown in dark red. *Adapted from* (Przewloka & Glover, 2009)

1.1.1. Centromere structure

The specific structure, architecture and scale of individual centromeres exhibit considerable variation among eukaryotes. At one extreme are holocentric centromeres where the centromere position spreads throughout the chromosome (Guerra et al 2010). These diffuse centromeres are typically found in nematodes, some insects and plants and are characterised by microtubules that attach over the length of the chromosome (Buchwitz et al., 1999; Dernburg 2001; Nagaki et al., 2005). The conventional type of centromere is the localised centromere in which microtubules attach to a single locus of the chromosome, a phenomenon known as monocentricity (McKinley & Cheeseman, 2016; Pluta et al., 1995). Localised centromeres fall into two categories. Point centromeres and regional centromeres. Point centromeres are defined as centromeres that require a specific DNA sequence for kinetochore assembly and DNA segregation (Carbon & Clarke, 1984; Clarke & Carbon, 1983; McGrew et al., 1986). Point centromeres, found in *Saccharomyces cerevisiae*, comprise of one centromere specific nucleosome that spans ~ 125 bp of DNA and contains three distinct centromeric elements CDEI, CDEII, CDEIII, that are functionally important for recruiting centromeric proteins, (Clarke & Carbon, 1980) (figure 1.2 A).

Most eukaryotes have regional centromeres, typically characterised by hundreds of kilobases of repetitive alpha satellite DNA sequences (reviewed in McKinley & Cheeseman, 2016). These repetitive DNA sequences are highly divergent among eukaryotes. However, despite this divergence, regional centromeres possess structural similarities among eukaryotes (reviewed in McKinley & Cheeseman, 2016). Regional centromeres typically consist of a central and an outer domain known as the pericentromere (reviewed in McKinley & Cheeseman, 2016). For example, in *Schizosacharomyces pombe* the centromere localises to a larger domain of 40-100 kb. This domain consists of a single copy of non-repetitive central core sequence, *cnt*, surrounded by two classes of repetitive sequences, the innermost repeats, *imr,* and the outermost repeats, *otr*, (figure 1.2 B) (Allshire & Ekwall, 2015; Clarke et al., 1986; Fishel et al., 1988). In mammalians, centromeres harbor A/T rich alpha satellite DNA repeats (~171 bp) organised into higher order arrays extending from 100 kb to several megabases in size (Manuelidis, 1978; Mitchell et al., 1985; Tyler-Smith et al., 1993;

Waye & Willard, 1987; Willard, 1985). The centromere chromatin is divided into two domains, a core domain for kinetochore assembly and domains of flanking pericentric heterochromatin, (reviewed in McKinley & Cheeseman, 2016), (figure 1.2 D).

Similarly in *Drosophila*, centromeres are embedded within highly repetitive DNA. The core centromere sequence was mapped to a 420 kb region (Murphy & Karpen, 1995), which consists of tandem arrays of simple 5bp satellite repeats, AATAT and CTCTT, (Sun et al., 2003) (figure 1.2 C).

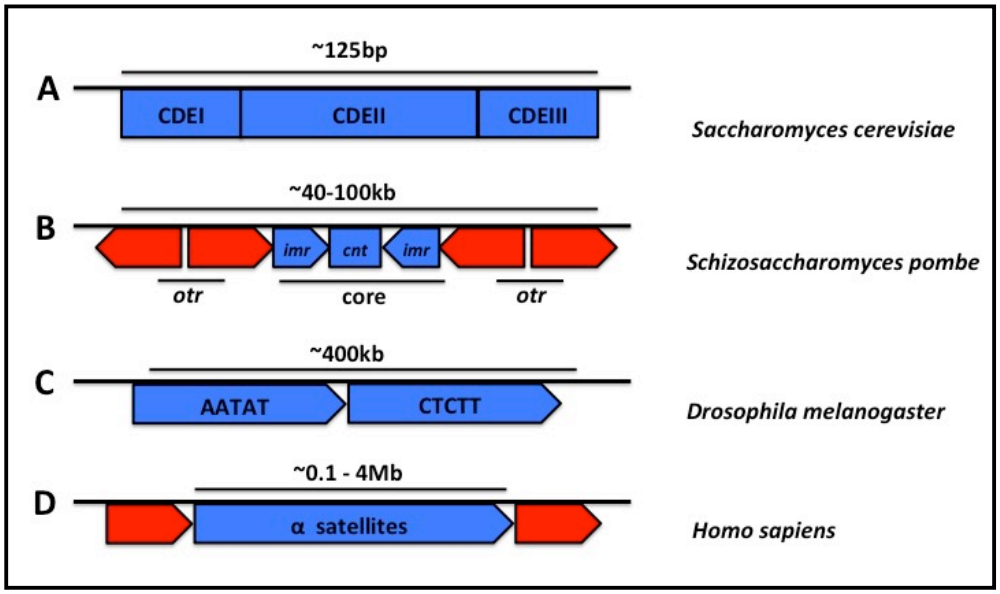


Figure 1.2. Centromere DNA structure. Organisation of centromeric DNA (blue) and pericentromeric DNA (red) between eukaryotes. **(A)** point centromere of *S. cerevisiae*, **(B)** regional centromere of *S. pombe*, **(C)** regional centromere of D. melanogaster **(D)** regional centromere of H. sapiens. Adapted from (Allshire & Karpen, 2008)

1.2. Epigenetic regulation of centromeric chromatin

In most eukaryotes, centromere identity is epigenetically defined, (Allshire & Karpen, 2008; Eichler, 1999; Karpen & Allshire, 1997). Epigenetics refers to inheritable properties of chromosomes, which are not determined by DNA sequence (Waddington 1942). Compelling evidence supporting epigenetic centromere specification came from analysis of dicentric chromosomes from patient samples (Earnshaw & Migeon, 1985; Higgins et al., 2005; Karpen & Allshire, 1997; Sato et al., 2012). In these early analysis, one centromere was functionally inactivated with no changes to the underlying DNA sequence (Earnshaw & Migeon, 1985; Higgins et al., 2005; Karpen & Allshire, 1997; Sato et al., 2012). Further evidence supporting epigenetic specification of centromeres comes from observations of centromere formation at unconventional sites, a phenomenon known as neocentromere formation (Amor et al., 2004; Depinet et al., 1997; Marshall et al., 2008; Tyler-Smith et al., 1999; Voullaire et al., 1993; Warburton et al., 1997). Both active centromeres of dicentric chromosomes and neocentromeres have been shown to recruit CENP-A and all other centromere proteins (Bassett et al., 2010; Lo et al., 2001; Voullaire et al., 1993; Warburton et al., 1997). Further demonstration of epigenetic inheritance of centromere identity came from studies that generated stable neocentromeres on chromosome fragments lacking typical centromeric loci in yeast and flies, (Ishii et al., 2008; Williams et al., 1998). These studies proved that underlying DNA sequence is insufficient for centromere specification.

Although precise DNA sequences are not necessary or sufficient for centromere identity and function, it has been shown that some centromere DNA sequences can indeed determine centromere function when exogenously introduced into some organisms (Haaf et al., 1992). Pioneering work integrated human alpha satellite DNA into African green monkey cells. This resulted in centromere protein binding at the sites of integration suggesting that alpha satellite DNA confers information for centromere protein binding (Haaf et al., 1992). In addition, neocentromere formation has been reported to favor certain regions within a given chromosome (Marshall et al., 2008). This non-random distribution of neocentromeres suggests existence of centromeric DNA signatures. Further evidence describes the interplay between transcription of centromeric DNA repeats and centromere function. Targeting of a

transcriptional silencer to alpha satellite repeats using human artificial chromosomes results in loss of centromere integrity (Nakano et al., 2008). Remarkably, emerging evidence reports identification of centromeric DNA transcripts in humans, plants and yeast vital for centromere integrity (Carone et al., 2009; Chan et al., 2012; Choi et al., 2011; Ohkuni & Kitagawa, 2011; Quénet & Dalal, 2014; Topp et al., 2004; Wong et al., 2007). For example, Chan and colleagues demonstrated that in the absence of alpha satellite RNA transcripts, centromere proteins delocalise and relocalise after alpha satellite replenishment (Wong et al., 2007). Moreover RNAPII has been shown to localise to centromeres in *Drosophila* (Rošić et al., 2014), and to interact with other centromeric proteins (Chen et al., 2015). Notably, facilitates chromatin transcription (FACT) a complex that allows movement of transcriptional machinery through chromatinised templates (Belotserkovskaya et al., 2003; Orphanides et al., 1999), has been reported to interact with a specific centromeric protein in *Drosophila* (Chen et al., 2015) to facilitate de novo centromere assembly (Chen et al., 2015). A separate study also demonstrated that RNAPI and RNAPII transcription is vital for meiotic centromere assembly using RNAP inhibitors, Actinomycin D and CX5461 (Kwenda et al., 2016).

Thus in some instances, α satellite DNA is sufficient to confer centromere formation thus revealing an underexplored role for ordered repeats of regional centromeres. The mechanism by which α satellite DNA initiates centromere formation is a subject of current investigation. One hypothesis is that α satellite DNA acquire chromatin marks that favor assembly of CENP-A nucleosomes (Bergmann et al., 2011; Ohzeki et al., 2012).

1.3. Centromere protein A (CENP-A): an epigenetic hallmark of centromeres

Despite the aforementioned contributions from DNA sequences, in eukaryotes centromere identity is defined epigenetically (reviewed in Black & Bassett, 2008) by the histone H3 variant CENP-A (reviewed in Karpen & Allshire, 1997; McKinley & Cheeseman, 2016). CENP-A has thus been labeled as the primary epigenetic mark required for demarcating centromere chromatin.

CENP-A was initially discovered as a centromere specific antigen from human patients with the autoimmune disease Calcinosis Raynauds Esophageal dismotility Sclerodactyly and Telangiectasia (CREST) syndrome (Earnshaw & Rothfield, 1985). Biochemical approaches demonstrated that CENP-A is a component of chromatin similar to histones (Palmer et al., 1987; Palmer et al., 1990; Palmer et al., 1991) which replaces histone H3 in centromere specific nucleosomes (Sullivan et al., 1994; Yoda et al., 2000)

Analysis of CENP-A at the sequence level revealed that CENP-A and histone H3 share a similar organisation comprising of a histone fold domain that has 60% sequence similarity with H3 and an N terminal tail that differs from H3 (Sullivan et al., 1994), (figure 1.3 A). The histone fold domain is necessary for targeting CENP-A to the centromere (Sullivan et al., 1994). This region contains the Centromere Targeting Domain (CATD) (Black et al., 2004; Black et al., 2007) (figure 1.3 B). The first loop and second α helix (L1- α2) of the histone fold domain are necessary for targeting CENP-A to the centromere (Black et al., 2007; Black et al., 2007). Mutations where the CATD is replaced with residues from histone H3 abolish efficient targeting of CENP-A to centromeres (Black et al., 2004; Shelby et al., 1997), further supporting the importance of this region in centromere targeting. The N terminus of CENP-A however differs significantly from that of H3; it is neither conserved in sequence nor length even among the centromeric H3 like molecules (figure 1.3 A). In recent years, the N terminus has been implicated in the recruitment of kinetochore proteins in different organisms (Chen et al., 2000; Fachinetti et al., 2013; Folco et al., 2015; Logsdon et al., 2015; Van Hooser et al., 2001).

CENP-A homologues have been identified in various eukaryotic model systems. In *Drosophila melanogaster*, CENP-A is known as CID, Cse4 in *Saccharomyces cerevisiae*, Cnp1 in *Schizosaccharomyces pombe* and CenH3 in *Arabidopsis thaliana* (Blower & Karpen, 2001; Buchwitz et al., 1999; Henikoff et al., 2000; Stoler et al., 1995; Takahashi et al., 2000; Talbert et al., 2002). Interestingly, CENP-A is poorly conserved across eukaryotes and divergence is extreme in the N terminus of CENP-A (figure 1.3 A) (reviewed in De Rop et al., 2012)

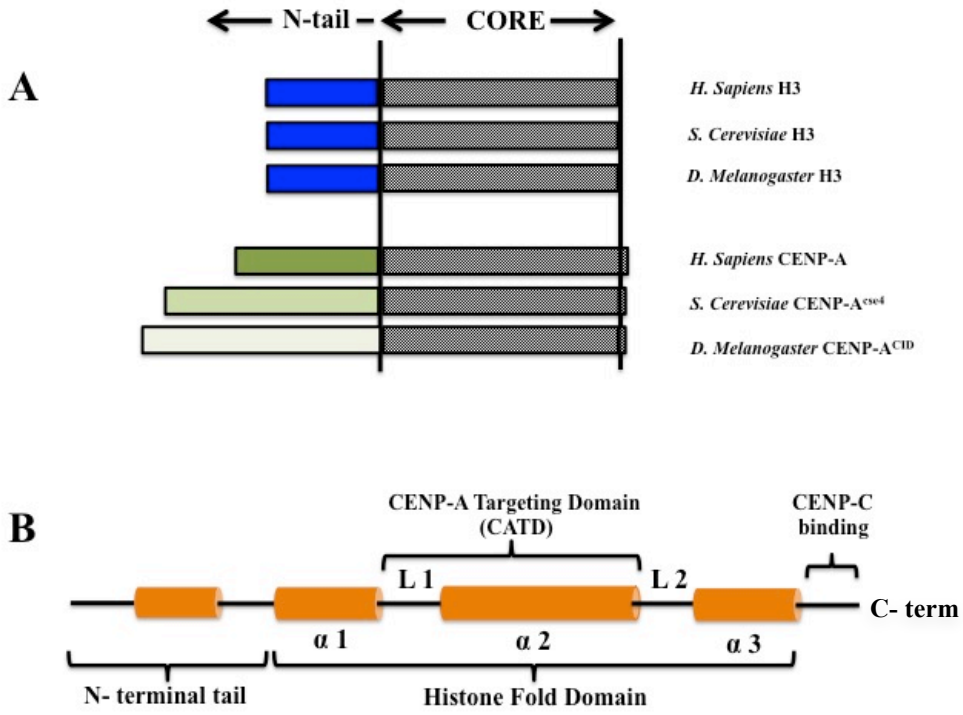


Figure 1.3. Schematic of histone H3 family of proteins. (A) The typical histone H3 proteins are well conserved across eukaryotes while the centromeric H3 like histones are more divergent in sequence and length particularly in the N-terminus. **(B)** Shows a schematic of the functional domains of CENP-A. Loop 1 separates α 1 and α 2. The CENP-A targeting domain contains key differences from H3 and is sufficient for centromeric localisation when substituted into canonical H3. CENP-C binds to the carboxy terminal region of CENP-A. Adapted from Malik & Henikoff 2001; Allshire & Karpen 2008.

Despite the structural variation among species, the function of CENP-A in defining centromere identity is highly conserved. Consistently, CENP-A is found at neocentromeres (Marshall et al., 2008), and active centromeres of dicentric chromosomes (Earnshaw & Migeon, 1985). Importantly, CENP-A is vital for assembly of kinetochore components (Chen et al., 2000; Fachinetti et al., 2013;

Howman et al., 2000; Liu et al., 2006; Régnier et al., 2005; Sullivan, 2001). Targeting of CENP-A to an unconventional chromosome sufficiently generates chromatin platforms capable of orchestrating microtubule attachment and ultimately chromosome segregation (Barnhart et al., 2011; Heun et al., 2006; Logsdon et al., 2015; Mendiburo et al., 2011). Remarkably, loss of CENP-A results in failure to define the attachment site for spindle microtubules in both mitosis and meiosis (reviewed in McKinley & Cheeseman, 2016). In addition, over expression of CENP-A leads to CENP-A mislocalisation and formation of multicentric chromosomes leading to chromosome mis-segregation events (Heun et al., 2006; Moreno-Moreno et al., 2006).

1.4. Centromere assembly

In most eukaryotes, centromere assembly is mediated by a chromatin based epigenetic mechanism involving the histone H3 variant CENP-A (reviewed in Allshire & Karpen, 1997; McKinley & Cheeseman, 2016). CENP-A assembly occurs in a replication independent manner (Shelby et al., 2000) differing from canonical histones that are deposited into chromatin during S phase. Indeed expression of canonical histones is predominant in S phase and distinct from CENP-A protein levels which peak in G2 phase (Shelby et al., 1997). In S phase CENP-A levels are halved and consequently partitioned to each daughter cell, (Jansen et al., 2007a). This results in a dilution of centromeric CENP-A. The transmission of CENP-A therefore identifies these chromosomal regions as centromeres following DNA replication. In addition, CENP-A is not replaced by protamines during spermatid differentiation in mammals, *Xenopus laevis* or *Drosophila melanogaster* (Dunleavy et al., 2012; Milks et al., 2009; Palmer et al., 1990; Raychaudhuri et al., 2012), and therefore provides a template for centromere location in the progeny. Fundamental to this transmission is CENP-A's stability revealing little exchange once incorporated at centromeres (Bodor et al., 2013; Falk et al., 2015; Jansen et al., 2007), thus making it a bona fide epigenetic mark for centromere identity.

1.4.1. Deposition or assembly of CENP-A nucleosomes

Assembly of new CENP-A requires coordinated activity of several key factors (reviewed in McKinley and Cheeseman, 2016), table 1. The CENP-A assembly pathway can be classified into three stages, each involving different protein complexes.

a) **Recognition and licensing**
b) **Assembly of newly synthesised CENP-A**
c) **Maintenance of newly incorporated CENP-A**

The initial stage, recognition and licensing, is characterised by sensing the location of the centromere and modification of underlying chromatin to maintain a permissive state for CENP-A assembly. CENP-A assembly factors then associate with the centromere allowing for newly synthesised CENP-A assembly into centromeric chromatin. Finally deposited CENP-A nucleosomes are maintained through chromatin remodeling to fully stabilise centromeric chromatin.

a) Recognition and Licensing

In mammalian cells, the earliest recognised step in the assembly of newly synthesised CENP-A is the association of the Mis18 complex with centromeres after mitotic exit (Fujita et al., 2007; Hayashi et al., 2004; Maddox et al., 2007). Mis18 was first discovered in fission yeast along with Mis16, homolog for RbAp48 and RbAp46 in humans, through a temperature sensitive screen for mutants that mis-segregated their chromosomes due to reduction of CENP-A in *S. pombe* (Hayashi et al., 2004). Sequence analysis identified mammalian homologs of Mis18, Mis18α and Mis18β (Fujita et al., 2007). In addition, RNAi screens in *C. elegans* uncovered a homolog of Mis18BP1 known as KNL-2 (Maddox et al., 2007). Knockdown of Mis18α, β and Mis18BP1 demonstrated that these proteins are dependent on each other for localising to the centromere (Fujita et al., 2007). Knockdown of the Mis18 complex as well as RbAp48 and RbAp46 further confirmed that the entire complex is necessary for assembly of CENP-A at the centromere (Fujita et al., 2007; Hayashi et al., 2004)

It has been proposed that the Mis18 complex promotes CENP-A deposition by recruiting chromatin modifying activity to centromeric chromatin which allows a permissive state, to 'prime' CENP-A recruitment (Fujita et al., 2007a; Hayashi et al., 2004). Consistently, the Mis18 complex interacts with chromatin modifying complexes (Fujita et al., 2007; Zhang et al., 2006).

Consistent with this hypothesis, temperature sensitive mutants of Mis16 and Mis18, that mis-segregate their chromosomes due to reduced CENP-A in fission yeast demonstrate an increase in the acetylation state of centromeric H3 and H4 (Hayashi et al., 2004), suggesting that Mis16 and Mis18 are necessary for inhibition of acetylation to generate a permissive state for CENP-A recruitment. More still, although contrary to findings in yeast, the mammalian Mis18 complex has been shown to promote acetylation to generate a permissive state for CENP-A recruitment (Fujita et al., 2007). Depletion of members of the Mis18 complex results in defective incorporation of newly synthesised centromeric CENP-A (Fujita et al., 2007). This phenotype however could be rescued by concurrently treating the Mis18 depleted cells with the HDAC inhibitor trichostatin (Fujita et al., 2007) suggesting a pivotal role for acetylation in mammalian CENP-A assembly. It has also been proposed that the Mis18 complex affects CENP-A assembly by altering DNA modifications. DNA methyltransferases have been demonstrated to interact with centromeric chromatin through Mis18α (Gopalakrishnan et al., 2009; Kim et al., 2012). Mis18α loss in conditional knockout studies in mouse embryonic fibroblasts resulted in reduced centromeric DNA methylation, leading to reduction in centromeric CENP-A (Kim et al., 2012). Surprisingly, the Mis18 complex is not conserved across eukaryotes (Hayashi et al., 2004; Maddox et al., 2007). *Drosophila melanogaster* for example has no Mis18 homologue (Phansalkar et al., 2012), suggesting that in other organisms, CENP-A assembly is not dependent on the presence of the Mis18 complex.

Thus key players involved in the initiation step of CENP-A recruitment include, but are not limited to, the Mis18 complex, which consists of Mis18α, Mis18β and Mis18BP1 along with RbAp48 and RbAp46.

b) CENP-A deposition

CENP-A deposition is achieved by the activity of chaperone proteins (Chen et al., 2014; Dunleavy et al., 2009; Foltz et al., 2009), defined as proteins that interact with histones and activate histone transfer and are not part of the final product (De Koning et al., 2007). A defining characteristic is the ability to form a complex with histones before assembly into nucleosomes (Dunleavy et al., 2009; Foltz et al., 2009).

Purification of non-nucleosomal CENP-A complexes in mammalian cells identified Holliday Junction Recognition Protein, HJURP, as a CENP-A specific chaperone (Dunleavy et al., 2009; Foltz et al., 2009). It has been demonstrated that CENP-A, histone H4 and HJURP form a complex that localises to centromeres to facilitate new CENP-A assembly (Bernad et al., 2011; Dunleavy et al., 2009; Foltz et al., 2009; Shuaib et al., 2010). This complex sufficiently determines the site of centromere formation. Notably retargeting of HJURP to non-centromeric sites leads to CENP-A incorporation into chromatin (Barnhart et al., 2011). In addition, HJURP knockdown leads to reduction of CENP-A at centromeres and perturbs assembly of newly synthesised CENP-A, which has been linked to severe segregation defects (Dunleavy et al., 2009; Foltz et al., 2009).

A fundamental feature characteristic for a CENP-A assembly factor is the capacity to discriminate its target from canonical histones (Chen et al., 2014). Structural studies of the mammalian CENP-A assembly factor HJURP reveal that HJURP distinguishes CENP-A from H3 through the CENP-A targeting domain, CATD, (Foltz et al., 2009; Zhou et al., 2011) and the amino terminal CENP-A binding domain of HJURP (Bassett et al., 2012; Hu et al., 2011; Shuaib et al., 2010; Zhou et al., 2011). Notably HJURP localisation to centromeres is dependent on the Mis18 complex. siRNA against Mis18α or Mis18BP1 eliminates HJURP recruitment to centromeres suggesting that the Mis18 licensing complex is required for the recruitment of HJURP to centromeres (Barnhart et al., 2011). However, direct interaction of the Mis18 complex and HJURP has not yet been reported (Barnhart et al., 2011; Fujita et al., 2007; Hayashi et al., 2004).

In both budding and fission yeast, Suppressor of chromosome mis-segregation (Scm3) is the CENP-A chaperone (Camahort et al., 2007; Cho & Harrison, 2011; Mizuguchi et al., 2007; Shivaraju et al., 2011; Stoler et al., 2007), that is homologous to HJURP, (Sanchez-Pulido et al., 2009). Co-purification and two-hybrid systems revealed that in fission yeast, Scm3 and CENP-A^{Cnp1} interact in vivo. Moreover chromatin immunoprecipitation studies demonstrate that Scm3, like CENP-A^{Cse4} is associated with centromeric DNA in budding yeast (Stoler et al., 2007). Deletion of Scm3 in budding yeast results in chromosome loss or mis-segregation as a result of defective CENP-A recruitment (Camahort et al., 2007; Mizuguchi et al., 2007; Stoler et al., 2007). Notably, the Mis18 complex is also required for Scm3 recruitment to centromeric chromatin in fission yeast (Hayashi et al., 2004; Pidoux et al., 2009), indicating an essential role for the licensing complex in the recruitment of CENP-A chaperones.

In *Drosophila melanogaster*, Chromosome Alignment Defect 1 (CAL1) is the CENP-A^{CID} chaperone required for assembly of newly synthesised CENP-A nucleosomes. (Chen et al., 2014; Dunleavy et al., 2012; Erhardt et al., 2008; Mellone et al., 2011). CAL1 associates with pre-nucleosomal CENP-A-H4 complex and discriminates between CENP-A and Histone H3 (Chen et al., 2014). When H3 is substituted for CENP-A in binding assays a trimeric complex fails to form (Chen et al., 2014). Moreover, tethering experiments using the LacI-LacO system, demonstrated that CAL1 is a potent CENP-A and kinetochore assembly factor in *Drosophila* cells (Chen et al., 2014). Notably, these CAL1 induced ectopic centromeres can recruit ectopic microtubule attachments suggesting full functionality (Chen et al., 2014). Taken together these findings demonstrate that CAL1 is a bona fide *Drosophila* CENP-A assembly factor.

Despite the functional similarity between yeast Scm3 and HJURP, sequence analysis reveal a limited 50 amino acid region of homology present within the N terminus (Sanchez-Pulido et al., 2009). Interestingly, secondary structure homology prediction servers (Kelley & Sternberg, 2009; Söding et al., 2005), have also identified a ~40 amino acid region of similarity between the N terminus of CAL1 and part of the *Kluyveromyces lactis* Scm3-domain (Phansalkar et al., 2012). This region is conserved in Scm3 and HJURP and facilitates interaction with CENP-A in yeast and

humans (Aravind et al., 2007; Barnhart et al., 2011; Bassett et al., 2012; Mizuguchi et al., 2007; Sanchez-Pulido et al., 2009; Shuaib et al., 2010). Deletion of this conserved patch, CAL1$^{\Delta 1\text{-}40}$, results in failure of CENP-A recruitment in *Drosophila* S2 cells. (Chen et al., 2014). Thus albeit lack of sequence conservation among the CENP-A chaperones, it is noteworthy that similar structures among these chaperones interact with CENP-A

c) Maintenance of CENP-A nucleosomes

Although the molecular players directing deposition of CENP-A nucleosomes into centromeric chromatin have been progressively characterised in different model systems over the years, knowledge of how CENP-A is maintained after initial deposition into centromeric chromatin remains limited.

Experimental evidence in mammalian cells support the hypothesis that CENP-A nucleosomes remain unstable following initial assembly, and thus require additional activity of remodeling complexes including GTP cycling to fully mature and stabilise (Lagana et al., 2010; Perpelescu et al., 2009). Immunoprecipitation of the CENP-A licensing factor, Mis18BP1 (KNL-2) led to the identification of MgcRacGAP, a GTPase activating protein (Lagana et al., 2010) and revealed a possible role for this GTPase in centromere identity (Izuta et al., 2006; Lagana et al., 2010; Perpelescu et al., 2009). Interestingly, MgcRacGAP briefly localises to centromeres following CENP-A deposition. This localisation is in line with a role in CENP-A maintenance and not licensing or deposition (Lagana et al., 2010). Knockdown of MgcRacGAP and its partner GEF, ECT2 required to regulate GTPase cycles results in loss of newly assembled CENP-A nucleosomes indicating a pivotal role of GTPase cycling in maintaining CENP-A at centromeres (Lagana et al., 2010). This maintenance mechanism however could potentially require downstream effector proteins that are not yet identified.

The Remodeling and Spacing Factor complex, (RSF), has also been implicated in maintenance of CENP-A stability (Perpelescu et al., 2009). The RSF complex associates with centromeres during CENP-A deposition and has been shown to confer stability of newly deposited CENP-A nucleosomes (Perpelescu et al., 2009).

Depletion of RSF reduced the stability of CENP-A. It is hypothesised that the in vivo activity of RSF might mainly be remodeling the preexisting CENP-A at centromeric regions to maintain stability (Perpelescu et al., 2009).

Recently, human Centromeric protein C, CENP-C, and Centromeric protein N, CENP-N, have been implicated in CENP-A maintenance in mammalian cells (Falk et al., 2015; Falk et al., 2016; Guo et al., 2017). Using rapid protein degradation (Auxin Inducible Degron tagging) and gene replacement, removal of CENP-C caused a dramatic decrease of the existing CENP-A pool at centromeres (Guo et al., 2017). Full length CENP-C as well as the CENP-C central domain rescued CENP-A retention. The stability of CENP-A nucleosomes following CENP-C replacement was exclusively attributed to the arginine anchor in the CENP-C central domain (Guo et al., 2017). Interestingly removal of CENP-N using an AID tagging approach reduces CENP-C levels at centromeres and evidently produces defects in CENP-A retention (Guo et al., 2017). Notably, dual removal of CENP-N and CENP-C severely compromised CENP-A retention thereby supporting a model wherein CENP-C and CENP-N partner in maintaining CENP-A nucleosome levels at centromeres (Guo et al., 2017). Notably, *Drosophila* CENP-C has also been implicated in CENP-A maintenance in spermatocytes. A C terminal missense mutation in CENP-C leads to reduction in CENP-A retained on mature sperm (Kwenda et al., 2016), indicating a role in maintenance of CENP-A on mature sperm bundles.

Although advances have been made in understanding how CENP-A is maintained on centromeric chromatin, many important questions still remain to gain thorough understanding of CENP-A maintenance following initial deposition, particularly among other eukaryotic model systems.

Table 1.1: Summary of the protein complexes required for CENP-A assembly in different model systems

Model system	Recognition and Licensing	Deposition	Maintenance	References
Human	Mis18 complex; RbAp48/RbAp46	HJURP	MgcRacGAP; CENP-C, CENP-N; RSF	(Dunleavy et al., 2009; Falk et al., 2015; Falk et al., 2016; Foltz et al., 2009; Fujita et al., 2007; Guo et al., 2017; Lagana et al., 2010; Perpelescu et al., 2009)
Yeast	Mis18; Mis16	Scm3	?	(Camahort et al., 2007; Hayashi et al., 2004; Mizuguchi et al., 2007; Sam Stoler et al., 2007)
Fruit flies	No known Mis18 homolog	CAL1	CENP-C	(Chen et al., 2014; Dunleavy et al., 2012; Erhardt et al., 2008; Kwenda et al., 2016; Phansalkar et al., 2012; Raychaudhuri et al., 2012; Schittenhelm et al., 2010)

1.5. Cell cycle timing of centromere assembly

1.5.1. The Cell cycle

The cell cycle encompasses a series of steps by which a cell coordinates the processes of DNA synthesis and cell division. It can be subdivided into M phase and interphase, which consists G1, S and G2 phases (Alberts, 1989; Hochegger et al., 2008; Murray & Hunt, 1993) (figure 1.4). G1 and G2 are gap phases that occur between S and M phases. In G1, the first gap phase the cells are ready for DNA synthesis. In S phase cells synthesise DNA and thus possess aneuploid DNA (Schafer, 1998). In the second gap phase, G2, the cell prepares for M phase, which is characterised by prophase, metaphase, anaphase and telophase, which results in the generation of two daughter cells (Alberts, 1989; Hochegger et al., 2008)

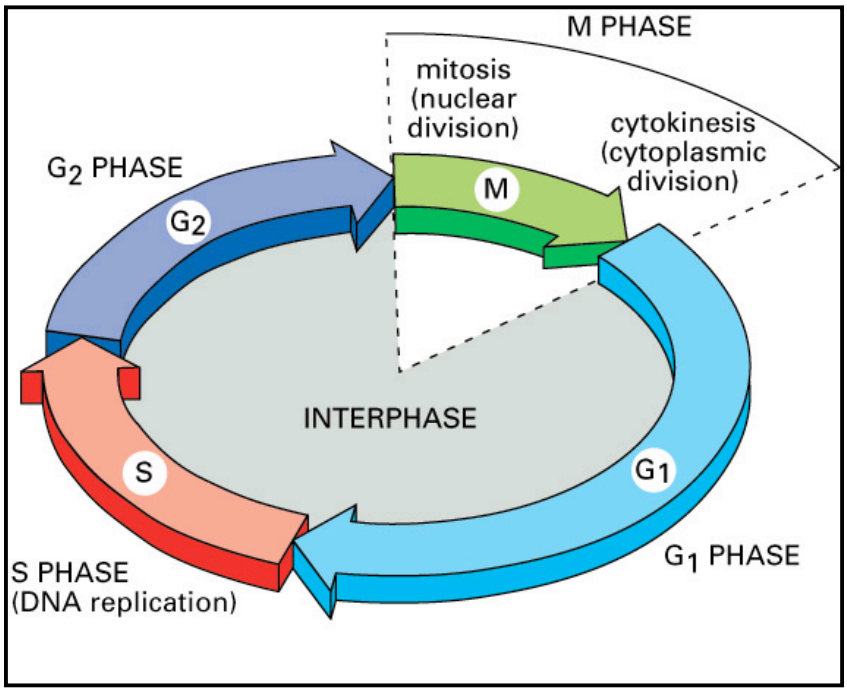


Figure 1.4. Phases of the cell cycle. Divided into M phase and interphase. M phase consists of two processes, mitosis, the process of nuclear division and cytokinesis, cytoplasmic division. Each mitotic phase culminates in the production of two cells that are genetically identical. The activation of each stage is dependent on the completion of the previous phase (cyclical). *Image from Alberts molecular biology of the cell 4th edition.*

1.5.2. Cyclins and Cyclin Dependent Kinases (Cdks)

The cell cycle is catalysed by a diverse family of proteins known as cyclins that display dramatic oscillations during the cell cycle (Hunt, 1991). Cyclins bind to and activate a family of Cyclin dependent kinases (Cdk), (figure 1.5). Cdks are a family of serine threonine protein kinases that mediate their effects on cell cycle events by phosphorylating target proteins on serine or threonine residues in a sequence context. The canonical consensus sequence for Cdks is (S/T) PX (K/R) (Hagopian et al., 2001; Holmes & Solomon, 1996). While cyclin concentration oscillates, the concentration of the kinase subunit remains constant. Importantly, the kinase remains inactive without cyclin binding (Hochegger et al., 2008). Thus together, cyclins and cyclin dependent kinases form a heterodimeric enzymatic complex, with a kinase subunit and a cyclin subunit, (Morgan, 2007) that act as universal motors in mediating cell cycle progression.

In addition to the cyclin binding, complete activation of normal Cdk function also requires Cdk activating kinase enzyme (CAK) to phosphorylate a threonine residue adjacent to the kinase active site (Drapkin et al., 1996; Harper & Elledge, 1998). Cyclins also perform multiple regulatory functions on Cdks. Because of their cyclical expression pattern, (Darzynkiewicz et al., 1996; Pines & Hunter, 1991), Cdks are only activated at specific times during cell cycle progression. It has also been reported that cyclins can aid targeting of Cdks to the nucleus as they contain nuclear localisation signals lacking in Cdks (David-Pfeuty & Nouvian-Dooghe, 1996). Moreover accumulating evidence indicates that cyclins confer substrate specificity for their Cdk partners via substrate recognition motifs, such the RxL motif on Cyclin A (Brown et al., 2007; Stankovic et al., 2017), or by directing Cdks to the subcellular compartment where they mediate their effects (Draviam et al., 2001)

In fission and budding yeast all cell cycle events are controlled by a single Cdk, Cdk1^{cdc28}. Cell cycle events in other eukaryotes are controlled by Cdk1 and Cdk2. Cdk1 and Cdk2 are promiscuous in their preferred cyclin partners and can bind cyclins A, B, D and E (Hochegger et al., 2008). However, Cdk1 binds to cyclin B with high affinity compared to the other cyclins, while Cdk2 can either bind to cyclin A or D with high affinity (Hochegger et al., 2008) (figure 1.5). In G1 phase and S

phase cyclins accumulate, resulting in activation of Cdk activity. By G2/M phase, Cdk levels peak thus maintaining the majority of Cdk substrates in a phosphorylated state.

The active enzyme complex can be turned off in various ways including expression of inhibitory gene regulatory proteins that suppress expression of cyclin genes (Morgan, 2007). In addition, rates of cyclin degradation increase, conferred by a region in their N terminus known as the cyclin destruction box (Glotzer et al., 1991). It is known that cyclin degradation is facilitated by the anaphase promoting complex (APC), a multisubunit ubiquitin protein ligase (Friedman & Snyder, 1994; Glotzer et al., 1991). APC associated destruction of cyclin A and cyclin B, leads to inactivation of all major Cdk activities, (Zachariae & Nasmyth, 1999). Cyclin A is degraded during metaphase while cyclin B is degraded at the metaphase-anaphase transition (Geley et al., 2001; Jacobs et al., 2001; White-Cooper et al., 1998; Zachariae & Nasmyth, 1999), thus maintaining low levels of Cdk activity until late in the onset of the next G1 phase.

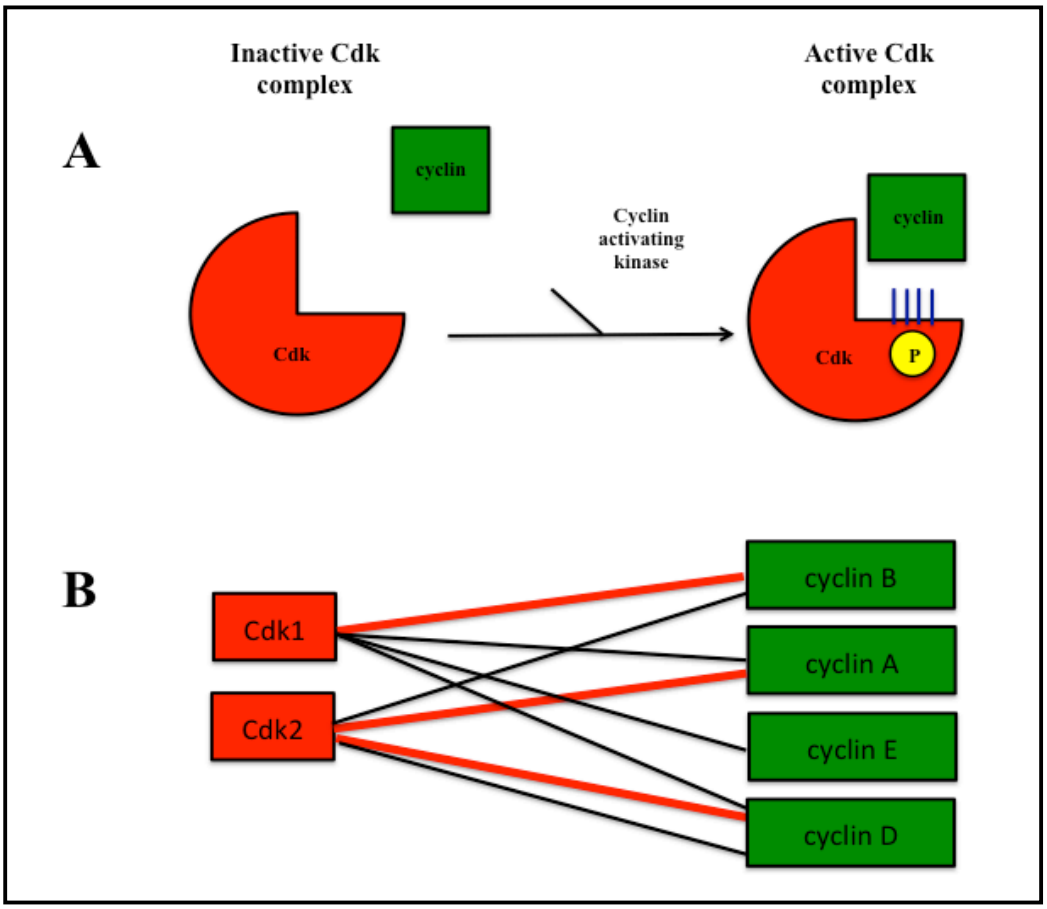


Figure 1.5. Activation of Cyclin dependent kinases. (A) Cyclin dependent kinases (Cdks) are activated by the binding of cyclins. When not complexed to cyclins, these kinases are inactive. **(B)** Cdk1 and Cdk2 both show promiscuity in their choice of cyclin partners. Thick red lines represent the preferred pairing for each kinase. *Adapted from essential cell biology 3rd edition Garland science 2010 (A) and Hochegger et al., 2008.*

1.6. Cell cycle timing of CENP-A assembly

1.6.1. Mitosis

The timing of assembly of CENP-A in relation to the cell cycle varies between organisms (Jansen, et al., 2007; Dunleavy et al., 2012; Hemmerich et al., 2008; Lermontova et al., 2006; Mellone et al., 2011; Pearson et al., 2004; Schuh et al., 2007; Takayama et al., 2008). Investigation in human cells demonstrated that assembly of newly synthesised CENP-A occurs in a replication independent process (Shelby et al., 2000). In *S. pombe* however, CENP-A levels peak in S phase, and newly synthesised CENP-A is deposited in S and G2 phase (Lando et al., 2012; Takayama et al., 2008). This is possibly because G1 in fission yeast is exceedingly short (Allshire & Karpen, 2008). This has led to the suggestion that CENP-A assembly in S phase merely coincides with replication rather than being dependent on it (Castillo et al., 2007).

Interestingly CENP-A is diluted in S phase in mammalian cells and partitioned evenly between replicated sister strands (Jansen, et al., 2007). The gaps that are generated during DNA replication are potentially filled by histone H3.3, which has been identified as a placeholder for subsequent CENP-A deposition, (Dunleavy et al., 2011) in late telophase/early G1 phase (Jansen, et al., 2007; Hemmerich et al., 2008). Similarly in *D. melanogaster*, dilution of CENP-A also occurs in S phase. However the onset of CENP-A assembly is between metaphase and anaphase in cultured fly cells (Mellone et al., 2011), although a recent study has challenged this finding (Lidsky et al., 2013), reporting CENP-A assembly in *Drosophila* cultured cells to be predominantly in G1, with only a transient increase being detected during early mitosis (Lidsky et al., 2013). Moreover, analysis of *Drosophila* brain cells show that CENP-A assembly begins in telophase and continues into G1 phase as reported for mammalian cells (Dunleavy et al., 2012). In *Drosophila* syncytial embryonic divisions cycling between S and M phases CENP-A assembly occurs during anaphase (Schuh et al., 2007). This difference in timing could be as a result of disrupted cell cycle regulation in cultured mitotic cells in comparison to tissues (Dunleavy et al., 2012; Schuh et al., 2007), or may reflect differences between the analysed cell lines (Lidsky et al., 2013). Moreover the differences could be due to the detection method used by Mellone and colleagues, SNAP-tag labeling compared to FRAP analysis

(Lidsky et al., 2013).

1.6.2. Meiosis

Interestingly cell cycle timing of CENP-A assembly differs between mitosis and meiosis (summarised in table 1.2). Because meiosis comprises two rounds of division, meiosis I and meiosis II, pioneering work has focused on determining CENP-A replenishment in both divisions. Studies in holocentric *C. elegans* using fixed and live analysis of oocytes revealed that unlike mitotic CENP-A, which shows little or no exchange once incorporated into centromeric nucleosomes, (Falk et al., 2015), meiotic CENP-A is dynamic. A drop in CENP-A fluorescence was reported between meiosis I and II, although the functional significance of this drop was not characterised (Monen et al., 2005). Surprisingly in striking contrast to mitosis (Buchwitz et al., 1999), CENP-A is not necessary for meiotic divisions in *C elegans* (Monen et al., 2005).

In *Drosophila* meiosis CENP-A assembly is biphasic, occurring at prophase I of meiosis I, a time of high Cdk activity and during spermatid differentiation after exit from meiosis II, (T1 spermatids) (Dunleavy et al., 2012; Raychaudhuri et al., 2012). CENP-A assembly in prophase I is also reported for *Drosophila* female meiosis (Dunleavy et al., 2012). Notably, CENP-A assembly is gradual occuring over a period of days with gradual increase in CENP-A intensity observed between early and late prophase I, (Dunleavy et al., 2012; Raychaudhuri et al., 2012). Intriguingly, similar to findings in *C. elegans*, a drop in CENP-A intensity is also observed between meiosis I and II in flies (Dunleavy et al., 2012). However, the significance of this reduction is still not yet understood.

Consistent with these findings, studies in meiocytes of the rye plant, *Secale cerale* also demonstrated CENP-A assembly in prophase I (Schubert et al., 2014). Again similar to *Drosophila* and worms, a drop in CENP-A intensity was also reported after the second meiotic division (Schubert et al., 2014). Collectively, these data reveal the unusual meiotic CENP-A dynamics. Compared to mitosis where CENP-A exhibits striking stability, meiotic CENP-A is dynamic characterised by coupling and uncoupling events that yet remain to be explored.

Table 1.2: Summary of key differences between timing of CENP-A assembly in mitosis compared to meiosis

Mitosis	**Meiosis**
CENP-A exhibits striking stability with little or no exchange once incorporated, (McKinley & Cheeseman, 2016).	CENP-A is dynamic exhibiting assembly and disassembly events during meiosis I and II, (Dunleavy et al., 2012; Monen et al., 2005; Schubert et al., 2014).
CENP-A assembly initiates upon loss of Cdk activity, (Dunleavy et al., 2012; Jansen et al., 2007; Lidsky et al., 2013; Schuh et al., 2007).	CENP-A assembly is coupled to high Cdk activity, (Dunleavy et al., 2012; Raychaudhuri et al., 2012; Schubert et al., 2014).
Assembly occurs in the order of minutes to hours, (Jansen et al., 2007; Lidsky et al., 2013; Schuh et al., 2007).	Assembly is gradual, occurring over a period of days in *Drosophila,* (Dunleavy et al., 2012).
CENP-A assembly occurs once per cell cycle, after division, with the exception of yeast, (Dunleavy et al., 2012; Hemmerich et al., 2008; Jansen et al., 2007; Schuh et al., 2007).	CENP-A assembly is biphasic occurs before division and after division, (Dunleavy et al., 2012; Raychaudhuri et al., 2012; Schubert et al., 2014).

1.6.3. Regulation of centromere assembly by the cell cycle machinery

(i) Cdk activity

Although the timing of CENP-A assembly differs slightly between systems, (reviewed in Valente et al., 2012), CENP-A assembly is intricately tied to cell cycle progression and suggests a hypotheses of cell cycle dependent regulation. In G1 and S phases, cyclins accumulate resulting in activation of Cdk activity. At G2/M phase, Cdk levels peak and maintain their substrates in a phosphorylated state. At the metaphase to anaphase transition, cyclins are degraded by the APC and the onset of the next G1 phase is characterised by minimal Cdk activity. This loss of Cdk activity coincides with the onset of CENP-A assembly in different systems (Dunleavy et al., 2012; Jansen et al., 2007; Lidsky et al., 2013; Schuh et al., 2007). This suggests that high Cdk activity directly prevents CENP-A assembly in S phase when Cdk activity increases. Moreover, assembly factors required for CENP-A assembly into centromeric chromatin associate with centromeres following mitotic exit when kinase activity is low (Dunleavy et al., 2009; Foltz et al., 2009; Fujita et al., 2007; Hayashi et al., 2004)

Recently, studies in mammalian cells have demonstrated that CENP-A deposition is indeed regulated by Cdk1 and Cdk2 activity (McKinley & Cheeseman, 2016; Müller et al., 2014; Stankovic et al., 2017; Silva et al., 2012). Brief inhibition of Cdk1/2 is sufficient to drive apparently normal, mis-timed CENP-A assembly in G2 prior to mitotic exit (Müller et al., 2014; Silva et al., 2012) suggesting that CENP-A assembly machinery is present but remain inactive throughout S, G2 and M phases due to high Cdk activity. It has also been demonstrated that key players necessary for CENP-A assembly in mammals, HJURP and Mis18BP1 are phosphoproteins regulated by kinase activity (Bailey et al., 2016; Dephoure et al., 2008; Fujita et al., 2007; Kato et al., 2007; McKinley & Cheeseman, 2016; Müller et al., 2014; Stankovic et al., 2017; Wang et al., 2014). When Cdk activity is high in late G1 and G2 phases, the licensing factor Mis18BP1, is unable to associate with centromeric chromatin (Müller et al., 2014; Silva et al., 2012). A decline in Cdk activity after anaphase onset results in alleviation of Mis18BP1 from inhibitory phosphorylation allowing for association with centromeres and recruitment of downstream CENP-A assembly factors (Müller

et al., 2014; Silva et al., 2012). Inhibition of Cdk1 and Cdk2 at other points of the cell cycle results in aberrant association of the licensing complex with downstream effectors leading to assembly of CENP-A outside of G1 phase (Silva et al., 2012). Recently, 4 putative Cdk motifs were identified on Mis18BP1 (Stankovic et al., 2017). Mutation of the four putative Cdk motifs, T4, T40, S110 and T653, to alanine leads to a loss of cell cycle controlled localisation of Mis18BP1 (Stankovic et al., 2017). Interestingly, residue T653 alone was sufficient to prematurely target Mis18BP1 to centromeric chromatin (Stankovic et al., 2017).

More still, the mammalian CENP-A assembly chaperone, HJURP features several putative Cdk sites, (Müller et al., 2014; Stankovic et al., 2017; Wang et al., 2014), making it a prime candidate for regulation by Cdks. Müller et al., characterised 3 phospho-residues on HJURP Ser412, Ser448 and Ser473. A non-phosphorylatable HJURP mutant localised prematurely to centromeres outside of telophase / early G1 phase, whereas a phosphomimic showed a reduction of centromeric localisation in early G1 phase in comparison to wild type, (Müller et al., 2014). Treatment of GFP-HJURP with the Cdk inhibitor roscovitine resulted in premature localisation of HJURP to S and G2 phases (Müller et al., 2014). This study suggests that HJURP recruitment specifically in telophase / early G1 phase relies on cumulative dephosphorylation of Ser412, Ser448 and Ser473 upon loss of Cdk activity.

In a separate study, Stankovic et al 2017 mapped 6 phospho-sites on HJURP, three of which are in line with two independent studies (Müller et al., 2014; Wang et al., 2014). Of the 6 phospho-sites identified, 5 displayed shorter Cdk consensus motifs, (S/T)P, instead of (S/T)PX(K/R). It has been reported that phosphorylation of these short motifs relies on added cyclin binding sites to allow substrate recognition (Adams et al., 1996). Indeed, a cyclin A binding motif, RXL was found to be located within the conserved domain (CD) of HJURP (Stankovic et al., 2017). Both cyclin A and cyclin B interact with HJURP. Cyclin A co-immunoprecipitates with GFP-HJURP, consistent with the hypothesis that CENP-A assembly is inhibited in mitosis (Jansen et al., 2007). Mutation of $HJURP^{RXL}$ motif to $HJURP^{AxA}$ disrupts the interaction of GFP-HJURP and cyclin A and also prematurely targets HJURP to centromeres in G2 phase (Stankovic et al., 2017). Thus CENP-A assembly favors low Cdk activity. It however remains obscure why in some organisms CENP-A assembly

is coupled to high kinase activity (Lermontova et al., 2006; Lando et al., 2012; Takayama et al., 2008; Dunleavy et al., 2012; Raychaudhuri et al., 2012).

Interestingly, mitotic cyclins have been localised to centromeres (Bentley et al., 2007; Erhardt et al., 2008; Nickerson et al., 2007; Touati et al., 2012). Cyclin A was reported to localise to centromeres in mouse spermatocytes (Nickerson et al., 2007), while in *Drosophila* cultured cells, cyclin A enriches at centromeres in interphase and mitosis. Analysis of cyclin A-GFP revealed that centromere enrichment was high after entry into mitosis and low after entry into anaphase, (Erhardt et al., 2008), coincident with degradation of Cyclin A, (Fuller, 1993; Lehner & O'Farrell, 1989), as well as CENP-A assembly, (Dunleavy et al., 2012; Jansen et al., 2007; Mellone et al., 2011; Schuh et al., 2007), raising the possibility that CYCA associated kinase activity may regulate CENP-A assembly in *Drosophila* mitosis. Notably, cyclin A RNAi resulted in CENP-A and CENP-C mislocalisation and a reduction in CENP-A protein levels (Erhardt et al., 2008).

In addition, CENP-A itself has been proposed to be phosphorylated on several residues, (Bailey et al., 2016; Yu et al., 2015; Zeitlin et al., 2001). Serine 68, is reported to be phosphorylated by Cdk activity to orchestrate assembly of CENP-A to centromeres (Yu et al., 2015). The significance of this proposed functionality is however disputed as mutation of CENP-A Serine 68 has been demonstrated to have no effect in the timing of CENP-A assembly (Fachinetti et al., 2017).

(ii) Proteasome mediated regulation

Additional cell cycle regulation of CENP-A assembly also stems from studies that have shown that CENP-A nucleosomes retain a capacity to mis-incorporate into non-centromeric regions (Heun et al., 2006). Therefore spatial regulatory mechanisms to restrict deposition of CENP-A only at the designated centromere locus must exist. Experimental evidence reveal that ubiquitin-mediated proteasome degradation spatially restricts CENP-A incorporation to a single locus (Collins et al., 2004; Moreno-Moreno et al., 2006).

The ubiquitin proteasome system consists of two parts (Ciechanover, 1994; Coux et al., 1996; Hilt & Wolf, 1996; Peters, 1994), one is the ubiquitinating enzyme system (ubiquitin system), while the other is the proteolytic machinery (the proteasome). Three mechanisms of the activation of the proteasome can be considered. The first mechanism involves the activation of the ubiquitin system where a ubiquitin protein ligase is activated (Hershko et al., 1994; King et al., 1995). The second involves activation at the level of the substrate by phosphorylation. In this case phosphorylation precedes degradation by the ubiquitin-proteasome system (Schwob et al., 1994; Tyers et al., 1992). Accumulating evidence demonstrates that proteins tagged for degradation by this mechanism contain PEST sequences, regions rich in Proline, Glutamine, Serine and Threonine, (Rechsteiner & Rogers, 1996; Rogers et al., 1986). It has been shown that PEST sequences are abundant in minimum consensus motifs for Cdks and other kinases (S/TP sequences) (Yaglom et al., 1995). This has been demonstrated for the yeast G1 cyclins Cln3 and Cln2, (Lanker et al., 1996; Yaglom et al., 1995). The third is the activation of the proteasome system by intracellular calcium signaling (Aizawa et al., 1996; Kawahara & Yokosawa, 1994).

Pioneering work from Collins et al., 2004, and Moreno-Moreno et al., 2006, demonstrate that proteolysis mediates formation of a single centromeric loci by degrading CENP-A that is non-centromeric in yeast and flies (Collins et al., 2004; Moreno-Moreno et al., 2006). Both yeast and *Drosophila*, E3 ubiquitin ligases have been implicated in CENP-A degradation. In *S. cerevisiae* the E3 ligase phs1 specifically provides a cue for CENP-A^{Cse4} degradation, (Hewawasam et al., 2010; Ranjitkar et al., 2010). Deletion of psh1 prevents CENP-A^{Cse4} ubiquitination and

increases the association of CENP-A^{Cse4} at non centromeric loci, (Hewawasam et al., 2010; Ranjitkar et al., 2010). Interestingly, Bade et al., 2014, identified a proteolysis-independent role of ubiquitination in centromere regulation. Remarkably, ubiquitination of CENP-A by the Cullin 3 (CUL3) ligase does not result in degradation but rather stabilises both CENP-A and its loading factor CAL1. CAL1 directly interacts with Roadkill (RDX), an adaptor for CUL3 mediated ubiquitination. Loss of RDX leads to degradation of both CAL1 and CENP-A (Bade et al., 2014), suggesting a mechanism in which ubiquitin conjugation is essential for the maintenance and stabilisation of both CAL1 and CENP-A. Taken together, the above data make a compelling case for cell cycle mediated regulation of CENP-A deposition.

1.7. *Drosophila melanogaster* as a model system

Drosophila melanogaster are fruit flies in the order Diptera and family Drosophilidae. *Drosophila,* has been used in research for over a century with pioneering work led by Thomas Morgan Hunt, 1910. Although the final architecture of fruit flies and humans differ greatly, the biological mechanisms and genetic pathways that regulate development are conserved. Comparisons between *Drosophila* and human genomes reveal that most human disease genes ~75% have a match in the fly genome (Reiter et al., 2001).

Advantages of using *Drosophila* over vertebrate models include but are not limited to, easy and inexpensive culturing, short life cycle, and genetic malleability. Importantly, ethical obstacles that limit the scope of experiments using human samples and vertebrate models do not apply in *Drosophila*. In addition *Drosophila* has a compact genome with a karyotype of 4 chromosomes. The first chromosome pair is the sex chromosomes X and Y, while the remaining three pairs are autosomes. The main limitation of using *Drosophila* is the inability to freeze down *Drosophila* gametes or embryos; therefore flies have to be maintained as living stocks.

1.7.1. *Drosophila melanogaster* as a model system for meiotic CENP-A assembly

Meiosis is a specialised form of cell division for the production of gametes with monoploid chromosome number. Meiosis consists of two successive events involving an accurate quantitative reduction in chromosome number, (homolog separation), Meiosis I, and sister segregation resulting in haploid cells, Meiosis II.

Errors in meiotic segregation lead to aneuploidy, developmental defects or sterility due to gene dosage imbalance, (McKee et al., 2012). Interestingly in humans, meiotic mis-segregation is quite common accounting for ~1/3 of miscarriages, developmental disability as well as mental retardation (Hassold & Hunt, 2001; Webster & Schuh, 2017).

1.7.2. *Drosophila* spermatogenesis

The *Drosophila* testis support and sustain sperm production in flies, (Fuller, 1993). Spermatogenesis is sustained by maintaining stem cells division once every 24 hours. Both germline and somatic cyst stem cells (GSC and CySC) are positioned at the apical tip of the testis, (Hardy et al., 1979). In the germline, the differentiating daughter cell is called a gonialblast (GB).

The gonialblast goes through 4 rounds of mitotic division coupled with incomplete cytokinesis to generate a 16 cell cyst (Fuller, 1993; Hardy et al., 1979). The 16 cyst germ cells initiate the meiotic program and together synchronously enter pre-meiotic DNA synthesis (figure 1.6), which is accomplished in approximately 3 hours, (Cenci et al., 1994). Pre-meiotic S-phase is followed by an extended specialised G2 phase lasting ~ 90 hours and characterised by a 25 fold increase in nuclear volume, attributed to extensive gene expression, (Cenci et al., 1994; Hiller et al., 2004; Hiller et al., 2001). This phase is succeeded by two meiotic divisions followed by spermatid differentiation, which culminates into production of haploid motile sperm that is stored in the seminal vesicle (Fabian & Brill, 2012; Fuller, 1993).

Drosophila larval testes are a good model system for studying meiotic centromere assembly as they present stages of CENP-A assembly in a sequential manner (figure 1.7). More still, the spermatocytes are highly abundant, large, easily accessible and easily identified as they form cysts. This system enables easy visualisation of meiosis using immunofluorescence techniques or tagged molecules. Because CENP-A assembly occurs in a biphasic manner, (Dunleavy et al., 2012), larval testes present an enrichment of cells undergoing the first phase of CENP-A assembly, prophase I of meiosis I, and thus are a 'cleaner' tissue sample compared to adult testis which shows enrichment of all stages of spermatogenesis, (Fuller, 1993). No meiotic recombination occurs in *Drosophila* males thus the canonical meiotic stages such as leptotene, zygotene and pachytene are absent. In 1994, examination of microtubule and chromatin organisation led to the stage subdivision of spermatocyte growth, meiotic divisions and early sperm differentiation where each stage is defined by morphological criteria.

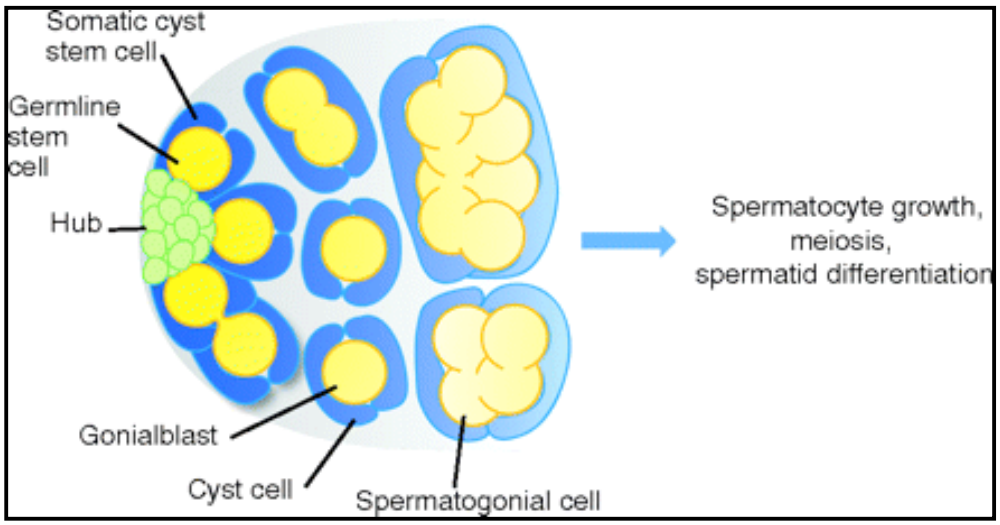


Figure 1.6. Schematic of *Drosophila* stem cell niche. Stromal hub cells (green) adhere to the apical tip of the testis. Germline stem cells (GSCs) yellow, surround the hub and give rise to spermatogonial cells (light yellow) which ultimately develop into sperm. Somatic cyst stem cells (CySC) blue, share the niche and give rise to cyst cells which encase the developing spermatogonial cells. : Cyst cells which wrap around the germ line creating cysts. *Image from de Cuevas & Matunis, 2011.*

1.7.3. Spermatocyte growth

S1 stage

Consists of 16 primary spermatocytes that have just undergone DNA synthesis. S1 spermatocytes are similar in size and morphology and the chromosomes fill the nucleus, (Cenci et al., 1994).

S2a/b stage:

Also known as the polar spermatocyte stage, (Tates, 1971), and is divided into S2a or S2b. During S2a, there is a substantial increase in nuclear diameter accompanied by changes in chromatin organisation within the nucleus (Cenci et al., 1994). As the polar spermatocytes grow, chromatin subdivides into three masses, (Cenci et al., 1994; Cooper, 1965), and becomes clearly trilobular. S2b nuclei possess 3 recognisable chromatin masses corresponding to the 3 major bivalents (figure 1.7).

S3 stage:

Characterised by further nuclear growth. Bivalents continue to separate and occupy distinct territories (Cenci et al., 1994).

S4 stage:

Characterised by a further increase in nuclear size (Cenci et al., 1994).

S5 stage:

Also known as the mature spermatocyte stage, (Cooper, 1965), this stage represents the largest cells produced during *Drosophila* spermatogenesis (Cooper, 1965; Cenci et al., 1994). The territories are separate and quite far apart (Cenci et al., 1994).

S6 stage:

Nuclei in this stage remain approximately the same size as mature spermatocytes (Cenci et al 1994). At this stage, the nucleolus disappears and the chromatin condenses in preparation for the first meiotic division (Cenci et al., 1994).

Meiotic division stages

The first and second meiotic divisions (MI and MII) occur in rapid succession upon completion of prophase I and are subdivided into M1-M11 (Cenci et al., 1994).

M1a:

Characterised by bivalents that are peripherally located near the nuclear envelope. M1a nuclei are round or ovoid (Cenci et al., 1994).

M1b:

Bivalents in M1b appear to move freely in the nucleoplasm. In the later M1b stage, the nuclei become irregularly shaped. The asters become more prominent and a few spindle fibers radiating from the asters appear to reach the bivalents (Cenci et al., 1994).

M2 stage:

M2 stage, as well as M1b correspond to prometaphase of the first meiotic division (Cenci et al., 1994), in which bivalents undergo complex motions necessary for proper metaphase orientation (Church & Lin, 1982; Church & Lin 1985)

M3 stage:

Metaphase I, bivalents congress to a metaphase plate (Cenci et al., 1994), the bivalents cluster in a single bright structure connected to spindle poles by two symmetrical sets of microtubules (Cenci et al., 1994). The metaphase spindle also exhibits bundles of microtubules that do not encounter the chromosomes, rather the microtubules run outside the congressed bivalents (Cenci et al., 1994).

M4a

Resembles early anaphase I. the segregating bivalents migrate a considerable distance towards the poles, suggesting that initial anaphase chromosome movement is very rapid (Cenci et al., 1994). The spindle organisation of M4a stage is very similar to that of M3 stage.

M4b:

Resembles mid anaphase, microtubules emanating from each pole overlap in the central region of the spindle such that the two opposed spindles of early anaphase are fused together in a unique oval structure (Cenci et al., 1994).

M4c:

Also known as late anaphase/ early telophase. There is a further reduction in the number of microtubules emanating from the centrosomes, accompanied by an increase of density of central spindle microtubules and a further increase in the distance between the two daughter nuclei, (Cenci et al., 1994).

M5:

In M5 stage, the dense bundle of microtubules in the central spindle is squeezed and attains an hourglass shape (Cenci et al., 1994). The two daughter nuclei become clearly demarcated from the cytoplasm and exhibit a dark dot (Cenci et al., 1994).

M6a/b:

Telophase I nuclei enlarge and the second division asters become apparent (Cenci et al., 1994). The central spindle progressively disappears.

M6b:

Secondary spermatocyte nuclei have considerably enlarged and the chromatin appears to be decondensed, occupying the centre of the nucleus (Cenci et al., 1994). The M6b cells are rare and correspond to interphase II.

M7:

Chromosomes condense in preparation for meiosis II. Three Hoescht-bright elements become apparent and lie close to each other (Cenci et al., 1994).

M8:

Additional spindle fibers penetrate the nucleus. Chromosomes undergo the prometaphase motions necessary for proper orientation and congression (Cenci et al.,

1994).

M9:

Chromosomes congregate into a metaphase plate in a manner analogous to the first meiotic division (Cenci et al., 1994).

M10a:

Resembles early anaphase II. The density of both kinetochore and polar microtubules increases. The chromosomes move rapidly towards the poles and the spindle retains a metaphase like configuration (Cenci et al., 1994).

M10b/c:

The daughter nuclei continue to move apart while the density of fibers in the central spindle continues to increase (Cenci et al., 1994).

M11:

Also known as telophase II. The spindle is progressively squeezed to an hourglass shape. The two daughter nuclei become demarcated from the cytoplasm (Cenci et al., 1994).

Meiosis I and II yield 64 haploid spermatids (Fuller, 1993). Differentiation and maturation of spermatids consequently follows culminating in mature sperm which is stored in the seminal vesicle (Fuller, 1993; Fabian & Brill, 2012).

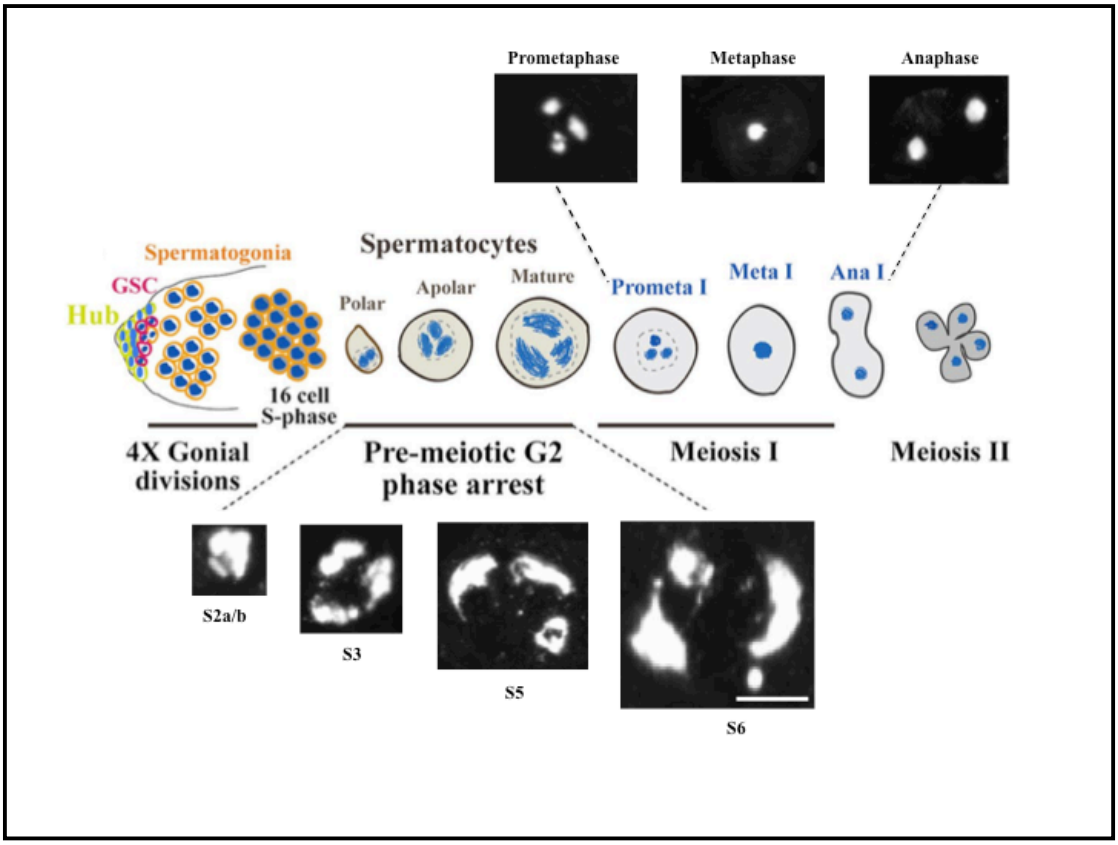


Figure 1.7. Schematic of *Drosophila* spermatocyte growth phases and meiotic division stages. A single gonialblast undergoes 4 rounds of mitotic divisions to produce 16 spermatocytes that enter into pre-meiotic G2 phase arrest, characterised by growth and increase in nuclear volume 25 x. This is followed by two rounds of meiotic divisions that produce 64 haploid spermatids. The highlighted stages of pre-meiotic G2 phase arrest were the focus of this study. Scale bar = 10 μm. *Figure adapted from Varadarajan et al., 2016 and Cenci et al., 1994.*

1.8. *Drosophila* centromeric proteins

Drosophila provides a fundamentally simplified centromere characterised by an interdependence between CAL1, CENP-A and CENP-C (Erhardt et al., 2008; Goshima et al., 2007). The functional overlap between CAL1, the Mis18 complex and HJURP has led to the proposal that CAL1, executes functions characteristic to both the Mis18 complex and HJURP despite lack of sequence homology, (Chen et al., 2014; Mellone et al., 2011). Moreover the absence of the constitutive centromere associated network (CCAN) in *Drosophila*, which plays an important role in kinetochore microtubule dynamics in vertebrates, has led to the proposal that CENP-C may take on the role of these 'missing' components. Thus *Drosophila* provides a simplified centromere architecture for dissecting the roles of these vital centromeric proteins.

1.8.1. Chromosome Alignment Defect 1 (CAL1)

Chromosome alignment defect 1 (CAL1) was first identified in a genome wide RNAi screen for mitotic defects in S2 cells, where CAL1 RNAi led to a severe misalignment phenotype (Goshima et al., 2007). In a subsequent genome wide RNAi screen for CENP-A^{CID} Localisation Deficient genes (CLD) in *Drosophila* tissue culture cells, CAL1 was also identified (Erhardt et al., 2008). CAL1 localises with CENP-A at centromeres, (Goshima et al., 2007; Schittenhelm et al., 2010), and also displays nucleolar staining (Erhardt et al., 2008; Schittenhelm et al., 2010). Cell cycle analysis of the timing of CAL1 assembly reveals that CAL1, similar to HJURP, displays unusual localisation dynamics, transient association and dissociation from centromeres with cell cycle progression, (Dunleavy et al., 2012; Raychaudhuri et al., 2012; Erhardt et al., 2008; Mellone et al., 2011). CAL1 is recruited to centromeres during prophase in both mitosis and meiosis (Dunleavy et al., 2012; Mellone et al., 2011). During mitosis, GFP-CAL1 levels are reduced in metaphase, coinciding with the timing of mitotic CENP-A assembly, and increased again in telophase (Erhardt et al., 2008). In meiosis, CAL1 localises to both centromeres and the nucleolus at the beginning of Prophase I; however foci at centromeres gradually reduce in intensity by the end of prophase I (S5 stage) and are almost undetectable at late prophase I (Dunleavy et al., 2012; Raychaudhuri et al., 2012), coinciding with the termination of

the first phase of meiotic CENP-A assembly.

Analysis of protein-protein interactions using the yeast three-hybrid system demonstrated that CAL1 links CENP-A and CENP-C via an N terminus interaction with CENP-A, amino acids 1-407, and a C terminus interaction with CENP-C, amino acids 699-979 (figure 1.8), (Schittenhelm et al., 2010). In the absence of CAL1 expression, no interaction between CENP-A and CENP-C could be forged, (Schittenhelm et al., 2010). The Middle region, amino acids 392-722, was assigned a role in CAL1 nucleolar localisation (Schittenhelm et al., 2010) (figure 1.8). Consistent with this, combining the N-M or C-M regions results in an enrichment of CAL1 in the nucleolus and not at centromeres (Schittenhelm et al., 2010). However, expression of a combination of the N-C terminal regions of CAL1 results in CAL1 recruitment to the centromere (Schittenhelm et al., 2010), indicating that CAL1 centromere localisation relies on functional interaction with CENP-A and CENP-C. Expression of this construct was also able to rescue *cal1* mutant embryos, (Schittenhelm et al., 2010).

Interestingly the N and C terminal regions of CAL1 are minimal for interaction with CENP-A or CENP-C. Both have been extensively conserved during *Drosophila* evolution in comparison to the middle region (Phansalkar et al., 2012). Notably, secondary structure homology prediction servers, (Kelley & Sternberg, 2009; Söding et al., 2005), reveal similarity between an amino terminal region of ~ 40 amino acids in CAL1 and part of the *Kluyveromyces lactis* Scm3 domain (Phansalkar et al., 2012). A CAL1 mutation lacking this Scm-like region is defective in recruiting CENP-A (Chen et al., 2014). This region is also conserved in mammalian HJURP and facilitates interaction with CENP-A (Aravind et al., 2007; Barnhart et al., 2011; Bassett et al., 2012; Mizuguchi et al., 2007; Sanchez-Pulido et al., 2009; Shuaib et al., 2010). Moreover, using CENP-A and CAL1 chimeras, it was demonstrated that successful CENP-A assembly into centromeric chromatin, requires residues 1-40 of CAL1 and CENP-A Loop 1 to be compatible (Rosin & Mellone, 2016). This suggests that this region mediates CAL1/CENP-A function (Rosin & Mellone, 2016)

In vivo imaging that quantitatively determined protein copy numbers at centromeres demonstrated that centromeric CAL1 levels are 40 times lower than those of CENP-A or CENP-C (Schittenhelm et al., 2010). Notably, this low amount of endogenous

CAL1 is necessary to prevent kinetochore failure and centromere expansion by limiting amounts of CENP-A and CENP-C incorporated at centromeres (Schittenhelm et al., 2010), thus CAL1 has also been assigned a 'limiting' role in centromere assembly (Schittenhelm et al., 2010). Consistent with this finding, pulse chase experiments demonstrate that CAL1 is less stably associated with centromeres, centromeric levels decrease by 66% after one cell cycle (Mellone et al., 2011), indicating that CAL1 displays a high turnover rate.

In 2014, Chen and colleagues demonstrated that CAL1 is the bona fide *Drosophila* CENP-A chaperone, (Chen et al., 2014). Using in vitro binding assays, CAL1 was shown to distinguish between CENP-A and histone H3. A trimeric complex failed to form when CENP-A was substituted by H3 (Chen et al., 2014). CAL1 was also shown to associate with pre-nucleosomal CENP-A in the cytoplasm (Chen et al., 2014). Moreover, CAL1 targeting is able to initiate ectopic centromere formation (Chen et al., 2014). Depletion of CAL1 abolishes CENP-A and CENP-C centromeric localisation resulting in errors in chromosome segregation (Dunleavy et al., 2012; Erhardt et al., 2008; Goshima et al., 2007).

Although CAL1 is important for centromere assembly and chromosome segregation in mitosis, little is known about its meiotic functions. Interestingly, using a *cal1* C terminal truncation mutant, Unhavaithaya et al 2013, demonstrated that a functional centromere is required for centromere clustering and pairing in *Drosophila* female meiosis, indicating that centromere proteins are necessary for nuclear positioning during this specialised division (Unhavaithaya & Orr-Weaver, 2013). In a separate study, using the same allelic mutation, CAL1 was shown to be important for meiotic chromosome segregation, CENP-A assembly and maintenance on mature sperm as well as fertility, (Kwenda et al., 2016).

Investigation into the molecular mechanism that regulate mitotic CAL1 demonstrated that the Cullin 3 (CUL3) ligase adaptor protein Roadkill (RDX) stabilises and maintains CAL1 protein levels as well as centromeric levels of CENP-A (Bade et al., 2014). CAL1 specifically binds to RDX via its C terminus through conserved RDX binding sites and acts as a bridging factor for CUL3/RDX mediated ubiquitination of CENP-A, which stabilises and maintains both CENP-A and CAL1 in a degradation-

independent ubiquitination pathway (Bade et al., 2014). Loss of RDX, or mutation of the conserved RDX binding sites triggers rapid degradation of CAL1 and CENP-A. Notably, CAL1 protein levels are restored when RDX depletion is combined with subsequent proteasome inhibition by MG132, indicating that in the absence of RDX, CAL1 is a substrate of proteasome mediated degradation (Bade et al., 2014). This study gives insight into the maintenance of CAL1 levels, however the mechanism by which CAL1 dissociates from centromeres in both mitosis and meiosis, (Dunleavy et al., 2012; Erhardt et al., 2008), still remains to be explored.

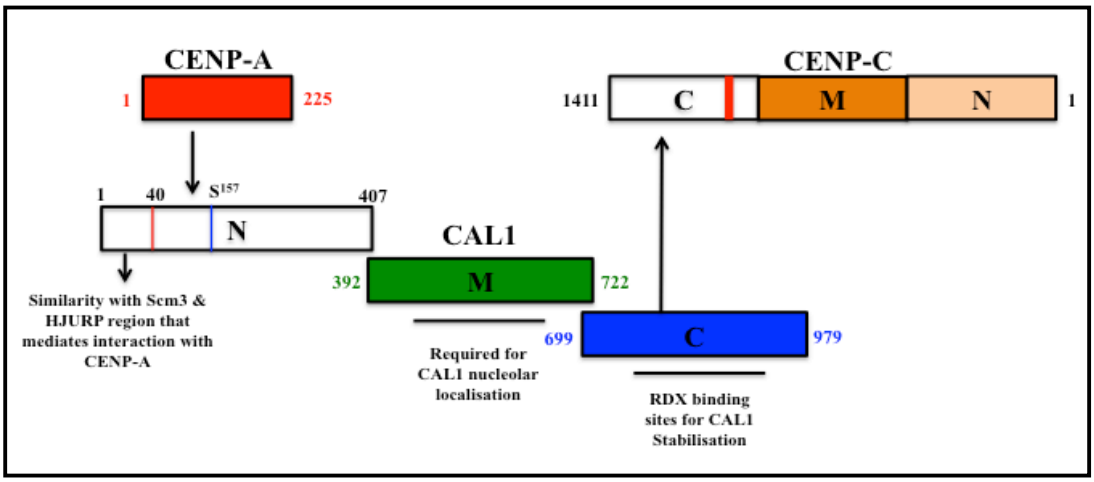


Figure 1.8. Schematic showing CAL1 domains and its interaction partners. The observed protein interactions are indicated in arrows. CAL1 is divided into N terminal, Middle and C terminal domains. CENP-A^{CID} interacts with CAL1s N-terminus. The region with amino acids 1-40 is similar to that of Scm3 and HJURP. The highlighted Serine 157 is a potential phosphorylation site (Bodenmiller et al 2007). The middle region of CAL1 is required for its nucleolar localisation. CENP-C interacts with CAL1s C terminus. CAL1 C terminus is also required for CAL1s stabilisation via binding to RDX. The red line on CENP-C indicates the position of the conserved CENP-C motif. Numbers indicate amino acid positions. *Figure adapted from Schittenhelm et al., 2010.*

1.8.2. Centromere Protein C (CENP-C)

CENP-C is a constitutive centromere protein identified originally as a human autoantigen that localises to centromeres (Earnshaw & Rothfield, 1985; Saitoh et al., 1992). Although there is extensive sequence divergence among eukaryotic centromeric proteins, CENP-C homologs have been identified in yeast, nematodes, plants and *Drosophila* (Brown, 1995; Dawe et al., 1999; Heeger et al., 2005; Meluh & Koshland, 1995; Moore & Roth, 2001; Oegema et al., 2001; Shibata & Murata, 2004; Talbert et al., 2004). Microinjection of antibodies in mammalian cells, (Tomkiel et al., 1994), RNA interference in C elegans and *Drosophila* mitosis and meiosis, (Dunleavy et al., 2012; Erhardt et al., 2008; Goshima et al., 2007; Moore & Roth, 2001; Oegema et al., 2001), and analysis in yeast, (Brown, 1995), demonstrate that CENP-C is essential for accurate chromosome segregation.

Importantly, previous reports in both mitosis and meiosis have demonstrated that *Drosophila* CENP-C is important for centromere localisation of both CAL1 and CENP-A, (Dunleavy et al., 2012; Erhardt et al., 2008; Goshima et al., 2007; Orr & Sunkel, 2011), as well as other centromeric proteins including the cohesion protector MEI-S332, (Kwenda et al., 2016; Orr & Sunkel, 2011), CENP-A, CAL1 and CENP-C interact and are dependent for centromere localisation. Notably, CENP-C is capable of initiating ectopic CENP-A recruitment albeit at low efficiency (Chen et al., 2014), further supporting the mutual dependencies of these centromeric proteins. Investigations into meiotic CENP-C functions demonstrate that CENP-C is essential for chromosome segregation, (Dunleavy et al., 2012; Kwenda et al., 2016), CENP-A maintenance as well as fertility (Kwenda et al., 2016). Furthermore, CENP-C has also been shown to be important for centromere clustering and pairing in *Drosophila* female meiosis (Unhavaithaya & Orr-Weaver, 2013).

Cell cycle analysis of mitotic *Drosophila* CENP-C reveals that pre-existing CENP-C is stably retained and distributed equally to daughter cells following one round of cell division, resulting in a dilution consistent with 50:50 segregation, (Mellone et al., 2011). Newly synthesised CENP-C recruitment occurs in interphase and mitosis in *Drosophila* tissue cultured cells, (Mellone et al., 2011), similar to reports in mammalian cells (Hemmerich et al., 2008). In *Drosophila* embryos, CENP-C is

assembled at centromeres along with CENP-A in anaphase, (Schuh et al., 2007). Interestingly in meiosis, CENP-C displays distinct localisation dynamics. CENP-C is found at centromeres through all the stages of meiosis I and meiosis II spermatocytes, but is progressively lost from centromeric loci after telophase of meiosis II, and is undetectable on mature sperm, (Dunleavy et al., 2012; Raychaudhuri et al., 2012).

In mammals, CENP-C is a component of the constitutive centromere- associated network (CCAN), (Przewloka & Glover, 2009; Santaguida & Musacchio, 2009), involved in the organisation of the inner kinetochore layers. CCAN proteins, except for CENP-C, are absent from centromeres in *Drosophila* raising the possibility that the functions of these 'missing' proteins may be combined in this one molecule. Expression of various CENP-C fragments fused to GFP in *Drosophila* embryos demonstrated that the N terminal third of CENP-C, amino acids 1-575, and the central third, amino acids 558-1038, fail to localise the fused GFP to centromeres, (Heeger et al., 2005). In contrast amino acids 1009-1411 are sufficient to direct centromere localisation. Further analysis demonstrated that amino acids 1009-1205 suffice to confer centromere localisation (Heeger et al., 2005) (figure 1.9). Interestingly, random mutagenesis using error prone PCR identified a single point mutation (R1101) that abolished centromere localisation (Heeger et al., 2005). Notably, R1101 is present in a short block ~24 amino acids, with a consensus sequence comparable to that of the CENP-C motif. This region is conserved in CENP-C proteins of vertebrates, yeast and plants (Talbert et al., 2004). The human CENP-C region encompassing the CENP-C motif has been demonstrated to be sufficient for centromere targeting (Lanini & McKeon, 1995; Song et al., 2002), thus the *Drosophilid* CENP-C appears to be a strongly diverged CENP-C ortholog.

Although the N terminal part of CENP-C does not confer normal centromeric localisation, the most pronounced conservation among *Drosophilids* is present within the N-terminal part in an arginine-rich region (Heeger et al., 2005) (figure 1.9). Interestingly, the N terminus was demonstrated to be vital for the recruitment of core kinetochore microtubule network (KMN) components, (Przewloka et al., 2011). The N terminus is not only sufficient to recruit core kinetochore components, but is also sufficient to recruit these components at ectopic sites, (Przewloka et al., 2011), supporting a model in which one side of CENP-C, C terminus, binds CENP-A rich

centromeric chromatin, (Heeger et al., 2005), while the other, N terminus, binds components of the KMN network, (Przewloka et al., 2011). CENP-C arginine rich region (Heeger et al., 2005), and its potential for post translational modifications, (Bodenmiller et al., 2007; Zhai et al., 2008) sparks real interest and suggests that CENP-C may also be a pivotal centre for regulating centromere and kinetochore function in both mitosis and meiosis.

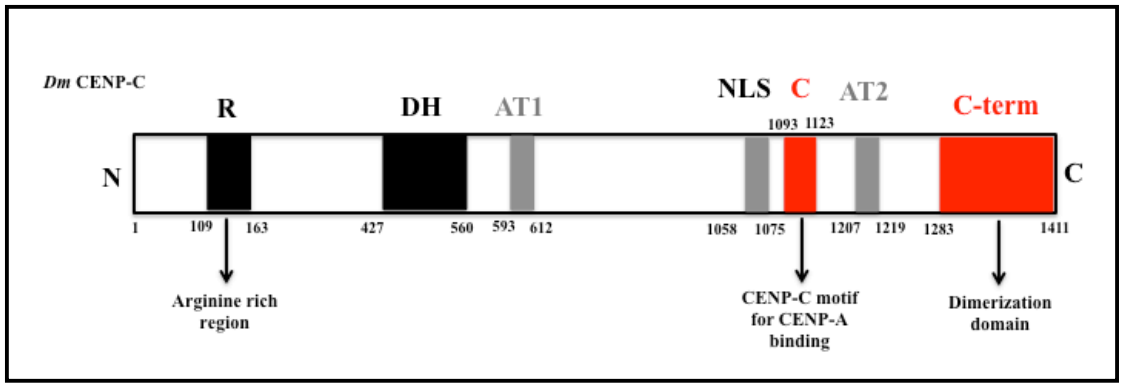


Figure 1.9. Schematic showing CENP-C domains. The boxes indicating the CENP-C motif and the C-terminal dimerisation region are filled in red. The arginine rich region (R) and another region (DH), are found to be maximally conserved among *Drosophilid* species and are highlighted in black boxes. Grey boxes indicate the poorly conserved AT hooks AT1 and AT2 and nuclear localisation signal (NLS). *Adapted from Heeger et al., 2005.*

1.9. Aims of study

Hypothesis:

We propose that in *Drosophila* male meiosis, CENP-A assembly via CAL1 and CENP-C is regulated by the cell cycle.

Aims:

(1) To understand the requirements of CAL1 and CENP-C in CENP-A assembly in male meiosis.

1.1: Assess the localisation of CENP-A, in flies mutant for *cal1* and *cenp-C.*

1.2: Assess chromosome segregation and fertility in flies mutant for *cal1* and *cenp-C.*

1.3: Assess the role of nucleolar transcription in CENP-A assembly.

Findings from Aim 1 have been published in Kwenda et al. 2016.

(2) Develop a better understanding of the role of CDK activity if any, in regulating meiotic CENP-A assembly.

2.1: Determine if CDK activity is required for CENP-A assembly in meiosis:

2.2: Determine if centromeric proteins (e.g. CAL1) are meiotic CDK substrates.

Chapter 2. Materials and methods

2.1. Fly Husbandry

Drosophila Nutri-Fly food #789210 (Flystuff Genesse) was made in 2 litre batches. Two packets of Nutri-Fly food were dissolved in 1800 ml milliQ water in a stainless steel beaker and cooked on a hot plate ~360°C for 20 minutes. The food was autoclaved followed by addition of 0.1% nipagin, anti-mold solution (tegosept) (Flystuff Genesee) and 0.5% propionic acid (Fisher Scientific). The fly food was poured into 25 x 95 mm vials or 6 oz square bottom vials using a funnel and allowed to cool at room temperature (RT). Cotton plugs (Flystuff Genesse) were added to the food once cooled and storage was at 4°C. Fly stocks were grown at 20°C in a humid incubator with day and night light cycle and passed into fresh media every 3 weeks.

2.1.1. Fly stocks

Below are listed the original fly stocks, used in this study. The fly strains are from Bloomington *Drosophila* Stock Center (BDSC), Vienna *Drosophila* Research Center (VDRC), donated by researchers as indicated or transgenics made by molecular cloning techniques followed by embryo injections by BestGeneInc.

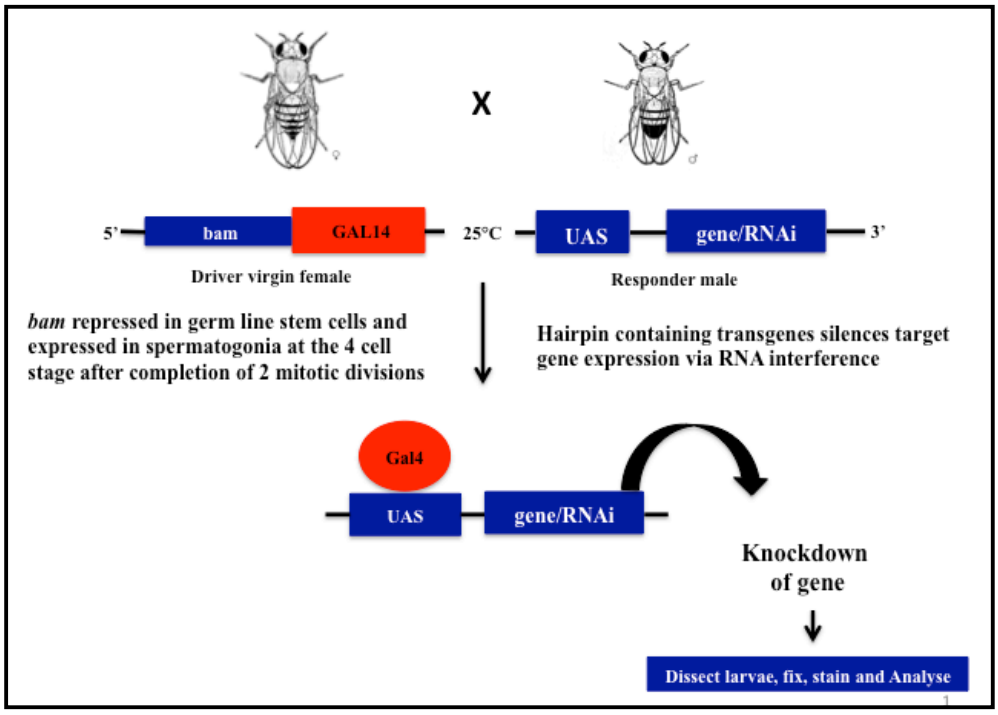
Table 2.1: *Drosophila* fly stocks

Fly strains	**Source**
wild type $y^{+}ry^{+}$	Lab
cenp-$C^{Z3\text{-}4375}$*/TM3*	(Unhavaithaya & Orr-Weaver, 2013)
cal1^{2k32}*/TM3*	(Unhavaithaya & Orr-Weaver, 2013)
P{w+ , gcal1-EGFP}II.2 (GFP-CAL1)	(Schittenhelm et al., 2010)
P{w+ , giEGFP-Cenp-C}II.1 (GFP-CENP-C)	(Schittenhelm et al., 2010)
P{w+ , gcid-EGFP-cid}III.2 (GFP-CID)	(Schuh et al., 2007)
(hs-Δ-cyclin A)	(Sigrist et al., 1995; Sprenger et al., 1997)
(hs-Δ-cyclin B)	(Sigrist et al., 1995)
y^{1} *sc** v^{1}*; P{TRiP.GLV21059}attP2 (UAS-Cyclin A-RNAi)*	BDSC # 35694
y^{1} *sc** v^{1}*; P{TRiP.HMS01897}attP2 (UAS-Cyclin B-RNAi)*	BDSC # 38981
w^{1118}; P{GD11553}v45248 *(UAS-cal1-RNAi)*	VDRC # 45248
y^{1} *sc** v^{1}*; P{TRiP.HMS01171}attP2 (UAS-cenp-C-RNAi)*	BDSC #34692
{w; bam-Gal4-VP16, UAS-dcr2} (bam-Gal4-VP-16/+)	(Chen & McKearin, 2003)
w[1118] (GD control: isogenic host strain for RNAi library)	VDRC # 60000

2.1.2. Gene knockdown (GAL4/UAS system)

To express a transgene or RNAi construct in a specific tissue the GAL4/UAS system was employed. This method expresses short inverted repeat RNA hairpins that target specific genes to knockdown expression of the genes messenger RNA (Kennerdell & Carthew, 2000). Flies carrying a 'driver' with a tissue specific promoter are placed 5' of the gene encoding the yeast GAL4 transcription factor, and the gene of interest placed 3' of the upstream activating sequence (UAS) which in turn is activated by binding to GAL4, fig 2.1. The tissue specific promoter / driver used in these studies was *bam (bag of marbles)* which is repressed in germline stem cells and expressed in spermatogonia at the 4 cell stage after completion of two rounds of mitotic divisions (McKearin & Spradling, 1990; Schulz et al., 2004). Thus the genes of interest were efficiently knocked down in the spermatocyte stages (S1-S6) that were analysed.

A



B

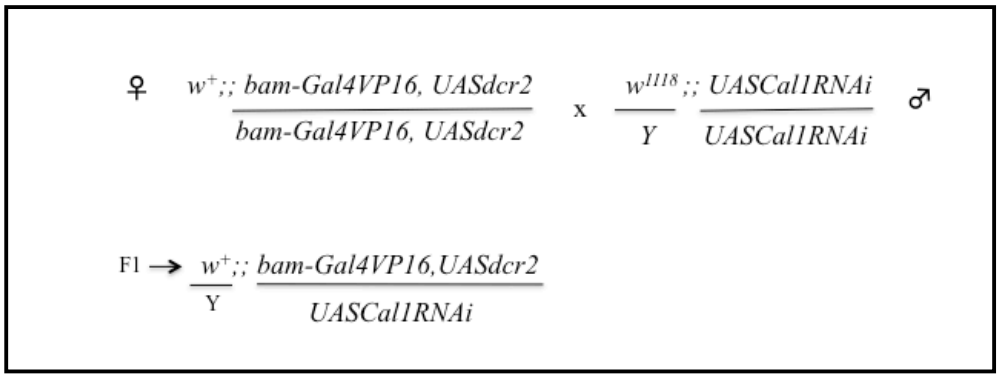


Figure 2.1. Schematic of the GAL4-UAS system and an example of a genetic cross. (A) Transgenic flies expressing GAL4 a yeast transcriptional activator with a tissue specific promoter placed 5' of GAL4 were crossed with transgenic flies carrying the gene of interest inserted 3' of the Upstream Activating Sequence (UAS). The gene of interest was effectively knocked down in the progeny. (B) An example of a standard RNAi cross. The cross was performed at 25°C homozygous *bam-Gal4* virgin females were crossed to homozygous *UAS-cal1-RNAi* males. All progeny were homozygous for *cal1-RNAi*. Knockdown of CAL1 was confirmed by immunofluorescence microscopy.

2.1.3. Fertility test

2- to 3-day-old virgin males and 2- to 3- day old virgin females were crossed at 20°C and passed to new tubes every third day to obtain 3 repeats per cross. All progeny were counted upon eclosion.

2.1.4. Larval and Adult testes dissections

Dissections were carried out in 500 µl 1 x PBS. Male larvae were identified by transparent gonads, (highlighted in green), fig 2.2 A, close to the rear anal end, while adult males were identified by a darkening on the genital plate, fig 2.2 B, and sex combs on fore legs. Forceps were firmly placed on either side of these defining structures followed by a gentle pulling to expose the testes. The testis were disconnected from fat layers to make staining and mounting easier.

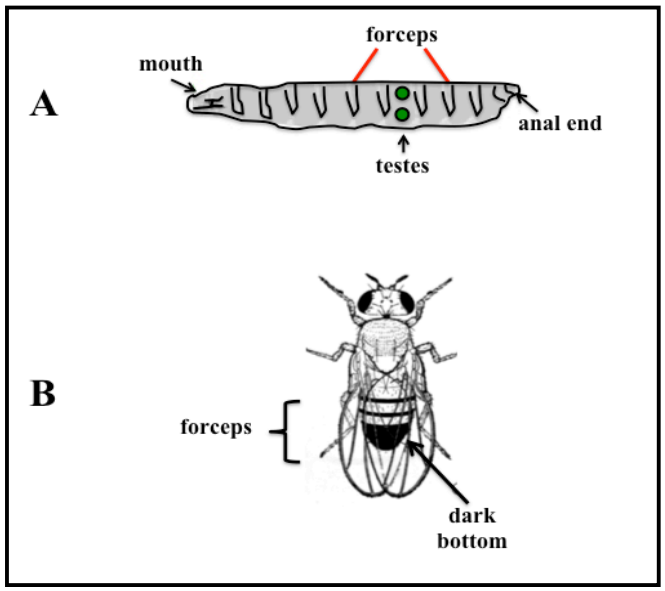


Figure 2.2. Schematic of *Drosophila* larval and adult males. (A) Third instar male larvae, identified by transparent gonads on the rear anal end, highlighted in green (B) *Drosophila* adult male, identified primarily by a dark bottom.

2.1.5. *Drosophila* Drug Treatments

Below are listed the drugs used in this study. The source and concentration of drugs used are also listed below

Table 2.1.1: Drug treatments

Name of drug	Use	Final Concentration	Source
CX5461	Inhibition of Pol I driven transcription of rRNA	300 nM	Millipore
Actinomycin D	Inhibition of DNA directed RNA synthesis	2 μg/ml	Sigma-Aldrich
RO-3306	Inhibition of Cdk1 and / or Cdk2	100 μM	Sigma-Aldrich
MG132	Inhibition of the proteasome	50 μM	Sigma-Aldrich

Dissected testes were incubated for 30 minutes in CX5461, actinomycin D, RO-3306 or MG132 diluted in 100 μl 1X PBS, at the above final concentrations. The drug was washed off in 1X PBS. Testes were pipetted onto polysine slides and fixed for immunofluorescence analysis or lysed for western blot analysis where stated.

2.1.6. Overexpression of Cyclin A or Cyclin B via Heat shock induction

Drosophila 3rd instar larvae, carrying the transgenes *hs-Δ-cyclin A or hs-Δ-cyclin B,* table 2.1, which allow the expression of *Δ-cyclins* lacking the region with the destruction box, fig 2.3 A, under the control of a heat shock promoter (HSP) were incubated for 30 minutes at 37°C followed by 30 minutes recovery at 25°C (figure 2.3 B). Larval testes were dissected using the method described above. Testes were fixed and subsequently stained for immunofluorescence microscopy.

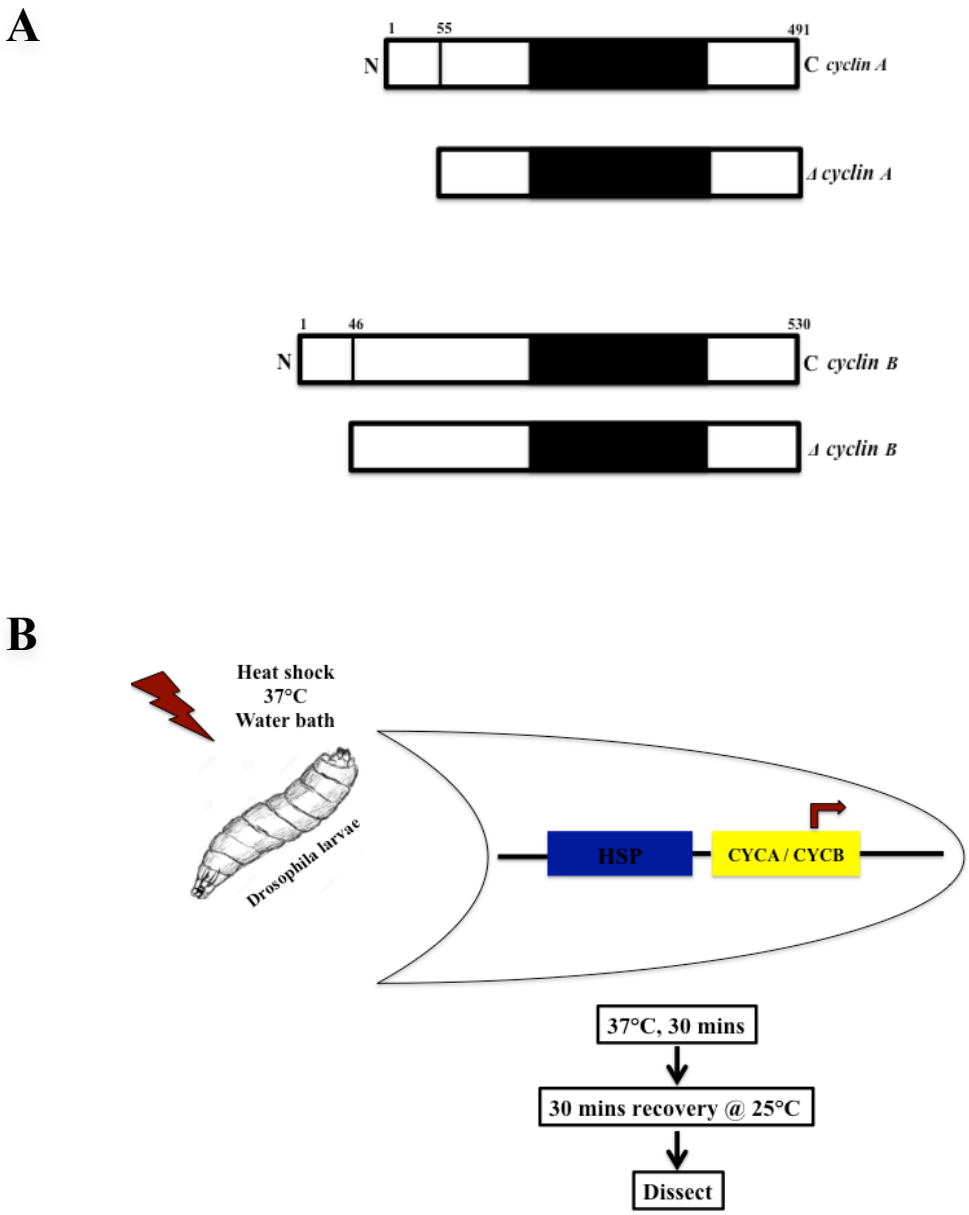


Figure 2.3. Schematic of the structure of *Δ-cyclins* and Heat shock protein induction method. (A) Transgenic lines were constructed allowing the expression of *Δ-cyclins,* which lack the region with the destruction box motif required for degradation (Kaspar et al., 2001; Sprenger et al., 1997) under the control of a heat shock promoter. The Structure of the *Δ-cyclins* is illustrated schematically. Numbers designate amino acid position. The black box represents the *cyclin* box which is required for association with Cyclin dependent kinase. (B) illustrates the heat shock induction method. Larvae carrying the *Δ-cyclins* transgenes under a heat shock promoter (HSP) were incubated in a 37°C water bath and allowed to recover at 25°C, followed by dissections. Adapted from (Sigrist et al., 1995).

2.2. Chemical reagents and Buffers

The chemicals used in this study were of analytical grade standard and purchased from Sigma-Aldrich, Fisher Scientific, Invitrogen or GE Healthcare, Solutions. The buffers used in this study were prepared using MilliQ- purified water (Millipore, Billerica) and were autoclaved before use when necessary. These are listed according to the technique used in table 2.2

Table 2.2.1: Reagents and Buffers used in this study

Immunofluorescence Microscopy	**Description**	**Notes and use**
Blocking solution, PFA fixation	1% Bovine Serum Albumin (BSA) + 1 x PBS + 0.1% Triton X - 100	For blocking cells and diluting antibodies for Paraformaldehyde fixation (PFA)
Blocking solution, methanol acetone fixation	1% Bovine Serum Albumin (BSA) + 1 x PBS + 0.4% Triton X - 100	For blocking cells and diluting antibodies for Methanol Acetone fixation
Bovine Serum Albumin (BSA)	1% BSA, pH 5.0	For blocking samples
Paraformaldehyde (PFA)	4% PFA diluted in 1 x PBS	For fixation of cells for immunofluorescence
Methanol/ Acetone	100% methanol and 100% acetone	For fixation of cells to preserve tubulin
Permeabelisation Buffer	1 x PBS + 1% Triton X-100 + 0.5% acetic acid	For permeabilisation of cells for methanol/ acetone fixation
Phosphate buffered Saline (PBS)	80 g NaCl, 2 g KCl, 14.4 g NaH_2PO_4, 2.4 g $NaHPO_4$, pH 7.45	For washing fixed cells
4',6' diamino-2-phenylindole-2HCL (DAPI)	1 µg / ml in 1 x PBS	For staining DNA
Wash buffer	1 x PBS + 0.1% Triton X-100	Washes for IF (4% PFA fix)
Wash buffer	1 x PBS +0.4% Triton X-100	Washes for IF (methanol acetone fix)
Slowfade gold antifade reagent	Glycerol based antifade solution	For mounting immunofluorescence slides

Molecular cloning	**Description**	**Notes**
4 x DNA loading dye	10 mM Tris, 30% glycerol, Bromophenol blue powder, fisher molecular grade water	Used for loading DNA gels
Tris-Acetate-EDTA (TAE) buffer	242 g Tris base, 57.1 ml 100% glacial acetic acid, 500 mM EDTA	For preparation and running of DNA gels
Luria-Bertani (LB) broth	10 g peptone, 5 g yeast, 5 g sodium chloride	Used as a growth medium for the cultivation of bacteria *Escherichia. coli*
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Used at 0.5 mM	For induction of β galactosidase activity
Buffer A (genomic DNA extraction)	100mM Tris pH 7.5, 100mM NaCl, 100 mM EDTA, 1% SDS	For lysing flies for genomic DNA preparation
TRIzol reagent		For RNA extraction

Fly husbandry and dissections	**Description**	**Notes**
Nutri-Fly food	Yellow cornmeal, corn syrups solids, soy flour, yeast, agar	For culturing *Drosophila melanogaster* stocks
Phosphate buffered Saline (PBS)	80 g NaCl, 2 g KCl, 14.4 g NaH_2PO_4, 2.4 g $NaHPO_4$, pH 7.45	For dissecting larvae or adult flies

Western blotting	**Description**	**Notes**
Blocking solution (Western Blotting)	5% non fat dry milk + 1 x TBS + 0.1% tween	For blocking the membrane and diluting antibodies
Bolt 4-12% Bis Tris Plus gradient SDS-PAGE gels	4-12% Bis Tris Plus SDS precast gels	For running protein gels
Enhanced Chemiluminescence (ECL) substrate	1 x peroxide solution, 1 x luminol enhancer solution	For detection of horse radish peroxidase (HRP) activities from antibodies
Lysis Buffer (Protein extraction)	20 mM Tris, 300 mM NaCl, 0.5% EDTA, 0.5% NP40, 0.05% titron, 1 mM Phenylmethylsulfonyl fluoride (PMSF)	Protein extraction for western blot analysis
Ponceau S	0.5% Ponceau s, 0.5% acetic acid	For staining western blot membranes to check efficiency of protein transfer
Running buffer, NuPAGE MOPS SDS (20X)	50 mM MOPS, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.7	For running protein gels
1 x Lithium Dodecyl Sulfate (LDS) buffer	Lithium dodecyl sulfate pH 8.4 + 2% β mercaptoethanol	Used for loading protein samples
Transfer Buffer (10X)	288 g glycine, 60.4 g Tris base 1.8 ml milliQ water (10% methanol was added to 1 x working stock)	For transfer of proteins from SDS-PAGE gels to nitrocellulose membranes
Tris Buffered Saline (TBS) 10 X	87.7 g NaCl, 1 M Tris-Cl pH 7.5, milliQ water	For washing western blots

Purification of TAQ polymerase	Description	Notes
Buffer B	50 mM Tris pH 7.9 50 mM glucose 1 mM EDTA	For purification of TAQ polymerase
Lysis Buffer	10 mM Tris pH 7.5 50 mM KCl 1 mM PMSF 0.5% Tween 0.5% NP40	For lysis of bacteria expressing TAQ polymerase
Pre-lysis Buffer	10 mM Tris pH 7.5 50 mM KCl 0.5% Tween 0.5% NP40 4 mg / ml lysozyme	Re-suspension buffer
Storage Buffer	20 mM Hepes 100mM KCl mM EDTA 0.5 mM PMSF 1 mM DTT 75% glycerol	Storage of purified recombinant TAQ polymerase

2.2.1. Molecular Biology Reagents

The restriction ensymes used for DNA digestion and ligation were obtained from New England Biolabs (NEB). The Q5 DNA polymerases used for polymerase chain reactions (PCR) was obtained from (NEB) and TAQ polymerase was homemade. Calf Intestinal Phosphatase (CIP) and the 1 kb DNA ladder was also supplied by NEB. The bacteria strain used for cloning was *E. coli* DH5α. *E. coli* clones were selected using the antibiotics ampicillin 50 μg/ml or kanamycin 30 μg/ml (Sigma). The plasmids used were from the *Drosophila* Genomics Rescource Centre (DGRC) or a gift where stated. The molecular biology kits used for this study are listed in Table 2.2.1

Table 2.2.2: Molecular Biology Reagents

Name	Use	Source
Click-iT chemistry	Detection of global RNA transcription	Life Technologies™
FuGENE HD Transfection Reagent	Transfection of *Drosophila* Schneider tissue culture cells	Promega
NucleoBond PC 100	Large scale Plasmid DNA extraction	Macherey-Nagel
NucleoSpin Plasmid	Small scale Plasmid DNA extraction	Macherey-Nagel
Superscript III First Strand Synthesis Kit	Synthesis of cDNA from purified RNA	Invitrogen
Q5 Site Directed Mutagenesis Kit	site specific mutagenesis of double stranded plasmid DNA	NEB

The plasmids used in this study are listed in table 2.2.3.

Table 2.2.3: Plasmids used in this study

Plasmid name	Use	Source
{pCopia-LAP-cal1}	Sub-cloning	Dr. Weiguo Zhang (Karpen lab)
pP{CaSpeR-5}	Destination vector for transgenic fly generation	DGRC

The antibodies used in this study were for immunodetection in western blot analysis and imaging of cells by immunofluorescence (IF) and are listed in Tables 2.2.3 and 2.2.4

2.2.2. Localisation and Affinity purification Tag (LAP-Tag)

The LAP-Tag contains a fluorescent protein fusion such as GFP or mCherry and an affinity purification S peptide that binds S protein with high affinity (Cheeseman & Desai 2005), separated by a Tobacco Etch Virus (TEV) cleavage site (figure 2.4). In this study we combined the LAP-Tag with our target protein CAL1.

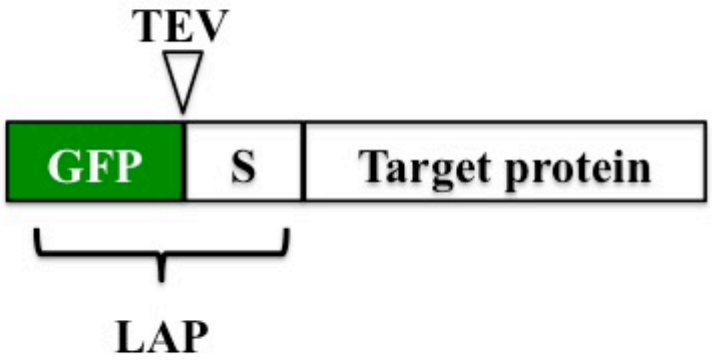


Figure 2.4. Schematic of the LAP-Tag system. The LAP-Tag contains GFP or mCherry and S peptide separated by a TEV cleavage site. In this study our target protein was CAL1.

Table 2.2.4: Primary antibodies

Antigen	Reference number	Host species	Dilution (W/B)	Dilution (IF)	Source
anti- Cyclin A	A12	Mouse	1:50	1:100	Developmental Studies Hybridoma Bank (DSHB)
anti- Cyclin B	F2F4	Mouse	1:50	1:100	DSHB
anti- CAL1	-	Rabbit		1:100	(Bade et al., 2014)
anti- CAL1	-	Rabbit	1:2000	-	(Bade et al., 2014)
anti- CENP-C	-	Guinea pig	1:1000	1:500	(Heeger et al., 2005)
anti- Fibrillarin	38F3	Mouse	1:1000	1:500	Abcam
anti- Modulo	-	Chicken	1:1000	1:100	(Mikhaylova et al., 2006)
anti- Tubulin	DM1A	Mouse	-	1:100	Sigma-Aldrich
anti- Tubulin	B512	Mouse	1:1000	-	Sigma-Aldrich
anti- Actin	AC15	Mouse	1:1000	-	Sigma-Aldrich
anti- CID	39713	Rabbit	1:1000	1:500	Active motif
anti- MEI-S332	-	Guinea pig	-	1:500	(Tang et al., 1998)

Table 2.2.5: (A) Immunofluorescence secondary antibodies

Antibody	Conjugation	Host species	Dilution	Source
anti- rabbit	Alexa 488 Alexa 546	Goat	1:500	Invitrogen
anti- mouse	Alexa 488 Alexa 546 Alexa 647	Goat	1:500	Invitrogen
anti- guinea pig	Alexa 488 Alexa 546 Alexa 647	Goat	1:500	Invitrogen
anti- chicken	Alexa 488	Goat	1:500	Invitrogen

Western blotting secondary antibodies

Antibody	Conjugation	Host species	Dilution	Source
anti- mouse	HRP	Goat	1:5000	Pierce
anti- rabbit	HRP	Goat	1:5000	Pierce
anti- chicken	HRP	Donkey	1:5000	Invitrogen

2.3. Cell biology techniques

2.3.1. Tissue culture aseptic technique

All cell culture was performed in the tissue culture hood to protect samples from aerosol contamination by filtering all of the air that enters. In addition, to prevent other contamination, the inside working surfaces were sprayed down by 70% ethanol and wiped dry using kimwipes before and after use of the hood. In addition, all items brought into the hood were sprayed down.

2.3.2. *Drosophila* tissue culture reagents and conditions

Drosophila Schneider (S2) cells were grown in Schneider's media supplemented with 10% inactivated fetal calf serum (Gibco) and antibiotic, streptomycin, to a final concentration of 1 μg/ml and maintained at 25°C. Stocks were grown in T75 flasks and were split ~ every 3 days.

2.3.3. S2 cell transient transfection

To count cells, cells were resuspended and 20 µl was loaded onto a hemacytometer counting grid. 6 squares were counted and an average was obtained, which was multiplied by 90,000 to get amount of cells per milliliter (ml). To a 6 well plate, 2 x 10^6 cells were added followed by 2 ml media with antibiotic. The cells were allowed to adhere for 30 minutes. 1 µg DNA was mixed with 6 µl FuGENE HD transfection reagent (Promega) in 90 µl sterile water and allowed to form complexes for 30 minutes. After cell adhesion, media with antibiotic was removed and replaced by 2 ml antibiotic free media. The transfection complex was added into the wells followed by a gentle swirl. The cells were incubated at 25°C for 48 hours. Cells were harvested in 2 ml tubes, spun down at RT for 5 minutes 300 relative centrifugal force (rcf). Media was discarded, leaving ~300 µl to resuspend pellet. Resuspended cells were pipetted onto a polysine slide and left at RT for 10 minutes to adhere, after which cells were fixed in PFA for 10 minutes and analysed by IF.

2.3.4. Immunofluorescence microscopy (IF)

Dissected testes were gently squashed to release spermatocytes from cysts, frozen in liquid nitrogen and fixed using methanol/acetone, to preserve microtubules, (Cenci et al 1994) or 4% paraformaldehyde (PFA) to enrich for nuclear signal. Cells were fixed for 10 minutes followed by primary antibody dilution in blocking solution (Table 2.2). Samples were incubated overnight at 4°C followed by 3 x 15 minute washes in wash buffer (Table 2.2). This was followed by secondary antibody incubations for 1 h at RT. Slides were washed 3 x 15 minute washes, followed by a single wash in 1X PBS 10 mins. Samples were stained in DAPI (5 mins) followed by a single 1x PBS wash 10 mins. Samples were mounted using slowfade antifade reagent (Invitrogen). Images were acquired using the DeltaVision Elite microscope system (Applied Precision). 20-30 *z*-sections were taken for each image at a constant exposure time. Raw images were deconvolved and projected using SoftWorx (Applied Precision) and uniformly scaled in Photoshop.

2.3.5. Quantification of fluorescence intensity using Image J

To determine the amount of CENP-A within meiotic nuclei, fluorescence intensity at centromeres was measured using ImageJ software (NIH) on TIFF images after maximal intensity projections. An intensity threshold was set on centromeric foci followed by a measurement of the fluorescence intensity. Using a defined region of interest (ROI) the background pixels from the image were subtracted. The corrected values of all signals within the nucleus was used to determine CENP-A content. The mean CENP-A amounts based on fluorescence intensity were then calculated. Standard Error of the Mean (SEM) was used for generation of error bars. Non-overlapping error bars were regarded as significant differences between the mean values. *P*-values were calculated by two-tailed *t*-test.

2.3.6. Click-iT chemistry

To detect global RNA transcription, Click-iT chemistry, (Life Technologies^{TM}) was used. Dissected testes were incubated in 100 mM 5-Ethynyl Uridine (EU), a nucleoside analog of uracil, diluted in 100 µl 1 x PBS. After which cells were fixed in 4% PFA. After the last secondary antibody wash, the slides were incubated in the Click-iT reaction mix (Table 2.3.1) for 30 minutes to detect incorporated EU.

Table 2.3.1: Click-iT reaction mix

Reagent	Final concentration
Na-L-ascorbate	1M
TEG-Azide	10mM
Copper Sulfate	0.1M

The reaction was followed by 3 x 5 minute washes in 1 x PBS, followed by DAPI staining and mounting. Slides were analysed using the DeltaVision Elite microscope system (Applied Precision).

2.4. Nucleic acid techniques

2.4.1. Agarose gel electrophoresis

To separate the mixed population of DNA fragments by size they were run on a 1% agarose gel. The gel was made in 1 x TAE buffer supplemented by 0.5 µg/ml ethidium bromide and were run in the mini sub tank (Bio-Rad), in TAE buffer at 100V for ~30 minutes or until the desired separation was achieved. DNA fragments were visualised using a UV light transilluminator. Images were captured with the attached digital camera. For DNA extraction, bands were excised with a scalpel blade followed by purification using the Macherey-Nagel Gel Extraction kit according to the manufacturer's instructions.

2.4.2. Purification of TAQ polymerase

To purify *Taq* polymerase, bacteria expressing recombinant *Taq* polymerase was added into 50 ml LB broth + ampicillin and incubated overnight at 37°C. 600 µl of overnight culture was added to 100mls LB broth + ampicillin 50 µg/ml. This was incubated at 37°C until OD_{600} (0.3-0.4) followed by addition of 0.5mM IPTG. The culture was incubated for 16 hrs at 37°C. The overnight culture was centrifuge at 4,000 rpm for 10 minutes. The pellet was washed in 5 ml Buffer A (table 2.2) and centrifuged at 4,000 rpm for 5 minutes. 5 ml pre-lysis buffer (table 2.1) was added to the pellet. The sample was left at RT for 20 minutes followed by addition of 5 ml lysis buffer, table 2.2. This was then incubated for 60 minutes at 80°C and centrifuged for 10 minutes at 4°C 4,500 rpm. The supernatant was recovered followed by addition of equal volume of storage buffer, table 2.2. The *Taq* polymerase was aliquoted into 1ml samples and frozen on dry ice or liquid nitrogen then stored at -80°C. The working stock was stored at -20°C.

2.4.3. Preparation of competent E. coli

~100 µl of DH5α cells, an *E.coli* cloning strain, were added to 10 ml LB broth and grown overnight. 1 ml of the overnight culture was added to 700 ml LB broth and grown until the culture reached OD_{600} of 0.5. All subsequent steps were carried out on ice to maximise the competency of the cells. *E. coli* were chilled on ice for 5 minutes

and centrifuged at 4°C, 3,200 rpm for 4 minutes. The pellet was resuspended in 20 ml of transformation buffer I, TFBI, table 2.2, and chilled on ice for 15 minutes. The suspension was centrifuged at 3,200 rpm for 10 minutes at 4°C and the supernatant was discarded. Pellets were resuspended in transformation buffer II, TFBII, table 2.2, and chilled on ice for 30 minutes. Cells were aliquoted into 50 µl volumes, snap frozen in liquid nitrogen and stored at -80°C.

2.4.4. Restriction enzyme digestion

Restriction enzymes used were supplied by New England Bio labs (NEB). The reactions were performed according to manufactures guidelines on a thermo-stable block for 1-16 hours depending on the sample.

2.4.5. DNA ligation

Digested DNA was purified using NucleoSpin Gel and PCR clean up kit (Macherey-Nagel) to remove restriction enzymes and buffers. Calf intestinal phosphatase, CIP, (NEB) was used to treat digested DNA to prevent self-ligation according to manufacturers instructions. The ratio of vector and insert fragment used in ligations was either 1:3 or 1:5 (DNA range 50 nanograms vector: 150 nanograms insert). The ligations were performed using T4 DNA ligase (NEB) in line with manufacturers guidelines. Reactions were subsequently transformed into competent *E. coli* cells with appropriate antibiotic selection.

2.4.6. *E. coli* transformation

To introduce foreign DNA into bacterial cells, 50 µl of competent DH5α cells were thawed on ice, mixed with ~50 – 100 ng plasmid DNA and incubated on ice for 30 minutes. This was followed by heat shock at 42°C for 90 seconds. The sample was placed on ice for ~1 minute to recover. Using aseptic conditions, 100 µl of LB broth was added to the sample followed by incubation at 37°C under agitation for 45 minutes. The sample was plated onto LB agar plates containing an appropriate antibiotic selection under aseptic conditions and incubated overnight at 37°C. Positive colonies, confirmed by colony PCR, were picked and grown overnight in LB broth cultures containing desired antibiotics at 37°C. Plasmid DNA was then extracted from

these cultures.

2.4.7. Plasmid DNA preparation

Bacterial cell cultures were grown overnight under gentle agitation at 37°C in the presence of a selective antibiotic. For mini plasmid preparations, a 4ml *E. coli* overnight culture was used while for midi plasmid preparations, a 100ml *E. coli* overnight culture was used. Mini and midi plasmid DNA isolation was carried out using NucleoSpin Gel and PCR clean up or NucleoBond PC 100 kit (Macherey–Nagel) respectively according to manufacturers instructions. The resultant DNA pellet was resuspended in molecular biology grade water (Fisher Scientific). Plasmid DNA concentration was quantified using Nanodrop 2000 (Thermo Fisher Scientific).

2.4.8. Sequencing

DNA samples at 100 ng/µl were sent to Source Bioscience (Waterford, Ireland) for commercial sequencing. Primers were also sent as per recommendation 3.2 pmol/µl. Sequencing results were analysed using SnapGene Software.

2.4.9. RNA extraction

For RNA extraction, ~30 whole adult *Drosophila* were collected in a 1.5 microcentrifuge tube and snap frozen in liquid nitrogen. 500 µl TRIzol reagent (Life Technogies™) was added followed by homogenisation using a plastic pestle. The sample was incubated at RT for 5 minutes followed by centrifugation at 12,000 rcf, 10 minutes at 4°C to pellet insoluble debri. Supernatant was transferred to a new microcentrifuge tube. 200 µl chloroform was added followed by rigorous shaking of the tube. The sample was incubated at RT for 3 minutes followed by centrifugation at 10,000 rcf, 15 minutes 4°C. The aqueous phase was transferred to a fresh RNase free microcentrifuge tube followed by addition of 250 µl isopropanol, to precipitate the RNA, and incubation at RT for 10 minutes. The sample was centrifuged at 12,000 rcf, 10 minutes at 4°C. The supernatant was discarded and the pellet was washed with 1 ml 75% ethanol followed by centrifugation at 7,500 rcf for 5 minutes at 4°C. The supernatant was discarded and the pellet was air dried for ~5-10 minutes. The pellet was resuspended in 100 µl RNase free water. RNA concentration was measured using

Nanodrop 2000 (Thermo Fisher Scientific). RNA was aliquoted into smaller fractions ~5 µl and stored at -80°C. Extracted RNA was mainly used to make cDNA.

2.5. Complementary DNA (cDNA) synthesis

cDNA synthesis was performed using Superscript III First Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. Briefly 5 µg of RNA was mixed with 50 µM oligo dT, 100 mM dNTP mix and sterile water up to 20 µl. The mixture was incubated at 65°C for 5 minutes and then incubated on ice for 1 minute followed by brief centrifugation to collect the contents. 5 X First Strand Synthesis Buffer, 0.1 M 1,4 Dithiothreitol, (DTT), 40 U/µl RNaseOUT and 200 U/µl Superscript III reverse transcriptase, all supplied by the manufacturer, were added to the centrifuged contents. This was mixed by pipetting gently and incubated at 50°C for 60 minutes. The reaction was inactivated by heating to 70°C for 15 minutes. The cDNA was stored at -20°C for use in PCR where required.

2.5.1. Genomic DNA extraction

Genomic DNA was extracted from whole flies to amplify genomic regions of interest. ~30 whole flies were anesthetised and collected into an eppendorf tube. Flies were homogenised in 200 µl Buffer A, table 2.2, with a plastic pestle. An additional 200 µl Buffer A was added followed by further homogenisation until only cuticles remained. The sample was incubated at 65°C for 30 minutes followed by addition of 800 µl Lithium Chloride / Potassium Acetate solution. This was followed by incubation on ice for 30 minutes. The sample was centrifuged for 15 minutes at 12,000 rpm RT. 1 ml of the supernatant, avoiding floating crude, was transferred into a new tube, followed by addition of 600 µl isopropanol to precipitate DNA. The sample was centrifuged at 12,000 rpm for 15 minutes RT. Supernatant was discarded and the pellet was washed with 1ml 70% ethanol. The sample was centrifuged again at 12,000 rpm, 15 minutes RT. The supernatant was removed and the pellet was dried at RT for ~5-10 minutes. The pellet was resuspended in 100 µl elution buffer. The concentration of genomic DNA extracted was measured using the Nanodrop 2000 (Thermo Fisher Scientific). Genomic DNA was stored at -20°C

2.5.2. Polymerase chain reaction (PCR)

PCRs were performed using the BioRad T 100 thermal cycler. Polymerases used were either homemade *Taq* polymerase, or Q5 polymerase (NEB) for high proof-reading activity. Tables show PCR conditions and concentrations of reagents used.

Table 2.5.1 PCR reagents and concentrations

Reagent	Final concentration/ reaction
PCR reaction buffer	1 x
dNTP's	200 µM
Primers	0.25µM
Template	1 – 4 ng/µl
$MgCl_2$	2 mM
Enzyme	0.02 U/µl
Fisher molecular biology grade water	Up to 25µl

Table 2.5.2 PCR conditions

PCR step	Temperature	Duration
Initial denaturation	95°C	3 mins
Denaturation	95°C	30sec
Annealing*	55°C	30 sec
Extension*	72°C	30 sec/ kb Q5 polymerase 1min/ kb *Taq* polymerase
Number of cycles	35	Varies depending on amplicon size
Final extension	72°C	5 minutes
Storage	4°C	-

*Adjusted with different primer pairs

Table 2.5.3 List of primers

Primer name	Sequence
5'-UTR-Fwd	CCGCATAGGCCACTAGTGGATCTG CAGCCATGTCAAGCACTCTGG
5'-UTR-Rev	GCTCCTCGCCCTTGCTCACCATGGT TATGTAGTTATCAGTTCACG
3'-UTR-Fwd	GAATAATTCCGGTGACAAGTAATT AAGTAGTTAGGTTTCATATTT
3'-UTR-Rev	AAGCTTTAGAGCTCTTCTTAAGAAGT ATGCTTCGCATGTGTATGT
LAP-CAL1-Fwd	TCCGTGAACTGATAACTACATAAC CATGGTGAGCAAGGGCGAGGA
LAP-CAL1-Rev	AATATGAAACCTAACTACTTAATT ACTTGTCACCGGAATTATTCT
SERINE_ALANINE_Fwd	CAGCGTCTACCTGGAGGCCGCACC ACCAAAGTACCTTCCAC
SERINE_ALANINE_Rev	TTGTGCCTTCGCATCCAGCGGCCCA CAT

2.6. Protein methods

2.6.1. Protein Extraction

For total protein extracts, larval testes or whole larvae were collected in a 1.5 ml tube and lysed in lysis buffer, table 2.2, containing 300 mM Sodium Chloride, (NaCl). Chromatin and nuclear soluble protein extracts were isolated by grinding samples with plastic pestles, followed by centrifugation at 4°C 10,000 rpm. Supernatant, containing the soluble protein extracts, was collected into a new tube and snap frozen in liquid nitrogen for storage at -20°C , while NuPAGE 4x LDS sample buffer (Invitrogen), supplemented with 10% β-mercaptoethanol was added to the pellet fraction, containing the chromatin bound protein. The concentration of soluble protein extracts was determined using the Bradford assay. Briefly, Bradford reagent (Sigma), was diluted in a 1:1 ratio with distilled water. 1 µl of the sample to be measured was pipetted into a cuvette followed by addition of 1 ml of the diluted Bradford reagent. Samples were incubated at RT for 5 minutes before measuring absorbance at 595 nm using the Nanodrop 2000 (Thermo fisher Scientific). A standard curve was used to convert the absorbance into protein concentration.

2.6.2. Sodium dodecyl-sulfate polyacramide gel electrophoresis (SDS-PAGE)

Protein samples were supplemented with 4 x NuPAGE LDS sample buffer (Invitrogen) containing 10% β-mercaptoethanol. Samples were boiled at 95°C for 10 minutes and loaded on a 4-12% SDS-PAGE gel (Life Technologies^{TM}) alongside a pre-stained broad range protein marker, Novex Sharp (Life Technologies^{TM}) to determine molecular weight. Gels were run at 120V constant for 30-45 minutes in running buffer, table 2.2, using a Bolt mini gel tank (Life Technologies^{TM}).

2.6.3. Wet protein transfer

Protein transfer following appropriate separation by electrophoresis was carried out using the mini Trans-Bolt tank (BioRad). Proteins were transferred onto a nitrocellulose blotting membrane (GE Healthcare), the SDS-PAGE gel was placed onto a nitrocellulose membrane, equilibrated in ice cold 1 x transfer buffer, table 2.2, between 2 sheets of Whatman paper that had also been soaked in 1 x transfer buffer. Transfer was performed at 100V constant, 350 milliamps for 1 hour in 1 x transfer buffer.

2.6.4. Western blotting

Post transfer, the membrane was stained in Ponceau S solution (Sigma-Aldrich) for 5 minutes to confirm the efficiency of the transfer. Ponceau was washed off in distilled water for 5 minutes followed by incubating the membrane in blocking solution, table 2.2, for 1 hour at room temperature on a roller. The membrane was then incubated in primary antibody diluted in blocking solution at 4°C overnight. Primary antibody was washed off, 3 x 15 minute washes in 1x TBS-Tween followed by 1 hour secondary antibody incubation with an HRP-conjugated antibody diluted in blocking solution at RT. This was followed by 3 x 15 minute washes, after which the membrane was incubated in a 1:1 mix of the enhanced cheminoluminiscent substrate solution (ECL) (Fisher Scientific) and incubated for 5 minutes followed by autoradiograph film exposure (CL-xposure film) (Fisher scientific) and development in the CP1000 AGFA film processor.

Chapter 3. Dissecting the role of CENP-C and CAL1 in *Drosophila* male meiotic CENP-A assembly

3.1 Introduction

In previous years, the cell cycle timing of CENP-A assembly and CENP-A specific assembly factors has been uncovered. In *Drosophila* mitosis, CENP-A assembly occurs at late M and early G1 phase of the cell cycle, (Schuh et al., 2007; Lidsky et al., 2013) and requires the CENP-A assembly factors, CENP-C and CAL1 (Erhardt et al., 2008; Goshima et al., 2007, Heeger et al., 2005). In *Drosophila* meiosis, a novel cell cycle timing for CENP-A assembly exists but yet requires the CENP-A assembly factors CENP-C and CAL1 (Dunleavy et al., 2012; Raychaudhuri et al., 2012). Given the differences in the timing of CENP-A assembly between mitosis and meiosis, we hypothesise that the CENP-A assembly factors, CENP-C and CAL1 may have specialised meiotic functions. We also hypothesise that CENP-C and CAL1 may also be differentially regulated between mitosis and meiosis. In 2013, Unhavaithaya and Orr-Weaver highlighted potential specific roles for both CAL1 and CENP-C in *Drosophila* female meiosis. Using *cal1* and *cenp-C* mutants it was shown that CENP-C and CAL1 are required for centromere clustering and pairing in female meiosis (Unhavaithaya & Orr-Weaver 2013).

We explored whether in *Drosophila* male meiosis CAL1 and CENP-C had specialised meiotic functions using these *cal1* and *cenp-C* mutants. The *cenp-C*$^{Z3\text{-}4375}$ mutation is a C terminal missense mutation resulting in a change from proline 1115 to a serine (figure 3.1 A). The *cal1*^{2k32} mutation is a C terminal truncation introducing a premature stop codon at glutamate 930 (figure 3.1 B). We uncover roles for CENP-C and CAL1 in CENP-A assembly, maintainence, chromososme segregation and fertility in *Drosophila* male meiosis.

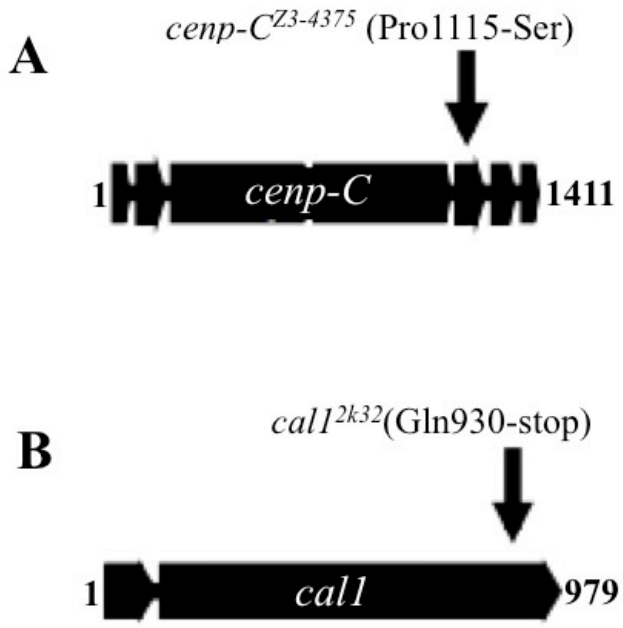


Figure 3.1 *cal1* and *cenp-C* centromere protein mutants. **(A)** Position change of the missense mutation in *cenp-C*$^{Z3\text{-}4375}$ indicated by the arrow. **(B)** Change of glutamate 930 to a stop codon in *cal1*^{2k32} is indicated by the arrow. *Adapted from Unhavaithaya & Orr-Weaver 2013*

Declaration

I declare that my contribution and the contribution of other authors to the presented work have been explicitly indicated. I confirm that appropriate credit has been given in this chapter where some experiments are the work of others.

My contribution involved initial analysis of the requirements of *cenp-C*$^{Z3\text{-}4375}$*/TM3* and *cal1*^{2k32}*/TM3* in meiotic CENP-A assembly, maintenance of CENP-A, chromosome segregation and relevance to fertility. I also analysed the requirements of RNA polymerase I and II transcription in CENP-A assembly using a drug based approach, and analysed nucleolar integrity of *cenp-C*$^{Z3\text{-}4375}$*/TM3* and *cal1*^{2k32}*/TM3* using fibrillarin and modulo as nucleolar markers. The figures that I contributed to the paper are as follows: (Figure 1 F, G and H); (Figure 2 E); (Figure 3 A, B, D, E and F); (Figure 5 A, B) and lastly (Figure 6 A-E).

The Fluorescence in situ hybridisation (FISH) experiments were carried out by Dr. Elaine Dunleavy and I had no input in these experiments. The manuscript was written by Dr. Elaine Dunleavy with my contributions given during verbal discussions and editing of manuscript.

The authored work has been published in the journal Development (2016) and is entitled 'Nucleolar activity and CENP-C regulate CENP-A and CAL1 availability for centromere assembly in meiosis'

Authors of the work are Kwenda L, Collins CM, Dattoli AA and Dunleavy EM

Lucretia Kwenda

Chapter 4. Cell cycle regulation of CENP-A assembly in *Drosophila* male meiosis

4.1. Introduction

The timing of CENP-A assembly invokes clear hypotheses of a cell cycle dependent regulatory mechanism. Although CENP-A has been identified in many eukaryotic species, the cell cycle timing of its incorporation into centromeric chromatin differs between organisms (reviewed in Valente et al., 2012). In mitosis CENP-A assembly is restricted to late M early G1 phases (Jansen et al., 2007; Schuh et al., 2007), a time of low Cdk activity. Interestingly in *Drosophila* and *Secale cerelae* meiosis, assembly occurs at prophase I of meiosis I a time of high Cdk activity (Dunleavy et al., 2012; Raychaudhuri et al., 2012; Schubert et al., 2014) . In recent years, progress has been made in understanding the molecular players involved in the incorporation and maintenance of CENP-A at centromeres in mitosis, with particular emphasis on the role of cell cycle regulatory components such as Cdks and the ubiquitin proteasome machinery (Collins et al., 2004; Erhardt et al., 2008; Moreno-Moreno et al., 2006; Müller et al., 2014; Stankovic et al., 2017)

Recently, small molecule inhibitior studies revealed that brief inhibition of Cdk1/2 activities uncouples CENP-A from its restricted loading window, (early G1), into S, G2 and M phases (Müller et al., 2014; Silva et al., 2012). This has led to a model where CENP-A assembly is inhibited in S, G2 and M phase due to high Cdk1/2 activity. Insights into key proteins necessary for CENP-A assembly revealed that HJURP and Mis18BP1, a member of the Mis18 complex, are phosphoproteins, with Cdk1/2 consensus sites (Bailey et al., 2016; Dephoure et al., 2008; Kato et al., 2007; McKinley & Cheeseman, 2014; Müller et al., 2014; Silva et al., 2012; Wang et al., 2014). Both HJURP and Mis18BP1 transiently associate, and dissociate from centromeres in a cell cycle dependent manner (Dunleavy et al., 2009; Foltz et al., 2009; Fujita et al., 2007; Maddox et al., 2007). Mutation of the HJURP Cdk1/2 consensus motifs Serine412, Serine448 and Serine473 to non phosphorylatable Alanine412, Alanine448 and Alanine473, is sufficient to prematurely drive CENP-A assembly into S and G2 phases (Müller et al., 2014). Moreover Cyclin A and Cyclin

B, the major drivers of Cdk activity, robustly co-immunoprecipitate with GFP-HJURP (Stankovic et al., 2017). HJURP also harbours a Cyclin A binding site (RXL motif), which upon mutation HJURP^{AxA} disrupts the interaction and is sufficient to prematurely target CENP-A to centromeres in G2 (Stankovic et al., 2017).

In *Drosophila* tissue cultured cells, Cyclin A localises to centromeres and its depletion results in CENP-A mislocalisation and reduction in centromeric CENP-A levels (Erhardt et al., 2008). Notably Cyclin A also localises to centromeres in mouse spermatocytes and oocytes (Bentley et al., 2007; Nickerson et al., 2007; Touati et al., 2012) though the relevance of this localisation has not yet been characterised. Additional cell cycle control of CENP-A assembly stems from studies that demonstrate that proteolysis facilitates formation of a single centromere by degrading non-centromeric CENP-A in yeast and flies (Collins et al., 2004; Moreno-Moreno et al., 2006). However the role of cell cycle regulatory elements in meiotic CENP-A assembly remains elusive.

Investigations into the requirements for meiotic CENP-A assembly in fly testes implicate mitotic CENP-A assembly factors CENP-C and CAL1, (Dunleavy et al., 2012; Kwenda et al., 2016; Raychaudhuri et al., 2012). CAL1 is the dedicated CENP-A chaperone and assembly factor in *Drosophila* (Chen et al., 2014). Notably, CAL1 depletion or mutation can lead to chromosome instability and thus segregation defects (Chen et al., 2014; Dunleavy et al., 2012; Erhardt et al., 2008; Kwenda et al., 2016; Raychaudhuri et al., 2012). Interestingly, quantitative in vivo imaging to determine protein copy numbers reveal that CAL1 centromeric levels are 40 times lower than that of CENP-A or CENP-C (Schittenhelm et al., 2010). Consistent with this finding, pulse chase experiments demonstrate that CAL1 is less stably associated with centromeres, centromeric levels decrease by 66% after one cell cycle (Mellone et al., 2011) indicating that CAL1 displays a high turnover rate. Indisputably, 'too much' CAL1 leads to excess incorporation of CENP-A at centromeres, further assigning CAL1 a 'limiting' role in the CENP-A assembly pathway (Schittenhelm et al., 2010).

Intriguingly, CAL1 like HJURP, is a phosphoprotein, with Cdk1/2 consensus sites (Bodenmiller et al., 2007) making it a prime candidate for cell cycle control. Moreover CAL1, comparable to HJURP dynamics, also transiently associates with

centromeres at the time of meiotic CENP-A assembly, and dissociates from centromeres towards the end of the first phase of CENP-A assembly (Dunleavy et al., 2012; Raychaudhuri et al., 2012). Given that CAL1 is functionally homologous to HJURP, (Phansalkar et al., 2012) this raises several mechanistic questions. How does CAL1 specifically target CENP-A strictly to centromeres? How is CAL1 dynamically kept low at centromeres? How does CAL1 associate and dissociate from centromeres? And more puzzling, why is the timing of meiotic CENP-A assembly coupled to high kinase activity?

Here we uncover the role of CYCA and CYCB associated kinase activity in cell cycle regulation of CENP-A assembly in *Drosophila* male meiosis. We identify a mechanism that is sufficient to restrict incorporation of CENP-A at centromeric loci. Our findings also provide a valuable explanation for why meiotic CENP-A assembly is coupled to high kinase activity. Our results provide first insight into the mechanism of meiotic regulation of CAL1, by Cdk1/2.

4.2. Cyclin A and Cyclin B localise to meiotic centromeres in prophase I

To test the hypothesis that meiotic CENP-A assembly in *Drosophila* spermatocytes is regulated by kinase activity, we dissected wild type larval testes and fixed them using 4% PFA followed by treatment in permeabilisation buffer to remove excess cytoplasmic material. Spermatocytes were then immunostained with antibodies against CYCA or CYCB, the major drivers of Cdk activity. We report that in addition to their expected cytoplasmic distribution, both CYCA and CYCB are enriched at centromeres, particularly at the early stages of prophase I (S1-S3) and decrease specifically from centromeres, as prophase I progresses, with much reduction observed by S4-S6 stage, the last stages of prophase I (figure 4.1). Although the centromere localisation of both CYCA and CYCB appeared similar, the overall staining pattern throughout the nucleus appeared different. CYCA was more nuclear and showed more punctuated foci in comparison to CYCB. This difference could be attributed to the differences of these cyclin proteins or to antibody specificity. Interestingly, CYCB also displayed nucleolar staining in some cells at S5 stage. Our data suggest that the reduction of both CYCA and CYCB at centromeres as prophase I progresses is centromere specific as other nuclear foci of CYCA or CYCB are detectable, (figure 4.1). More still in *Drosophila* spermatocytes the degradation of both CYCA and CYCB begins at the onset of metaphase I for CYCA and anaphase I for CYCB (White-Cooper et al., 1998). Interestingly, loss of Cyclin A and Cyclin B at centromeres at S6 stage coincides with the termination of the first phase of CENP-A assembly and with removal of CAL1 (Dunleavy et al., 2012; Raychaudhuri et al., 2012). We next questioned whether this centromere localised CYCA or CYCB is of functional significance.

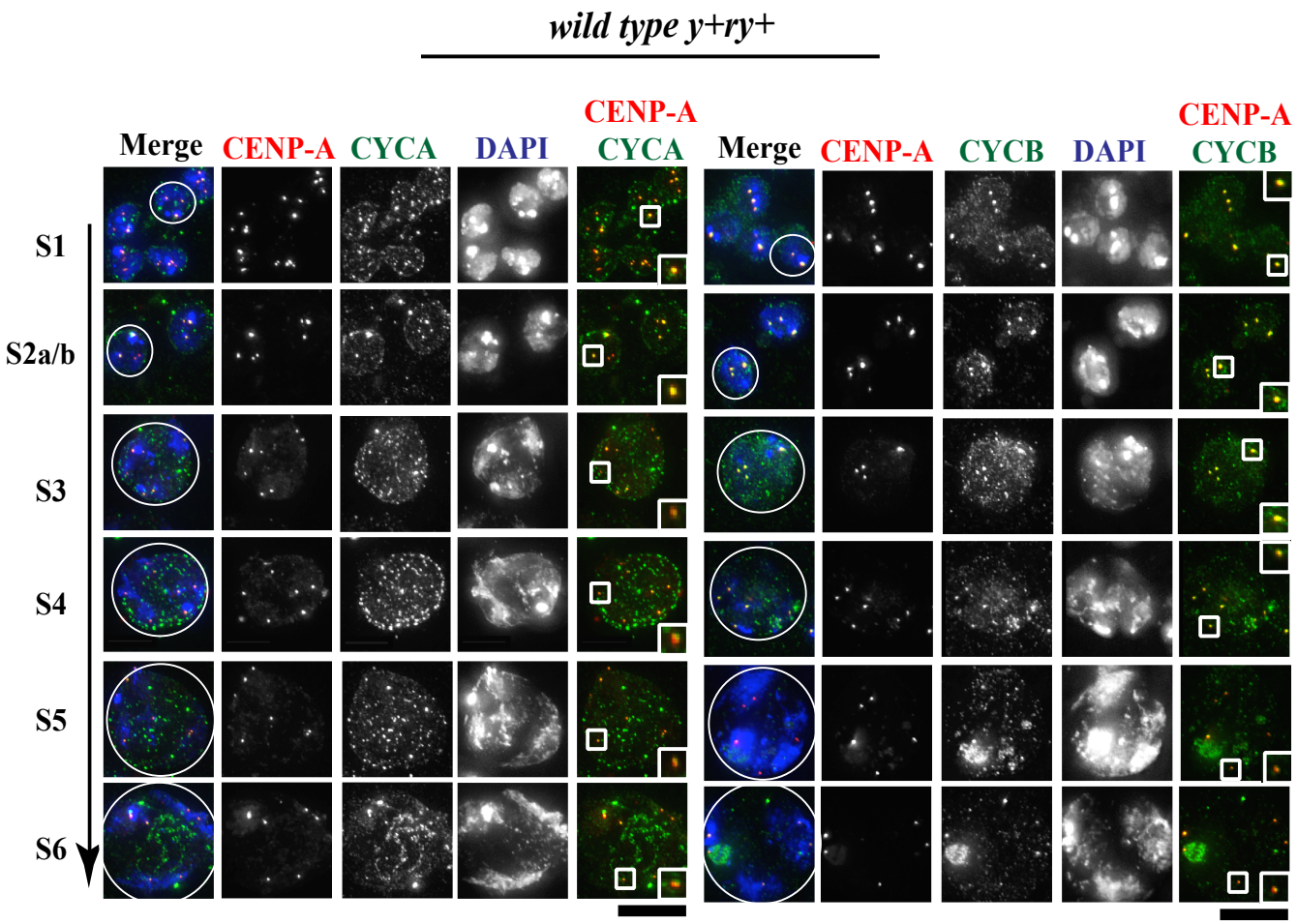


Figure 4.1. CYCA and CYCB localise to meiotic centromeres. wild type $y^{+}ry^{+}$ larval testes were fixed and stained for CENP-A (red), CYCA (green) or CYCB (green) and DNA (blue). Stages S1-S6 of meiotic prophase I are shown. CYCA or CYCB are enriched at centromeres in early prophase I inset (S1-S3). Centromeric CYCA and CYCB decrease as prophase I progresses inset (S5-S6). Scale bar 10 µm.

4.3. CYCA or CYCB Cdk-associated activity limits CENP-A assembly

To determine if localisation of CYCA or CYCB at meiotic centromeres is of functional significance, we tested whether altering centromeric as well as overall levels of either CYCA or CYCB was sufficient to perturb meiotic CENP-A assembly. Deletion of the degradation box in the N terminus of either *cyclin A* or *cyclin B* stabilises the protein by preventing its APC mediated ubiquitination and destruction by the proteasome (Kaspar et al., 2001; Lehner & O'Farrell, 1989; Sigrist et al., 1995). Transgenic larvae lacking the destruction box under the control of a heat shock promoter, *hs-Δ-cyclin A or hs-Δ-cyclin B* (Sigrist et al., 1995), were subjected to heat shock at 37°C in a water bath to allow the expression of non-destructible CYCA or CYCB (figure 4.2 A). To test that CYCA or CYCB was overexpressed, immunostaining of spermatocytes with antibodies against CYCA or CYCB, in combination with CENP-A revealed that total as well as centromeric levels of CYCA and CYCB were increased in heat shocked *hs-Δ-cyclin A or hs-Δ-cyclin B* spermatocytes particularly at the S2 stage compared to no heat shock control (figure 4.2 B), moreover, western blot analysis on whole larvae following CYCB overexpression also revealed that total protein levels were indeed increased (figure 4.2 A). Intriguingly, we observed that at early stages of prophase I, overexpression of CYCA or CYCB at centromeres corresponded to reduced levels of CENP-A, (figure 4.2 B and C), while CYCA or CYCB centromeric levels were strikingly elevated, (figure 4.2 D). Our data suggest that CYCA or CYCB associated kinase activity limits CENP-A assembly in early prophase I possibly via local inhibition. Thus we have focused on the S2 stage as this stage revealed more sensitivity to CYCA or CYCB perturbations.

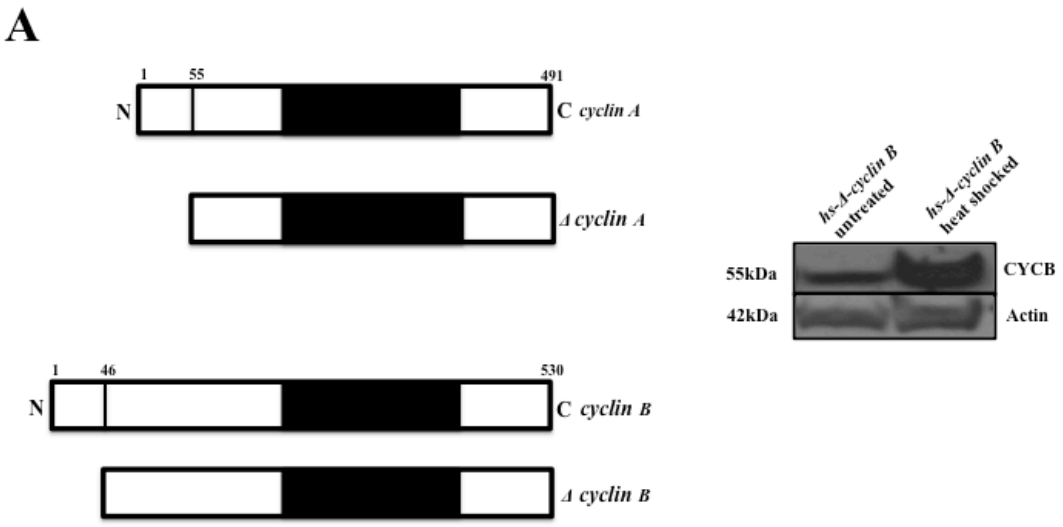


Figure 4.2 A. Schematic showing the structure of *Δ-cyclin A and Δ-cyclin B* and western blot analysis of *Δ-cyclin B* following heat shock. *Δ-cyclin A* and *Δ-cyclin B* lack the region with the destruction box in the N terminus, amino acids 1-55 for *Δ-cyclin A* (Kaspar et al., 2001) and amino acids 1-46 for *Δ-cyclin B* (Sprenger et al., 1997) and are under the control of a heat shock promoter. Numbers designate amino acid positions. The black box represents the conserved cyclin box, which is required for association with Cdk. **Right**, western blot on whole larvae following overexpression of *hs-Δ-cyclin B*. Overall CYCB levels were increased upon overexpression of *hs-Δ-cyclin B*.

B

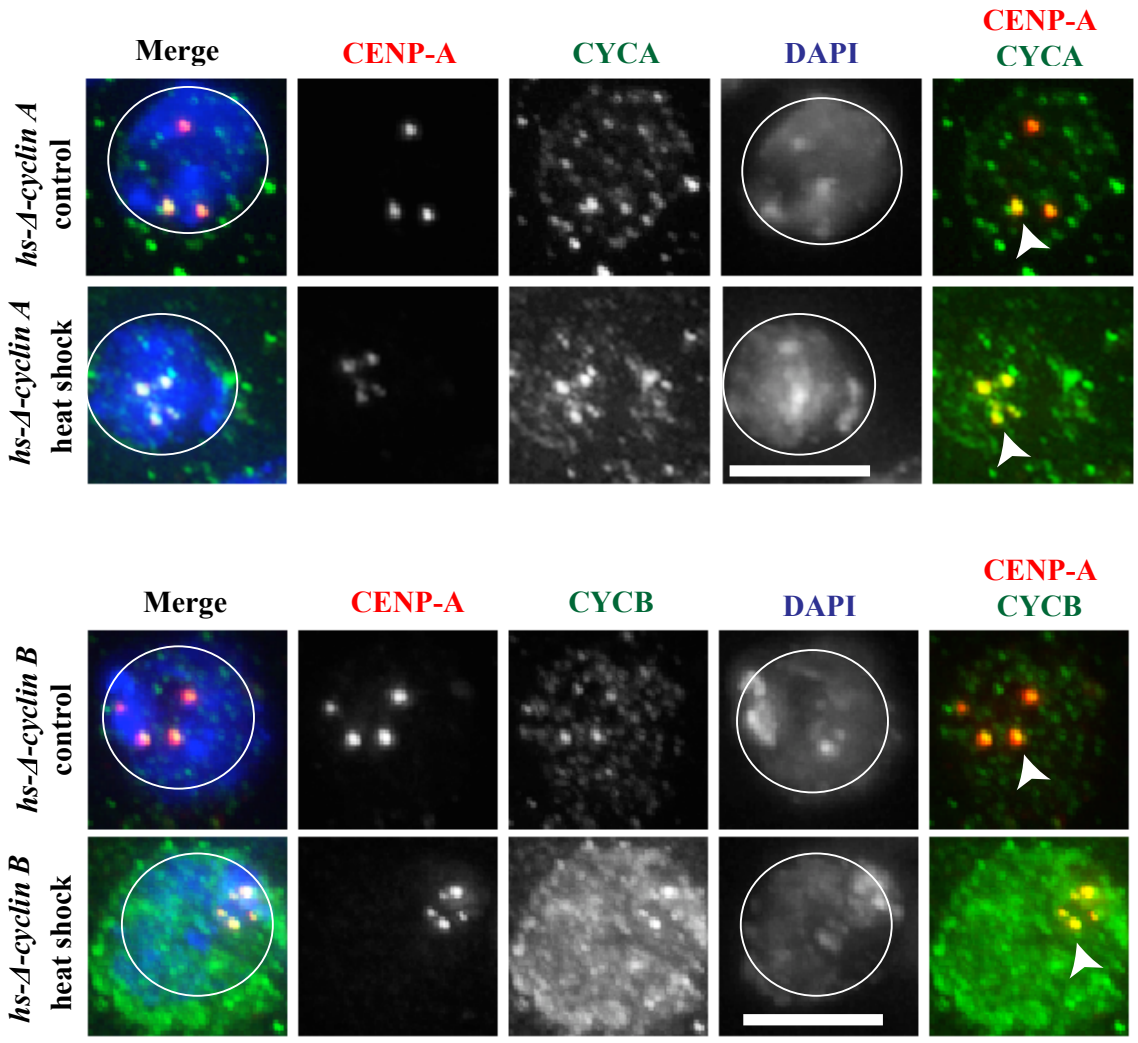


Figure 4.2 B. CYCA or CYCB Cdk-associated activity limits CENP-A assembly. *hs-Δ-cyclin A or hs-Δ-cyclin B* larval testes were heat shocked at 37°C and allowed to recover at 25°C for 30 minutes. Spermatocytes were fixed and stained for CENP-A (red), CYCA (green) or CYCB (green) and DNA (blue). Centromeric CENP-A decreases upon overexpression of either stable CYCA or CYCB (CENP-A channel), while CYCA and CYCB levels at centromeres are increased compared to controls (white arrows). Scale bar: 5 μm.

C

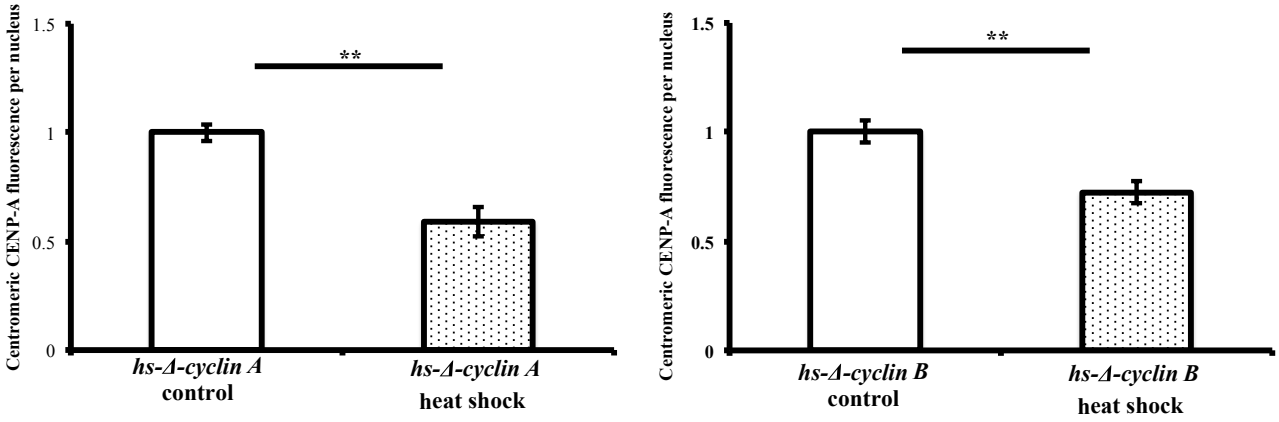


Figure 4.2 C. Quantification of centromeric CENP-A fluorescence per nucleus following *hs-Δ-cyclin B* or *hs-Δ-cyclin A* overexpression. Error bars = SEM, p value ≤ 0.01 number of cells quantified = 25 per experiment n=3 independent experiments

D

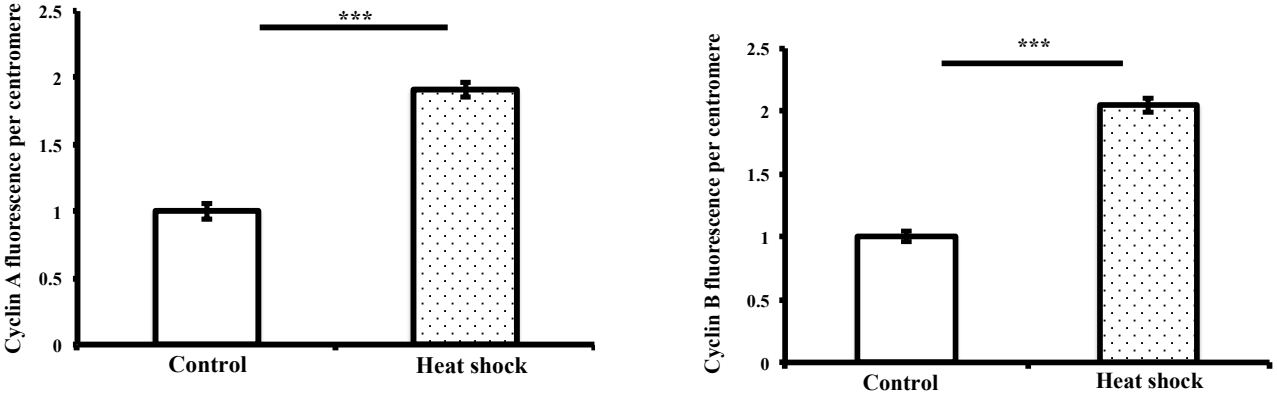


Figure 4.2 D. Quantification of centromeric CYCA and CYCB fluorescence per nucleus following *hs-Δ-cyclin B* or *hs-Δ-cyclin A* overexpression. Error bars = SEM, p value ≤ 0.001 number of cells quantified = 25 per experiment n=3 independent experiments.

4.4. Inhibition of Cdk1/2 results in loss of CYCA or CYCB at centromeres and enhanced CENP-A incorporation at centromeres

To further characterise the role of CYCA or CYCB associated kinase activity in meiotic CENP-A assembly, we used a small molecule inhibitor study. If Cdk activity limits CENP-A assembly, we hypothesised that exposure of *Drosophila* spermatocytes to Cdk inhibition would result in increased CENP-A levels. Thus wild type larval testes were dissected and briefly treated for ~30 minutes, with RO-3306 a selective Cdk1 inhibitor. RO-3306 has an inhibitory constant of 35nM for Cdk1 nearly 10 fold selectivity relative to Cdk2 (Vassilev et al., 2006). We exposed *Drosophila* spermatocytes to varying concentrations of RO-3306 starting at 35 nM to 300 μM and quantified centromeric CENP-A intensity at each concentration, (figure 4.3 A). We found that the optimum concentration for Cdk1 inhibition alone, 35 nM showed the least change in centromeric CENP-A intensity, while a higher dose 100 μM, possibly inhibiting both Cdk1 and Cdk2 (Cdk1/2 henceforth), showed the most significant change (figure 4.3 A). To confirm if indeed we were inhibiting Cdk activity, we carried out western blot analysis on *Drosophila* spermatocytes after treatment with 100 μM RO-3306 and examined the levels of CYCA an APC substrate. Because the APC remains inactive during initial RO-3306 induced G2 block, APC substrates accumulate (Ma, 2009). Therefore, as expected total CYCA levels accumulated following RO-3306 treatment, (figure 4.3 B), validating that the inhibition was indeed working in our system.

Immunofluorescence analysis of spermatocytes treated with RO-3306 and staining with antibodies against CENP-A and CYCA or CYCB showed that Cdk1/2 inhibition resulted in increased centromeric CENP-A incorporation (figure 4.3 C, D and E). Interestingly CYCA or CYCB levels at centromeres were significantly reduced compared to the control (figure 4.3 C, D and F). This reduction of CYCA or CYCB from centromeres coincided with uncontrolled, non gradual centromeric CENP-A recruitment in early prophase I, further suggesting that the presence of centromere localised CYCA or CYCB is functional. It is possible that the overall reduction in CYCA observed (figure 4.3 C) may be due to the difference in the number of z

sections projected. This may also explain the mismatch between figure 4.3 B and 4.3 C.

We also observed increased number of centromeric foci in spermatocytes treated with RO-3306 compared to control samples, (figure 4.3 C and D). Quantification revealed that in the control the average number of centromeric foci per nucleus was between 3 and 4 centromeres (figure 4.3 G). In comparison, the average number of centromeric foci per nucleus in spermatocytes treated with RO-3306 increased to an average of between 5 and 6 centromeres per nucleus, (figure 4.3 G). We speculate that this could be due to loss of sister centromere cohesion, clustering, or homolog pairing. It is also possible that this phenotype may arise from off target effects of Cdk1/2 inhibition. However this phenotype was not explored further.

We conclude that centromere localised CYCA or CYCB associated kinase activity 'limits' meiotic CENP-A assembly as Cdk1/2 inhibition is sufficient to drive aberrant, non gradual CENP-A loading dynamics.

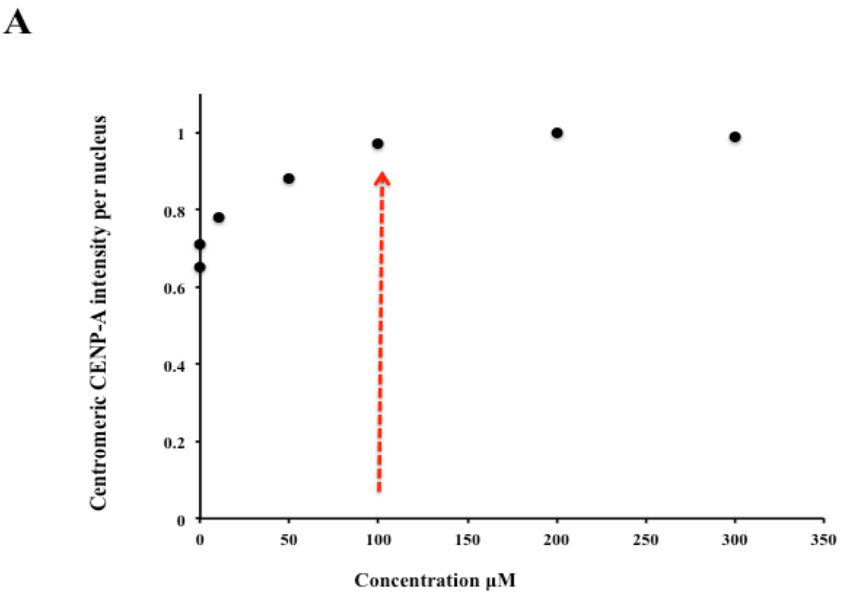


Figure 4.3 A. Dose response curve showing the optimum concentration for Cdk1/2 inhibition in *Drosophila* spermatocytes. *wild type* $y^{+}ry^{+}$ larval testes were dissected and briefly treated ex vivo with increasing concentrations of RO-3306 for 30 minutes. Testes were fixed and stained for CENP-A. CENP-A levels at S2 stage were quantified and plotted against the concentration of drug used. 100 µM (red arrow) showed the best response.

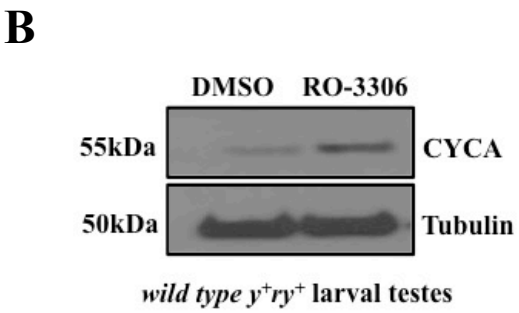


Figure 4.3 B. Western blot on *wild type* $y^{+}ry^{+}$ larval testes showing stabilisation of CYCA following Cdk1/2 inhibition compared to DMSO treated spermatocytes.

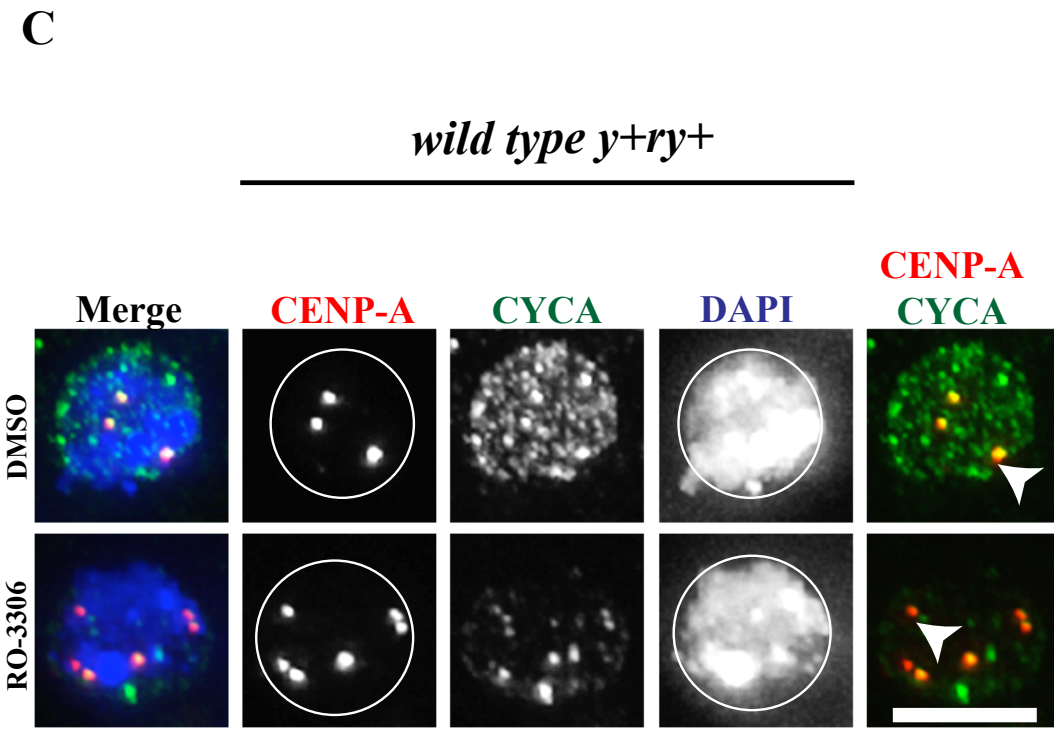


Figure 4.3 C. Cdk1/2 inhibition results in reduced CYCA at centromeres and increased CENP-A levels in early prophase I. *wild type* $y^{+}ry^{+}$ larval testes were dissected and briefly treated ex vivo with DMSO or 100 µM RO-3306 for 30 minutes. Testes were fixed and stained for CENP-A (red), CYCA (green) and DAPI (blue). Stage S2 of meiotic prophase I is shown. CYCA is enriched at centromeres in the DMSO control (white arrow). RO-3306 treated samples show reduced centromeric CYCA (white arrow) and increased CENP-A levels and foci (circle in CENP-A channel). Scale bar = 5 µm.

D

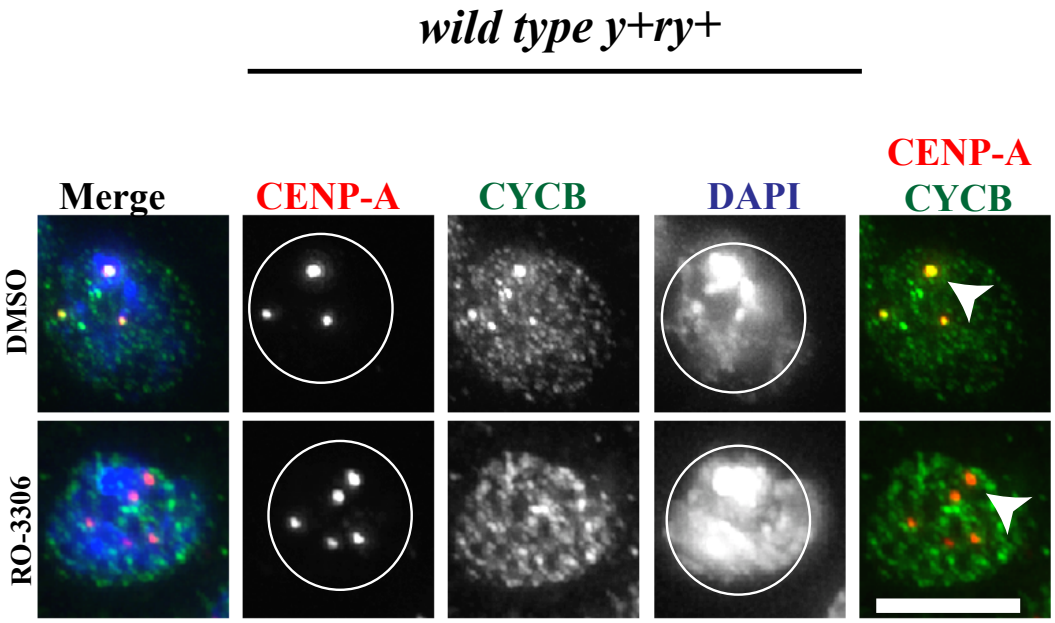


Figure 4.3 D. Cdk1/2 inhibition results in reduced CYCB from centromeres and increased CENP-A levels in early prophase I. *wild type* $y^{+}ry^{+}$ larval testes were dissected and briefly treated ex vivo with DMSO or 100 µM RO-3306 for 30 minutes. Testes were fixed and stained for CENP-A (red), CYCB (green) and DAPI (blue). Stage S2 of meiotic prophase I is shown. CYCB is enriched at centromeres in the DMSO control (white arrow) while CENP-A levels remain unaffected (circle in CENP-A channel). RO-3306 treated samples show reduced centromeric CYCB (white arrow) and increased CENP-A levels and foci (circle in CENP-A channel). Scale bar = 5 µm

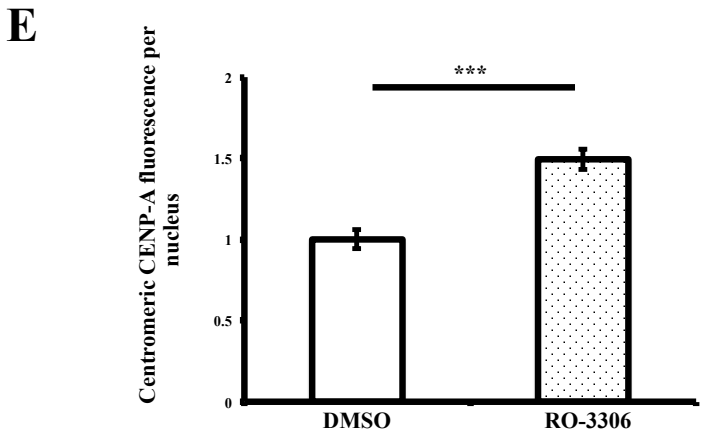


Figure 4.3 E. Quantification of centromeric CENP-A levels per nucleus following Cdk1/2 inhibition. A significant increase in CENP-A levels is observed in the RO-3306 treated samples compared to DMSO controls. Error bars = SEM, number of cells quantified = 25 per experiment n = 3, p value ≤ 0.001.

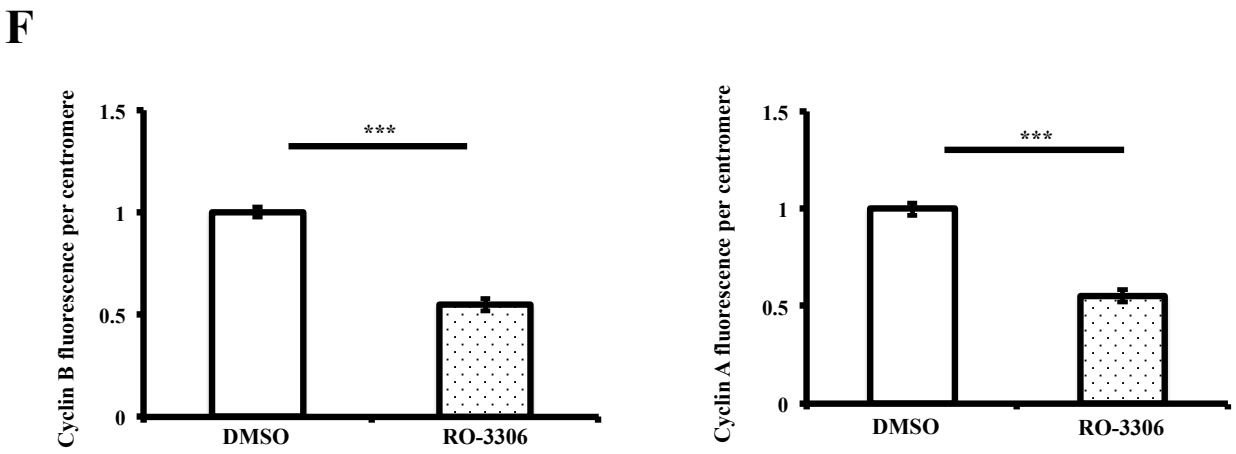


Figure 4.3 F. Quantification of centromeric CYCA or CYCB levels per centromere following Cdk1/2 inhibition. A significant decrease in CYCA or CYCB levels is observed in the RO-3306 treated samples compared to DMSO controls. Error bars = SEM, number of cells quantified = 30 per experiment, n = 3, p value ≤ 0.001

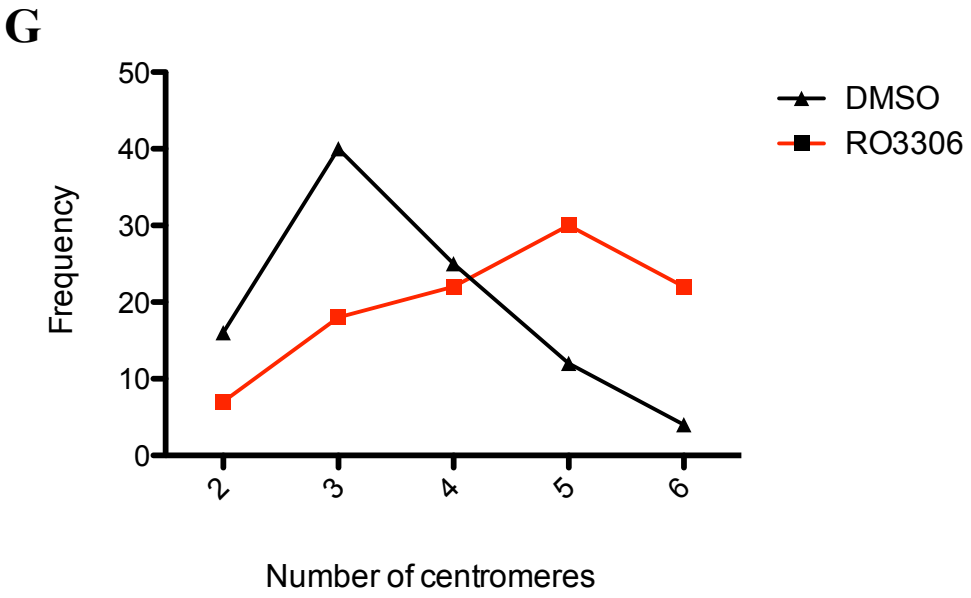


Figure 4.3 G. Quantification of number of CENP-A foci per nucleus. The average number of centromeric foci per nucleus in the control lies between 3 and 4 centromeres while the average number of centromeric foci per nucleus in spermatocytes treated with RO-3306 increases to an average of between 5 and 6 centromeres per nucleus, number of cells quantified = 30 per experiment, n = 3.

4.5. Testis specific Cyclin A or Cyclin B RNAi leads to CENP-A mistargeting

To reduce CYCA or CYCB levels at centromeres using a different method, we performed testis specific RNAi of either *cyclin A* or *cyclin B* using the GAL4/UAS system. Briefly, *UAS-cyclin A-RNAi* or *UAS-cyclin B-RNAi* lines were crossed to a line expressing GAL4 under the control of the *bam* promoter (*bam-Gal4*), which is repressed in germline stem cells and expressed in spermatogonia at the four-cell stage after completion of two mitotic divisions (McKearin & Spradling, 1990; Schulz et al., 2004). The RNAi efficiency was confirmed by immunostaining larval testes from F1 males with antibodies against CYCA or CYCB. In RNAi testes, levels of CYCA or CYCB were reduced compared to *bam-Gal4* control (figure 4.4 A), confirming that the RNAi was indeed efficient.

Next, we examined CENP-A localisation. Immunostaining of CYCA or CYCB depleted spermatocytes with antibodies against CENP-A revealed that CENP-A promiscuously incorporates throughout chromatin to non- centromeric sites in both CYCA and CYCB depleted spermatocytes (figure 4.4 B and C). CENP-A mis-incorporation was however more pronounced in the *cyclin A RNAi* particularly at S2 stage compared to the *cyclin B RNAi*. CYCA depleted spermatocytes however showed a more severe mislocalisation compared to CYCB depleted spermatocytes suggesting that early prophase I cells are more sensitive to CYCA depletion.

Levels of CENP-A particularly at non- centromeric regions were evidently increased in both CYCA or CYCB depleted spermatocytes in the IF analysis (figure 4.4 B and C) further supporting that CYCA or CYCB associated kinase activity limits and spatially restricts localisation of CENP-A to centromeric loci. Surprisingly, CENP-A centromeric localisation was unaffected in the CYCA or CYCB depleted spermatocytes. It is possible that CYCA or CYCB depletion did not interfere with centromeric CENP-A localisation possibly because at this stage, CYCA or CYCB may specifically function to restrict incorporation of CENP-A to specific centromeric loci. More still, centromeric CENP-A localisation may remain unaffected because of the existence of a template-governed mechanism where old CENP-A maintains centromere location or position during cell proliferation (Raychaudhuri et al., 2012). Quantification revealed that CENP-A fluorescence intensity in CYCA or CYCB

depleted spermatocytes was almost similar to control samples (figure 4.4 D). We speculate that centromeric CENP-A levels remain unchanged in the CYCA or CYCB RNAi because in these conditions, CAL1 is mislocalised (figure 5.2 B). Collectively our data suggest that CYCA or CYCB associated kinase activity is important for spatially restricting and limiting CENP-A incorporation into centromeric chromatin.

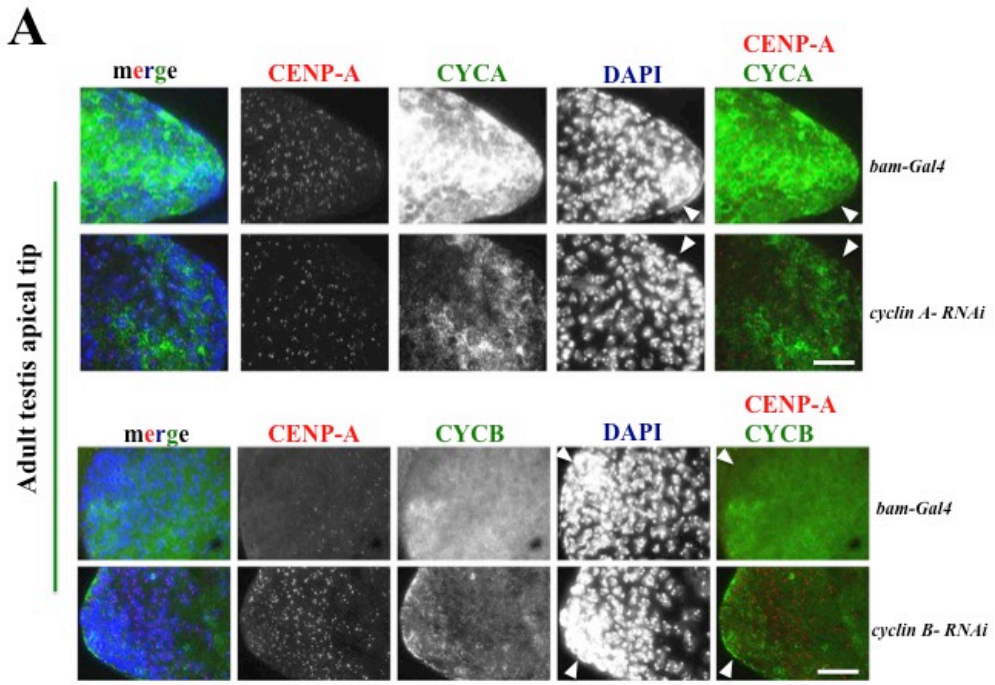


Figure 4.4. A. Confirmation of testis specific Cyclin A or Cyclin B RNAi (A) *bam-Gal4* virgin females were crossed to *UAS-cyclin A-RNAi* or *UAS-cyclin B-RNAi* males. Adult testes were dissected, fixed and stained for CENP-A (red), CYCA (green) or CYCB (green) and DNA (blue). The apical tip of the testes is shown (white arrows). CYCA and CYCB levels are reduced in knockdown spermatocytes compared to the controls. Scale bar = 20 μm

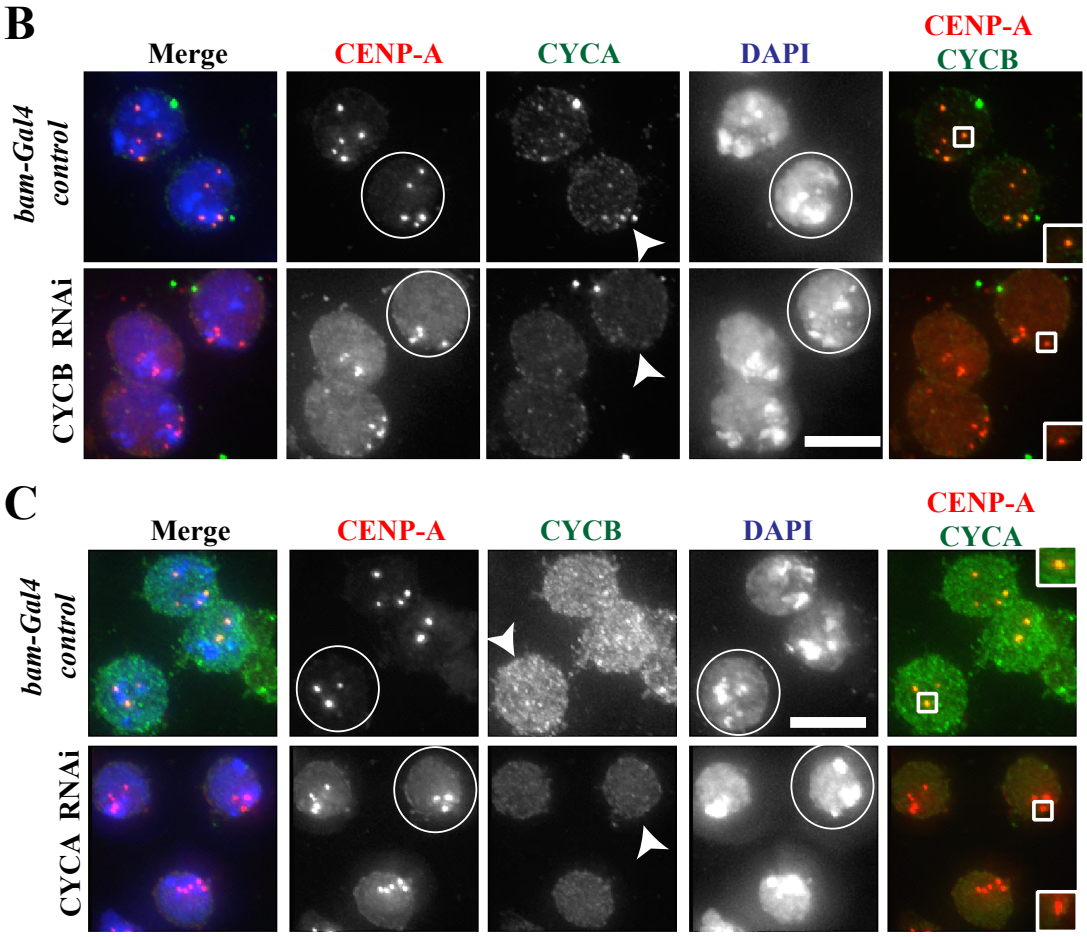


Figure 4.4. B and C. Testis specific Cyclin A or Cyclin B RNAi leads to promiscuous CENP-A localisation. *bam-Gal4* virgin females were crossed to *UAS-cyclin A-RNAi* or *UAS-cyclin B-RNAi* males. 3rd instar larval testes were dissected, fixed and stained for CENP-A (red), CYCA (green) or CYCB (green) and DNA (blue). CENP-A is visibly spread in the entire nucleus, (circles in CENP-A channel). CYCA and CYCB levels are reduced in knockdown spermatocytes compared to the controls (white arrows). Stage S2 of meiotic prophase I is shown. Scale bar = 5 μm

D

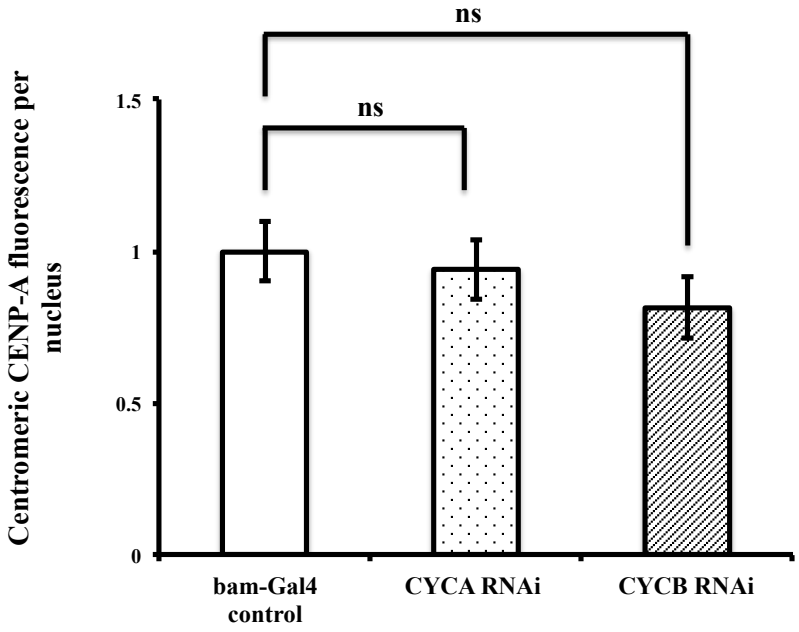


Figure 4.4 D. Quantification of centromeric CENP-A levels per nucleus following CYCA or CYCB RNAi. No significant centromeric increase following CYCA or CYCB knockdown is observed. Error bars = SEM, p value > 0.05, number of cells quantified = 40 per experiment, n=3.

4.6. Regulation of CENP-A assembly by Cdk1/2 requires the canonical assembly factors

We next determined whether regulation of CENP-A assembly by Cdk1/2 activity depended on the canonical CENP-A assembly machinery. CAL1 and CENP-C are essential for CENP-A assembly in both *Drosophila* mitosis and meiosis (Chen et al., 2014; Dunleavy et al., 2012; Erhardt et al., 2008; Kwenda et al., 2016; Raychaudhuri et al., 2012). To test whether CAL1 or CENP-C were in the pathway leading to increased CENP-A assembly following Cdk1/2 inhibition (figure 4.3 A), we performed testis specific RNAi of either *cal1 or cenp-C* using the GAL4/UAS system. Following CAL1 or CENP-C RNAi, we investigated the ability of CAL1 or CENP-C depleted spermatocytes treated with RO-3306 to 'promote' CENP-A assembly. Thus, *UAS-cal1-RNAi* or *UAS-cenp-C-RNAi* lines were crossed to a line expressing GAL4 under the control of the *bam* promoter (*bam-Gal4*), which is repressed in germline stem cells and expressed in spermatogonia at the four-cell stage. Larval testes from F1 males were subsequently treated with 100 μM RO-3306 for 30 minutes. The RNAi efficiency was confirmed by immunostaining spermatocytes with antibodies against CAL1 and CENP-C. In spermatocytes depleted of either *cal1* or *cenp-C*, levels of CAL1 or CENP-C were reduced respectively compared to the isogenic host strain for the RNAi library (GD isogenic control) and *bam-Gal4* driver control; (figure 4.5 A).

Analysis of CAL1 and CENP-C depleted spermatocytes treated with RO-3306 revealed that centromeric CENP-A levels were reduced compared to *bam-Gal4* and *GD* isogenic controls which displayed increased CENP-A levels upon Cdk1/2 inhibition (figure 4.5 B). Cdk1/2 inhibition, particularly in CAL1 depleted spermatocytes, had little effect on increasing CENP-A intensity compared to controls and CENP-C depleted spermatocytes. In contrast, although CENP-A intensity did not revert back to wild type levels following Cdk1/2 inhibition in CENP-C depleted spermatocytes, quantification of CENP-A fluorescence revealed a statistically significant accumulation in CENP-A intensity of about 20% in CENP-C depleted spermatocytes compared to CAL1 depleted spermatocytes which showed a fold change of 10% that was not statistically significant (figure 4.5 C). Strikingly similar to previous observations, spermatocytes treated with RO-3306 also displayed an

increase in the number of centromeric foci visible, suggesting a loss in cohesion, a phenotype that we have not fully characterised in this study. Taken together, these data indicate that Cdk1/2 mediates CENP-A regulation possibly through CAL1.

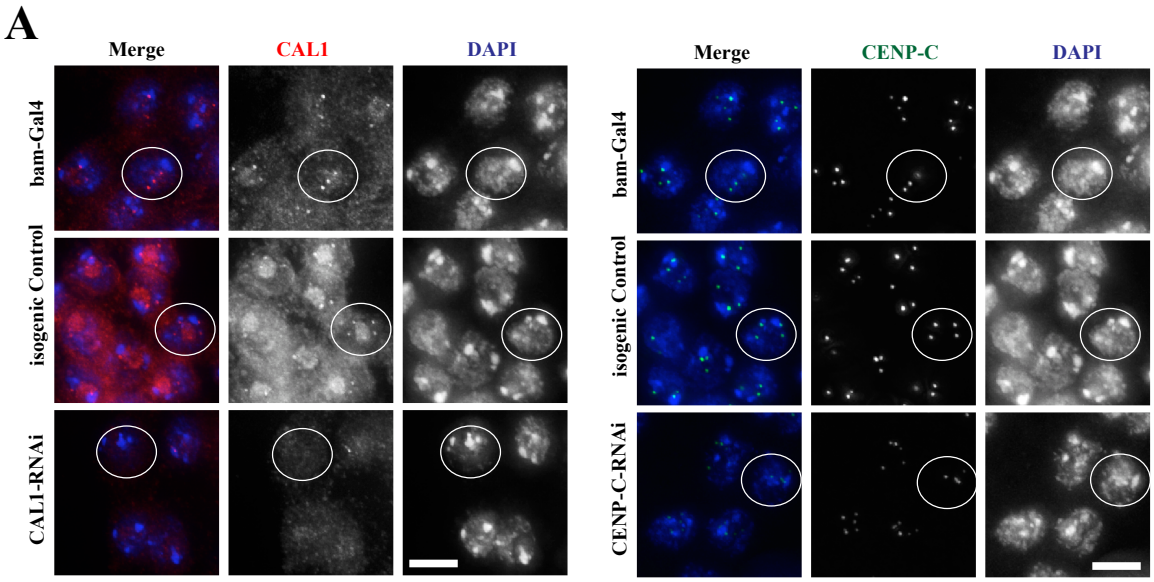


Figure 4.5 A. Confirmation of CAL1 and CENP-C RNAi. *bam-Gal4* virgin females were crossed with *UAS-cal1-RNAi* or *UAS-cenp-C-RNAi* males. 3rd instar larval testes from the F1 progeny were dissected fixed and stained for CAL1 (red), CENP-C (green) and DNA (blue). CAL1 and CENP-C levels were reduced in the CAL1 and CENP-C RNAi compared to the *bam-Gal4* control and the GD isogenic host strain for the RNAi library (white circles). Scale bar = 5 μm.

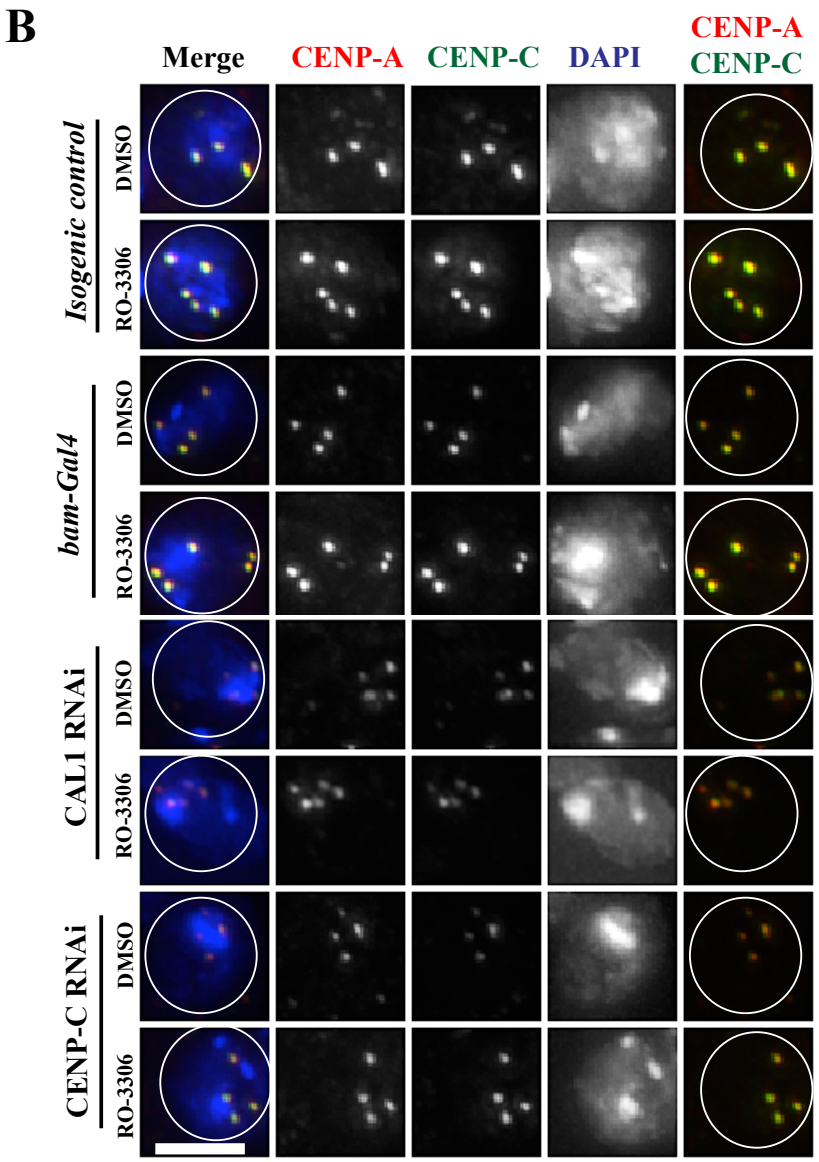


Figure 4.5 B. Negative regulation of CENP-A assembly by Cdk1/2 requires the canonical CENP-A assembly factors. 3rd instar larval testes from F1 progeny of either CAL1 RNAi or CENP-C RNAi were dissected and briefly treated ex vivo with DMSO or RO-3306 fixed and stained for CENP-A (red) CENP-C (green) and DNA (blue). *bam-Gal4* and *GD isogenic* controls showed increased CENP-A levels following treatment with RO-3306. *UAS-cal1-RNAi* spermatocytes showed the least response to Cdk1/2 inhibition. Circles represent the outline of the nucleus. *Scale bar* = 5 μm

C

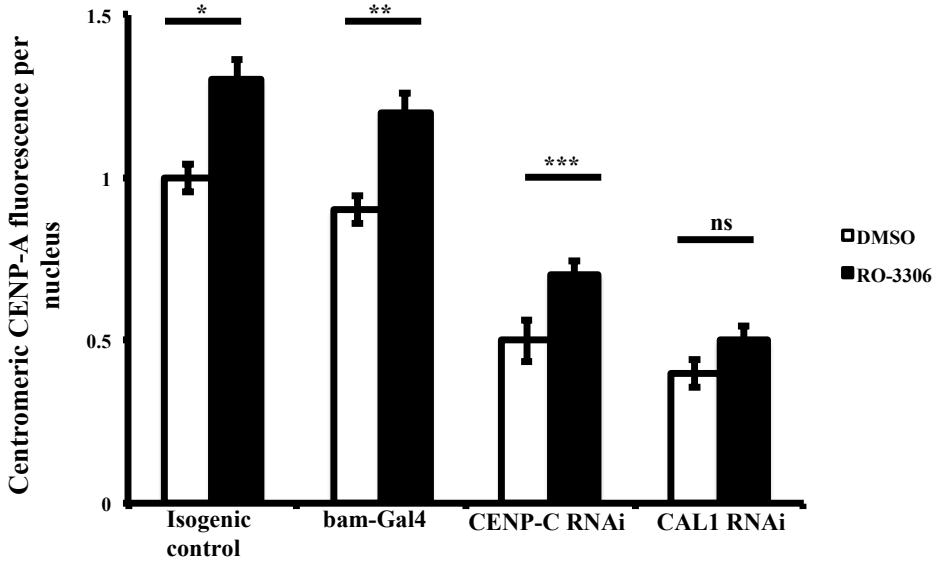


Figure 4.5 C. Quantification of centromeric CENP-A levels per nucleus following Cdk1/2 inhibition. A significant increase of about 30% in centromeric CENP-A fluorescence intensity is observed in RO-3306 treated spermatocytes in the *GD isogenic* control $p \leq 0.05$. *bam-Gal4* control shows a significant increase of ~ 20% $p \leq 0.01$ and CENP-C depleted spermatocytes reveal an increase in CENP-A intensity of ~ 20%, $p \leq 0.001$. The increase observed in the CAL1-RNAi of ~10% is not significant $p > 0.05$. Error bars = SEM, number of cells quantified = 25 per experiment, n=3.

4.7 Discussion

The requirement for exit from mitosis to trigger CENP-A assembly is broadly conserved across species (Bernad et al., 2011; Hemmerich et al., 2008; Jansen et al., 2007; Moree et al., 2011; Schuh et al., 2007) where loss of Cdk activity releases the CENP-A assembly machinery from inhibitory phosphorylation allowing CENP-A assembly to ensue (Müller et al., 2014; Silva et al., 2012; Stankovic et al., 2017). Intriguingly, meiotic CENP-A assembly occurs gradually over a period of days, at a time of high Cdk activity. How CENP-A assembly is regulated during high kinase activity and over such extended time periods in meiosis is currently unknown. Here we provide first insight into the regulation of meiotic CENP-A assembly via centromere localised CYCA and CYCB associated kinase activity.

We show that CYCA or CYCB associates and dissociates from centromeres in *Drosophila* spermatocytes, at prophase I of meiosis I during the first phase of CENP-A assembly (Dunleavy et al., 2012; Raychaudhuri et al., 2012). We show that CYCA and CYCB associate with centromeres at the beginning of prophase I, stages S1-S3, and dissociate from centromeres at later stages of prophase I, S4-S6. Intriguingly, loss of CYCA and CYCB from centromeres coincides with the termination of the first phase of CENP-A assembly, (Dunleavy et al., 2012; Raychaudhuri et al., 2012) and with the removal of the CENP-A assembly factor CAL1 (Dunleavy et al., 2012; Raychaudhuri et al., 2012), suggesting that centromere localised CYCA or CYCB may functionally regulate CENP-A assembly factors. Both CYCA and CYCB appear to share similar localisation dynamics at the centromere, suggesting a possible redundancy in function. However, although the centromere localisation of both CYCA and CYCB appeared similar, the overall staining pattern throughout the nucleus was different. CYCA was more nuclear and showed more punctuated foci in comparison to CYCB. This difference could be attributed to the differences of these cyclin proteins or to antibody specificity

Although cyclins have been localised to meiotic centromeres (Bentley et al., 2007; Nickerson et al., 2007; Touati et al., 2012), neither CYCA nor CYCB have been implicated in centromere assembly or maintenance in meiosis. In *Drosophila* mitosis however, CYCA associated kinase activity has been shown to be important for

centromere assembly (Erhardt et al., 2008). Depletion of CYCA results in CENP-A and CENP-C mislocalisation as well as significant reduction in CENP-A protein levels (Erhardt et al., 2008; Mellone et al., 2011). Using a heat inducible system to overexpress CYCA or CYCB, we report for the first time that the amount of centromere localised CYCA or CYCB is critical for meiotic CENP-A assembly as increased levels of centromeric CYCA or CYCB leads to a dramatic reduction of CENP-A levels to almost half. Consistently, using a pharmacologically based approach, we show that brief inhibition of Cdk1/2 promotes increased centromeric CENP-A recruitment. However because the drug concentration used in this study, 100 μM is very high, it is possible that the excess incorporation of CENP-A observed may be due to off-target effects. Nonetheless, these findings are indicative that offsetting kinase activity impacts on centromeric CENP-A recruitment. Interestingly, we could correlate increased centromeric CENP-A incorporation to a reduction in CYCA or CYCB at centromeres following Cdk1/2 inhibition. We therefore propose that centromere localised CYCA or CYCB associated kinase activity 'limits' meiotic CENP-A assembly over a long time period possibly via local inhibition. Consistently, depletion of CYCA or CYCB from spermatocytes promotes promiscuous CENP-A incorporation to non-centromeric regions. This argues that meiotic CENP-A assembly is coupled to high kinase activity in order to spatially restrict and limit CENP-A incorporation specifically into centromeric loci. This spatial restriction may represent an important mechanism to maintain a proper centromere size and architecture.

In addition, we show that negative regulation of CENP-A assembly by Cdk1/2 requires the CENP-A assembly factor CAL1. CAL1 depleted spermatocytes fail to promote CENP-A incorporation at centromeres following Cdk1/2 inhibition in comparison to controls and CENP-C depleted spermatocytes. Although CENP-A levels were reduced in CENP-C depleted spermatocytes, CAL1 depleted spermatocytes displayed a more pronounced effect, failing to significantly promote centromeric CENP-A incorporation upon Cdk1/2 inhibition. From this, we propose that CAL1 could be a direct target for Cdk1/2 regulation. We conclude that in this pathway, CAL1 may be rate limiting for CENP-A assembly.

Taken together, our data implicates the major drivers of cell cycle progression in guaranteeing gradual, limited and stable incorporation of CENP-A at specific

centromeric loci. In addition, we implicate the *Drosophila* CENP-A assembly factor CAL1 in cell cycle mediated regulation of CENP-A assembly in *Drosophila* male meiosis.

Chapter 5. CAL1 as a potential substrate for CYCA or CYCB associated kinase activity

5.1. Introduction

In recent years it has been demonstrated that key proteins necessary for CENP-A deposition in mammals, HJURP and Mis18BP1 are phosphoproteins regulated by kinase activity to temporally restrict and isolate CENP-A deposition from the deposition of canonical H3 in S phase (reviewed in McKinley & Cheeseman, 2016). Ongoing work indicates that Cdk activity negatively regulates CENP-A incorporation at numerous steps. In mammalian cells, Cdks phosphorylate Mis18BP1 to reduce its centromere localisation (Silva et al., 2012), and to prevent the recruitment of the Mis18α and Mis18β subunits (reviewed in McKinley & Cheeseman, 2016) outside of G1 phase. In addition Cdk phosphorylation of HJURP disrupts its localisation to centromeres (Müller et al., 2014), while Cdk phosphorylation of CENP-A itself on Ser68 has been reported to inhibit the CENP-A/HJURP interaction (Yu et al., 2015), however the role of Ser68 in CENP-A assembly is controversial (Fachinetti et al., 2017). By passing the Cdk regulation of the Mis18 complex and HJURP results in CENP-A deposition outside of the canonical G1 loading phase. Thus the timing and localisation of these assembly factors can be manipulated by controlling Cdk activity (Müller et al., 2014; Silva et al., 2012; Stankovic et al., 2017). Collectively these studies have led to a model whereby temporal restriction of CENP-A assembly to late M early G1 phases is achieved by Cdk-dependent phosphorylation of key deposition factors.

We therefore investigated whether CAL1, a key CENP-A assembly factor in *Drosophila* is a Cdk regulated substrate in meiosis. The transient association and dissociation of CAL1 from centromeres supports the idea that CAL1 is actively regulated. Notably it has been reported that centromeric CAL1 levels are 40 times lower than those of CENP-A or CENP-C (Schittenhelm et al., 2010) and that CAL1 is less stably associated with centromeres compared to CENP-A and CENP-C, decreasing by 66% after one cell cycle (Mellone et al., 2011). These reports indicate

that CAL1 displays a high turnover rate. In addition, a previous phosphoproteome screen on *Drosophila* Kc152 tissue culture cells found that CAL1 is phosphorylated on at least 3 positions, 1 of these corresponds to a Cdk consensus site (Bodenmiller et al., 2007).

In this chapter we have made advances in understanding how CAL1 is regulated in a cell cycle dependent manner. We have also uncovered vital clues to explain why the timing of CAL1 assembly is coupled to high kinase activity.

5.2. CAL1 and CYCA display similar localisation dynamics at the centromere

In previous years, it has been demonstrated that CAL1 transiently associates and dissociates from centromeres. In meiosis CAL1 levels at centromeres are gradually reduced during prophase I through to the end of spermatogenesis (Dunleavy et al., 2012; Raychaudhuri et al., 2012). Using immunofluorescence microscopy and live cell imaging, CAL1-GFP foci were observed to be enriched at centromeres in early stages of prophase I (S1-S3). By S5, CAL1-GFP foci at centromeres were gradually reduced (Dunleavy et al., 2012; Raychaudhuri et al., 2012). Our initial analysis of CYCA and CYCB localisation, (figure 4.1 A) suggest that both CYCA and CYCB share similar localisation dynamics at centromeres. Association of either CYCA or CYCB with centromeres is predominant in S1-S3 stages and similar to CAL1, as prophase I progresses, levels of either CYCA or CYCB are dramatically reduced at centromeres.

We therefore sought to determine whether CYCA uncouples from centromeres at the same time as CAL1 at prophase I S4-S6 stages. We dissected and fixed larval testes from transgenic flies expressing CAL1-GFP (Schittenhelm et al., 2010) and immunostained spermatocytes with antibodies against CYCA. In line with a previous report (Dunleavy et al., 2012), CAL1-GFP localised at centromeres in early stages of prophase I (S1-S3) and also displayed nuclear staining associated with the nucleolus where centromeres cluster (figure 5.1). Also in line with previous reports, CAL1-GFP foci at centromeres gradually reduced in intensity by S4 stage and remained almost undetectable by S5 stage (figure 5.1). Analysis of CYCA localisation showed that CAL1-GFP and CYCA colocalised at centromeres in early stages of prophase I (S1-S3) (figure 5.1). Interestingly, centromere localised CYCA, like CAL1, was also dramatically reduced as prophase I progressed. By S4 stage CYCA was almost undetectable at centromeres (figure 5.1). This localisation of both CAL1 and CYCA at meiotic centromeres coincides with the onset of CENP-A assembly (S1), and the removal of both CAL1 and CYCA from centromeres is concomitant with the termination of the first phase of CENP-A deposition (Dunleavy et al., 2012; Raychaudhuri et al., 2012). It is therefore tempting to speculate that CYCA and CYCB may be directly regulating the first phase of CENP-A assembly via CAL1. We

conclude that CAL1 and CYCA share similar localisation dynamics at the centromere.

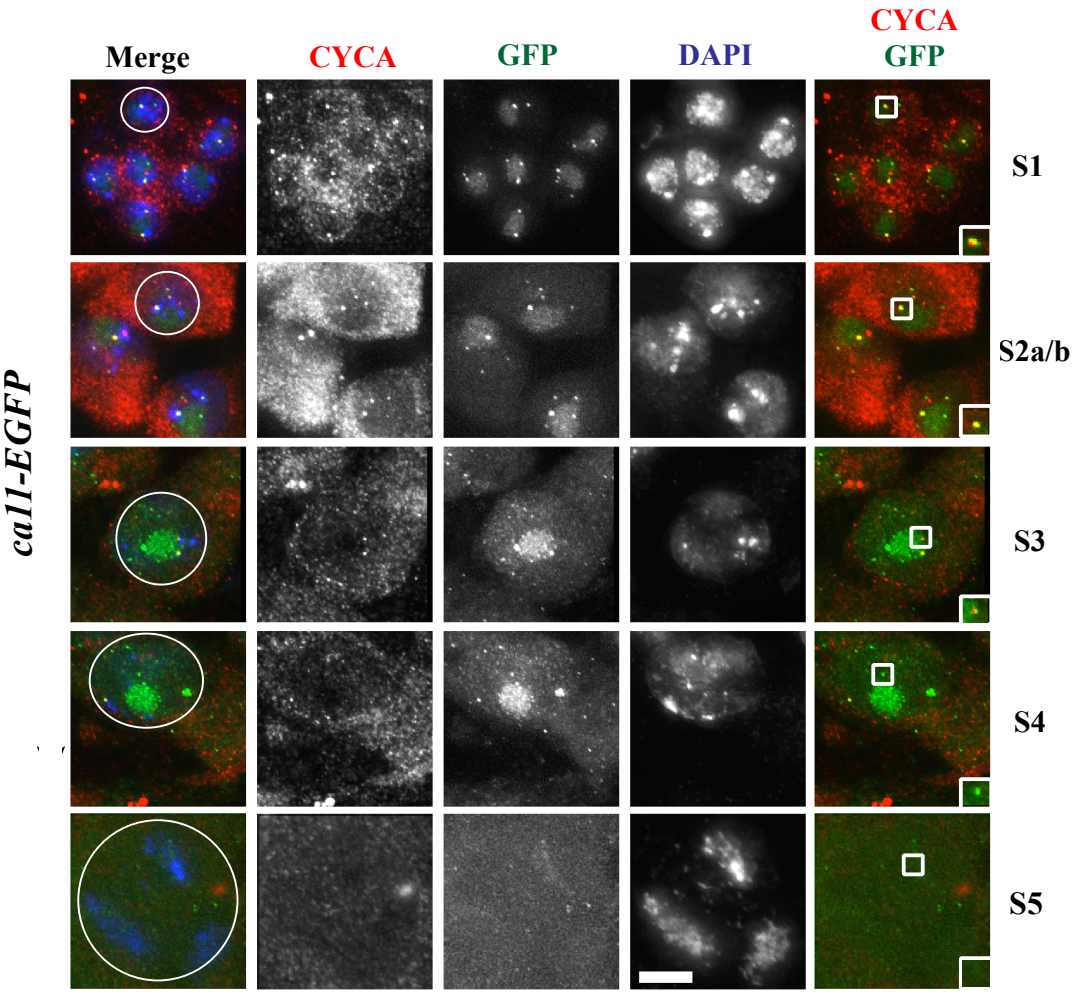


Figure 5.1. CAL1 and CYCA display similar localisation dynamics at meiotic centromeres. CAL1-GFP larval testes were dissected, fixed and stained with anti-CYCA antibody (red) and DAPI (blue). The GFP signal was directly visualised. Both CYCA and CAL1 associate with centromeres in the early stages of prophase I (S1-S3 inset) and dissociate from centromeres as prophase I progresses (S5 inset). The circles indicate the outline of the nucleus. Scale bar = 5 µm.

5.3. Cyclin A or Cyclin B RNAi leads to gross mislocalisation of CAL1

Following our findings that CAL1 and CYCA share similar localisation dynamics at centromeres, we next investigated the functionality behind this association. We therefore analysed CAL1 localisation following testes specific RNAi depletion of *cyclin A* or *cyclin B* using the GAL4/UAS system. *bam-Gal4* virgin females were crossed to *UAS-cyclin A-RNAi* or *UAS-cyclin B-RNAi* males. Larval testes form F1 males were dissected and knockdown was confirmed by immunofluorescence staining with anti-CYCA or anti-CYCB antibodies. Analysis of the apical end of the testes (figure 4.4 A), revealed that controls displayed normal staining of either CYCA or CYCB while CYCA or CYCB depleted spermatocytes displayed reduced total protein levels (figure 4.4 A).

Immunofluorescence staining of spermatocytes depleted of either *cyclin A* or *cyclin B* with antibodies against CAL1 and CENP-C revealed a gross mislocalisation of CAL1 to the cytoplasmic compartment (figure 5.2) (white circles). This mislocalisation was strongly evident at the S2 stage of prophase I (figure 5.2), reinforcing the sensitivity of this stage to CYCA or CYCB disruption as observed in Chapter 4. In comparison, controls showed normal staining of CAL1 at both centromeric and nucleolar pools (figure 5.2). In CYCA depleted spermatocytes however, centromeric localisation of CAL1 appeared to be impaired, data not quantified, (figure 5.2 inset) and a diffuse nuclear distribution of CAL1 (figure 5.2), could also be observed. However because centromeric CENP-C levels in the CYCA or CYCB depleted spermatocytes appeared to be reduced, data not quantified, it is unclear whether centromeric localisation of CAL1 in these spermatocytes is truly impaired or merely difficult to visualise.

Because CAL1 can form a bridge between CENP-A and CENP-C, we also closely analysed CENP-C localisation following *cyclin A* or *cyclin B* RNAi. Interestingly, CENP-C still localised to centromeres albeit at reduced levels. We could not detect any increase in CENP-C or mis-incorporation as observed for both CENP-A and CAL1. This indicates that regulation of CAL1 and CENP-A may be coupled while that of CENP-C is not. Collectively, our data suggest that CYCA or CYCB associated kinase activity is vital for spatial and temporal localisation of CAL1. We conclude

that CAL1 is grossly mis-targeted if CYCA or CYCB meiotic Cdk activity fails to accumulate at prophase I.

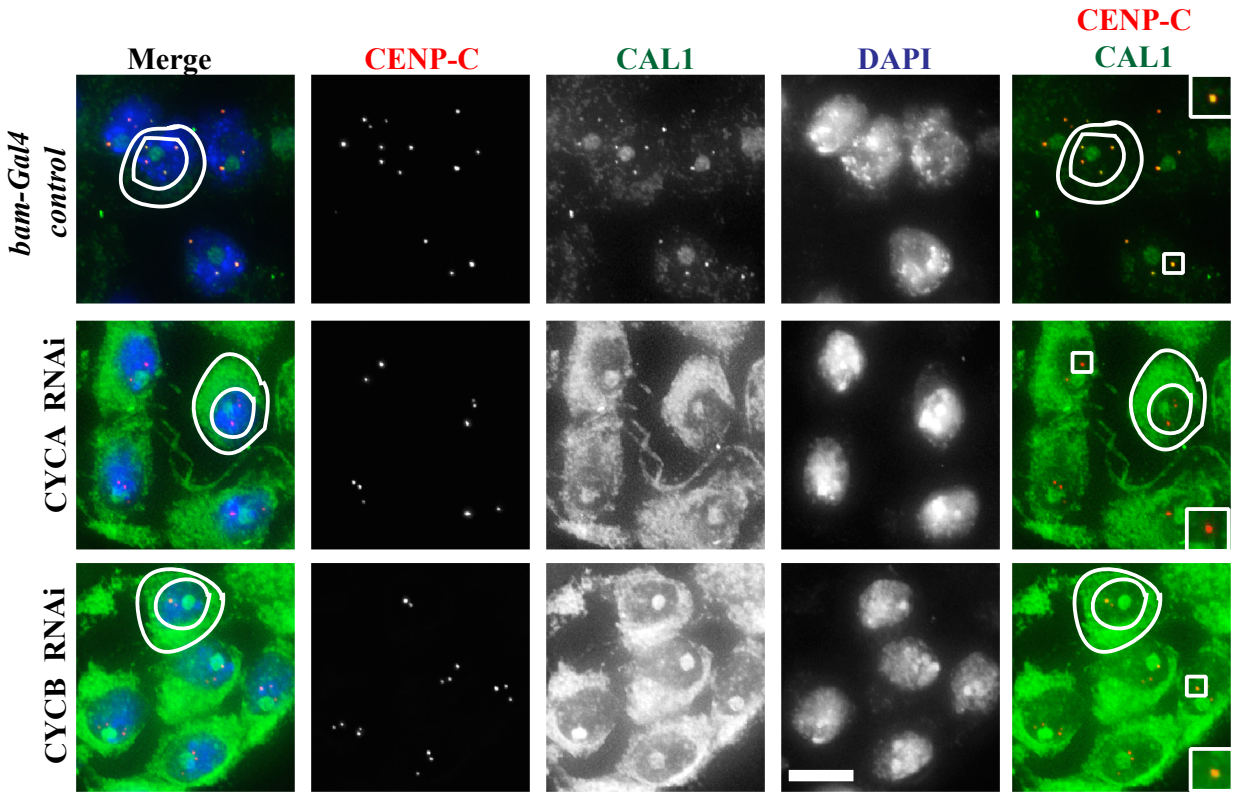


Figure 5.2. Cyclin A or Cyclin B RNAi leads to gross mislocalisation of CAL1. *bam-Gal4* virgin females were crossed to *UAS-cyclin A-RNAi* or *UAS-cyclin B-RNAi* males. 3rd instar larval testes from the F1 progeny were dissected, fixed and stained for CENP-C (red) and CAL1 (green). CAL1 is largely mislocalised in the cytoplasm. Inner circle marks the nuclear membrane, outer circle marks the cell membrane . CAL1 is almost undetectable at centromeres in the CYCA RNAi (inset) compared to CYCB RNAi and controls (inset). CENP-C (red) can still localise to centromeres Scale bar = 5 μm.

5.4. CAL1 Serine157 limits CENP-A assembly in *Drosophila* Schneider (S2) tissue culture cells

To determine whether CAL1 is a centromeric substrate for Cdk activity we turned to a phosphoproteome database published in 2007 (Bodenmiller et al., 2007), and mapped 3 potential phosphorylation sites. Serine 157 in the N terminus (figure 5.3 A) (blue box) followed the canonical consensus site for Cdks (S/T) PX (K/R) as previously described (Hagopian et al., 2001; Holmes & Solomon, 1996). Using site directed mutagenesis; we mutated this residue to a non- phosphorylatable mutant, expressed with a localisation and affinity purification (LAP) tag, LAP-CAL1^{S157A} (figure 5.3 B) and assessed the efficiency of CAL1 and CENP-A incorporation at centromeres. Success of the site directed mutagenesis was confirmed by sequencing (figure 5.3 B). Thus, LAP-CAL1 (control) and LAP-CAL1^{S157A} constructs both expressed from the constitutive *copia* promoter were transiently transfected into *Drosophila* Schneider S2 tissue culture cells. Following 48 hours transfection, cells were fixed and immunostained with antibodies against CENP-A while the GFP signal was directly visualised.

Strikingly in comparison to the wild type control, overexpression of the non-phosphorylatable mutant, LAP-CAL1^{S157A}, resulted in an increase in CENP-A recruited to centromeric loci in these mitotic cells (figure 5.3 C). This phenotype corroborates our small molecule inhibitor experiments in meiotic cells where Cdk inhibition results in excess CENP-A incorporation at centromeres (figure 4.3 A). We however do not exclude the possibility that the observed increase in centromeric CENP-A following transient transfection may reflect differences in the expression levels of the plasmids used or may arise from non-uniformity of the expression pattern or poor transfection efficiency. These data however suggest that CAL1 may be regulated similarly in both mitosis and meiosis via Cdk activity. Interestingly, CAL1 is first detected at centromeres in mitotic cells during prophase (Mellone et al., 2011), a time of high kinase activity. Quantification of CENP-A fluorescence intensity per nucleus (figure 5.3 D) suggests that CAL1 Serine 157 phosphorylation provides a signal that promotes CAL1 turnover. Taken together, our data suggest that

CAL1 Serine 157 embedded in the N terminus is an essential negative regulator of CAL1 and CENP-A assembly.

A

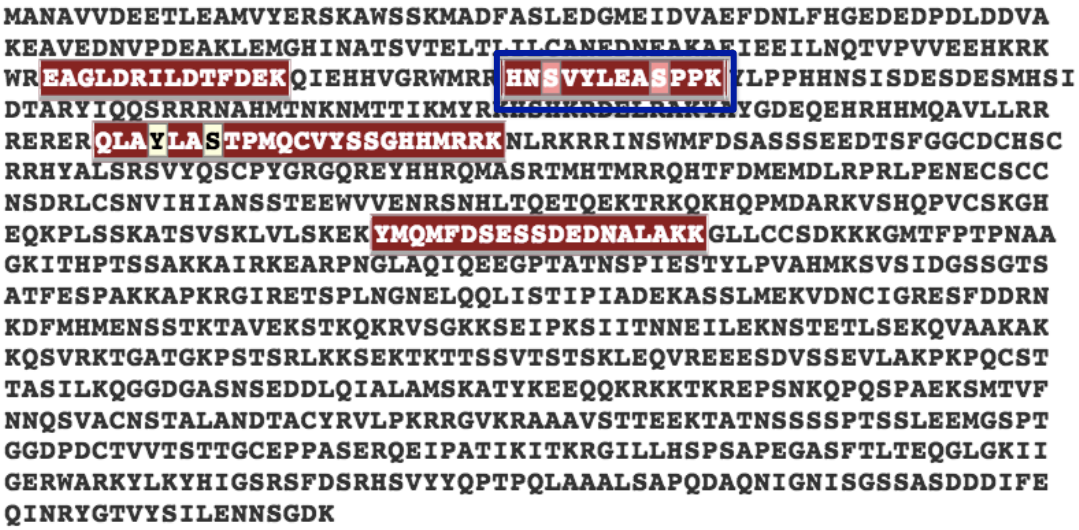


Figure 5.3 A. Map of potential phosphorylation sites on CAL1. CAL1 possesses 3 potential phosphorylation sites, one of these sites, Serine 157 in the N terminus, (blue box) corresponds to a Cdk1/2 consensus site. This region likely interacts with CENP-A (Schittenhelm et al., 2012)

B

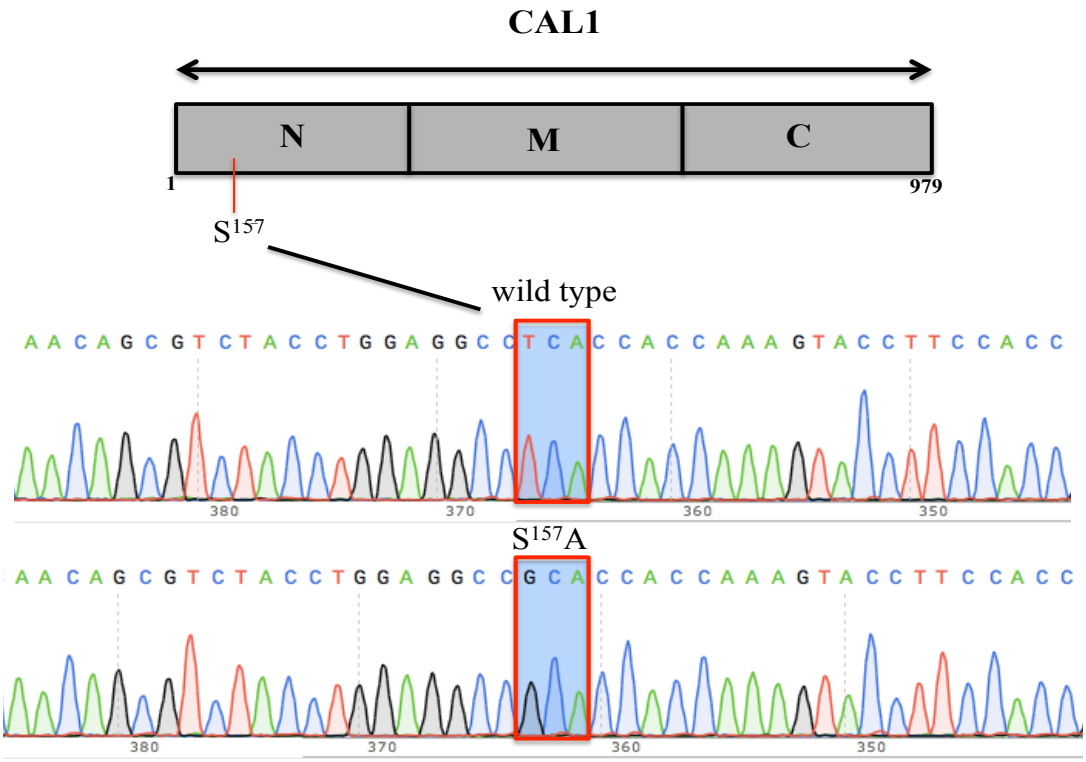


Figure 5.3 B. Schematic of CAL1 showing the location of Serine 157 in the N terminus. The inserts directly represent a screenshot of the sequencing results confirming the mutation.

C

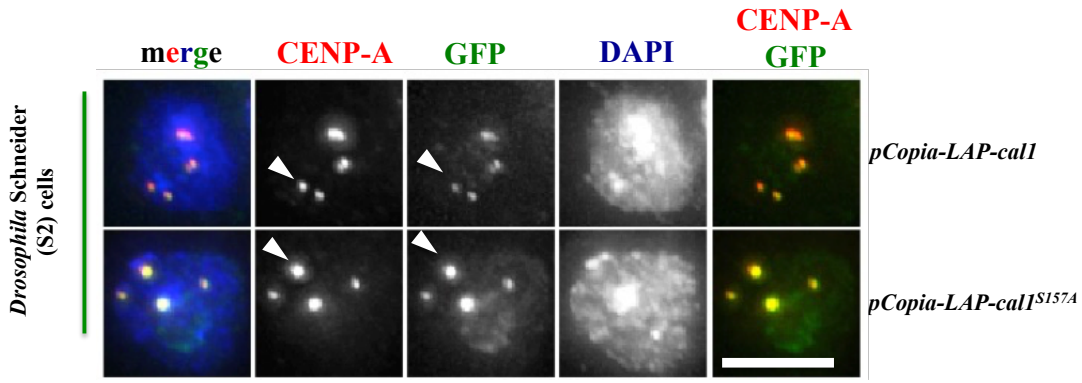


Figure 5.3 C. Phosphorylation of CAL1 Serine 157 limits CENP-A levels in *Drosophila* Schneider (S2) cells. *Drosophila* Schneider S2 cells were transiently transfected with LAP-CAL1 (control) or LAP-CAL1^{S157A} fixed and immunostained with anti-CENP-A (red) and DAPI (blue). The GFP signal (green) was directly visualised. CENP-A and GFP is increased in LAP-CAL1^{S157A} compared to the LAP-CAL1 control (arrows) scale bar = 5 µm.

D

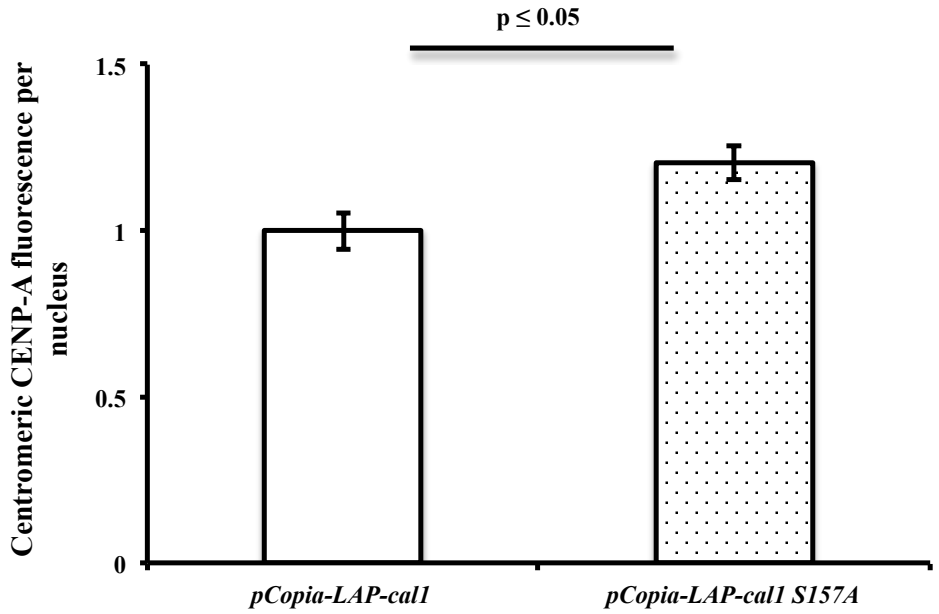


Figure 5.3 D. Quantification of centromeric CENP-A fluorescence per nucleus following transfection of S2 cells with LAP-CAL1 or LAP-CAL1^{S157A} constructs. Centromeric CENP-A levels are increased in the LAP-CAL1^{S157A} mutant compared to the LAP-CAL1 control, p value ≤ 0.05. Error bars = SEM, number of cells quantified = 40 per experiment, n = 3.

5.5. Inhibition of the 26S proteasome and Cdk1/2 reveal a potential role for the proteasome and phosphorylation in facilitating CAL1 turnover

Next we speculated the mechanism by which the phosphorylation of CAL1 at Serine 157 might dynamically regulate CAL1 levels. In recent years, proteolysis has been shown to facilitate formation of a single centromere by degrading non-centromeric CENP-A in yeast and flies (Collins et al., 2004; Moreno-Moreno et al., 2006). We therefore determined if CAL1 is a potential substrate of the 26S proteasome using MG132, a 26S proteasome inhibitor, which is known to be effective in insect cells (Lundgren et al., 2005; Muro et al., 2002). Larval testes from transgenic flies expressing GFP-tagged CAL1, *gcal1-EGFP* (Schittenhelm et al., 2010), were dissected and briefly treated with 50 μM MG132 for 30 minutes, fixed and stained with DAPI to visualise the DNA.

Strikingly and although not quantified, spermatocytes treated with MG132 displayed an accumulation of CAL1-GFP in the nucleolus and at centromeres compared to controls (figure 5.4 A). These observations suggest that proteasome mediated degradation contributes to 'limit' levels of available CAL1 at both nucleolar and centromeric pools. Interestingly western blotting on protein extracts from larval testes treated with a Cdk1/2 inhibitor and immunoblotting with antibodies against CAL1 and tubulin as a loading control, reveal an increase in total CAL1 levels upon Cdk1/2 inhibition (figure 5.4 B). These data suggest the importance of CAL1 phosphorylation as a signal that promotes its degradation. Interestingly and although not quantified, YFP-CENP-C spermatocytes, treated with 50 μM MG132 displayed no visible changes in YFP-CENP-C abundance in comparison to CAL1-GFP spermatocytes (figure 5.4 A right). This suggests that CAL1 is specifically recognised and degraded by the proteasome while CENP-C is not, at least at the S2 stage.

We conclude that CAL1 is actively regulated via phosphorylation-mediated degradation during cell cycle progression to restrict and facilitate accurate targeting of CENP-A as well as limit amounts of CENP-A deposited into centromeric chromatin.

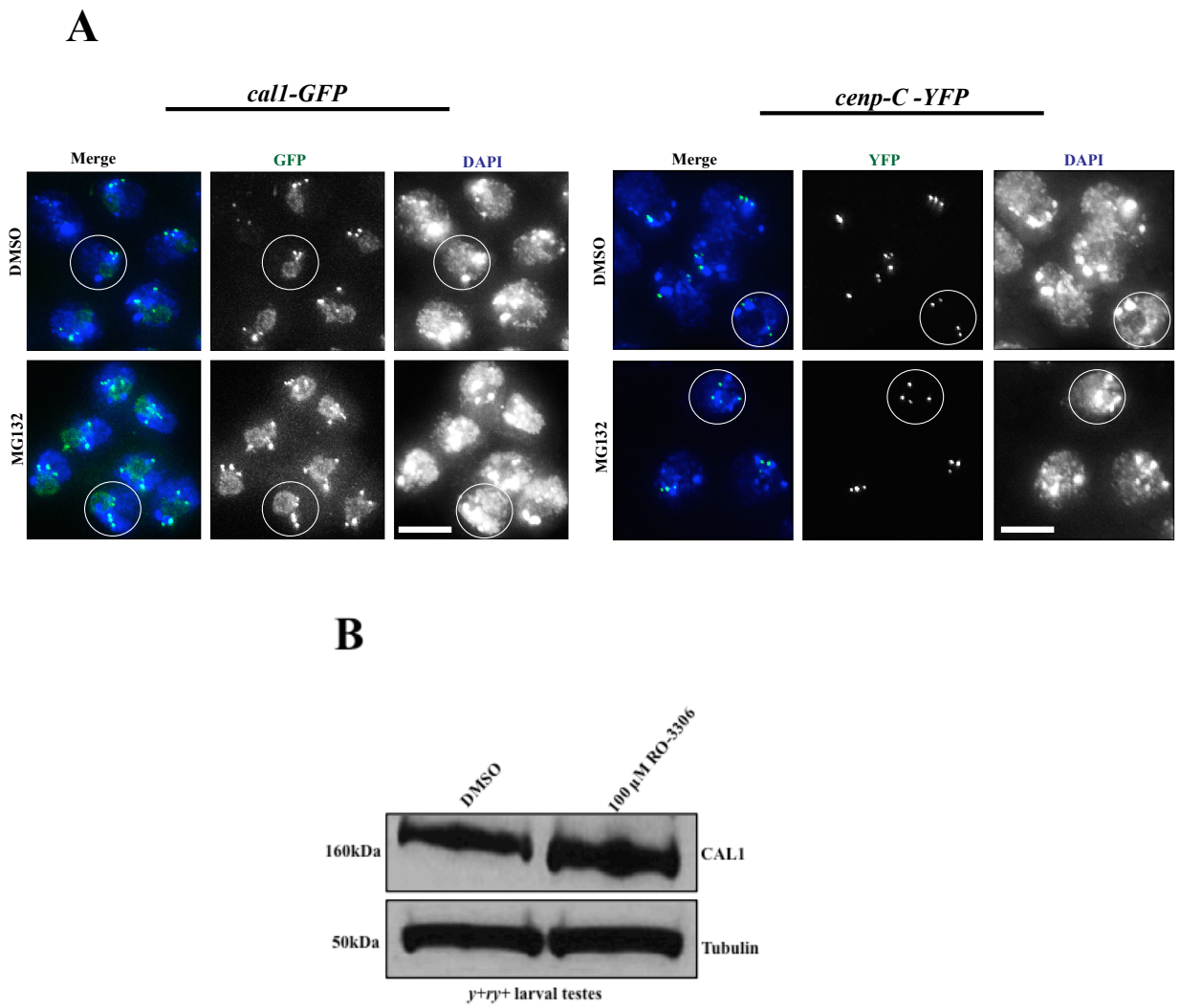


Figure 5.4 A and B. Inhibition of the 26S proteasome and Cdk1/2 reveal a potential role for the proteasome and phosphorylation in facilitating CAL1 turnover. (A) Larval testes from flies expressing CAL1-GFP or CENP-C-YFP were dissected and briefly treated ex vivo with 50 µM MG132 for 30 minutes. Testes were fixed and stained for DAPI to visualise DNA. The GFP signal was directly visualised. Stage S2 of meiotic prophase I is shown. Proteasomal inhibition results in accumulation of nucleolar and centromeric CAL1-GFP (circles in GFP channel). CENP-C levels at centromeres however remains unaffected (circles in YFP channel). Scale bar = 5 µm, n = 3 **(B)** Western blot on *wild type* y^+ry^+ larval testes showing stabilisation of CAL1 following Cdk1/2 inhibition compared to DMSO treated control.

5.6 Discussion

Our data so far implicates the major drivers of cell cycle progression in guaranteeing gradual, limited and stable incorporation of CENP-A at centromeric loci. In this chapter, we have further uncovered a potential Cdk substrate and the mechanism of CENP-A mistargeting in *Drosophila* spermatocytes. We show that CAL1 and CYCA display similar localisation dynamics at the centromere, with both CAL1 and CYCA coupling to centromeres in the early stages of prophase I (S1-S2b) and uncoupling from centromeres by S4 stage. We show that in the absence of CYCA or CYCB, CAL1 is grossly mislocalised in the cytoplasmic compartment, suggesting that overlapping CYCA or CYCB associated kinase activity may regulate CAL1 spatial localisation.

Notably, CAL1 contains a Cdk1/2 consensus site within the N terminus, the region known to interact with CENP-A. Site directed mutagenesis of a CAL1 at Serine 157 shows that regulation of CAL1 by CYCA or CYCB associated kinase activity is made possible via this residue in *Drosophila* mitotic tissue cultured cells. In the absence of CAL1 phosphorylation at Serine 157, CENP-A levels at centromeres are increased. Although this data corroborates our small molecule inhibitor study, (figure 4.3 C and D), we do not exclude the possibility that the observed increase in CENP-A intensity following expression of LAP-CAL1^{S157A} could arise from cell to cell differences in the uptake and expression level of the plasmid. It is also important to note that although CAL1 Serine 157 may be an important CAL1 regulatory phospho residue, other predicted phosphorylation sites within the CAL1 peptide sequence also exist. Using in silico approach, GPS 3.0 for prediction of posttranslational modification sites, (Zexian et al., 2015) we uncovered numerous potential phospho-residues within CAL1 (data not shown). However, whether these phosphorylation sites are important for regulation of CAL1 we do not know.

Nevertheless, this data suggest that CAL1 Serine 157 acts as a pivotal switch in regulating CENP-A levels. Importantly, our data suggest that regulation of CENP-A assembly in *Drosophila* Schneider cells may be analogous to meiotic regulation. Given that the timing of CENP-A assembly differs between *Drosophila* mitotic

cultured cells (Lidsky et al., 2013; Mellone et al., 2011) and *Drosophila* meiotic tissue (Dunleavy et al., 2012; Raychaudhuri et al., 2012), this result is surprising. However, in agreement the timing of CAL1 assembly in *Drosophila* mitotic cultured cells is coupled to high kinase activity where new CAL1 is first detectable during prophase (Mellone et al., 2011). Thus, it becomes clear why the observed temporal distinction of CAL1 assembly in *Drosophila* cultured cells might be important. It is possible that incorporation of CAL1 at centromeres during prophase in mitosis is regulated by high kinase activity, which could promote limited and restricted incorporation of CENP-A only at centromeric loci as observed for our meiotic data.

We further show that brief inhibition of the 26S proteasome in spermatocytes using MG132, results in rapid accumulation or stabilisation of CAL1 suggesting a vital role for the proteasome in facilitating CAL1 turnover. In addition, we show that this degradation is made effective when Cdk activity is unperturbed, (figure 5.4 B). Inhibition of Cdk by RO-3306 results in stabilisation of CAL1 as revealed by our western blot analysis on total protein levels.

Because CAL1 Serine 157 conforms to a consensus Cdk site, we propose that phosphorylation of this residue by Cdk1/2 provides a signal that possibly tags CAL1 for degradation by the 26S proteasome to spatially restrict and limit CAL1, and ultimately CENP-A. This data provides a mechanism that explains CAL1 dynamic association and dissociation from centromeres. Additionally this mode of regulation could also be one of the mechanisms employed to keep CAL1 levels far lower than those of CENP-A or CENP-C at centromeres (Schittenhelm et al., 2010). Moreover CAL1 dissociation from centromeres by S6 may also be facilitated by this proposed degradatory pathway. Failure to remove CAL1 by S6, may dupe cells into sensing that assembly is still ongoing. Thus coupling CENP-A assembly to high kinase activity could provide a cue for gradual, stable incorporation of CENP-A into centromeric chromatin further ensuring that CENP-A localises exclusively to centromeric chromatin and ultimately switch off assembly.

Our findings lead to interesting questions in the regulation of the human CENP-A chaperone HJURP. Similar to CAL1, HJURP localises transiently at centromeric chromatin at the time of CENP-A assembly and dissociates at later points in G1 phase

coinciding with termination of assembly (Dunleavy et al., 2009; Foltz et al., 2009). How HJURP dissociates from centromeres still remains unclear. It would be interesting to assess if proteasomal mediated degradation is one of the mechanisms employed by HJURP to transiently and dynamically associate and dissociate from centromeres during G1 phase. An interesting question that arises in light of these findings however is how then are centromeric and nucleolar pools of CAL1 required for CENP-A assembly maintained, stabilised or protected from the phosphorylation-mediated degradation we propose?

Recently in mitosis, the CUL3 ligase adaptor protein Roadkill (RDX) has been implicated in stabilisation and maintenance of CAL1 protein levels and centromeric levels of CENP-A (Bade et al., 2014). CAL1 specifically binds to RDX via its C terminus through conserved RDX binding sites and acts as a bridging factor for CUL3/RDX mediated ubiquitination of CENP-A, which stabilises and maintains both CENP-A and CAL1 in a degradation-independent ubiquitination pathway (Bade et al., 2014). Loss of RDX, or mutation of the conserved RDX binding sites triggers rapid degradation of CAL1 and CENP-A. Notably, CAL1 protein levels were restored when RDX depletion was combined with subsequent proteasome inhibition by MG132, indicating that in the absence of RDX, CAL1 is a substrate of proteasome mediated degradation (Bade et al., 2014). Because our data suggest that in *Drosophila* mitosis CAL1 is regulated in a manner analogous to meiosis, it will be important to determine if RDX may also maintain and stabilise CAL1 during meiosis. Taken together, these data suggest a dual mechanism for regulation of CAL1, wherein stabilisation (mediated via the C terminus) and degradation (mediated via the N terminus) of CAL1 corporately fine-tune CAL1 availability and ultimately CENP-A levels. By coupling both the stabilisation and degradation event of CENP-A to CAL1, the cell might be able to differentiate between CENP-A protein required for assembly and excess protein that must be degraded to prevent centromere expansion and ectopic kinetochore formation.

It would be interesting to manipulate gene expression of both *cyclin A* and *cyclin B* after the second meiotic division to assess if these proteins are also necessary for the second phase of CENP-A assembly during spermatid differentiation (Dunleavy et al., 2012). However lack of a strong specific reliable binary system such as the

GAL4/UAS for probing spermatid differentiation has limited the approaches available. The development of efficient genetic manipulation during these stages would be beneficial for further investigation into the requirements of *cyclin A* and *cyclin B* in aiding the second phase of CENP-A assembly or in maintenance of CENP-A assembled in the first deposition phase.

We conclude that CAL1 Serine 157 is an essential cell cycle control element that effectively tags CAL1 for proteasomal degradation. This residue possibly provides a 'switch-like' cue for spatial deposition of CENP-A and CAL1 at *Drosophila* centromeres.

Chapter 6. Dissecting the role of CAL1 Serine 157 *in vivo*

6.1. Introduction

In recent years, it has been demonstrated that key proteins necessary for CENP-A assembly in mammals, HJURP and Mis18BP1, are phosphoproteins regulated by kinase activity to temporally restrict and isolate CENP-A deposition from that of canonical H3 in S phase, (McKinley & Cheeseman, 2016). Recently, Stankovic et al., 2017, uncovered a Cyclin A binding site, (RxL), in the mammalian CENP-A chaperone, HJURP, mediating specific inhibitory phosphorylation. Mutating this Cyclin A binding site from $HJURP^{RXL}$ to $HJURP^{AxA}$ resulted in HJURP targeting to centromeres prematurely in G2 phase. In addition $HJURP^{AxA}$ resulted in precocious deposition of nascent CENP-A in G2 phase (Stankovic et al., 2017). This report provides evidence that CYCA associated kinase activity negatively regulates HJURP by sequestering it away from the centromere to prevent its untimely recruitment and that of CENP-A (Stankovic et al., 2017).

In line with this data, our findings from Chapter 5 strongly suggest that Cyclin A or CYCB associated kinase activity negatively regulates the *Drosophila* CENP-A assembly factor, CAL1. When CYCA or CYCB associated kinase activity fails to accumulate, CAL1 is grossly mislocalised (figure 5.2 A). Moreover site directed mutagenesis of the CAL1 Cdk consensus motif reveal that mutation of CAL1 at Serine 157 to a non-phosphorylatable Alanine is sufficient to alleviate this negative regulation as $CAL1^{S157A}$ recruits excess CENP-A and CAL1 to centromeric loci *in vitro* in S2 cultured mitotic cells (figure 5.3 B). We therefore wanted to dissect the role of $CAL1^{S157A}$ *in vivo* and characterise the localisation of this CAL1 mutant during meiosis I and spermatid differentiation.

Spermatid differentiation is characterised by dramatic morphological changes to the 64 haploid spherical interconnected spermatids generated from meiosis II (Fuller 1993, Fabian & Brill, 2012). Gross chromatin modification including the recruitment of protamines to condense the sperm chromatin ensues and the spermatids differentiate from small round cells into thinner needle like long motile sperm capable

of participating in fertilisation (Fuller 1993, Fabian & Brill, 2012) (figure 6.1). In this chapter we will focus on the round stage spermatids (T4) for assessing chromosome segregation. The T4 stage is readily identifiable due to the presence of a dense cytoskeleton (Cenci et al., 1994) and provides a large sample number for quantifications. We will also focus on the early canoe stage spermatids to assess the progress of the second phase of CENP-A assembly.

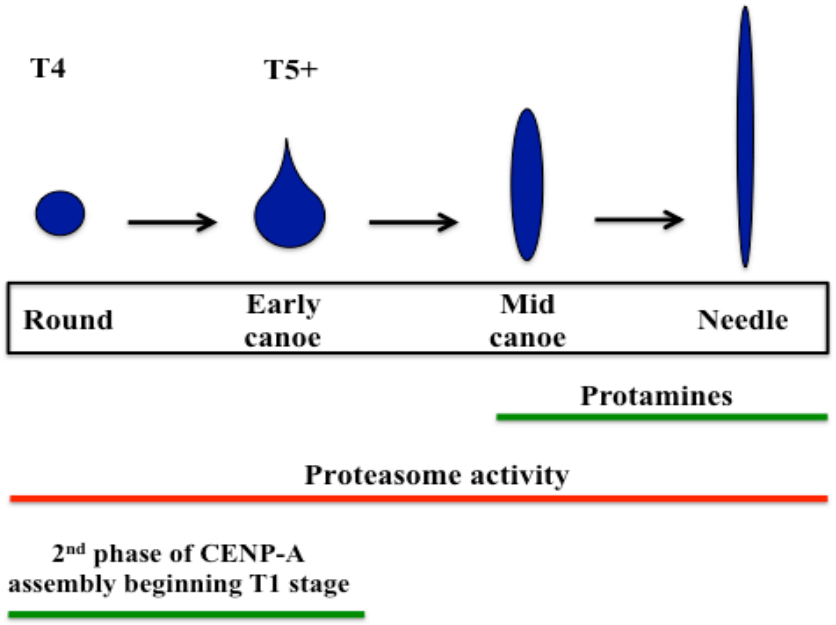


Figure 6.1. Schematic showing *Drosophila* spermatid differentiation. Elongating spermatid nuclei go through round, early canoe, mid canoe, late canoe (not shown) and needle like shaped stages. Inside the nucleus, the chromatin is reorganised and histones are replaced by transition proteins, and then by protamines. Notably, proteasome activity is high from as early as the round stage to the needle stage. Adapted from (Fabian & Brill, 2012).

6.2. Characterisation of LAP-CAL1 localisation in comparison to endogenous CAL1

A previous report using a transgenic fly line expressing C terminally tagged CAL1-GFP showed that in meiosis, CAL1 localises at centromeres and in the nucleolus and that CAL1 levels at centromeres gradually decrease as meiosis progresses, (Dunleavy et al., 2012; Raychaudhuri et al., 2012). By S5 stage, CAL1-GFP foci at centromeres were almost undetectable and could not be observed in cells that had completed meiosis I or II, (Dunleavy et al., 2012). In line with our experiments in *Drosophila* Schneider (S2) cells (figure 5.3 A and B), we generated a transgenic line expressing N terminally tagged LAP-CAL1 under the control of its native promoter. The endogenous promoter was amplified from genomic DNA using specific primers listed in table 2.5.3. The 3' UTR was also amplified using the primers listed in table 2.5.3. *LAP-cal1* was amplified from plasmid DNA. The fragments were assembled in a pCasper empty vector using Gibson assembly to yield LAP-CAL1 under the control of its endogenous promoter, (figure 6.2 A).

Because the LAP-Tag contains GFP (figure 6.2 A), we visualised GFP fluorescence to assay CAL1 localisation. Firstly we compared the localisation of GFP-CAL1 to that of endogenous CAL1. Interestingly in comparison to endogenous CAL1 which is enriched at centromeres in early prophase I, and dissociates from centromeres at S5 stage (figure 6.2 B) GFP-CAL1 was enriched at centromeres in early prophase I cells (S1-S3) as well as in late prophase I cells (S5-S6) however no nucleolar association was observed (figure 6.2 C). The centromeric signal for GFP-CAL1 was still detectable as late as M5 stage (telophase I) and T4 spermatids (figure 6.2 C inset). By early canoe stage, association of GFP-CAL1 with centromeres was undetectable (figure 6.2 C inset). In comparison, although endogenous CAL1 could be detected in the nucleus as late as M5 stage (telophase I), red arrows, association with centromeres was not observed (figure 6.2 B).

Taken together this analysis demonstrates that the localisation pattern of GFP-CAL1 differs slightly from that of endogenous CAL1 localisation and what has been previously reported in literature. Dunleavy and colleagues report dissociation of CAL1-GFP from centromeres earlier, at S5 stage of prophase I, whereas dissociation

of GFP-CAL1 was later, after meiosis II at the T4 stage. These differences could be reconciled by the knowledge that the transgenic line used in Dunleavy et al., 2012 is C terminally tagged whereas the construct used in this study is N terminally tagged, which may impact the stability of the protein.

A

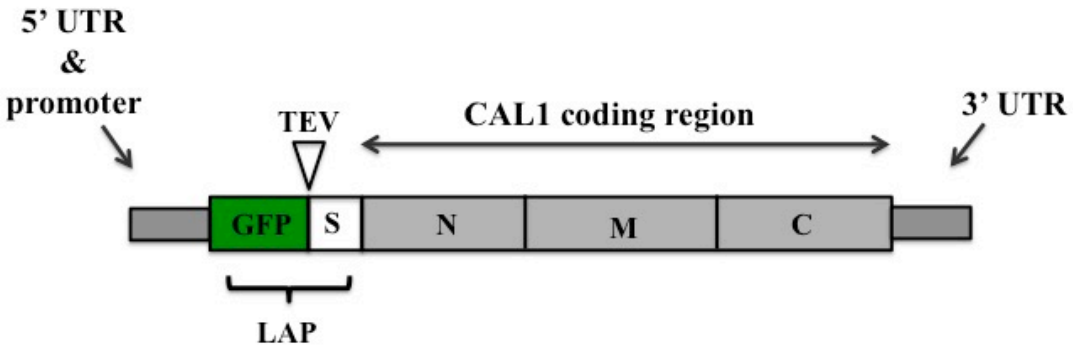


Figure 6.2 A. Schematic showing the LAP-CAL1 transgene. LAP-CAL1 was amplified from plasmid DNA and is under the control of its native promoter. The promoter was amplified from the 5' UTR (1-1000 bp) region upstream of CAL1 using genomic DNA as a template. The 3' UTR was also amplified from genomic DNA (1-600bp) downstream of CAL1. The EGFP coding sequence (green box) is inserted immediately before the start codon. The horizontal double arrow represents CAL1 coding region.

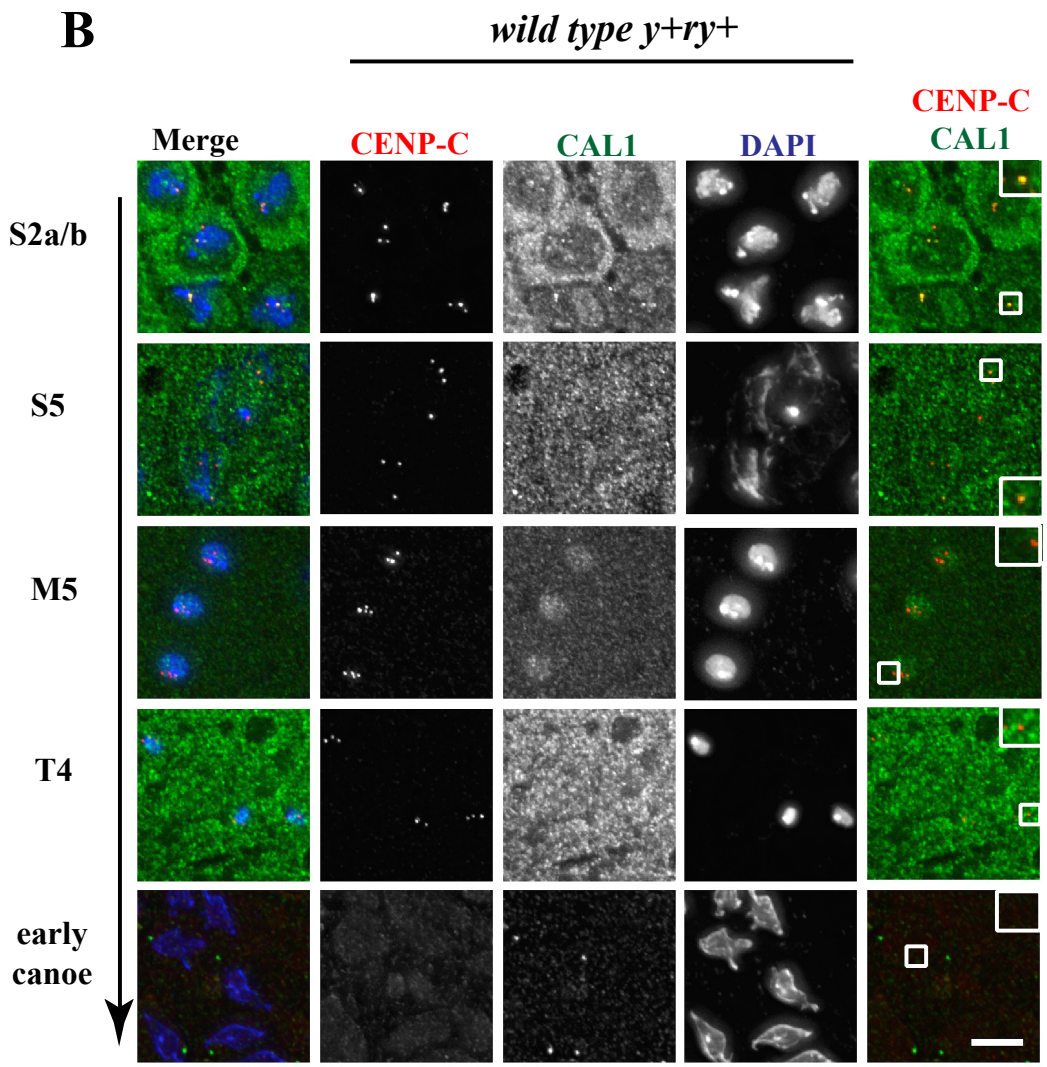


Figure 6.2 B. Characterisation of endogenous CAL1 localisation in a wild type fly line. Adult testes from *wild type* $y^{+}ry^{+}$ flies were dissected and immunostained with antibodies against CENP-C (red), CAL1 (green) and DAPI (blue) to visualise DNA. Stages S2, S5, M5, T4 and Early canoe are shown. Endogenous CAL1 localises to centromeres from S2 to S5 (inset) and is undetectable at centromeres at M5, T4 and early canoe stages (inset). Scale bar = 5 μm.

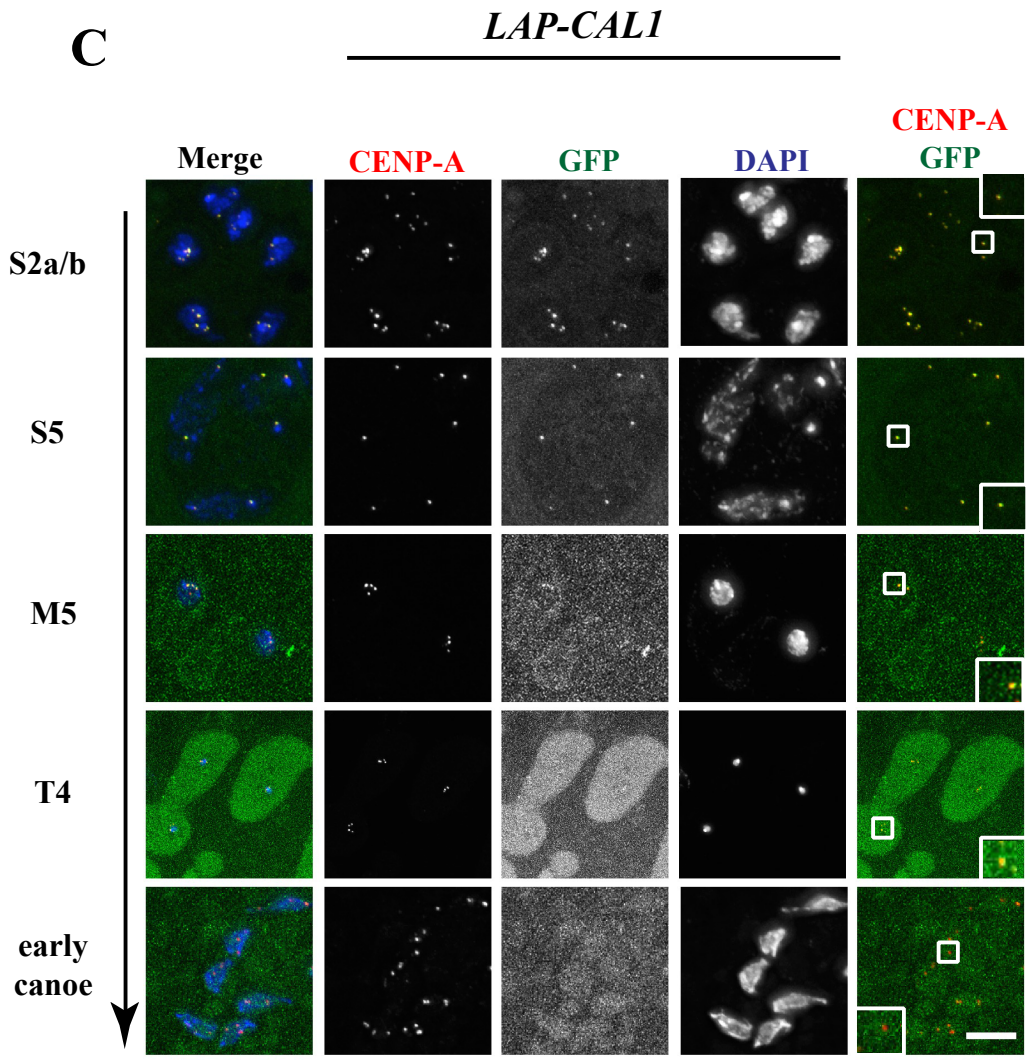


Figure 6.2 C. Characterisation of LAP-CAL1 localisation. Adult testes from flies expressing LAP-CAL1 were dissected and immunostained with antibodies against CENP-A (red), DAPI (blue) to visualise DNA. The GFP signal was directly visualised. Stages S2, S5, M5, T4 and Early canoe are shown. GFP-CAL1 localises to centromeres from S2 to T4 spermatids (inset) and is undetectable at centromeres at early canoe stage (inset). Scale bar = 5 μm.

6.3. Transgenic flies expressing LAP-CAL1^{S157A} incorporate excess CAL1, CENP-A and CENP-C at centromeres in meiosis I

A previous study reported that CAL1 limits incorporation of centromeric CENP-A and CENP-C to mitotic centromeres (Schittenhelm et al., 2010). In agreement, up to four fold overexpression of CENP-A under UAS control resulted in a marginal increase in the intensity of centromeric anti-CENP-A signal. However, when CENP-A and CAL1-EGFP were simultaneously overexpressed, a significant increase in centromeric CENP-A signal, ($p < 0.001$), was observed suggesting that CAL1 levels are limiting (Schittenhelm et al., 2010). Our site directed mutagenesis in vitro results implicate CAL1 Serine 157 as a residue that could be critical in the execution of this limiting role of CAL1 (figure 5.3 C). We therefore sought to determine whether CAL1 Serine 157 is responsible for CAL1 limiting role *in vivo* in male meiosis.

Using a transgenic line expressing LAP-CAL1^{S157A}, we determined whether CAL1 Serine 157 is also a limiting element *in vivo*. Because our previous findings show that early prophase I cells are mostly sensitive to Cdk1/2 inhibition, we focused our analysis on the S2 stage. Thus adult testes expressing LAP-CAL1 (control) and LAP-CAL1^{S157A} were dissected and the GFP signal within the LAP-Tag was directly visualised. Testes were immunostained with antibodies against CENP-A or CENP-C to mark centromeres. Interestingly, LAP-CAL1^{S157A} displayed a significant increase in the intensity of centromeric CAL1, CENP-A and CENP-C signals at S2 stage of meiosis I compared to the control (figure 6.3 A and C inset). Quantification of centromeric CAL1 fluorescence at S2 stage indicated an increase of ~ 33% in the LAP-CAL1^{S157A} mutant flies compared to the control (figure 6.3 E). Notably, quantification of CENP-A and CENP-C levels revealed an increase in the LAP-CAL1^{S157A} mutant although slightly higher than those of CAL1 (figure 6.3 B and D). This result was quite intriguing as it is in line with findings that CAL1 levels are lower than those of CENP-A and CENP-C at centromeres (Schittenhelm et al., 2010). Collectively, our data suggest that CAL1 Serine 157 is a key residue in ensuring that limited amounts of CENP-A and CENP-C are incorporated at centromeres during meiosis I.

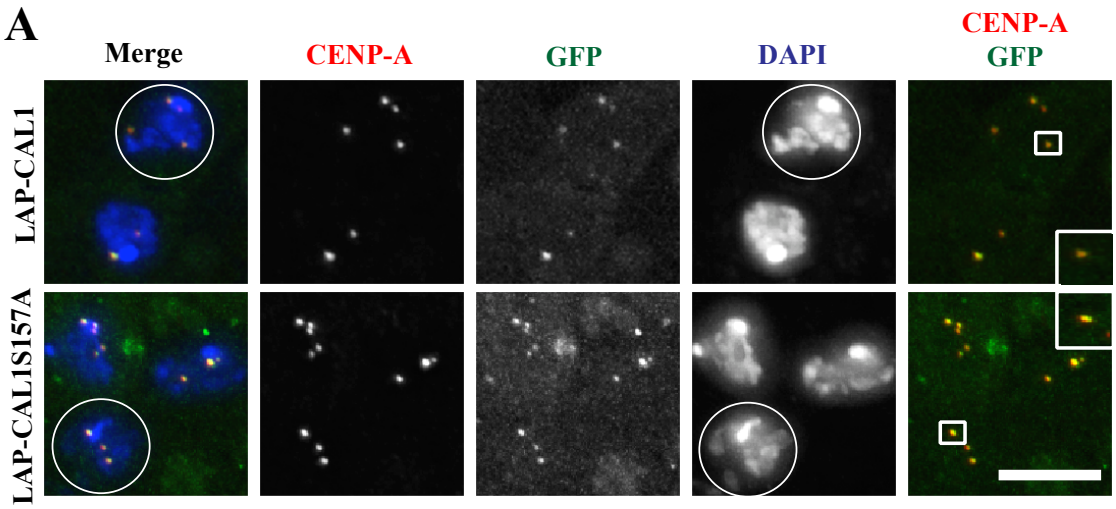


Figure 6.3 A. Transgenic flies expressing LAP-CAL1^{S157A} incorporate excess CAL1 and CENP-A at centromeres in meiosis I. Adult testes from flies expressing *LAP-CAL1* or *LAP-CAL1^{S157A}* were dissected and immunostained with antibodies against CENP-A (red) and DAPI (blue) to visualise DNA. The GFP signal (green) was directly visualised. Stage S2 of meiotic prophase I is shown. *LAP-CAL1^{S157A}* incorporates excess CENP-A and CAL1 at centromeres (inset). The circle represents the outline of the nucleus. Scale bar = 5µm.

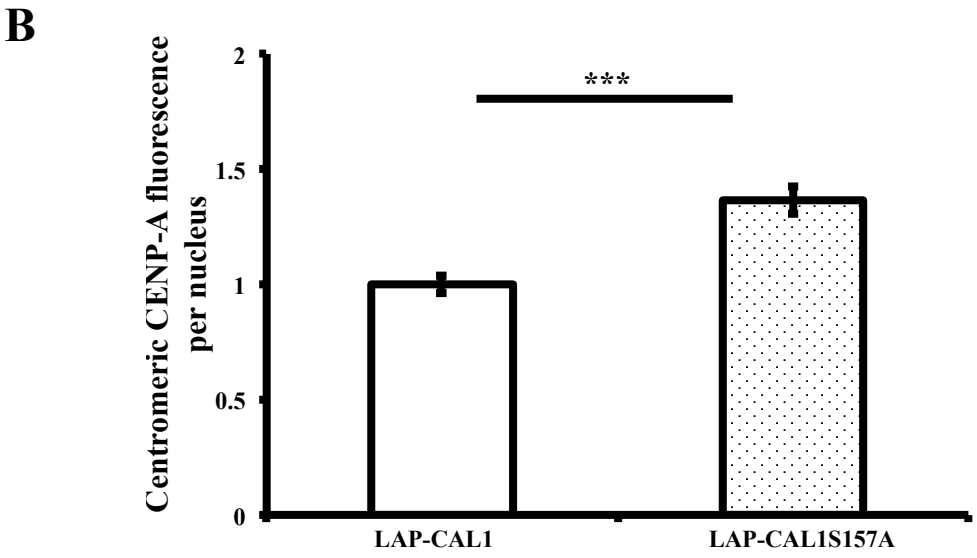


Figure 6.3 B. Graph showing quantification of centromeric CENP-A fluorescence per nucleus.

An increase of ~36% in CENP-A is observed number of cells quantified = 25 per experiment n = 3, Error bars = SEM, $p \leq 0.001$.

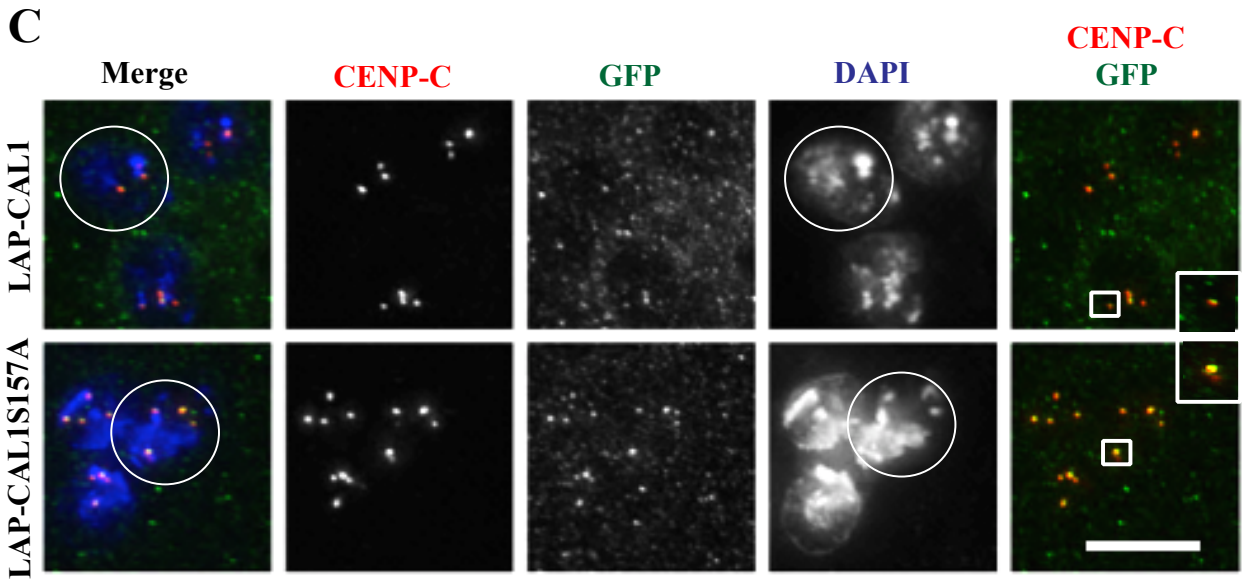


Figure 6.3 C. Transgenic flies expressing LAP-CAL1^{S157A} incorporate excess CAL1 and CENP-C at centromeres in meiosis I. Adult testes from flies expressing *LAP-CAL1* or *LAP-CAL1^{S157A}* were dissected and immunostained with antibodies against CENP-C (red) and DAPI (blue) to visualise DNA. The GFP signal (green) was directly visualised. Stage S2 of meiotic prophase I is shown. *LAP-CAL1^{S157A}* incorporates excess CENP-C and CAL1 at centromeres (inset). Circles represent the outline of the nucleus. Scale bar = 5μm.

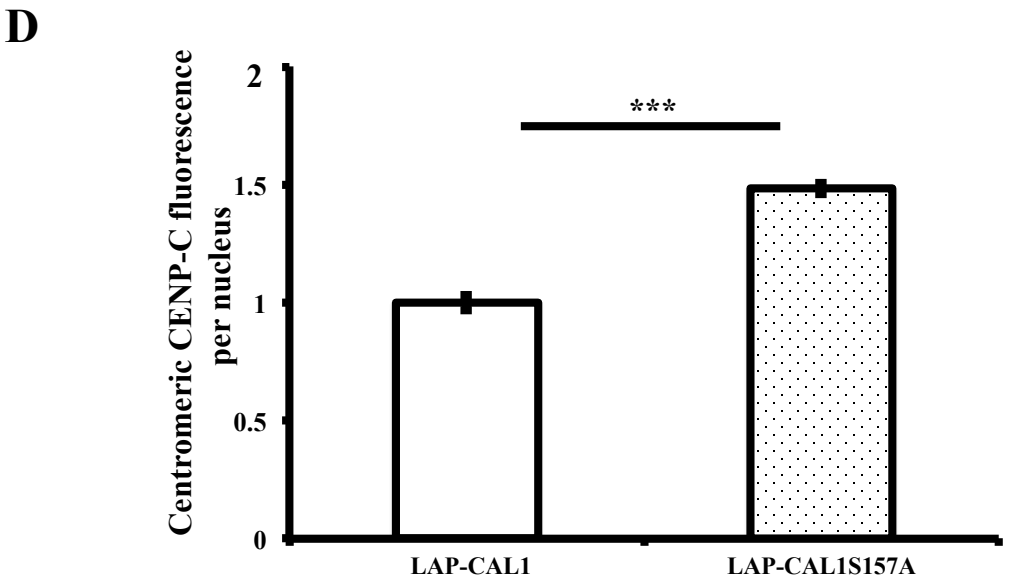


Figure 6.3 D. Graph showing quantification of centromeric CENP-C fluorescence per nucleus. An increase of ~48% in CENP-C is observed, number of cells quantified = 30 per experiment, n= 3. Error bars = SEM, $p \leq 0.001$.

E

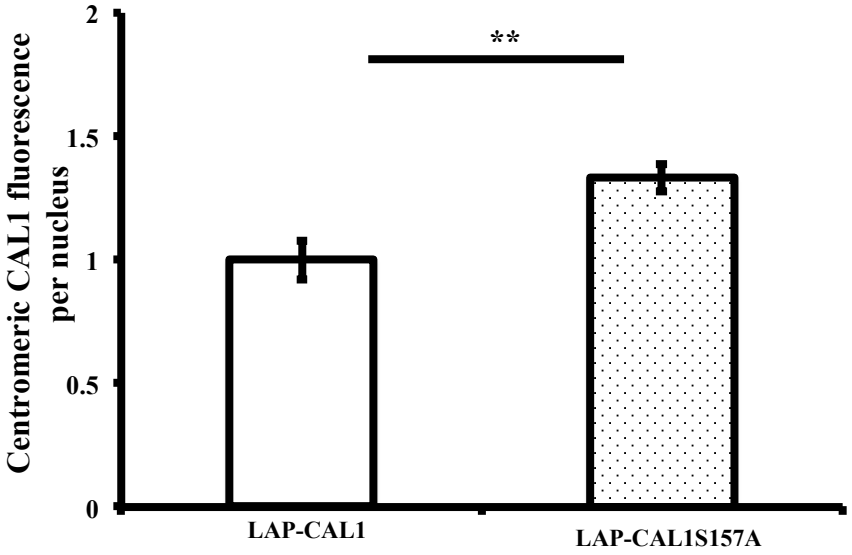


Figure 6.3 E. Graph showing quantification of centromeric GFP-CAL1 fluorescence per nucleus. An increase of ~33% in GFP-CAL1 is observed, number of cells quantified = 25 per experiment, n = 3, Error bars = SEM, $p \leq 0.01$

6.4. Transgenic flies expressing LAP-CAL1^{S157A} retain CAL1 and incorporate excess CENP-A on differentiating spermatids.

A key observation from literature is that centromeric CAL1 shows rapid changes in abundance, with levels decreasing as meiosis I progresses (Dunleavy et al., 2012), and levels decreasing by 66% after one cell cycle (Mellone et al., 2011). This demonstrates that CAL1 is a short lived protein with a high turnover rate. In agreement, Bade et al., 2014 identified the adaptor protein (RDX) to be essential for the maintenance and stabilisation of both CAL1 and CENP-A. Loss of RDX led to a rapid degradation of both CAL1 and CENP-A further testifying that CAL1 is significantly unstable (Bade et al., 2014). Our results so far argue that CAL1 Serine 157 is a critical element in mediating CAL1 stability. We therefore next sought to characterise the localisation and abundance of GFP-CAL1 in the LAP-CAL1^{S157A} transgenic line during spermatid differentiation.

Adult testes from lines expressing either LAP-CAL1 (control) or LAP-CAL1^{S157A} were dissected, fixed and immunostained with antibodies against CENP-A to mark centromeres. As already reported for the LAP-CAL1 (control) GFP-CAL1 localises to centromeres from early prophase I and dissociates from centromeres at T4 spermatids and is undetectable at centromeres at the early canoe stage (figure 6.2 B). Interestingly and in contrast to the control, in LAP-CAL1$^{\mathbf{S157A}}$ mutants GFP-CAL1 localises to centromeres throughout meiosis I and II and post meiosis II (figure 6.4 A). At mid canoe stage, we clearly observed GFP-CAL1 foci spaced along the length of the nucleus. This data suggest that CAL1 removal is perturbed in LAP-CAL1^{S157A} mutants. This observation was quite surprising because the onset of sperm nuclei differentiation is characterised by an extensive period of gross chromatin remodeling to facilitate protamine exchange, (Jayaramaiah et al., 2005; Rathke et al., 2007). Moreover, it has also been reported that early canoe stage nuclei are abundant in ubiquitin and proteasomes, (Rathke et al., 2007), yet LAP-CAL1^{S157A} bypasses removal at these stages. This data suggest that LAP-CAL1^{S157A} is possibly protected from removal as it lacks the phospho posttranslational modification on Serine 157.
Notably, the presence of CAL1 at these stages positively affects CENP-A maintenance during spermatid differentiation. We observed that LAP-CAL1^{S157A}

early canoe stage spermatids maintain almost double amounts of CENP-A, in comparison to LAP-CAL1 (control) (figure 6.4 B and C). We also cannot exclude our observation that LAP-CAL1^{S157A} displayed abnormal sperm morphologies (figure 6.4 B inset). These sperm nuclei exhibited decondensed chromatin in comparison to the control suggesting an error in chromatin packaging.

We conclude that CAL1 Serine 157 controls the timing of CAL1 removal and that if not removed; CAL1 is a potent CENP-A maintenance factor during spermatid differentiation.

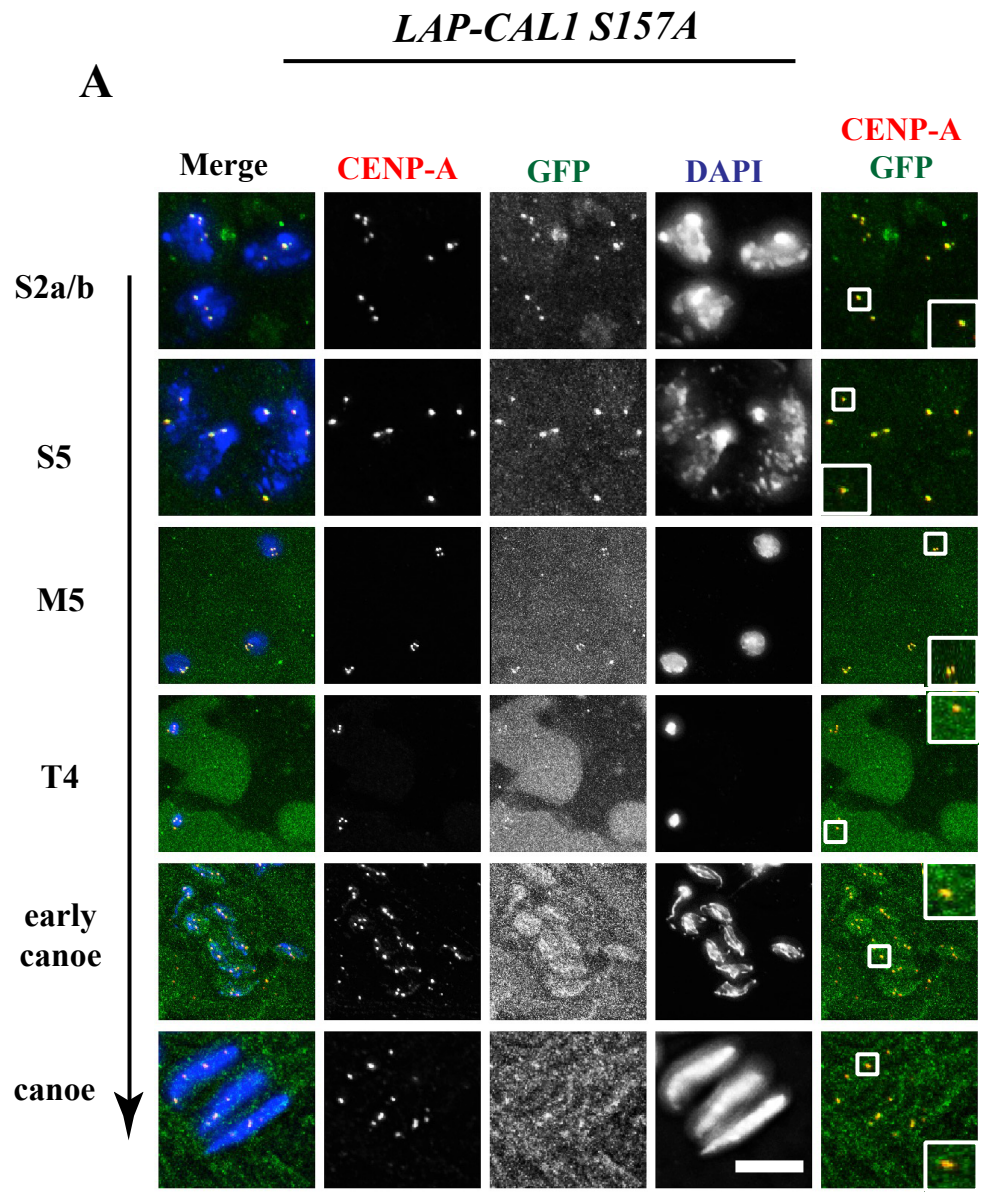


Figure 6.4 A. Transgenic flies expressing LAP-CAL1^{S157A} retain CAL1 and CENP-A on differentiating spermatids. Adult testes from flies expressing *LAP-CAL1^{S157A}* were dissected and immunostained with antibodies against CENP-A (red) and DAPI (blue) to visualise DNA. The GFP signal (green) was directly visualised. Stages S2, S5, M5, T4 Early canoe and Canoe are shown. LA*P-CAL1^{S157A}* retains CENP-A and CAL1 at centromeres (insets) through all the shown stages during spermatogenesis. Scale bar = 5 μm.

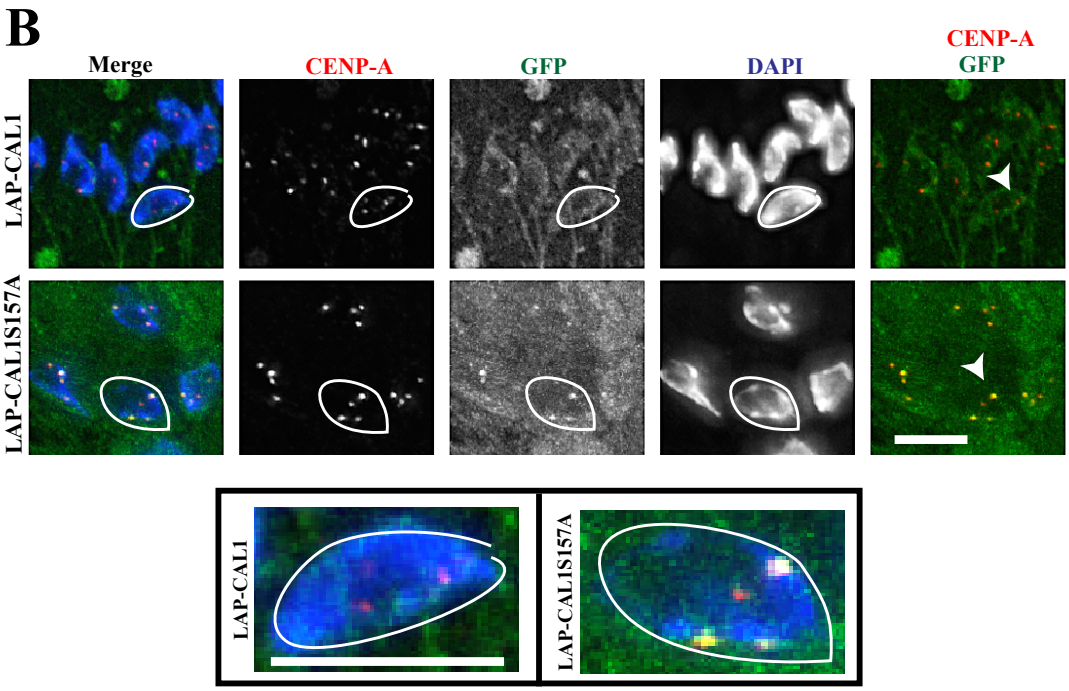


Figure 6.4 B. Transgenic flies expressing *LAP-CAL1*^{S157A} incorporate excess CENP-A on differentiating spermatids. Adult testes from flies expressing *LAP-CAL1* or *LAP-CAL1*^{S157A} dissected and immunostained with antibodies against CENP-A (red) and DAPI (blue) to visualise DNA. The GFP signal (green) was directly visualised. Early canoe and stage is shown. *LAP-CAL1*^{S157A} incorporates excess CENP-A and GFP CAL1 at centromeres (arrowheads) and exhibit decondensed chromatin (inset). Scale bar = 5 µm.

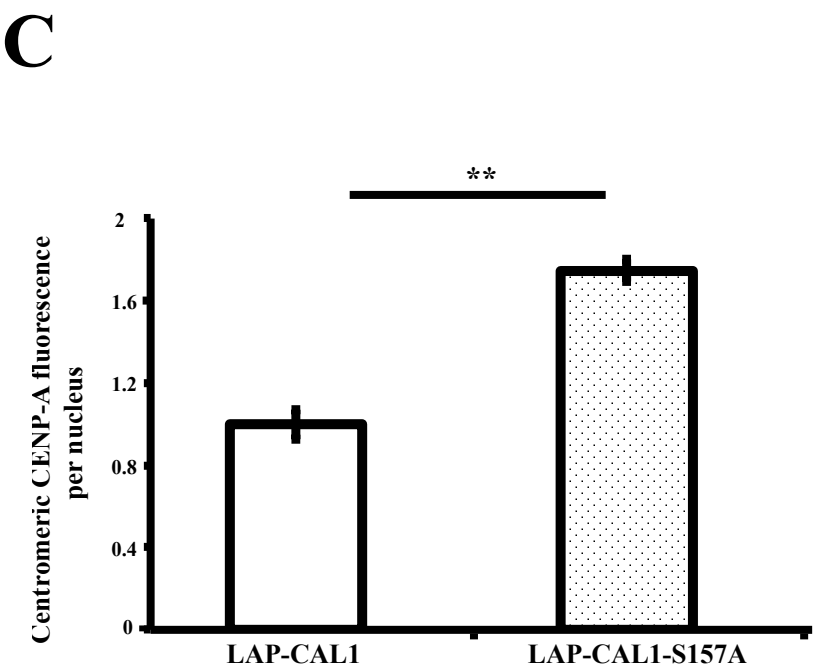


Figure 6.4 C. Graph showing quantification of centromeric CENP-A fluorescence per nucleus at the early canoe stage. An increase of almost twofold in CENP-A intensity is observed. Number of cells quantified = 30 per experiment, n = 3. Error bars = SEM, $p \leq 0.01$.

6.5. Regulation of CAL1 incorporation at centromeres is dependent on Cdk mediated regulation of CAL1 Serine 157.

The degradation of a number of eukaryotic proteins appears to be activated by their phosphorylation (Lanker et al., 1996; Lin & Desiderio, 1993). Our small molecule inhibitor study suggest that CAL1 is recognised as a substrate for the proteasome only in its phosphorylated state as Cdk1/2 inhibition results in the stabilisation of CAL1 by ~30% (figure 5.4 B). Given that accumulation of Cdk1/2 activity coincides with the timing of CAL1 localisation at meiotic centromeres, prophase I of meiosis I (White-Cooper et al., 1998) and that CAL1 Serine 157 is a strongly predicted phosphorylation site for Cdk1/2 (Bodenmiller et al., 2007) we hypothesised that Cdk1/2 may directly regulate CAL1 activity via inhibitory phosphorylation of CAL1 Serine 157. To test our hypothesis, we exploited the LAP-CAL1^{S157A} point mutant, which interferes with the predicted Cdk1/2 consensus site and assessed whether this mutant is sensitive to Cdk1/2 inhibition. As previously shown in chapter 4, Cdk1/2 inhibition results in increased incorporation of CENP-A at centromeres at S2 stage. Thus if CAL1 Serine 157 is indeed the residue responsible for this excess incorporation of centromeric CENP-A, we hypothesised that LAP-CAL1^{S157A} spermatocytes would not be sensitive to this inhibition. To this end, larval testes from lines expressing either LAP-CAL1 or LAP-CAL1^{S157A} were dissected and treated with DMSO or 100 μM RO-3306 for 30 minutes. After treatment, the testes were fixed and the GFP signal was directly visualised.

Upon Cdk1/2 inhibition, centromeric GFP-CAL1 levels in LAP-CAL1 spermatocytes treated with 100 μM RO-3306 were increased in comparison to LAP-CAL1 DMSO treated spermatocytes, (figure 6.5 A, circles, and B). This data supports that CAL1 is sensitive to Cdk1/2 inhibition. Strikingly, GFP-CAL1 levels in the LAP-CAL1^{S157A} mutant, although already higher than those of the LAP-CAL1 control, remained unchanged upon Cdk1/2 inhibition (figure 6.5 A circles). Quantification of this data revealed that there was no significant difference, $p \geq 0.05$, in CAL1 levels between the LAP-CAL1^{S157A} DMSO control compared to the LAP-CAL1^{S157A} mutant treated with 100 μM RO-3306. This suggests that perturbation of the Cdk1/2 consensus site results in no detectable hyperaccumulation of CAL1 as observed for the control.

Collectively, our results demonstrate that phosphorylation of CAL1 at Serine 157 provides a signal that is necessary for CAL1 degradation. Thus the ability of Cdk1/2 to regulate CAL1 can be correlated to possessing an intact Cdk1/2 consensus motif.

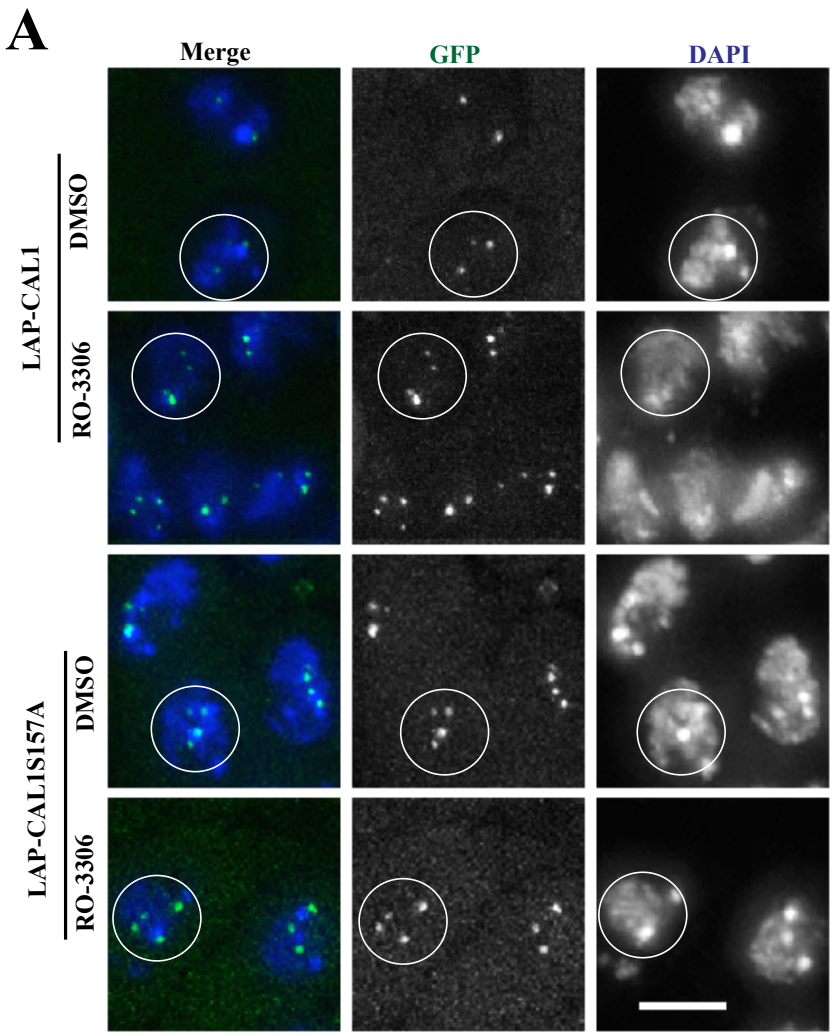


Figure 6.5 A. Regulation of CAL1 incorporation at centromeres is dependent on Cdk1/2 mediated modulation of CAL1 Serine 157. Adult testes from flies expressing *LAP-CAL1* or *LAP-CAL1*^{S157A} were dissected and treated with DMSO or 100 µM RO-3306 for 30 minutes. Testes were fixed and immunostained with DAPI (blue) to visualise DNA. The GFP signal (green) was directly visualised. S2 stage of meiosis I is shown. GFP-CAL1 levels remain unchanged in the *LAP-CAL1*^{S157A} mutant following treatment with RO-3306 (circles in GFP channel) compared to LAP-CAL1 control levels, which are increased (circles in GFP channel). Scale bar = 5 µm.

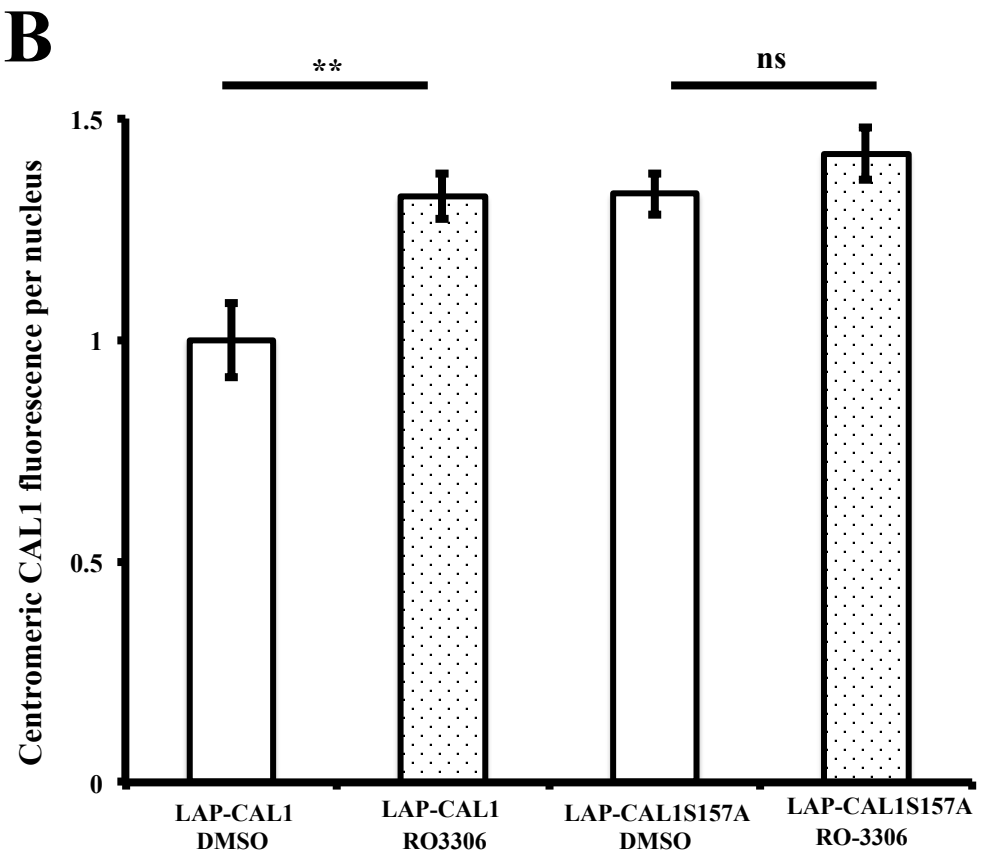


Figure 6.5 B. Quantification of GFP-CAL1 fluorescence following Cdk1/2 inhibition. GFP-CAL1 levels remain unchanged in the *LAP-CAL1*^{S157A} mutant following Cdk1/2 inhibition $p > 0.05$ compared to LAP-CAL1 control levels, which are significantly increased $p \leq 0.01$. Number of cells quantified = 25 per experiment, n = 3, Error bars = SEM.

6.6. LAP-CAL1^{S157A} transgenics display micronuclei and nuclear budding at T4 spermatids, a hallmark of chromosomal instability.

Given the central role of centromeres in the engagement of chromosomes with the spindle, we next tested whether the LAP-CAL1^{S157A} mutant flies had any defects in chromosome segregation. A study by Schittenhelm and colleagues analysing the simultaneous overexpression of CAL1, CENP-A and CENP-C on the dynamics of progression through mitosis in fixed embryos, showed subtle to strong abnormalities in chromosome segregation during anaphase compared to controls which did not overexpress the centromeric proteins. The most prominent defects observed were abnormal anaphase and telophase figures with chromatin bridges containing lagging centromeres, (Schittenhelm et al., 2010). These data suggested that increased levels of centromeric CAL1 CENP-A and CENP-C compromise kinetochore function during mitosis. Because LAP-CAL1^{S157A} recruits excess CENP-A and CENP-C to centromeres, we analysed if chromosome segregation in this mutant was disrupted. We examined round spermatids (T4 stage) (figure 6.1) to score nuclear anomalies following the two rounds of meiotic divisions. The T4 stage is readily identifiable due to the presence of a dense cytoskeleton (Cenci et al., 1994) and provides a large sample number for quantifications.

Thus LAP-CAL1 and LAP-CAL1^{S157A} adult testes were dissected, fixed and immunostained with antibodies against CENP-A to mark centromeres and tubulin to mark the spindles. Interestingly, LAP-CAL1^{S157A} spermatocytes displayed aberrant spindle morphology (figure 6.6 A arrowheads) compared to the control, suggesting a possible disintegration of the spindle in the LAP-CAL1^{S157A} spermatocytes. Analysis of the DNA staining revealed that LAP-CAL1^{S157A} spermatocytes had prominent defects in nuclear morphology. Micronuclei, typical of displaced chromosomes or chromosome fragments were observed at the T4 stage in the LAP-CAL1^{S157A} spermatocytes in comparison to controls (figure 6.6 A). Quantification of T4 spermatids displaying micronuclei revealed that 30% of the LAP-CAL1^{S157A} T4 spermatids were affected in comparison to 2.7% of the LAP-CAL1 control (figure 6.6 B). The LAP-CAL1^{S157A} T4 spermatids also displayed another nuclear anomaly which has been characterised in lymphocytes as nuclear budding, (Fenech et al.,

2011). Nuclear budding is characterised by the constriction of the parent nucleus (figure 6.6 A). Such nuclear budding may possibly represent entrapment of DNA that has been left in the cytoplasm after nuclear division or from excess DNA that is being extruded from the nucleus (Fenech et al., 2011). Quantification of LAP-CAL1^{S157A} T4 spermatids displaying nuclear budding revealed that 42% of the LAP-CAL1^{S157A} T4 spermatids were affected in comparison to 10.4% of the LAP-CAL1 control (figure 6.6 C). These data suggest that excess incorporation of centromeric CENP-A, CAL1 and CENP-C compromise chromosome segregation during meiosis and culminates in defective sperm nuclei consisting of abnormally segregated chromosomes.

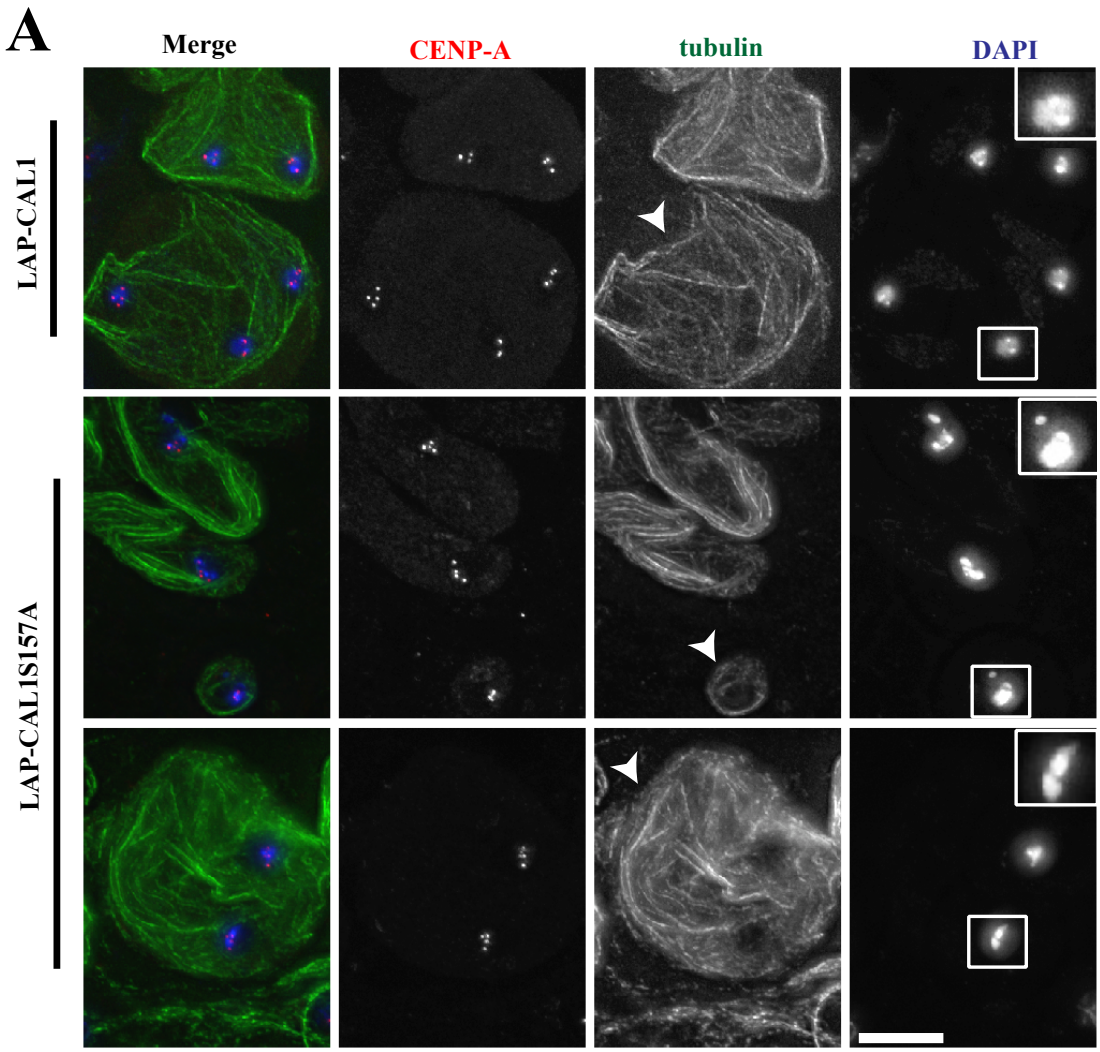


Figure 6.6 A. LAP-CAL1^{S157A} flies display micronuclei and nuclear budding at T4 spermatid stage. Adult testes from flies expressing LAP-CAL1 or LAP-CAL1^{S157A} were dissected and immunostained with antibodies against CENP-A (red) tubulin (green) and DAPI (blue) to visualise DNA. T4 spermatid stage is shown. LAP-CAL1^{S157A} display micronuclei (inset) and exhibit abnormal spindle morphology (arrowheads). Scale bar = 5 μm.

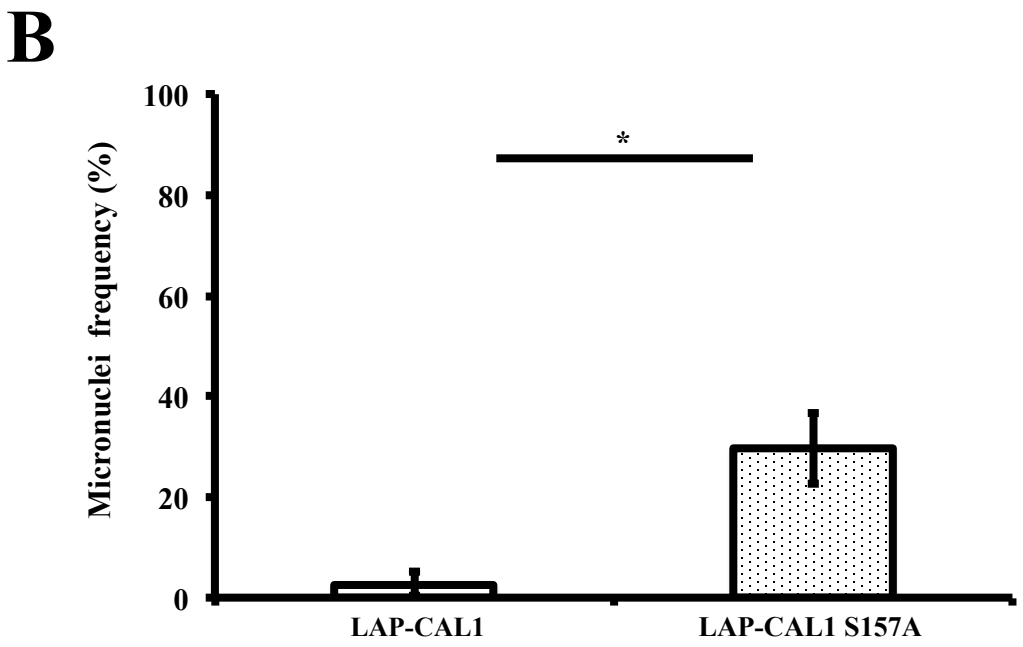


Figure 6.6 B. Graph showing quantification of micronuclei frequency. LAP-CAL1^{S157A} T4 spermatids display 30% micronuclei compared to 2.7% in the LAP-CAL1 control. Number of cells quantified = 150 pooled, n= 3, Error bars = SEM, $p \leq 0.05$

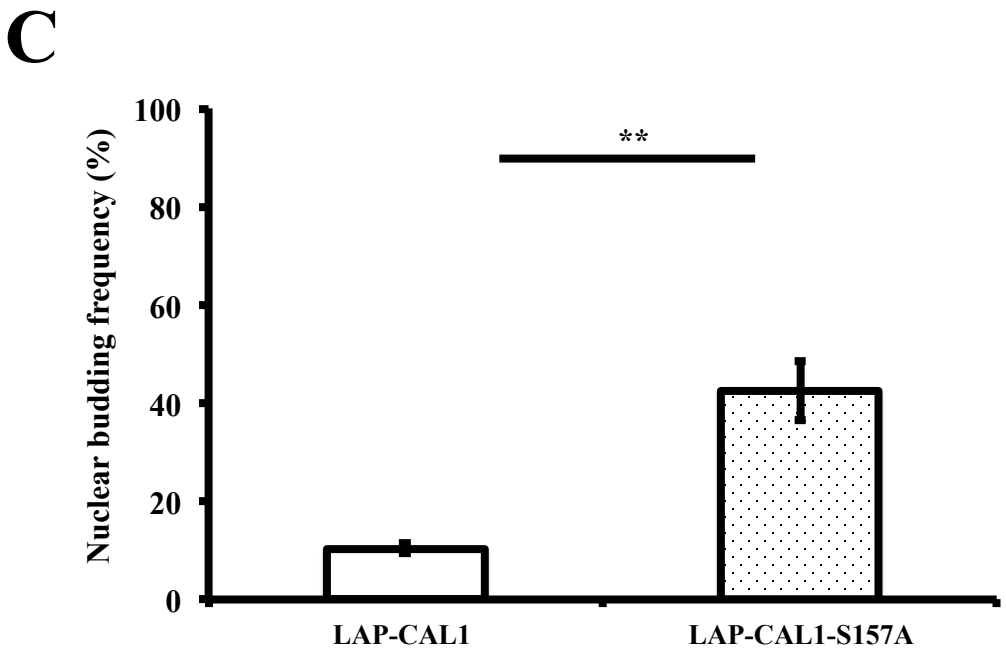


Figure 6.6 C. Graph showing quantification of nuclear budding frequency. LAP-CAL1^{S157A} T4 spermatids display 47% nuclear budding compared to 10.4% in the LAP-CAL1 control. Number of cells quantified = 150 pooled, n = 3. Error bars = SEM, $p \leq 0.01$

6.7 Discussion

Our data suggest that mutation of CAL1 Serine157 under the control of its endogenous promoter produces a dominant negative effect for regulation of CAL1. We find that transgenic flies expressing LAP-CAL1^{S157A} incorporate excess CAL1, CENP-A and CENP-C to meiotic centromeres. These data suggest that CAL1 Serine 157 is an important residue for limiting association of CAL1, CENP-A and CENP-C with centromeric chromatin. One limitation however is that the transgenes used in this study do not localise in a manner comparable to endogenous CAL1. Endogenous CAL1 localises to centromeres at early prophase I and dissociates before the onset of the first meiotic division, at S5/S6 stage. In comparison, the LAP-CAL1 control transgene localises to centromeres throughout meiosis I and II and only dissociates from centromeres at early canoe stage. Even more surprising, the mutant LAP-CAL1^{S157A} transgene localises to centromeres until late canoe stage. Thus, one could argue that in these transgenic lines CAL1 becomes more stabilised which could account for the phenotypes observed. Arguably if this was indeed the case, we would be unable to observe any clear differences in the recruitment of CAL1, CENP-A and CENP-C in the LAP-CAL1 control compared to the mutant LAPCAL1S157A (figure 6.3 A and C; figure 6.4 B). Because transgenic flies expressing the mutant CAL1 protein also carry a normal wild type copy, it is possible that CAL1 could undergo an even higher turnover and our findings could be an underestimation. It would be interesting to assess CAL1 localisation and CENP-A assembly in a background that does not produce any endogenous CAL1 and assess if LAP-CAL1$^{\mathbf{S157A}}$ elicits stronger negative effects in the absence of the wild type copy. More still, it is important to note that although CAL1 Serine 157 may be an important CAL1 regulatory phospho residue, other predicted phosphorylation sites within the CAL1 peptide sequence also exist. Using in silico approach, GPS 3.0 for prediction of posttranslational modification sites, (Zexian et al., 2015) we uncovered numerous potential phospho-residues within CAL1 (data not shown). However, whether these phosphorylation sites are important for regulation of CAL1 we do not know.

Our findings further show that transgenic flies expressing LAP-CAL1$^{\mathbf{S157A}}$ retain CAL1 on differentiating spermatids, particularly at the early canoe stage. This finding

was surprising as CAL1 has been reported to dissociate from centromeres at stage S5/S6 of meiosis I and is undetectable at centromeres during spermatid differentiation (Dunleavy et al., 2012). Remarkably, these early canoe stage spermatids also display highly uncondensed chromatin suggesting a failure in DNA packaging. These findings are especially important as it has been shown that semen with defective chromatin packing has a significantly lower fertilisation capacity (Bianchi et al., 1996). It is tempting to speculate that retaining CAL1 on these differentiating spermatids may lead to disturbances in the maturation process of sperm nuclei by interfering with the recruitment of protamines to effectively condense down the sperm nuclei. Given that CAL1 interacts with the chromatin remodeler FACT (Chen et al., 2015) it is possible that CAL1 could recruit chromatin-remodeling activity at these late stage spermatids resulting in decondensed sperm. Our findings could thus provide insight into the factors that may negatively influence chromatin reorganisation during sperm maturation.

Notably, we also observe micronuclei and nuclear budding at T5 spermatids. These nuclear anomalies are biomarkers of chromosomal instability and are indicative of genome damage events that could increase the risk of developmental diseases. It is possible that the micronuclei observed at the T4 spermatid stage (figure 6.6 A) arise from acentric chromatid fragments or whole chromosomes that fail to be included in the daughter nuclei at the completion of telophase. Given the central role of centromeres in the engagement of chromosomes with the spindle, it is probable that the CAL1 Serine 157 mutation leads to centromeric dosage imbalance events and ultimately defects in kinetochore and microtubule interactions leading to chromosome loss. Because sex chromosomes contribute to the majority of chromosome loss events, (Norppa & Falck, 2003), it would be interesting to determine using chromosome specific DNA probes the definitive chromosome loss event, whether it is the X or Y chromosome. Collectively these data indicate that CAL1 is an essential rate-limiting CENP-A assembly factor whose degradation involves Cdk1/2 dependent phosphorylation events and ultimately recognition by the 26S proteasome.

We conclude that mutation of CAL1 Serine 157 leads to increased centromeric CENP-A and CENP-C levels due to CAL1 dosage. In principle, CAL1 could be rate

limiting in the canonical CENP-A assembly pathway. It is possible that the phosphorylated forms of CAL1 may be intermediates in CAL1 turnover.

Chapter 7. General discussion and future directions

7.1. Differences in the cell cycle timing of CENP-A assembly in mitosis compared to *Drosophila* meiosis

Key differences in the timing of CENP-A assembly between mitosis and meiosis have been reported in literature (Dunleavy et al., 2012). In mitosis CENP-A assembly initiates at a time of low Cdk activity (Jansen et al., 2007; Lidsky et al., 2013; Schuh et al., 2007) while in meiosis CENP-A assembly begins at a time of high Cdk activity (Dunleavy et al., 2012; Raychaudhuri et al., 2012; Schubert et al., 2014) (figure 7.1). The relevance of this temporal disconnect however remains elusive. In this thesis, I have shed some insight into the potential purpose for this disconnect.

In mitosis it has been demonstrated that CENP-A assembly occurs in a matter of minutes to hours and initiates upon loss of Cdk activity. This is because the CENP-A assembly factors Mis18BP1 and HJURP are phosphoproteins which are sequestered away from the centromere due to high kinase activity (Bailey et al., 2016; Dephoure et al., 2008; Fujita et al., 2007; Kato et al., 2007; McKinley & Cheeseman, 2016; Müller et al., 2014; Stankovic et al., 2017; Wang et al., 2014). Inhibition of Cdk activity alleviates these CENP-A assembly factors from inhibitory phosphorylation. This allows CENP-A assembly to occur prematurely and promiscuously into S, G2 and M phases of the cell cycle.

In comparison, meiotic CENP-A assembly occurs gradually over a period of 90 hours, at a time of high Cdk activity. Our findings from chapters 4, 5 and 6 suggest that CENP-A assembly is coupled to high kinase activity to allow for gradual limited and spatially restricted CENP-A incorporation to specific centromeric loci. We find that the CENP-A assembly factor CAL1 is a substrate for Cdk1/2 activity. CAL1 Serine 157 follows the Cdk consensus motif, (SPPK). In chapter 5 and 6 we find that mutation of CAL1 Serine 157 to a non-phosphorylatable Alanine results in CAL1, CENP-A and CENP-C being rapidly incorporated at centromeres. These data suggest that high Cdk activity negatively regulates CAL1 to facilitate gradual and limited CENP-A incorporation at centromeres.

Unlike mitosis where CENP-A assembly occurs in a matter of minutes to hours, CENP-A assembly in *Drosophila* meiosis occurs over a number of days. We therefore suggest that high Cdk activity provides a cue for gradual and spatially limited assembly over this long time course. In chapter 5 we provide evidence that CAL1 may be recognised as a substrate for degradation by the 26S proteasome. This in turn results in degradation of any excess CAL1 to facilitate limited centromeric incorporation of CENP-A and CENP-C. We conclude that meiotic CENP-A assembly is coupled to high Cdk activity to allow for gradual, limited and spatially restricted CENP-A.

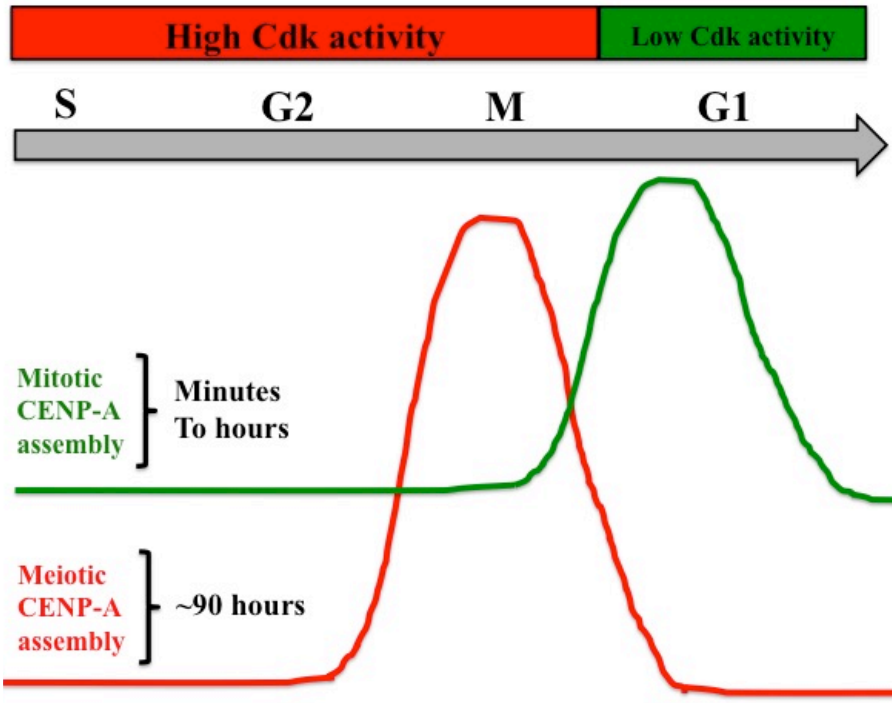


Figure 7.1. Differences in the cell cycle timing of CENP-A assembly between mitosis and meiosis
In mitosis, CENP-A assembly represented by the green line, begins at late telophase / early G1 phase coupled to low Cdk activity. Assembly occurs in a matter of minutes to hours. In meiosis, CENP-A assembly represented by the red line begins in M phase and is coupled to high Cdk activity. Assembly occurs over a period of ~90 hours.

7.2 CAL1, the most rapidly turned over *Drosophila* centromeric protein

Our findings from chapters 3, 5 and 6 clearly point to a recurring theme that CAL1 is a rapidly turned over centromeric protein. In chapter 3 we find that immunostaining of *Cenp-C*$^{Z3\text{-}4375}$ heterozygotes at prophase I stages S2-S3 reveal increased nucleolar and centromeric CAL1 levels when CENP-C is compromised. Western blotting of total or fractionated extracts from *Cenp-C*$^{Z3\text{-}4375}$ larval testes confirmed this increase mostly in nucleoplasmic CAL1. In chapter 5 we find that CAL1 accumulates in the cytoplasmic compartment when CYCA or CYCB meiotic Cdk activity fails to accumulate. In addition we show that CAL1 Serine 157 is a key residue in regulating CAL1 stability. Site directed mutagenesis of CAL1 Serine 157 to a non phosphorylatable Alanine mutant leads to CAL1 stabilisation at centromeric pools. Moreover, inhibition of the 26S proteasome leads to rapid accumulation of CAL1 at both centromeric and nucleolar pools. Additional findings from literature also show that CAL1 is less stably associated with centromeres decreasing by 66% after one cell cycle (Mellone et al., 2011). More still, a separate study by Schittenhelm et al., 2010 reveals that CAL1 levels at centromeres are far lower than those of CENP-A and CENP-C. Dunleavy and colleagues also shows that CAL1 transiently associates and dissociates from centromeres in *Drosophila* meiosis, with rapid loss from centromeres observed extensively by S5-S6 stages of prophase I. Collectively these rapid changes in CAL1 abundance clearly argue that CAL1 is a short-lived protein.

Furthermore, our site directed mutagenesis results from Chapters 5 and 6 are in excellent agreement. Both these chapters demonstrate that CENP-A incorporation at centromeres is sensitive to CAL1 levels. In Chapter 5 and 6 we report that the turnover of CAL1 is dependent on a Cdk consensus motif located in the N terminus of CAL1. A point mutation on CAL1 Serine 157 which interferes with a Cdk1/2 consensus motif on CAL1 inhibits CAL1 degradation and causes approximately 0.3-fold stabilisation of CAL1 and consequently promotes the incorporation of excess CENP-A throughout meiosis I and during spermatid differentiation. Because CAL1 Serine 157 contains the only stringent representation of the consensus site for the Ser/Thr kinase Cdk1/2, our data strongly suggests that regulation of CAL1 incorporation at centromeres is dependent on these kinases. Interestingly Cdk1/2

regulatory subunits, CYCA and CYCB colocalise with centromeres and manipulation of these cyclins by either overexpression or depletion (RNAi) impacts negatively on CAL1 levels and localisation. We therefore propose that control over CAL1 levels may be achieved through coupling degradation to Cdk1/2 mediated phosphorylation. This could provide a mechanism by which cell cycle regulators, CYCA and CYCB, affect CAL1 stability via local inhibition as summarised in our proposed model (figure 7.2). Our findings suggest that CAL1 turnover may be fundamental to its role in executing limited CENP-A and CENP-C incorporation at centromeres during meiosis I and spermatocyte maturation. CAL1 turnover could effectively serve as a temporal cue for spermatids to shut off CENP-A assembly as CAL1 dosage clearly modulates the termination of CENP-A incorporation at centromeres (figure 6.4 B).

How then is CAL1 rapidly extinguished from centromeric chromatin? Over the years peptide motifs that target proteins for destruction have been identified. Among these motifs PEST sequences (Proline, Glutamic acid, Serine and Threonine) have been shown to be critical proteolytic signals (reviewed in Rechsteiner & Rogers, 1996). Using an algorithm EMBOSS:epestfind, http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind, we were able to identify 9 potential PEST sequences on CAL1. Although 8 of these are poor PEST motifs, 1 of these motifs at position 820-862, sparks real interest. Because many PEST sequences are conditional signals, there are a number of ways to activate them. One of these includes phosphorylation where phosphorylation precedes degradation, (Lin & Desiderio, 1993; Yaglom et al., 1995). A considerable body of evidence supports the idea that PEST sequences target proteins for degradation by the 26S proteasome. Thus taken together, we propose a mechanism by which CAL1 abundance and transient association and dissociation from centromeres is regulated via phosphorylation-mediated degradation.

We thus propose a model in which CAL1 is the relevant substrate for CYCA or CYCB associated kinase activity (figure 7.2). We propose that the unusual timing of CAL1 assembly at centromeres in prophase I is coupled to high Cdk activity to regulate CAL1 availability via phosphorylation of CAL1 Serine 157. This phospho moiety in turn provides a signal that tags CAL1 for degradation by the 26S proteasome to spatially restrict and limit CAL1, and ultimately CENP-A (figure 7.2).

Although it seems logical to look among ubiquitin pathways (E2 or E3 ubiquitin ligases) for components that would lead to CAL1 recognition by the 26S proteasome, one cannot exclude the possibility that phosphorylation alone may lead to recognition of CAL1 by the 26S proteasome in a ubiquitin independent manner. A ubiquitin independent degradation pathway mediated via the 26S proteasome has previously been reported in literature for Ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis (Murakami et al., 1992). In this study purified 26S proteasome complex was shown to catalyse ODC degradation in the absence of ubiquitin (Murakami et al., 1992). This finding led to a shift in current opinion where the 26S proteasome once believed to be specific for degradation of ubiquitinated proteins (Driscoll & Goldberg, 1990; Eytan et al., 1989; Kanayama et al., 1992; Orino et al., 1991) now has a more general function in selective removal of short-lived proteins by recognising degradation signals other than ubiquitination (Murakami et al., 1992).

Alternatively, it is also possible that phosphate addition to CAL1 Serine 157 might induce conformational changes that expose CAL1 for interaction with other proteolytic components, in particular, ubiquitin conjugation enzymes (figure 7.2). Using an algorithm for prediction of ubiquitination sites; UbPred (data not shown), we identified 4 high confidence sites for CAL1 ubiquitination. This suggests that CAL1 may be a direct substrate for ubiquitin conjugation. Analysis of CAL1 stability in *Drosophila* mutant for ubiquitin activating enzymes may provide conclusive results on the involvement of ubiquitin conjugation in CAL1 removal by the proteasome. It would also be interesting to mutate or delete CAL1 amino acid residues 820-862 and determine whether in the absence of this PEST motif if CAL1 Serine 157 is still effective at regulating CAL1 turnover.

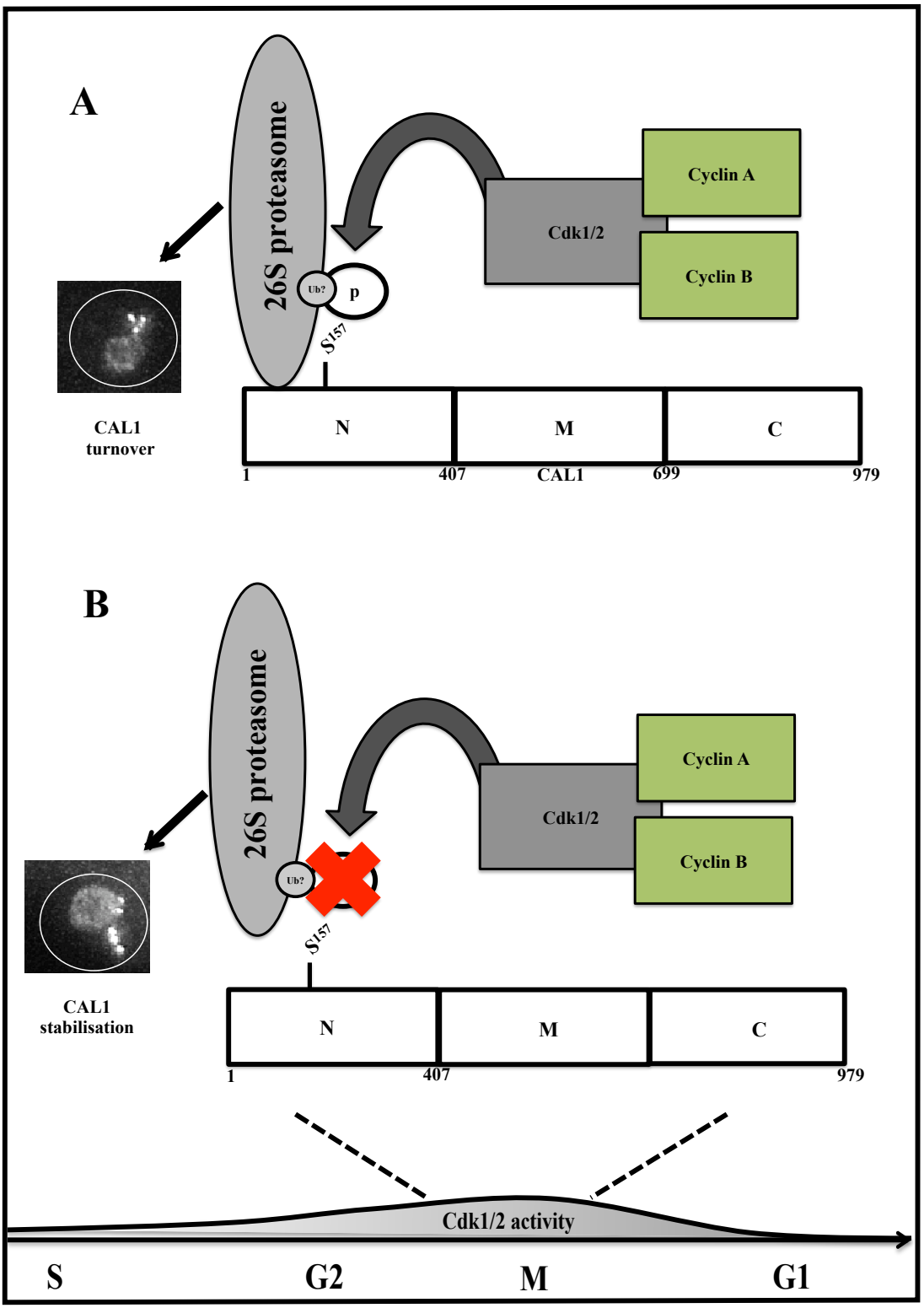


Figure 7.2. Potential model for the regulation of CAL1 through CYCA or CYCB associated kinase activity. (A). Accumulation of CYCA or CYCB associated kinase activity leads to phosphorylation of CAL1 at Serine 157, which potentially tags CAL1 for turnover by the 26S proteasome leading to normal CAL1 localisation at both centromeric and nucleolar pools (inset). **(B).** Failure of Cdk1/2 activity to accumulate leads to a dephosphorylation event where CAL1 is left 'unmarked' for recognition by the 26S proteasome leading to CAL1 stabilisation at both centromeric and nucleolar pools (inset).

7.3. Centromeric chromatin in mitosis: Relevance to genome instability

During the past few years, our understanding of the components required for CENP-A assembly and regulation has vastly improved. The identification of CENP-A assembly factors; Cdks as molecular triggers facilitating CENP-A assembly, as well as proteolysis mediated degradation of excess CENP-A to prevent mistargeting, represent a few of the many milestones that provide a more complete understanding of how centromeres work and how they are regulated during the cell cycle (reviewed in McKinley & Cheeseman 2016). These findings raise novel questions in the applicability of this knowledge to a common mitotic disease state such as cancer.

As CENP-A epigenetically marks the central platform for kinetochore assembly, its tight regulation is instrumental to avoid mis-segregations and genomic instability, a major contribution of tumorigenesis and cancer progression. Noteworthy, CENP-A is overexpressed in colorectal, breast and lung cancer tissues (Tomonaga et al., 2003; Zhang et al., 2017). Immunostaining with anti-CENP-A antibodies in colorectal cancer tissues showed mistargeting of CENP-A to non-centromeric chromatin suggesting a deregulation in CENP-A targeting and an important role for CENP-A in colorectal cancer possibly by contributing to ectopic centromere formation. How CENP-A is overexpressed in these cancer cells remains unclear.

Interestingly, this level of CENP-A mistargeting has been reproduced in different model systems, (Heun et al., 2006; Mendiburo et al., 2011) and a mechanism for removal of mistargeted CENP-A via ubiquitin mediated proteolytic degradation from non-centromeric loci has been proposed in yeast and flies (Collins et al., 2004; Moreno-Moreno et al., 2006). It would therefore be interesting to tease out if in cancers overexpressing CENP-A, this mistargeting is due to a failure in the proteolytic machinery to rid the cells of excess CENP-A. Moreover insights into post translational modifications of CENP-A assembly factors via phosphorylation by Cdks (reviewed in McKinley & Cheeseman, 2016) to regulate timing of CENP-A assembly could also be an interesting avenue to explore. In primary cancer tissues overexpressing CENP-A it would be interesting to investigate whether assembly factors such as HJURP or Mis18BP1 are constantly switched on (via dephosphorylation events) to promote ectopic CENP-A incorporation. If CENP-A is

directly or indirectly the driving force of tumor progression suppression of its expression, or its assembly factors in cancer cells, might open potential therapeutic avenues.

It is interesting to note that pioneering work by Zhang et al., 2016 developed a novel Centromere and kinetochore gene Expression Score (CES) signature that quantifies centromere and kinetochore gene misexpression in cancers. High CES values correlate with increased levels of genomic instability, adverse tumor properties and poor prognosis for patient survival for early-stage breast and lung cancers. The CES signature also forecasts patient response to adjuvant chemotherapy or radiotherapy (Zhang et al., 2016). These results demonstrate the importance of applying knowledge of basic biological functions to cancer research.

7.4. Centromeric Chromatin in Meiosis: Relevance to fertility

In meiosis, chromosomal aneuploidy is the leading cause of pregnancy loss and developmental disabilities in humans (Hassold & Hunt, 2001). It has become apparent that infertile men have significantly higher levels of sperm aneuploidy compared to their fertile counterparts. Because CENP-A is vital for chromosome segregation, it sparks real interest to characterise CENP-A dynamics in human sperm. Interestingly, it has been shown that CENP-A is quantitatively retained on mature spermatozoa in tissue of bovine origin (Palmer et al., 1990). This finding is quite striking, as a prominent feature of spermatogenesis in mammals is the replacement of most somatic and testes specific histones with protamines (Palmer et al., 1990), sperm specific peptides that pack DNA into a highly compacted structure. Indirect immunofluorescence studies further revealed that CENP-A is retained in sperm nuclei in discrete foci rather than being dispersed throughout the sperm head (Palmer et al., 1990). These observations suggest that CENP-A is a functionally crucial component of centromeres and that pre-existing CENP-A:DNA interactions are likely vital in organising the centromeres of the paternal genome during early embryogenesis (Palmer et al., 1990). In agreement, Raychaudhuri et al., 2012 demonstrated that in *Drosophila* when normal oocytes are fertilised with sperm lacking centromeric

CENP-A, paternal chromosomes fail to recruit the maternally provided CENP-A and cannot generate functional kinetochores during mitosis. As a result, gynogenetic haploid embryos develop (Raychaudhuri et al., 2012). Moreover partial depletion of centromeric CENP-A in sperm resulted in reduced centromeric CENP-A being maintained throughout development of the next generation without being restored to normal amounts (Raychaudhuri et al., 2012). It is therefore pressing to uncover potential roles for this epigenetic mark in fertility. CENP-A may present an unexplored molecular signature that drives sterility and could thus present potential prognostic and diagnostic markers for fertility.

In apparent contrast to mitosis, very little is known about regulation of meiotic CENP-A assembly. In this thesis I have shed light into the requirements of the CENP-A assembly factors, CAL1 and CENP-C in CENP-A assembly in *Drosophila* spermatocytes. I have also uncovered the molecular trigger facilitating specific CENP-A incorporation into centromeric chromatin via Cdk mediated proteolytic degradation of the CENP-A assembly factor CAL1. These findings raise novel questions in the applicability of this knowledge to fertility. It would be interesting to firstly characterise CENP-A assembly in human donor sperm and assess if assembly is coupled to high kinase activity as in plants and flies (Dunleavy et al., 2012; Schubert et al., 2014). From this it would then be interesting to investigate CENP-A levels in fertile donors compared to subfertile patients. It would also be interesting to assess the level of CENP-A in subfertile patients undergoing treatment for infertility who achieve pregnancy compared to those who do not. Our published work from *Drosophila* spermatocytes in chapter 3 gives insight into some of these questions. We demonstrate that reduced CENP-A levels in spermatocytes correlate to reduced sperm quality, with ~ 30-50% T5 spermatid nuclei displaying abnormal decondensation, characteristic of aberrant DNA packaging, as well as reduced fertility (Kwenda et al., 2016)

In light of the proposed experimental design, if CENP-A levels are lower in subfertile or infertile patient samples, it would be imperative to speculate the possible causes for reduced CENP-A on mature sperm. Again our findings in chapters 3, 4 and 5 point to CENP-A assembly factors, CENP-C and CAL1, as well as cell cycle machinery components, Cdks and the proteasome as detrimental for the maintenance of CENP-A

on spermatocytes and mature sperm. Thus, our presented findings in this thesis would be key to answering these novel questions for translational research, where assessing regulation of CENP-A assembly may prove essential in understanding the underlying factors leading to deregulated CENP-A dynamics in disease states.

7.5 Conclusion

In summary, we conclude that in *Drosophila* spermatocytes, CENP-A assembly is achieved by CAL1 and CENP-C and is regulated in a cell cycle dependent manner via Cyclin dependent kinases and the proteasome. Future efforts in understanding CENP-A regulation in primary tumor samples as well as in human sperm samples will undoubtedly extend our insight on the importance of CENP-A in these biological conditions with implications for diagnostics and treatment.

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