Mechanistic Understanding Of PCNA-based Interactions: The Interplay Between CAF-I and Rrm3p for the DNA Sliding Clamp PCNA

By

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The Proliferating Cell Nuclear Antigen (PCNA) is a homotrimeric ring that encircles DNA at the replication fork and acts as a docking hub for numerous replication related proteins. Chromatin Assembly Factor I (CAF-I) and rDNA Recombination Mutation 3 (RRM3) have been implicated in the conversions of the epigenetic state during DNA replication and interact with PCNA through a PCNA-Interacting Peptide (PIP) sequence. The exchange of these factors at the replication fork must play a key role in the coordination of successful replication and the maintenance of chromatin structure. I used a co-immunoprecipitation assay and a GST-based pulldown assay to determine the interaction profile of these proteins and observed that a co-operative tri-partite complex seems plausible. Furthermore, I investigated the significance of CDC7 and CDC28 phosphorylation on CAF-I association with PCNA using a Yeast-Two Hybrid assay. My results show that eliminating putative phosphorylation sites reduced association to differing degrees.
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List of Abbreviations

ASF-1 – Anti-Silencing Function-Protein-1
BSA – Bovine Serum Albumin
CAC – Chromatin Assembly Complex
CAF-I – Chromatin Assembly Factor-I
CDC28 – Cell Division Cycle-28
CDC7 – Cell Division Cycle-7
ChB – Chromatography Buffer
CpG – Cytosine-guanine dinucleotide
DDK – Dbf4 Dependent Kinase
EDTA – EthyleneDiamineTetraacetic Acid
FACT – FAcilitates Chromatin Transcription
FOA – 5-Fluoro-Orotic Acid
FOB1 – FOrk Blocking less-1
GST – Glutathione S-Transferase
HAT – Histone AcetylTransferase
HDAC – Histone DeACetylase
IPB – Immunoprecipitation Buffer
LB – Luria-Bertani broth
MCM – Mini-Chromosomal Maintenance
Ni-NTA – Nickle-NitriloTriacetic Acid
OD – Optical Density
ONPG – Ortho-NitroPhenyl-β-Galactoside
ORF – Open Reading Frame
PBS – Phosphate-Buffered Saline
PCNA – Proliferating Cell Nuclear Antigen
PCR – Polymerase Chain Reaction
PEV – Positional Effect Variegation
PIP – PCNA-Interaction Peptide
PMSF – PhenylMethylSulfonyl Fluoride
RAP1 – Repressor Activator Protein 1
RFB – Replication Fork Barrier
RRM3 – Ribosomal DNA Recombination Mutation-3
rDNA – Ribosomal DNA
SC – Synthetic Complete
SDS – Sodium Dodecyl Sulfate
SIR – Silent Information Regulator
TG1-3 – Telomeric Repeats
TPE – Telomere Position Effect
U – Units of β-Galactosidase
Y2H – Yeast Two-Hybrid
YPD – Yeast Peptone Dextrose
1.0 Introduction

■ 1.1 Epigenetics

The field of epigenetics is defined by the study of heritable changes in gene expression that are not coded for in the DNA sequence but by chromatin structures that are transmitted from mother to daughter cells (1). Within a multicellular organism, most cells harbour the same genetic material; however, different cells can express distinctive proteins and carry out unique functions. This is possible through epigenetic modifications that confer activation or silencing of appropriate genes without altering the genetic code (2). Epigenetic modifications (often referred to as epigenetic marks) include covalent modifications to DNA (i.e. methylation) and post-translational modifications to histones (3). The establishment of these modifications results in the alteration of chromatin structure, which can be categorized as euchromatin or heterochromatin (4). Euchromatin is a region of DNA that has its genes loosely packaged, and therefore more accessible to transcription factors. Comparatively, heterochromatin is more densely condensed and does not contain as many genes but harbours many repetitive DNA sequences (5). The epigenetic marks on chromatin can change the nature of the interaction of regions of DNA with cell factors, or entirely block access to the gene (6). Moreover, these epigenetic marks can be faithfully inherited from mother to daughter cells through replication. The fundamental differences between the states of chromatin form the crux of epigenetics and gene silencing.

■ 1.2 Nucleosomes and Post-Translational Modification of Histones

The nucleosome, a protein complex comprised of an octamer of histone proteins, serves to organize and package DNA. Each nucleosome is bound by 147 bp of DNA and contains pairs of the following core histones: H2A, H2B, H3 and H4 (7, 8). H1 is another histone that is not part of this core but interacts with the octameric nucleosome (9). H2A/H2B histones are found in
dimer units while H3/H4 histones exist as tetramer units. Nucleosomes were formerly believed to only serve an organizational role in their interactions with DNA. However, recent findings suggest they also have a major functional role (10). The N-terminal tails of the core histones and the post-translational modifications on them are significant for the regulation of gene expression, regulation of origin firing during DNA replication, and for the suppression of transposon mobility (10–12).

The N-terminal tails of the histones are exposed outside of the nucleosome complex where they can be chemically modified in several ways, including acetylation, methylation or phosphorylation (12). Acetylation of specific lysine residues on histone N-terminal tails have been shown to neutralize the positive charge of the histone, which reduces its affinity to bind DNA (9, 12). This change is postulated to lead to the unraveling of the nucleosome, allowing transcription factors to access the genes that were once tightly bound (9, 12). Therefore, the addition of an acetyl group generally leads to decreased DNA compaction and increased gene expression. Histone acetyl-transferases (HATs) carry out this reaction and these HATs are evolutionarily conserved from yeast to humans (13). Acetylation is a dynamic yet reversible process which is countered by the activities of histone deacetylases (HDACs) (14).

Histone phosphorylation is another post-translational modification that significantly affects transcription in the cell. Studies analyzing serine or threonine protein kinases have shown that phosphorylation is possible on all H1, H2A, H2B, H3, and H4 histones (15). Interestingly, Serine 10 on H3, when acetylated, was less likely to be phosphorylated. When histone phosphorylation is increased globally using calyculin A, a serine/threonine phosphatase inhibitor, DNA fragmentation occurs through unknown mechanisms (16). Furthermore, loss of H2AX phosphorylation site S139A; a common DNA repair modification, also leads to DNA
fragmentation (17). These observations suggest that histone phosphorylation is a key caretaker process that is linked to genome integrity and DNA repair. Despite being poorly understood, it seems that histone phosphorylation has a crucial role in chromatin structure maintenance and overall cellular wellbeing.

The act of methylation constitutes the covalent addition of a methyl group and can occur on DNA or on histones. In vertebrates, DNA methylation occurs mostly on CpG islands, which are comprised of a series of a cytosine nucleotides adjacent to guanine nucleotides along a stretch of DNA (18). The mammalian genome contains a high density of CpG islands, but predominantly upstream of gene promoter sites (19). DNA methylation leads to heterochromatin formation and compaction of chromatin, which restricts access to transcription factors. The model organism, *Saccharomyces cerevisiae*, is found not to have DNA methylation on its genome. In plants, DNA methylation occurs at sequences and in patterns that are distinct from vertebrates (20). Therefore, DNA methylation is not uniform across various forms of life.

Histone methylation is well studied at certain residues such as H3 lysine 9 (H3K9) or H3 lysine 27 (H3K27). Lysine methyltransferases and demethylases are known to dynamically affect both of these key residues on H3 (21). Histone methylation patterns have been found to establish long-term effects and affect neural plasticity of neurons in studies addressing cocaine addiction (21).

In summary, post-translational modifications of histones and DNA are diverse and plentiful; however, their effects are not mutually exclusive of one another. Contemporary research has demonstrated that different modifications appear to be conversing with one another. Each alteration reinforces its effects through a self-perpetuating cycle that leads to long-term silencing of a gene of interest (22). DNA methylation machinery has been found to recruit
complexes that contain HDACs, which implies DNA methylation lays the foundation for silencing (23). Conversely, H3K9 methylation by Suv39h H3K9 methyltransferases, was required for DNA methylation, suggesting the opposite series of events (24). Furthermore, transcriptionally silent heterochromatin was also found to contain de-acetylated histones which reinforces this notion of synergy (23, 25, 26).

1.3 Establishment and Maintenance of Silencing

The regulation of gene expression through restricting access of transcription factors to the locus is termed gene silencing. Long-term or transient silencing of specific genes is crucial to the wellbeing of a cell. In *S. cerevisiae*, the transcription factor Repressor Activator Protein 1 (Rap1p) acts as a sequence-specific transcriptional repressor at the silent mating type locus and functions to establish heterochromatin and silencing (27). Rap1p recognizes DNA using the consensus sequence 5’-ACACCCATACATTT-3’ and a similar site within the telomeric repeat 5’-ACACCCACACACCC-3’, respectively (28). Rap1p is believed to determine where the formation of heterochromatin is nucleated and initiates a cascade of histone modification that leads to the silencing of nearby genes.

To silence the target locus *S. cerevisiae* uses a class of genes that encode for components of the silent information regulator (Sir) complex. This Sir silencing complex is composed of three proteins, Sir2p, Sir3p, and Sir4p respectively (29). Sir2p is classified as a class III deacetylase and is recruited to genes and regions near the telomeres to confer silencing (30). Sir3p and Sir4p are recruited to DNA by Rap1p and also read the N-termini of H3 and H4 histones as it spreads across the chromatin (29) (Figure 1).

Specifically at the telomere, Rap1p reads and recognizes the telomeric repeats (TG1-3) to bind to a telomere consensus sequence (31). Rap1p then recruits Sir3p and Sir4p to the telomere
to initiate heterochromatin formation. The final component to be recruited is Sir2p, which deacetylates adjacent nucleosomes at specific lysine residues on the histones, with the H3-K16 residue being critical for silencing (32). For example, it is believed that Sas2p which encodes for a HAT opposes the activity of Sir2p by acetylating the very same H3-K16 thus countering the spreading of heterochromatin and to establish a dynamic chromatin boundary away from the telomeres (Figure 2).
Figure 1. General schematic for the spread of heterochromatin from a telomere to sub-telomeric regions. Rap1p reads and binds to TG1-3 telomeric repeats (black arrows), which will signal the recruitment of Sir3p/Sir4p silencing protein to initiate silencing complex formation. The histone de-acetylase, Sir2p is recruited by Sir3p/Sir4p and deacetylates the adjacent nucleosomes at specific lysine residues on the histone. De-acetylated histones (green) are shown to be spreading from the telomere into the sub-telomere where there are acetylated histones (white).
1.4 Position Effect Variegation and Telomere Position Effect

Unidirectional spreading of heterochromatin from the centromere or the telomeres and the challenging of this spreading by HATs and chromatin boundaries allow for the occasional switch between active and silent epigenetic states. This phenomenon is called Position Effect Variegation (PEV) (33). The expression profile of genes located at such putative heterochromatin/euchromatin boundaries or transition regions is not stable, but is quasi-stable (34). The heterochromatic marks can flex over the genes and we term these transitions between active and silent state, epigenetic conversions.

A classic example of the PEV phenomenon has been observed in *Drosophila melanogaster*. The *WHITE* gene in fruit flies forms a component of an ABC transporter which allows for the intracellular transport of guanine and tryptophan (35). This gene is functional in wild type flies and contributes to the development of the red eye phenotype. The eyes will appear red when the *WHITE* gene is in the euchromatic region, and white pigmented eyes when found in a heterochromatic region (36). A chromosomal translocation event in this organism causes the *WHITE* gene to be moved to a heterochromatic boundary region close to a centromere. A combination of red and white patchy coloured eyes is observed in these flies due to random switches between silent and active state of the *WHITE* gene (36).

Gene silencing at telomeres can produce similar epigenetic changes in neighbouring genes. This phenomenon is termed Telomere Position Effect (TPE). In this situation, the genes within the vicinity of telomeres are repressed by the spreading and retraction of Sir proteins, and similarly to the *WHITE* gene in PEV, variegate in their expression (Figure 2) (35, 37). In Figure 2 an example of TPE using the *ADE2* gene is presented. Similar epigenetic instability is observed in the subtelomeric regions of many other organisms (35).
Figure 2. Changes in gene expression at a heterochromatin boundary at the sub-telomere. Heterochromatin (dark green circles) spreads from the telomere (black arrows) into the sub-telomeric region. Three scenarios are presented: the ADE2 gene is placed in the heterochromatin (maroon rectangle), in the euchromatin (white rectangle), or at the boundary between heterochromatin and euchromatin (maroon and white rectangle). The resulting phenotypes of placing the ADE2 gene in the heterochromatin, at the heterochromatin/euchromatin boundary and euchromatin region respectively are presented. The ADE2 gene is responsible for producing adenine, and when it is placed in the heterochromatic region, it cannot be expressed. This results in accumulation of a red pigment that is produced in the adenine biosynthetic pathway. When the ADE2 gene is placed in the euchromatin, it can be expressed and will not build up the red pigment, resulting in a white colony. If the ADE2 gene is placed at the heterochromatin/euchromatin boundary, the heterochromatin flexes over the gene and back, and we observe sectored colonies.
1.5 – DNA Replication and Nucleosome Reconstitution

During eukaryotic DNA replication, DNA and nucleosomes and their respective modifications must be faithfully replicated and reproduced. Histone chaperone proteins coordinate with other replication factors to confer nucleosome re-formation in the wake of the fork. Nucleosome assembly proteins (Nap) are a family of histone chaperones that regulate interactions between histones and other proteins and DNA (11). In yeast, octamers of histones lacking N-terminal tails were unable to interact with yeast Nap proteins, signifying an essential need for these residues (11). It is important to mention that the N-terminal tails of the histones protrude away from the core nucleosomal structure and are exposed to interactions with a significant number of proteins, including the NAPs. To ensure maintenance of chromatin silencing or activation, it is essential that epigenetic modifications in these N-termini are re-established after each passage of the replication fork.

1.6 Maintenance of Epigenetic Marks: Histone Transfer at the Replication Fork

Histone transfer at the replication fork is a necessity for the faithful inheritance of the epigenetic state. Figure 3 depicts a current model for the transfer of histones and histone marks across the replication fork. The figure emphasizes the role of several key histone chaperones and their role in the disassembly and reassembly of nucleosomes. The actual DNA replication is not presented in detail (38).

The DNA replication helicase, Minichromosome Maintenance Protein Complex (MCM), is a key component of the pre-replication complex that assembles at the origin of replication (39). The MCM acts as a hub for other factors such as Cdc18 and Cdt1 to bind to initiate the pre-replication complex formation. MCM also associates with a histone chaperone called Facilitates Chromatin Transcription (FACT). FACT binds H2A/H2B histones and transfers them behind the
replication fork (40). DNA is tightly wrapped around the nucleosomes, hence FACT destabilizes the interactions between DNA and the nucleosome (41). This MCM/FACT activity allows the fork to progress smoothly through the chromatin. Anti-Silencing Function 1 protein (Asf1p) has a global role in chromatin disassembly, and more specifically acts as a histone chaperone for H3/H4 histones at the replication fork (42). It also associates with the MCM complex and can co-chaperone an H3/H4 dimer at the replication fork (43). Both FACT and Asf1p in yeast work in tandem to systematically disassemble and transport parental histones to chaperones associated with the daughter stands of DNA.
Figure 3. The replication fork and corresponding nucleosome reassembly via chaperone proteins. Histone chaperones FACT and Asf1p disassemble the parental nucleosomes found upstream of the fork and deposit them onto the nascent strand with the help of the CAF-I complex. CAF-I associates with PCNA (orange ring) at the replication fork to function. Newly synthesized histones (yellow circle), are deposited on the nascent strand and histone modifying enzymes replicate the post-translational modification from the parental histones (green circles).
During DNA replication, newly synthesized histones also need to be chaperoned to the replication fork. The parental nucleosome is randomly segregated onto the nascent strands while the delivery of new histones and *de novo* nucleosome assembly completes the histone replication process. Several histone chaperones that are not tethered to the fork, including Asf1p and FACT, bring the newly synthesized histones behind the replication fork and interact with Chromatin Assembly Factor I (CAF-I) (44, 45). CAF-I is capable of accepting both parental and newly synthesized histones. The interplay between these different components allows for the deposition of the new histones. After the assembly of the nucleosome, histone modifying enzymes will recognize the new histones and will begin to modify them to copy the histone marks of the parental histones. H3K9 and H3K27 trimethylation are epigenetic marks that are not significantly found on new histones (46). Histone modifying enzymes act upon these new histones with the help of replication factors at these locations to impart their respective epigenetic marks and maintain the state of the chromatin. This process is not so well understood and the timing and specificity of the acting complexes awaits further characterization.

**1.7 Nucleosome Reconstitution and H3/H4 Tetramer Splitting at the Replication Fork**

As already discussed, the nucleosomes at the advancing replication fork need to be disassembled and reassembled onto the two nascent strands. One complete set of histones comes from the parental chromatin, and a new set of histones is synthesized and deposited behind the fork (47). Here emerges the question, do the parental nucleosomes disassemble into their individual histone units and constitute a proportion of each nascent strand (semi-conservatively replicating) or do the parental histones conservatively move to a nascent strand?

A study by Prior *et al.* (1980) argues that the parental H3/H4 do not split into dimers but are ferried as a tetramer across the replication fork. This study linked iodoacetoxypyrrene to H3
histones. When two pyrene residues are of close proximity, they fluoresce green, and when there is no secondary pyrene residue within a close vicinity, it appears blue (48). The authors allowed enough time for the majority of nucleosomes in the *Physarum* to incorporate the labelled histone. They observed consistently green fluorescence for 90 hours, inferring a long-term stability of H3/H4 tetramers (48). Other studies have also supported the notion that there is a conservative transfer of intact H3/H4 tetramers to newly synthesized DNA (49, 50).

In contrast to these findings, Xu *et al.* (2010) state that the old tetramer is distributed onto the nascent daughter strands intermittently with new histones to form a more semi-conservative nucleosome assembly (51). Xu *et al.* (2010) observed increased tetramer splitting with DNA replication-dependent deposition and that newly synthesized H3-H4 dimers were incorporated with old dimers (51).

■ **1.8 Proliferating Cell Nuclear Antigen – A Processivity Factor**

Proliferating Cell Nuclear Antigen, (PCNA) is a homotrimeric DNA clamp found at the replication fork (Figure 3). PCNA has a positively-charged inner surface and an outer surface composed of β-sheets, and similar replication clamps can be found in all branches of life (52). It encircles the double-stranded DNA and recruits crucial factors in DNA replication and replication-linked processes to the replication fork (53). PCNA tethers polymerases to DNA and increases processivity from 10 to thousands of nucleotides per second (54). Earlier studies have found that a single PCNA trimer is present on both the leading and lagging strand during replication (55). Contemporary findings suggest that more PCNA trimers can be found on the lagging strand than at the leading strand (56).
To interact with PCNA, many but not all PCNA-interacting proteins possess a PCNA-Interacting Peptide (PIP) box consensus sequence: \((Q/M)\)-X-X-(I/L/M)-X-(F/Y)-(F/Y) or a KA-Box: K-A- (A/L/I)-(A/L/Q)-X-X-(L/V) \((57)\). A trimer of PCNA possesses three inter-domain connecting loops, which is where the PIP or KA sequence interacts with PCNA \((58)\). PCNA-interacting proteins only bind to the C-side of the trimeric ring \((58)\). Many PIP-containing proteins have been found to bind the inter-domain connecting loop. Provided that there are only three available sites on a trimer, one can predict that there can be competition or cooperation for these sites during replication.

As replication proceeds, the DNA clamp slides along with its bound replication factors. PCNA has been described as a conductor of replication, faithfully switching its associated protein factors at appropriate time points \((54)\). The mechanism underlying the switch of interacting partners is currently unknown, however it is postulated that affinity-driven competition, phosphorylation, sumoylation or a combination of modifications could be the cause of this coordinated switch. Amongst the binding partners of PCNA, we are interested in the histone chaperone CAF-I and DNA helicase Rrm3p.

In addition to implications in DNA replication/repair and histone reconstitution, PCNA plays a role in the pathogenesis of Parkinson’s disease and Lupus \((59, 60)\). Recent findings suggest that S-Nitrosylation causes signalling changes in neurons that can be linked to the neurodegenerative disease Parkinson’s \((59)\). PCNA is typically found in the nucleus, but S-Nitrosylation causes it to be found in the cytosol. It was found that PCNA can interact with caspase 9 to block the apoptotic pathway, and that S-Nitrosylation prevents this interaction. Hence, chemical modifications on PCNA promote initiation of the apoptotic cascade resulting in the clinical manifestations of Parkinson’s Disease \((59)\). Lupus is a term used to define a broad
spectrum of diseases that cause inflammation of the skin. Typically, Lupus is caused by autoantibodies, which are antibodies created by an individual that target the host’s own proteins. Anti-PCNA has been observed in the sera of systemic lupus erythematosus (SLE) and in CNS lupus (60). The antibody has been found to bind to the primary epitope found on PCNA’s C-terminal domain. The effects of anti-PCNA are currently not known because Lupus patients possess many autoantibodies in conjunction with anti-PCNA (61).

1.9 Chromatin Assembly Factor I – A Histone Chaperone Complex

CAF-I is a histone chaperone complex composed of three subunits: Cac1p, Cac2p and Cac3p (62). Cac1p is the largest of the three subunits at 70.2 kDa and it mediates association with PCNA via its single consensus PIP box. Strains of Saccharomyces cerevisiae, in which the CAC1 gene has been deleted, show decreased epigenetic switching at the telomeres and are more sensitive to ultraviolet radiation (63, 64). CAF-I has also been found to facilitate the repair of single-stranded breaks in DNA and nucleotide excision repair (38). These results indicate that CAF-I is involved in DNA repair, but the precise mechanisms are not known. The mechanisms of the loss of epigenetic conversions in cac1Δ mutants is even less well understood. As mentioned before, CAF-I assembles nucleosomes behind the replication fork. It has a preference for H3/H4 histones over H2A/H2B (44). CAF-I deposits H3-H4 histones onto newly synthesized DNA and it works in tandem with Asf1p at nascent daughter strands (38). Asf1p has been found to interact directly with Cac2p, and this interaction could allow for the transfer of histones from Asf1p to the CAF-I complex. The DNA clamp PCNA slides along the nascent daughter strands and serves as a hub for the binding of CAF-I and other replication-related proteins (65). PCNA is the key factor in this process, and it is strongly believed that the PCNA-association allows CAF-I to carry out the coordinated assembly of the nucleosomes. Homologs of the CAF-I complex can
be found in all eukaryotes. In humans, the Cac1p, Cac2p and Cac3p subunits are found as p150, p60, p48 respectively (38). Human Cac1p possesses two PIP consensus sequences, but the yeast counterpart only possesses one (66). The activities of CAF-I in both organisms functionally overlap and therefore studies carried out in yeast can be related to its counterparts in humans, with discretion.

- **1.10 Cell Cycle Kinases**

  Cyclin-dependent kinases, or CDKs, phosphorylate residues on substrates to affect their activity and coordinate it to the eukaryotic cell cycle (67). Cdc7p in particular associates with Dbf4p cyclin to form a DDK-complex, which acts upon pre-replication complexes (pre-RCs) to initiate replication (68). Studies have shown that Cdc7p is critical in the transition from G1 to S phase in the cell cycle. For example, Cdc7p-Dbf4p phosphorylates the MCM helicase complex found at the origin of replication. This modification triggers DNA replication by stimulating the DNA helicase activity of MCM, unwinding the origin DNA and allowing access to the remaining replication factors (68).

  Cdc28p is called the master regulator of the cell cycle in *Saccharomyces cerevisiae* and is regulated by *Cln* and *Clb* cyclins (69). Cdc28p also functions to initiate the transition from G1 to S phase by interacting and activating an array of transcription factors (67). Pre-replicative complexes form during G1 phase and do not form again until after completion of mitosis. Re-initiation of replication is prevented by Cdc28p and Clb cyclins, which work together to prevent pre-RC formation till after the end of mitosis (70).
1.11 The Effect of Phosphorylation of CAF-I

It is not known how the activity of CAF-I is coordinated with the cell cycle and with specific events during DNA replication. For example, it is not clear precisely how the complex is loaded onto the replication fork and how the binding of CAF-I to PCNA is regulated. CAF-I has been found to be phosphorylated \textit{in vivo} by Cdc28p and Cdc7p and the sites of phosphorylation have been determined via mass spectrometry analysis (66, 71). Based on this analysis, site-directed mutagenesis (SDM) studies from our lab addressed the role of the phosphorylated residues on Cac1p. It was found that serine residues 94 and 515 are putative substrates for Cdc28p (66). Similarly, serine residues 238, 501, and 503 are speculated to be phosphorylated by Cdc7p. These chemical modifications are implemented in early S phase and contribute to Cac1p’s ability to associate with chromatin and PCNA.

These studies showed with significant level of confidence that serine residues 94 and 515 are phosphorylated by Cdc28p however, the effects of Cdc7p are not as well elucidated (66). The mutant strains containing mutations at S94 or S515 did not associate with chromatin to the same degree as their \textit{wild type} counterparts, but their ability to bind PCNA was unaffected. On the other hand, the S238, S501 and S503 mutants were able to bind to chromatin. Only the S238A mutation slightly decreases the association of Cac1p with PCNA. These observations raised the question, does phosphorylation of Cac1p by Cdc7p regulate the binding of CAF-I to chromatin and the replication fork or regulate some other, yet unknown, aspect of the activity of CAF-I? Further research into these putative phosphorylation sites may reveal their relation to CAF-I and chromatin/PCNA association at the replication fork.
1.12 Rrm3p - A DNA Helicase

Rrm3p is a 5’ to 3’ DNA helicase that has been found to interact with PCNA via a PIP consensus sequence found at its N-terminus (72). Deletions of the N-terminus and in turn the PIP box, have abolished its PCNA interaction, however DNA helicase functionality persists (72). Given that it is a 5’ to 3’ helicase, it is postulated that it only functions on the lagging strand. Rrm3p plays a significant role in the replication of subtelomeric DNA, in the stabilization of ribosomal DNA and in the recommencement of paused replication forks. The pausing of replication forks occurs commonly due to protein-DNA barriers formed by tightly bound non-histone proteins (73) (Figure 4). During replication, the replisome complex cannot be paused indefinitely, as it may cause the replication fork to become unstable and collapse. To overcome this, replisomes deploy a helicase to disrupt the protein-DNA barrier upstream of the replication fork and to allow for resumption of replication (73). Currently it is not well understood whether Rrm3p is travelling with the fork or only is recruited when a protein-DNA barrier is encountered. Interestingly, some of the most potent replication pausing sites are found in the subtelomeric regions of the chromosomes and these are the same positions where epigenetic meta-stability leads to variegation.

It has been proposed that pausing of replication forks can be linked in epigenetic conversions and histone turnover (34). When the replication fork is stalled, nucleosome disassembly ahead of the fork is also stalled. Without contributions from the parental strand, the nascent daughter strands will only possess newly synthesized histones to reconstitute the nucleosome. Figure 5 shows the incorporation of new histones that lack the appropriate post-translational modifications found on the parental strands. Rrm3p association with PCNA allows
it to displace bound proteins ahead of the replication fork, allowing DNA replication and histone turnover to resume (72).
Figure 4. Depiction of Rrm3p DNA helicase mediated resumption of a paused replication fork. A) A tight protein-DNA interaction upstream of the replication fork presents a barrier for the progress of the fork. This leads to indefinite pausing of the replication fork till the recruitment of the DNA helicase Rrm3p. B) Rrm3p is recruited to the replication fork where it binds to PCNA and unwinds the DNA ahead of the fork to resume progression.
1.13 Summary: Implications of CAF-I/Rrm3p and PCNA

Numerous PCNA-interacting proteins have been identified, however how they interact and exchange positions is not understood. Prior findings in the Yankulov lab show a loss of variegation phenotype for both deletion strains of Cac1p (cac1Δ) and Rrm3p (rrm3Δ) using the URA3/5-FOA TPE assay. This assay can select for the reporter URA3 gene to be active or silent expression depending on selective growth pressure. Once the pressure is relieved, the wild type BY4742 strain has been found to return to ~50/50 proportions for expressing the reporter. Contrarily, the cac1Δ and rrm3Δ strains were impaired in their ability to epigenetically switch from their initially selected condition. The presence of a PIP-box on both Cac1p and Rrm3p and the similar epigenetic phenotype found in both strains suggest an interplay between Cac1p, Rrm3p and PCNA. Furthermore, the interaction profile between Cac1p and PCNA can be dependent on both Cdc7p or Cdc28p. I intend to characterize the interaction between these three proteins and elucidate whether Cac1p and Rrm3p can cooperate to bind to PCNA. These experiments will help answer whether CAF-I must be displaced when a replication fork reaches a barrier to accommodate Rrm3p loading. The maintenance of epigenetic state and histone modifications are mechanisms that are currently poorly understood, however studies investigating these mechanisms can be applied to humans.
2.0 Hypotheses

1. Cac1p and Rrm3p compete with one another to bind to the PCNA homotrimer.

2. Single and double mutations in Cdc7p and Cdc28p putative phosphorylation sites on Cac1p will decrease CAF-I’s association with PCNA.

Research Objectives:

1. Perform co-immunoprecipitation assay with PCNA, Cac1p, and Rrm3p expressed in S. cerevisiae to assess tripartite complex formation plausibility.

2. Clone, express and purify Cac1p, Rrm3p and PCNA in Escherichia coli and perform an in vitro pulldown assay.

3. Test the binding of PCNA to Cac1p, mutated at single or double putative Cdc7p/Cdc28p phosphorylation sites.
3.0 Materials and Methods

3.1 – Growth Media and Conditions

3.1.1 – *Escherichia coli* DH5α and Rosetta™ (DE3)

DH5α and Rosetta cells were cultured at 37°C in a shaking incubator in Luria-Bertani (LB) broth (1% NaCl, 1% tryptone and 0.5% yeast extract) for 18-24 hours. To maintain the expressing plasmids in both liquid and solid media; ampicillin, kanamycin and/or chloramphenicol were supplemented at final concentrations of 100µg/mL, 50µg/mL and 35µg/mL, respectively.

3.1.2 – *Saccharomyces cerevisiae*

Yeast strains were grown at 30°C in a shaking incubator in Yeast Peptone Dextrose (YPD) 1% yeast extract, 2% tryptone, and 2% glucose) media. The doubling time for the laboratory strain *BY4742* was approximately 1.5 hours in YPD. Glucose is prepared as a 20% w/v stock solution and sterilized by autoclaving prior to addition to YPD liquid media or plates. Yeast seed cultures were typically cultured overnight in 3 mL of liquid YPD in test tubes. These seed cultures were then inoculated into YPD media of 50-100 mL volumes. Synthetic Complete (SC) media is composed of 2.32 g/L yeast nitrogen base, 2% sterilized glucose, and essential nutrients listed below in Table 1. All amino acids and nucleobase stocks were made at 100X concentrations and were filter-sterilized using a 0.22 µm membrane prior to its addition.

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Table 1. Final Concentrations of Amino Acids and Nucleobases in SC Media
3.2 – Plasmid Isolation from *Escherichia coli*

Three mL cultures of *E. coli* were grown in the conditions described above. Cells were pelleted at 1000g for 2 minutes and the cells were resuspended in 100 µL of Buffer 1 (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 7.5). 100 µL of Buffer 2 (0.2 M NaOH, 1% SDS) was added to lyse the cells. This reaction was neutralized with 150 µL of Buffer 3 (3 M C₂H₃KO₂, 11.5% v/v glacial acetic acid) for 10 minutes on ice. To separate the genomic DNA and cellular debris from the plasmid, the reaction was pelleted at 10000g for 5 minutes. The supernatant containing the plasmid was collected and moved to a new microcentrifuge tube. 400 µL of Phenol Chloroform (1:1 v/v) was added and vortexed for 1 minute to remove proteins, and then the reaction was centrifuged for 6 minutes at 12000g. The top phase containing the plasmid DNA is collected and transferred to a microcentrifuge tube containing 95% ethanol and 5 M NaCl (1:50 v/v) and incubated at -80°C for 1 hour. The DNA was pelleted at 12000g for 10 minutes and the pellet was washed with 1 mL 70% v/v ethanol. The DNA was then resuspended in 40µL of dH₂O and 2 µL of RNase (10 mg/mL) was added to the tube before being placed in a 37°C incubator for an hour. After the residual RNA was degraded, quality and purity was assessed by loading 2.5 µL of the isolated sample and run on a 1% w/v TAE-agarose gel.

3.3 – Restriction Enzyme Digest

Vector DNA (10 µg) was digested in 50 µL reactions with 5 U of restriction enzymes of interest in buffer supplied by Thermo-Fisher. Reactions were incubated at 37°C for 1 hour and then the restriction enzymes were heat inactivated at 65°C for 20 min. The reaction was cleaned up using a phenol chloroform purification and ethanol precipitation of DNA as described in Section 3.2 The digested DNA was re-suspended in 20 µL of dH₂O and 5 µL was run on a 1% TAE-agarose gel to confirm complete digestion.
3.4 – Purification of PCR Products

Omega® Bio-Tek E.Z.N.A.® was used to purify PCR amplicons. Five volumes of purchased CP Buffer was mixed with the PCR reaction and transferred to HiBind DNA Mini Column. The column was centrifuged at 11000g for 1 minute and washed twice with 700 µL of supplied DNA Wash Buffer. The column was centrifuged once more at 11000g for 1 minute to remove any residual liquid. 50 µL of the supplied Elution Buffer was added to the column and incubated at 55°C and eluted via centrifugation at 10000g for 1 minute. The purity and quantity of the sample was verified by loading 2.5 µL on a 1% TAE-agarose gel and electrophoresed for 40 minutes at 140V.

3.5 – Cloning of PCR Amplicon into Vectors

PCR amplicons of interest were produced by amplifying the gene of interest with primers harboring the appropriate restriction enzyme cut sequences. The amplicon was purified as described in Section 3.4. The purified PCR amplicon was digested as described in Section 3.3 as per the manufacturer’s instructions. The vector was digested with appropriate restriction enzymes and cleaned up with 400 µL of Phenol Chloroform (1:1 v/v) and vortexed for 1 minute to remove proteins. The reaction was centrifuged for 6 minutes at 12000g and the top phase containing the plasmid DNA was collected and transferred to a microcentrifuge tube containing 95% ethanol and 5 M NaCl (1:50 v/v) and incubated at -80°C for 1 hour. The vector was then treated with FastAP Thermosensitive Alkaline Phosphate from ThermoFisher to prevent religation. The digested insert and vector were combined in a 20 µL reaction with insert-vector ratios of 1:1, 3:1, 5:1 and 7:1. The reactions were incubated overnight at 15°C and were transformed into chemically competent DH5α cells with 2-4 µL of the ligation reaction as
described in Section 3.11. The transformation culture was spread on LB agar containing the appropriate antibiotic(s).

■ 3.6 – DNA Sequencing

DNA sequencing was performed at the Advanced Analysis Center, University of Guelph. DNA and primer concentrations were followed as per their instructions located online: (http://www.uoguelph.ca/aac).

■ 3.7 – Quantifying Total Protein Concentration via Bradford Assay

10 µL of the whole cell extract was pipetted into single wells of a 96-well plate in triplicate and serially diluted. Standards of Bovine Serum Albumin (BSA) was prepared at 2 mg/mL, 1.75 mg/mL, 1.5 mg/mL, 1 mg/mL, 0.75 mg/mL, 0.5 mg/mL, 0.25 mg/mL and 0.125 mg/mL. Bio-Rad® Protein Assay Concentrated Dye Reagent was diluted 1:4 v/v as per the manufacturer’s instructions and 200 µL was dispensed into all wells including three blanks. The plate was incubated for 5 minutes at room temperature and the plate was read at OD595 using the Molecular Devices VMax® Kinetic ELISA Microplate Reader. A standard curve was generated in Microsoft Excel using the inputted BSA standards. A line of best fit was applied and basic linear regression was used to calculate the concentration of the unknown test samples.

■ 3.8 – Transformation of S. cerevisiae by Electroporation

A single yeast colony was selected from a YPD plate for inoculation into a 3 mL seed culture of YPD at 30°C. Once this culture reached saturation overnight, it was inoculated into 50 mL of YPD to grow to OD$_{600}$=1.0 at 30°C. Cells were pelleted at 3000g for 3 minutes and washed twice with ice-cold sterile dH$_2$O. Cells were washed in 3 mL of ice-cold 1 M sorbitol followed by centrifugation and resuspension in approximately 3X the pellet volume of ice-cold 1M sorbitol. 40 µL of the sorbitol-cell suspension was added to Eppendorf tubes containing ~
100 ng DNA of interest. These components were transferred to a 2 mm gapped cuvette and electroporated using a BioRad Micropulser at setting “Fungi Sc2”. Every transformation had a positive control plasmid and a negative control using water instead of DNA. After electroporating the cells, 1 mL of recovery media (1 M sorbitol, 1% yeast extract, 2% tryptone, 2% glucose) was added to the cells. The suspension was transferred from the cuvette to a sterile Eppendorf tube and placed in a 30°C incubator for 1.5 hours with a lid lock. The cells use this time to recover and re-establish their cell walls. The cells were then pelleted by centrifugation at 3000g for 2 minutes and the aqueous layer was decanted to a final volume of 120 µL. Cells were then re-suspended in this volume and plated on the appropriate SC dropout plates. Plates were allowed to dry for 15 minutes before being incubated at 30°C for 2-3 days until colonies emerged.

3.9 – Yeast Genomic DNA Isolation

Single yeast colonies were grown to saturation at 30°C in 3 mL YPD. All of the contents were transferred to 1.5 mL Eppendorf tubes and pelleted at 300g for 2 minutes before the supernatant was decanted. 500 µL of dH2O was used to wash the pellet which was decanted after centrifugation. Approximately 0.4 g of chilled glass lysis beads were added to the residual cells with 200 µL of lysis buffer (1M Tris pH 7.5, 10% SDS, 0.5M EDTA pH 8). The tubes are taken to the fumehood where 200 µL of phenol chloroform was added and lid locks are placed on the tubes to prevent spillage. Cells were vortexed for 30 minutes at 4°C to mechanically lyse the cells using the glass lysis beads. Before spinning the contents down at 4°C, 200 µL of 1X Tris-EDTA buffer (1M Tris pH 7.5, 0.5M EDTA pH 8) was added. The aqueous layer was then transferred to a new Eppendorf tube containing 1 mL of 95% ethanol and 0.5 M NaCl and moved to -80°C for 30 minutes. The tubes were then centrifuged for 10 minutes at 12000g and the DNA
pellet is washed with 1 mL of 70% ethanol. The pellet was resuspended in 400 µL of dH₂O and 2 µL of RNase A was added to the tube followed by incubation at 37°C for 1 hour. Tubes were recovered from the incubator and 1 mL 95% ethanol and 40 µL of NaCl were added to the tubes followed by incubation at -20°C for 1 hour. Once the ethanol precipitation was complete, the tubes were spun at 12000g for 10 minutes and the ethanol was aspirated. Another 70% ethanol wash is performed followed by re-suspension of the DNA pellet in a final volume of 40 µL dH₂O. The purity and concentration of genomic DNA was checked by loading 2 µL prepped sample onto a 1% TAE-agarose gel. Prepped DNA samples were stored at -20°C indefinitely till required for downstream purposes.

■ 3.10 – Preparation of chemically competent E. coli DH5α and Rosetta Cells

A 3 mL seed culture of E. coli DH5α or Rosetta was grown for 18-24 hours on a 37°C shaker. One mL was inoculated into 100 mL of LB media and incubated in a 37°C shaker until they reached an OD₆₀₀ of 0.3. Cells were then pelleted at 4500g for 10 minutes at 4°C and then resuspended in 40 mL of ice-cold 0.1 M CaCl₂ and incubated on ice for 30 minutes. The cells were centrifuged as before and the cell pellet was resuspended in 6 mL of 0.1 M CaCl₂ containing 15% v/v glycerol and aliquoted in 50, 100 and 150 µL volumes. The aliquots were snap frozen on dry ice and stored at -80°C until needed.

■ 3.11 – Transformation of E. coli DH5α and Rosetta Cells

Prepared competent E. coli cells were thawed on ice for 10 minutes and 2 µL of DNA (~100 ng/µL) was added to 50 µL of competent cells. The contents were incubated on ice for 30 minutes before heat shocking at 42°C for 2 minutes, followed by incubation on ice for 2 minutes. One mL of LB pre-warmed to 37°C was added, so cells could recover at 37°C for 1 hour with gentle shaking. Cells were then centrifuged at 4000g for 4 minutes, 900 µL of LB media was
removed followed by resuspending in the residual media. The cell suspension was plated on a prewarmed 37°C plate with the appropriate antibiotic and incubated at 37°C for 18-24 hours.

### 3.12 – Western Immunoblotting

Protein samples were electrophoresed through 10%, 12% or 4-12% gradient denaturing polyacrylamide gels at 140V for ~1.5-2 hours. The proteins were then transferred to a PVDF membrane (Immobilon-P™ [Millipore®]) that had previously been soaked in 100% methanol (CH₄O) prior to the transfer. Both the membrane and the gel were equilibrated for 15 minutes in Transfer Buffer (48 mM Tris base, 39 mM glycine, 10% v/v methanol, 0.03% w/v SDS). A semidry electrotransblot machine (Bio-Rad® 25 Trans-blot® SD Semi-Dry Transfer Cell) was used to transfer the proteins from the gel onto the membrane at a constant voltage of 18V for 15-20 minutes. The membrane was then moved into a blocking solution of 5% w/v non-fat dry milk powder in TBST (25 mM Tris-HCl, pH 7.5, 140mM NaCl, 0.2% v/v Tween-20) for 1 hour with shaking. The membrane was incubated overnight at 4°C with the appropriate primary antibody in a solution composed of 1% w/v BSA in TBST and 0.05% w/v sodium azide. The membrane was washed three times with 10 mL of TBST for 5 minutes each, followed by incubation with the appropriate secondary antibody coupled to horseradish peroxidase in 1% w/v nonfat dry milk powder in TBST with shaking for 1 hours at room temperature. The membrane was washed again three times with 10 mL of TBST for 5 minutes each. The immunoblots were developed using Clarity™ Western ECL Substrate (Bio-Rad®) and exposed to X-Ray Film and developed in a dark room.
3.13 – Yeast Two-Hybrid Analysis of Protein Interactions

Strains used are summarized in Table 6. Cells were grown in 50 mL of SC Leu'/Ura'/His' + 2% w/v Glucose at 30°C to OD\textsubscript{600} 0.8-1.0 with shaking. Cells were harvested by centrifugation at 3000g for 4 minutes and resuspended in SC Leu'/Ura'/His' + 2% w/v Galactose + 1% w/v Raffinose and incubated at 30°C for 4 hours. Cells were centrifuged at 4000g and washed with 25 mL of sterile, ice-cold dH\textsubscript{2}O and resuspended in 4 mL of Buffer P (50mM sodium phosphate, pH 7.7, 300mM sodium acetate, 10% v/v glycerol, 1 mM 2-Mercaptoethanol, 500 nM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF] and 1% v/v protease inhibitor cocktail from Biotool® [CAT# B14002-10ML]); split into three 1.5mL microcentrifuge tubes. Cells were pelleted at 4000g for 4 minutes and resuspended in 200µL of Buffer P and 200µL of 0.55mm ice-cold glass beads. A pulsing vortex was used to reach complete lysis with 10 minutes of pulsing at 4°C. The three replicate tubes were pierced with a hot needle and placed over a newly labelled 1.5 mL microcentrifuge tube. Centrifugation at 100g at 4°C allowed for the lysate to be collected in the new tube where it was incubated on ice for 15 minutes. Centrifugation at 14000g for 15 minutes at 4°C allowed for the pelleting of cellular debris and the supernatant was transferred to a new 1.5 mL microcentrifuge tube. 50 µL of the whole cell lysate was collected and stored at -20°C to measure total protein concentration by Bradford describe in Section 3.7.

200 µL of Buffer P + 4 mg/mL ortho-Nitrophenyl-β-galactoside (ONPG) was added to the collected whole cell lysate and inverted to mix. This reaction was incubated in a 30°C water bath to allow for the enzyme reaction to take place optimally. A timer was started once the tubes were placed in the water bath and the reaction would take place till a “medium-yellow colour” was obtained between a OD\textsubscript{420} ~0.3-0.7. Once this endpoint was achieved, 500µL of 1M Na\textsubscript{2}CO\textsubscript{3} was added to stop the enzyme reaction and the timer was stopped and recorded at this point. The
reaction tube was then centrifuged at 1000g to clear any precipitation and the supernatant was analyzed at OD$_{420}$ and OD$_{550}$. A Bio-Rad® SmartSpec™ 3000 Spectrophotometer was used for these readings and the reading was averaged between the triplicates. The units of β-galactosidase were calculated using the formula below:

$$ V = \frac{1000 \times [(OD_{420}) - (1.75 \times OD_{550})]}{(t) \times (v) \times (OD_{600})} $$

### 3.14 – Co-immunoprecipitation of Tagged Yeast Proteins

Strains and respective growth media used are summarized in Table 4. Strains were grown in their respective SC dropout media to maintain their expression plasmids. A colony was selected from a SC plate and inoculated into 50 mL of SC media. Culture was grown at 30°C in a shaker till it reaches an OD$_{600}$ of 1.2. Cells were spun down at 3000g for 3 minutes and washed with 2 mL ice cold IPB (150 mM NaCl, 50 mM Tris (pH7.5), 2 mM EDTA, 5 mM NaF, 5 mM β-Glycerophosphate, 0.1 mM NaVO3). The cells were split into two microcentrifuge tubes of 1 mL volumes each. Cells were centrifuged again and resuspended in 400 µL of IPB + 2% v/v protease inhibitor cocktail from Biotool® [CAT# B14002-10ML]). An equal volume (400 µL) of ice-cold glass lysis beads was added and vortexed at 4°C for 15 minutes. The microcentrifuge is then pierced with a hot 25G needle and placed over a new microcentrifuge tube and centrifuged for 30 seconds at 3000g. An additional 200 µL of IPB + 2% v/v protease inhibitor cocktail was added to the beads to collect all the extract. The microcentrifuge is then spun at 14000g for 15 minutes at 4°C to clear the extract of cellular debris. A sample of the extract is taken (25 µL) to verify the tagged protein’s expression. The rest of the extract is split into four tubes of approximately 300 µL each as indicated below.
Tube 1 and Tube 3 served as “no antibody” controls while Tube 3 and Tube 4 contained the antibody to immunoprecipitate the proteins of interest. 1 µg of MYC and FLAG antibody was added to the tubes as illustrated in Table 2 and placed on a nutator at 4°C for 3 hours. 30 µL of Protein G resin (Genscript CAT No: Z02007) was added to each tube and incubated on the nutator for 1.5 hours at 4°C. The resin was washed five times with 1 mL of IPB + 0.2% Triton X100 for five minutes each. The last wash was collected to verify the purity of the immunoprecipitated sample. For elution of tagged proteins, 30 µL of IPB + 1% v/v protease inhibitor cocktail + 2 µg of FLAG peptide (Genscript) was added to Tube 1 and Tube 2 and 30 µL of IPB + 1% v/v protease inhibitor cocktail + 2 µg of MYC peptide (Genscript) was added to Tube 3 and Tube 4. All tubes were incubated on ice for 45 minutes with occasional agitation followed by centrifugation at 3000g for two minutes. The supernatants were collected and were labelled as elution samples. Load, wash, elution samples were all protein prepped with SDS loading buffer and analyzed by SDS-PAGE and Western blotting. Presence of Cac1p-FLAG, Rrm3p-MYC and PCNA was analyzed by anti-FLAG (Genscript), anti-MYC (Genscript) and anti-PCNA (Requested From Moldovan Lab).

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Table 2. Summary of the different immunoprecipitation conditions used in each experiment.
3.15 – Protein Expression and Purification of His$_6$-Tagged Proteins in *E. coli* Rosetta

Cells harboring the respective *pET11a* or *pET30a* cloned vector were inoculated into 3 mL of LB broth with the appropriate antibiotics indicated in Table 3. This seed culture was grown overnight at 37°C to saturation on a shaker and was sub-inoculated into a 400 mL LB broth with the appropriate antibiotic. It was grown from an OD$_{600}$ of ~0.05 at 37°C to an OD$_{600}$ of 0.4-0.6. Protein expression was induced by addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 – 1 mM and was shaken vigorously at 30°C for 3 hours. Cells were harvested by centrifugation at 8000g for 15 minutes at 4°C (SLC-3000). Cells were snap frozen at -80°C to crack the cell walls and resuspended in 2 mL ice-cold ChB Buffer (50 mM NaCl, 20 mM HEPES pH 7.9) plus 1% v/v protease inhibitor cocktail. Cell membranes and DNA were sheared by sonication (Misonix® XL-2020) for 2 cycles – 10 seconds on, 2 minutes off on ice at 30% output. Triton X100 was added to a final concentration of 0.1% v/v and was incubated on a rocker at 4°C for 15 minutes to aid cell lysis. Cellular debris was cleared by centrifugation at 14000g for 10 minutes at 4°C. A sample of the supernatant was boiled in 5X SDS loading buffer for SDS-PAGE and Western analysis. The remainder of the supernatant was poured over 0.5 mL Ni-NTA (Genscript: L00223-25) in a chromatography column and incubated at 4°C for 10 minutes to allow for His$_6$ protein binding. The flow-through from the column was collected for SDS-PAGE/Western analysis. The protein-bound resin was washed with 10X resin volume with ChB + 0.1% Triton X100. This was followed by elution with 5X resin volume of increasing concentrations of imidazole in ChB Buffer as indicated: 5 mM, 25 mM, 50 mM, 150 mM, 250mM, 400 mM. 1 µL of each fraction was spotted on nitrocellulose and compared to BSA standards of known concentrations. Peak concentrations are pooled and 15% glycerol is added prior to freezing at -80°C. All purification steps were performed at 4°C and on ice. Protein
fractions were analyzed via SDS-PAGE and Western blot analysis to gauge protein concentration and purity before downstream applications.

3.16 – Protein Expression and Purification of GST-Tagged Proteins in E. coli Rosetta

Cells harboring the respective pGEX4T-3 cloned vector was inoculated into 3 mL of LB broth with the appropriate antibiotics indicated in Table 3. This seed culture was grown overnight at 37°C to saturation on a shaker and was sub-inoculated into 400 mL LB broth with the appropriate antibiotic. It was grown from an OD$_{600}$ of ~0.05 at 37°C to an OD$_{600}$ of 0.6. Protein expression was induced by addition of IPTG to a final concentration of 1 mM and was shaken vigorously at 30°C for 3 hours. Cells were harvested by centrifugation at 8000g for 15 minutes at 4°C (SLC-3000). Cells were snap frozen at -80°C to crack the cell walls and resuspended in 2 mL ice-cold PBS Buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na$_2$HPO$_4$, and 2 mM KH$_2$PO$_4$) at pH 7.4 + 1% v/v protease inhibitor cocktail. Cell membranes and DNA were sheared by sonication (Misonix® XL-2020) for 2 cycles – 10 seconds on, 2 minutes off on ice at 30% output. Triton X100 was added to a final concentration of 0.1% v/v and was incubated on a rocker at 4°C for 15 minutes to aid cell lysis. Cellular debris was cleared by centrifugation at 14000g for 10 minutes at 4°C. A sample of the supernatant was boiled in 5X SDS loading buffer for SDS-PAGE and Western analysis. The remainder of the supernatant was poured over 0.5 mL Glutathione Resin (Genscript) in a chromatography column and incubated at 4°C for 10 minutes to allow for GST protein binding. The flow-through from the column was collected for SDS-PAGE/Western analysis. The protein-bound resin was washed with 10X resin volume with PBS Buffer + 0.1% Triton X100. This was followed by elution with 5X resin volume of 10 mM Glutathione in PBS Buffer. 1 µL of each fraction was spotted on nitrocellulose and compared to BSA standards of known concentrations. Peak concentrations were pooled and 15% glycerol was
added prior to freezing at -80°C. All purification steps were performed at 4°C and on ice. Protein fractions were analyzed via SDS-PAGE and Western blot analysis to gauge protein concentration and purity before downstream applications.

<table>
<thead>
<tr>
<th>Cloned Vector</th>
<th>Antibiotic</th>
<th>Induction OD600</th>
<th>IPTG Induction Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNA-pET11a</td>
<td>Ampicillin/Chloramphenicol</td>
<td>0.4</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>CAC1-pET30a</td>
<td>Kanamycin/Chloramphenicol</td>
<td>0.6</td>
<td>1 mM</td>
</tr>
<tr>
<td>RRM3-pET28a</td>
<td>Kanamycin/Chloramphenicol</td>
<td>0.6</td>
<td>1 mM</td>
</tr>
<tr>
<td>CAC1-pGEX4T-3</td>
<td>Ampicillin/Chloramphenicol</td>
<td>0.6</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

Table 3. Summary of growth conditions for *E. coli* Rosetta expression strains.

- 3.17 – Native Protein Complex Analysis of His$_6$-Proteins via Native Gels

Cells expressing recombinant His$_6$-tagged proteins were grown, induced, lysed and purified as described prior in Section 3.15. Purified protein concentrations were determined using the Bradford Assay as described in Section 3.7. Purified proteins were mixed and incubated for 30 minutes on ice at 4°C at specific ratios as required by the experiment. Mixed samples were loaded onto a 4-15% BioRad Mini-PROTEAN TGX™ Precast gradient native polyacrylamide gel. The running buffer (25 mM Tris, 192 mM Glycine, pH 8.3) was maintained at or below 4°C by placing the gel apparatus in an ice bath in the 4°C cold room. The gel was run for 4-6 hours at 25V till the dye front reached the bottom 10% of the gel. The gel was then stained in Coomassie R250 dye to stain for the presence of protein bands.
3.18 – GST-Resin Pulldown of Non-GST Tagged *E. coli* Proteins

Cells expressing recombinant GST-tagged proteins were grown, induced, lysed and purified as described prior in Section 3.16. After the GST resin was charged with the GST-tagged protein, purified His<sub>6</sub>-PCNA and His<sub>6</sub>-Cac1p extract was added and incubated in the column for 15 minutes at 4°C in the cold room. The GST resin was washed with 10X bed volume of 1X PBS plus protease inhibitors followed by 10 mM glutathione elution as described in Section 3.16. All purification steps were purified at 4°C and on ice. Protein fractions were analyzed via SDS-PAGE and Western blot analysis.
4.0 Results

To test whether Cac1p and Rrm3p interact with PCNA individually and in a complex, I designed two separate lines of experiments. A co-immunoprecipitation experiment was used to test the interaction between tagged Cac1p-FLAG and Rrm3p-Myc recombinant proteins that were co-expressed in yeast cells. This experiment maintains the two recombinant proteins in their natural host which is likely to preserve endogenous modifications and interacting cofactors. Some drawbacks of this experiment is the inability to remove endogenous PCNA from this experiment to definitively conclude its contribution to the direct interaction between Cac1p and Rrm3p, if any. Endogenous yeast co-factors will also be present in the extract, therefore possibly introducing confounding variables to the interactions.

The second experiment employed recombinant Cac1p, Rrm3p and PCNA proteins expressed in E. coli, purified and used to test for interactions in vitro. Protein complex formation was analyzed by native gel electrophoresis and alternatively, by GST-affinity chromatography. This experiment allows for the removal of PCNA and addressing the issue of whether any observed interaction between Cac1p and Rrm3p is direct or mediated by PCNA or other proteins present in the yeast extracts. These two experiments provide a strategy that is likely to produce a better understanding of the interactions between Cac1p, Rrm3p and PCNA.

In a separate set of experiments, I investigated the importance of Cdc7p and Cdc28p putative phosphorylation sites on Cac1p and its effect on PCNA association via a modified Yeast Two-Hybrid (Y2H) assay. Cdc7p and Cdc28p both possess consensus sequences for Cac1p as seen in its amino acid sequence. Furthermore, a temperature-sensitive cdc28-1 mutant demonstrated reduced Cac1p phosphorylation and mutations in cdc7 target sites reduce Cac1p’s
stability (74). This leads me to believe these putative phosphorylation sites will decrease Cac1p-PCNA association.

4.1 Evaluation of the PCNA-mediated Interaction Between Cac1p and Rrm3p in S. cerevisiae

To evaluate the interaction of Cac1p and Rrm3p, I transformed the well-established laboratory BY4742 strain with the pRS315-Cac1-FLAG and pRS313-Rrm3-Myc plasmids (Table 4). These plasmids express tagged copies of the proteins from their natural promoters. In the BY4742 strain, the genomic copies of the CAC1 and RRM3 genes are also present. It should be considered that under these circumstances both proteins could be expressed twice as much as compared to their normal levels. In addition, the untagged copies of the proteins could interfere with the co-immunoprecipitation approach. Nevertheless, a positive outcome of this experiment would indicate if in cell extracts, Cac1p and Rrm3p are found in complexes containing PCNA. Strains were grown in the corresponding selective drop-out media to ensure the maintenance of the plasmids and the expression of the tagged proteins. Cell extracts were prepared as described in Section 3.14.

<table>
<thead>
<tr>
<th>Strains</th>
<th>SC Selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4742 + Cac1-FLAG + Rrm3-Myc</td>
<td>His⁺/Leu⁻</td>
</tr>
<tr>
<td>BY4742 + Cac1-FLAG</td>
<td>Leu⁻</td>
</tr>
<tr>
<td>BY4742 + Rrm3-Myc</td>
<td>His⁻</td>
</tr>
</tbody>
</table>

Table 4. Summary of all strains made for co-immunoprecipitation experiments.
In my co-immunoprecipitation experiments, I used anti-FLAG resin (Genscript) to immunoprecipitate Cac1p-FLAG and test if Rrm3p-Myc and endogenous PCNA are found as co-immunoprecipitated proteins. I also used anti-MYC resin (9E10 Hybridoma) to determine whether the reverse approach would reproduce the same result. Cac1p and Rrm3p both possess a PIP-box and therefore theoretically should be able to interact with a trimer of PCNA. Cac1p and Rrm3p’s ability to interact with PCNA was also tested individually using BY4742 strains expressing only one of the tagged proteins.

I grew each strain in a 50 mL liquid culture to an OD$_{600}$ of 1.2 to ensure that the cells were in exponential phase. This is important because Cac1p and Rrm3p are expected to interact at the replication fork in dividing cells. Whole cell extracts were prepared in parallel from the three strains. Each lysate was divided into three aliquots: 1) FLAG immunoprecipitation, 2) MYC immunoprecipitation, 3) “No antibody” control. From this point on, each aliquot is processed separately as indicated in Section 3.14. Once the resin had been added to the cell extracts, 3 hours was given for incubation followed by washing the resin in the tubes with IP Buffer. The FLAG or MYC resin were then incubated with 500 molar excess of FLAG or MYC peptide, respectively, for 20 minutes. The co-eluted proteins were collected and then the resins were boiled in 1X SDS Loading Buffer to recover any protein that was not eluted by the peptide. Three independent technical replicates of this experiment were conducted and the best images are displayed below in Figure 5.

When analyzing the anti-FLAG immunoprecipitation of BY4742 transformed with only Cac1-FLAG, the anti-FLAG antibody western shows that Cac1p-FLAG was present in the Load at the start of the experiment and that a significant amount has been successfully immunoprecipitated as shown in both the peptide elution and the boiled resin lane. The anti-
PCNA antibody Western shows that PCNA was also co-immunoprecipitated with Cac1p-FLAG. I also performed an anti-MYC immunoprecipitation with the same extracts to confirm that the presence of PCNA depends on the anti-FLAG antibody. As evident in Figure 5A, the anti-MYC-immunoprecipitation failed to precipitate detectable levels of any FLAG, Myc or PCNA proteins. Furthermore, the “No Antibody” immunoprecipitation control and Wash lanes were also free of FLAG, Myc or PCNA proteins.

I followed up by testing the interaction of Rrm3p with PCNA by using BY4742 + Rrm3-Myc in the same experiment. As seen in Figure 5B, Rrm3p-Myc was successfully immunoprecipitated in the anti-MYC IP and endogenous PCNA was also successfully precipitated in those samples. The anti-FLAG immunoprecipitation and the “No Antibody” control of the same strain lacked any detectable levels of FLAG, Myc or PCNA proteins. From Figure 5A/B we observe that Cac1p-FLAG and Rrm3p-Myc can be successfully immunoprecipitated and endogenous PCNA also co-immunoprecipitates in each of those samples. Figure 5C depicts co-immunoprecipitation experiments when both Cac1p-FLAG and Rrm3p-Myc were expressed in the same strain. In the anti-FLAG IP in Figure 5C, very little amounts of Rrm3p-Myc is detectable in the elution sample, however the anti-MYC IP detected significant amounts of Cac1p-FLAG in the resin sample. In all samples where Cac1p-FLAG or Rrm3p-Myc were detected, the anti-PCNA antibody western shows that PCNA was present in substantial amounts. The “No Antibody” control did not immunoprecipitate any of the three analyzed proteins.

Based on these results, I have shown that I can detect the interactions of PCNA with both Cac1p and Rrm3p individually as reported earlier (72, 75). In addition, I detect all three proteins in the co-immunoprecipitations with anti-FLAG and anti-MYC antibodies as shown in Figure
5C. Therefore, Cac1p, PCNA, and Rrm3p are able to form a complex within the yeast cell extracts. Of the three proteins analyzed, PCNA appears to be the most abundant in the co-immunoprecipitation which leads me to propose that the interaction between Cac1p and Rrm3p could be mediated by PCNA. To determine whether the Cac1p-Rrm3p interaction is direct or mediated by PCNA, this experiment must be taken out of the host cell and PCNA should be removed to verify its necessity to this interaction.
A) **BY4742 + Cac1-FLAG**

- **Immunoprecipitation:** anti-FLAG, anti-MYC, and No Antibody
- **Western Blot:**
  - anti-MYC
  - anti-FLAG
  - anti-PCNA

B) **BY4742 + Rrm3-Myc**

- **Immunoprecipitation:** anti-FLAG, anti-MYC, and No Antibody
- **Western Blot:**
  - anti-MYC
  - anti-FLAG
  - anti-PCNA

C) **BY4742 + Cac1-FLAG + Rrm3-Myc**

- **Immunoprecipitation:** anti-FLAG, anti-MYC, and No Antibody
- **Western Blot:**
  - anti-MYC
  - anti-FLAG
  - anti-PCNA

**Figure 5:** Western blot analysis of FLAG, MYC, or “No Antibody” resins based co-immunoprecipitations from: A) **BY4742 + Cac1-FLAG:** The anti-FLAG and anti-PCNA western blot of FLAG IP shows the immunoprecipitation of Cac1p-FLAG and PCNA in the Elution and Resin fractions. B) **BY4742 + Rrm3-Myc:** The anti-MYC and anti-PCNA western blot of MYC IP shows the immunoprecipitation of Rrm3p-Myc and PCNA in the Elution and Resin fractions. C) **BY4742 + Cac1-FLAG + Rrm3-Myc:** The western blot of the FLAG IP shows the immunoprecipitation of Cac1p-FLAG, Rrm3p-Myc and PCNA in the Elution fraction. The western blot of MYC IP shows the immunoprecipitation of Rrm3p-Myc, Cac1p-FLAG and PCNA in the Resin fractions. Samples were separated on 10% and 12% SDS-PAGE gels and analyzed by Western blotting with the antibodies shown on the left axis of the figure. The mobility of 100, 75, and 25 kDa molecular weight markers are shown on the right axis of the figure.
4.2 Cloning of CAC1, RRM3 and POL30 (PCNA) for Expression in E. coli

To assess the interaction between Cac1p, Rrm3p and PCNA, I needed to clone, express and purify these proteins and then use them in an in vitro interaction assay. I used PCR to amplify the ORFs of CAC1 and RRM3 from the genomic DNA of the laboratory yeast strain BY4742. The amplified DNA fragments were cloned into the pET30a plasmid in frame with the N-terminal His6 tag provided by the vector. CAC1 was also separately cloned into the pGEX4T-3 plasmid in frame with the N-terminal GST tag. The primers used in the PCR amplification are listed in Appendix A. All cloned vectors were sequenced for further verification. pET11a-PCNA was already available in the lab and was used directly in the downstream assays.

4.3 Expression and Purification of Cac1p, Rrm3p and PCNA in E. coli Rosetta.

I grew E. coli carrying the respective pET11a/pET30a/pGEX4T-3 plasmids with selective antibiotic pressure in 400 mL cultures at 37°C. Protein expression was induced with 0.5 mM IPTG at an OD600 =0.4-0.6 and the cultures were vigorously shaken at 30°C for 3 hours. Optimization experiments showed better yields at 30°C as compared to 37°C, 15°C and 4°C. This slightly reduced temperature may provide time for proper protein folding and a possible reduction of inclusion body formation. His6- and GST-tagged proteins were purified as detailed in Section 3.15/3.16.

The recombinant His6-Cac1p and His6-Rrm3p proteins were collected and analyzed via Western blot as shown in Figure 6 below. The extract from cells harboring pET30a-CAC1 showed a band at around 75 kDa when analyzed via anti-His6 Western blot. The extract from cells expressing His6-Rrm3p shows a prominent band around the 75 kDa mobility marker. Both His6-Cac1p and His6-Rrm3p crude extracts display significant degradation products despite the
presence of protease inhibitors. Cells harboring empty *pET30a* served as a negative control and showed no cross-reacting bands at the positions of His$_6$-Cac1p and His$_6$-Rrm3p. All three extracts were loaded on Ni-NTA columns (Genscript) and eluted by a stepwise gradient with increasing imidazole concentrations. Imidazole elution samples collected from both 150 mM and 250 mM imidazole fractions were pooled and aliquots were analyzed by anti-His$_6$ Western blot. In these experiments His$_6$-Cac1p was successfully purified as indicated by the presence of a 75 kDa band, however significant degradation products were observed in the eluates. The His$_6$-Rrm3p protein was lost during the purification and the empty vector failed to produce any detectable proteins in the eluates as seen in Figure 6. The lack of His$_6$-Rrm3p bands in the elution could be due to degradation or improper elution conditions. The His$_6$-PCNA purification was performed as described in the literature and produced significant amounts of intact PCNA (76)(Figure 6).

I repeated the expression and purification of His$_6$-Rrm3p with a wider range of imidazole concentrations as shown in Figure 7. His$_6$-Rrm3p was successfully eluted off the Ni-NTA resin at an imidazole concentration of 400 mM as demonstrated by the presence of the anti-His$_6$ interacting band in this fraction slightly above the 75 kDa marker. However, a significant number of degradation products were observed again.
Figure 6: Anti-His6 Western blot analysis of purified His6-Cac1p, His6-Rrm3p and His6-PCNA. The full-length His6-Cac1p band is shown in both the Cac1p Extract and in the Elution fraction at about 72 kDa. His6-PCNA has a mobility of about 29 kDa as shown in the pET11a Elution fraction. The samples labelled as “extract” represent the supernatants collected immediately after cell lysis. Samples labelled “eluate” were the pooled fractions of the 150 mM and 250 mM imidazole elution samples. Lanes labelled “Empty” indicate cells harboring the empty pET30a vector. Samples were separated on 10% SDS-PAGE gels and analyzed by anti-His6 Western blot.
Figure 7: Anti-His\textsubscript{6} Western blot analysis of purified His\textsubscript{6}-Rrm3p. His\textsubscript{6}-Rrm3p is expressed in the Extract and purified in the 400 mM Eluate fraction at about 80 kDa. The sample collected immediately after lysing the cells is labelled as Extract and imidazole based elution samples were loaded as indicated on the top axis. Sample labelled Resin represents the remaining resin used in the purification after the elution steps. Samples were separated on 10% SDS-PAGE gels and analyzed by anti-His\textsubscript{6} Western blot.
I expressed \textit{pGEX4T-3-CAc1} in the same manner as \textit{pET30a-CAc1} and purified it using GST Sepharose resin (Genscript) as indicated in Section 3.16. I eluted GST-Cac1p with 5X resin bed volume of 10 mM glutathione and each of the 5 fractions were collected separately to be analyzed via anti-GST Western blot. As seen in Figure 8, GST-Cac1p was successfully expressed and eluted in the sequential 10 mM glutathione elutions as demonstrated by the presence of a 100 kDa anti-GST antibody interacting band (Figure 8). The addition of the GST tag increases Cac1p’s total size from 72 kDa to around 100 kDa. Compared to the expression level of the other recombinant proteins, GST-Cac1p appears to have less expression of full length Cac1p with increased degradation products.
Figure 8: Anti-GST Western blot analysis of GST-Cac1p purification. Purified GST-Cac1p is shown at a mobility of 100 kDa in the Extract, Flowthrough and Eluate fractions. The sample collected immediately after lysing the cells is labelled as “Extract” and the sequential 10 mM glutathione elution samples were loaded as indicated on the top axis. The flowthrough fraction collected from the column was also loaded. Samples were separated on 10% SDS-PAGE gels and analyzed by Western blot with anti-GST antibodies.
4.4 Assessing the Interaction of Recombinant Cac1p, Rrm3p and PCNA via Native Gels

To assess the interaction of these proteins and determine whether the formation of a triple-partite complex could be detected, I decided to employ gradient non-denaturing gels (BioRad). This approach could allow the detection of native protein-protein interactions by means of a band shift which indicates a larger complex has been formed by the analyzed proteins. The first experiment was only to investigate the interaction between His₆-Cac1p and His₆-PCNA as it was well documented (75) and would determine whether this experimental approach was worth pursuing. I purified His₆-Cac1p and His₆-PCNA as described in Section 3.17 and incubated the purified proteins on ice at 4°C. Multiple incubation reactions were set up to titrate His₆-Cac1p with His₆-PCNA to determine what protein concentration ratio would produce a detectable interaction. The experiment was designed so that His₆-Cac1p’s concentration was consistent at about 5 µg per reaction and His₆-PCNA was added on a decreasing gradient being diluted by a factor of 2 from 20 µg to about 19.5 ng as shown in Figure 9.

The His₆-PCNA and His₆-Cac1p loading controls in lanes 2 and 3 show clear bands representing each individual protein purification. His₆-Cac1p has significant degradation products as I had observed in past purifications via Western Blot (Figure 6). Based on these loading controls, I can observe the lack of any detectable band shifts in any of the PCNA titrations from lanes 5-15. I concluded that this technique cannot detect an interaction between Cac1p and PCNA. This experimental approach could have failed for many reasons, some of which I will discuss below. The purified His₆-Cac1p obtained and used in this experiment may not be sufficient to detect the interaction. Despite optimizations of the His₆-Cac1p purification protocol, I was not able to improve the overall protein yield and, more importantly, the stability of the recombinant protein. Also, since the proteins are not denatured, they could also be...
degrading during the 6-hour run time of the gel at 4°C. This finding suggests that an alternative method that is able to detect interactions at a lower protein concentration and reduced processing time could be more successful.
Figure 9: Coomassie stain of a 4-12% gradient native gel assessing the interaction between His$_6$-Cac1p and His$_6$-PCNA. Purified His$_6$-PCNA and His$_6$-Cac1p were loaded in lanes 2 and 3, respectively. His$_6$-PCNA was incubated with His$_6$-Cac1p at increasing concentrations as indicated on the top axis. Lanes 5-15 have His$_6$-PCNA at concentrations of 20 µg, 10 µg, 5 µg, 2.5 µg, 1.25 µg, 0.625 µg, 0.313 µg, 0.156 µg, 78.1 ng, 39.1 ng, 19.5 ng respectively. Samples were separated in non-denaturing conditions at 4°C followed by Coomassie staining.
4.5 Assessing the Interaction of Cac1p and PCNA via GST Pulldowns

I designed a GST-Cac1p based pulldown experiment to assess the interaction between GST-Cac1p, His6-PCNA and His6-Rrm3p in a more rapid and sensitive way as compared to the native gel experiment. The recombinant proteins were expressed and purified as described in Section 3.15/3.16. Cells harboring *pGEX4T-3-CAC1* were grown and processed in parallel with cells harboring an empty *pGEX4T-3* vector as a negative control. 20 µg of purified His6-PCNA was added directly to the cell extracts and incubated for 10 minutes at 4°C. Both extracts plus purified His6-PCNA were passed through the GST resin and 10X bed volume of PBS buffer containing protease inhibitors was used to wash both columns. The column was washed again with wash buffer, followed by 10 mM glutathione elutions which were collected in separate aliquots.

As shown in Figure 10, GST-Cac1p is successfully eluted from the GST resin and His6-PCNA successfully co-elutes with it. The strain harboring the empty *pGEX4-T3* vector failed to retain His6-PCNA which supports the notion that GST-Cac1p is mediating this interaction. This experiment shows that interactions between Cac1p and PCNA, which has been observed in my immunoprecipitation experiments (Figure 5) can be successfully reproduced by recombinant proteins expressed in *E. coli*. I followed up this finding by challenging this complex with His6-Rrm3p.
Figure 10: Western blot analysis of GST based pulldown of GST-Cac1p and His₆-PCNA.
Cell extracts from *E. coli* Rosetta containing an expression plasmid for GST-Cac1p and an empty *pGEX4T-3* vector were mixed with purified His₆-PCNA. Samples were separated on 10% SDS-PAGE gels and analyzed by Western blot with anti-His₆ and, anti-GST antibody as indicated on the left axis.

The experiment with an empty *pGEX4T-3* extract and purified His₆-PCNA is shown on the left. Lanes 1 – 6 show the sequential samples collected by 10 mM Glutathione. Lane 7 is the last wash from the column before the elution. The experiment with GST-Cac1p and purified His₆-PCNA is shown on the right. Lane 8 is a loading control for purified His₆-PCNA. Lanes 9 – 14 indicates the sequential samples collected by 10 mM Glutathione elution. Lane 15 is the last wash before the elution.
4.6 Assessing the Interaction of Cac1p, PCNA and Rrm3 via GST Pulldowns

The GST pulldown experiment in Section 4.4 was carried out with His₆-Rrm3p to investigate whether Rrm3p can form a complex with Cac1p and PCNA. Cells harboring pGEX4T-3-CAC1 and an empty pGEX4T-3 vectors were grown and processed in parallel. The produced extracts were split and loaded on 4 columns. After washing the resins with 10X bed volumes of PBS buffer plus protease inhibitors, each of these columns were loaded with different combinations of purified His₆-PCNA and His₆-Rrm3p extract. Additionally, cellular extracts harboring empty pET28a vector was added to columns that lacked His₆-Rrm3p extract. The columns were then washed again with 10X bed volumes of PBS buffer plus protease inhibitors followed by the elution of the bound proteins with 10 mM glutathione. The various reaction combinations and the concentrations of the constituents are summarized in Table 5 below.

<table>
<thead>
<tr>
<th>pGEX4T-3-CAC1 Columns</th>
<th>Empty pGEX4T-3 Columns</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEX4T-3-CAC1 + empty pET28a</td>
<td>Empty pGEX4T-3 + empty pET28a</td>
</tr>
<tr>
<td>pGEX4T-3-CAC1 + His₆-PCNA + empty pET28a</td>
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<tr>
<td>pGEX4T-3-CAC1 + His₆-PCNA + His₆-Rrm3p Extract</td>
<td>Empty pGEX4T-3 + His₆-PCNA + His₆-Rrm3p Extract</td>
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<tr>
<td>pGEX4T-3-CAC1 + His₆-Rrm3p Extract</td>
<td>Empty pGEX4T-3 + His₆-Rrm3p Extract</td>
</tr>
<tr>
<td>His₆-PCNA – 5 μl – (4 μg/μl)</td>
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</tr>
<tr>
<td>His₆-Rrm3p Extract – 50 μl (250 μg/μl)</td>
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</tr>
<tr>
<td>Empty pET28a – 50 μl (250 μg/μl)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Summary of the different purification constituents in the pGEX4T-3-CAC1 and Empty pGEX4T-3 GST columns and their relative concentrations.
The interaction between GST-Cac1p and His6-PCNA was successfully reproduced as seen in Lane 4 in Figure 11. When His6-Rrm3p extract was mixed with His6-PCNA, PCNA was no longer retained on the column. Importantly, His6-Rrm3p was not retained on the GST-Cac1p beads either. The resin loaded with the extract from the cells with empty pGEX4T-3 did not retain His6-Rrm3p or His6-PCNA, as expected. These experiments suggest that His6-Rrm3p may be competing with GST-Cac1p for interaction with PCNA. In addition, it is clear Cac1p and Rrm3p do not interact with each other under these experimental conditions. Based on the co-immunoprecipitation experiments, we could conclude that Cac1p and Rrm3p require PCNA to form a complex with one another. However, under the conditions of this experiment a tri-partite complex between Cac1p, Rrm3p and PCNA was not observed. I will address this issue in the discussion.
Figure 11: GST-Cac1p and GST affinity chromatography with His$_6$-PCNA and His$_6$-Rrm3p. His$_6$-PCNA has been pulled down in lane 4 through interactions with GST-Cac1p. Cell extracts from *E. coli* Rosetta containing an expression plasmid for GST-Cac1p and an empty pGEX4T-3 vector were lysed and immobilized on Glutathione-Sepharose resin. Purified His$_6$-PCNA, and His$_6$-Rrm3p extracts were added in different combinations to the columns as indicated above the lanes. Bound proteins were eluted using 10 mM glutathione buffer and labelled as eluates on this figure. Samples were separated on 10% SDS-PAGE gels and analyzed.
4.7 Assay to Investigate the Interaction Between Cac1p and PCNA in vivo

I used a modified yeast two-hybrid (Y2H) assay to assess the interaction between PCNA and Cac1p with S→A mutations at the positions of known phosphorylation sites. These experiments were conducted in a strain where the genomic copy of CAC1 is deleted (cac1Δ) to minimize the interference of the endogenously expressed Cac1p. The cac1Δ strain was transformed with both the reporter plasmid (pSH18-34) and the PCNA-Gal4AD prey plasmid (pBL240). This strain was then individually transformed with either plasmid expressing wildtype Cac1-LexA<sub>BD</sub> (WT), pEG202 (Empty Bait plasmid), Cac1-ΔPIP-LexA<sub>BD</sub>, or one of the serine to alanine point mutants summarized in Table 6. A diagram of the major plasmids used in this assay and how they function is presented in Figure 12. The empty bait plasmid with only the LexA<sub>BD</sub> intact was used as a negative control. The bait plasmid with a destroyed PIP-box serves as a control for the loss of interaction between Cac1p and PCNA. The strains were grown on SC/Ura<sup>−</sup>/Leu<sup>−</sup>/His<sup>−</sup> to properly maintain all three plasmids and processed as described in Section 3.13. This experiment was conducted using one biological replicate which was repeated three individual times with three technical replicates. The results from these experiments were pooled, averaged and analyzed.
Table 6. Plasmids and strains used in yeast two-hybrid for Cac1p/PCNA interaction

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Reporter</th>
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</tr>
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<td></td>
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Figure 12. A diagram of the Yeast Two-Hybrid plasmids and how they are used in this experiment.

A) The Prey plasmid (1) has POL30 (PCNA) fused to an HA epitope fused to a Gal4AD (pBL240). The Bait plasmid (2) is CAC1 (pEG202-CAC1) fused to the LexABD. Site-directed mutagenesis was performed on this Bait plasmid to produce the Cac1p mutants. The Reporter plasmid (pSH18-34) consisting of 8 LexA Operators upstream of a minimal GAL1 promoter which drives the expression of the LacZ reporter gene producing β–galactosidase. The elements presented are not to scale.

B) Cac1p functions as the bait and will bind to the reporter plasmid via the fused LexABD to the LexAOP. If the Prey, PCNA-Gal4AD interacts with the bait, the Gal4AD will be brought into close proximity of the minimal GAL1 promoter and drive the expression of the LacZ reporter gene as indicated by the black arrow. LacZ encodes for β-galactosidase and the abundance of this enzyme can be quantified by a colourimetric assay as described in Section 3.13.
Figure 13. The Cac1p serine to alanine mutations decrease the binding of Cac1p to PCNA. All single and double mutations show a statistically significant reduction in Cac1p-PCNA association relative to wildtype (WT). cac1Δ cells containing a LexAOP driven reporter plasmid containing the reporter gene LacZ was co-transformed with the Prey plasmid expressing PCNA-Gal4AD and the Bait plasmid expressing either wildtype Cac1p (WT) or one of the mutants indicated on the x axis. The average β-galactosidase activity in whole cell extracts/mg of total protein from three technical replicates was measure and plotted. Significance was determined using an unpaired two-tailed T-test assuming unequal variance and samples showing statistically significant difference from wildtype (WT) samples (p-values less than 0.05) are indicated by the asterisks. The error bars represent the standard deviation.
■ 4.8 Cac1p Point Mutations Reduce the Cac1p/PCNA Interaction

Relative to the wildtype Cac1p-LexABD, the interaction of PCNA-Gal4AD and Cac1-\DeltaPIP-LexABD was reduced by 28.4-fold which indicates that the PIP-box consensus sequence is critical for this interaction (Figure 13). Interestingly, all mutants showed a statistically significant decrease (p<0.05) in the production of β-galactosidase relative to the wildtype WT control. The single mutations that most affected β-galactosidase production were the S238A and S503A at 5.1 and 4.6-fold reduction, respectively. When analyzing the double mutants, S94A+S515A and S238A+S503A had the most significant effects in β-galactosidase production at 7.7 and 4.5-fold reduction, respectively. It is noteworthy that the S238 mutation that had the most significant effect on the Cac1p-PCNA interaction is proximal to the PIP box which spans from residues 227-234. However, the S238A double mutants do not display any additional reduction in PCNA association compared to the single mutant. Comparatively, the S94A+S515A double mutant showed a more synergistic reduction of β-galactosidase production compared to its single mutant counterparts. These two sites are known targets of the Cdc28p kinase while S238 is believed to be a Cdc7p/Dbf4p target. Collectively, it seems that both cdc28 and cdc7 appear to play a role in Cac1p association with PCNA.
5.0 Discussion

5.1 Cac1p-PCNA-Rrm3p: Co-operation of competition?

The role of CAF-I in the proper disassembly of parental nucleosomes and reassembly of nucleosomes on the nascent strands is reasonably well-understood. Asf1p works with FACT to systematically disassemble parental nucleosomes using MCM as an anchor. CAF-I, through its association with PCNA, works alongside Asf1p to receive and transfer H3-H4 histones from the parental to daughter strands (38). Disruptions to this well-coordinated process can result in chromatin aberrations and dis-regulation of genes or significant DNA damage (77). Disruptions to replication fork progression can be in the form of protein barriers in front of the fork (77). The replication fork cannot pause indefinitely, due to the forks instability and the eventuality of fork collapse. In these situations, DNA helicases such as Rrm3p are deployed to unwind the DNA ahead of the fork, which allows for resolution of the tightly bound region (72). When independently assessed, both of these processes function to maintain genome stability however there is a caveat. Both CAF-I and Rrm3p possess PIP-box domains which allow them to interact with PCNA at the replication fork. When Rrm3p is recruited, does it compete with CAF-I for PCNA binding and displace it during its function? If CAF-I is displaced, what happens to the preservation of post-translational modifications by shuttling of parental histones to daughter strands? Studies investigating epigenetic switching at the sub-telomere have shown that Cac1p is critical to preserving the ability to switch freely under no selective pressure. *cac1Δ* cells showed inability to epigenetically switch after being repeatedly selected for one expression type (79). The same study assessing Rrm3p’s effects on this system reproduced the same epigenetic phenotype result which leads me to believe that both CAF-I and Rrm3p work concurrently (possibly through PCNA) to maintain epigenetic flexibility (79).
5.2 Evidence for a Cooperative Tri-Partite Complex in *S. cerevisiae*

Previous work from our lab showed that deletions in either *CAC1* or *RRM3* produced the same epigenetic phenotype, a loss of variegation (79). Furthermore, both Cac1p and Rrm3p contain a PIP-box domain which mediates their interaction with PCNA at the replication fork (66, 72). Based on these observations, I hypothesized that Cac1p and Rrm3p could either cooperatively bind PCNA as a tri-partite complex or compete for the limited binding sites on the PCNA trimer. To address this hypothesis, I established a co-immunoprecipitation experiment based on tagged versions of Cac1p and Rrm3p as described in Section 4.1.

When *BY4742* transformed with *CAC1-FLAG* and *RRM3-MYC* was immunoprecipitated with FLAG resin, Rrm3p-Myc was co-immunoprecipitated in those samples along with endogenous PCNA. A similar result was observed when Myc resin was used with the same cellular lysate. Therefore, regardless which tagged protein is immunoprecipitated, PCNA and the other protein of interest is also precipitated out of the lysate. My experiments strongly suggest that a cooperative complex is possible between Cac1p, PCNA and Rrm3p. Although not quantitative, we can observe significantly less Rrm3p-Myc co-immunoprecipitated in the anti-FLAG immunoprecipitation. Rrm3p recruitment to the replication fork is not well understood. Its recruitment could be PCNA-dependent or it could be tethered to other replication factors allowing it to travel alongside PCNA and function whenever it is needed. Rrm3p is also found to interact with a subunit of the pre-replicative complex, Mcm4p and a catalytic subunit of DNA polymerase ε, DNA pol II (80). These observations lead me to hypothesize that, Rrm3p could be travelling with the replication fork through Mcm4p or DNA pol II and if a tightly bound protein was encountered, Rrm3p could be recruited onto PCNA to carry out its function. In doing so, Rrm3p could be dislodging CAF-I from PCNA for a brief period before being ejected by CAF-I.
so regular replication can progress. Contrarily, Rrm3p could cooperatively bind to PCNA, without needing CAF-I’s eviction to function. Given that PCNA is found as a homotrimer at the replication fork, cooperative binding is theoretically possible (81). A PCNA homotrimer presents three interdomain connecting loops, which hypothetically can be docked onto by up to three PIP-domain possessing proteins.

This assay was carried out in the endogenous yeast organism, which promotes the spontaneous formation of a PCNA homotrimer. Given that both Cac1p-FLAG and Rrm3p-Myc were immunoprecipitated using either FLAG or Myc resin, it leads be to believe that a PCNA homotrimer could have been formed. Additionally, the immunoprecipitated samples showed substantial amounts of PCNA which further suggests that the interaction between Cac1p and Rrm3p could be PCNA mediated. To test whether PCNA is critical for this interaction, I needed to remove PCNA from the system which required a shift to a bacterial expression system. Some caveats to using the host of origin include the presence of confounding variables that could be contributing to the interaction of interest that would not be detected under the experimental design being executed. Post-translational modifications to Cac1p or other co-factors that we may be unaware of could be required for the interaction of these three replication-related proteins. Some possible avenues to further investigate these variables would be to use variants of Cac1p and Rrm3p that are mutated at key residues to control for the effects of post-translational modifications. Additionally, co-immunoprecipitation-coupled mass spectroscopy could be employed to identify novel proteins that contribute to the complex formation. This method would help broaden the study and shed light on the complex interactions at the replication fork.
5.3 Possible Competition Between Cac1p and Rrm3p for PCNA

Based on my experiments using bacterially expressed proteins, GST-Cac1p successfully interacted with His6-PCNA as expected. When His6-Rrm3p was added to this experiment, there was no detectable tri-partite complex formation. Interestingly, however, the addition of His6-Rrm3p also abolished the interaction between GST-Cac1p and His6-PCNA we had seen earlier. This indicates a more competitive interaction profile between GST-Cac1p and His6-Rrm3p. The addition of His6-Rrm3p could be sequestering all the available His6-PCNA and therefore preventing any from being retained on the GST resin through interactions with GST-Cac1p. If we are detecting competition in this bacterial system that lacks any post-translational modifications or endogenous co-factors, it suggests that the interaction between Cac1p, Rrm3p and PCNA could be predominantly affinity-based. It is well understood that CAF-I, PCNA and Rrm3p are post-translationally modified in their host of origin, so this result provides some knowledge on how these proteins function without those variables (74, 82).

Provided that I was not able to detect a complex between Cac1p, PCNA and Rrm3p, the question of whether we are working with a PCNA homotrimer or a monomer must be asked. If PCNA is not spontaneously forming trimers, then the outcomes of this experiment become easily interpretable (72). A PCNA monomer will interact with GST-Cac1p and be retained on the resin while not providing any additional binding sites for the free His6-Rrm3p. Additionally, His6-Rrm3p could have bound all the available monomeric His6-PCNA preventing His6-PCNA from interacting with GST-Cac1p as seen in the Western blot. A major drawback to the design of this experiment is that unless a three-protein complex was detected, we cannot verify whether a monomer or trimer was formed during the experiment.
Prior studies in the Yankulov lab had shown that the loss of either Cac1p or Rrm3p yielded the same epigenetic phenotype: loss of switching. This observation led me to investigate whether both proteins needed to interact with one another for the maintenance of normal gene expression state. An interesting finding from this experiment was that GST-Cac1p did not interact with His6-Rrm3p directly. Despite both proteins possessing PIP-box sequences, it was not known whether there are any underlying mechanisms that would allow them to interact without PCNA. This result leads me to believe that PCNA is important for this interaction. Additionally, other endogenous yeast proteins could mediate interaction between Cac1p and Rrm3p that we are unable to detect in a bacterial system.

### 5.4 Cdc7p and Cdc28p Contribute to Cac1p Association with PCNA

The largest subunit of CAF-I, Cac1p is highly phosphorylated at several serine residues as identified by mass spectroscopy (71). Recently, Cac1p was shown to be phosphorylated by Cdc7p and Cdc28p in vitro in *S. cerevisiae* (74). This finding coincided with prior work that showed that the human homologue of Cac1p (p150) was phosphorylated by Cdc7/Dbf4 (83). Cdc28p phosphorylation was found to be important to recruiting Cac1p to chromatin but did not show any effect on association with PCNA (66). Five putative phosphorylation residues were determined: S94, S238, S501, S503, S515. Cdc28p sites important for the recruitment of Cac1p to chromatin were S94 and S515, while S238, S503, and S515 matched Cdc7p’s consensus sequence (84, 85). Given that Cac1p’s PIP-box domain spans 227-234 amino acid residues, I believed that S238 would most affect Cac1p association with PCNA due to the modification’s proximity to the PIP-box.
My experiment shows that all the putative phosphorylation sites investigated resulted in a statistically significant reduction in Cac1p-PCNA association. The two most significant single mutants were S238 followed by S503 which are both Cdc7p sites. S238’s proximity to the PIP-box domain could allow for a phosphorylation-based conformational change of the PIP, allowing for more space to interact with PCNA’s interdomain connecting loop. Interestingly, Cdc28p does not appear to only affect Cac1p recruitment to chromatin but also its association with PCNA. When analyzing the effect of the double mutants, S94 + S515 and S238 + S503 had the most significant reduction of Cac1p-PCNA association. S94 + S515 had a synergistic effect on Cac1p-PCNA association which indicates that Cdc28 and Cdc7p could be working in tandem. These findings can be further supported by performing this Y2H in temperature sensitive cdc28-1 and cdc7-1 strains that can evaluate Cac1p-PCNA association without the need for biochemical mutants. If both cdc28-1 and cdc7-1 strains reproduce a significant reduction association, it would further support the notion that Cdc7p and Cdc28p are critical modifiers of this interaction. Additionally, co-immunoprecipitation experiments could be done with strains expressing Cac1p mutants and the amount of PCNA precipitated from those mutants can be analyzed by Western blot and quantified by densitometry.
6.0 Conclusions and Future Directions

This study aimed to investigate the interaction between three vital components of the replication fork and by extension, their influence on the epigenetic expression state. The findings suggest that Cac1p, PCNA and Rrm3p can form a tri-partite complex which proposes that this complex could be formed cooperatively at the replication fork. Furthermore, the interaction between Cac1p and PCNA appears to be controlled by many factors including, but not limited to phosphorylation by Cdc7p and Cdc28p. My studies help shed light onto the complex nature of DNA replication-mediated proteins and how they function during the progression of the fork. Here I have shown that Cac1p and Rrm3p interaction requires a mediating factor such as PCNA and that it is not a direct interaction.

Another in vivo experiment that could help investigate this Cac1p-PCNA-Rrm3p association, would be to employ a Yeast Three-Hybrid assay. My findings suggest the notion that Cac1p and Rrm3p do no interact directly, which would indicate that Cac1p and Rrm3p be cloned as bait and prey. The third protein, PCNA is sub-cloned as a “bridge” protein to mediate the interaction (if it exists in vivo) which yields a detectable output. This experiment would help determine if this tri-partite interaction is feasible in an in vivo setting. The majority of findings put forth in this thesis were in vitro biochemical analyses of the proteins of interest. For a holistic view into how these proteins function at the replication fork, the Cac1p/Rrm3p interaction at a paused replication fork must be investigated.

Fork pausing is usually a result of epigenome aberrations such as DNA damage, histone/nucleotide deprivation; however does fork pausing as a result of a tightly bound protein constitute a stress? Rrm3p is needed for normal fork progression and as such, protein-DNA complexes should also be seen as natural impediments (86). The lack of Rrm3p has been shown
to slow replication fork progress through telomeres and increase the internal tracts of C_1.3A/TG_1.3 telomeric DNA (86). The slowing down of fork progression in the absence of Rrm3p suggests a role for this helicase in TPE and possibly other epigenetic changes during development and differentiation. To begin answering these questions, we must first investigate the function of these proteins \textit{in vivo}. An experiment to assess these questions is currently undergoing in our lab by another student. The experiment uses a rDNA locus and Fob1p to induce protein-mediated replication fork pausing at a specific loci. This pausing of the replication fork allows for the capture and detection of proteins at the pause site. This system would allow for the detection of Cac1p, PCNA and Rrm3p at a paused replication fork and determine whether Cac1p is evicted from the site during pausing. Once these experiments are completed, we should get a more insightful understanding of what transpires at the replication fork.
7.0 References


8.0 Appendix

**Appendix A**: Primer sequences (5’ to 3’) and respective melting temperatures for all PCR reactions used in this study.

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