THE ANAPHASE-PROMOTING COMPLEX INTERACTS WITH HISTONE MODIFICATION PROTEINS AND CHROMATIN ASSEMBLY FACTORS

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ABSTRACT

The Anaphase-Promoting Complex (APC) plays an important role in cell cycle progression. This evolutionarily conserved multi-subunit ubiquitin ligase is responsible for targeting proteins that hinder passage through mitosis and G1 progression for ubiquitination and proteasome-dependent degradation. Our laboratory has previously linked the APC with mitotic chromatin metabolism, as APC mutants were shown to exhibit impaired chromatin assembly. Chromatin assembly occurs when appropriately acetylated histones are deposited onto DNA. To date the only chromatin assembly factor linked to the cell cycle is the evolutionarily conserved CAF-I, a three-subunit complex of Cac1, Cac2 and Msi1. CAF-I associates with the histone chaperone Asf1, which first presents histones H3 and H4 to the histone acetyltransferases (HATs), Gcn5 and Rtt109, for acetylation. Following acetylation the histones are then passed on to CAF-I, which facilitates chromatin formation. Defective chromatin assembly has been linked to mitotic defects, leading to chromosomal rearrangements and aneuploidy. In addition to chromatin assembly, histone modifications have been linked to transcriptional activity and mitotic progression. The molecular mechanisms employed by the APC to govern chromatin biogenesis are unknown. In this thesis project, a modified genetic screen was performed to identify HAT and histone deacetylase (HDAC) mutants that interacted with APC mutants in the budding yeast *Saccharomyces cerevisiae*. This thesis focuses on the genetic and biochemical interactions observed between the APC and the HATs, Elp3 and Gcn5. As the majority of the proteins involved in chromatin assembly and histone modification are evolutionarily conserved, the insights obtained from the studies presented here utilizing the budding yeast *S. cerevisiae* should be directly applicable to research in human cells.

Via Western assays, this thesis demonstrates that yeast cells harboring mutations to the APC exhibit altered histone acetylation levels as well as altered total histone levels. Our genetic screen found that the temperature sensitive *apc5*^{CA} (chromatin assembly) mutant genetically interacted with a number of HATs and HDACs. Combining the *apc5*^{CA} allele with deletion of the genes *ELP3*, *GCN5*, *HDA1* or *SAS3* worsened the growth of the *apc5*^{CA} mutant, whereas deletion of *HOS1*, *HOS2*, *HOS3* or *SAS2* improved the growth of the *apc5*^{CA} mutant. Consistent with the genetic interaction results, increased expression of genes encoding the HATs Elp3, Gcn5 and Rtt109 (binds to Asf1) rescued the *apc5*^{CA} temperature sensitive phenotype. The temperature sensitive phenotype of the *apc5*^{CA} mutant was also rescued by increased expression of the genes encoding the CAFs Asf1 and Msi1 (a CAF-I subunit), as well as those encoding histones H3 and H4. These results suggest that increased deposition of acetylated histones is beneficial to APC function. Further analysis demonstrated that the APC and the HATs Elp3 and Gcn5 interact in the same pathway: cells lacking *ELP3* or *GCN5* accumulated in mitosis, whereas cells lacking both accumulated in G1 regardless of whether the APC was mutated or not. Additionally, increased *APC5* expression partially rescued the severely slow growing *elp3*Δ *gcn5*Δ double mutant. Elp3 and Gcn5 do not activate the APC, as the APC target Clb2 remained unstable in *elp3*Δ *gcn5*Δ cells. Our analysis suggests that Elp3, Gcn5 and the APC work together to promote mitotic progression. However, as increased expression of *ELP3* or *GCN5* causes cells to arrest in G1 this may reflect a need to degrade Gcn5 and/or Elp3 to exit G1. This is consistent with previous findings that show Gcn5 is required to transcribe genes necessary for mitotic exit. Using protein degradation assays we determined that Gcn5 is unstable during G1 in an APC dependent manner. Furthermore, wild-type Elp3 modification patterns are dependent on various APC subunits, the E2 Ubc1 and the proteasomal ubiquitin receptor Rpn10.

This thesis presents a model where the activities of Elp3 and Gcn5, along with the APC, promote mitotic exit and G1 progression, but that Gcn5, and possibly Elp3, must be degraded to allow progression into S-phase. The APC is further linked to chromatin assembly in that the APC physically interacted with the CAFs Asf1 and Cac2 (a CAF-I subunit). This interaction with Cac2 still occurred in the absence of Asf1. The literature has genetically linked Cac2 with Gcn5 and here my findings demonstrate that Cac2 and Gcn5 physically associate. Taken together, the data presented in this thesis suggest that the APC may bring the proteins involved in chromatin assembly and histone modification into close proximity in order to facilitate and possibly optimize chromatin assembly and subsequently genomic stability.

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Δ	gene deletion
ADA	transcriptional adaptor
Ahc	Ada histone acetyltransferase complex component
Ama	Activator of meiotic APC/C
amp^R	ampicillin
APC	Anaphase-promoting complex
Asf	Anti-silencing function
Bdf	Bromodomain factor
bp	base pairs
Bub	Budding uninhibited by benzimidazole
BubR	Bub1-related
Cac	Chromatin assembly complex
CAF	Chromatin assembly factor
CAF-I	Chromatin assembly factor -I
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
Cdc	Cell division cycle
Cdh	Cdc20 homolog
Cdk	Cyclin dependent kinase
CHX	cycloheximide
Clb	Cyclin B
CoIP	Co-immunoprecipitation
CREB	cAMP-response element-binding protein
D box	destruction box
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
DTT	dithiothreitol
E. coli	*Escherichia coli*
E1	ubiquitin-activating protein
E2	ubiquitin-conjugating proteins
E3	ubiquitin-protein ligases
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
Elp	Elongator protein
Emi	Early meiotic induction
Esa	Essential sas2-related acetyltransferase
FACS	Fluorescence Activated Cell Sorting
FACT	Facilitates chromatin transcription

Fas	FASCIATA
Gcn	General control nonderepressible
GNAT	GCN5-N-acetyltransferase
HAT/Hat	Histone acetyltransferase
Had	Histone deacetylase-A
HDAC	histone deacetylase
HECT	homologous to E6-AP carboxyl-terminus
HIR(A)	Histone Information Regulation
Hos	HDA1 one similar
HP	Heterochromatin Protein
Hpa2	Histone and other protein acetyltransferase
IP	Immunoprecipitation
K	lysine residue
LB	Luria Broth
LBA	Luria Broth Ampicillin
Mad	Mitotic arrest-deficient
MBD3	Methyl-CpG-binding domain protein
MMS	methyl methanesulfonate
Mnd	Meiotic nuclear divisions
mRNA	messenger RNA
Msi1	multicopy suppressor of Ira1
MYST	MOZ, Ybf2/Sas3, Sas2 and Tip60
Nap	Nucleosome assembly protein
NASP	Nuclear autoantigenic sperm protein
Npr	Nitrogen permease reactivator
Nup	Nuclear pore
OD	optical density
PCAF	p300/CREB-binding protein-associated factor
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PEG	polyethylene glycol
PKA	Protein kinase A
Plk1	polo-like kinase
PTEN	phosphatase and tensin homolog
Rad	Radiation sensitive
Rae	mRNA export factor
RAS	Rat sarcoma
Rb	retinoblastoma
RCAF	replication-coupling assembly factor
rDNA	ribosomal DNA
RING	really interesting new gene
RNA	Ribonucleic acid
RNAi	RNA interference

Rpd	Reduced potassium dependency
rpm	rotations per minute
Rpn	regulatory particle non-ATPase
RSC	Remodel the structure of chromatin
Rsp5	Reverses Spt-phenotype
Rtt	Regulator of Ty1 transposition
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
SAGA	Spt-Ada-Gcn5 acetyltransferase
SAS/Sas	something about silencing
SCF	Skp1/Cdc53/F-box
SD	synthetic dextrose
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SGA	synthetic genetic array
Sir	silent information regulator
Skp	Suppressor of kinetochore protein mutant
SLIK	SAGA-like
SV40	Simian vacuolating virus 40
Swm	Spore wall maturation
TAP	tandem affinity purification
TCA	trichloroacetic acid
TEMED	tetramethylethylenediamine
Tlk	tousled-like kinase
TPR	tetratricopeptide repeats
Ubc	Ubiquitin conjugating
U-boxes	UFD2 homolgy
UV	ultra violet
Vps	Vacuolar protein sorting
WT	wild-type
YPD	yeast extract, peptone, dextrose

CHAPTER ONE

INTRODUCTION AND BACKGROUND

1.1. Chromatin metabolism

Chromatin metabolism begins with the proper post-translational acetylation of histones followed by the deposition of those acetylated histones onto DNA to form one unit of nucleosomal DNA, or the nucleosome. The nucleosome, which is the fundamental structural component of chromosomes, consists of 147 bp of DNA wrapped twice around a nucleosome core particle (2 copies each of histones H2A, H2B, H3 and H4; Luger *et al*., 1997; Richmond & Davey, 2003). Multiple nucleosomes strung together like 'beads on a string' make up chromatin (Kornberg, 1974). Chromatin is progressively packaged and/or folded to eventually form a fully condensed chromosome. Chromosomes undergo severe gross morphological changes throughout each cell cycle and it is during mitosis that the segregation of properly folded chromosomes plays a key role in maintaining genomic stability. This process is necessary in order for a perfect copy of the genome to be passed from mother cell to daughter cell (reviewed in Felsenfeld, 1978; Belmont, 2006; Thompson *et al*., 2010). Without the highest level of fidelity in chromatin metabolism, genetic inheritance would not be possible. The temporally ordered sequence of chromatin packaging, and virtually all events that involve DNA thereafter, are governed by histone modifications (reviewed in Kurdistani & Grunstein, 2003; Krebs, 2007). In higher eukaryotes, defects in chromatin metabolism are associated with cancer and other disease states (reviewed in Misteli, 2010; Thompson *et al*., 2010). This thesis deals with the first steps in chromosome assembly, specifically how histone modification and deposition is regulated using a model organism, the budding yeast *Saccharomyces*

cerevisiae. As many of the proteins involved in chromatin assembly and histone modification are evolutionarily conserved, the findings from *S. cerevisiae* can be directly applied to research in higher organisms. Previous work in our laboratory has identified the critical cell cycle regulator, the Anaphase Promoting Complex (APC), as a regulator of both histone modification and mitotic chromatin assembly (Harkness *et al*., 2005; Turner *et al*., 2010). The role of the APC, an evolutionarily conserved ubiquitin-protein ligase, in regulating chromatin metabolism will be the major focus of this thesis.

1.1.1 Early understanding of Chromatin Assembly

As early as 1947, chromatin was known to be composed of DNA and proteins called histones (Mirsky & Ris, 1947). In 1965 Littau and Colleagues demonstrated that histones were responsible for holding chromatin threads together (Littau *et al*., 1965). Acid extraction of histones from calf thymus nuclei resulted in loosening of the chromatin, while the re-addition of the extracted histones was observed to restore its compact structure. At this time it was also understood that the core histones existed in chromatin in equimolar numbers. However the mechanisms of how, or even if, histones interacted with each other remained elusive. In 1974 Kornberg and Thomas determined that histones exist in solution as heterocomplexes (Kornberg & Thomas, 1974). Histone H2A was shown to form dimers with histone H2B while histones H3 and H4 came together as tetramers. These heterocomplexes were formed in the absence of DNA yet it was thought that these associations would also occur in chromatin as a solution of DNA, H2A/H2B dimers and H3/H4 tetramers formed complexes that had X-ray patterns identical to that of native chromatin (Kornberg & Thomas, 1974). Furthermore, work performed using electron microscopy demonstrated repeating units in chromatin (Olins & Olins, 1974; Oudet *et al*., 1975). In 1974 it was hypothesized that, like ‘beads on a

string', particles consisting of DNA wrapped tightly around histone complexes were evenly spaced along the DNA strand (Kornberg, 1974; Olins & Olins, 1974).

The experiments that demonstrated that histones and DNA could come together to form chromatin were performed under non-physiological conditions, and suggested that chromatin was a static self-assembling structure (Kornberg & Thomas, 1974; Oudet *et al*., 1975). Prior to the first demonstration of chromatin assembly under physiological conditions in 1977, experiments were performed using salt gradient dialysis starting at non-physiological ionic strengths (≥1M NaCl) (reviewed in Laskey & Earnshaw, 1980). As the salt was dialyzed away, nucleosomes formed on the DNA strand (Peterson, 2008). Using a cell-free system of SV40 DNA combined with purified histones and unfertilized *Xenopus* egg supernatant, chromatin assembly was accomplished under physiological ionic strength (Laskey *et al*., 1977). Micrococcal nuclease digestion of the assembly products produced DNA segments of multiples of 200 bp, which is identical to the digestion pattern of native eukaryotic chromatin. The requirement of an additional factor was demonstrated as histones, DNA and a nick-closing enzyme were unable to form chromatin under physiological conditions until a small amount of egg extract was added to the reaction. By fractionating the cell-free supernatant it was determined that an acidic protein binds histones before transferring them to the DNA (Laskey *et al*., 1978). Subsequently, this protein was named 'nucleoplasmin' as it is localized in the nucleoplasm (reviewed in Laskey & Earnshaw, 1980).

It has been noted that chromatin assembly factors are required for structured chromatin assembly to occur. Early *in vitro* experiments exhibited highly disordered insoluble aggregates as they contained only DNA and excess histones. It was theorized that chromatin assembly factors are required to mediate the interaction between the DNA

and histones. In fact, the addition of simple acidic molecules such as RNA, poly(glutamic acid) or poly(aspartic acid) led to ordered nucleosome arrays and prevented insolubility. Under physiological conditions, *in vitro* reactions containing excess purified chicken erythrocyte histones and SV40 plasmid DNA were fully assembled in the presence of poly(glutamic acid) or poly(aspartic acid) (Stein *et al*., 1979). Assays using *Drosophila* embryo extracts found that addition of fractions containing large amounts of various types of RNA were capable of assembling chromatin (Nelson *et al*., 1981). Interestingly, the most efficient assembly of chromatin occurred when RNA, poly(glutamic acid) or poly(aspartic acid), were present at a weight equal to that of histones, suggesting that it is the competition for histones that results in the ordered assembly (Stein *et al*., 1979; Nelson *et al*., 1981).

The early experiments using *Xenopus* egg and *Drosophila* embryo extracts demonstrated chromatin assembly in the absence of DNA replication and therefore outside of S-phase. In addition, further characterization showed that this chromatin contained basal variants of histones but not S-phase specific histone variants (Wu & Bonner, 1981; Dilworth *et al*., 1987). In 1985 it was reported that a nuclear extract was required for supercoiling of replicated DNA (Stillman & Gluzman, 1985). It was later discovered that a specific factor from these nuclear extracts was required for DNA synthesis coupled to chromatin assembly (Smith & Stillman, 1989). Chromatin Assembly Factor-I (CAF-I) was purified from the human 293 cell line and was shown to be required for chromatin assembly associated with DNA synthesis. An additional factor, termed RCAF (replication-coupling assembly factor), was later identified to also be required for replication-associated chromatin assembly (Tyler *et al*., 1999). RCAF was shown to be comprised of Asf1 (Anti-silencing function 1) and acetylated histones H3 and H4. Since

then Asf1 has been shown to participate in both replication-dependent and replication-independent chromatin assembly (Green *et al*., 2005).

1.1.2 Regulation of Chromatin Assembly

1.1.2.1 Role of Histones in Chromatin

Chromatin, repeating units of histone octamers each wrapped with 147 bp of DNA (Luger *et al*., 1997; Richmond & Davey, 2003), is organized into increasingly complex structures until the completely folded metaphase chromosome is created (Kornberg & Lorch, 1999; Woodcook & Ghosh, 2010). Histones are highly basic proteins with two domains, a globular core and a "highly dynamic N-terminal tail" and are some of the most conserved proteins across evolutionary boundaries (Mardian & Isenberg, 1978; Kornberg & Lorch, 1999). The globular core is responsible for histone/histone and histone/DNA binding while the protruding tails are heavily modified by a variety of post-translational modifications: acetylation, methylation, phosphorylation, ubiquitination, sumoylation and ADP-ribosylation (reviewed in Peterson & Laniel, 2004). These tail modifications have been associated with a variety of cellular processes such as DNA synthesis, transcriptional control, DNA repair, chromatin assembly and chromosome condensation. The occupancy of nucleosomes has been found to be lower at active promoters than at inactive promoters (Bernstein *et al*., 2004; Lee *et al*., 2004; Pokholok *et al*., 2005) with nucleosomes being removed from the DNA upon gene promoter activation (Reinke & Hörz, 2003; Boeger *et al*., 2004). This removal likely increases the accessibility of the transcription machinery by exposing naked DNA. Unregulated chromatin formation has been associated with aging and a variety of diseases, such as cancer. Therefore understanding the mechanisms that regulate chromatin formation is

crucial to understanding the onset and progression of disease and aging (reviewed in Enomoto & Berman, 1998; Kurdistani & Grunstein, 2003).

Following DNA replication during S-phase, naked DNA is an available target for every DNA binding protein in the cell. Thus, replication-dependent chromatin assembly is a vital process within the cell that establishes a silent, closed chromatin conformation that helps to prevent aberrant gene expression (discussed in Loyola & Almouzni, 2004). Replication-independent chromatin assembly can also occur, which is tightly associated with DNA repair (reviewed in Linger & Tyler, 2007). Interestingly, replication-dependent and -independent chromatin assembly is believed to follow clearly different pathways (Tagami *et al*., 2004). CAF-I is active in the presence of DNA synthesis while the HIR (Histone regulation) family of chromatin assembly factors are active in its absence. *S. cerevisiae* encodes two HIR genes while there is only one gene, HIRA, present in higher eukaryotes such as *Xenopus* and human. This division of labor was first demonstrated in experiments where *Xenopus* extracts were depleted of either HIRA or the CAF-I subunit p150 and incubated with intact or UV-treated plasmid DNA. The newly repaired UV-treated DNA represents replicated DNA while the intact plasmid represents DNA not undergoing replication. Extracts lacking CAF-I were defective in replication-dependent chromatin assembly but were still able to assemble chromatin in a replication-independent manner. The opposite occurred in extracts lacking HIRA (Tagami *et al*., 2004). Using HeLa cells the same study found that the replication-dependent assembly complex was comprised of all three CAF-I subunits (p150, p60 and p48) as well as histones H3.1 and H4. The replication-independent assembly complex contained HIRA and the histone variant H3.3, but did not contain p150, p60 or H3.1 (Tagami *et al*., 2004). It should be noted that even though *S. cerevisiae* does contain some histone variants, the genome only contains one form of histone H3, which is similar to the

vertebrate histone H3.3 variant. Interestingly both complexes contained p48 and the histone acetyltransferase Hat1. The finding of both Hat1 and p48 coexisting in the same complexes highlights the importance of histone acetylation to histone deposition. In fact, it was shown that acetylation of the histone H4 tail is necessary, as mutations that prevent acetylation resulted in defective deposition and growth (Ma *et al*., 1998).

The majority of chromatin assembly occurs immediately following DNA synthesis. This is the most important step in chromosome building as it establishes proper histone/DNA interactions that allow higher order chromatin packaging and segregation later in the cell cycle. Chromatin assembly occurs in a two-step manner with the H3/H4 tetramer being deposited onto the DNA first, followed by the addition of two H2A/H2B heterodimers (Smith & Stillman, 1991). Interestingly, solutions of DNA and H3/H4 have been found to form complexes that appear similar to chromatin; whereas solutions lacking either H3 or H4 are unable to form any chromatin-like structures (reviewed in Felsenfeld, 1978). Chromatin assembly assays using *Drosophila* extracts identified two fractions capable of, as well as required for, efficient assembly of chromatin (Bulger *et al*., 1995). One fraction contained CAF-I, the other contained the evolutionarily conserved nucleosome assembly protein 1 (Nap1; Bulger *et al*., 1995; Ito *et al*., 1996). NAP1 binds H2A/H2B dimers in the cytoplasm and acts as a histone chaperone (reviewed in Adams & Kamakaka, 1999; Tyler , 2002). H2A/H2B dimers are transported from the cytoplasm to the nucleus as cells progress through the G1/S transition (Ito *et al*., 1996). The assembly of the H2A/H2B dimers into the maturing nucleosome is likely facilitated by their binding affinity for DNA/H3/H4 complexes being higher than their affinity for Nap1 or naked DNA (Nakagawa *et al*., 2001).

1.1.2.2 Regulation of Histone Levels

Cells need to actively monitor histone levels as excess levels of free histones are toxic (Gunjan & Verreault, 2003). Experiments that alter the stoichiometry of histone pairs result in chromosome loss (Meeks-Wagner & Hartwell, 1986). This is possibly because unregulated DNA/histone binding leads to aggregated complexes, aberrant chromosome segregation and inhibited cell growth (Meeks-Wagner & Hartwell, 1986; Gunjan & Verreault, 2003; Groth *et al*., 2005). The levels of free histones within the cell can be controlled in several different ways, such as regulation of histone gene transcription, histone storage proteins or degradation of excess histones. For the purposes of this thesis, only histone storage proteins will be briefly described.

1.1.2.3 Histone Chaperones

Excess free histones are believed to be toxic to the cell due to formation of insoluble DNA/histone aggregates, but constantly degrading and retranslating these proteins is thought to require a significant amount of energy. As a solution to this problem, the cell possesses proteins that sequester histones to prevent their premature or inappropriate interaction with the DNA. There are several proteins believed to act as histone chaperones, such as nucleoplasmin, N1/N2, Nap1 and Asf1.

The H2A/H2B and H3/H4 binding proteins, nucleoplasmin and N1/N2, respectively, were theorized to allow progressive release of histones during rapid cell division in early development as both DNA replication and cell division occur too quickly to allow sufficient histone synthesis to occur (Dutta *et al*., 2001; Loyola & Almouzni, 2004). Nucleoplasmin is believed to act as a histone storage molecule, as it is capable of simultaneously binding up to five histone octamers by directly interacting with the

H2A/H2B dimers (Dutta *et al*., 2001). Nap1 preferentially binds H2A/H2B and is believed to shuttle histones from the cytoplasm into the nucleus (Ito *et al*., 1996; Mosammaparast *et al*., 2002). Asf1 has been shown to bind acetylated histone H4 in the cytoplasm prior to H3/H4 dimer transportation into the nucleus by importin-4 (Campos *et al*., 2010). Asf1 may also play a role as a histone reservoir in mammalian cells as the presence of Asf1/H3/H4 complexes increases in cells when DNA synthesis is blocked (Groth *et al*., 2005). These complexes also contained the p48 subunit of CAF-I and NASP, a member of the N1/N2 family (Groth *et al*., 2005; Campos *et al*., 2010).

1.1.3 Replication-dependent Chromatin Assembly

1.1.3.1 CAF-I

Chromatin assembly factors (CAFs), have been found in almost every organism studied (reviewed in Loyola & Almouzni, 2004). Currently, the only CAF believed to be linked to the cell cycle is CAF-1, which has been shown to be necessary for nucleosome deposition following DNA replication (Smith & Stillman, 1989; Gaillard *et al*., 1996). CAF-I is composed of three subunits that are conserved in almost all eukaryotic systems (Smith & Stillman, 1989). In *S. cerevisiae* these subunits are known as Cac1, Cac2 and Cac3/Msi1 (Multicopy suppressor of IRA1) (Kaufman *et al*., 1997), whereas in humans, the CAF-I orthologs are known as p150, p60 and p48 (Smith & Stillman, 1989). Human CAF-I has been shown to associate *in vitro* with histones H3 and H4 once lysines 5 and 12 of H4 have been acetylated (Verreault *et al*., 1996; Smith & Stillman, 1989; Sobel *et al*., 1995). This acetylation is rapidly removed once the histones are incorporated into chromatin in order to provide a blank slate for the rest of the cellular machinery to act upon (Annunziato & Seale, 1983). All three *S. cerevisiae* CAF-I subunits have been

shown to bind histones; however, only the two largest subunits (Cac1 and Cac2) are required for efficient chromatin assembly *in vitro* (Kaufman *et al*., 1995; Tyler *et al*., 2001). There are redundant activities among the CAFs since *S. cerevisiae* cells are still viable when all three CAF-I subunits are deleted (Kaufman *et al*.,1997; Harkness *et al*., 2005). In fact, yeast cells are still able to grow at the permissive temperature of 30°C even when 6 of the various CAF genes are deleted in one cell (CAC1, CAC2, MSI1, ASF1, HIR1, and HIR2; Harkness, unpublished data).

When CAF-I was first characterized as a replication-dependent chromatin factor it was shown to be required as soon as DNA replication begins. Chromatin was only partially assembled when CAF-I was added to reactions after DNA replication had already begun, suggesting that DNA synthesis and chromatin assembly occur in tandem (Smith & Stillman, 1989). In support of a coupling with replication, CAF-I localizes to DNA replication forks and has been shown to physically interact with the proliferating cell nuclear antigen (PCNA), a component of the replication machinery (Krude, 1995; Shibahara & Stillman, 1999; Moggs *et al*., 2000; Krawitz *et al*., 2002). This interaction was also shown to be localized to sites of DNA replication (Shibahara & Stillman, 1999). In cell-free systems, CAF-I activity was impaired during DNA repair after depletion of PCNA and CAF-I was no longer targeted to newly replicated DNA once its interaction with PCNA was disrupted (Moggs *et al*., 2000; Krawitz *et al*., 2002). Mutation of either CAF-I subunits or PCNA also caused defects in the silencing of the DNA located at the telomeres and these defects were exacerbated by deletion of either *ASF1* or *HIR1* (Sharp *et al*. 2001; Krawitz *et al*., 2002). This supports the finding that Asf1 and the HIR family of proteins are involved in an alternative silencing pathway from CAF-I (Kaufman *et al*., 1998; Sharp *et al*., 2001).

1.1.3.1.1 Role of CAF-I in maintaining genomic stability

Several studies suggest that CAF-I plays a role in genomic stability via DNA replication-dependent chromatin assembly. As naked DNA is thought to be more prone to damage, it would stand to reason that a defect in histone deposition would lead to DNA damage and genomic instability resulting in mutations and chromosomal rearrangements (Loyola & Almouzni, 2004). In fact, yeast mutants lacking *CAC1* exhibited increased gross chromosomal rearrangements such as telomere additions and translocations (Myung *et al*., 2003). Expression of a dominant negative variant of the *Xenopus* p150 CAF-I subunit inhibited *in vitro* chromatin assembly and resulted in defective cell cycle passage (Quivy *et al*., 2001), while in human cells, a dominant negative p150 inhibited chromatin assembly and progression through S-phase while inducing double strand breaks (Ye *et al*., 2003). Additionally, silencing of p60 resulted in death of proliferating cells, which was likely due to a decrease in nucleosome assembly and accumulation of double strand breaks (Nabatiyan & Krude, 2004). These effects on chromatin assembly and cell cycle progression resulted in deleterious organismal effects, as *Xenopus* embryos expressing dominant negative p150 did not survive past the mid-blastula stage (Quivy *et al*., 2001). Furthermore, silencing of the *Arabidopsis* orthologs of p150 and p60 (Fas1 and Fas2, respectively) resulted in cellular disorganization of the apical meristem (Kaya *et al*., 2001). These findings point to a role for CAF-I during DNA synthesis, which when impaired can lead to genomic instability during mitosis.

A review of the literature suggests that CAF-I subunits can function outside of the CAF-I complex as well as in functions other than acetylated histone deposition following DNA replication. While the majority of this research has focused on the smallest CAF-I subunit (Msi1 in yeast, p55 in *Drosophila*, p48 in many other eukaryotes) some evidence suggests that the other subunits may have roles outside of the complex as p150 and p60

associate with nucleotide excision repair sites in humans (Martini *et al*., 1998). Several studies have also shown Msi1/p55/p48 to individually associate with other proteins, including those responsible for histone acetylation and deacetylation. Studies of Msi1 orthologs in human, *Xenopus*, *Drosophila* and *Bos taurus* all showed that this subunit associates with the histone deacetylase Rpd3 (Taunton *et al*., 1996; Tyler *et al*., 1996; Vermaak *et al*., 1999; Tie *et al*., 2001) while chicken p48 has been shown to interact with a variety of histone modifying proteins, such as HAT1 and the HDACs 1, 2 and 3 (Ahmad *et al*., 1999; Ahmad *et al*., 2001). Studies in plant and human cells have also demonstrated p48 to interact with the retinoblastoma (Rb) protein (Qian *et al*., 1993; Ach *et al*., 1997). It is likely that yeast Msi1 also interacts with Rpd3 as Rb-dependent-gene repression was dependent on the presence of both Rpd3 and Msi1 in yeast cells containing recombinant human Rb protein (Kennedy *et al*., 2001). Since newly deposited histones are rapidly deacetylated to allow chromatin maturation, an interaction between Msi1/p48 and Rpd3, as observed in Xenopus, would provide a mechanism coupling chromatin assembly with deacetylation. CAF-I subunits have also been implicated in DNA methylation as p48, along with Rpd3, associated with the methyl-CpG binding protein Mbd3 in *Xenopus* extracts (Wade *et al*., 1999). Methyl-CpG binding proteins are responsible for recruiting HDACs to chromatin, suggesting that p48 plays a role in DNA methylation as well as histone acetylation (Wade *et al*., 1999). Furthermore, studies in the mouse have shown that the largest CAF-I subunit, p150, interacts with the methyl-CpG binding protein, Mbd1 (Reese *et al*., 2003).

The interaction of mouse p150 with heterochromatin is further supported by its interaction with the heterochromatin protein HP1 (Murzina *et al*., 1999). Whether the interactions with these DNA methylation proteins are due to CAF-I complex function or to the functions of the individual sub-units remains to be seen. Nevertheless, these results

suggest that CAF-I subunits are not only able to act independently of one another but also in a non S-phase dependent manner which greatly expands their role within the cell. In fact, our laboratory has shown the individual CAF-I subunits to be involved in mitotic chromatin assembly, in conjunction with the APC, as expression of the individual CAF-I subunits rescued the temperature sensitive phenotype of the chromatin assembly mutant *apc5*^{CA} (Harkness *et al*., 2005). Further supporting roles for the individual CAF-I subunits is the finding that overexpression of Msi1 is able to inhibit the RAS/cAMP signaling pathway in yeast cells lacking either *CAC1* or *CAC2* by interacting with Npr1, a cytoplasmic kinase (Zhu *et al*., 2000; Johnston *et al*., 2001).

1.1.3.1.2 Regulation of CAF-I

The intricacies of CAF-I biology are still not fully understood; however, it appears that phosphorylation of CAF-I may play a role in human cells. Phosphorylation of p60 occurs after DNA damage, while hyperphosphorylation occurs upon entry into mitosis (Marheineke & Krude, 1998; Martini *et al*., 1998). Interphase cells exhibited both a phosphorylated form and a hypophosphorylated form. The phosphorylated form was demonstrated to be more active in promoting chromatin assembly than the hypophosphorylated form (Martini *et al*., 1998). Thus, it would appear that CAF-I activity may be controlled through the reversible phosphorylation of p60. The hyperphosphorylated p60 in mitotic cells was inactive for replication-dependent chromatin assembly (Marheineke & Krude, 1998) and resulted in a large proportion of p60 being exported to the cytosol while only a small proportion of p150 is exported (Marheineke & Krude, 1998; Martini *et al*., 1998). Therefore, not only may hyperphosphorylation cause the dissociation of CAF-I from chromatin but it may also result in dissolution of the complex.

One of the reasons CAF-I may not be essential in yeast is the existence of other redundant factors. *CAC1* mutants have been shown to genetically interact with other CAF mutants, such as *asf1*Δ and *hir*Δ mutants. Asf1 was first identified in yeast due to its overexpression being able to inhibit the silencing of genes near the telomeres (Le *et al*., 1997) and was shown to bind H3/H4 dimers (Tyler *et al*., 1999). Cells lacking both the genes *ASF1* and *CAC1* were shown to exhibit UV sensitivity as well as gene silencing and growth defects that were more severe than either of the mutations alone, suggesting overlapping but independent activities (Tyler *et al*., 1999; Sharp *et al*., 2001; Krawitz *et al*., 2002). It was theorized that these UV dependent defects were due to errors occurring during DNA replication as DNA checkpoints were activated in the absence of exogenous DNA damage (Myung *et al*., 2003). As naked DNA is thought to be more damage-prone, it would stand to reason that a defect in histone deposition may lead to DNA damage (Loyola & Almouzni, 2004). Mutating the *HIR* genes along with *CAC1* resulted in sensitivity at low growth temperature (16˚C), exacerbated silencing and slowed progression through G2/M (Kaufman *et al*., 1998; Sharp *et al*., 2001; Krawitz *et al*., 2002). Thus, studies in yeast clearly suggest an intricate network of CAFs that function together in a redundant manner to ensure that proper progression through the cell cycle occurs.

CAF-I has also been shown to be involved in replication-independent chromatin assembly pathways. CAF-I preferentially assembles newly replicated DNA; however, excess levels will target bulk DNA for assembly (Kamakaka *et al*., 1996). As will be discussed in greater detail below, CAF-I has also been found to be involved with mitotic chromatin assembly in addition to DNA repair (Harkness *et al*., 2005; reviewed in Linger & Tyler, 2007).

1.1.3.2 ASF1

Unlike in budding yeast, *ASF1* is essential in many organisms suggesting that yeast either has redundant factors involved or is more tolerant of incomplete chromatin assembly in S-phase (Mousson *et al*., 2007). Redundant factors are a more plausible explanation as nucleosome density does not change drastically in *ASF1* mutants (Adkins *et al*., 2004; Prado *et al*., 2004). Deletion of *ASF1* in budding yeast does result in increased DNA damage and slowed growth. However, double mutants of *ASF1* and *CAC1* are still viable with levels of nucleosome density similar to that of wild-type, which suggests that there are redundant factors that allow H3/H4 replication-dependent chromatin formation (Le *et al*., 1997; Tyler *et al*., 1999; Adkins *et al*., 2004; Prado *et al*., 2004). This is likely the case as additional CAFs that deposit H3/H4 dimers onto DNA have since been described in yeast, such as Vps75 and Rtt106 (Huang *et al*., 2005; Selth & Svejstrup, 2007).

Asf1 has been shown to participate in both replication-dependent and replication-independent chromatin assembly by interacting with CAF-I and the Hir proteins (Green *et al*., 2005). Both human and *Drosophila* Asf1 were able to stimulate CAF-I replication dependent chromatin assembly *in vitro.* Nevertheless, Asf1 can also contribute to histone deposition in the absence of CAF-I and DNA replication, suggesting that Asf1 also plays a role in replication-independent chromatin assembly (Tyler *et al*., 1999; Munakata *et al*., 2000; Sharp *et al*., 2001; Green *et al*., 2005). Asf1 has also been shown to bind to the Hir1 and Hir2 chromatin assembly factors which are involved in replication-independent chromatin assembly (Sharp *et al*., 2001; Ray-Gallet *et al*., 2002; Ahmad *et al*., 2004). *In vitro* experiments using mutant CAF-I unable to bind to PCNA showed that Asf1/H3/H4 complexes were able to assemble bulk DNA that had not been replicated (Krawitz *et al*., 2002). However, Asf1 is not known to deposit histones onto chromatin

itself during DNA replication (Tyler *et al*., 2001), thereby highlighting the importance of additional chromatin assembly factors.

Human Asf1 has been found to be involved with the DNA damage response as it is phosphorylated by the tousled-like kinase (Tlk1) (Groth *et al*., 2003). This kinase does not appear to exist in yeast, which indicates that a different regulatory network is at play. The C-terminal domain of Asf1, where the phosphorylation is believed to occur, differs between yeast and higher eukaryotes (Silljé & Nigg., 2001). Along with the type of histone variant utilized, phosphorylation of Asf1 may help to dictate which pathway Asf1 participates in. Yeast Asf1 is bound by the Rad53 checkpoint kinase, but this interaction appears to be dependent on Asf1 being phosphorylated by a protein other than Rad53 (Emili *et al*., 2001; Schwartz *et al*., 2003). Cac2/p60 and Asf1 have been shown to physically interact and this interaction is evolutionarily conserved (Tyler *et al*., 2001; Krawitz *et al*., 2002; Mello *et al*., 2002). Asf1 phosphorylation did not affect its physical interaction with the CAF-I subunit p60, which is also phosphorylated (Mello *et al*., 2002). Like CAF-I, Asf1 has also been shown to localize to DNA replication forks (Krude, 1995; Schulz & Tyler, 2006). Additional activities have also been ascribed to yeast Asf1, such as chromatin disassembly during gene transcription (Adkins *et al*., 2004). In fact, cells lacking Asf1 show delayed gene activation (Adkins *et al*., 2004; Korber *et al*., 2006). This could link into the idea that Asf1 can also act as a histone storage protein (Groth *et al*., 2005). Alternatively, Asf1's role in transcription could also be explained by its role in histone acetylation. Asf1 is responsible for presenting the H3/H4 histone dimer to Gcn5 and Rtt109 for acetylation prior to deposition onto the DNA (Fillingham *et al*., 2008; Burgess *et al*., 2010). See Figure 1.1 for a simple schematic of chromatin assembly involving Asf1 and CAF-I. Gcn5 and Rtt109 are each capable of acetylating histone H3 at residue K9 while Rtt109 also acetylates H3K56. Acetylation of these residues is

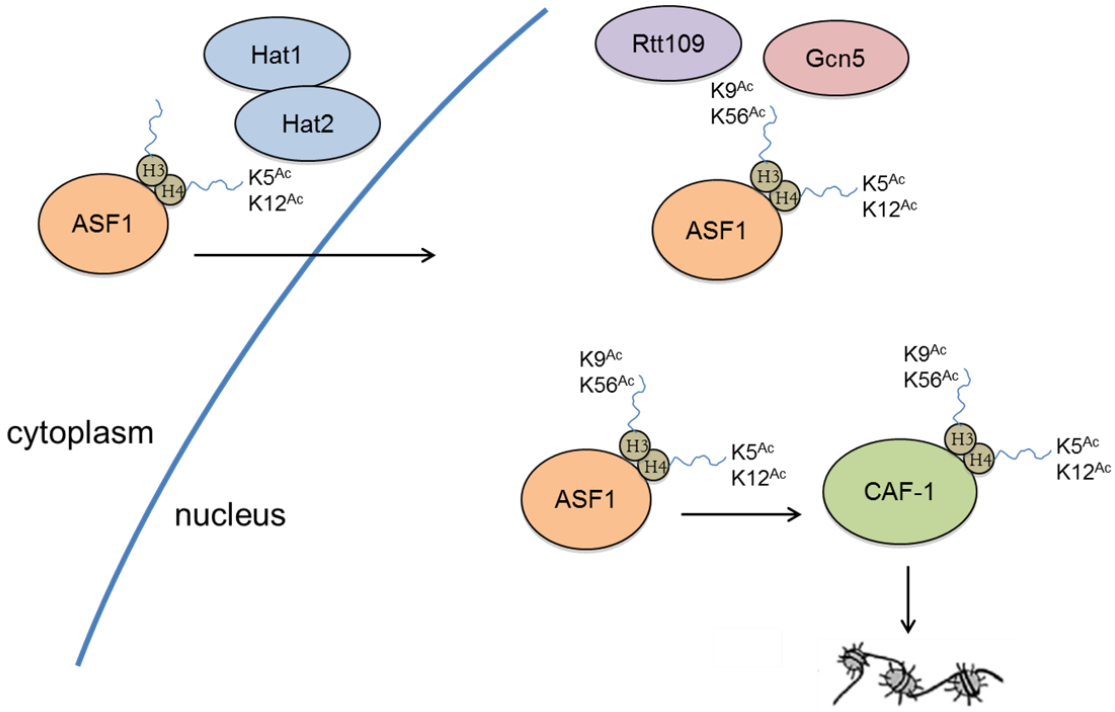

Figure 1.1. **Role of Asf1 and CAF-I in chromatin assembly**. Asf1 presents histone H3/H4 dimers to Hat1/Hat2 which acetylate K5 and K12 on histone H4. The Asf1/H3/H4 complex is then transported from the cytoplasm into the nucleus. The histone H3/H4 dimer is then presented to the HATs Rtt109 and Gcn5 to acetylate K9 and K56 on histone H3. The acetylated histones are then passed on to CAF-I which assembles them onto replicated DNA. Adapted from Fillingham *et al*., 2008.

associated with newly deposited histones. Acetylation of the K9 residue has also been associated with transcription and acetylation of K56 is also associated with DNA repair (Kuo *et al*., 1996; Masumoto *et al*., 2005; Millar & Grunstein, 2006).

1.1.4 Replication-independent Chromatin Assembly

Studies in *Xenopus* show that the H3/H4 histone chaperone HIRA (histone information regulator) is involved in replication-independent chromatin assembly. HIRA-depleted extracts could not assemble chromatin on unreplicated DNA: in contrast, chromatin was assembled in the presence of DNA synthesis (Ray-Gallet *et al*., 2002). In yeast, the HIR genes *HIR1* and *HIR2* are part of the HIR complex and are both required for gene repression when there is an overabundance of the histones H2A/H2B (Recht *et al*., 1996). Overexpression of the single human HIR gene, *HIRA,* resulted in a decrease of histone transcription (Nelson *et al*., 2002). The HIR family may also act as histone storage proteins as they have been shown to interact with the yeast form of FACT (facilitates chromatin transcription), an RNA polymerase II transcription elongation factor proposed to remove H2A/H2B dimers from the chromatin prior to transcription (Orphanides *et al*., 1999; Formosa *et al*., 2002). The yeast HIR complex is required in cells with a defective FACT complex, perhaps compensating for the loss of FACT histone chaperone function. This further highlights the role of the HIR family of proteins as histone chaperones outside of S-phase.

CAF-I is also able to participate in replication-independent chromatin assembly *in vitro*. Human and *Drosophila* studies showed that increased amounts of p150 and p60 subunits resulted in assembly of bulk DNA as well as newly replicated DNA (Kamakaka *et al*., 1996; Tyler *et al*., 2001; Krawitz *et al*., 2002). CAF-I's preference for newly

replicated DNA is concentration dependent as excessive amounts of human CAF-1 completely assembled bulk DNA to the point of inhibiting DNA synthesis (Kamakaka *et al*., 1996). The specificity of CAF-I also depends on interaction with other proteins involved in DNA replication, as CAF-I complexes that were unable to bind to PCNA preferentially assembled bulk chromatin over newly synthesized DNA (Krawitz *et al*. , 2002). CAF-I has also been found to be involved with mitotic chromatin assembly as progressive deletion of the CAF-I subunits produces progressively more severe phenotypes in the APC mutant *apc5*^{CA}, which exhibits compromised progression through mitosis and impaired chromatin deposition (Harkness *et al*., 2002). Overexpression of any single CAF-I subunit is enough to rescue the temperature sensitivity of an *apc5*^{CA} mutant lacking all three CAF-I subunits (Harkness *et al*., 2005). Thus, the individual CAF-I subunits appear to be functional outside of the CAF-I complex, and this activity may be specific to mitosis.

1.1.5 Histone modification

Proper acetylation and deacetylation of the nucleosome bound histone N-terminal tails results in timely and appropriate gene expression and repression (Grunstein, 1997; Kurdistani & Grunstein, 2003; Peterson & Laniel, 2004; Peserico & Simone, 2011). An example of this is the requirement of acetylation of the histone H3 and H4 N-terminal tails to allow transcription and *in vitro* chromatin assembly to occur. Histones are acetylated on lysine residues in a reversible manner. Histone acetyltransferases (HATs) are responsible for attaching acetyl groups onto histones while HDACs remove acetyl groups. As a general rule, acetylation of the histone tails promotes gene transcription while deacetylation inhibits it (Durrin *et al*., 1991; Vogelauer *et al*., 2000). An exception to this rule is the HDAC Hos2 as it was found that yeast cells lacking Hos2 had

decreased levels of *GAL1* and *INO1* mRNA (Wang *et al*., 2002). Hos2 may be unique among HDACs, as its deacetylation of histones aids transcription. This may be a result of Hos2 restoring the coding regions of recently transcribed genes back to an unaltered state that is permissive for transcription initiation to occur again (Wang *et al*., 2002).

The majority of histone synthesis is coupled to DNA synthesis during S-phase to ensure an adequate supply of histones for chromatin assembly (Plumb *et al*., 1983). Histone synthesis does occur during the rest of the cell cycle but at a lower level. In addition, the majority of histones synthesized outside of S-phase are the histone variants, such as H3.3, H2A.X, H2A.Z, CENP-A (humans) and CEN3 (yeast) (Loyola & Almouzni, 2004). H3 itself is associated with newly replicated or repaired DNA while the histone variants are associated with different aspects of chromatin assembly. Notable examples are H3.3, which is predominantly found at actively transcribed genes, and CENP-A, which is found in centromeres (Palmer *et al*., 1991; Stoler *et al*., 1995; Ahmad & Henikoff, 2002). The variants may play a role in cellular functions by either marking specific regions or recruiting specific factors that increase or decrease transcription and/or chromatin condensation.

The nucleosome is a major inhibitor of gene transcription initiation (Wasylyk & Chambon, 1979; Lorch *et al*., 1987). Each of the core histones contains a domain that contributes to proper DNA folding resulting in the repression of basal transcription (Lenfant *et al*., 1996). The interaction of nucleosomal histones with DNA can be altered by their post-transcriptional modification. Acetylation, methylation, phosphorylation, ubiquitination, sumoylation and ADP-ribosylation all affect gene expression and repression, silencing, DNA replication, DNA damage repair and apoptosis (reviewed in Peterson & Laniel, 2004). The fact that each histone has multiple residues that can be modified in a

multitude of combinations has led to the hypothesis called the 'histone code'. The histone code proposes that each modification (or lack thereof) plays a role in controlling cellular functions such as gene expression, DNA repair and chromatin packaging (Strahl & Allis, 2000; Peterson & Laniel, 2004; Lee *et al*., 2010). In addition, there are many different histone modifying enzymes within the cell, each with their own preference of residue and chromosomal region.

1.1.5.1 Histone acetylation

The acetylation state of histone tails is important for chromatin structure and the regulation of transcription (Allfrey *et al*., 1964; Horn & Peterson, 2002). There are two main hypotheses to explain this; the charge-neutralization model and the histone code model. One hypothesis suggests that acetylation weakens the bond between DNA and histones allowing easier access of transcription factors to gene promoters (Verreault et al., 1996; Grunstein, 1997; Kurdistani & Grunstein, 2003). An alternative theory states that acetylation acts as a signal to recruit transcription factors and other proteins to that specific site (Kurdistani & Grunstein, 2003).

The charge-neutralization model theorizes that negatively charged acetyl groups neutralize the positive charge on histone tails to decrease the attraction to negatively charged DNA (Hong *et al*., 1993). This loosening of the chromatin would allow transcription factors access to promoters in the DNA (Mutskov *et al*., 1998). The reverse would occur after deacetylation; the positively charged histone tails would cause the chromatin to tighten up, thereby preventing access to transcription factors (reviewed in Kurdistani & Grunstein, 2003). A slightly different hypothesis is that histone deacetylation causes chromatin to fold into a structure that prevents access to transcription factors

(Mutskov *et al*., 1998). Acetylation of the histones would prevent this folding (Tse *et al*., 1998). Both variations of this hypothesis result in gene activity being regulated as acetylation provides access to specific binding sites within the chromatin while deacetylation prevents that access. Acetylated histones have been associated with transcriptional activation thereby supporting this hypothesis (Hebbes *et al*., 1988).

A second hypothesis, the Histone Code, proposes that specific histone modifications induce functions such as gene expression, silencing and DNA repair by recruiting effector proteins that recognize these specific modifications in a sequence dependent manner (Strahl & Allis, 2000; Agalioti *et al*., 2002). The theory states that histone modifications act as signals for protein binding. In support of this, many proteins and protein complexes associated with chromatin contain domains that recognize and bind to acetylated lysines (Dhalluin *et al*., 1999; Jacobson *et al*., 2000). Bromodomains bind to acetylated lysines (Dhalluin *et al*., 1999).The proteins Bdf1 and Bdf2 (Bromodomain factor-1 and -2) each contain two bromodomain motifs and bind to the TFIID subunit TAF67 (Matangkasombut *et al*., 2000). The complexes SAGA (Spt-Ada-Gcn5 acetyltransferase) and RSC (remodel the structure of chromatin) also each contain multiple bromodomains. The large multi-subunit transcriptional activator SAGA contains two bromodomain proteins, Gcn5 and Spt7 (Hassan *et al*., 2002), while the remodeler RSC has eight (Kasten *et al*., 2004). The bromodomain found in Gcn5, the histone acetylase (HAT) component of SAGA, has been shown to directly interact with the N-terminal tails of H3 and H4 (Ornaghi *et al*., 1999) and is required for SWI/SNF mediated nucleosome remodeling (Syntichaki *et al*., 2000). Patterns of acetylation and deacetylation may provide specific binding sites for repressors and activators (Johnson *et al*., 1990; Carmen *et al*., 2002). For example, the silencing information regulator SIR3 (Silent information regulator 3) will only bind to H4 when Lys16 (K16) is deacetylated. In

experiments where K16 was mutated to glutamine, which mimics the acetylated state, Sir3 binding and heterochromatin formation was abolished/inhibited (Johnson *et al*., 1990; Carmen *et al*., 2002). The charge-neutralization and histone code hypotheses are not mutually exclusive and it is highly likely that transcription and chromatin structure are controlled in part by both.

The site specific recruitment of HATs and histone deacetylases (HDACs) is often accomplished through the association of sequence specific DNA binding proteins. Some examples of this involve the HDACs Hst1 and Rpd3, as well as the HAT Esa1. Rpd3 associates with the transcriptional repressor Ume6 at the INO1 gene, while Hst1 is recruited to sporulation genes by the transcription factor Sum1 (Kurdistani *et al*., 2002; Robert *et al*., 2004). The HAT Esa1 is targeted to the promoters of ribosomal genes by two different transcriptional factors, Rap1 and Abf1 (Reid *et al*., 2000). A similar mode of specificity occurs in other histone modifying proteins as the methyltransferase Dot1 is recruited to active genes, to methylate H3K79 (Shahbazian *et al*., 2005). In contrast to this specificity, some HAT and HDAC activity appears to be global. Rpd3 has been detected on a global level regardless of the presence of Ume6 (Kurdistani *et al*., 2002). Esa1 and Gcn5 have also been observed to act globally (Robert *et al*., 2004). The differences seen in specificity may be due to the fact that many histone modification proteins can be found in multiple complexes. Other proteins are targeted to specific domains in the chromatin. For example, Sir2 has been shown to deacetylate histones at the mating type loci and telomeric regions, both of which are large silenced areas (reviewed in Grunstein, 1998).

There are a large number of HATs and HDACs, even within the relatively small yeast genome (reviewed in Sterner & Berger, 2003). Many appear to be redundant to one

another in the role of acetylation and deacetylation but likely play important roles due to their presence in many different protein complexes. Esa1 is present in two separate complexes, with the larger NuA4 being targeted to promoters while the smaller piccolo NuA4 acts on a more global basis (Boudreault *et al*., 2003). The HATs Gcn5 and Elp3 are both capable of acetylating H3 lysine 14, however, that is the only activity they are known to share. Gcn5 acetylates histones H2B and H3 when part of the SAGA complex while Elp3 acetylates histones H3 and H4 as a part of the Elongator complex (Grant *et al*., 1997; Li *et al*., 2005).

1.1.5.2 Histone acetyltransferases (HATs) relevant to this study

There are many HATs within the yeast genome, each with their own preference for specific lysines as well as chromosomal region (see Table 1.1; reviewed in Millar & Grunstein, 2006). This study will focus primarily on Gcn5 and Elp3, both of which target histone H3K14 (Wittschieben *et al*., 2000). Histones are acetylated on lysine residues in a reversible manner. HATs are responsible for attaching acetyl groups onto histones while HDACs remove acetyl groups. As a general rule, acetylation of the histone tails promotes gene transcription while deacetylation inhibits it (Durrin *et al*., 1991; Vogelauer *et al*., 2000).

All HATs use acetyl-coenzyme A as a donor for histone acetylation. The acetyl group is first removed from coenzyme A by the catalytic domain of the HAT then transferred to the ε-amino group of the target lysine (Loidl, 1994; Lin *et al*., 1999). This formation of an amide bond can be removed by the action of an HDAC. Additionally, the manner in which the lysine is acetylated depends on the type of HAT involved. The GCN5/PCAF family of HATs must bind to both acetyl-coenzyme A and the histone substrate at the

Table 1.1 Yeast HATs used in this study

HATs	Known Complexes	Histones targeted in vivo	Known Function
Elp3	Elongator	H3/H4	Transcriptional elongation
Gcn5	SAGA	H2B/H3/H4	Coactivator/ nucleotide excision repair
	ADA SLIK HAT-A2	H3 H2B/H3/H4 H3	nucleotide excision repair
Hat1	HAT-B, HAT-A3	H4	Replication-dependent chromatin assembly
Hpa2	May exist as a dimer or tetramer	H3	unknown/possibly transcription
Rtt109	Copurifies with Vps75	H3	Replication-dependent chromatin assembly
Sas2	SAS	H4	Silencing
Sas3	NuA3	H3	Silencing

Information obtained and modified from Angus-Hill, etal. 1999; Wittschieben, et al. 1999; Sterner & Berger 2000; Marmorstein 2001b; Suka, et al. 2002; Lee & Workman 2007; Fillingham, et al. 2008.

same time to allow lysine acetylation to occur (Tanner *et al*., 2000). In contrast, the MYST family of HATs first transfers the acetyl group from acetyl-coenzyme A to a cysteine residue within the HAT itself. Co-enzyme A must then be released before the substrate is acetylated (Yan *et al*., 2002). This acetylation neutralizes the basic charge of the lysine and can therefore alter both its, and the histone's, interaction with the DNA (Tse *et al*., 1998).

HATs can be broken up into two basic categories; A-type HATs acetylate nucleosomal histones within chromatin and B-type HATs act predominantly within the cytoplasm where they acetylate histones (reviewed in Sterner & Berger, 2000). It should be noted that since many HATs are part of several different complexes, these categories can become blurred (Ruiz-Garcia *et al*., 1998). In addition, there are several families of HAT each with their own histone lysine specificities: GCN5-N-acetyltransferase (GNAT)-related, MYST, CBP/p300, TAFII250, SCR and ATF-2 families (reviewed in Sterner & Berger, 2000; Marmorstein, 2001a; Marmorstein, 2001b). The following three pertain to this thesis:

1. GCN5-N-acetyltransferase (GNAT)-super family: grouped together based on similarity of 4 different acetylation-related motifs of 15-33 amino acids. This group includes HATS such as Gcn5, PCAF, Hat1, Hpa2 and Elp3 (Neuwald & Landsman, 1997). The A motif is required for acetyl-coenzyme A binding, is the most evolutionarily conserved (Dutnall *et al*., 1998; Wolf *et al*., 1998; Lin *et al*., 1999).
2. MYST family: named for its founding members; MOZ, Ybf2/Sas3, Sas2 and Tip60 (reviewed in Sterner & Berger, 2000). Members of this group are included

due to their sequence similarities and the presence of the A motif, which is also found in the GNAT superfamily (Neuwald & Landsman, 1997).

3. CBP/p300 family: global coactivators, containing a bromodomain, like many other transcription factors (Eckner *et al*., 1994). In addition to the ability to acetylate histones CBP/p300 proteins are also capable of acetylating transcription factors to modulate their activity (reviewed in Marmorstein, 2001a). Although there have been no proteins with a sequence similar to that of CBP/p300 found in the *S. cerevisiae* genome, crystal structure analyses have demonstrated that Rtt109 (Regulator of Ty1 transposition) is structurally similar (Liu *et al*., 2008; Tang *et al*., 2008; Wang *et al*., 2008).

1.1.5.2.1 Gcn5

Gcn5 (General control nonderepressible 5) was first shown to have HAT activity in the ciliate *Tetrahymena thermophila* and to be homologous to a transcriptional adaptor in yeast (Brownell *et al*., 1996). Since then Gcn5 orthologs have been identified in numerous eukaryotes, suggesting that it is highly evolutionarily conserved (Candau *et al*., 1996; Smith *et al*., 1998; Xu *et al*., 1998; Hettmann & Soldati, 1999). The conservation of this protein is highlighted by human Gcn5 being found to have the same activity as yeast Gcn5 (Candau *et al*., 1996; Wang *et al*., 1997). In addition, the p300/CREB-binding protein-associated factor (PCAF; cAMP response element binding), a protein with high homology to mammalian Gcn5, has also been shown to target H3K14 and H4K8 *in vitro* (Schiltz *et al*., 1999).

Gcn5 is able to acetylate free histones, but not nucleosomal histones *in vitro* (Grant *et al*., 1997). Gcn5 specificity *in vivo* is a complex matter as the activity of Gcn5 is required

in several different complexes (Grant *et al*., 1997; Grant *et al*., 1999). For example, when part of the ADA (transcriptional adaptor) complex, Gcn5 acetylates H3 lysine residues 14 and 18, but when incorporated into SAGA Gcn5 acetylates H3 lysine residues 9, 14, 18 and 23 (Grant *et al*., 1999). Gcn5 has also been shown to acetylate histone H2B when incorporated into the SAGA complex (Wang *et al*., 1998). Gcn5's *in vitro* binding of H3K14 was shown to be increased by the phosphorylation of H3S10 (Cheung *et al*., 2000; Lo *et al*., 2000). This has also been observed *in vivo*, as addition of epidermal growth factor to mammalian cells results in S10 phosphorylation, quickly followed by K14 acetylation (Cheung *et al*., 2000).

SAGA is a multi-subunit protein complex involved in transcriptional activation. In addition to acetylating histone H3, SAGA also allows Gcn5 to target histone H2B which it is unable to do by itself at physiological levels (Grant *et al*., 1997). When incorporated into SAGA Gcn5 is also able to acetylate H3 at residues K9, K14, K18 and K23 while alone it is only able to target K14 (Grant *et al*., 1999). This ability is likely due to both the presence of other proteins within the complex and to Gcn5's bromodomain. Deletion of this domain resulted in a decrease of the nucleosomal acetylation associated with SAGA as well as *HIS3* transcription (Sterner *et al*., 1999). The complexes SAGA and ADA both contain Gcn5, Ada2 and Ada3, but that is where the similarity ends (Grant *et al*., 1997; Grant *et al*., 1999). ADA is a separate complex, not just a subcomplex of SAGA, as it contains Ahc1 (Ada histone acetyltransferase complex component), which is not found in SAGA (Eberharter *et al*., 1999). While a part of ADA, the activity of Gcn5 is more restricted than in SAGA, as only the residues K14 and K18 are targeted on H3 (Grant *et al*., 1999). ADA does not seem to play a role in transcription, as it does not interact with activation domains (Utley *et al*., 1998).

Gcn5 was first identified as a transcriptional co-activator required for Gcn4 mediated transcriptional activation (Georgakopoulos & Thireos, 1992). In fact, the HAT domain of Gcn5 is required for adaptor mediated transcriptional activation (Candau *et al*., 1997), cell growth, and histone H3/H2B acetylation (Wang *et al*., L1998). Mutants lacking Gcn5 exhibit temperature sensitivity and slow growth on minimal media, accumulation in G2/M and altered nuclear migration (Zhang *et al*., 1998; Vernarecci *et al*., 2008; Turner *et al*., 2010). Gcn5 is involved in the regulation of several genes. For example, the expression of *HIS3* and *PHO5* are decreased in strains harboring Gcn5 mutants incapable of acetylating histones (Gregory *et al*., 1998; Kuo *et al*., 1998). In addition, promoter DNA was shown to be remodeled due to Gcn5 HAT activity (Gregory *et al*., 1998). Taken together, these studies provide a persuasive argument for a link between histone acetylation and transcription.

1.1.5.2.2 Elp3

Elp3 (Elongator protein 3) is the HAT component of Elongator, a six subunit complex associated with the RNA polymerase II holoenzyme (Otero *et al*., 1999; Wittschieben *et al*., 1999). Once transcription is initiated RNA polymerase II is phosphorylated at its carboxy-terminal domain to allow elongation to occur (Payne *et al*., 1989). This phosphorylation is required for Elongator/RNA polymerase II interaction (Otero *et al*., 1999). As nucleosomes inhibit transcriptional elongation (Wasylyk & Chambon, 1979) acetylation of these histones should allow greater access of the transcriptional machinery to the DNA (Tse *et al*., 1998). Recombinant Elp3 is able to acetylate all four core histones on their N-terminal tails *in vitro* when presented with them individually (Wittschieben *et al*., 1999). When part of Elongator, Elp3 preferentially targets H3K14 and H4K8 (Winkler *et al*., 2002). Genetic studies of the deletion mutants *elp1*Δ or *elp3*Δ

showed salt and temperature sensitivity, slow growth adaptation and decreased activation of certain genes (Otero *et al*., 1999; Wittschieben *et al*., 1999). An *elp1*Δ *elp3*Δ double mutant exhibited phenotypes identical to the single mutants suggesting that these subunits do not act individually from each other. Human Elp3 is functionally similar to Yeast Elp3 and can rescue, at least partially, deletion mutants (Li *et al*., 2005).

Elp3 has been shown to have an overlapping role with Gcn5 as they both have been implicated in the control of transcription, Gcn5 with initiation and Elp3 with elongation (Georgakopoulos & Thireos, 1992; Wittschieben *et al*., 1999). Additionally, they each preferentially target histone H3K14 for acetylation (Grant *et al*., 1999; Wittschieben *et al*., 2000; Winkler *et al*., 2002). The fact that neither deletion is lethal may be attributed to redundancy of HAT activity. Deletion of both Elp3 and Gcn5 results in increased temperature sensitivity relative to the single deletions and the inability to grow on alternate carbon sources such as galactose, raffinose and sucrose (Wittschieben *et al*., 2000; Turner *et al*., 2010). By deleting various subunits specific to Elongator, ADA or SAGA, it was determined that the phenotype of the *elp3*Δ *gcn5*Δ mutant was due to their activities within Elongator and SAGA. A double deletion mutant, *gcn5*Δ *hat1*Δ, exhibited phenotypes similar to the *gcn5*Δ single mutant suggesting that the Elp3/Gcn5 interaction is specific for their roles in transcription and not due to other activities (Ruiz-Garcia *et al*., 1998; Wittschieben *et al*., 2000).

1.1.5.2.3 Other HATs

In addition to Gcn5 and Elp3 there are many other HATs within the yeast genome (reviewed in Millar & Grunstein, 2006). Each of these HATs has their own, specific lysine targets but as with Gcn5 and Elp3 there is sometimes overlap (Wittschieben *et al*.,

2000). For example, this overlap of function can be highlighted by the finding that a double deletion of *GCN5* and *SAS3* is synthetically lethal due to the loss of HAT function (Howe *et al*., 2001). Both Sas3 (Something about silencing) and Gcn5 are recruited to similar active gene pools and both are capable of acetylating histone H3K14 (Kuo *et al*., 1996; Rosaleny *et al*., 2007). Sas3 was originally found in a screen for silencing defects in a *sir1* deletion background (Reifsnyder *et al*., 1996). Sas3 was also shown to target histones H3 and H4, and to a lesser extent H2A, for acetylation *in vitro* (Takechi & Nakayama, 1999). Also found in this screen was the related protein Sas2, which is the catalytic component of the yeast SAS complex involved in transcriptional silencing and has been shown to acetylate histones H3K14 and H4K16 *in vitro* (Sutton *et al*., 2003). SAS is thought to counteract heterochromatin spreading at the subtelomeric regions by the HAT activity of Sas2 (Suka *et al*., 2002; Oki *et al*., 2004). For a complete list of the various HATs used in this study and their preferred histone lysine targets within the budding yeast genome see Table 1.1.

1.1.5.3 Histone deacetylases (HDACs)

Like HATs, the many HDACs within the *S. cerevisiae* genome have preferential targets (see Table 1.2 for HDACs relevant to this study). HDACs play an antagonistic role to HATs as they are responsible for removing acetyl groups from histones and are generally considered to be transcriptional repressors. This is demonstrated by the finding that the mammalian histone deacetylase protein HD1 was related sequence-wise to the transcriptional regulator Rpd3 in yeast (Taunton *et al*., 1996). Relevant to this study are the five related HDACs Rpd3, Hda1, Hos1, Hos2 and Hos3. These HDACs all share sequence homology within an enzymatic domain (Taunton *et al*., 1996; Marmorstein, 2001c). Hda1 (histone deacetylase-A 1) is a component of the HDA

Table 1.2 Yeast HDACs used in this study

HDACs	Histones targeted in vivo	Known Complexes	Genes targeted
Hda1	H2B/H3	HDA	Global, preference for genes involved in carbohydrate utilization, stress response, detoxification, drug transport and cell wall function
Hos1	H2B/H4	unknown	rDNA locus
Hos2	H4	SET3C	ribosomal protein-encoding genes
Hos3	H2B/H4	unknown complex/ self-dimerizes	rDNA locus
Rpd3	H2A/H2B/H3/H4	HDB/Sin3	Global, slight preference for genes involved in carbohydrate utilization, sporulation, germination and meiosis

Modified from Carmen, et al. 1999.; Pim Pijnappel, etal. 2001.; Robyr, et al. 2002.

complex and targets histones H2B and H3 for deacetylation *in vivo* (Rundlett *et al*., 1996; Wu *et al*., 2001). Hos1, Hos2 and Hos3 (HDA one similar) are all involved in ribosomal biogenesis. Hos1 and Hos3 preferentially target the rDNA locus while Hos2 targets ribosomal protein-encoding genes (Robyr *et al*., 2002). Interestingly the APC appears to antagonize these three HDACs as deletion of any one of these HDACs rescued the temperature sensitive growth of an APC mutant background (Turner *et al*., 2010). HDACs, and their transcription repression activities have also been implicated in the progression of human disease, such as cancer (Mahlknecht & Hoelzer, 2000). As such, the inhibition of HDACs has recently become a target of clinical interest as a treatment for some cancers (reviewed in Drummond *et al*., 2005). As perturbations in acetylation levels can result in improper gene activation or repression it is imperative that this modification is tightly controlled.

1.1.5.4 Histone methyltransferases

Even though it has been known for a long time that histone methylation occurs, it was only recently that the activity of these proteins has been studied in any depth. In 2002 the first family of histone methyltransferases was discovered (Rea *et al*., 2002). The proteins in this family each contain the conserved catalytic SET domain (named for three *Drosophila* gene regulators, SUVAR3-9, E(Z) and TRX). Unlike the majority of the acetyltransferases, many histone methyltransferases only have one known residue target. For example, Set8 targets H4K20, Dim5 targets H3K9, and Dot1 targets H3K79 (Tamaru & Selker, 2001; Fang *et al*., 2002; van Leeuwen *et al*., 2002). Unlike acetylation, histone methylation does not change the charge of the histone. Histone methylation likely acts as a recruitment signal as methylation of different residues within the same histone can confer different results. For example, methylation of histone H3K4

is associated with sites of histone acetylation and transcriptional activation (Strahl *et al*., 1999) while methylation of histone H3K9 induces DNA methylation and gene repression (Tamaru & Selker, 2001).

1.2 Ubiquitin signaling pathway

1.2.1 Ubiquitin

Ubiquitin (Ub) is a 76 amino acid protein that is covalently attached to lysine residues within proteins (Ciechanover *et al*., 1980; Wilkinson *et al*., 1980). MonoUb and polyUb chains can be formed on proteins, each with a distinct cellular outcome. Proteins targeted with monoUb are generally involved in signal transduction events. A notable example of this is the monoUb of histone H2B that is required for methylation of H3K4 within promoters of transcribed genes (Henry *et al*., 2003). PolyUb chains can be generated through one of 7 Ub lysines (K). Chains built through K48 typically trigger ubiquitin- and proteasome-dependent degradation of the targeted protein (Chau *et al*., 1989; Finley *et al*., 1994). On the other hand, chains built through K63 are generally involved in stress response and can result in internalization of membrane proteins (Arnason & Ellison, 1994). The longer the polyUb chain, the more likely it is that it will be recognized by the proteasome for subsequent degradation.

1.2.2 Ubiquitination pathway and components

Ubiquitination of a protein involves an assembly line of several enzymes: a ubiquitin-activating protein (E1), one of several ubiquitin-conjugating proteins (E2) and one of a series of functionally and structurally diverse ubiquitin-protein ligases (E3) that play a

role in substrate specificity (see Figure 1.2; reviewed in Hochstrasser, 1996). This is an evolutionarily conserved network of proteins from yeast to humans.

S. cerevisiae encodes a single E1 enzyme, Ubc1 (Ubiquitin conjugating 1), while higher eukaryotes encode several. Ubiquitin is first activated by the adenylation of its C-terminal glycine by the E1 (Ciechanover *et al*., 1981; Hershko *et al*., 1983). The E1 then transfers the ubiquitin molecule to an internal sulfhydryl group to form a thiol ester bond. An E2 then removes the ubiquitin molecule from the E1 by forming a new thiol ester linkage with the activated ubiquitin. At this point the E2 either attaches the ubiquitin molecule directly onto the substrate or passes it on to a specific E3. Both E2s and E3s can attach ubiquitin to the substrate with an amide isopeptide linkage. Substrates are ubiquitinated by E3s in one of two ways; either the E3 forms its own thiol ester bond with ubiquitin or it brings the E2 and the substrate into close proximity of each other (reviewed in Pickart & Eddins, 2004). The large number of E2 and E3 enzymes allows specificity in substrate ubiquitination; however, additional protein interactions are required. For example, the E2 Cdc34 self-associates to allow Ub chain formation, the E2 Ubc13 requires Ubc variants to direct its activity to different pathways, and the E3 APC requires association of activators to determine substrate selectivity (Visintin *et al*., 1997; Cooper *et al*., 2000; Schwab *et al*., 2001; Varelas *et al*., 2003; Andersen *et al*., 2005). The requirement of additional proteins for targeting substrates highlights both the complexity and flexibility of the ubiquitin pathway.

1.2.2.1 E3s

E3s are an important part of this pathway as they confer substrate specificity. E3s are characterized into three groups based on their domains; HECT, RING-finger and U-box (reviewed in Pickart & Eddins, 2004). The HECT (homologous to E6-AP carboxyl-

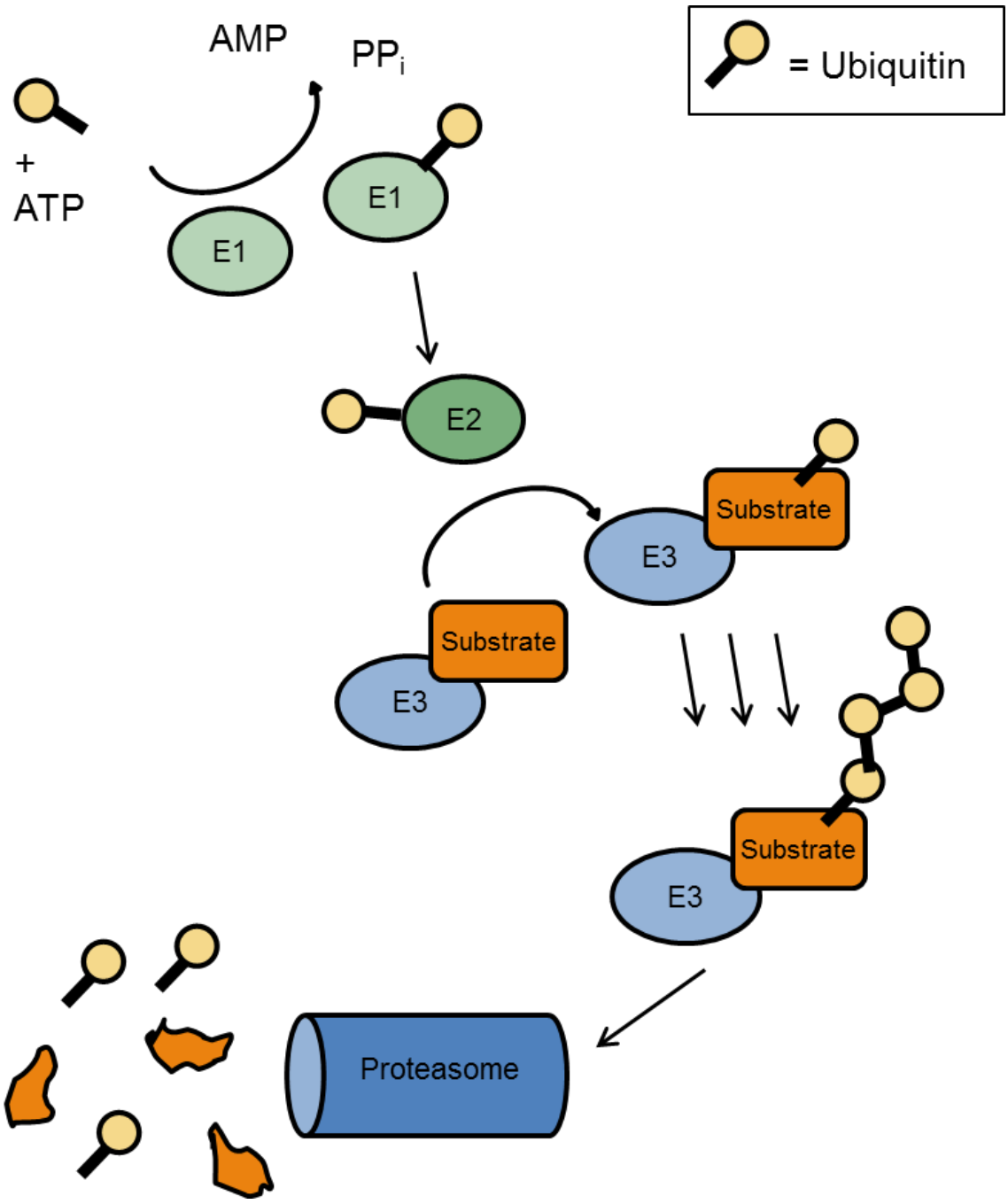

Figure 1.2. **Ubiquitination**. Ubiquitin must first be activated by adenylation using ATP as a donor. The activated ubiquitin molecule is then covalently attached to the E1 through a thiolester bond. The ubiquitin molecule is then transferred from the E1 to an E2. The E3 associates with the E2 to facilitate substrate ubiquitination. Polyubiquitination then results in the substrate being targeted to the proteasome and subsequently degraded. Information obtained and adapted from Hochstrasser, 1996; Hershko & Ciechanover, 1998, Wäsch & Engelbert, 2005.

terminus) domain contains a conserved cysteine that directly binds the ubiquitin molecule through a thiol ester linkage before passage to the substrate. The RING (really interesting new gene) domain is comprised of a cysteine and histidine rich motif that coordinates two zinc ions. RING E3s do not physically bind the ubiquitin molecule but are responsible for bringing the E2 and substrate into close proximity to each other. U-boxes (UFD2 homology) are similar to RING domains except that they do not contain the two zinc coordinating residues. The APC, on which this thesis focuses, is a member of the RING finger family.

1.2.2.2 RING finger E3s

Two multi-subunit RING finger E3 complexes important for cell cycle control are the APC and SCF (Skp1/Cdc53/F-box). Both the APC and SCF are large multi-subunit complexes (the APC is larger) that are structurally and functionally conserved across evolutionary boundaries (reviewed in Tyers & Jorgensen, 2000). They both contain a RING subunit and a cullin subunit. Cullin family members contain a specific domain that associates with the RING finger domain (Ohta *et al*., 1999). The APC is responsible predominately for mitotic progression and exit as well as G1 maintenance, while the SCF controls the G1/S and G2/M transitions (see Figure 1.3; Deshaies, 1999; Castro *et al*., 2005; Skaar & Pagano, 2009). Both the APC and SCF target a number of proteins for degradation with specificity being conferred by association of different proteins. Targets are recruited to the SCF by the binding of different F-box proteins to the two core subunits Skp1 (RING domain) and Cdc53 (Cullin) (reviewed in Patton *et al*., 1998a). Similarly, APC targets are specified by the binding of the co-activators Cdc20, Cdh1 and Ama1 to the core APC complex (Visintin *et al*., 1997; Cooper *et al*., 2000; Schwab *et al*., 2001; McLean *et al.*, 2011).

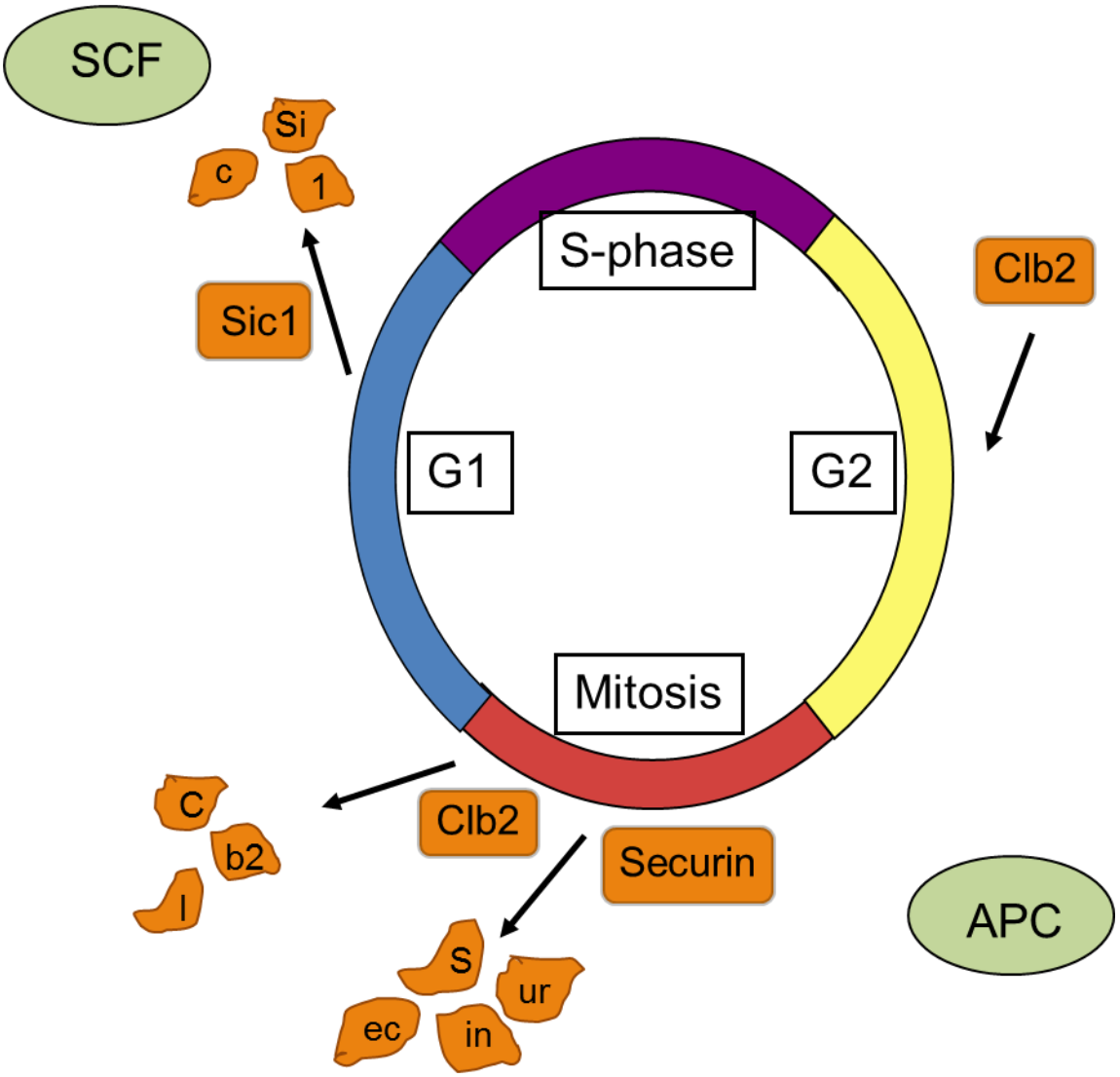

Figure 1.3. **Schematic of cell cycle progression**. Certain proteins required for specific cell cycle stages must be degraded to allow progression into the next stage of the cell cycle. For example, Clb2 is required for progression through G2 but must be degraded for passage through mitosis. E3s (SCF and APC) are responsible for targeting these proteins to the proteasome for degradation.

Cell cycle progression depends on the coordinated action of the APC and SCF. Both the APC and SCF are known to antagonize one another. In humans, APC^{Cdh1} maintains G1 by targeting the SCF F-box Skp2 for degradation (Bashir *et al*., 2004). Elevated levels of Skp2 have been associated with human cancer (Zhu *et al*., 2004), as Skp2 targets the Cdk inhibitors p21 and p27 for degradation (Carrano *et al*., 1999; Bornstein *et al*., 2003). The activity of p21 and p27 prevents cyclin-Cdk phosphorylation of Cdh1 and Rb, and premature S-phase entry. Premature entry into S-phase was also induced by RNA interference of Cdh1 (Bashir *et al*., 2004). On the other hand, examples exist where the APC and SCF work together, such as through the degradation of Emi1 by the SCF to allow APC mediated cell cycle progression (Margottin-Goguet *et al*., 2003). Emi1 accumulates in late G1 and inactivates APC activity by binding to its activators Cdc20 and Cdh1 to prevent substrate recognition (Reimann *et al*., 2001a; Reimann *et al*., 2001b; Hsu, Reimann, Sørensen, Lukas, & Jackson, 2002). This binding can occur before or after they physically associate with the APC (Reimann *et al*., 2001b). Emi1 is phosphorylated in early mitosis by Plk1 which induces $SCF^{\beta TrCP}$ to target Emi1 for degradation (Margottin-Goguet *et al*., 2003), thus allowing APC^{Cdc20} activation. Emi1 is a key mitotic regulator as overexpression in p53-deficient cells results in tetraploidy and chromosomal instability (Lehman *et al*., 2006). Understanding how the APC and the SCF function is extremely important, as impairing either activity, especially APC activity, ultimately leads to genomic instability and cancer.

1.3 The APC promotes mitotic progression and genomic stability

1.3.1 Anaphase-Promoting Complex (APC)

As introduced above, the APC is an essential evolutionarily conserved E3 ubiquitin ligase protein complex required for mitotic passage (Castro *et al*., 2005; Peters, 2006;

Wäsch *et al*., 2010; Kim & Yu, 2011; McLean *et al*., 2011). Composed of 13 subunits, the yeast APC controls cell cycle progression by ubiquitinating regulatory proteins such as Pds1 (securin) and mitotic and S-phase cyclins for destruction (Castro, 2005; Peters, 2006). Recognition of substrates by the APC is dependent on its activators Cdc20, Cdh1 and Ama1 (Visintin *et al*., 1997; Cooper *et al*., 2000; Schwab *et al*., 2001). Ama1 will not be discussed as it appears to be specific to meiosis (Cooper *et al*., 2000). Both Cdc20 and Cdh1 direct APC activity during mitosis but Cdh1 is also active throughout G1. See Figure 1.4 for an overview of APC activity throughout the cell cycle. There is a brief period in the cell when both APC^{Cdc20} and APC^{Cdh1} species exist. APC^{Cdc20} has been shown necessary to allow the initiation of anaphase by targeting securin/Pds1 for degradation while APC^{Cdh1} targets Clb2 for degradation, which triggers mitotic exit (King *et al*., 1995; Thornton & Toczyski, 2003). As soon as APC^{Cdh1} is activated it begins to target Cdc20 for ubiquitination and subsequent degradation (Pfleger & Kirschner, 2000). Many of the subunits are essential as their deletion leads to cell cycle arrest at the metaphase/anaphase junction and cell death in a variety of organisms, from yeast to mice (Irniger *et al*., 1995; Wirth *et al*., 2004; Peters, 2006).

The APC was first associated with cell cycle control in the mid 1990's. Fractionation of clam oocyte extracts produced a large protein complex capable of targeting cyclin for ubiquitination. This complex, inactive during interphase, was activated by Cdk1 during mitosis (Hershko *et al*., 1983; Sudakin *et al*., 1995). Simultaneously, a genetic screen looking for cyclin degradation mutants in *S. cerevisiae* showed the involvement of Cdc23 and Cdc16 (Irniger *et al*., 1995). These genes, in addition to Cdc27, had been previously shown to be required for passage through metaphase (Hartwell *et al*., 1970). Another extract study, this time using *Xenopus* eggs, found a complex containing orthologs of the yeast Cdc16 and Cdc27 that was both sufficient and necessary for ubiquitination of

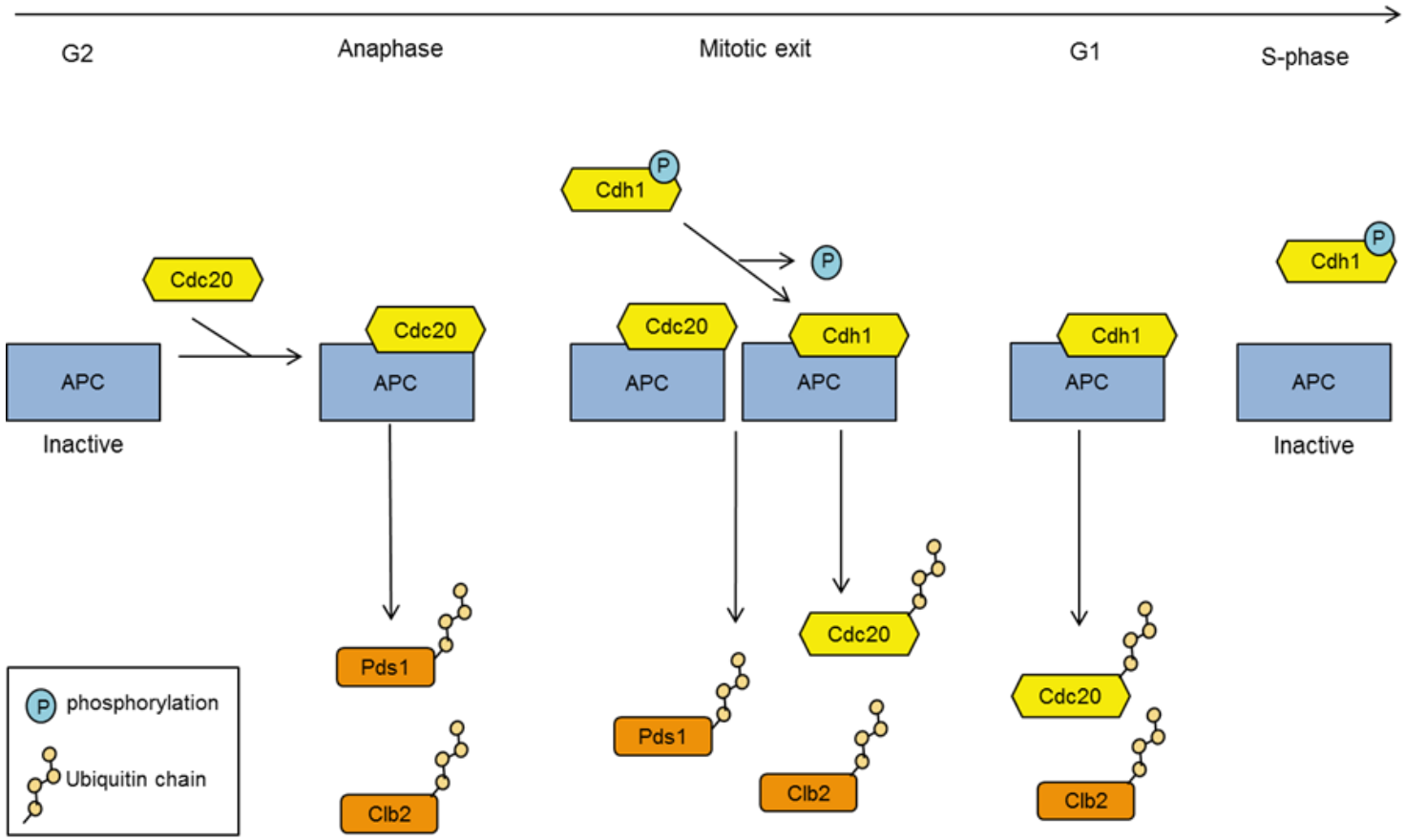

Figure 1.4. **APC activity throughout the cell cycle**. APC activity is coordinated throughout the cell cycle via interactions with the two coactivators Cdc20 and Cdh1. Cdc20 regulates early mitotic events, such as targeting Pds1 and Clb2 (Cyclin B1 in humans) for degradation. Cdh1 then drives degradation of factors that block mitotic exit as well as Cdc20. Information obtained and adapted from Eckerdt and Strebhardt, 2006; Thornton & Toczyski, 2006; McLean *et a*l. 2011.

cyclin B (King *et al*., 1995). In preparations where this complex had been removed by Cdc27 immunoprecipitation, cyclin B was not ubiquitinated. In contrast, addition of the immunoprecipitated complexes to interphase extracts induced cyclin B ubiquitination. As this complex, and the homologous yeast Cdc16 and Cdc27, were all required for anaphase onset, it was termed the Anaphase-promoting complex (APC; King *et al*., 1995). The role of the APC in ubiquitination was further solidified by a mass spectrometric analysis of the budding yeast APC that identified the presence of a RING-finger protein and a cullin subunit, both of which are associated with E3 ubiquitin ligases (Zachariae *et al*., 1998b). The evolutionary conservation of the APC was shown by the simultaneous finding that the Apc2 sequence was similar in human, *Xenopus* and budding yeast (Yu *et al*., 1998).

Since then many more targets of the APC have been discovered in various organisms. For example, in order for the polar migration of the sister chromatids to occur, *Xenopus* Xkid and budding yeast Kip1 and Cin8 kinesins must be degraded (Funabiki & Murray, 2000; Gordon & Roof, 2001; Hildebrandt & Hoyt, 2001). Xkid maintains chromosomes at the metaphase plate ready for separation while Kip1 and Cin8 are involved in mitotic spindle assembly (Hoyt *et al*., 1992; Funabiki & Murray, 2000). The kinases Cdc5/Plk1 and Aurora A are targeted by APC^{Cdh1} to allow entry into G1 (Charles *et al*., 1998; Castro *et al*., 2002; Lindon & Pines, 2004). The re-accumulation of these proteins is prevented by the continued activity of APC^{Cdh1} during G1 (Shirayama *et al*., 1998; Castro *et al*., 2002).

1.3.2 Control of the cell cycle

Anaphase is defined by the separation of sister chromatids. Sister chromatids are held together by a multi-protein complex called cohesin that must be cleaved to allow

separation (Darwiche *et al*., 1999; Uhlmann *et al*., 1999). This cleavage is accomplished by an enzyme called separase (Uhlmann *et al*., 1999). Separase activity is inhibited by securin (Pds1 in *S. cerevisiae*) binding and cyclin B/Cdk activity (Zou *et al*., 1999).

The cyclical nature of cyclins was first discovered by Tim Hunt in 1983 using sea urchin eggs. It was observed that cyclin levels decreased every cell cycle during division (Evans *et al*., 1983). The term cyclin refers to the cyclical rise and fall of these proteins throughout the cell cycle. Cyclins associate with the protein kinase Cdk1/Cdc28 (Cyclin dependent kinase 1/Cell division cycle 28) to target phosphorylation of specific substrates (Arellano & Moreno, 1997). Cdk1/Cdc28 phosphorylation is essential to activate proteins needed to move the cell through G2 and into mitosis. Additionally, it must be deactivated during anaphase and telophase to allow chromosomes to decondense, the nuclear envelope to reform, and cells to divide. The cell accomplishes this deactivation of Cdk by degrading its activating cyclins (Peters, 2006).

The destruction of securin and the B-type cyclins have been shown to be required for mitotic passage (King *et al*., 1995; Thornton & Toczyski, 2003). A genetic screen in yeast identified APC^{Cdc20} mediated destruction of securin and Clb5 as being essential for mitotic passage. Clb2 is also targeted for degradation by both APC^{Cdc20} and APC^{Cdh1} to allow exit from mitosis and maintenance of G1 (Shirayama *et al*., 1999; Yeong *et al*., 2000).

Given the number of proteins marked for proteolysis by the APC, it is understandable that APC activity would be vital to the cell (see Table 1.3). For example, overexpression of the human kinase Aurora A, an APC target, results in mitosis occurring without cytokinesis (Meraldi *et al*., 2002; Castro *et al*., 2005). Both polyploidy and increased

Table 1.3 APC substrates in various organisms

Target	Stage degraded	Activator required	Organism
Cdc5/Plx1/Plk1	before mitotic exit	Cdh1	Sc, Xl, Human
Clb2/cyclin B	metaphase	Cdc20 and Cdh1	Sc, Xl, Human
Cdc20/Fzy/p55CDC	G1	Cdh1	Sc, Human
Cdh1/Hct1/Fzr1	before G1 exit	Cdh1	Human
Pds1/Securin	metaphase	Cdc20 and Cdh1	Sc, Human
Xkid	metaphase	Cdc20 and Cdh1	Xl, Human
Kip1	metaphase	Cdc20	Sc, Human
Cin8	metaphase	Cdh1	Sc, Human
Geminin	metaphase		Human
Aurora A	before mitotic exit	Cdh1	Xl, Human
Cdc6	before G1 exit	Cdh1	Human
Ase1/Prc1	before mitotic exit	Cdh1	Sc, Xl, Human
Skp2	before mitotic exit	Cdh1	Sc, Human

Sc = *Saccharomyces cerevisiea*

Xl = *Xenopus leavis*

Information obtained and modified from Castro*, et al*. 2005; Baker, et al. 2007; Wäsch, et al. 2010.

levels of Aurora A have been found in cancer cells indicating that appropriate ubiquitination by the APC may be crucial to a cell's health (Castro *et al*., 2005). Yeast and vertebrates share many APC targets in common, so it is reasonable to conclude that results from research on yeast can be directly applied to vertebrate systems (Castro *et al*., 2005).

1.3.3 APC subunits

There are 13 core subunits and three activators known to comprise the APC in *S. cerevisiae* (reviewed in McLean *et al*., 2011). The majority of these proteins have orthologs in other organisms including humans (see Table 1.4 for a list of subunits). The binding of the activators is cell cycle dependent while the core subunits appear to stay associated throughout the cell cycle, even when inactive (Peters *et al*., 1996; Fang *et al*., 1998; Grossberger *et al*., 1999; Cooper *et al*., 2000). A representative schematicof the structure of the APC is illustrated in Figure 1.5. For more detailed structures see Buschhorn *et al*. 2011; da Fonseca *et al*. 2011; Schreiber, *et al*. 2011.

1.3.3.1 Apc2, Apc11

The catalytic function of the APC is provided by the Apc2 and Apc11 subunits. As a RING-H2 finger protein Apc11 is responsible for mediating the passage of ubiquitin from the E2 to the substrate (Thornton & Toczyski, 2006). The RING-H2 domain is thought to recognize and bind to the E2. Apc2 is a member of the Cullin family, members of which contain a specific 180-residue domain (Zachariae *et al*., 1998b) that associates with the RING-H2 finger domain (Ohta *et al*., 1999). Cullin and RING-H2 finger domains are also

Table 1.4 APC subunits in various organisms

S. cerevisiae	*S. pombe*	*Drosophila melanogaster*	Human	Domain	Function	Essential in yeast?
Apc1	Cut4	Shattered A/B	Apc1	PC repeats	Scaffold	Yes
Apc2	Apc2	Morula	Apc2	Cullin-like	E2 binding	Yes
Cdc27*	Nuc2*	Makos	Apc3	TPR repeats	Protein binding	Yes
Apc4	Lid1	Apc4	Apc4		Scaffold	Yes
Apc5	Apc5	Ida A/B	Apc5	TPR repeats	Scaffold	Yes
Cdc16*	Cut9*	Cdc16	Apc6	TPR repeats	Subunit binding	Yes
-	-	Apc7 A/B	Apc7	TPR repeats		-
Cdc23*	Cut23*	Cdc23	Apc8	TPR repeats	Subunit binding	Yes
Apc9	-	-	-		Complex stabilization	No
Doc1/Apc10	Apc10	Apc10	Apc10	Doc domain, IR motif	Substrate recognition	No
Apc11	Apc11	Lemming	Apc11	RING finger	Catalytic subunit/E2 binding	Yes
Cdc26*	Hcn1	-	Cdc26		Induced by heat shock	No
Swm1	Apc13	-	Apc13		Complex stabilization/ Role in meiosis	No
-	Apc14	-	-			-
Mnd2	Apc15	-	-		Role in meiosis	No
-	-	-	Apc16			-

PC = proteasome-cyclosome
TPR = tetratricopeptide repeats
S. pombe = *Schizosaccharomyces pombe*

*, Two copies of each subunit are present in each complex
-, Subunit has not been identified in that species.

Information obtained and modified from Thornton & Toczyski, 2006.; McLean *et al.,* 2011.; Schreiber *et al*., 2011.

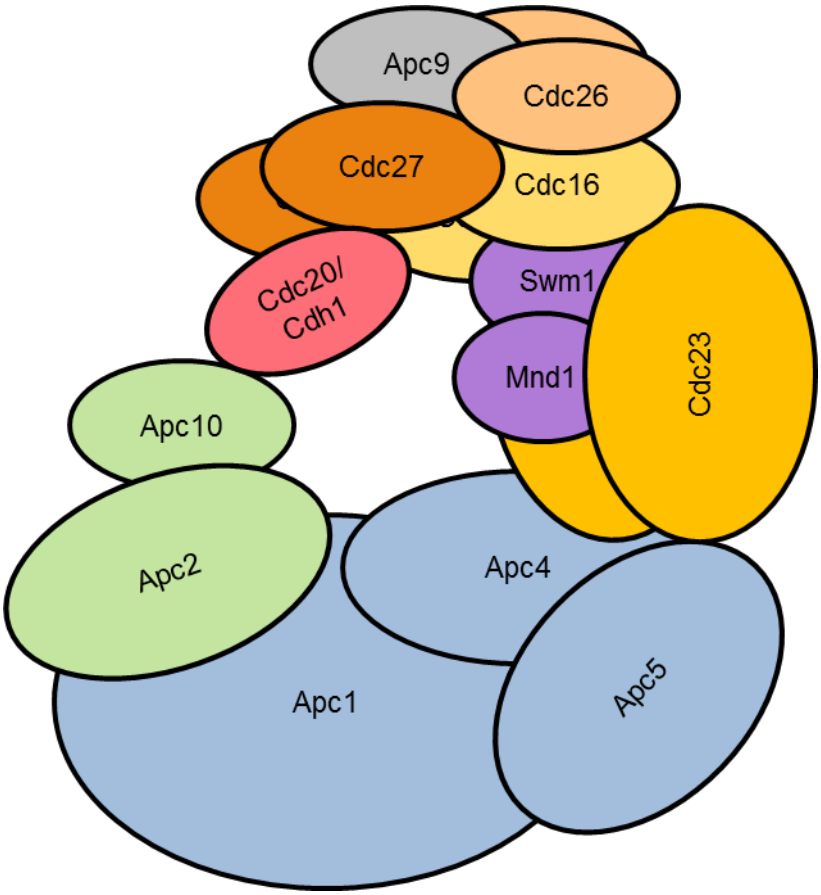

Figure 1.5. **Representative schematic of the APC**. Green indicates catalytic proteins, Blue indicates structural, Orange indicates TPR subunits, Purple indicates proteins involved in meiosis, Grey indicates exact function unknown. The location of the adaptors Cdc20 and Cdh1, which associate with Cdc27 and Apc10, is shown in pink. Information obtained and modified from Thornton & Toczyski, 2006; Thornton, *et al*. 2006.; Schreiber, *et al*. 2011. For more detailed EM structures of *S. cerevisiae* and human APCs see Buschhorn *et al*. 2011; da Fonseca *et al*. 2011; Schreiber, *et al*. 2011.

found in subunits of the SCF complex. Apc2 is responsible for tethering Apc11 (and Apc10) to the rest of the APC complex (Thornton & Toczyski, 2006). Together Apc2 and Apc11 have been shown *in vitro* to be capable of ubiquitinating proteins but this activity is not substrate specific and requires the presence of an E2. For example, Apc2 (Cullin) and Apc11 (RING domain) together with the E2 Ubc4, can ubiquitinate targets *in vitro* (Tang *et al*., 2001b). The RING-H2 finger domain on Apc11 is responsible for associating with the cullin domain of Apc2, as well as the human E2 UbcH10 and UbcH5 enzymes (Tang *et al*., 2001b).

1.3.3.2 Cdc16, Cdc23 and Cdc27

The essential Cdc16, Cdc23 and Cdc27 (Cell-division cycle) proteins all contain tetratricopeptide repeats (TPR motif; Sikorski *et al*., 1990; Lamb *et al*., 1994). These tandem repeats of 34-residue sequences are believed to facilitate protein-protein interactions (D'Andrea & Regan, 2003). TPR motifs are found in a wide range of proteins associated with a large variety of cellular processes, such as cell cycle regulation, organelle protein transport, transcriptional control and protein folding (D'Andrea & Regan, 2003). Cdc16, Cdc23 and Cdc27 are all phosphorylated during mitosis by Cdc28, which then activates the APC to enable mitotic progression (Lahav-Baratz *et al*., 1995; Peters *et al*., 1996; Kotani *et al*., 1998; Rudner & Murray, 2000; Kraft *et al*., 2003). It has also been shown that Cdc27 binds the APC activators Cdh1 and Cdc20 by recognizing a C-terminal IR (Isoleucine-Arginine) dipeptide motif and a short internal C-box (conserved in Cdc20 family proteins) motif (Schwab *et al*., 2001; Passmore *et al*., 2003; Vodermaier *et al*., 2003). The phosphorylation of Cdc27 increases the binding affinity of Cdc20 (Kraft *et al*., 2003). In addition, the deletion of any one of Cdc16, Cdc23 or Cdc27 greatly decreases the binding of Cdh1 (Thornton *et al*., 2006).

1.3.3.3 Apc1, Apc4 and Apc5

The largest APC subunit, Apc1, was discovered in 1996 by two different studies. A screen of yeast mutants identified a role for Apc1 in Clb2 degradation as mutants had stabilized levels of Clb2 (Zachariae *et al*., 1996), while a *Xenopus* egg extract purification study determined that Apc1 was a conserved member of the APC (Peters *et al*., 1996). Apc1 is stable throughout the cell cycle but its activity seems to be controlled by phosphorylation during mitosis (Peters *et al*., 1996). Apc1 is believed to be responsible for binding proteins, suggesting a role in substrate binding or scaffolding for the complex itself (Lupas *et al*., 1997). Supporting a role in scaffolding, the deletion of *APC1* results in the APC separating into two distinct complexes; one containing Apc2, Apc11 and Apc10, the other containing Cdc27, Cdc16, Cdc23 and Cdc26 (Thornton *et al*., 2006).

Though essential, little is known regarding the roles Apc4 and Apc5 play within the APC complex. These proteins were shown to be tightly associated with Apc2/Apc11 as they, along with Apc1, remained bound to the Apc2/Apc11 subcomplex after washing with high salt while the TPR proteins did not (Vodermaier *et al*., 2003). As such, they were believed to connect the ubiquitin-ligase activity of the Apc2/Apc11 subunits with the protein binding capability of the TPR proteins. A recent study designed to allow deletion of essential APC subunits supports this hypothesis as the deletion of either Apc4 or Apc5 resulted in the loss of the three TPR-containing subunits Cdc16, Cdc23 and Cdc27 from the rest of the APC (Thornton *et al*., 2006). Interestingly, deletion of any one of Apc1, Apc4, Apc5 or Cdc23 resulted in the loss of the other three, suggesting that a complex binding pattern is required for maintaining APC structure.

1.3.3.4 Apc10

Apc10 is believed to interact with substrates in order to inhibit their dissociation from the APC, thereby allowing efficient ubiquitination (Carroll & Morgan, 2002; Passmore *et al*., 2003). Apc10, also known as Doc1 (destruction of cyclin B), contains a 'Doc' domain which is involved in protein-protein binding (Grossberger *et al*., 1999; Carroll & Morgan, 2002; Passmore *et al*., 2003; Carroll *et al*., 2005). This motif has been found in other proteins of the ubiquitin-proteasome system containing cullin and HECT motifs, suggesting that this motif is tightly linked with ubiquitination (Grossberger *et al*., 1999). Apc10 mutants exhibit temperature sensitivity (Hwang & Murray, 1997; Irniger *et al*., 2000, Harkness *et al*., 2002, Turner *et al*., 2010). APC complexes purified from strains lacking Apc10 contained all other subunits found in wild-type cells suggesting that Apc10 likely plays a role in substrate recognition and not complex integrity (Passmore *et al*., 2003). A deletion assay by Thornton and colleagues supports this as only the deletion of Apc2 resulted in a loss of Apc10, suggesting that Apc10 is located at the periphery of the complex (Thornton *et al*., 2006). In fact, two recent studies have further defined the structure of the APC to show Apc10 to be at the periphery of the catalytic arm (Buschhorn *et al*., 2011; Schreiber *et al*., 2011).

Interestingly, Apc10 has been shown to physically associate with both Cdc27 and Apc11 (Tang *et al*., 2001b; Wendt *et al*., 2001). Similar to Cdc20 and Cdh1, the Apc10 subunit also possesses a C-terminal IR motif that is required for the binding of Cdc27 (Wendt *et al*., 2001; Vodermaier *et al*., 2003). The Apc10 subunit has been shown to be necessary for substrate recognition and binding as complexes lacking it are unable to bind or ubiquitinate substrates in an effective manner (Carroll & Morgan, 2002; Passmore *et al*., 2003). Apc10 has been proposed to act as a processivity factor to promote APC substrate ubiquitination as APC complexes lacking Apc10 are still able to ubiquitinate

substrates albeit at a much slower rate (Carroll & Morgan, 2002). This possibility is supported by *in vitro* studies showing that Cdh1 is unable to bind substrates in Apc10 mutants (Passmore *et al*., 2003; Carroll *et al*., 2005).

1.3.3.5 Cdc26 and Apc9

Apc9 is the only subunit shown to be unique to yeast; no known ortholog has been discovered yet (Zachariae *et al*., 1998b; McLean *et al*., 2011). Additionally, Apc9 is not essential and mutants do not exhibit temperature sensitivity (Page *et al*., 2005). Cdc26 is an evolutionarily conserved heat shock inducible protein involved in the retention of Cdc16 and Cdc27 at high temperatures (Zachariae *et al*., 1996; Yamada *et al*., 1997; Zachariae *et al*., 1998b). Not much is known about these two proteins except that they play a role in APC structure. Immunoprecipitations of the APC in Apc9 mutants showed decreased levels of associated Cdc27 while mutation of Cdc26 decreased APC association of Cdc27, Cdc16 and Apc9 (Yamada *et al*., 1997).

1.3.3.6 Swm1 and Mnd2

Mnd2 (Meiotic nuclear divisions 2) has no known ortholog in vertebrates and is believed to play a role in meiosis (Hall *et al*., 2003). In fact, it is believed to prevent APC activation by the meiosis-specific activator Ama1 (Oelschlaegel *et al*., 2005). The evolutionarily conserved Swm1 (Spore wall maturation 1) is required for efficient growth at elevated temperatures (Schwickart *et al*., 2004). Swm1 helps to stabilize the association of Apc9, Cdc16, Cdc26 and Cdc27 to the APC, as deleting SWM1 resulted in the loss of these subunits (Schwickart *et al*., 2004). Swm1 has also been shown to bind Cdc23 and Apc5 (Yoon *et al*., 2002; Hall *et al*., 2003).

1.3.4 Regulation of APC

1.3.4.1 Regulation of APC by two activators, Cdc20 and Cdh1

The specificity of APC substrate recognition is regulated by the transient binding of several activators (see Table 1.5). Both Cdc20 (cell-division cycle 20) and Cdh1 (Cdc20 homolog 1) direct APC activity during mitosis but Cdh1 is also active throughout G1 (see Figure 1.4). Each activator targets several different proteins for degradation, though, only the destruction of securin and the B-type cyclins is absolutely required for mitotic passage (Thornton & Toczyski, 2003). APC^{Cdc20} has been shown necessary to allow the initiation of anaphase by targeting securin/Pds1 for degradation while APC^{Cdh1} targets Clb2 for degradation, triggering mitotic exit (Castro *et al*., 2005). One study using mouse cells has challenged this, as APC^{Cdc20} was found to be responsible for targeting cyclin B while APC^{Cdh1} targeted securin (Jeganathan *et al*., 2005). This work suggests that the targeting of substrates for ubiquitination is even more complicated than previously thought.

Cdc20 and Cdh1 are members of the WD40 family of proteins. WD40 repeats are believed to be involved in protein binding. For example, the protein binding capability of the SCF protein Skp1 is mediated by its WD40 domain (Patton *et al*., 1998b). While Cdc20 and Cdh1 help to regulate APC activity, the activity levels of Cdc20 and Cdh1 are themselves heavily regulated. Phosphorylation, cellular localization and activator degradation all co-operate to ensure a tightly controlled process. APC activity is controlled by phosphorylation of its subunits and activators by the kinases Cdc28, polo-like kinase (Cdc5) and PKA (Protein kinase A) (see Figure 1.6 for a synopsis of APC activity regulation; Kotani *et al*., 1998; Rudner & Murray, 2000; Golan *et al*., 2002). Cdc20 binding and APC activation are both increased in response to Cdc28-dependent

Table 1.5 Co-activators of the APC

S. cerevisiae	*S. pombe* *	*Drosophila melanogaster*	Human	Phase
Cdc20	Slp1	Fizzy	Cdc20/Fzy/p55CDC	M/Me
Cdh1/Hct1	Ste9/Srw1	Fizzy-related	Cdh1/Fzr1	M/Me/G1
Ama1	-	-	-	Me
-	Mfr1	-	-	Me
-	-	Cortex	-	Me

M=mitosis; Me=meiosis; G1=G1 phase

* *Schizosaccharomyces pombe*

Information obtained and modified from Thornton & Toczyski, 2006; McLean, et al. 2011.

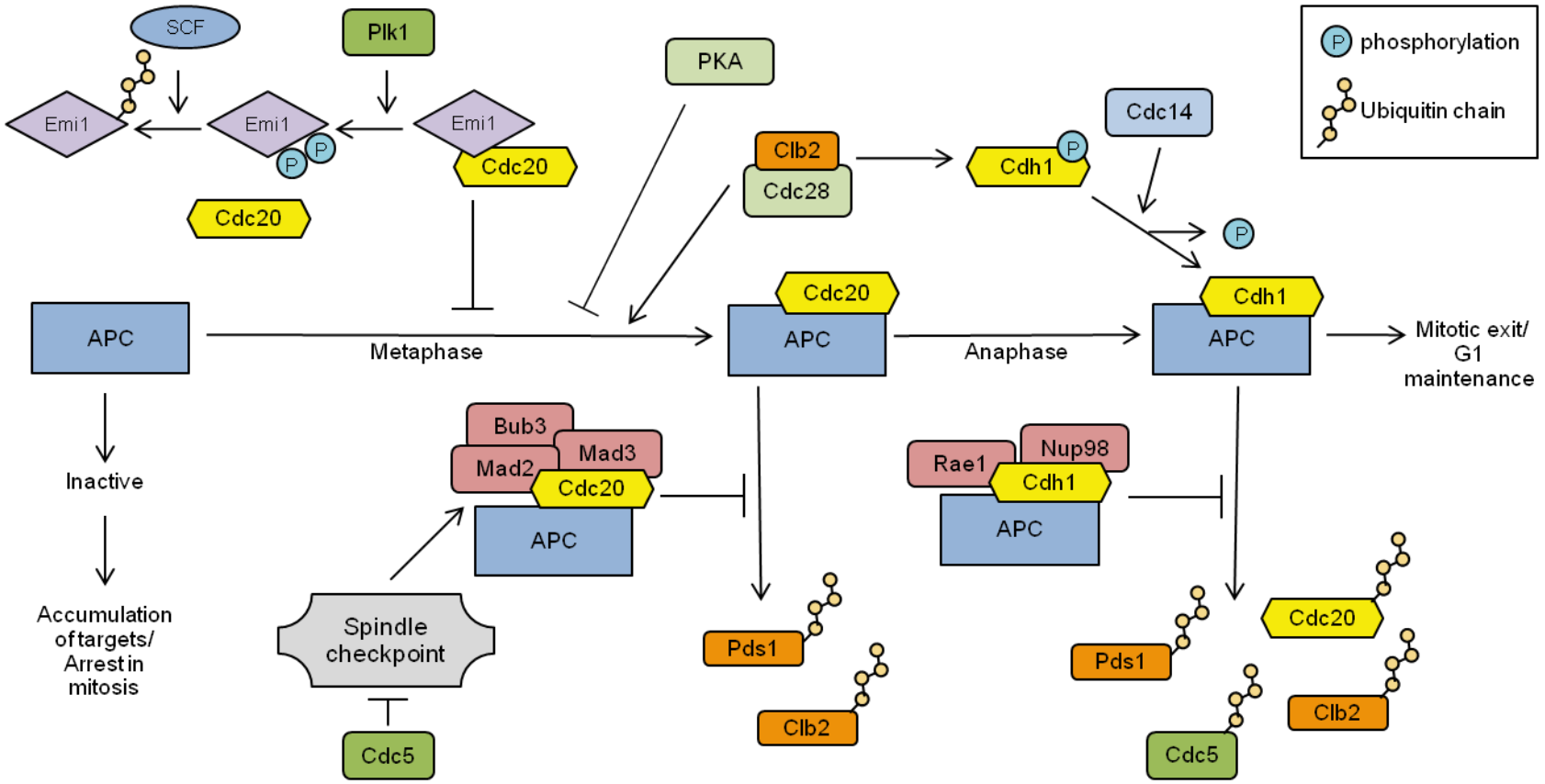

Figure 1.6. **Regulation of APC activity**. APC activity is coordinated via interactions with 2 binding partners, Cdc20 and Cdh1. Cdc20 regulates early mitotic events, such as targeting Pds1 and Clb2 (Cyclin B1 in humans) for degradation. Cdh1 then drives degradation of factors that block mitotic exit. Cdc20 is negatively impacted by the Spindle checkpoint. Plk1 (Cdc5 in *S. cerevisiae*) activates the APC by blocking the Spindle checkpoint and by mediating the SCF-dependent degradation of the APC inhibitor Emi1. Information obtained and adapted from Eckerdt and Strebhardt, 2006; Visintin *et al*., 2008.

phosphorylation of Cdc16, Cdc23 and Cdc27 (Kotani *et al*., 1998; Kramer *et al*., 2000; Rudner *et al*., 2000; Rudner & Murray, 2000; Kraft *et al*., 2003). Phosphorylation of the APC by Cdc5 is also able to increase APC activity, but only in conjunction with Cdc28 phosphorylation (Golan *et al*., 2002; Kraft *et al*., 2003). In contrast, Cdc28-dependent phosphorylation of Cdh1 prevents its association with the APC (Zachariae *et al*., 1998a; Rudner & Murray, 2000; Crasta *et al*., 2008). Dephosphorylation of Cdh1 is induced by inactivation of Cdc28. Dephosphorylated Cdh1 associates with the APC to regulate substrate degradation throughout mitosis and G1 (Zachariae *et al*., 1998a; Rudner & Murray, 2000). Once APC^{Cdh1} is activated it immediately targets Cdc20 for ubiquitination and subsequent degradation (Pfleger & Kirschner, 2000). PKA inhibits APC activity through the phosphorylation of Cdc23 and Cdc27 (Kotani *et al*., 1998). This phosphorylation prevents both the ubiquitination of cyclin B as well as the ability of Cdc5 to activate the APC (Kotani *et al*., 1998).

Cdh1 activity is not just regulated by phosphorylation but also by its abundance and localization. Cdh1 levels are at their highest during mitosis with a gradual decline during G1 possibly due to it targeting itself for ubiquitination, at least in mouse fibroblasts (Kramer *et al*., 2000; Listovsky, 2004). Cdh1 also changes its cellular localization throughout the cell cycle. From S-phase until the end of mitosis Cdh1 is located in the cytoplasm then stays nuclear throughout G1 (Jaquenoud *et al*., 2002; Zhou *et al*., 2003). Cyclin-Cdk (Cdc28) phosphorylation promotes Cdh1 nuclear export, which likely aids in the prevention of APC and substrate binding by Cdh1, resulting in the accumulation of proteins required for entry into mitosis (Jaquenoud *et al*., 2002; Zhou *et al*., 2003). The maintenance of Cdh1 phosphorylation from G1/S-phase to early mitosis is likely due to cooperation between G1 cyclins and S-phase B-type cyclins (Zachariae *et al*., 1998a).

Cdc20 and Cdh1 are capable of directly binding substrates in the absence of the APC suggesting that they may mediate the interaction of the substrate with the ubiquitination machinery (Pfleger *et al*., 2001). In support of this is the finding that most substrates do not bind to the APC when it is not activated by Cdh1 or Cdc20 (Passmore & Barford, 2005). One exception is human Nek2A NIMA-related kinase 2A, whose binding to the APC occurs before Cdc20 is able to drive APC activity (Hayes *et al*., 2006). Once APC^{Cdc20} is released by Emi1, Nek2A is rapidly ubiquitinated, directing it to the proteasome for degradation. Binding of Cdc20 and Cdh1 are selective as they do not always bind the same substrates as is the case for Clb2. For example, Pds1 is targeted for degradation by APC^{Cdc20} and Cdc20 has been shown to directly bind with Pds1, but Cdh1 does not (Hilioti *et al*., 2001). This specific recognition of substrates depends on the presence of degradation signals in their amino acid sequence. Several different sequences have been discovered to date; the two predominant ones being the D box and the KEN box (McLean *et al*., 2011). The D box is composed of the sequences RxxLxxxN or RxxLxxxxN/D/E (where x is any amino acid residue), while the KEN box is composed of KENxxxE/D/N (Glotzer *et al*., 1991; Pfleger & Kirschner, 2000, McLean *et al*, 2011). Cdh1 will bind to proteins containing either a KEN box or a D box, while Cdc20 does not recognize KEN boxes (Glotzer *et al*., 1991; Pfleger & Kirschner, 2000). Mutations in the D box and KEN boxes of Clb2 results in stabilization, but, only mutation to the D box actually prevents mitotic exit (Wäsch & Cross, 2002). It was demonstrated that APC^{Cdc20}-mediated degradation was sufficient to allow mitotic exit, suggesting that APC^{Cdh1}'s role is to maintain the low levels of Clb2 during M/G1. Low mitotic cyclin activity during G1 may delay S-phase to allow proper assembly of pre-replication complexes and fidelity of DNA synthesis (Wäsch & Cross, 2002).

1.3.4.2 Control of the APC by the Spindle Checkpoint

The spindle checkpoint is responsible for ensuring that all sister chromatids are properly attached to the mitotic spindle (Zhou *et al*., 2002; Kops *et al*., 2005). Attachment of the mitotic spindle to the spindle poles and the kinetochore of a sister chromatid creates tension. It is the lack of tension on any one of the mitotic spindles resulting from improper attachment that activates the spindle checkpoint to arrest the cell until all sister chromatids are properly attached (Zhou *et al*., 2002).

One of the ways the cell is prevented from continuing through the cell cycle is through the spindle checkpoint mediated inactivation of the APC. The Bub3/BubR1/Mad2 complex binds to Cdc20 to inhibit APC^{Cdc20} activity (Fang *et al*., 1998; Tang *et al*., 2001a) Phosphorylation of Cdc20 allows the direct binding of Mad2 and BubR1, the vertebrate ortholog of yeast Mad3, to APC^{Cdc20} (Fang *et al*., 1998; Hardwick *et al*., 2000; Chang & Chen, 2003). Please see Figure 1.6 for an overview of APC regulation. The spindle checkpoint proteins bind to kinetochores and inhibit the cell cycle until tension is restored (Shah *et al*., 2004; Kops *et al*., 2005). Once all kinetochores are attached to the mitotic spindle the inhibitory complex releases APC^{Cdc20}, allowing degradation of its substrates to continue (Mao *et al*., 2003; Kim & Yu, 2011). Inhibition of APC^{Cdh1} occurs in a manner similar to APC^{Cdc20}, albeit by different proteins. One line of research has demonstrated that the nucleocytoplasmic transport factors Nup98 (nuclear pore 98) and Rae1 (mRNA export factor 1) bind to APC^{Cdh1} to prevent premature degradation of securin in early mitosis (Jeganathan *et al*., 2005). This inhibition occurs even though APC^{Cdh1} is already bound to securin (Jeganathan *et al*., 2006). Mouse cells with decreased levels of Nup98 and Rae1 exhibited premature sister chromatid separation and aneuploidy, likely due to securin being degraded during prometaphase instead of anaphase (Jeganathan *et al*., 2005). The release of APC^{Cdc20} and APC^{Cdh1} by the inhibitory complexes both occur at

the metaphase/anaphase transition to allow entry into anaphase. The degradation of securin and cyclin B then allow separase to cleave cohesin resulting in sister chromatid separation (Uhlmann *et al*., 1999).

1.4 APC and Chromatin

1.4.1 Involvement of APC in genomic stability

Checkpoint defective cells that cannot properly inhibit APC activity enter anaphase prematurely and often exhibit chromosomal instability. Premature sister chromatid separation can lead to lagging chromosomes and anaphase bridges resulting in daughters inheriting incorrect chromosomes (Baker *et al*., 2007). As genomic instability appears to be a leading cause of cancer and aging, it is highly likely that the APC plays an important role and that this role is evolutionarily conserved. Indeed decreased Cdh1 expression has been linked to cancer (Wang *et al*., 2000; Engelbert *et al*., 2008). Downregulation of Cdh1 results in defective cell cycle control as well as inefficient chromosome separation. For example, mouse cells lacking Cdh1 showed activation of the DNA damage response and premature senescence (Engelbert *et al*., 2008; García-Higuera *et al*., 2008; Li *et al*., 2008). Furthermore, embryos lacking Cdh1 died at embryonic day 9.5, while heterozygous mice exhibited an increase in spontaneous tumor growth (Wang *et al*., 2000; García-Higuera *et al*., 2008). Cdh1 has also been shown to be downregulated in prostate, ovary, liver and brain tumors (Bassermann *et al*., 2008). As such, there is a large pool of APC substrates that when not degraded can lead to defects in cytokinesis, such as aberrant spindle pole organization, premature cytokinesis, polyploidization and multi-polar mitosis in the following cell cycle (Hildebrandt & Hoyt, 2001; Meraldi *et al*., 2002; Anand *et al*., 2003; Stewart & Fang, 2005). Additionally, the protein levels of APC targets such as Aurora A and B, Cdc20,

cyclin B, Nek2, Plk1 and Skp2 have been shown to be increased in a variety of cancers (Carter *et al*., 2006; Lehman *et al*., 2007).

Genomic integrity depends on both error-free DNA replication and sister chromatid separation. When errors occur they can result in uncontrolled cell proliferation or cell death. The activity of the APC is required to prevent the accumulation of proteins that can affect this fidelity. Studies encompassing the entire evolutionary range of species (yeast, mouse and human) all show that accumulation of this type of protein leads to defective DNA replication and chromosome separation (Wäsch *et al*., 2010). The role the APC plays in genomic stability has been linked to the tumor suppressor protein Rb (Binné *et al*., 2007). Rb has been shown to bind to and inhibit members of the E2F transcription factor family (Wu *et al*., 1995). Many cancers exhibit mutated Rb pathways, resulting in increased gene transcription activity by E2F proteins. E2F transcription factors are responsible for activating the spindle checkpoint protein Mad2, which has been shown to be increased in several human tumor samples (Hernando *et al*., 2004). Mad2 inhibits APC activity and these cells progress through mitosis slowly with elevated levels of APC targets (Fang *et al*., 1998). Cells that spend a prolonged time with an inactive APC and increased levels of its targets will eventually proceed with chromosome segregation, often resulting in defects (Zou *et al*., 1999; Meraldi *et al*., 2002; Hernando *et al*., 2004). This highlights the fact that activity of the APC needs to be tightly regulated in order to progress through mitosis properly and to maintain genomic stability. Interestingly, Rb has also been shown to physically bind to human APC^{Cdh1} and to induce cell cycle arrest in an APC^{Cdh1}-dependent manner (Binné *et al*., 2007). Induction of Rb is unable to cause cell cycle arrest in cells where Cdh1 has been depleted. While Rb binds to active APC^{Cdh1} it is not targeted by the APC for degradation. Instead, Rb binding increased the rate of ubiquitination and degradation of the APC^{Cdh1}

targets Plk1 and Skp2. While the exact mechanism of Plk1 recruitment is unknown, Skp2 recruitment is likely facilitated through Rb-Skp2 binding (Ji *et al*., 2004). The interaction of APC^{Cdh1} with Rb is specific, as two familial proteins, p107 and p130, did not bind to the APC, nor was cell cycle arrest by p107 dependent on the presence of Cdh1 (Binné *et al*., 2007). The ability of Rb to influence the activity of the APC, in multiple ways, such as recruitment of substrates and by preventing expression of APC inhibitors, highlights the complexity of cell cycle control.

1.4.2 Involvement of APC with Transcription Factors

Two transcription factors, CBP and p300, have been shown to physically interact with the APC and to increase its activity during mitosis (Turnell *et al*., 2005). The human APC components Apc5 and Apc7 (a TPR repeat protein not present in *S. cerevisiae*) share a conserved binding domain with E1A, a protein that associates with the transcription factors CBP and p300 (Arany *et al*., 1995; Turnell *et al*., 2005). Immunoprecipitation data showed that CBP and p300 physically interact with the subunits Apc2, Apc5, Apc6 (Cdc16 in *S. cerevisiae*) and Apc7, indicating that the association likely occurs with the entire APC complex. Interaction with the APC has been shown to occur through the binding of Apc5 and Apc7, as these subunits are able to bind CBP and p300 in isolation from the complex. Increased expression of Apc5 or Apc7 resulted in elevated p300 transcriptional activity, while expression of APC5/7 mutants did not. CBP/p300 binds p53, which then activates the p21 promoter in response to DNA damage (Dulić *et al*., 1994; Lill *et al*., 1997). APC5 or APC7 knockdown using RNAi (RNA interference) resulted in decreased p21 mRNA levels but did not affect p53 levels (Turnell *et al*., 2005). Protein levels of CBP and p300 were also elevated when cells were treated with APC5 or APC7 RNAi constructs, suggesting that they may actually be targets of the

APC. Interestingly, treatment with RNAi against CBP resulted in increased protein levels of the APC targets cyclinB1 and Plk1, and cells were stalled in mitosis. Further highlighting a possible role of the APC in transcriptional control is the finding that certain promoters exhibited decreased histone H4 acetylation levels in cells treated with APC5 or APC7 RNAi. Conversely, APC overexpression increases H4 acetylation. In addition, Apc5 and Apc7 co-localized with CBP and acetylated H3, suggesting it has a role in transcription (Turnell *et al*., 2005).

A recent study has identified another tumor suppressor protein that binds to the APC and results in increased activity, PTEN (phosphatase and tensin homolog, Song *et al*., 2011). As a consequence of its role in repressing the P13K/AKT pathway and cell growth, PTEN is found to have a high mutation rate in cancer (Li *et al*., 1997; Maehama & Dixon, 1998; Sun *et al*., 1999). Using mass spectrometry and immunoprecipitations, nuclear PTEN was found to bind to the APC core complex. Similar to Rb, PTEN binds to active APC complexes, as demonstrated by the *in vitro* ubiquitination of Cyclin B (Song *et al*., 2011). PTEN was shown to promote APC-Cdh1 binding and activity. In fact, induction of PTEN resulted in rapid degradation of APC targets, while PTEN silencing led to their stabilization. Additionally, the growth-suppressive ability of PTEN was dependent on APC^{Cdh1}, as *cdh1* null cells failed to arrest. The phosphatase activity of PTEN has been found to be dispensable for its ability to induce APC activity as the phosphatase inactive form is still capable of inducing degradation of APC targets. This finding of an additional role for the tumor suppressor protein PTEN highlights the complexities in cell growth regulation and genomic stability.

1.4.3 Role of the APC in Chromatin Assembly

Previous research from our laboratory using *S. cerevisiae* suggests that the ubiquitin-targeting pathway plays a role in chromatin assembly, and may do so in a cell cycle-dependent manner. *In vitro* chromatin assembly assays identified mutations in the genes encoding the E2 proteins Ubc7 and Cdc34, as well as the E3 proteins APC, SCF and Rsp5 (Reverses Spt-phenotype; Harkness *et al*., 2002; Arnason *et al*., 2005). Interestingly, Rsp5 is responsible for plasma membrane transporter turnover (Rotin *et al*., 2000; Morvan *et al*., 2004). One of the mutations recovered was in the essential *APC5* subunit of the APC. This mutation (*apc5*^{CA}, chromatin assembly) also increased chromosome loss and reduced lifespan (Harkness *et al*., 2002; Harkness *et al*., 2004). The *apc5*^{CA} allele grows normally at 30˚C but at the restrictive temperature of 37˚C it grows slowly and accumulates at the metaphase/anaphase junction. A 2 bp deletion in the 5' end of the gene results in a premature stop codon. An N-terminally truncated protein is likely produced due to an internal start site as we are able to detect a C-terminally tagged protein in apc5^{CA} cells (Harkness *et al*., 2002; unpublished data). Similar defects are seen with mutations in other APC subunits, indicating that this is an APC phenotype and not isolated to *APC5* mutants (Harkness *et al*., 2002; Harkness *et al*., 2004). The demonstration that the APC plays a role in regulating chromatin assembly suggests that chromatin assembly plays an important role during mitosis. Segregation of chromosomes during mitosis is a complex process and improperly assembled chromatin may result in genomic instability. As aberrations in APC activity have been linked to genomic instability and cancer in higher eukaryotes (Wäsch *et al*., 2010) the link between yeast APC and chromatin assembly is likely evolutionarily conserved.

Further evidence supporting a role for the APC in chromatin assembly is the genetic associations between the APC and several CAFs. The *apc5*^{CA} allele has been shown to genetically interact with the CAFs *ASF1*, *CAF-I*, *HIR1* and *HIR2* (Harkness *et al*., 2005). Interestingly, the presence of the CAF-I complex is required for suppression of the *apc5*^{CA} temperature sensitive phenotype by *ASF1*, *HIR1* or *HIR2* overexpression. To date Asf1 and CAF-I are the only chromatin assembly factors required for a specific stage of the cell cycle. As noted above, cell cycle specific phosphorylation patterns of the p60 subunit (the orthologous subunit in yeast, Cac2, has not yet been found to be phosphorylated) have been shown to regulate the replication-dependent assembly activity, with hyperphosphorylation occurring during mitosis, causing inhibition (Marheineke & Krude, 1998; Martini *et al*., 1998). Thus it appears that CAF-I activity may be controlled through the reversible phosphorylation of p60. Nonetheless, the CAF-I subunit Msi1 was shown to independently suppress *apc5*^{CA} defects in the absence of the rest of the CAF-I complex (Harkness *et al*., 2005). This indicates that the CAF-I subunits can function independently from one another to facilitate mitotic chromatin assembly. In fact, progressive deletion of the CAF-I subunits resulted in an exacerbation of the temperature sensitive growth of the *apc5*^{CA} strain. Additionally, *in vitro* studies show that each individual CAF-I subunit is capable of binding histones (Kaufman *et al*., 1995; Verreault *et al*., 1996; Shibahara *et al*., 2000). Taken together, these results suggest that the individual CAF-I subunits can function independently during mitosis to facilitate chromatin assembly.

1.4.4 APC and Histone Modifications

In addition to transcriptional control, the acetylation pattern of histones is important for deposition onto DNA. CAF-I has been shown to associate with histones H3 and H4 to

allow replication-dependent chromatin assembly to occur once H4 has been acetylated (Verreault *et al*., 1996). Conversely, histones involved in replication-independent chromatin assembly during mitosis and G1 are hypoacetylated on the H4 amino-terminal tail and hyperphosphorylated on H3 Serine 10 (Altheim & Schultz, 1999). The APC has been linked to the chromatin assembly factor CAF-I in yeast, as well as the transcription factors CBP and p300 in humans, suggesting a role in both histone deposition and modification (Harkness *et al*., 2005; Turnell *et al*., 2005). CBP and p300 have been shown to acetylate histone H3K56, a residue associated with histone deposition by CAF-I (Das *et al*. , 2009). Physical interaction of CBP and p300 with the APC results in an increase in p300 transcriptional activity and H4 acetylation (Turnell *et al*., 2005). Furthermore, increased expression of histones H3 and H4 suppresses the temperature sensitivity of *apc5*^{CA} cells irrespective of an intact CAF-I complex (Harkness *et al*., 2005). Thus, it is clear that a strong link between the APC and histone metabolism exists.

1.5 Rationale and Hypothesis

The findings presented above link CAF-I, APC and histone modifications together with mitotic chromatin assembly. CAF-I is required for passage through S-phase with properly assembled chromatin. Condensation of chromatin must occur to allow mitotic segregation that is controlled by the APC. It is possible that CAF-I activity during mitosis is required to maintain chromatin assembly to allow proper sister chromatid separation and prevent genomic instability. In addition to transcriptional control, the modification levels of histones play a role in chromatin assembly throughout the cell cycle and the APC can influence these modifications (Verreault *et al*., 1996; Grunstein, 1997; Altheim & Schultz, 1999; Ramaswamy *et al*., 2003; Turnell *et al*., 2005). Thus, it is likely that a

tightly controlled pattern of histone modification and chromatin assembly occurs to allow cell cycle progression, and that the APC plays a very important role in this activity.

Work from our laboratory has demonstrated that the APC is involved with both histone deposition and modification (Harkness *et al*., 2005; Turner *et al*., 2010; Islam *et al*., 2011). The hypothesis of this thesis is that the APC interacts with chromatin assembly factors and histone modification proteins to regulate cell cycle progression See Figure 1.7 for a model of these interactions. This thesis deals with how histone modification and deposition are regulated using the budding yeast *S. cerevisiae*. We hypothesize that the APC directs mitotic chromatin assembly though Asf1 and CAF-I and that mitotic progression is promoted by the redundant activities of the APC with the HATs Gcn5 and Elp3. We propose that the APC facilitates interaction between the HATs and Asf1 in order to deliver properly acetylated histones to the CAF-I complex for deposition into chromatin. Finally, we propose that Gcn5, and possibly Elp3, are targeted by the APC for degradation during G1. The rapidly growing and easily manipulated *S. cerevisiae* is an excellent model organism as many of the proteins involved in chromatin assembly and histone modification are evolutionarily conserved. In light of this, the findings from studies on *S. cerevisiae* should be able to be directly applied to higher organisms.

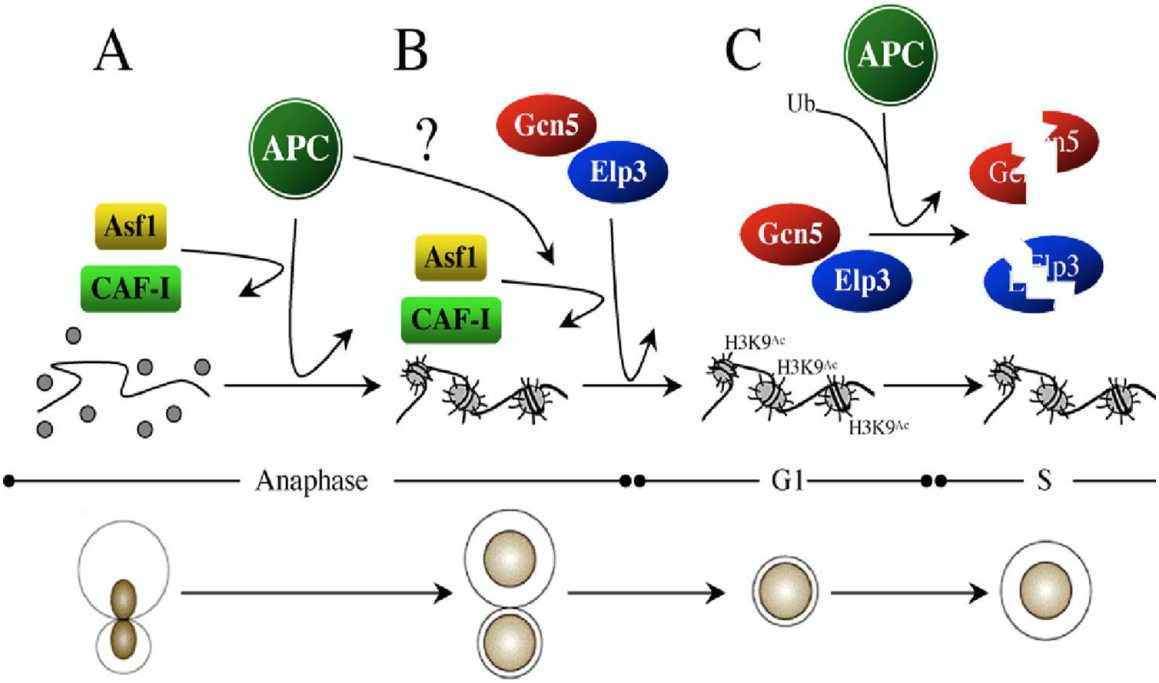

Figure 1.7. **Model of APC/HAT/CAF interactions. (A)** The APC directs mitotic chromatin assembly though Asf1 and CAF-I. **(B)** Our data indicate that Gcn5 and Elp3 independently work redundantly with the APC to promote mitotic progression. The establishment of a transcriptional profile required for G1 progression by Gcn5/Elp3 is likely necessary for APC function. We propose that the APC facilitates the interaction between the HATs and Asf1 in order to deliver acetylated histones to the CAF-I complex for deposition into chromatin. **(C)** Finally, to exit G1 and enter S, we propose that the transcriptional profile established by Elp3/Gcn5 must be reset. This is likely accomplished by APC-dependent targeting of Gcn5, and possibly Elp3, for degradation during G1. Figure from Turner *et al*., 2010.

1.6 Specific aims

The specific aims of this thesis are as follows:

1. To assess the molecular network involved in histone modification and deposition pertaining to the possible involvement of the APC.
    - Genetic screens, Western assays and Fluorescence Activated Cell Sorting (FACS) will be used to determine the interaction of the APC with histone modification proteins and chromatin assembly factors.
2. To determine the mode of regulation of HATs by the APC.
    - It is predicted that the APC will play a role in targeting at least some of these proteins for degradation.
3. To determine the physical association of the APC with chromatin assembly factors and HATs.
    - Immunoprecipitations will be used to determine any physical interaction between the chromatin assembly factors CAF-I and Asf1, the HAT Gcn5 and the APC.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Yeast genetics

2.1.1 Yeast strains and cell culture

All yeast strains used in this study were haploid and are listed in Table 2.1. The nomenclature used in this thesis to identify each mutant strain refers to the specific protein/subunit affected within the strain. *apc5*^{CA} and *apc16-1* refer to specific mutations within the APC5 and APC16 genes respectively. Deletion of an entire gene is indicated by Δ following the gene name, for example, *apc10*Δ and *gcn5*Δ refer to deletions of the entire *APC10* and *GCN5* genes, respectively. Yeast cells were cultured at 30˚C in rich medium [YPD - 1% yeast extract (VWR, CA9000-726), 2% peptone (VWR, CA07224-1000), 2% glucose (dextrose)] or in synthetic dextrose (SD) medium (0.67% yeast nitrogen base without amino acids (VWR, CA99501-686), 2% glucose, galactose or sucrose, plus supplementation of necessary amino acids at recommended concentrations). Omission of specific amino acids allowed selection pressure for the maintenance of transformed plasmids. Selection of the KanMX marker was obtained by supplementing YPD with 0.2 mg/ml G418 (Geneticin, Gibco, #11811). To make plates, 2% agar was added to the liquid medium prior to autoclaving. The molten agar was cooled to approximately 55-60˚C before pouring into petri dishes (VWR, 25384-302). For long term storage cells were grown to log phase in YPD, suspended in 1.5% glycerol and stored at -80˚C.

Table 2.1 List of strains used in this study

Strains	Genotype	Source (reference)
ResGen		
YTH1029	MATa	Research Genetics
YTH3638	*MATa rpn10::kanMX6*	W. Xiao
YTH3785	*MATa cdh1::kanMX6*	W. Xiao
S288c derivatives		
YTH5	*MATa ade2 his3 lys2 ura3*	Harkness et al. 2002
YTH457	*Mata rmc1 ade2 leu2 ura3 his3*	Harkness et al. 2002
YTH1049	*MAT? cdc16-1 leu2 his3*	This study
YTH1085	*MATa ura3-52 lys2-801 ade2-101 trp1-D63 his3-D200 leu2-D1::apc11-13::LEU2*	T. Hunter (YAP201)
YTH1149	*MATα ade2 his3 leu2 ura3 asf1::HIS3*	Harkness et al. 2005
YTH1235	*Mata ade2 his3 leu2 lys2 ura3*	Harkness et al. 2004
YTH1377	*MAT? ade2 his3 leu2 lys2 ura3 apc5CA-PA::His5 apc9::kanMX6*	Harkness et al. 2004
YTH1387	*MAT? ade2 his3 leu2 lys2 ura3 apc5CA-PA::His5 cdc26::kanMX6*	Harkness et al. 2004
YTH1410	*MAT? ade2 his3 leu2 lys2 ura3 apc5CA-PA::His5 apc10::kanMX6*	Harkness et al. 2004
YTH1636	*MATa ade2 his3 leu2 lys2 ura3*	Harkness et al. 2004
YTH1637	*MATα ade2 his3 leu2 lys2 ura3 apc5CA-PA::His5*	Harkness et al. 2004
YTH1648	*MAT? ade2 his3 leu2 lys2 ura3 apc9::kanMX6*	Turner *et al.* 2010
YTH1669	*MAT? ade2 his3 leu2 lys2 ura3 cdc26::kanMX6*	Turner *et al.* 2010
YTH1693	*MATα ade2 his3 leu2 lys2 ura3 apc10::kanMX6*	Turner *et al.* 2010
YTH1992	*MAT? apc5CA-PA::His5 hpa2::kanMX6*	Turner *et al.* 2010
YTH1994	*MAT? hpa2::kanMX6*	Turner *et al.* 2010
YTH1997	*MAT? sas2::kanMX6*	Turner *et al.* 2010
YTH1998	*MAT? apc5CA-PA::His5 sas2::kanMX6*	Turner *et al.* 2010
YTH2001	*MAT? apc5CA-PA::His5 rpd3::kanMX6*	Turner *et al.* 2010
YTH2003	*MAT? rpd3::kanMX6*	Turner *et al.* 2010
YTH2061	*MAT? apc5CA-PA::His5 gcn5::kanMX6 hpa2::kanMX6*	Turner *et al.* 2010
YTH2072	*MATa gcn5::kanMX6*	Turner *et al.* 2010
YTH2075	*MAT? apc5CA-PA::His5 gcn5::kanMX6*	Turner *et al.* 2010
YTH2076	*MAT? gcn5::kanMX6 hpa2::kanMX6*	Turner *et al.* 2010
YTH2216	*MAT? apc5CA-PA::His5 elp3::kanMX6*	Turner *et al.* 2010
YTH2217	*MAT? elp3::kanMX6*	Turner *et al.* 2010

YTH2219	*MAT? elp3::kanMX6*	Turner *et al.* 2010
YTH2260	*MAT? hos1::kanMX6*	Turner *et al.* 2010
YTH2261	*MAT? apc5CA-PA::His5 hos1::kanMX6*	Turner *et al.* 2010
YTH2305	*MAT? hda1::kanMX6*	Turner *et al.* 2010
YTH2306	*MAT? apc5CA-PA::His5 hda1::kanMX6*	Turner *et al.* 2010
YTH2389	*MAT? hos2::kanMX6*	Turner *et al.* 2010
YTH2390	*MAT? apc5CA-PA::His5 hos2::kanMX6*	Turner *et al.* 2010
YTH2504	*MAT? hat1::kanMX6*	Turner *et al.* 2010
YTH2507	*MAT? apc5CA-PA::His5 hat1::kanMX6*	Turner *et al.* 2010
YTH2525	*MAT? sas3::kanMX6*	Turner *et al.* 2010
YTH2526	*MAT? apc5CA-PA::His5 sas3::kanMX6*	Turner *et al.* 2010
YTH2804	*MAT? hos3::kanMX6*	Turner *et al.* 2010
YTH2805	*MAT? hos3::kanMX6*	Turner *et al.* 2010
YTH2806	*MAT? apc5CA-PA::His5 hos3::kanMX6*	Turner *et al.* 2010
YTH2807	*MAT? apc5CA-PA::His5 hos3::kanMX6*	Turner *et al.* 2010
YTH3037	*MAT? gcn5::kanMX6*	Turner *et al.* 2010
YTH3038	*MATα apc5CA-PA::His5 gcn5::kanMX6*	Turner *et al.* 2010
YTH3606	*MAT? elp3::kanMX6 gcn5::kanMX6*	Turner *et al.* 2010
YTH3607	*MAT? apc5CA-PA::His5 elp3::kanMX6 gcn5::kanMX6*	Turner *et al.* 2010
YTH3644	*MATa ade2 his3 leu2 lys2 ura3*	This study
YTH4001	*MATa ade2 his3 leu2 lys2 ura3 bar1::kanMX6* PCR integration	This study
YTH4004	*MATa rmc1 ade2 leu2 ura3 his3 apc5CA-PA::His5 bar1::kanMX6* PCR integration	This study
YTH4040	*MAT?* 1693 + *Gcn5-TAP::HIS* PCR integration	This study
YTH4044	*MATa ade2 his3 leu2 lys2 ura3 cdc16-1 bar1::kanMX6* PCR integration	This study
YTH4048	*MATa* 4001 + *Gcn5-TAP::HIS* PCR integration	This study
YTH4051	*MATa* 4004 + *Gcn5-TAP::HIS* PCR integration	This study
YTH4062	*MATa* 4044 + *Gcn5-TAP::HIS* PCR integration	This study
YTH4179	*MATa* 4001 + *Elp3-TAP::HIS* PCR integration	This study
YTH4180	*MATa* 4004 + *Elp3-TAP::HIS* PCR integration	This study
YTH4181	*MATα* 1693 + *Elp3-TAP::HIS* PCR integration	This study
YTH4182	*MATa* 4044 + *Elp3-TAP::HIS* PCR integration	This study
YTH4202	*MAT? apc10::kanMX6 Elp3-TAP::HIS ubc1::kanMX6*	This study
YTH4268	*MATa ura3 ade2 leu2 lys2 his3*	This study
YTH4269	*MATα ura3 ade2 leu2 lys2 his3*	This study

W303 derivatives

YTH1	*MATa ade2 his3 leu2 trp1 ura3*	H. Steiner
YTH370	*MATa ade1 bar1 his3 leu2 trp1 ura3 cdc16-1*	D. Stuart
YTH371	*MATa ade1 his2 trp1 ura3 cdc23-1*	D. Stuart
YTH448	*MATa cdc34-2*	D. Stuart

YTH602	*MATa ade1 bar1 his2 leu2 trp1-1 ura3Δns cdc4-3*	This study
YTH1007	*MAT? ade1 bar1 his2 leu2 trp1-1 ura3Δns*	D. Stuart
YTH1096	*MATa cdc53-1 bar1 his2 leu2 trp1 ura3*	D. Stuart
YTH1273	*MATa cac2::CAC2-13Myc-kanMX6 leu2 ura3 his3 trp1 ade2 can1*	B. Stillman
YTH3775	*MATa, ade2-1 his3-11 leu2-3,112 trp1-1::(SIC1-trp1)10x ura3-1can1-100 clb5::HIS3*	D. Toczyski
YTH3776	*MATa, ade2-1 his3-11 leu2-3,112 trp1-1::(SIC1-trp1)10x ura3-1can1-100 clb5::HIS3 apc11::kanMX6*	D. Toczyski

MAT? = mating type was not determined

2.1.2 Yeast transformation

Cells to be transformed were inoculated into 5 ml of YPD and grown overnight at 30˚C. The next day, cells were diluted to an optical density of OD_{600} 0.5 in 5 ml of YPD and allowed to double in density (approximately 2 hours). Cells were then pelleted and washed in sterile water. All centrifugation steps were performed at room temperature for 30 seconds at 14,000 rpm. Cells were resuspended in 0.5 ml of 100 mM LiAc solution [0.1 M lithium acetate, 10 mM Tris-HCl pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA)] and incubated at 30˚C for fifteen minutes. Cells were pelleted, the LiAc solution removed and 5 µl of denatured salmon sperm DNA was added. The solution was mixed by pipetting and then 3-5 µl of transforming DNA was added. Samples were then mixed by vortexing. Following the addition of 300 µl PEG (polyethylene glycol) solution (40% PEG (3500), 100 mM LiAc, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) samples were mixed by pipetting. Transformations were incubated at 30˚C for twenty minutes followed by heat shock at 42˚C for fifteen minutes. Following the incubations, cells were pelleted and the transformation mixture was removed. Cells were washed in 100 µl of 1 M sorbitol and then resuspended in sorbitol again. The cell/sorbitol solution was then spread on selection plates, allowed to dry and incubated at room temperature or 30˚C until colonies were observed; typically two to five days.

2.1.3 Yeast genomic DNA extraction (Smash and Grab)

The desired strain was inoculated into 5 ml of YPD or SD liquid media and allowed to grow to logarithmic phase. Cells were then centrifuged for three minutes at 4000 rpm. The pellet was then resuspended in 200 µl SCE (1 M sorbitol, 100 mM sodium citrate, 60 mM EDTA) plus 20µl of 12.5units/µl of lyticase (Sigma #L4025) and transferred to a microcentrifuge tube. Cells were then incubated at 37˚C for an hour. Following

incubation, tubes were placed on ice and 400 µl of freshly made SDS/NaOH lysis buffer (0.2 N NaOH, 1% SDS) was added. The tubes were mixed by inverting and then placed back on ice. After five minutes, 300 µl of 3 M NaAC (pH 4.8) was added; tubes were inverted to mix and kept on ice for five minutes. To precipitate DNA, 600 µl of isopropanol was added and the tube was placed at -80˚C for a minimum of ten minutes to overnight. Tubes were then centrifuged for ten minutes at 14,000 rpm and the supernatant discarded. The DNA pellet was washed with 500 µl 70% ethanol and allowed to dry for five to ten minutes to allow evaporation of remaining ethanol. Pellets were then resuspended in 50-100 µl of distilled water, depending on pellet size. If required, DNA extractions were purified using a phenol/chloroform extraction.

2.1.4 New strain formation

The majority of strains used in this study were obtained by performing genetic crosses. Mutants were repeatedly backcrossed with our S288c background strain until multiple isolates displayed identical phenotypes (typically 5 or 6 crosses were needed). Strains of opposite mating type were combined on a YPD plate and allowed to mate and form diploids for two days. Diploids were then transferred to a sporulation plate (1% potassium acetate, 0.1% yeast extract, 0.05% glucose/dextrose, 2% agarose, 5 ml adenine) and incubated at room temperature. After one to two weeks, the formation of tetrads was determined by visualization using a light microscope. Sporulated tetrads were suspended in 100 µl of distilled water with 10 µl of lyticase added. 25 µl of the tetrad/lyticase solution was applied to a YPD plate and allowed to dry for twenty to thirty minutes. Once dry the individual tetrads were separated using a micromanipulator (Singer MSM) and allowed to form colonies. After the colonies were grown, they were struck onto selection plates to determine desired genotype. Double mutants where both

mutations were marked by the same selection criteria were identified by a 2:2 segregation of the tetrads.

2.1.5 Spot dilutions

Strains of interest were grown overnight to log phase in liquid media and checked for bacterial contamination using a light microscope. Solutions were then prepared for each strain so that they contained approximately 10^7 cells: 10-fold serial dilutions were then made from this starting solution. Volumes of 3 µl from each dilution were pipetted onto selection plates in a grid pattern and allowed to grow at the indicated temperatures. Plates were grown from two to ten days until differences in growth were easily discernable. Plates were scanned once cells began to grow and the computer files were saved. Methyl methanesulfonate (MMS) was used to induce DNA damage and was purchased from Sigma (#1299-25).

2.1.6 Protein extraction

2.1.6.1 TCA protein extraction

Cells to be used for protein analysis (typically 3–5 ml) were transferred to a microcentrifuge tube. The tubes were centrifuged for three minutes at 4000 rpm and the supernatant was removed. The cells were then resuspended in 240 µl of freshly made, ice cold, solution C (1.85 M NaOH, 7.4% 2-Mercaptoethanol) and incubated on ice. Five minutes later 250 µl of 100% trichloroacetic acid (TCA; VWR, CATX1045) was added and the tubes were vortexed to mix and put back on ice for five minutes. A ten minute room temperature centrifugation was then performed at 14,000 rpm and the supernatant discarded. Pellets were carefully washed in 1 ml of sterile distilled water and

resuspended in equal amounts of solution A (13% SDS and 1 M Tris) and solution B (30% glycerol plus a small amount of Bromophenol Blue), according to pellet size. Samples were then stored at -80˚C or prepared for Western analysis.

2.1.6.2 Bead beat lysis

The samples that were used for immunoprecipitations were obtained by bead beat lysis. Cultures were centrifuged for three minutes at 4000 rpm, the supernatant removed and cell pellets washed in 5-10 ml of sterile distilled water. Pellets were then resuspended in 300 µl of RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 4.8, 5 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% (v/v) NP-40, 0.5% deoxycholate) with 10 µl/ml Protease Inhibitor Cocktail (Sigma, P8215) and 1 µl/ml DTT (dithiothreitol; VWR, CA99501-684) added and transferred to a microcentrifuge tube. Approximately 300 µl of 0.5-1 mm glass beads were added to the cell mixture. Tubes were then subjected to three rounds of bead beating lasting one minute each, for which a bead beater (Scientific Industries, Disruptor Genie) was used. In between rounds of beating, tubes were kept on ice for intervals of one minute. After the third round, tubes were centrifuged at a speed of 14,000 rpm for fifteen minutes at 4˚C. Following centrifugation, the top layer of solution was pipetted off, transferred to a new microcentrifuge tube and stored at -80˚C.

2.1.7 Cell cycle dependent stability analysis

All cell cycle stability analyses were performed in essentially the same manner. Arresting agent, media used and time of sampling is indicated in Figure Legends.

2.1.7.1 Cell cycle arrest

Strains of interest were inoculated into 15 ml of liquid media and allowed to grow. Once cultures had reached logarithmic phase the density was determined and cells were inoculated into 50 ml of liquid media at a concentration optimal to obtain an OD_{600} of 1.0 the following morning. The next day cultures were set back to an OD_{600} of 0.5 and the appropriate arresting agent was added. For arresting cells in: G1, alpha factor (Zymo Research, #Y1001) was used at a concentration of 0.1 µM. For arresting cells in S-phase, hydroxyurea (Sigma, #H8627) was used at a concentration of 0.3 M. For arresting cells in mitosis, nocodazole (Sigma, #M1404) was used at a concentration of 15 µg/ml. Following arrest, cells were pelleted and washed twice in 10 ml of fresh media. Cells were then resuspended in fresh media at a volume equal to that removed following arrest. As indicated in the Figure Legends, some experiments had cycloheximide (Calbiochem, #239763) added during resuspension to a concentration of 10 µg/ml to prevent further protein synthesis. Samples for protein analysis and Fluorescence Activated Cell Sorting (FACS) were collected at the indicated times.

2.1.7.2 FACS (fluorescence activated cell sorting)

FACS was used to determine cell cycle profiles of yeast cultures during logarithmic growth and stability assays. Samples taken for FACS were adjusted to have a density equivalent to 1 ml of OD_{600} 0.4. Cells were pelleted, washed with 1 ml 50 mM Tris-HCl (pH 8.0) and resuspended in 1 ml 70% ethanol. All centrifugation steps were performed for 30 seconds at 14,000 rpm. Samples were incubated at room temperature until day of analysis. Prior to analysis cells were pelleted, resuspended in 500 µl of 50 mM Tris-HCl (pH 8.0) and 5 µl of RNase A (20 mg/ml) and incubated at 37˚C for a minimum of two hours, maximum overnight. Cells were then pelleted and the Tris/RNase A solution was

removed. Samples were then washed with 1 ml of 50 mM Tris and resuspended in 500 µl of propidium iodide solution [1 mg/ml propidium iodide (Sigma, #P4170) in PBS (phosphate-buffered saline: 8% (w/v) NaCl, 0.2% (w/v) KCl, 1.44% (w/v) Na2HPO4, and 0.24% (w/v) KH2PO4)]. Samples were transferred to FACS tubes (12 X 75 mm polystyrene round-bottom tubes) and incubated for one hour in the dark. FACS analyses were performed at the Saskatoon Cancer Center.

2.2 Molecular biology techniques

2.2.1 Bacterial culture and storage

The *Escherichia coli* (*E. coli*) strain DH5α was used for propagation of plasmids used in this study. See Table 2.2 for a list of the plasmids. Transformed strains were grown in Luria Broth (LB) (1% tryptone (VWR, CA9000-282), 0.5% yeast extract (VWR, CA9000-726), 0.5% NaCl) containing 50 µg/ml ampicillin (LBA). All plasmids contained the ampicillin resistance marker gene amp^R to ensure retention. For long term storage, transformed strains were inoculated into 5 ml LBA and grown overnight. The following day 813 µl of bacterial culture was mixed with 187 µl of 80% glycerol in a microcentrifuge tube and stored at -80˚C.

2.2.2 Preparation of competent cells

To make *E. coli* cells competent for transformation cells were inoculated into LB liquid media and allowed to grow at 37˚C to an OD_{600} of 0.4-0.5. The culture flask was swirled in an ice water bath for ten minutes, then an equal volume of ice cold TSS solution [LB with 10% PEG8000, 5% DMSO, and 50 mM Mg^{2+} ($MgSO_4$ or MgCl, pH 6.5)] was added. Aliquots of 500 µl were pipetted into microcentrifuge tubes and stored at -80˚C.

Table 2.2 List of plasmids used in this study

Plasmid	Vector	Insert	Source
YCp50	*CEN-URA3*		M. Ellison
YCplac111	*CEN-LEU2*		W.Neupert
BG1805-*APC5*	2µ-*URA3*	$GAL1_{prom}$-*APC5-HA*	W. Xiao
BG1805-*APC10*	2µ-*URA3*	$GAL1_{prom}$-*APC10-HA*	W. Xiao
BG1805-*ELP3*	2µ-*URA3*	$GAL1_{prom}$-*ELP3-HA*	W. Xiao
BG1805-*GCN5*	2µ-*URA3*	$GAL1_{prom}$-*GCN5-HA*	W. Xiao
BG1805-GCN5-LEU2	2µ-*LEU2*	$GAL1_{prom}$-*GCN5-HA*	This study*
BG1805-*RTT109*	2µ-*URA3*	$GAL1_{prom}$-*RTT109*	W. Xiao
pRM102	*CEN4-ARS1-URA3*	$GAL10_{prom}$-*H3* $GAL1_{prom}$-*H4*	M. Grunstein
pYEX-*ASF1*	2µ-*URA3*	CUP_{prom}-*GST-ASF1*	Exclone library
pYEX-*MSI1*	2µ-*URA3*	CUP_{prom}-*GST-MSI1*	Exclone library

* plasmid created by A. Islam.

2.2.3 Chemical bacterial transformation

Transformation solution was prepared by adding 1-5 µl of plasmid DNA and 20 µl of 5 X KCM (0.5 M KCl, 0.15 M $CaCl_2$ and 0.25 M $MgCl_2$) to distilled water to make up a total volume of 100 µl. An equal volume of competent cells was added to the transformation mixture and incubated on ice for 20 minutes. The cells were heat shocked for five minutes at 37˚C. 1 ml of LB was added and cells were shaken at 37˚C for one hour. Cells were then plated onto LBA plates and incubated overnight at 37˚C.

2.2.4 Plasmid DNA isolation

2.2.4.1 LiCl plasmid Mini-prep

LBA was inoculated with bacterial cells transformed with the plasmid of choice and incubated overnight at 37˚C. The following day the sample was centrifuged for five minutes at 4000 rpm and the supernatant discarded. The pellet was resuspended in 200 µl of cold GTE (50 mM glucose, 25 mM Tris-HCl PH 8.0 and 10 mM EDTA) and incubated for five minutes at room temperature. 400 µl of fresh lysis buffer (0.2 N NaOH, 1% SDS) was added, the tube inverted to mix contents and incubated on ice. Five minutes later, 300 µl of NaAc was added, tube inverted to mix, incubated on ice for another five minutes and then centrifuged at 14,000 rpm for five minutes. All following centrifugation steps were performed at room temperature for 30 seconds at 14,000 rpm. The supernatant was transferred to a new tube, 450 µl of isopropanol was added and the new tube put on ice. After five minutes, the tube was centrifuged, the supernatant discarded, and the pellet allowed to dry. The pellet was then resuspended in 100 µl of distilled water containing 5 µl of 20 mg/ml RNase A and the tube was incubated at room temperature for twenty minutes. To further isolate the plasmid DNA, 100 µl of 10 M LiCl

and 100 µl of chloroform were added and the tube was vortexed to mix. The tube was then incubated at room temperature for ten to twenty minutes and then centrifuged. The upper layer was transferred to a new tube and 600 µl of 95% ethanol was added. The tube was incubated at -80˚C for a minimum of fifteen minutes. The tube was centrifuged, the pellet washed in 500 µl of 70% ethanol, resuspended in 50 µl of distilled water and stored at -20˚C.

2.2.4.2 Large scale DNA isolation (Maxi-prep)

A 5 ml aliquot of LBA was inoculated with bacterial cells transformed with the plasmid of choice and incubated overnight at 37˚C. The following day the entire 5 ml culture was added to 500 ml of fresh LBA and again incubated overnight at 37˚C. Cells were transferred to two 250 ml screw-capped bottles and centrifuged at 4000 rpm in 4˚C. Supernatant was poured off and cells in each bottle were resuspended in 50 ml of ice cold STE (0.1 M NaCl, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0) and centrifuged again. The supernatant was discarded and cells were resuspended in 9 ml of GTE (50 mM Glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA pH 8.0). 40 ml of fresh lysis buffer (0.2 N NaOH, 1% SDS) was added. Bottles were mixed by gentle inversion and incubated at room temperature. After five minutes 20 ml of ice cold KAc solution (5 M potassium acetate, 11.5% glacial acetic acid) was added, bottles were mixed by shaking and then incubated on ice for ten minutes. The bottles were then centrifuged for fifteen minutes at 4000 rpm and the rotor was allowed to come to a complete stop without braking. The supernatant was filtered through cheesecloth into two new 250 ml plastic bottles. A 0.6 volume of isopropanol was added and bottles were incubated for ten minutes at room temperature. Bottles were centrifuged at 5000 rpm for fifteen minutes at room temperature and the supernatant removed. The nucleic acid pellets were washed

with 70% ethanol and allowed to dry. The pellets were then suspended in 3 ml of distilled water and transferred to microcentrifuge tubes for indefinite storage at -80˚C.

2.2.5 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to amplify DNA used for transformation of yeast using genomic yeast DNA obtained by Smash and Grab (described in 2.1.3) as template. Primers of 18 – 22 bp were designed against sequences 500 bp upstream and 500 bp downstream of the DNA sequence of interest. See Table 2.3 for a list of primers used in this study. Primers were then tested for specificity using the BLAST program on the Saccharomyces Genome Database website (www.yeastgenome.org/cgi-bin/blast-sgd.pl). A typical 25 µl PCR reaction mixture contained 1 µl dNTPs (10 mM each deoxyribonucleotide triphosphate; Bio Basic Inc., #D0056), 1 ng of each primer , 1 µl of genomic DNA, 2.5 µl 10X PCR Buffer (Sigma, #P2317), 4 µl of 15 mM $MgCl_2$ (Sigma, #M8787), 16.5 µl of distilled water and 0.5 µl *Taq* Polymerase (New England Biolabs, #M0267). Volumes of reagents were adjusted to optimize reactions as necessary. A programmable thermocycler (Eppendorf Mastercycler) was used to perform the amplifications. The standard PCR protocol was as follows: 95˚C for four minutes, followed by thirty repeats of one minute of denaturing at 95˚C, one minute of annealing at 55˚C, and two minutes of elongation at 72˚C. A final ten minute incubation of 72˚C was followed by long-term storage at 4˚C.

2.2.6 Agarose gel electrophoresis and DNA fragment isolation

Analysis of plasmid and genomic DNA was performed using a 0.8% agarose gel containing 0.5 µg/ml ethidium bromide for visualization under UV light. The gel was immersed in 1 X TAE (24% Tris-base, 5.7% glacial acetic acid, 10% EDTA pH 8.0) prior

Table 2.3 List of primer sequences used in this study.

Name	Sequence
BAR1-500	CTGATTGAGTTAGACAGTAT
BAR1+500	CCAACATTCCGACACAACAA
GCN5-500TAP	CGGATGATGGTTATCAAC
GCN5+500TAP	TGGACGCAGGTAAGATTC
ELP3-500TAP	GCCCTAGTGCCCCCATGGACA
ELP3+500TAP	TGGGGATTTTTCCCTGAGCTGG

to sample loading. A 5 µl aliquot of DNA ladder (Fermentas, #SM0313) was pipetted into the left most lane to allow size determination. To isolate DNA fragments of interest the area of gel containing the DNA fragment was removed from the rest of the gel and placed into a microcentrifuge tube and weighed. DNA fragments were then extracted from the agarose gel using a DNA gel extraction kit (Qiagen, #28704). According to the mass of the agarose gel, three volumes of Buffer QG were added and the tube was incubated at 50˚C until the agarose had completely dissolved. The solution was then pipetted into a QIAquick spin column and centrifuged for thirty seconds at 10,000 rpm to remove the liquid. All centrifugation steps were performed for one minute at 10,000 rpm. 500 µl of Buffer QG was pipetted into the QIAquick spin column and centrifuged again to remove any remaining agarose solution. The QIAquick spin column and DNA were washed by the addition of 750 µl Buffer PE and centrifuged twice, discarding the filtered solution after each centrifugation. To release DNA from the QIAquick spin column 30-50 µl of distilled water was pipetted directly onto the filter pad, followed by thirty seconds of centrifugation. DNA was then kept at -20˚C indefinitely.

2.2.7 Phenol/chloroform extraction of DNA

Phenol/chloroform extraction was performed to remove proteins from the nucleic acid samples. DNA samples obtained by DNA extraction (described in 2.1.3) were incubated at 37˚C for thirty minutes with 2 µl of 20 mg/ml of RNase A. Following incubation, 100 µl of phenol/ chloroform/isoamyl alcohol mixture (24:25:1; Sigma #77617) was added and the tube inverted several times to mix. The tube was then centrifuged at 14,000 rpm for five minutes and the upper layer was transferred to a new microcentrifuge tube. To precipitate the DNA 100 µl of distilled water, 20 µl of 3M NaAc (pH 4.8) and 500 µl of 95% ethanol were added. The sample was mixed by inverting and incubated on ice for

ten minutes. Following incubation, the sample was centrifuged at 14,000 rpm for ten minutes and the supernatant removed. DNA was washed with 70% ethanol, allowed to dry and resuspended in 50-100 µl of distilled water.

2.2.8 Co-immunoprecipitations (CoIPs)

Protein concentrations of beat bead lysates were determined via the Bradford assay. Lysates were kept on ice at all times. Briefly, 200 µl of Bradford Reagent (BioRad, #500-0006) was pipetted into a disposable cuvette (VWR, #97000-586) and 1 µl of the protein lysate was mixed into the Bradford reagent. For a control, 1 µl of sterile distilled water was used. Each cuvette had 800 µl of sterile distilled water added. Cuvettes were covered with parafilm (Parafilm M, #PM-996), inverted twice to mix contents and allowed to sit at room temperature. After two minutes, the protein concentration was determined using a spectrophotometer set at OD_{595} (BioRad SmartSpec^{TM} 3000). A previously prepared calibration curve was used to quantify protein concentrations.

Each immunoprecipitation (IP) required 1 mg of protein. Volumes of bead beat lysate needed were calculated using the protein concentration. RIPA buffer that contained 10 µl/ml Protease Inhibitor Cocktail (Sigma, P8215) and 1 µl/ml DTT (dithiothreitol; VWR, CA99501-684) was added to make each sample up to a final volume of 300 µl. Samples were pre-cleared by the addition of 17 µl of washed salmon sperm DNA/Protein A agarose beads (Upstate, #16-157) and rotated at 4˚C for thirty minutes to one hour. The salmon sperm helps to block non-specific interactions with the beads when used in chromatin immunoprecipitations. Beads were washed by suspension in three volumes of fresh RIPA buffer. The suspension was centrifuged and the buffer removed. All centrifugation steps were performed at 4˚C for 30 seconds at 10,000 rpm. This process

was repeated three times. Following the third removal of RIPA buffer, an equal volume of fresh RIPA buffer containing protease inhibitors and DTT was added to the beads. Following the pre-clear incubation, tubes were centrifuged and the supernatant was transferred to a fresh microcentrifuge tube. Input samples were created by combining 20 µl of this supernatant with 20 µl of 2X SDS loading buffer [100 mM Tris-HCl pH6.8, 2% β-mercaptoethanol (Sigma #M7154), 4% sodium dodecyl sulfate (VWR, CA99501-538), 0.2% bromophenol blue (VWR, CA-EM2830), 20% glycerol (VWR, CAGX0185)]. To the remaining 280 µl, 1 µl of stock bait antibody was added. Samples were then rotated at 4˚C for forty-five to ninety minutes. Samples were then centrifuged and the supernatant was discarded. The remaining beads were washed three times in the same manner described above, the exception being that all three RIPA washes contained protease inhibitors and DTT. After the washes, 30 µl of 2X SDS loading buffer was added to the beads to dissociate the proteins. Samples were then frozen or prepared for Western blot analysis.

2.2.9 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Depending on the degree of protein separation needed, 10-15% separating gels [4–2.4 ml distilled water, 3.3–5 ml 30% Acrylamide (BioRad #161-0156), 2.5 ml 1.5% Tris-HCl pH8.8, 100 µl 10% SDS (VWR #CA99501-538), 100 µl ammonium per sulfate (APS; Sigma A-7460), 10 µl tetramethylethylenediamine (TEMED; VWR CA-EM8920)] were used, each topped with a 5% stacking gel (4.2 ml distilled water, 1 ml 30% Acrylamide, 76 µl 1.0% Tris-HCl pH 6.8, 60 µl SDS, 60 µl APS, 6 µl TEMED). The separating gel solution was mixed and then immediately poured

between two glass plates immobilized in a casting apparatus. Gels were cast and run using the BioRad Mini-PROTEAN®-2 and -3 Electrophoresis systems (BioRad). The gel solution was topped with a thin layer of isopropanol to ensure a level surface. Once the separating gel solidified, the isopropanol was poured off and a 5% stacking gel solution was poured on top of the separating gel between the plates and a comb was inserted to create wells. Once the stacking gel had solidified, the gels were removed from the casting apparatus, locked into the electrophoresis apparatus and then placed in the buffer tank. Combs were removed once the gel and connecting wires were covered with 1 X SDS-Page running buffer (25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS). Samples were kept on ice prior to loading into wells. A 2 µl aliquot of pre-stained protein ladder (Fermentas, #SM0671) was pipetted into the left most lane. Unused wells were filled with 2X SDS loading buffer to a volume equaling that of the samples to prevent sample spreading. Gels were run at a constant 150 Volts until desired proteins were sufficiently separated, approximately one to two hours. Gels were then removed from the glass plates and proteins were visualized by agitating in Coomassie blue staining solution [(0.25% (w/v) Coomassie brilliant blue R250 dissolved in 40% (v/v) methanol, 10% (v/v) acetic acid in water)] for minimum of thirty minutes. Excess stain was removed by agitating in de-stain solution (40% methanol, 10% acetic acid) until protein bands were visible (one hour to overnight). Gels were then examined to determine volumes required for equal protein load. In order to detect proteins via Western blot, new gels were made as described above and equal protein loads were pipetted into wells. Once protein migration was complete gels were removed from the electrophoresis apparatus and glass plates, and then assembled into ‘sandwiches’. Sandwiches were composed of Wattman paper, acrylamide gel, nitrocellulose membrane, and another layer of Wattman paper. The sandwiches were then placed into a semi-dry protein transfer apparatus and ran for one hour at 12 Volts. Sandwiches used in wet-transfer were themselves

sandwiched between two fiber pads and inserted into the BioRad Mini Trans-Blot Electrophoretic Transfer Cell Cassette. The cassette was placed into the buffer tank and covered with transblot buffer (1.8% glycine, 0.4% Tris and 40% ml methanol). Transfers were performed at 400 milliAmps for two hours, or equivalent current. Membranes were removed from the transfer apparatus and stained with Ponceau S staining solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid) by agitating until proteins were visible, approximately five minutes, to confirm equal protein load. Ponceau S strained blots were scanned for a permanent record and the stain then removed by rinsing with distilled water. Membranes were then incubated in 5% PBST blocking milk (PBS, 0.01% Tween (v/v), 5% non-fat milk) for one hour at room temperature or overnight at 4˚C. Following blocking, membranes were incubated with primary antibody diluted in 1% PBST/non-fat milk powder for one hour at room temperature or overnight at 4˚C, depending on antibody. Primary antibodies were typically diluted at 1:1000 in 1% PBST blocking milk; the only exception being anti-H3 K14^{Ac}, which was diluted in 5% bovine serum albumin in PBS (suppliers are listed in the next paragraph). After incubation, primary antibody was removed and membranes were washed three times for five to fifteen minutes with PBST. Membranes were then incubated with secondary antibodies conjugated with horseradish peroxidase for thirty to forty-five minutes at room temperature. Secondary antibodies were diluted at 1:10,000 in 1% PBST/non-fat milk powder. Membranes were then washed with PBST for five minutes, twice. Proteins were detected by rinsing the membranes with a 1:1 dilution of Chemiluminescence Substrate (PerkinElmer, Inc. #NE104001EA) that allows visualization of horseradish peroxidase-conjugated secondary antibodies by exposing the membranes to X-ray film (Kodak, BioMax, #165-1496).

Primary antibodies used in this study were purchased from the indicated suppliers: rabbit anti-H3K9^{Ac} (Upstate, 07-352), rabbit anti-H3K56^{Ac} (Abcam, ab76307), rabbit anti-H3K14^{Ac} (Abcam ab52946), rabbit anti-H3K79^{me2} (Abcam ab3594), rabbit anti-H3^{total} (Abcam ab1791), rabbit anti-H2B^{total} (Upstate 07-371), rabbit anti-H4^{total} (Abcam ab16483), rabbit anti-H3S10^{phos} (Santa Cruz sc-8656-R), rabbit anti-Clb2 (Santa Cruz sc-9071), mouse anti-GAPDH (Sigma-Aldrich G8795), rat anti-HA (Roche 3F10), rabbit anti-tandem affinity purification (TAP) (Genscript Cat# A00683), rabbit anti-GST (generated by T.A.A. Harkness and M.C. Schultz), mouse anti-myc (Stressgen MSA-110), and mouse anti-UB (Cell signaling P4D1). Secondary antibodies used in this study were goat anti-mouse (BioRad #170-6516), goat anti-rabbit (BioRad #170-6515) and rabbit anti-rat (Sigma #A5795).

CHAPTER THREE

THE APC IS REQUIRED FOR HISTONE METABOLISM [1]

3.1 Introduction

Chromatin assembly has been shown to play a role in chromosome segregation during mitosis (Sharp *et al*., 2002). Defects in chromatin assembly can result in DNA damage, gross chromosomal rearrangements and defects in cell cycle progression (Quivy *et al*., 2001; Myung *et al*., 2003; Ye *et al*., 2003). One of the critical controllers of chromosome segregation, the APC, targets proteins that prevent progression through mitosis and G1 for ubiquitination and proteasome-dependent destruction (Castro *et al*., 2005; Peters, 2006). Our laboratory has shown that the APC is required for efficient chromatin assembly during mitosis in cooperation with the chromatin assembly factors Asf1, Hir1 and Hir2, as well as the individual subunits of CAF-I - Cac1, Cac2 and Msi1 (Harkness *et al*., 2005).

Recent studies have shown that the APC physically interacts with the transcription factor Rb and the HAT CBP (Turnell *et al*., 2005; Binné *et al*., 2007). In the case of CBP, interaction with the APC increased CBP's HAT activity and subsequently, its ability to drive gene transcriptional activity (Turnell *et al*., 2005). To date, only two HATs, Gcn5 and Rtt109, have been linked to progression through mitosis. Yeast Gcn5 has been shown to be the HAT component of multiple transcriptional activators, such as SAGA, ADA and SLIK (Grant *et al*., 1997; Pray-Grant *et al*., 2002), and is linked to mitotic

[1] The majority of this chapter has been published in Turner *et al*., 2010, *Eukaryotic Cell 9*, 1418-1431, Figure 3.3A is published in Islam *et al*., 2011, *Cell Div 6*, (Epub ahead of print), and Figure 3.8B is published in Harkness *et al*., 2005, *Eukaryotic Cell 4,* 673-684.

progression as yeast cells lacking *GCN5* show difficulty in segregating chromosomes and in passing through mitosis (Zhang *et al*., 1998; Krebs *et al*., 2000). These cells accumulate in G2 with unsegregated nuclei and exhibit increased loss of centromere based plasmids. Gcn5 has also been shown to localize to centromeres in addition to acetylating promoters of genes required for mitotic exit and passage though G1/S (Krebs *et al*., 2000; Vernarecci *et al*., 2008). In addition to transcriptional activation, efficient gene expression also requires elongation of the mRNA transcript. As such, the HAT component of Elongator, Elp3 has been shown to genetically interact with Gcn5. Elp3 and Gcn5 both target H3K9 and H3K14 for acetylation; cells lacking both of these HATs are temperature sensitive and show severe histone hypoacetylation (Wittschieben *et al*., 2000; Kristjuhan *et al*., 2002).

Rtt109, which is structurally similar to CBP, has been shown to acetylate histones prior to their deposition onto DNA by the Asf1/CAF-I pathway (Fillingham *et al*., 2008; Tang *et al*., 2008). Rtt109 is capable of targeting H3K9 and H3K56 for acetylation, both of which are markers for newly deposited histones in yeast (Kuo *et al*., 1996; Han *et al*., 2007; Fillingham *et al*., 2008). Asf1 has been shown to genetically interact with the APC as the *apc5*^{CA} phenotype was exacerbated by *ASF1* deletion (Harkness *et al*., 2005). The genetic interaction of Asf1 with the APC in yeast and the physical association of CBP with the APC in humans suggest that the interaction between histone modifying enzymes and the APC may be evolutionarily conserved. Cells lacking Rtt109 are delayed in passing through mitosis and exhibit DNA damage susceptibility. Evidence linking Gcn5 with Rtt109 comes from double deletion mutants exhibiting severely hypoacetylated H3K9 and further increases in DNA damage susceptibility than in single mutants (Fillingham *et al*., 2008). It was proposed that Gcn5, in conjunction with Rtt109, acetylates Asf1-bound histones prior to their passage to CAF-I for deposition onto DNA.

As histones are acetylated in specific patterns prior to deposition and the APC appears to play a role in chromatin assembly, studies were initiated to determine what role the APC plays in this process. This chapter provides evidence that the APC is involved in maintaining total histone levels and in establishing post-translational modifications. Total and modified histone levels were reduced in various APC mutants and the APC genetically interacted with multiple genes encoding histone-modifying proteins. Furthermore, I show that the HATs Elp3 and Gcn5 play an important role in mitotic exit and cell cycle progression by functioning in a single pathway with the APC.

3.2 Results

3.2.1 The APC is required for histone metabolism

To extend our laboratory's previous findings demonstrating that the APC is required for efficient chromatin assembly (Harkness *et al*., 2002; Harkness *et al*., 2005), studies were initiated to examine whether the APC is involved in the maintenance of total and modified histone levels (Figure 3.1A). The nomenclature used to identify each mutant strain refers to the specific subunit affected within the strain. For example, *apc5*^{CA} and *cdc16-1* refer to specific point mutations within the genes for the APC subunits APC5 and CDC16, respectively. Deletion of an entire gene is indicated by Δ following the gene name, for example, *apc10*Δ and *gcn5*Δ refer to deletions of the entire *APC10* and *GCN5* genes, respectively. Cells harboring the indicated mutations were grown to mid log phase at 30˚C, or shifted to 37˚C for an additional 3 hours prior to protein isolation and Western analysis. The APC mutants used here have been shown to grow slowly and accumulate at the metaphase/anaphase junction at 37˚C (data not shown). Even at the permissive temperature of 30˚C many of the mutants had decreased levels of total histone H3 as compared to wild-type (Figure 3.1A). Exceptions were the *apc5*^{CA} and

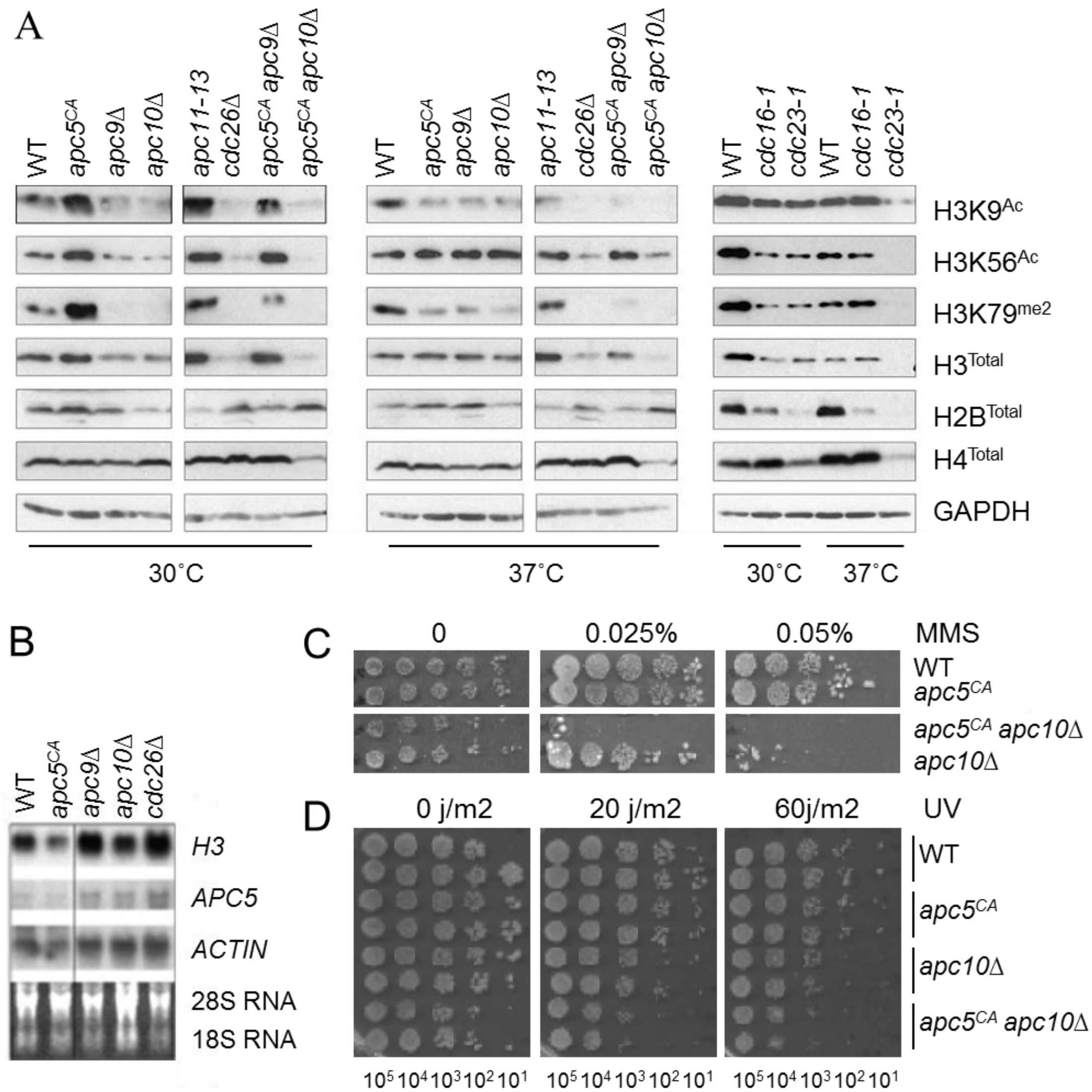

Figure 3.1. **The APC is required for maintenance of total and modified histone H3 levels.** (**A**) Extracts prepared from the APC mutants shown at the top of the lanes, grown at 30°C or after a 3 hour shift to 37°C, were analyzed by Western analysis with the antibodies indicated on the right. Antibodies against GAPDH were used to control for protein load. (**B**) Northern analysis of the APC mutants indicated at the top of the lanes was performed on extracts from cells grown at room temperature overnight to early log phase. PCR fragments corresponding to ORFs of *histone H3*, *APC5* and *ACTIN* were prepared, labeled using [α-^{32}P]dCTP, and hybridized to purified and separated total RNA. (**B**) prepared by G. Davies (Turner, *et al*. 2010). (**C** and **D**) The mutants shown were grown overnight to early log phase in rich glucose media at 30°C. The next day, a 10-fold dilution series was prepared and spotted onto plates containing increasing concentrations of methyl methanesulfonate (MMS) (**C**) or plated onto YPD plates and exposed to increasing ultraviolet radiation (UV) doses (**D**) to induce DNA damage. The plates exposed to UV were wrapped in aluminum foil and grown in the dark for 3 days. The approximate number of cells in each diluted spot is indicated below the plates. WT = wild-type. Images shown are representative of routinely observed results. Published in Turner *et al*., 2010.

apc11-13 single mutants and the *apc5*^{CA} *apc9*Δ double mutant. Interestingly total histone H3 levels appear elevated in *apc11-13* and *apc5*^{CA} *apc9*Δ mutants. At the restrictive temperature of 37˚C total histone H3 levels were similar to that at 30˚C except for *cdc23-1*, where total histone H3 was undetectable. It should be noted that the mutants in the right most panel in Figure 3.1A are a different background (W303) than the rest of the mutants (S288c). This may account for the differences exhibited by the two wild-types. At 30˚C, the majority of total H2B levels were similar to that of wild-type, exceptions being the *apc10*Δ, *apc11-13*, *cdc16-1* and *cdc23-1* single mutants. Reductions in total H2B levels were also seen at 37˚C in the *cdc16-1* and *cdc23-1* mutants. Total levels of histone H4 were essentially unchanged at both 30°C and 37°C, the only exceptions being *cdc23-1* and the *apc5*^{CA} *apc10*Δ double mutant. The decreased levels of histone H3 are not due to impaired transcription as the levels of histone H3 mRNA were increased in APC mutants exhibiting decreased histone H3 protein levels (Figure 3.1B).

Next it was determined whether APC mutants affect the modification levels of histone H3 (Figure 3.1A). Acetylation of H3 lysine 56 (H3K56^{Ac}) was chosen as it is associated with histone deposition by Asf1 and CAF-1 while H3K9^{Ac} was chosen as it is associated with both active genes and histone deposition (Kuo *et al.*, 1996; Pokholok *et al*., 2005; Li *et al*., 2008). Dimethylation of H3K79 (H3K79^{me2}) was chosen due to its association with genes required for progression through mitosis and G1 (Schulze *et al*., 2009). Antibodies specifically raised against these modifications were purchased and used for this analysis. H3K56^{Ac} mirrored the pattern of total histone H3 levels both at 30˚C and 37˚C, suggesting that H3K56^{Ac} is not affected by the APC mutations. H3K9^{Ac} and H3K79^{me2} modifications were both reduced beyond that observed for total H3 in most mutants, indicating that these modifications may be specifically regulated by the APC.

3.2.2 APC mutants are sensitive to DNA damaging conditions

Our laboratory has reported that Apc5 and Apc10 play redundant roles in lifespan and cell growth (Harkness *et al*., 2002; Harkness *et al*., 2004). The experiments presented above indicate that *apc5*^{CA} *apc10*Δ double mutants have decreased levels of total histone H3 and H4. As naked DNA is believed to be more damage prone than correctly assembled DNA an assay was performed to determine if the decrease in histone levels would result in sensitivity to DNA damage (Loyola & Almouzni, 2004). Serial dilutions of wild-type, *apc5*^{CA}, *apc10*Δ and *apc5*^{CA} *apc10*Δ strains were exposed to either methyl methanesulfonate (MMS; Figure 3.1C) or UV radiation (Figure 3.1D). The double mutant was more sensitive to DNA damage than either of the single mutants. This suggests that Apc5 and Apc10 may play complementary roles in stress response.

3.2.3 Interaction of HAT and HDAC mutants with the *apc5*^{CA} allele

To further determine the relationship between the APC and histone metabolism a modified genetic screen was performed where non-essential HAT and HDAC genes were deleted in combination with the *apc5*^{CA} mutation to discern their effects on a simple measurable phenotype, temperature sensitivity. The hypothesis is that if the APC is required for histone acetylation, and if histone acetylation is tightly linked to gene activation, then mutation of genes involved in gene activation, such as HATs, would worsen the *apc5*^{CA} phenotype. Conversely, deletion of genes involved in gene silencing may rescue the *apc5*^{CA} phenotype. The *apc5*^{CA} allele was used as it has allowed the effective screening of genetic interactions (Harkness *et al*., 2004; Arnason *et al*. 2005; Harkness *et al*., 2005). At 30˚C the *apc5*^{CA} allele grows in a similar manner as wild-type but at the restrictive temperature of 37˚C it grows slowly and accumulates at the metaphase/anaphase junction. This may be due to a 2 bp deletion in the 5’ end of the

gene resulting in an internal start site that produces an N-terminally truncated protein (Harkness *et al*., 2002; unpublished data). Even though related by sequence, the yeast HDACs Rpd3, Hda1, Hos1, Hos2 and Hos3 have all been shown to target specific regions and gene subsets within the genome (see Table 1.2; Robyr *et al*., 2002). The data presented in this thesis agree with this division of labor as a variety of phenotypes were observed when deletions were combined with the *apc5*^{CA} background. Deleting any of the *HOS* genes completely suppressed the temperature sensitive phenotype of the *apc5*^{CA} allele (Figure 3.2A). As Hos1 and Hos3 preferentially target the rDNA locus and Hos2 targets ribosomal protein-encoding genes it would make sense that all three would affect the APC in the similar manner (Zhang *et al*., 1998). A recent synthetic genetic array (SGA) screen observed that suppression of the *apc5*^{CA} temperature sensitive phenotype occurred when genes involved in mRNA decay and quality control were deleted (Costanzo *et al*., 2010). Taken together, these data suggest that high levels of mRNA, and the subsequent increased translation, may contribute to the suppression of the *apc5*^{CA} temperature sensitive phenotype. This is also consistent with the observation that the human APC5 protein physically interacts with the ribosome and inhibits the translation of certain mRNAs, mostly encoding viral and growth promoting proteins (Hellen & Sarnow, 2001; Koloteva-Levine *et al*., 2004). Evolutionary conservation of this interaction may occur as the SGA screen also identified genetic interactions between the *apc5*^{CA} allele and ribosomal subunits (Costanzo *et al*., 2010). Mutation to the *APC5* allele may prevent the inhibition of translation of certain mRNAs in yeast, resulting in inappropriate cell growth.

Further supporting the division of labor of HDACs, deleting *HDA1* in an *apc5*^{CA} background resulted in increased temperature sensitivity while deletion of Rpd3 did not affect the *apc5*^{CA} phenotype (Figure 3.2A). Rpd3 and Hda1 have been shown to exhibit

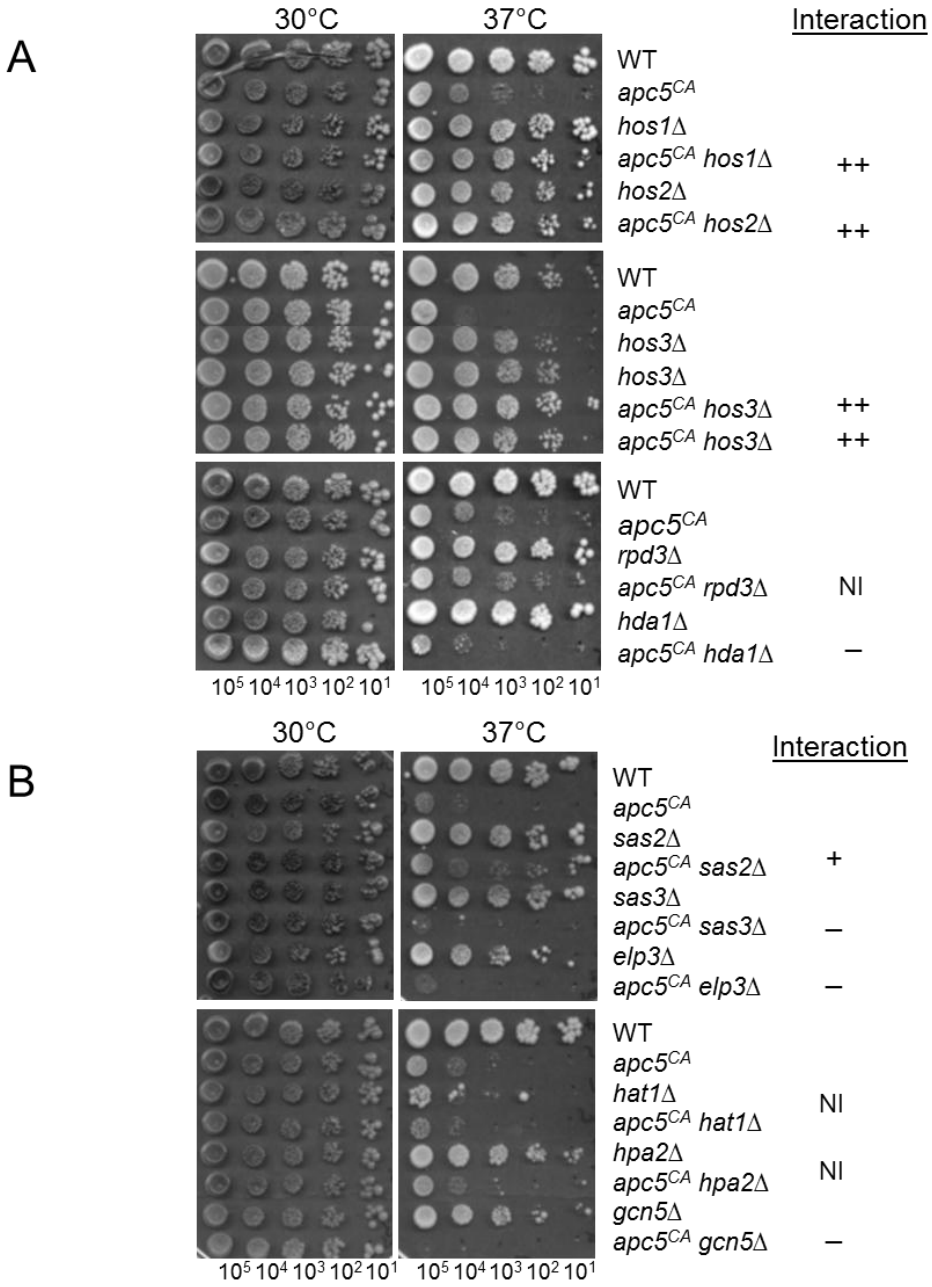

Figure 3.2. **Genetic interactions of HDAC and HAT mutants with the *apc5^{CA}* allele.** (**A**) *apc5^{CA}* cells were repeatedly crossed with the HDAC mutants shown to generate double mutants. Tenfold serial dilutions of the different mutants were spot diluted onto rich media, and growth levels were compared at the temperatures shown. (**B**) *apc5^{CA}* cells were repeatedly crossed with the HAT mutants shown to generate double mutants. The mutants were prepared and analyzed as described above. The effect of the interaction is indicated on the right. ++, strong suppressive interaction in double mutant that grows like wild type; +, double mutant grows better than single mutants but not at wild-type level; -, double mutant grows more poorly than the single mutants. NI, no interaction. WT = wild-type. Representative images of routinely observed results are shown. Published in Turner *et al*., 2010.

the greatest effect on promoter deacetylation; however, each enzyme targets distinct regions within the genome (Zhang *et al*., 1998). These results demonstrate that it is not simply the level of acetylation that regulates processes within the cell but that it is the specific region and histone residue acetylated that plays an important part.

Next, it was asked whether deletions of the genes encoding the HATs Sas2, Sas3, Elp3, Hat1, Hpa2 and Gcn5 would also alter the *apc5*^{CA} temperature sensitive phenotype (Figure 3.2B). Similar to the deletion of HDACs, various phenotypes were observed. Deletion of *ELP3*, *GCN5* or *SAS3* each slightly impaired growth of *apc5*^{CA} cells at 37˚C, while deletion of *SAS2* suppressed temperature sensitivity to almost wild-type levels. It is logical that deletion of the various HATs in the *apc5*^{CA} background affects the temperature sensitive growth in different ways as HATs also play specific roles within the cell. For example, deletion of *GCN5* results in changes in the expression of only ~5% of genes throughout the genome (Holstege *et al*., 1998), while deletion of *SAS2* results in the silencing of sub-telomeric regions (Kimura *et al*., 2002; Suka *et al*., 2002). As such, deletion of *GCN5* or *SAS2* resulted in opposite effects in the *apc5*^{CA} strain. This could be due to the different targets of these two HATs. Gcn5 has been implicated in the transcription of mitotic specific genes while Sas2 maintains expression of sub-telomeric genes (Krebs *et al*., 2000; Kimura *et al*., 2002; Suka *et al*., 2002; Krebs , 2007). Reduced mitotic specific gene expression in an *apc5*^{CA} mutant due to *GCN5* deletion would further inhibit cell cycle progression while the deletion of Sas2 may result in the silencing of genes that contribute to the *apc5*^{CA} temperature sensitive phenotype. Deletion of *ELP3* or *SAS3* resulted in a slight exacerbation of the *apc5*^{CA} temperature sensitive phenotype. As the histone acetylation and genetic interaction patterns of Elp3 and Sas3 overlap with that of Gcn5 (Rosaleny *et al*., 2007; Wittschieben *et al*., 2000) it is not surprising that they would exhibit similar phenotypes. Both Hat1 and Hpa2 appear to

have no effect on the *apc5*^{CA} temperature sensitive phenotype as the double mutants were similar to the single *apc5*^{CA} mutant. The predominantly cytoplasmic Hat1 targets H4 for acetylation prior to chromatin deposition (Ruiz-Garcia *et al*., 1998) while Hpa2 preferentially targets K14 on histone H3 (Angus-Hill *et al*., 1999). Hpa2 may not play an major role in the cell as H3K14 is targeted by several other HATs and gene expression was only mildly affected in *hpa2*Δ mutants (Durant & Pugh, 2006). The lack of interaction between the APC and Hat1 suggests that APC does not play a role in histone acetylation prior to nuclear import while the lack of interaction with Hpa2 might be due to the redundancy among HATs and the ability to acetylate H3K14.

3.2.4 *GCN5* and *HDA1* interact antagonistically when combined with the *apc5*^{CA} allele

To further study the interactions of histone modifiers with the APC, triple mutants were created with the *apc5*^{CA} allele (Figure 3.3A). In the *apc5*^{CA} background, deletion of either *GCN5* or *HDA1* exacerbated the temperature sensitive phenotype while deletion of both partially suppressed this exacerbation back to the level of the *apc5*^{CA} single mutant. Both Gcn5 and Hda1 target a variety of promoters and have opposing affects on acetylation levels (Zhang *et al*., 1998; Durant & Pugh, 2006). These results suggest that the exacerbation of the *apc5*^{CA} temperature sensitive phenotype is in part due to an imbalance of Gcn5/Hda1 activity as deletion of both cancels out the increased sensitivity. Importantly, deletion of *GCN5* or *HDA1* in wild-type cells had no effect on growth, indicating that *apc5*^{CA} cells have difficulty coping with this imbalance. Interestingly, in two separate SGA genome-wide screens a growth phenotype associated with *gcn5*Δ was suppressed by deletion of *HDA1* (Lin, *et al*., 2008; Costanzo *et al*., 2010). The SGA screen utilizes plates lacking many amino acids and containing

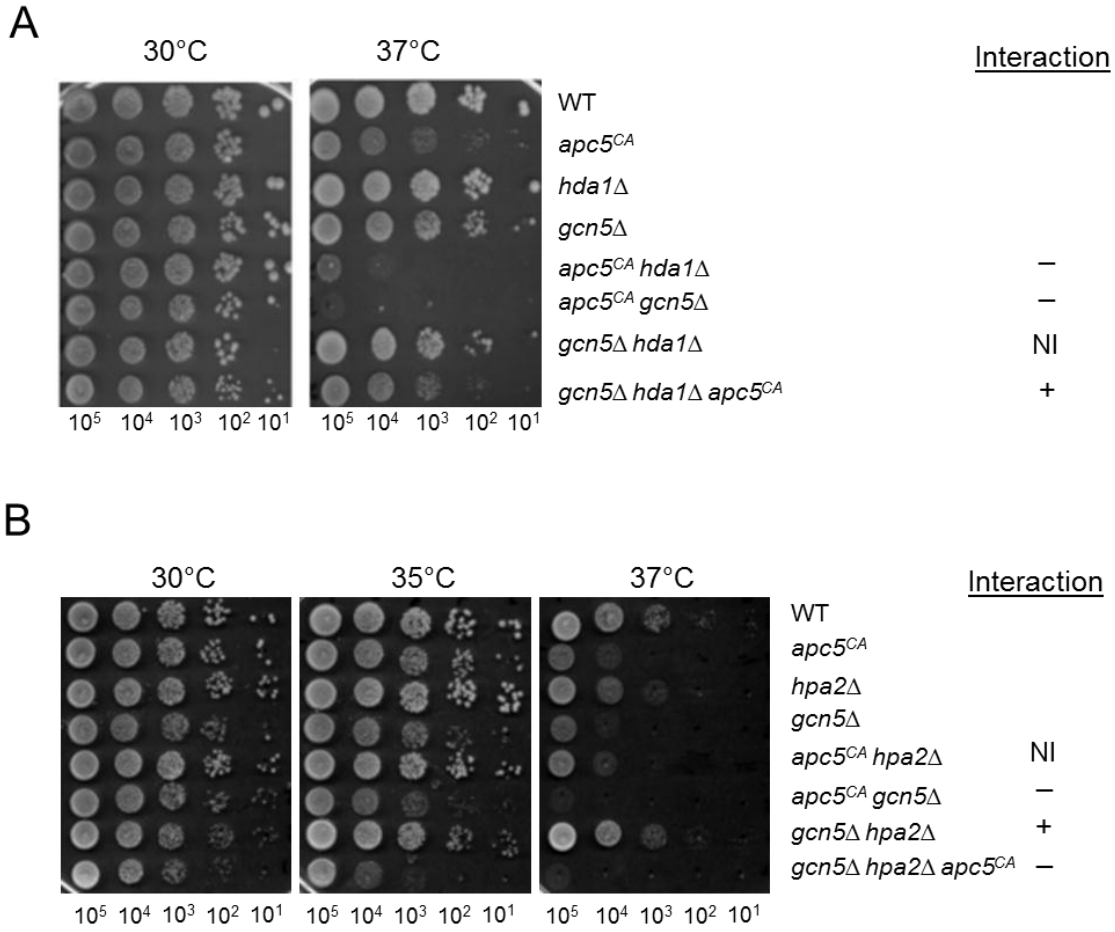

Figure 3.3. **Genetic interactions between histone modifiers and the APC. (A) A genetic interaction between *hda1*Δ and *gcn5*Δ mutants is revealed in *apc5*^{CA} cells**. The mutants shown were constructed by genetic crosses and tested for temperature sensitivity using spot dilutions. Please see the legend of Figure 3.2 for explanation of interaction symbols. Serial dilutions were prepared as in Figure 3.2, samples were then spotted onto YPD and grown at 30°C and 37°C. (**A**) prepared by A. Islam (Islam, *et al*. 2011). **(B) A genetic interaction between *hpa2*Δ and *gcn5*Δ mutants is revealed in *apc5*^{CA} cells**. Cells were treated in a similar manner as in (A). WT = wild-type. Representative images of routinely observed results are shown. Published in Turner *et al*., 2010.

specific antibiotics for selection purposes (Lin *et al*., 2008). Therefore, under certain conditions Gcn5 is required, and its loss can be compensated for by deletion of *HDA1*. Under normal growth conditions, such as on YPD, this phenomenon is only observed when the APC is compromised. This work was continued in Islam *et al*., 2011.

3.2.5 *GCN5* and *HPA2* interact antagonistically alone but synergistically with the *apc5*^{CA} allele

To further study the interactions of HATs with the APC, triple mutants with the *apc5*^{CA} allele and the HAT mutations *gcn5*Δ and *hpa2*Δ were created (Figure 3.3B). Although deletion of *HPA2* did not affect the temperature sensitivity of the *apc5*^{CA} mutant it did negatively influence the *gcn5*Δ *apc5*^{CA} double mutant, as indicated by reduced growth of the triple mutant at 30˚C and 35˚C, compared to the double mutant. Interestingly, when *HPA2* was deleted in *gcn5*Δ cells, *gcn5*Δ temperature sensitive growth was suppressed, indicating an antagonistic relationship. Gcn5 and Hpa2 are both capable of acetylating H3K14; however, they may target different gene promoters (Kuo *et al*., 1996; Angus-Hill *et al*., 1999). This result indicates that Gcn5 and Hpa2 counteract each other's activities in wild-type cells but possibly cooperate in an *apc5*^{CA} background.

3.2.6 Genetic interactions between *apc5*^{CA} and the HAT mutants, *elp3*Δ and *gcn5*Δ

The genetic screen described in 3.2.3 identified two HAT mutants, *gcn5*Δ and *elp3*Δ, that together exhibit a genetic interaction. Deletion of *ELP3* and *GCN5* together has been shown to result in slow growth rates and reduced acetylation of H3 lysine residues (Wittschieben *et al*., 2000). I have also observed these phenomena, as *elp3*Δ *gcn5*Δ double mutants grow slowly at 30°C and display very little H3K9 or H3K14 acetylation (Figures 3.4A, 3.5A). The observed redundancy is due to the fact that Gcn5 and Elp3,

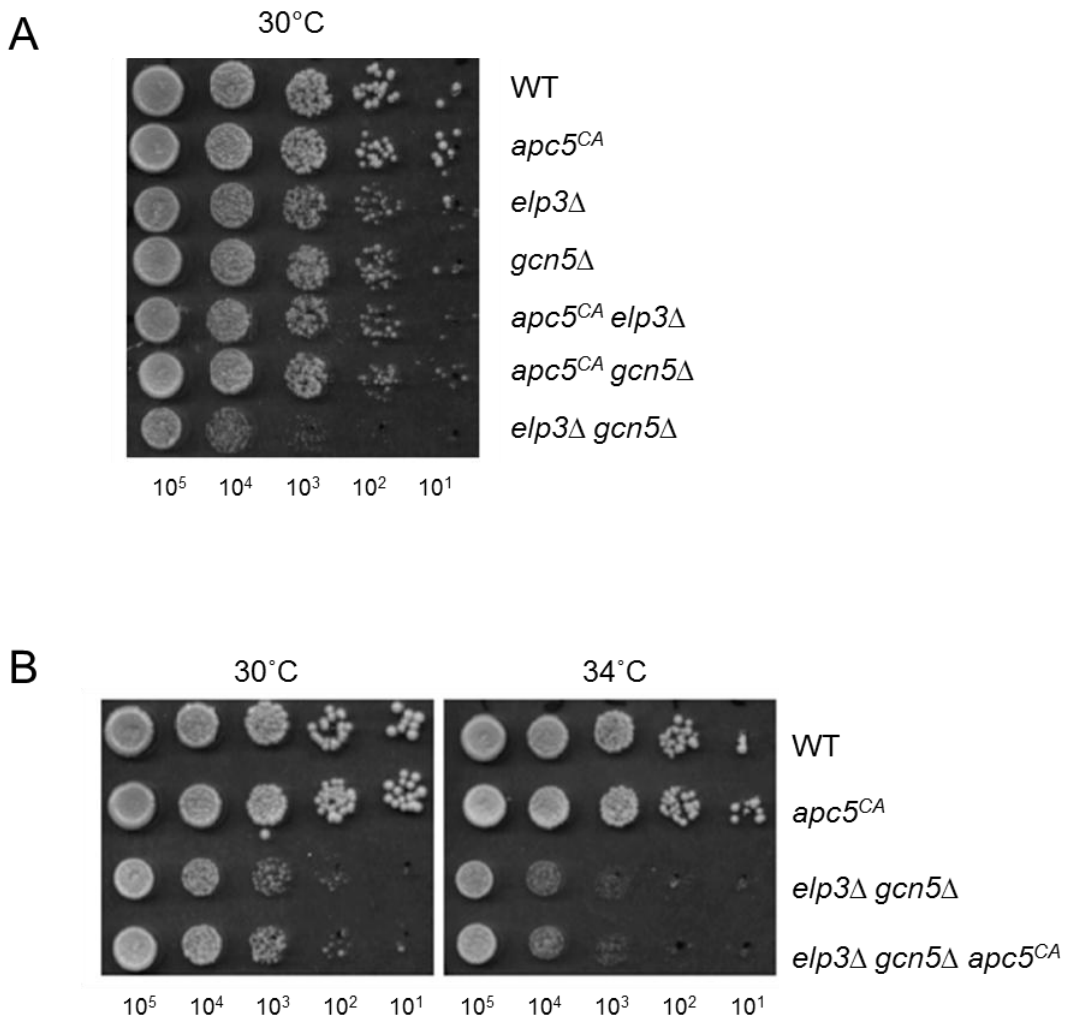

Figure 3.4 **The APC genetically interacts with Elp3 and Gcn5. (A**) The *elp3*Δ *gcn5*Δ defect is more severe than that of strains containing *apc5*^{CA}, as determined by spot dilutions. (**B**) The APC is involved in the same pathway as Elp3 and Gcn5. The mutants shown were created through multiple rounds of backcrossing and characterized using spot dilutions. The strains were spot diluted onto YPD plates and grown at the indicated temperatures. 34°C was used as the double HAT mutants are extremely slow growing at 37°C. WT = wild-type. Images shown are representative of routinely observed results. Published in Turner *et al*., 2010.

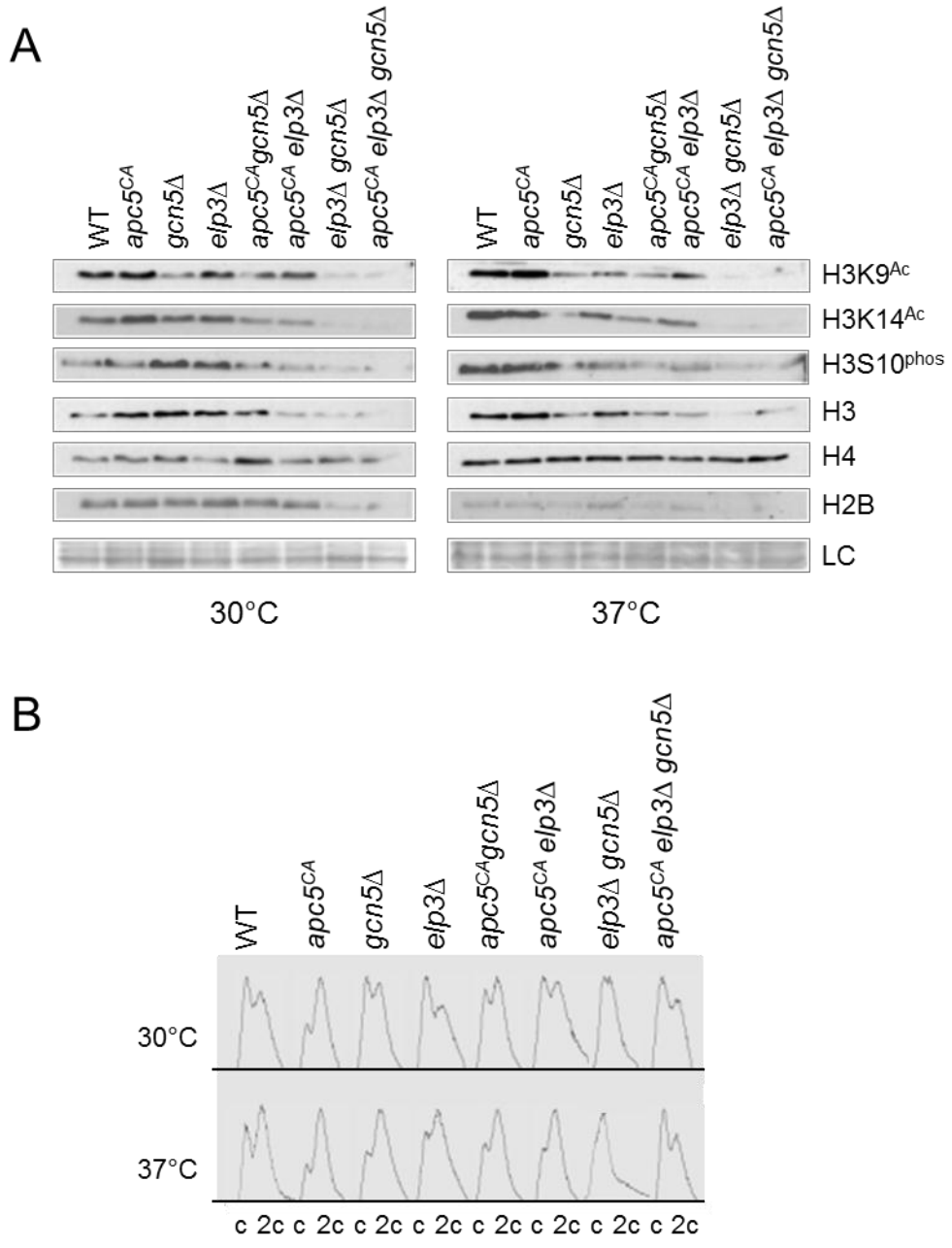

Figure 3.5. **Histone posttranslational modifications and cell cycle profiles in *apc5*^{CA}, *elp3, and gcn5* single, double, and triple mutants**. (**A**) *apc5*^{CA}*, elp3,* and *gcn5* mutants were used to characterize histone profiles. Extracts were prepared after growth at 30°C or following a shift to 37°C for 3 h. Proteins were separated by SDS-PAGE, and Western analysis was performed using the antibodies indicated. Ponceau S staining was used as a loading control (LC) (**B**) Flow cytometry was conducted on the asynchronous cultures described for panel A. c, unreplicated DNA, 2c, replicated DNA. Images shown are representative of results observed from multiple experiments. Published in Turner *et al*., 2010.

the HAT components of SAGA and Elongator, respectively, acetylate H3K14 in order to initiate (SAGA) transcription, and then to ensure the progression of the elongating transcript (Elongator) (Wittschieben *et al*., 2000). The *apc5*^{CA} allele was combined with the *elp3*Δ and *gcn5*Δ deletions through genetic crosses. Interestingly, the addition of the *apc5*^{CA} allele to the *elp3*Δ *gcn5*Δ double deletion mutant did not affect the growth phenotype observed in the *elp3*Δ *gcn5*Δ mutant as it did with the single HAT deletions (compare Figure 3.4B with Figure 3.2B). This suggests that while *apc5*^{CA} interacts synergistically with the single *elp3*Δ and *gcn5*Δ mutants, an epistatic relationship exists when *apc5*^{CA} is combined with the double deletion, suggesting that these three proteins may act in the same pathway.

3.2.7 Deletion of *ELP3* and *GCN5* in *apc5*^{CA} cells does not affect histone modification profiles

Reduced histone levels are associated with impaired cell growth in APC mutants and the deletion of *ELP3* or *GCN5* exacerbates the *apc5*^{CA} temperature sensitive phenotype. As such histone levels were examined in *elp3*Δ, *gcn5*Δ and *apc5*^{CA} double and triple mutants (Figure 3.5A). Protein lysates were prepared from cells grown at 30˚C, or following a shift to 37˚C for 3 hours, and Westerns were performed using the antibodies shown. At 30˚C all three double mutants containing *elp3*Δ exhibited decreased total histone H3. Total histone H2B was unaffected in the single HAT mutants regardless of the presence of Apc5 as only the two double HAT mutants exhibited decreased levels. Histone H4 was essentially unchanged at either 30˚C or 37˚C. It was next asked if modifications to histone H3 were also affected in these mutants. The levels of H3K9^{Ac}, H3K14^{Ac} and H3S10^{phos} were assessed and were found to essentially follow the pattern

of total histone H3. This suggested the synergistic interaction between *apc5*^{CA} and the HAT single mutants does not involve histone modifications per se. This may be a result of the redundancy among HAT acetylation activity as multiple HATs have been shown to target the histone H3 residues K9 and K14 (Grant *et al*., 1997; Winkler *et al*., 2002; Rosaleny *et al*., 2007; Fillingham *et al.* 2008). It is interesting to note that total histone H3 and H4 protein levels are not the same in the mutants. It has been shown that altered histone stoichiometry in yeast cells leads to cell cycle defects (Meeks-Wagner & Hartwell, 1986). Thus, the growth defects observed in these studies may in fact be due to altered histone stoichiometry.

As the Western analysis of the various mutants showed similar histone modification levels when both *GCN5* and *ELP3* were deleted regardless of whether *APC5* was mutated, cell cycle progression was characterized (Figure 3.5B). Using Fluorescence Assisted Cell Sorting (FACS) it was observed that many of the mutants spend a prolonged time in G2/M as compared to wild-type, indicating a defect in mitotic passage. The main exceptions were the double HAT mutants, as *elp3*Δ *gcn5*Δ cells were predominantly in S-phase while *elp3*Δ *gcn5*Δ *apc5*^{CA} cells had a larger proportion of cells in G1. This suggests that cells lacking *GCN5* or *ELP3* have difficulty progressing through mitosis, while cells lacking both are impaired at transiting through G1/S.

3.2.8 Clb2 levels do not accumulate in *elp3*Δ *gcn5*Δ mutants

The above observations suggest that cells lacking *ELP3* or *GCN5* have mitotic passage defects while cells lacking both *ELP3* and *GCN5* are defective in G1/S-phase progression. The epistatic interaction observed between *apc5*^{CA} and *elp3*Δ *gcn5*Δ could be interpreted to imply that Gcn5 and Elp3 work together upstream of the APC, which

raises the question of whether an APC target, Clb2, is stable in the *elp3*Δ *gcn5*Δ double mutant. Clb2 has been shown to be ubiquitinated by the APC to target it for degradation, thereby allowing progression through mitosis (Wäsch & Cross, 2002). If Clb2 is stable in these mutant cells, this would suggest that APC activity is impaired when both *GCN5* and *ELP3* are deleted. Thus, endogenous Clb2 protein levels were examined by Western blotting in various *elp3*Δ and *gcn5*Δ mutants grown asynchronously to early log phase (Figure 3.6). The cells were grown at 30°C and then shifted to 37°C for 3 hours. Protein extracts were prepared following the 30°C and 37°C treatments. As expected, the *apc5*^{CA} mutant strain exhibited an increased level of Clb2 as compared to wild-type, especially at the restrictive temperature of 37˚C. At 30°C *gcn5*Δ and *apc5*^{CA} *elp3*Δ cells exhibited lower levels of Clb2 than observed in wild-type while *elp3*Δ and *apc5*^{CA} *gcn5*Δ cells had similar levels as wild-type. At 37°C *gcn5*Δ and *apc5*^{CA} *elp3*Δ cells had levels of Clb2 similar to wild-type cells while Clb2 levels were higher in *elp3*Δ and *apc5*^{CA} *gcn5*Δ. Interestingly, at both 30°C and 37°C, The finding that Clb2 is still degraded in the majority of these mutants could be an indirect effect of them spending a prolonged time in G2, a time in the cell cycle when Clb2 is targeted for degradation. Strains lacking both Gcn5 and Elp3 exhibited similar levels of Clb2 regardless of whether Apc5 was mutated or not, again highlighting an epistatic relationship. These mutants both exhibited very low levels of Clb2 when compared to wild-type and this may be attributed to the fact that these strains can pass through mitosis to G1, but spend a prolonged amount of time in G1/S, perhaps allowing for increased time to degrade Clb2. These results indicate that Elp3 and Gcn5 are not upstream activators of the APC.

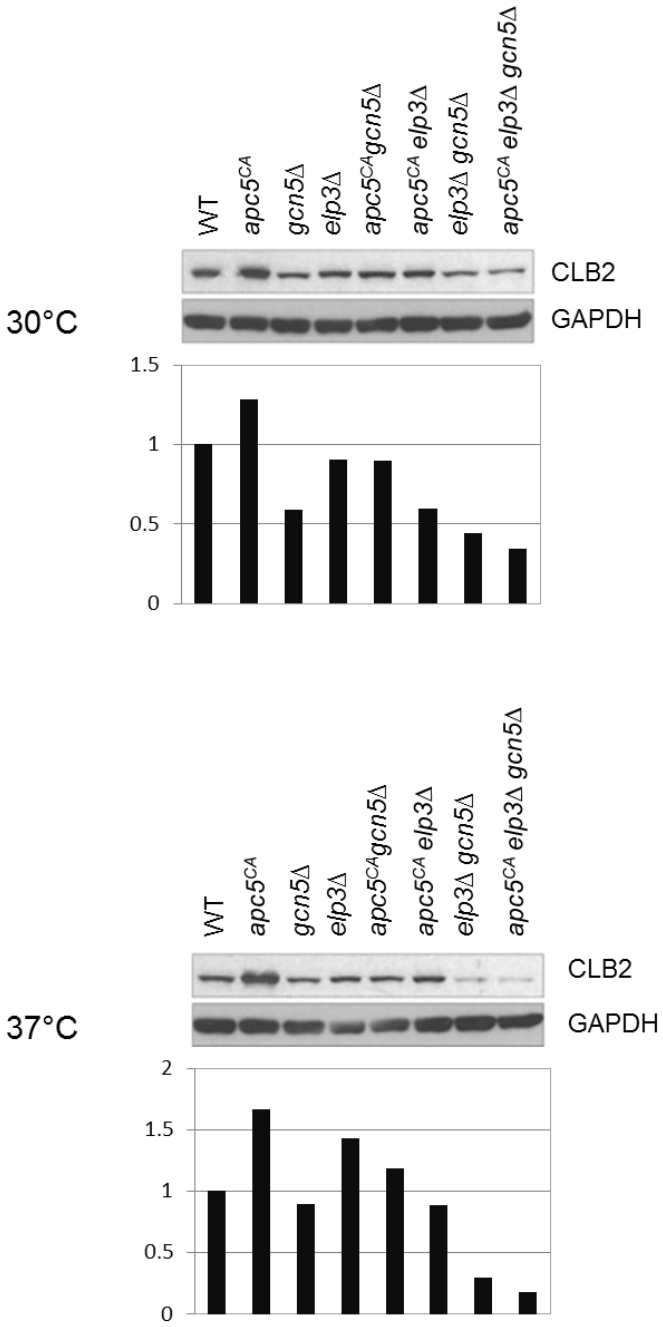

Figure 3.6. **Clb2 stability in *apc5^{CA}*, *elp3, and gcn5* single, double, and triple mutants**. Clb2 stability was assessed in *apc5^{CA}, elp3,* and *gcn5* mutants. Extracts were prepared after growth at 30°C or following a shift to 37°C for 3 h. Proteins were separated by SDS-PAGE, and Western analysis was performed using the antibodies indicated. Western analyses were performed using antibodies against endogenous Clb2 and as a load control (LC), GAPDH. Densitometry of Clb2 bands was normalized to GAPDH bands and is graphed relative to wild-type (WT). Images shown are representative of results observed from multiple experiments. Western blots are published in Turner *et al*., 2010.

3.2.9 Increased expression of *ELP3* and *GCN5* rescues the *apc5*^{CA} temperature sensitive growth phenotype while overexpression impairs growth of all cells

To further characterize the relationship between the APC and the Elp3/Gcn5 HATs, the effect of increased levels of Elp3 and Gcn5 on the growth of wild-type and *apc5*^{CA} mutant strains was examined by using plasmids producing galactose driven HA-tagged Elp3 or Gcn5 (Figure 3.7A). As the galactose promoter produces basal levels of protein when in glucose media, the effects of both low and high levels of protein on the cell is able to be determined (Figure3.7B). On 2% glucose-supplemented plates the low level increase of both Elp3 and Gcn5 protein allowed *apc5*^{CA} cells to grow at wild-type levels (Figure 3.7A). On 2% galactose-supplemented plates, overexpression of Elp3 was only able to partially rescue the *apc5*^{CA} mutant while overexpression of Gcn5 was toxic to all cells tested. Such an extreme difference in the growth assay led us to further analyze the effect of increased Gcn5 and Elp3 expression. When cells were grown in glucose-supplemented media, the levels of both Elp3-HA and Gcn5-HA were moderately increased and the cells accumulated in G1 (Figures 3.7B, 3.7C). When grown in galactose-supplemented media, the protein levels of both Elp3 and Gcn5 rapidly accumulated. We were unable to determine how overexpression of Elp3 or Gcn5 influenced the cell cycle as even cells containing an empty vector rapidly accumulated in G1 when in galactose (data not shown). Taken together, this data indicates that Elp3 and Gcn5 levels must be reduced to exit G1, a hypothesis that will be tested below.

Elp3 and Gcn5 may link the APC with histone metabolism. Since increased expression of Elp3 rescued the temperature sensitive *apc5*^{CA} growth defect, the effect of increased Elp3 protein on histone H3 levels was examined. Both low (glucose) and high-level (galactose) expression of Elp3 resulted in an increase of total histone H3 levels in APC

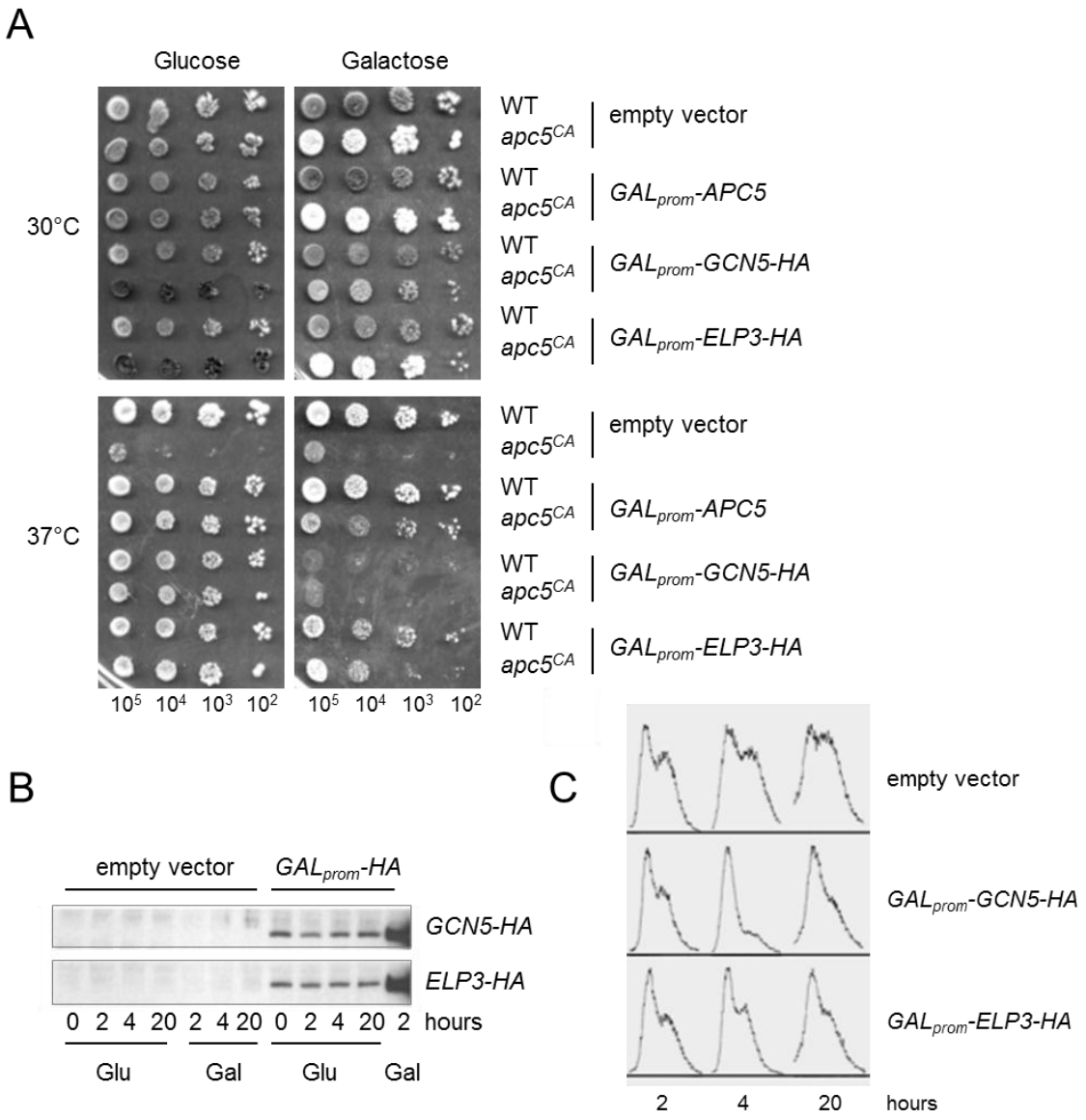

Figure 3.7. **Low-level expression of the HAT genes *ELP3* or *GCN5* suppresses the *apc5^{CA}* temperature sensitive defect.** (**A**) Wild-type (WT) and *apc5^{CA}* cells were transformed with plasmids expressing *GCN5 or ELP3* under the control of the *GAL1* promoter. Transformants were spot diluted onto glucose- and galactose-supplemented media and grown at either 30°C or 37°C. The plates were then scanned. Image shown is representative of routinely observed results. (**B**) Western analyses were performed on extracts obtained from wild-type cells expressing either empty vector, *GAL$_{prom}$-GCN5-HA, or GAL$_{prom}$-ELP3-HA.* The cells were grown overnight to early log phase at 30°C. The next morning, the cultures were divided, with 2% galactose added to one and the other left in 2% glucose. Samples were taken at the times indicated and probed using antibodies against hemagglutinin (HA). (**C**) Low-level expression of *GCN5* or *ELP3* results in cells exiting the cell cycle early in G1. Cells were grown overnight to early log phase and then diluted back in glucose-supplemented media. Samples were taken at the times indicated and prepared for flow cytometry. Published in Turner *et al*., 2010.

mutants (Figure 3.8A). Increased total H3 was even observed in wild-type cells grown in galactose. This increased histone content may be responsible for the Elp3-based suppression of the temperature sensitive phenotype of *apc5*^{CA} (Figure 3.7A), as we previously observed that increased expression of histones alone could suppress the *apc5*^{CA} defect (Figure 3.8B; Figure produced by E. Turner; Harkness *et al*., 2005).

3.2.10 Increased expression of Apc5 partially suppresses the *elp3*Δ *gcn5*Δ temperature sensitive growth defect while overexpression impairs growth

Observations thus far suggest that Elp3 and Gcn5 are not upstream activators of the APC, since the APC target Clb2 is still degraded in cells lacking *ELP3* and *GCN5* (Figure 3.6). The moderate over-expression of Elp3 or Gcn5 being able to rescue the temperature sensitivity of the *apc5*^{CA} mutant suggests that Elp3 and Gcn5 may in fact be downstream of the APC. To test this further, it was assessed whether increased expression of *APC5* would rescue the double *elp3*Δ *gcn5*Δ temperature sensitive phenotype. *APC5* was expressed in Wild-type and *elp3*Δ *gcn5*Δ cells using a plasmid containing galactose driven *APC5* (Figure 3.9). The excessive increase of *APC5* from being grown on 2% galactose-supplemented plates prevented the growth of *elp3*Δ *gcn5*Δ cells at both 30˚C and 37˚C and even wild-type cells at 37˚C (data not shown). When grown on 2% glucose-supplemented plates, the low-level increase in Apc5 protein was sufficient to partially rescue the growth of *elp3*Δ *gcn5*Δ cells at both 30˚C and 37˚C, providing support for the hypothesis that the APC works in a positive, and perhaps redundant, manner with both Elp3 and Gcn5.

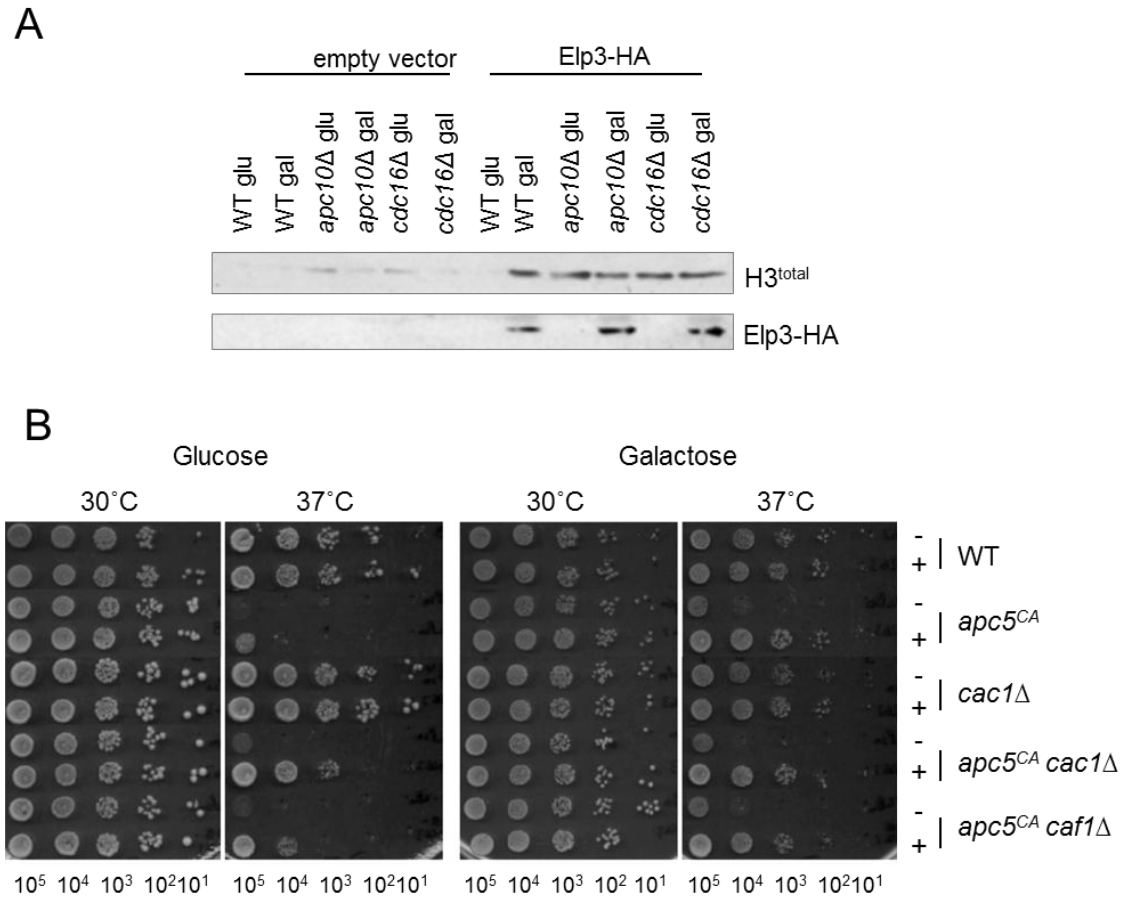

Figure 3.8. (**A**) **Overexpression of the HAT gene *ELP3* results in increased histone H3.** Western analyses were performed on extracts obtained from wild-type (WT), *apc10 and cdc16-1 cells* expressing either an empty vector *or* GAL_{prom}*-ELP3-HA.* Samples were grown overnight at 30°C. The next morning the cultures were divided and grown for 4 hours in media containing either 2% galactose or 2% glucose. Antibodies against HA and total H3 were used. (B) **Increased levels of histones H3 and H4 suppresses the *apc5*^{CA} temperature sensitive phenotype**. Wild-type, *apc5*^{CA}, *cac1*Δ, *apc5*^{CA} *cac1*Δ, and *apc5*^{CA} *caf1*Δ cells were transformed with empty vector (-) or pRM102 (+), which overexpresses histones H3 and H4 under control of the *GAL1* promoter and grown on either glucose- or galactose-supplemented plates. (**B**) prepared by E. Turner, (Harkness, *et al*. 2005).

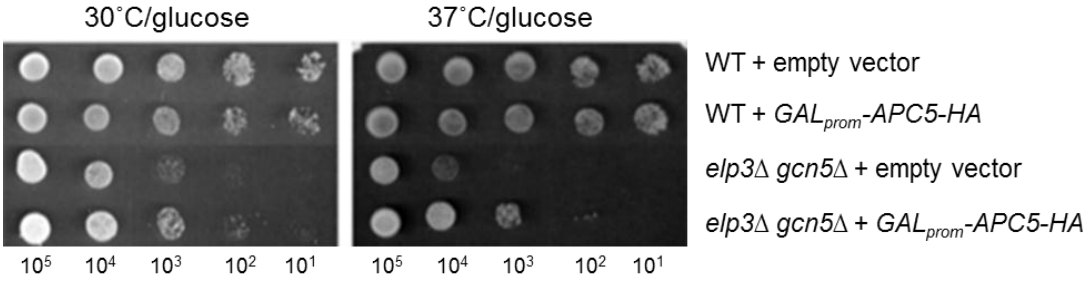

Figure 3.9. **Increased expression of *APC5* partially rescues the *elp3*Δ *gcn5*Δ temperature sensitive growth**. Wild-type (WT) and *elp3*Δ *gcn5*Δ cells were transformed with either an *APC5*-expressing construct under the control of the *GAL1* promoter or the empty vector control. The transformants were spot diluted onto 2% glucose- or 2% galactose-supplemented plates and grown at the indicated temperatures. Representative image of results observed. Published in Turner *et al*., 2010.

3.2.11 Apc5 overexpression toxicity is partially suppressed by overexpression of Gcn5

Since Gcn5 overexpression caused cells to accumulate in G1 (Figures 3.7B, 3.7C), and APC^{Cdh1} is active during G1, it was determined whether there is a functional interaction between the APC and Gcn5. It is possible that since increased Gcn5 causes cells to accumulate in G1, the APC may be involved in Gcn5 turnover. To begin the analysis of this concept, a *LEU2*-based GAL_{prom}-*GCN5*-*HA* plasmid was co-expressed in wild-type cells with galactose driven plasmids that expressed *APC5* or *APC10* (Figure 3.10). Unlike the *URA*-based *GCN5* plasmid the *LEU2*-based *GCN5* plasmid was not toxic when over-expressed with 2% galactose-supplemented media. Overexpression of *LEU2*-based *GCN5* partially rescued the toxicity of *APC5* overexpression. This suggests that *APC5* overexpression toxicity may be due to increased targeting of Gcn5 for degradation, causing insufficient amounts of Gcn5 within the cell. Thus, by increasing the Gcn5 content within cells overexpressing *APC5*, the toxic effects are partially reduced. It is possible that Apc5 overexpression toxicity may be further overcome by overexpression of both *ELP3* and *GCN5*.

3.2.12 *RTT109* overexpression rescues the $apc5^{CA}$ temperature sensitive growth phenotype

While levels of histone H3 were decreased in many of the APC mutants tested, Lys9 acetylation was further compromised in several mutant strains. Gcn5 and Rtt109 are the only two HATs known to acetylate this residue (Fillingham *et al*., 2008). As low-level expression of *GCN5* was able to rescue the $apc5^{CA}$ temperature sensitive phenotype, it was determined whether or not Rtt109 would also be able to do so. Similar to the *GCN5* expression data, low-level expression of the *URA*-base d galactose driven *RTT109*

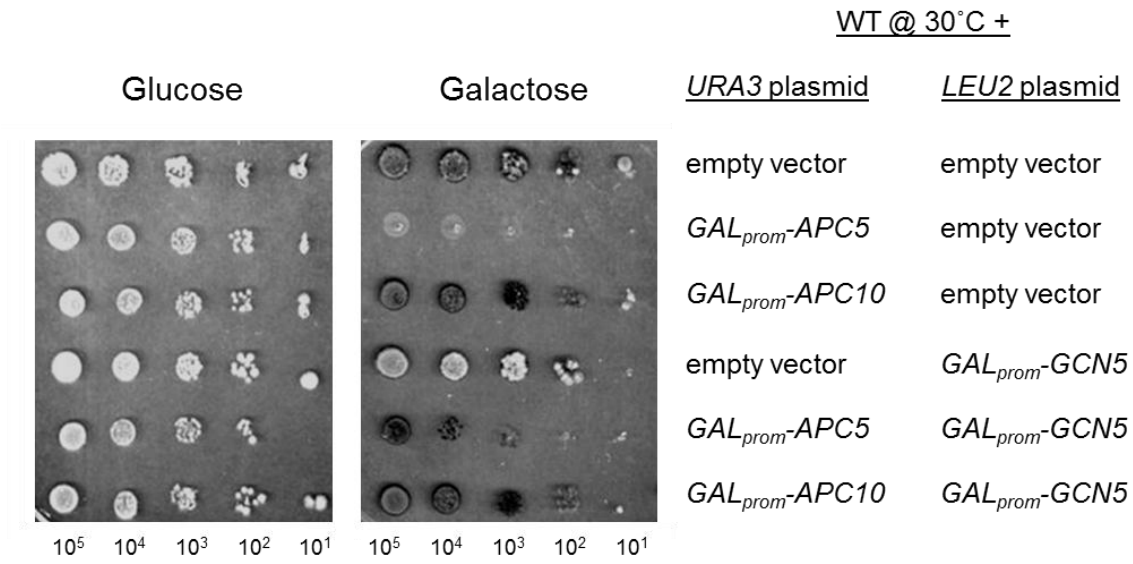

Figure 3.10. **Overexpression of *GCN5* partially suppresses the *APC5* overexpression toxicity**. Wild-type (WT) cells were co-transformed with the plasmids shown and grown at 30°C on 2% glucose- or galactose supplemented plates. Published in Turner *et al*., 2010.

plasmid did indeed rescue the *apc5*^{CA} phenotype to wild-type levels (Figure 3.11). This suggests that Rtt109 may function with the APC by contributing to H3K9 acetylation together with Gcn5. Rtt109 is also known to acetylate H3K56, but acetylation of this residue was not affected in APC mutant cells, thus uncoupling the action of Rtt109 on H3K9 with that of H3K56.

3.3 Discussion

The APC is an important regulator of cell cycle control as it is required for targeting the proteins that inhibit mitotic progression for degradation (Castro *et al*., 2005; Peters, 2006). Our laboratory has also shown it to be likely involved with mitotic chromatin assembly (Harkness *et al*., 2002; Harkness *et al*., 2005). Since chromatin assembly involves the deposition of specifically acetylated histones onto DNA, the role the APC may play in histone acetylation was investigated. Contrary to our expectations, a modification associated with newly deposited histones, H3K56^{Ac} did not appear to be affected by mutation of the APC. Alterations in H3K9^{Ac} and H3K79^{me2} were observed, both of which are associated with gene activation, indicating the APC may play a role in mitotic gene activation (Pokholok *et al*., 2005; Schulze *et al*., 2009). A role for the APC in gene transcriptional activation is supported by the observation that the APC genetically interacts with a variety of histone modification proteins involved in gene transcriptional control. Interestingly, the *apc5*^{CA} temperature sensitive phenotype was exacerbated by deletion of genes involved in transcriptional activation (*ELP3, GCN5*, *SAS3*) but suppressed by deletion of genes involved in silencing (*HOS1*, *HOS2*, *HOS3* and *SAS2*). Both Elp3 and Sas3 target promoters that are also targeted by Gcn5, and Gcn5 has also been shown to predominantly target genes transcribed during late mitosis (Krebs *et al*., 2000; Rosaleny *et al*.,2007). Interactions, both physical and functional, have been reported for the APC and transcriptional regulators, such as CBP/p300 and Rb (Turnell

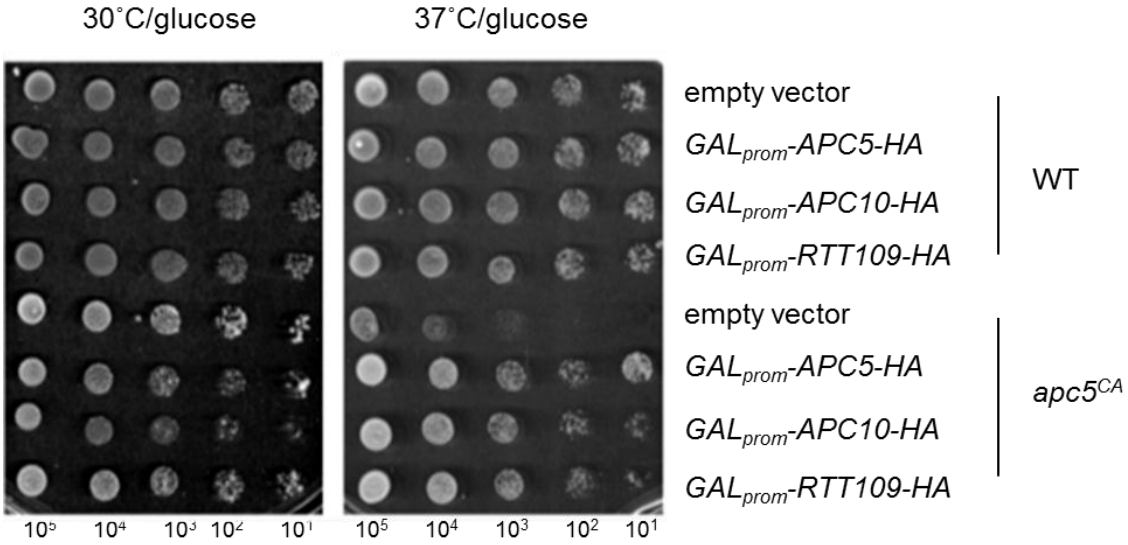

Figure 3.11. **Low-level expression of the HAT gene *RTT109* suppresses the *apc5*^{CA} temperature sensitive defect**. *RTT109* under the control of the *GAL1* promoter was expressed in wild-type (WT) and *apc5*^{CA} cells. The transformants were spot diluted onto glucose-supplemented plates and grown at 30°C or 37°C. Published in Turner *et al*., 2010.

et al., 2005; Binné *et al*., 2007), indicating that the molecular networks identified in this thesis are evolutionarily conserved.

The HDACs Hos1, Hos2 and Hos3 are each targeted to ribosomal genes and deletion of these may result in an increase in overall mRNA transcription. A recent genetic screen identified a number of genes involved in mRNA regulation as interacting with the *apc5*^{CA} allele (Costanzo *et al*., 2010). See Table 3.1 for genes involved in histone, mRNA and ribosome metabolism that genetically interact with the *apc5*^{CA} allele. For example, deletion of genes involved in mRNA decay or degradation (*NAM7*, *UPF3*, *EBS1*, *NMD2*, *AIR2* and *LSM1*) all suppressed the *apc5*^{CA} temperature sensitive defect. Taken together, this indicates that an increase in mRNA abundance is of benefit to cells with a compromised APC. The literature supports this as human Apc5 was found to bind to the ribosome and inhibit the translation of specific proteins involved in apoptosis and uncontrolled cell growth (Koloteva-Levine *et al*., 2004). These proteins include growth factors, oncogenes and apoptotic activators (Hellen & Sarnow, 2001). It is possible that an increase in overall mRNA translation within the cell may result in a more balanced transcriptome profile and that the increased expression of proteins that counteract this uncontrolled growth can rescue the *apc5*^{CA} temperature sensitive phenotype. The involvement of Apc5 with the ribosome and translational inhibition of proteins involved in uncontrolled cell growth may slow cell growth enough to allow the DNA damage and mitotic-spindle checkpoints time to ensure that the chromatin is ready to go through with sister chromatid separation. The APC is usually prevented from targeting proteins for degradation until the cell is ready to go through mitosis. Nevertheless, if enough time passes, the cell may still proceed with sister chromatid separation (Zou *et al*., 1999; Meraldi *et al*., 2002; Hernando *et al*., 2004). Premature progression of separation

Table 3.1 SGA analysis of the *apc5*^{CA} allele

histones	score	P-value	**histones**	score	P-value	**mRNA decay**	score	P-value
SAGA			**transcription factors**			*LSM1*	0.2	1.00E-03
AHC2	-0.2	1.30E-03	*SWI5*	-0.49	5.20E-15	*NAM7*	0.2	2.60E-54
CHD1	-0.17	1.20E-03	SAC3	-0.42	3.30E-42	*AIR2*	0.2	5.40E-03
SPT3	0.2	2.00E-09	*RPN4*	-0.33	1.20E-10	*UPF3*	0.2	8.50E-19
ISW1			*RTT103*	-0.29	5.40E-25	*EBS1*	0.2	7.80E-04
ISW1	-0.22	6.20E-99	*UME6*	-0.28	4.50E-13	*NMD2*	0.2	3.20E-03
IOC2	-0.19	4.00E-32	*HAP4*	-0.11	1.20E-02			
IOC4	-0.18	2.10E-04	*TEC1*	-0.1	4.00E-02			
ISW2			*IMP2*	0.1	2.50E-04	**ribosomes**	score	P-value
ITC1	0.2	2.40E-36	*BAS1*	0.1	3.90E-12			
RPD3L			**chromatin assembly**			**40S**		
SIN3	-0.18	4.90E-04	*CAC2*	0.1	1.10E-03	*RPS7B*	-0.19	5.70E-04
SAP30	-0.12	5.30E-03	*HIR3*	0.1	6.10E-03	*RPS24A*	-0.18	1.50E-04
RPD3S			*HPC2*	0.1	5.70E-04	*RPS27A*	-0.12	1.70E-02
RCO1	-0.13	1.80E-16	*CAC1*	0.1	1.60E-07	*RPS21B*	-0.11	5.20E-10
Elongator						*ASC1*	0.1	2.10E-02
IKI3	0.1	4.20E-02				*RPS18B*	0.1	2.50E-06
ELP2	0.1	2.10E-02				**60S**		
NuA3						*RPL40B*	-0.15	2.10E-13
NTO1	-0.12	4.60E-05				*RPL22A*	-0.1	4.80E-02
NuA4						*RPL38*	-0.09	2.00E-02
EAF3	-0.22	4.20E-04				*RPL43A*	0.1	4.40E-02
SWR1						*RPL11B*	0.1	1.40E-02
VPS72	-0.17	4.50E-05				*RPL14A*	0.1	1.90E-03
SWC3	-0.15	4.40E-03				**stalk**		
SWC7	-0.11	6.60E-03				*RPP1B*	-0.14	1.20E-05
SWR1	-0.08	3.40E-02				**ribo biogenesis**		
SWI/SNF						*TMA64*	-0.6	1.00E-51
SNF5	0.1	6.80E-03				*PML39*	-0.16	1.20E-09
COMPASS						*NOP6*	-0.11	4.40E-04
SDC1	0.1	2.40E-03				*MRN1*	-0.1	1.30E-03
CCR4-NOT						*ESL2*	0.1	6.30E-11
CAF130	-0.23	1.80E-04				*ARX1*	0.1	4.60E-05
Histones						**rDNA interactions**		
HHT2	0.1	3.40E-03				*YBR246w*	-0.13	8.00E-06
HTA1	0.1	2.50E-02				*YCL060c*	-0.12	1.30E-05
histone methylation						*FOB1*	0.1	1.30E-03
SET2	-0.18	1.90E-35				*PAT1*	0.2	2.10E-04

Red scores indicate synergistic or complimentary roles

Green scores indicate suppressive or antagonistic roles

frequently results in mitotic catastrophe so regulating this process is extremely important to cell health.

A striking example of how the end readout of histone acetylation is important, rather than the enzyme itself, was provided by the deletion of the HDAC, *HDA1*, and the HAT, *GCN5*, which each individually exacerbated the *apc5*^{CA} temperature sensitive defect. According to the hypothesis presented above that increased mRNA abundance improves APC phenotypes, Hda1 must be involved in activation of some set of genes involved in APC function. Investigation of this point by our laboratory showed that it may be the repression of genes encoding APC inhibitors, such as *PDS1* and *BCY1*, which is important (Islam *et al*., 2011). A most interesting interaction was observed when both *HDA1* and *GCN5* were deleted in *apc5*^{CA} cells. Rather than being further impaired, perhaps even lethal, this combination of mutations grew much better than either double mutant. Of note, the two single mutants, *gcn5*Δ and *hda1*Δ, and the double mutant *gcn5*Δ *hda1*Δ showed no temperature sensitive phenotype. It was recently reported by two groups that deletion of *GCN5* could suppress phenotypes associated with *hda1*Δ mutations (Lin *et al*., 2008; Costanzo *et al*., 2010). These observations were made while performing genome-wide Synthetic Genetic Array (SGA) screens, which utilize plates lacking multiple amino acids and supplemented with antibiotics for selection purposes. Interestingly, these interactions could not be replicated when the various mutations were grown on rich YPD media, as done in the experiments presented in this thesis. Thus, under suboptimal growth conditions, such as those when selecting for interactions in the SGA screen, *gcn5*Δ phenotypes can be suppressed by *HDA1* deletions. This suggests that Gcn5 and Hda1 antagonize one other and that wild-type cells under normal growth conditions are able to cope with this unbalanced activity, whereas *apc5*^{CA} cells cannot.

Gcn5 also genetically interacted with Hpa2, another HAT capable of acetylating H3K14. The exact role of Hpa2 within the cell is not yet known, but it has been shown to target a small proportion of genes for transcriptional activation (Rosaleny *et al*., 2007). Gcn5 and Hpa2 interact antagonistically in a wild-type background as deletion of *HPA2* partially rescued the temperature sensitive *gcn5*Δ phenotype (Figure 3.3B). Interestingly, deletion of both HATs in the *apc5*^{CA} background grew worse than the individual single and double mutants. This suggests that Gcn5 and Hpa2 may have redundant functions that are necessary in *apc5*^{CA} but dispensable in wild-type cells. The same mechanism envisioned for Gcn5/Hda1 interactions may also be working for Gcn5/Hpa2; it is possible that genes required for APC function are disproportionately influenced in *gcn5*Δ and/or *hpa2*Δ cells, and this becomes a liability when APC activity is compromised.

Elp3 was also identified as a protein that functionally interacts with the APC and Gcn5. Combining the *apc5*^{CA} allele with *elp3*Δ or *gcn5*Δ resulted in further temperature sensitivity. Elp3 and Gcn5 have been shown to have overlapping functions in gene activation as the HAT components of Elongator and SAGA, respectively. Gcn5 is required for SAGA-mediated transcriptional activation, while Elongator is required for transcriptional elongation following activation. *ELP3* and *GCN5* have previously been shown to interact as the double mutant results in a more severe phenotype than either single mutation alone (Wittschieben *et al*., 2000). Both HATs target H3K14 and deletion results in accumulation of cells with unsegregated DNA at elevated temperatures. The data presented in this thesis agree with the literature. Not only was the *elp3*Δ *gcn5*Δ double mutant more temperature sensitive than either single mutant, but it was also more impaired than either single mutant combined with the *apc5*^{CA} allele (compare Figure 3.4A with 3.2B). To further characterize these genetic interactions triple mutants were constructed. Interestingly, the temperature sensitivity was similar regardless of

whether APC was mutated or not. This finding was initially interpreted as defining an epistatic relationship, where Elp3 and Gcn5 together act upstream of the APC to allow progression through G1/S. Consistent with this hypothesis, moderate overexpression of *APC5* rescued the *elp3*Δ *gcn5*Δ temperature sensitive phenotype (Figure 3.9). Surprisingly, the *elp3*Δ *gcn5*Δ double mutants accumulate in G1 while the single HAT mutants each accumulate in G2, which indicates that Elp3 and Gcn5 have individual functions in G2/M but act redundantly to pass through G1 (Figure 3.5B). The finding that Clb2 levels were drastically reduced from wild-type levels in *elp3*Δ *gcn5*Δ double mutants may be attributed to these cells progressing through G1 at a slow rate, thereby allowing for increased time to degrade Clb2 (Figure 3.6). Nonetheless, these results indicate that Gcn5 and Elp3 are not upstream activators of the APC. The finding that the toxic overexpression of *APC5* can be mediated by overexpression of *GCN5* supports a model where the APC is upstream. Since the APC is predominately involved in targeting proteins for degradation, the toxic effects of *APC5* overexpression may in part be due to insufficient amounts of Gcn5 within the cell; as cells overexpressing Gcn5 accumulate in G1 (Figure 3.10), the APC may be required to reduce Gcn5 levels and allow cell cycle progression.

The APC promotes genomic stability and progression through mitosis and G1. The effects on genomic stability are likely linked to a requirement for a specific transcriptional profile to progress through the cell cycle. This is highlighted by the finding that levels of $H3K9^{Ac}$ and $H3K79^{me2}$, modifications associated with mitotic gene activation, are altered in APC mutants (Figure 3.1A). Results presented here suggest that the APC interacts with histone modification proteins and may play a role in transcriptional control. The APC is also genetically involved with Rtt109, Asf1 and CAF-I, suggesting a role in histone deposition (Figure 3.11; Harkness *et al*., 2005). Taken together, these data indicate that

the APC links histone modification, and possibly mitotic gene transcription, with genomic stability. Specifically, this chapter demonstrates that the APC and the HATs, Elp3 and Gcn5, work together to allow cells to progress effectively through mitosis.

CHAPTER FOUR

THE APC IS REQUIRED FOR GCN5 PROTEIN INSTABILITY [2]

4.1. Introduction

The APC is required for the turnover of proteins that perform cell cycle specific functions during mitosis and G1. By targeting such proteins for degradation, cell cycle progression is ensured because the blocks imposed by these proteins are now removed. Our laboratory has previously demonstrated that the APC plays a role in mitotic chromatin assembly while the work presented in this thesis suggests that it also plays a role in histone metabolism in actively growing cells (Figure 3.1A; Harkness *et al*., 2002). As described in the previous chapter, *elp3*Δ and *gcn5*Δ genetically interact with the APC and work in the same pathway to control mitotic events. In an attempt to determine whether an ordered pathway involving APC/Elp3/Gcn5 exists, reciprocal experiments were performed where *ELP3* or *GCN5* were overexpressed in *apc5*^{CA} cells, or *APC5* was overexpressed in *elp3*Δ *gcn5*Δ cells (Figures 3.7A and 3.9). Overexpression of a downstream factor may be able to rescue the temperature sensitive phenotypes expressed by upstream components. Surprisingly, overexpression in both experiments rescued the temperature sensitivity phenotypes. Further experiments using the APC target Clb2 demonstrated that Clb2 remained unstable in *elp3*Δ *gcn5*Δ cells, suggesting that Elp3 and Gcn5 do not necessarily control APC activity (Figure 3.6). Thus, a straight forward interpretation of the overexpression suppression data is not possible. Nonetheless, the observation that *elp3*Δ and *gcn5*Δ accumulate with G2/M cells

[2] A portion of this chapter has been published in Turner *et al.,* 2010*, Eukaryotic Cell 9,* 1418-1431.

indicates that Elp3/Gcn5 and the APC may at least work redundantly to ensure mitotic progression (3.5B).

Gcn5 has been shown to acetylate the promoters of genes required for mitotic progression and entrance into G1 (Krebs *et al*., 2000). Cells lacking both Elp3 and Gcn5 are temperature sensitive and exhibit slow growth (Figure 3.4B; Wittschieben *et al*., 2000). The data presented in Chapter Three demonstrates that cells lacking *ELP3* or *GCN5* have difficulty in progressing through mitosis into G1 (Figure 3.5B). The activities of Elp3 and Gcn5 that are required for mitotic progression appear to be inhibitory to the passage through G1 into S-phase. Given that overexpression of either HAT results in cells accumulating in G1 (Figure 3.7A), it may be that the transcriptional profile needed to pass through mitosis is not able to be reset to allow the G1/S transition. For example, in human cells, the phosphorylation of Cdc6 and its subsequent nuclear export and degradation is dependent on the HAT activity of Gcn5 as increased expression of GCN5 resulted in elevated Cdc6 phosphorylation and relocalization to the cytoplasm (Paolinelli *et al*., 2009). This accelerated export of Cdc6 likely results in DNA pre-replication complexes not being formed properly, if at all, resulting in a lack of DNA replication. Additionally, the toxic effects of *APC5* overexpression are mitigated by *GCN5* overexpression, suggesting that the growth defect may be due to inadequate amounts of Gcn5 within the cell (Figure 3.10). Thus, the hypothesis is that Gcn5 (and possibly Elp3) must be degraded once the mitotic and G1 specific transcript profile is established in order for progression from G1 into S-phase to occur.

4.2. Results

4.2.1 Elp3 and Gcn5 may be targeted for degradation in a proteasome- and ubiquitin-dependent manner

The APC plays a major role in genomic stability as it regulates progression through mitosis and G1. This is accomplished by targeting the proteins that prevent sister chromatid separation and exit from mitosis for destruction. Several yeast proteins involved in controlling APC activity are themselves targeted for destruction by the APC, such as Cdc5 (Plk1 in humans), Clb2 and Cdc20 (Castro, *et al*. 2005; Wäsch *et al*., 2010). Since Elp3 and Gcn5 appear required for mitotic progression, it may be that their function must be stopped to allow progression further through the cell cycle. This is consistent with the finding that increased expression of *ELP3* or *GCN5* causes cells to accumulate in G1 (Figure 3.7C). To determine whether Elp3 and Gcn5 may be APC targets, the stability of Elp3 and Gcn5 was examined. This analysis began by using a proteasome mutant, *rpn10*Δ. Ubiquitinated proteins degraded by the proteasome are first unfolded and then threaded through the centre of the structure to systematically remove groups of amino acids (Sorokin *et al*., 2009). The proteasome subunit Rpn10 (regulatory particle non-ATPase) is a multi-ubiquitin chain receptor and mutation to this gene results in the stabilization of some, but not all, proteasomal targets (Sorokin *et al*., 2009). Consistent with the idea that Elp3 and Gcn5 must be reduced to allow cell cycle progression, it was observed that *rpn10*Δ cells exhibit increased levels of both larger and smaller species of Elp3 and Gcn5 than does the wild-type (Figure 4.1A). As the proteasome is involved in degrading ubiquitinated proteins, the larger species are likely ubiquitinated while the smaller species may be partially processed species. As controls,

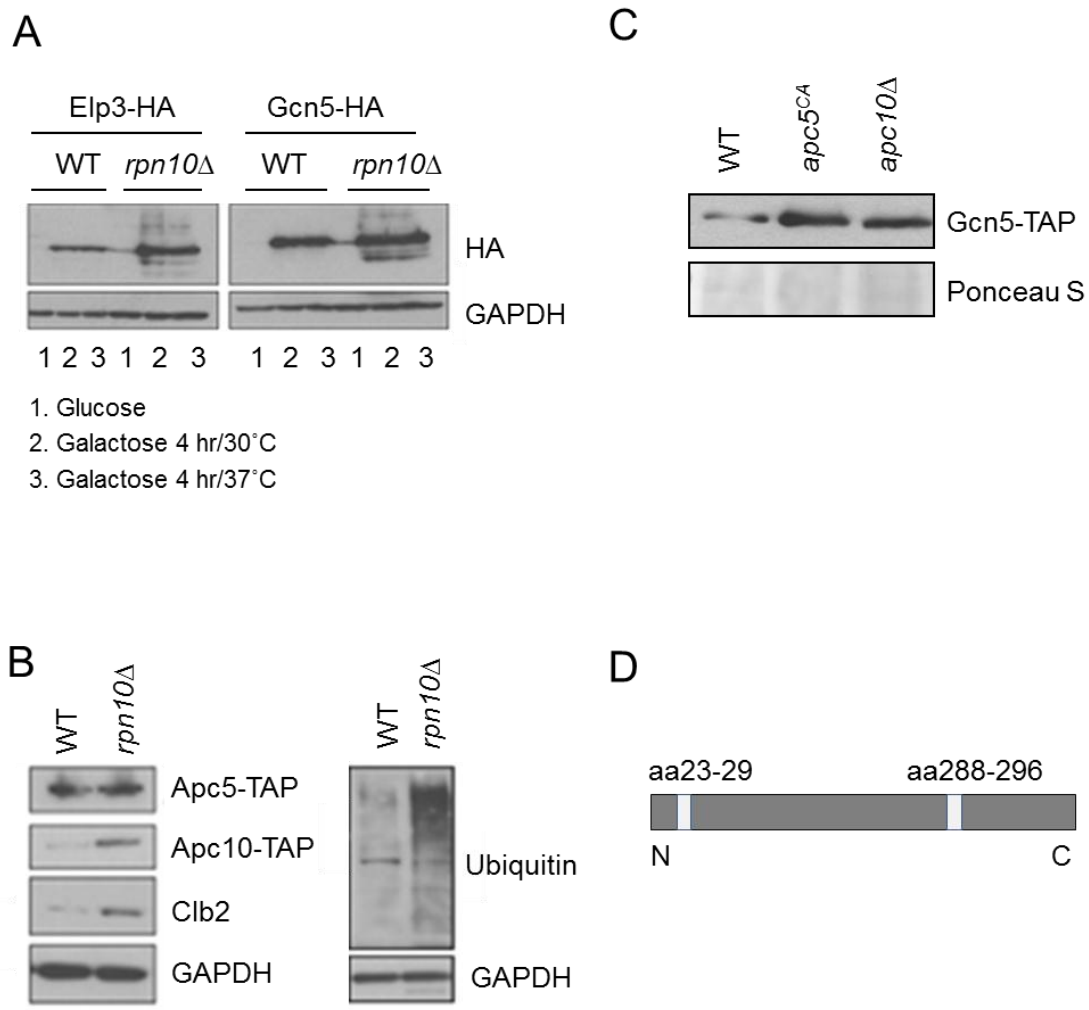

Figure 4.1**. Elp3 and Gcn5 may be targeted for degradation in a proteasome- and ubiquitin-dependent manner. (A)** Wild-type (WT) and *rpn10*Δ cells expressing *GAL*$_{prom}$*ELP3-HA* or *GAL*$_{prom}$*GCN5-HA* were grown to early log phase, then split. One half was harvested immediately, while the other half was resuspended in 2% galactose-supplemented media. This sample was split again and incubated at 30° and 37°C for 4 hours. Proteins were analyzed with antibodies against HA and GAPDH. Figure provided by J. Menzel. **(B)** Wild-type and *rpn10*Δ cells were harvested at early log phase. Proteins were analyzed with the indicated antibodies. Figure provided by M. Malo. **(C)** Asynchronous steady-state levels of endogenous *GCN5-TAP* were followed in the cells shown. Antibodies against TAP were used with a Ponceau S stained membrane used as a load control. Images shown are representative of routinely observed results. Published in Islam *et al*., 2011. (**D**) Locations of putative D-boxes in the amino acid sequence of Gcn5 are indicated by the white boxes.

the levels of ubiquitin and the APC target Clb2 were analyzed (Figure 4.1B). These were also increased in the *rpn10*Δ mutant, as was Apc10. Increased Apc10 may be the cell's response to the accumulation of proteasomal targets. Apc5 levels were similar in wild-type and *rpn10*Δ cells. The levels of Apc5 in the *rpn10*Δ mutant may already be at a high enough level to allow formation of additional APC complexes without further expression. Gcn5-TAP levels were also increased in APC mutants relative to wild-type (Figure 4.1C). Analysis of the amino acid sequence of the S. cerevisiae Gcn5 revealed two putative D-boxes, located at amino acid residues 23-29 and 288-296 suggesting that Gcn5 may be a target of the APC (Figure 4.1D). Thus, data presented in this thesis supports the idea that Gcn5 and/or Elp3 are targeted for degradation, perhaps to allow cell cycle progression through G1/S.

4.2.2 SCF mutants do not exhibit changes in steady state levels of Gcn5-TAP

The APC has been documented to be active during mitosis and G1 but not S-phase and the APC is required for degradation of proteins that block S-phase initiation. The other major ubiquitin-ligase active throughout the cell cycle is the SCF (Willems *et al*., 2004). For example, Sic1, a Cdk inhibitor, must be degraded by the SCF to allow the G1/S transition (Schwob *et al*., 1994). To determine whether the SCF is involved in Gcn5 turnover, levels of Gcn5-TAP (tandem affinity purification) in asynchronously growing SCF mutants were compared to wild-type and *apc5*^{CA} cells. No differences in Gcn5-TAP levels were observed in SCF mutants (Figure 4.2), suggesting that Gcn5-TAP instability may be linked to a particular cell cycle phase and to the activity of the APC.

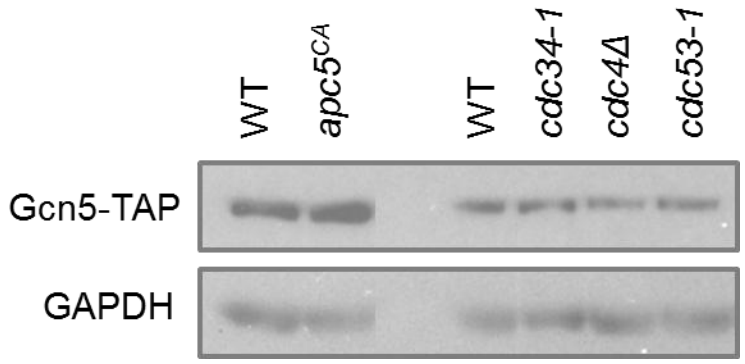

Figure 4.2. **SCF mutants do not exhibit changes in steady state levels of Gcn5-TAP**. Log phase *GCN5-TAP* expressing cells were grown overnight to log phase, then set back into fresh glucose containing media for 4 hours at 30˚C. Samples were taken immediately following the 4 hour incubations. Protein extracts were analyzed using antibodies against TAP and GAPDH. Images shown are representative of routinely observed results. WT = wild-type.

4.2.3 Gcn5-HA and Gcn5-TAP are unstable during G1 and S, but are stabilized in *apc5*^{CA}, *apc10*Δ and *apc11*Δ cells

Gcn5 may be directed towards ubiquitin- and proteasome-dependent degradation. Targeted degradation of Gcn5 by the APC may allow passage through the G1/S-phase transition. To test this hypothesis Gcn5 stability was examined in wild-type, *apc5*^{CA} and *apc10*Δ strains containing endogenously TAP-tagged Gcn5. Cells were grown overnight to log phase, set back to OD_{600} 0.5, alpha factor was added to arrest cells in G1, and followed with incubation at 30˚C for 3 hours when full arrest was achieved. Cells were then washed and resuspended in fresh media containing cycloheximide to prevent further protein synthesis. Samples were taken at the indicated time points and protein extracts were prepared. Gcn5-TAP was rapidly degraded in wild-type cells but was stable in both *apc5*^{CA} and *apc10*Δ cells (Figure 4.3A). This strongly suggests that the APC is required for the degradation of Gcn5. Similar results occurred when wild-type, *apc5*^{CA}, *cdc16-1* and *apc11*Δ cells containing galactose-induced Gcn5-HA were arrested in S-phase and released into glucose to allow cell cycle progression (Figures 4.3B and 4.3C). After 3 hours of galactose induction in the absence of cell cycle arrest, these cells were still cycling asynchronously. In these asynchronous cells Gcn5-HA levels were stable regardless of APC being mutated or not, suggesting that the Gcn5 instability observed in wild-type cells arrested with alpha factor or hydroxyurea is cell cycle specific to passage through G1/S (Figure 4.4).

4.2.4 Modified levels of Gcn5-TAP decrease during S-phase in *apc10*Δ and during G1 and S-phase in *cdc16-1* cells

The data suggests that Gcn5 is targeted for degradation in a cell cycle specific manner. To follow this hypothesis further, wild-type, *apc5*^{CA}, *apc10*Δ and *cdc16-1* were arrested

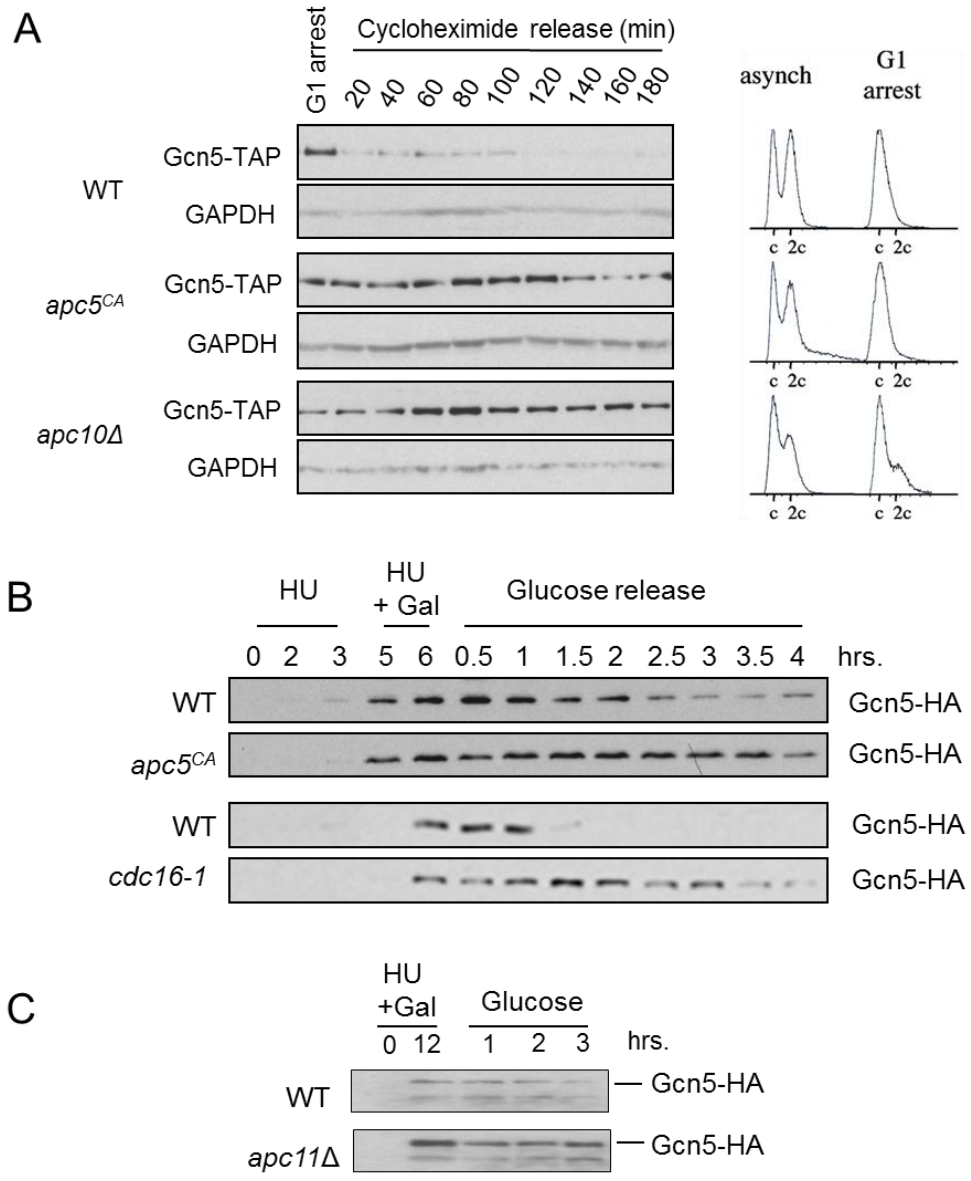

Figure 4.3. **Gcn5 is unstable during G1 and S phase but stabilized in APC mutants**. **(A)** Wild-type (WT), *apc5*^{CA}, and *apc10*Δ cells expressing endogenous *GCN5-TAP* were arrested in G1 using alpha-factor. Following arrest, cells were washed and added to fresh media containing cycloheximide to block all further protein synthesis. Samples were taken as indicated to assess Gcn5-TAP protein stability. GAPDH Western analyses were conducted to control for protein load. Cell cycle progression was followed using Flow cytometry. Figure from Turner *et al*. 2010. **(B)** Early log phase WT and *cdc16-1* cells transformed with *GAL*$_{prom}$*-GCN5-HA* were arrested in S-phase using 0.3 M hydroxyurea. 2% galactose-supplemented media was added to induce protein induction. Following arrest, cells were washed and added to fresh media containing glucose and allowed to grow. Samples were taken at the indicated times to assess Gcn5-HA protein stability. **(C)** WT and *apc11*Δ cells were transformed with *GAL*$_{prom}$*-GCN5-HA* and treated as in (A).

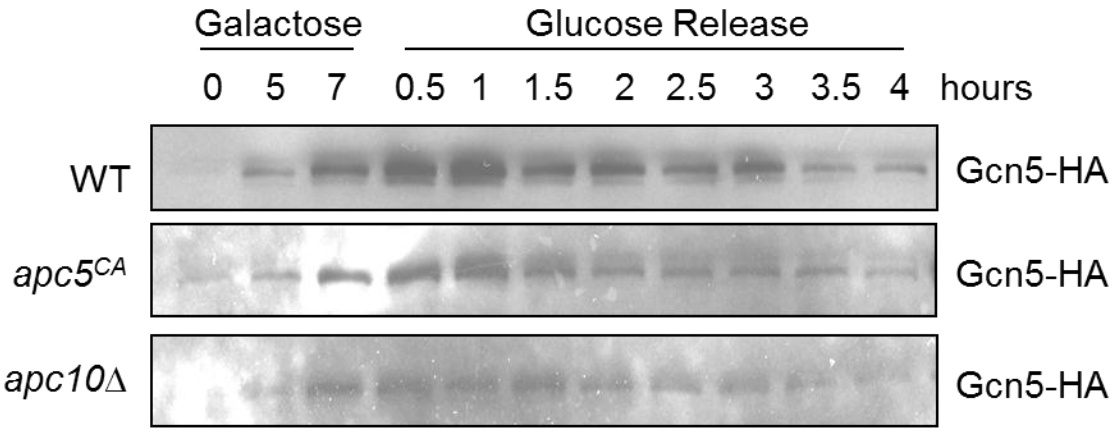

Figure 4.4. **Gcn5 is stable in asynchronous cells.** Strains transformed with *GAL$_{prom}$-GCN5-HA* were grown to log phase, then set back to OD 0.5. Proteins were induced for 4 hours in 2% galactose-supplemented media. Following induction, cells were washed and added to fresh media containing glucose and allowed to grow. Samples were taken at the indicated times to assess Gcn5-HA protein stability. Representative image of routinely observed results is shown. WT = wild-type.

in several different cell cycle phases at the permissive temperature of 30˚C (Figure 4.5). Compared to cells arrested in M phase, decreased levels of Gcn5-TAP were observed in both the *apc10*Δ and *cdc16-1* strains when cells were arrested in G1 or S-phase. An accumulation of higher molecular weight species was observed in *apc10*Δ and *cdc16-1*, suggestive of higher levels of modified Gcn5-TAP, regardless of cell cycle stage. This may be explained by the fact that Apc10 has been shown to be involved in protein substrate recognition (Grossberger *et al*., 1999; Buschhorn *et al*., 2011; da Fonseca *et al*., 2011), while Apc5 has been shown to interact with other factors such as the mitotic checkpoint complex, the ribosome and the transcription factors CBP and p300 (Koloteva-Levine *et al*., 2004; Turnell *et al*., 2005; Herzog *et al*., 2009).

4.3 Elp3-TAP modification levels are altered by mutation to the ubiquitin machinery

4.3.1 Elp3-TAP is unstable in G1

As Elp3 appears to interact with the APC in a manner similar to Gcn5, Elp3 may also be targeted for APC and proteasomal-dependent degradation. Targeted degradation of Gcn5 by the APC may allow passage through the G1/S-phase transition. To test this hypothesis the stability of Elp3-TAP was examined in wild-type cells. Cells were grown overnight to log phase and set back to an OD_{600} of 0.5. Alpha factor was then added for 3 hours to arrest cells in G1. Cells were then washed and resuspended in fresh media containing cycloheximide to prevent further protein synthesis. Samples were taken at the indicated time points and protein extracts prepared. Elp3-TAP was observed to be degraded in wild-type (Figure 4.6A). As Elp3 genetically interacts with the APC in a similar manner as does Gcn5 it may also be targeted for ubiquitination in an APC-dependent manner.

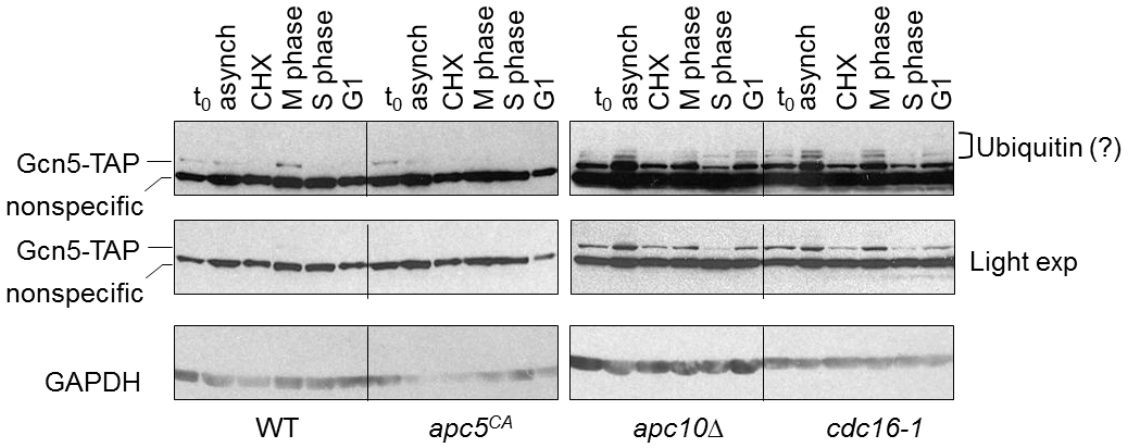

Figure 4.5. **Modified Gcn5-TAP levels are increased in APC mutants**. Log phase *GCN5-TAP* expressing cells were diluted and arrested in M using nocodazole, S using hydroxyurea or G1 using alpha factor. Controls included 4 hours in the presence and absence (asynch) of cycloheximide (CHX). Samples were taken immediately following the 4 hour incubations. Protein extracts were analyzed using antibodies against TAP and GAPDH. A lighter exposure of the membranes to X-ray film is also shown (Light exp). In this experiment, the TAP antibody recognized a nonspecific band that was also observed in untagged wild-type (WT), *apc5*^{CA}, ***elp3***Δ, and ***gcn5***Δ cells (data not shown). Images shown are representative of routinely observed results.

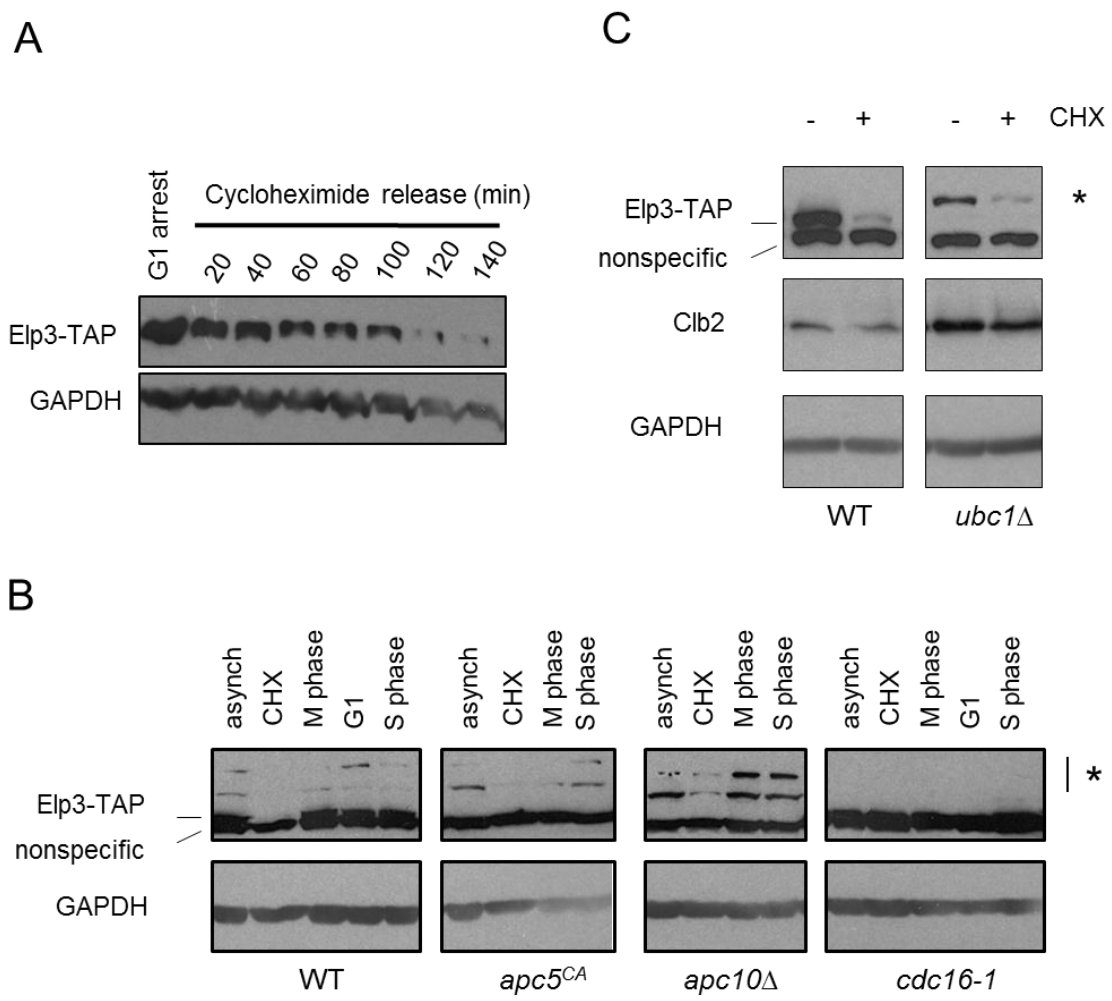

Figure 4.6**. Elp3 instability depends on the APC and Ubc1. (A) Elp3 is unstable during G1.** The cells shown expressed endogenous *ELP3-TAP* and were of the **a** Mating type. The cells were grown to early log phase, then arrested in G1 with alpha factor. Cycloheximide (CHX) was added to stop all protein synthesis and samples were taken every 20 minutes for 3 hours for protein analysis with antibodies against TAP and GAPDH as a load control. **(B) Elp3 instability may be cell cycle independent, but depends on the APC.** Log phase *ELP3-TAP* expressing cells were diluted and arrested in M using nocodazole, S using hydroxyurea or G1 using alpha factor. Controls included 4 hours in the presence (CHX) and absence (asynch) of CHX. Samples were taken immediately following the 4 hour incubations. Protein extracts were analyzed using antibodies against TAP and GAPDH. In this experiment, the TAP antibody recognized a nonspecific band that was also observed in untagged wild-type (WT), *apc5*^{CA}, *elp3*Δ, and *gcn5*Δ cells (data not shown). * denotes potentially modified Elp3-TAP protein. **(C) Elp3 instability may depend on Ubc1.** WT and *ubc1*Δ *ELP3-TAP* cells were incubated for 3 hours in the presence and absence of CHX at 30°C. Samples were taken immediately following the treatment. The nonspecific band recognized by the TAP antibody is shown.

4.3.2 Elp3-TAP is modified in *apc5*^{CA} and *apc10*Δ cells but not in *cdc16-1* cells

Elp3-TAP containing strains were arrested in the various cell cycle phases in order to detect any cell cycle specific molecular weight changes by SDS PAGE (Figure 4.6B). Surprisingly, Elp3-TAP stability and molecular weight changes were not cell cycle-dependent, but APC subunit-dependent. In wild-type cells, Elp3-TAP was not observed at all following the cycloheximide (CHX) treatment of asynchronous cells. Furthermore, wild-type cells exhibited several higher molecular weight bands, suggesting that Elp3-TAP exists in both non-modified and modified forms, while in *cdc16-1* cells, only the non-modified form was observed. Interestingly, only the modified forms of Elp3-TAP were observed in *apc5*^{CA} and *apc10*Δ cells. This perhaps suggests diverse functions for the different APC subunits. Additional study will be required to fully understand the specific functional activities of the different APC subunits.

4.3.3 *ubc1*Δ cells exhibit modified Elp3-TAP while wild-type cells do not

The results obtained from the above mentioned cell cycle arrest experiments using Elp3-TAP strains suggest that different APC subunits may perform alternative tasks when modifying substrates. The APC has been shown to interact with the ubiquitin-conjugating enzymes (E2s) Ubc1 and Ubc4 in a step-wise fashion to fully ubiquitinate substrate proteins (Rodrigo-Brenni & Morgan, 2007). First, the substrate is mono-ubiquitinated by Ubc4, and then becomes a target for Ubc1 dependent poly-ubiquitination. Wild-type and *ubc1*Δ cells containing Elp3-TAP were arrested with cycloheximide for 3 hours to prevent further protein synthesis in order to follow protein turnover (Figure 4.6C). It was found that the *ubc1*Δ cells harbored only higher molecular weight Elp3-TAP species, while wild-type only exhibited endogenous Elp3-TAP. The inconsistency in detection of modified Elp3-TAP in wild-type cells may be due to the inherent instability of

ubiquitinated proteins or as a result of extra time in cycloheximide (compare Figures 4.6B with 4.6C). Considering Ubc1 is responsible for poly-ubiquitinating APC substrates the *ubc1*Δ deletion strain is likely accumulating only mono-ubiquitinated substrates.

4.4 Discussion

The work presented thus far has demonstrated that the APC genetically interacts redundantly with Elp3 and Gcn5 to promote mitotic passage, and possibly upstream during G1 to target at least Gcn5 for ubiquitin- and proteasome-dependent degradation. An excellent example of coordinated cell cycle control is that several upstream regulators of the APC are themselves targeted by the APC for destruction (Castro *et al*., 2005; Wäsch *et al*., 2010). Some examples are Cdc5, Clb2 and Cdc20, and we propose that Gcn5, and perhaps Elp3, may also fall into this category. We do not believe that Elp3 and Gcn5 directly activate the APC, but rather propose that a mitotic transcriptional profile established by Elp3- and Gcn5-dependent promoter acetylation creates transcripts and proteins necessary to enable APC mitotic activity. Alternatively, and not necessarily mutually exclusive, Gcn5 and/or Elp3 may acetylate histones prior to deposition into chromatin during mitosis. This in itself may be what is important for APC function considering its role in mitotic chromatin assembly. Increased levels of Elp3 or Gcn5 result in cells having increased difficulty passing through G1/S. Whether this is due to increased transcription of proteins blocking cell cycle progression, or some other phenomenon, remains to be determined. Indeed, increased histone acetylation activities of Elp3 and Gcn5 may prevent progression through the cell cycle as hyperacetylation in human cells has been shown to prevent sister chromatid separation and result in segregation defects (Cimini *et al*., 2003). On the other hand, the cell cycle exit/arrest may be due to additional activities. For example, in human cells it has recently been

shown that Gcn5 acetylates Cdc6, a component of the pre-replicative complex (Paolinelli *et al*., 2009). Acetylation of Cdc6 leads to its release from chromatin and subsequent degradation. Excess levels of Gcn5 may result in the inactivation of Cdc6 before the pre-replicative complexes are properly formed, thus preventing the cell from proceeding with DNA replication. Additionally Gcn5 has been implicated with increasing CAF-I's association with the histone H3/H4 tetramer by acetylating the N-terminal tail of H3 (Burgess *et al*., 2010). Excess CAF-I has been demonstrated to assemble bulk DNA to the point of inhibiting DNA synthesis (Kamakaka *et al*., 1996), so increased histone affinity catalyzed by excess Gcn5 may contribute to this process. Gcn5 levels may need to be kept at appropriate levels to allow DNA replication and cell cycle progression to occur in a timely manner.

The finding that both Elp3 and Gcn5 are required for efficient progression through mitosis while only one is required for G1/S-phase progression raises the question of what exact role they play. Presumably, they have redundant roles pertaining to progression through G1/S-phase as both genes must be mutated for accumulation to occur (Figure 3.5B). Their redundant functions may in fact be associated with the APC as there was no difference in temperature sensitivity between wild-type or *apc5*^{CA} cells once both HATs were deleted (Figure 3.4B). Single HAT deletions did exacerbate the *apc5*^{CA} suggesting that the presence of Elp3 was unable to compensate for the loss of Gcn5, and vice versa (Figure 3.2B). Exactly how the APC associates with Elp3 and Gcn5 still remains to be determined. The data presented here indicate that the APC targets Gcn5 (and possibly Elp3) for degradation to allow progression into S-phase. The finding that deletion of *RPN10*, a component of the proteasome, results in alteration of the modification patterns of Elp3 and Gcn5 further supports this hypothesis (Figure 4.1A), as does the effect of deleting components of the APC or Ubc1, on Elp3

modification patterns (Figure 4.6). Gcn5 does contain two putative D boxes, suggesting that it may be a target of the APC. The APC is involved in mitotic chromatin assembly and histone metabolism (Figure 3.1; Harkness *et al*., 2002; Ramaswamy *et al,* 2003). Since HATs play a role in both processes, it stands to reason that they would also interact with the APC. While the hypothesis is that the APC targets for degradation some of the proteins involved in these processes, it may also act to bring them all together to promote chromatin assembly. The results presented in Chapter Five further link the APC with CAFs and histone metabolism. Chapter Five introduces the idea that the APC may affect mitotic chromatin assembly by acting as a scaffold or facilitator to bring together the proteins required for histone deposition during mitosis.

CHAPTER FIVE

THE APC INTERACTS WITH PROTEINS INVOLVED IN CHROMATIN MODIFICATION [3]

5.1. Introduction

As discussed in Chapter One, the APC is required for mitotic progression and genomic stability (Castro *et al*., 2005; Wäsch *et al*., 2010; Kim & Yu, 2011; McLean *et al*., 2011). The APC has also been shown to play a role in chromatin assembly during mitosis (Harkness *et al*., 2002; Arnason *et al*., 2005). To date, the only chromatin assembly factor shown to be associated with a specific stage of the cell cycle is CAF-I; it is required for replication-dependent chromatin assembly (Smith & Stillman, 1989). CAF-I has also been shown to be involved in DNA repair throughout the cell cycle (Gaillard *et al*., 1996). Previous work from our laboratory has genetically linked the APC with the individual subunits of CAF-I (Harkness *et al*., 2005). This was demonstrated by the sequential deletion of CAF-I subunits resulting in progressively increased temperature sensitivity in the *apc5*^{CA} mutant. Additionally, the overexpression of individual CAF-I subunits was able to rescue the *apc5*^{CA} temperature sensitive growth even in cells lacking intact CAF-I. Apc5 was also shown to interact with Asf1 as *apc5*^{CA} *asf1*Δ cells grew slower than either single mutant at both permissive and restrictive temperatures, 30˚C and 37˚C, respectively. In addition, overexpression of Asf1 was able to rescue *apc5*^{CA} temperature sensitive growth. Together, this suggests that the chromatin assembly defect associated with *apc5*^{CA} may impinge on Asf1/CAF-I-dependent histone deposition.

[3] A portion of this chapter has been published in Turner *et al*., 2010, *Eukaryotic Cell 9,* 1418-1431.

Prior to deposition onto DNA, histones must be properly acetylated. Asf1 aids in this by binding histones H3/H4 and presenting them to Gcn5 and Rtt109 for acetylation (Fillingham *et al*., 2008; Burgess *et al*., 2010). Asf1 then passes the acetylated histones to CAF-I for deposition (Tyler *et al*., 2001). A defect in the processes of histone modification and deposition in *apc5*^{CA} cells may result in the mitotic chromatin assembly defect. APC mutants exhibited altered total and modified histones as well as genetically interact with a number of histone modification proteins (Figures 3.1A, 3.2 and 3.11; Turner *et al*., 2010). Increased expression of the HAT genes *ELP3*, *GCN5* and *RTT109*, as well as a variety of CAFs, were each able to rescue the temperature sensitivity in *apc5*^{CA} cells. The overexpression of histones also rescued the temperature sensitive phenotype suggesting that the increased ability to deposit histones onto the DNA is of benefit to *apc5*^{CA} cells (Figure 3.8B). A model that explains these varied genetic interactions involves the physical association of the APC with the CAFs and HATs shown to genetically interact together. The rationale for such physical associations would involve optimizing the acetylation of histones by Asf1/Rtt109/Gcn5 and passage of the acetylated histones to CAF-I for chromatin assembly (Figure 1.7). It is possible that all these proteins use the APC as a scaffold in order to bring all the players together for efficient transfer of acetylated histones to CAFs. Alternatively, individual proteins, such as Asf1, may recruit other factors to the APC (Figure 5.1). The role of the APC in this model may be to regulate, via targeted degradation, which factors are associated with HATs, histones and CAFs. In this chapter experiments to test this model are presented.

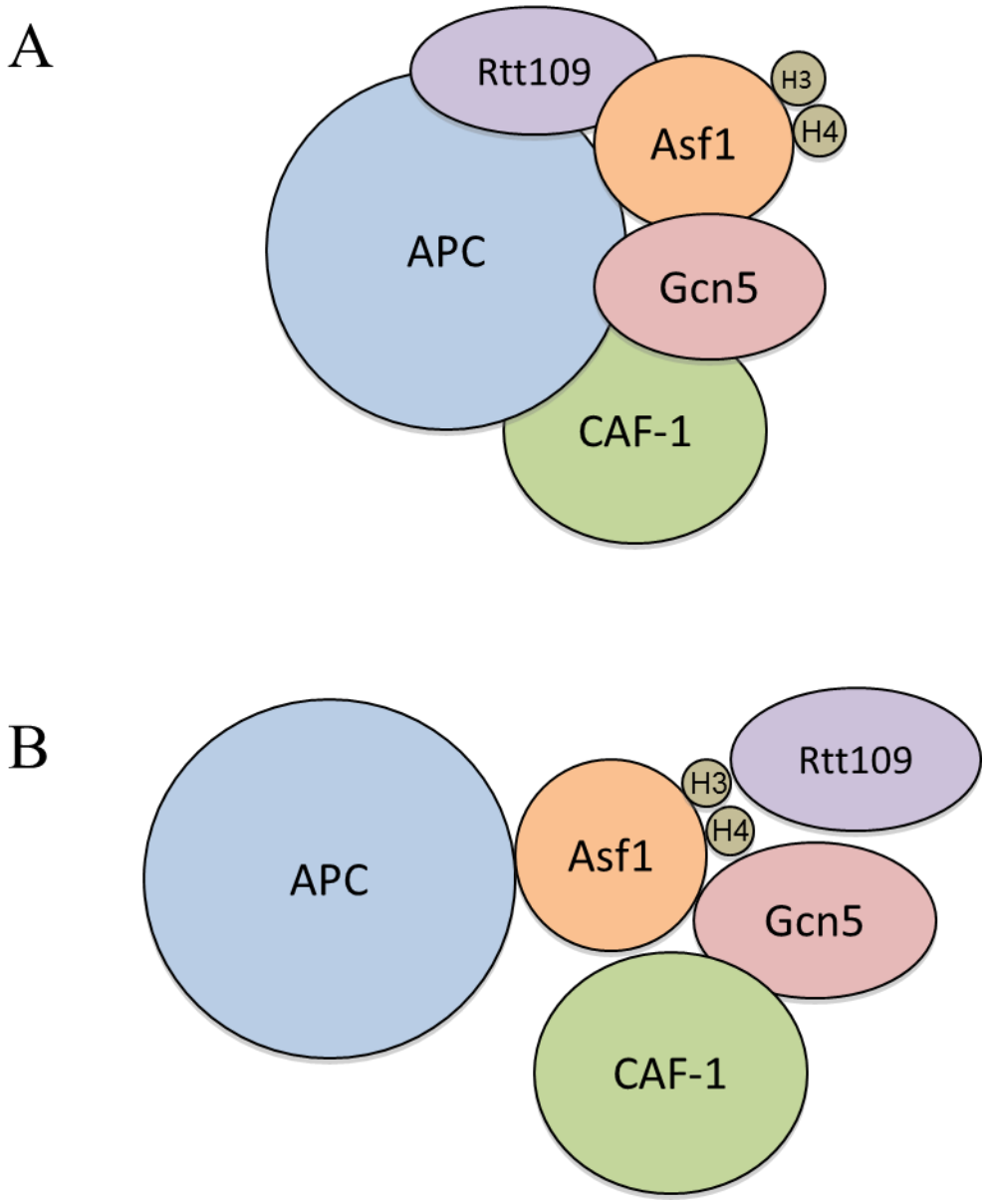

Figure 5.1. **Interaction models for the APC with the CAFs and HATs. (A)** The APC may act as a scaffold to bring together the proteins involved in histone modification and deposition in order to facilitate the modification and transfer of histones. The unique requirement for the APC in this complex may reflect the cell cycle-dependent nature of the interactions, whereby the APC can target players for degradation when their role is complete. **(B)** Alternatively, the APC may interact with only one protein, for example Asf1, which then recruits other HATs and CAFs to the APC. It is known that Asf1 physically interacts with Rtt109 and CAF-I.

5.2 Results

5.2.1 Influence of *ASF1-GST* or *MSI1-GST* overexpression on histone modifications and *apc5*^{CA} growth

Previous work conducted in our laboratory demonstrated that the APC genetically interacted with the chromatin assembly factors CAF-1 and Asf1 (Harkness *et al*., 2005). Work presented in this thesis (published in Turner *et al*., 2010) demonstrated that the APC interacts with Rtt109 (Figure 3.11). Rtt109 is a HAT responsible for acetylating Asf1-bound histones prior to their passage to CAF-I and subsequent deposition into chromatin (Chen & Tyler, 2008; Fillingham *et al*., 2008). Thus, it was investigated whether the reduced histone levels in APC mutant cells were linked to the chromatin assembly defects (Harkness *et al*., 2005). To test this, plasmid borne *GST-ASF1* and *GST-MSI1* (the smallest subunit of CAF-1) under the control of the copper induced promoter of *CUP1* were overexpressed in both wild-type and *apc5*^{CA} cells at 30˚C and 37˚C (Figure 5.2A). Similar results occurred at both temperatures. Increased *ASF1* expression resulted in increased levels of acetylation at K9 and K56 on histone H3 but decreased levels of K79 dimethylation. Overexpression of *MSI1* had no effect on these modifications. Others have provided data that supports our model that Asf1 presents histones H3 and H4 to the HAT Rtt109 (and possibly Gcn5) for acetylation prior to passing them on to CAF-I, of which Msi1 is a subunit, for chromatin deposition (Fillingham *et al*., 2008; Burgess *et al*., 2010). In addition, despite the differences in histone modification, overexpression of either *ASF1* or *MSI1* was able to rescue the *apc5*^{CA} temperature sensitive growth phenotype to that of wild-type (Figure 5.2B). This rescue occurred regardless of temperature or expression levels (moderate or excessive). It is likely that increasing the ability to deposit acetylated histones onto DNA is able to rescue APC mutants. This is supported by the finding that overexpression of

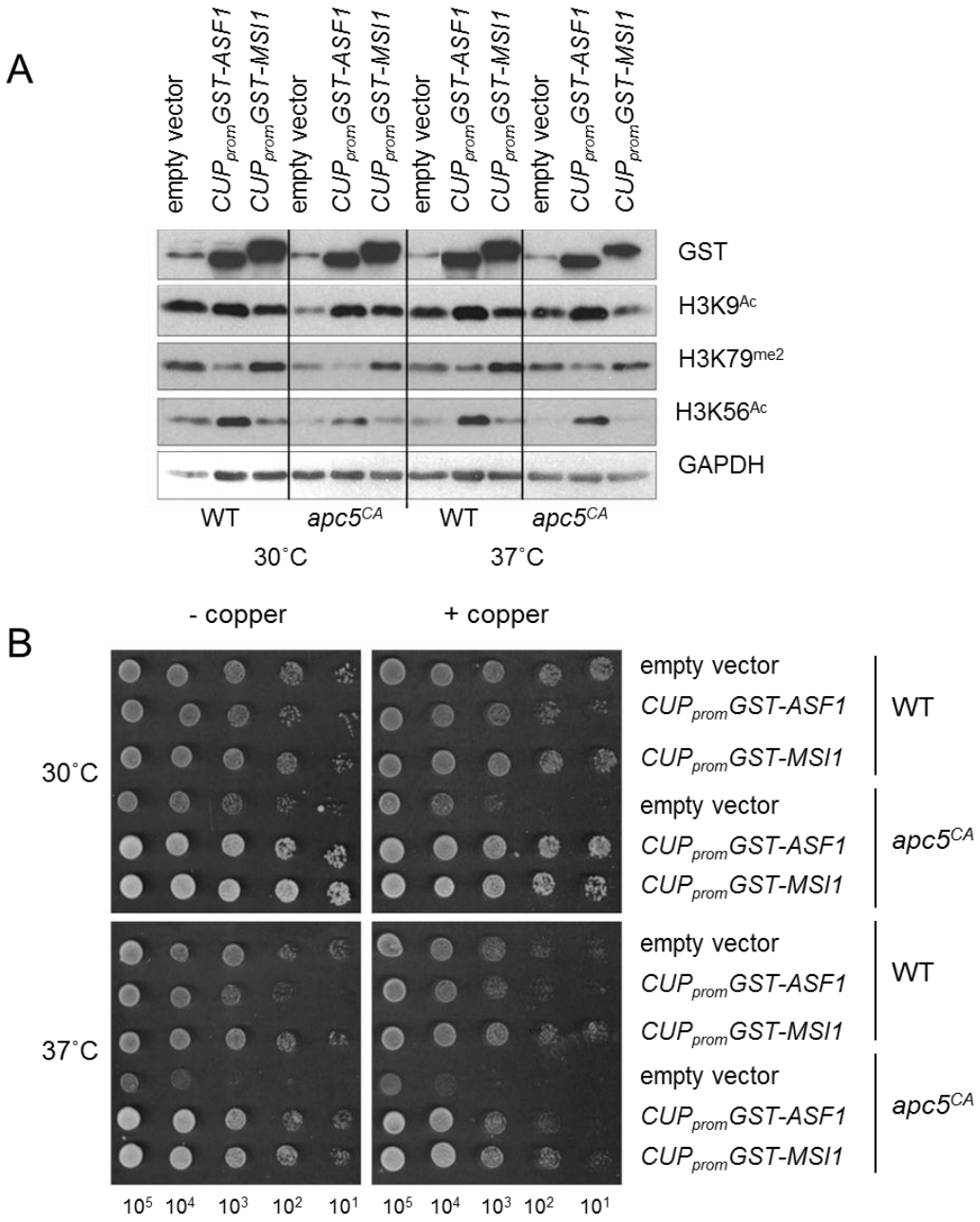

Figure 5.2. **Influence of increased *ASF1 or MSI1* expression on histone modifications**. **(A)** Histone modifications were assessed in wild-type (WT) and *apc5*^{CA} cells overexpressing *GST-ASF1* or *GST-MSI1* from the *CUP1* promoter. Protein extracts were prepared from cells following growth at 30°C or after a shift to 37°C for 3 h. Proteins were induced by the addition of 100 M $CuSO_4$ for 3 h. Extracts were then analyzed using the antibodies indicated. **(B)** The cells described for panel A were spot diluted onto control plates, or plates containing 100 M $CuSO_4$, and grown for 3 days at 30°C and 37°C to confirm that *apc5*^{CA} temperature sensitive defects were suppressed. This figure was published in Turner *etal*., 2010.

histones is able to rescue the temperature sensitive phenotype of *apc5*^{CA} (Figure 3.8B). This further supports a role for the APC in histone modification and deposition.

5.2.2 Physical interaction of chromatin assembly factors with the APC and Gcn5-HA

Recent research in humans has demonstrated that Apc5 and Apc7 both physically associate with the HATs CBP and p300 (Turnell *et al*., 2005). The yeast Rtt109 has been shown to be structurally similar to CBP (Liu *et al*., 2008; Tang *et al*., 2008; Wang *et al*., 2008). Rtt109 interacts with the chromatin assembly factor Asf1 and acetylates histone H3 residues K9 and K56 (Chen & Tyler, 2008; Fillingham *et al*., 2008; Das *et al*., 2009). Our laboratory has previously shown Apc5 to genetically interact with CAF-1 and Asf1 and that these proteins play a role in mitotic chromatin assembly (Harkness *et al*., 2005). Progressive deletion of CAF-I subunits in the *apc5*^{CA} background resulted in further exacerbation of the temperature sensitive phenotype, indicating that the individual CAF-I subunits have function, previously believed to not be the case, while overexpression of any one CAF-I subunit was capable of rescuing the *apc5*^{CA} phenotype. The APC is also shown to genetically interact with Rtt109, as increased expression of *RTT109* restored the growth of *apc5*^{CA} cells to wild-type levels (Figure 3.11).

To study the interaction of the APC with chromatin formation further, immunoprecipitations (IPs) were performed using a variety of tagged proteins, some endogenous, some plasmid borne. Western blot assays were used to detect physical

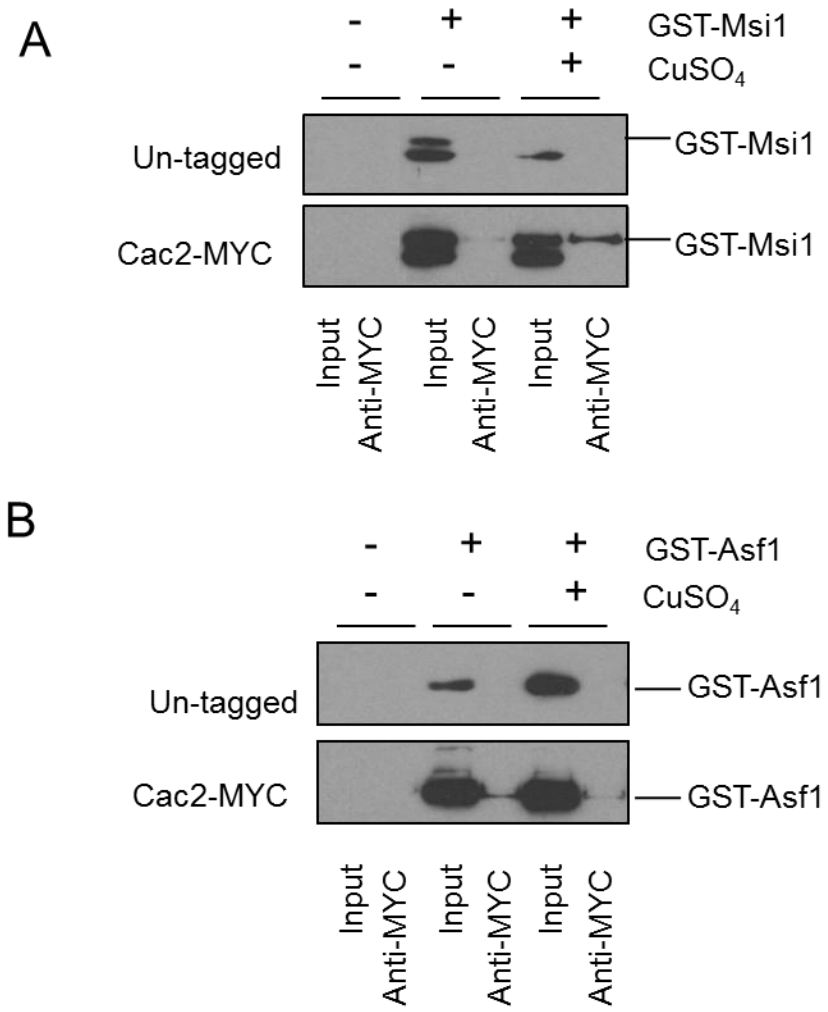

Figure 5.3. **Cac2-myc physically associates with GST-Msi1 and GST-Asf1**. **(A)** Wild-type and an endogenously C-terminal MYC-tagged *CAC2* strain were transformed with *CUP_{prom}GST-MSI1*. The cells were grown overnight to early log phase, induced with $CuSO_4$ for 4 hours, then subjected to bead-beating to prepare whole cell lysates. 1 mg of protein was incubated with antibody against MYC overnight, then mixed with pre-cleared protein A agarose beads. The beads were pelleted and the bound proteins were washed three times. 67% of the bound sample, along with 10 µg of input was separated by SDS-PAGE, transferred to membrane and detected with antibodies against the GST epitope. **(B)** Immunoprecipitations were performed as in (A) with the exception that the *CUP_{prom}GST-ASF1* plasmid was used.

association. Using an endogenous *CAC2-MYC* allele, Asf1 and Msi1 expressed from *CUP1*-induced plasmids were co-immunoprecipitated (Figure 5.3). This was to be expected, as Cac2 and Msi1 are both subunits of the chromatin assembly factor CAF-I, while Asf1 has been shown to physically bind to Cac2 to facilitate replication-dependent chromatin assembly (Kaufman *et al*., 1995; Tyler *et al*., 2001; Krawitz *et al*., 2002; Mello *et al*., 2002).

CAF-I genetically interacts with the APC and together they promote mitotic chromatin assembly (Harkness *et al*., 2005). We predict that they may also physically associate. Immunoprecipitations were performed using endogenously TAP-tagged *APC5*. GST-Asf1 was observed to physically associate with Apc5 (Figure 5.4A). Cac2-Myc was observed to physically associate with Apc5-TAP (Figure 5.4B). GST-Msi1 was not detected in association with APC5-TAP (Figure 5.4A); since Cac2 and Msi1 are both subunits of CAF-I this may indicate that Cac2 binds the APC independently of the complex. This may provide additional evidence to support the hypothesis that the CAF-I subunits interact in an independent manner with the APC to facilitate mitotic chromatin assembly. In humans, Apc5 has been shown to have functions outside of the APC, as it associates with the ribosome independently from the rest of the complex (Koloteva-Levine *et al*., 2004). To determine if the physical interactions observed were to be attributed to Apc5 alone or as part of the APC Apc10-HA was used as bait. Cac2-Myc also physically associated with Apc10-HA, suggesting that the interactions detected by Apc5 are likely due to CAF-I subunits associating with the APC complex and not necessarily the Apc5 subunit itself (Figure 5.4B).

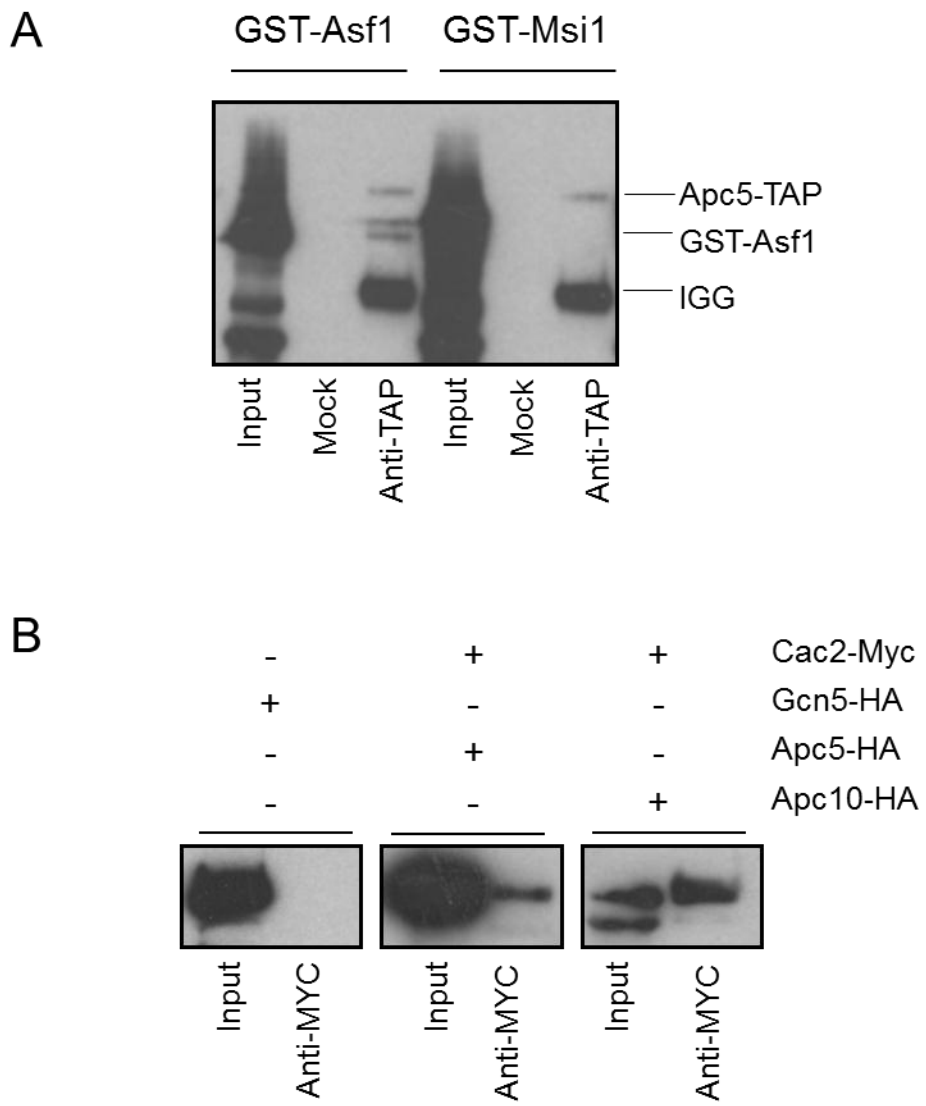

Figure 5.4**. Chromatin assembly factors physically associate with the APC**. **(A) Apc5-TAP physically interacts with GST-Asf1**. Strains harboring an endogenous C-terminal TAP-tagged *APC5* were transformed with *CUP$_{prom}$GST-ASF1* or *CUP$_{prom}$GST-MSI1* plasmids. The cells were grown overnight to early log phase, induced with $CuSO_4$ for 4 hours, then subjected to bead-beating to prepare whole cell lysates. 1 mg of protein was incubated with antibody against TAP overnight, then mixed with pre-cleared protein A agarose beads. The beads were pelleted and the bound proteins washed three times. 67% of the bound sample, along with 10 µg of input was separated by SDS-PAGE, transferred to membrane and detected with antibodies against the GST epitope. The GST antibody recognizes TAP because of the Protein A motif incorporated into the TAP-tag. **(B) Cac2-MYC physically associates with Apc5-HA and Apc10-HA**. The Cac2-MYC strain was transformed with *GAL$_{prom}$-APC5-HA* and *GAL$_{prom}$-APC10-HA* and treated as in (A), exceptions being induction in 2% galactose-supplemented media and IPs were performed using antibodies against HA. A wild-type strain transformed with the *GALprom-GCN5-HA* plasmid is used as a control (B) provided by M. Dash. Images shown are representative of routinely observed results.

One of the roles that the APC may be playing in chromatin assembly is as a scaffold or facilitator to bring together the proteins required for chromatin assembly (Figure 5.1). A recent study provides support for our model, as Gcn5 may be involved in regulating the interaction of CAF-I with histone H3 (Burgess *et al*., 2010). Further support is provided by a report suggesting Asf1 presents histones to Gcn5 (and Rtt109) for acetylation prior to passing them to CAF-I for deposition onto DNA (Fillingham *et al*., 2008). In support of this role, Cac2-Myc is demonstrated to physically associate with Gcn5-HA (Figure 5.5). This provides additional support for our model, but does not distinguish between a scaffold or facilitator role of the APC.

The finding that the APC physically associates with Cac2-Myc and GST-Asf1 does not necessarily signify that it actually binds to each of these proteins; for example, APC's association with Cac2-Myc may be through direct binding with Asf1 or some other protein. In an attempt to further resolve the details of the binding patterns of the APC with Asf1 and CAF-I, CoIPs were performed in an *asf1*Δ mutant strain. If Asf1 is responsible for linking Cac2 with the APC then deletion of Asf1 should abolish the Cac2/Apc5 interaction; however, it did not. Cac2-Myc was still able to co-immunoprecipitate Apc5-HA, which indicates that this interaction is not mediated by Asf1 (Figure 5.6). More CoIPs in cells containing deletions of these interacting proteins will need to be performed to truly elucidate the intricacies of APC interaction with these histone binding proteins.

5.3 Discussion

The APC is most commonly associated with regulating cell cycle progression by targeting proteins that block passage though mitosis for ubiquitin- and proteasome-

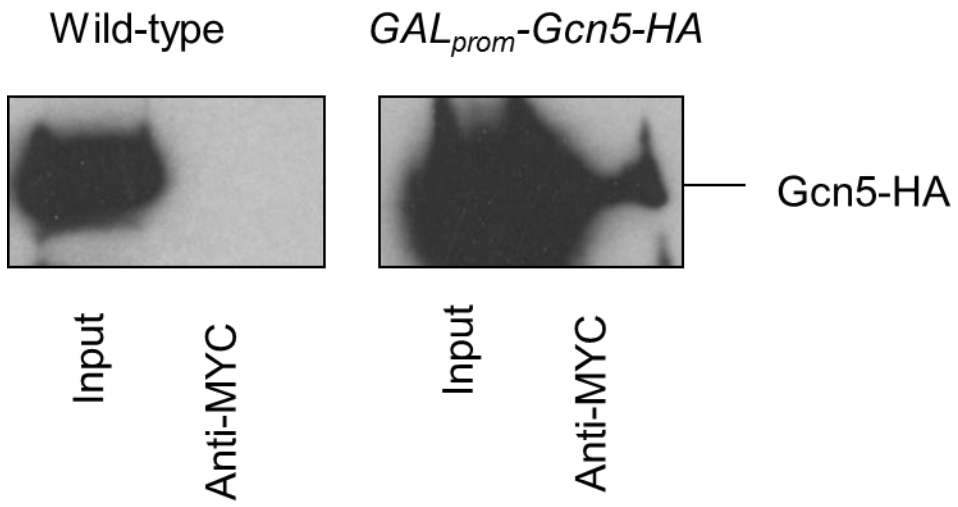

Figure 5.5**. Cac2-MYC physically associates with Gcn5-HA**. The Cac2-MYC strain was transformed with *GAL$_{prom}$-GCN5-HA* plasmid. Once cells reached log phase, proteins were induced in 2% galactose-supplemented media for 4 hours. Whole cell lysates were prepared by bead beating. 1 mg of protein was incubated with antibody against MYC overnight, then mixed with pre-cleared protein A agarose beads. The beads were pelleted and the bound proteins washed three times. 67% of the bound sample, along with 10 µg of input was separated by SDS-PAGE and transferred to membrane. Proteins were then detected with HA antibody. Figure provided by M. Dash

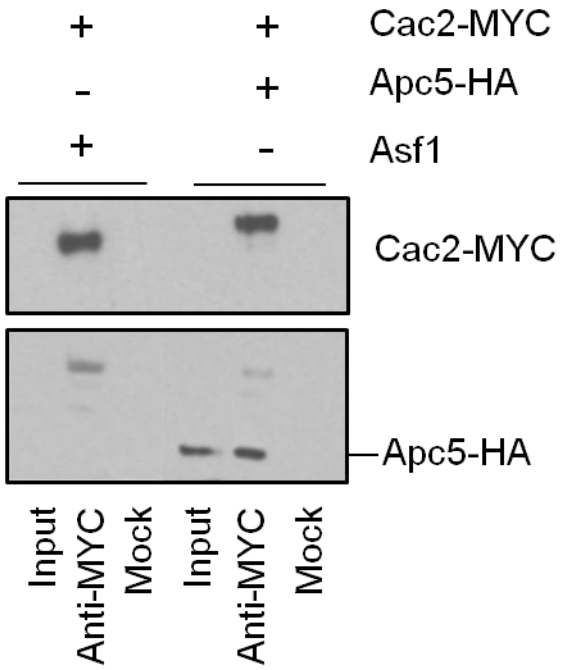

Figure 5.6**. Apc5-HA physically associates with Cac2-MYC in the absence of Asf1**. Wild-type and ***asf1*Δ** strains containing Cac2-MYC were transformed with empty vector or *GAL*$_{prom}$*-APC5-HA*. The cells were grown overnight to early log phase and then resuspendend in 2% galactose-supplemented media. After 4 hours, whole cell lysates were prepared by bead-beating.1 mg of protein was incubated overnight with antibody against MYC, then mixed with pre-cleared protein A agarose beads. The beads were pelleted and the bound proteins washed three times. 67% of the bound sample, along with 10 µg of input was separated by SDS-PAGE. Proteins were then transferred to a membrane and detected with antibodies against the HA epitope.

dependent destruction (Castro *et al.*, 2005; McLean *et al.*, 2011). APC activity is prevented if the cell is not ready to proceed with sister-chromatid segregation as premature separation can lead to mitotic catastrophe. Mitotic stability is vital to the survival of the cell and chromatin assembly is known to play an important role in maintaining this stability. For example, mutations to CAF-I results in chromosomal rearrangements and altered cell cycle progression (Quivy *et al.*, 2001; Myung *et al.*, 2003; Nabatiyan & Krude, 2004). Research from our laboratory has shown the APC to play a role in mitotic chromatin assembly through its genetic interaction with the individual subunits of CAF-I (Harkness *et al.*, 2005). Through *in vitro* immunoprecipitation studies, each CAF-I subunit has been shown capable of binding histones independently of the CAF-I complex (Verreault *et al.*, 1996; Shibahara *et al.*, 2000). Furthermore, Asf1 may be responsible for presenting histones to Gcn5 and Rtt109 prior to their deposition by CAF-I (Fillingham *et al.*, 2008; Burgess *et al.*, 2010). Moreover, Gcn5 has recently been implicated in acetylating histone H3 to facilitate its binding by CAF-I (Burgess *et al.*, 2010). Consistent with a role for all these proteins in chromatin assembly, they have each been shown to genetically interact with the APC (Figures 3.2B, 3.11, 5.2A; Harkness *et al.*, 2005). Overexpression of any one of the CAFs *CAC1*, *CAC2*, *MSI1*, *ASF1*, *HIR1* and *HIR2*, and the HATs *GCN5*, *ELP3* and *RTT109* were each capable of rescuing the $apc5^{CA}$ temperature sensitive growth phenotype (Figures 3.7A, 3.11, 5.2A; Harkness *et al.*, 2005). In fact, a recent study reported that Elp3 genetically interacted with Asf1, Cac1 and Rtt109 in response to the DNA damaging agents HU and MMS (Li *et al.*, 2009). Elp3 was also found to genetically interact with PCNA, as double mutants were more sensitive to DNA damage than were single mutants (Li *et al.*, 2009). PCNA physically associates with Asf1 and CAF-I to ensure effective coupling of DNA synthesis with histone deposition (Shibahara &

Stillman, 1999; Sharp *et al.*, 2001; Krawitz *et al.*, 2002). Thus, these observations tightly link Elp3 with the replication-dependent chromatin assembly that utilizes Asf1 and CAF-I.

The temperature sensitivity of the *apc5*^{CA} may be a result of altered histone metabolism and defective chromatin assembly. The overexpression of components that facilitate assembly rescues the *apc5*^{CA} temperature sensitive phenotype. Further linking the *apc5*^{CA} phenotype with aberrant histone modifications is the finding that overexpression of Asf1 increases the acetylation levels of H3K9 and H3K56 (Figure 5.2A); these modifications are associated with newly deposited histones in yeast. The increase in Asf1 likely enhances the association of histone H3 with the HATs that make these modifications, Gcn5 and Rtt109 (Chen & Tyler, 2008; Fillingham *et al.*, 2008; Burgess *et al.*, 2010). CAF-I preferentially deposits appropriately acetylated H3/H4 tetramers onto DNA, so it seems that increased histone acetylation may lead to improved histone deposition. Elevated histone deposition is a likely cause of suppression of the temperature sensitive phenotype in *apc5*^{CA} cells, as increased levels of CAF-I subunits and histones both suppress the temperature sensitive phenotype (Figure 3.8B; Harkness *et al.*, 2005). Excess free histones are detrimental to cells as they form uncontrolled aggregates with the DNA; thus, cells have developed methods of controlling histone levels (Gunjan & Verreault, 2003). In Chapter Three it was demonstrated that many APC mutants exhibit decreased levels of histones H2B and H3 (Figure 3.1). This decrease may in fact hinder chromatin assembly. The overexpression of histones in an *apc5*^{CA} background may be able to counteract this deficiency and allow sufficient chromatin assembly to occur to suppress the *apc5*^{CA} temperature sensitive defect.

In the past few years the APC has been shown to be involved in additional cellular functions. For example, binding of the human APC to the transcription factor p300

increases transcriptional activity while association with the ribosome results in specific mRNA translational repression (Koloteva-Levine *et al.*, 2004; Turnell *et al.*, 2005). Several other proteins have been shown to physically associate with the APC to regulate its activity, including CBP, PTEN and Rb (Turnell *et al.*, 2005; Binné *et al.*, 2007; Song *et al.*, 2011). Interestingly, the activity of these proteins also requires the presence of the APC. Obviously, many proteins come and go, but are necessary for timely APC function. Our laboratory has previously shown that the APC genetically interacts with the CAF-I subunits and the results presented in this thesis demonstrate that APC physically associates with at least one of these subunits, Cac2. APC physically interacts with Asf1 but whether the APC directly binds to Asf1 remains to be determined. Asf1 has been shown to bind to Cac2 and this may then lead to APC association. The interaction of Cac2 with the APC is not mediated by Asf1 as Cac2 still associated with Apc5 in cells lacking Asf1 (Figure 5.6). The hypothesis is that the APC acts as a scaffold to bring together the proteins necessary for chromatin assembly, namely chromatin assembly factors and the HATs required for acetylating histones prior to deposition (Figure 5.1). Two recent studies suggest Gcn5 may acetylate histones bound to Asf1 to facilitate their association with CAF-I and subsequent histone deposition (Fillingham *et al.*, 2008; Burgess *et al.*, 2010). This interaction may be mediated by Asf1, which will be tested in future studies by assessing Cac2-Myc/Gcn5-HA interactions in *asf1*Δ cells. The finding that Cac2 and Asf1 both associate with the APC is novel and may explain why chromatin assembly is defective in the $apc5^{CA}$ mutant. As many of the subunits, activators and targets of the APC are evolutionarily conserved, so too may the protein interaction ability of Apc5.

CHAPTER SIX

DISCUSSION

The results of this study provide many avenues for further research into the role of the APC in the cell. I will discuss the involvement of the APC with various HATs and HDACs, the effect of cellular mRNA content on APC mutants and the possible role APC may play in scaffolding to bring together the proteins needed for effective chromatin assembly.

6.1 The APC genetically and physically interacts with histone modifying enzymes

Increased levels of CAF-I subunits are capable of rescuing the *apc5*^{CA} temperature sensitivity (Harkness *et al*., 2005). This could be due to sequestration or increased chromatin assembly of free histones. If the CAF-I subunits were responsible for sequestering histones then overexpression of histones in cells lacking CAF-I should be toxic (Figure 3.8B). This does not appear to be the case, however, as CAF-I mutants grow similarly to wild type when expressing increased levels of histones (Figure 3.8B). Increased chromatin assembly is more likely, as the overexpression of histones is capable of rescuing the temperature sensitivity of *apc5*^{CA} and *apc5*^{CA} *caf1*Δ cells (Figure 3.8B). Histone levels are decreased in APC mutants (Figure 3.1A), as is chromatin assembly (Harkness *et al.*, 2002). The absence of elevated total histone levels in *apc5*^{CA} cells (Figure 3.1A) suggests that they are as capable of degrading any excess free histones that exist within the cell as is wild-type. As expression of histones in cells harboring the *apc5*^{CA} allele rescued the temperature sensitive phenotype, it may be that the increase in histone expression results in enhanced chromatin formation. Decreased histone levels have been linked to loss of silencing so an increase in histone levels may

result in a return of the genomic stability that is normally promoted by the APC (Feser *et al.*, 2010). Histone deposition may be slow in APC mutant cells, leading to the speculation that excess free histones are degraded before they can be deposited onto chromatin. The increase in histone levels may allow for appropriate levels of deposition to occur; despite excess degradation; as would increasing the levels of the CAFs or HATs involved.

Our laboratory has shown a link between the APC and chromatin dynamics. The APC genetically interacts with the genes that encode the CAFs CAF-I, Asf1, Hir1 and Hir2 (Harkness *et al.*, 2005). Data suggests Asf1 may couple histone acetylation with chromatin assembly, and the results provided in this thesis may demonstrate that the APC genetically interacts with numerous HATs (*ELP3*, *GCN5*, *RTT109*, *SAS2* and *SAS3*) and HDACs (*HDA1*, *HOS1*, *HOS2* and *HOS3*). Asf1 and Rtt109 physically interact with each other, while CAF-I and Gcn5 genetically interact (Fillingham *et al.*,2008; Burgess *et al.*, 2010). Identifying each of these factors as interacting with the *apc5*^{CA} allele is compelling evidence for a role in modifying and/or depositing histones onto DNA, at least for Apc5. The finding presented in Chapter Three that increased expression of Rtt109 rescues the *apc5*^{CA} phenotype points to an evolutionarily conserved interaction, as human Apc5 physically and functionally interacts with the Rtt109 ortholog CBP (Turnell *et al.*, 2005).

While one modification associated with newly deposited histones, H3K56^{Ac}, appears not to be affected by APC mutation, another modification, H3K9^{Ac}, is affected. H3K9^{Ac} has also been shown to increase during mitosis and is associated with mitotic specific genes. H3K9^{Ac} is affected by APC mutation, as is H3K79^{me2}, a modification associated with genes expressed during mitosis. Both of these modifications were reduced in many of

the APC mutants tested. The major H3K9 targeting HATs are Gcn5 and Rtt109, but Elp3 has also been shown to target this residue for acetylation (Kristjuhan *et al.*, 2002; Fillingham *et al.*, 2008). Thus, this thesis demonstrates compelling evidence to suggest that the gap between histone modification and histone deposition is bridged by the APC.

6.2 Interaction of the APC with HATs and HDACs

As a result of the APC genetic interaction with genes encoding chromatin assembly proteins, it was determined whether the APC also interacted with genes encoding histone modification proteins. By performing a genetic screen using the *apc5*^{CA} temperature sensitive allele it was determined that a wide variety of genes encoding HATs and HDACs genetically interacted with the APC. Based on the prevailing premise that HATs drive transcription, and HDACs silence it, the initial hypothesis was that deleting any HAT would exacerbate the *apc5*^{CA} temperature sensitive phenotype, while deleting any HDAC would improve cell growth. Surprisingly, approximately half of the genes tested did not support this hypothesis (Figure 3.2). This suggests that it is not the overall level of acetylation that impacts the *apc5*^{CA} temperature sensitive phenotype but the specific function of the enzyme involved that matters. While the majority of the HATs and HDACs tested in this study have specific preferences for which histone and chromosomal region they target (see Tables 1.1 and 1.2), exactly how their activity is controlled remains largely unknown.

The data presented in this thesis support the theory that the APC plays a role in the initiation of the transcription of genes required for cell cycle passage. Deletion of genes that facilitate transcription (*ELP3*, *GCN5* and *SAS3*) impairs the *apc5*^{CA} temperature sensitive phenotype, while deletion of genes involved in silencing (*HOS1*, *HOS2*, *HOS3*,

and *SAS2*) suppressed the *apc5*^{CA} phenotype (Figure 3.2). We hypothesize that Elp3 and Gcn5 are independently required for progression through mitosis but share an overlapping function that allows passage through G1. It is predicted that Elp3 and Gcn5 contribute to a transcriptional profile that enables cells to proceed through mitosis and G1. Overexpression of either *ELP3* or *GCN5* results in rapid accumulation in G1 (Figure 3.7C), suggesting that this profile must be reset to allow cells to exit G1. The APC may assist in the resetting of this profile as Elp3 and Gcn5 are unstable during G1 and, at least in the case of Gcn5, in an APC-dependent manner. The involvement of the CAFs CAF-I and Asf1, along with the associated HAT Rtt109 requires further investigation. More recently, studies have linked Gcn5 with CAF-I, and Elp3 with PCNA, indicating Elp3 may also associate with CAF-I (Li *et al.*, 2009; Burgess *et al.*, 2010). The acetylation levels of H3K9 and the methylation status of H3K79 were reduced in APC mutants beyond that observed for total H3, suggesting a role for the APC in facilitating these specific modifications (Figure 3.1A). As *apc5*^{CA} cells demonstrate a defect in chromatin assembly (Harkness *et al.*, 2002), it is possible that the APC plays a role in deposition of the properly acetylated histones.

The interactions between the APC and the HATs Gcn5 and Hpa2 warrant further investigation. Deletion of *HPA2* in *apc5*^{CA} cells did not affect the temperature sensitive phenotype, while the deletion of *GCN5* did. Gcn5 has been shown to target histones within actively transcribed genes (Krebs *et al.*, 2000). Hpa2 is also known to target H3K14 for acetylation *in vitro*; however, its exact role within the cell is still unknown, as *HPA2* deletions do not alter gene expression dramatically (Angus-Hill *et al.*, 1999; Rosaleny *et al.*, 2007). Interestingly, deletion of *HPA2* impaired the growth of *gcn5*Δ *apc5*^{CA} cells but increased the growth of *gcn5*Δ cells (Figure 3.3B). It has previously been shown that *gcn5*Δ *hpa2*Δ cells are viable (Howe *et al.*, 2001). This suggests the

individual activities of Gcn5 and Hpa2 counteract each other in wild-type cells and they each target different gene pools. Why, and how, this antagonistic behavior is cancelled out by mutation to *APC5* remains to be determined. It is possible that the accumulation of an APC target is detrimental to cells lacking both Gcn5 and Hpa2. Further research may show Hpa2 to also interact with Elp3, as Elp3 and Gcn5 have been shown to have overlapping roles within the cell in relation to H3K14 (Wittschieben *et al.*, 2000).

An excellent example highlighting the importance of specific roles for each HAT is the finding that deletion of *SAS2* or *SAS3* affects the *apc5*^{CA} temperature sensitive phenotype differently. Deletion of *SAS2* improved *apc5*^{CA} cell growth while deletion of *SAS3* exacerbated it (Figure 3.2B). Even though they both acetylate histones the different outcomes can be explained due to their specific roles within the cell. Sas2 maintains expression of sub-telomeric genes by preventing the spreading of heterochromatin through acetylation of H4K16 (Kimura *et al.*, 2002; Suka *et al.*, 2002). Sas3 is targeted to actively transcribed genes throughout the genome and acetylates histone H3 on residues K14 and K23 (Howe *et al.*, 2001). Sas3 has been shown to functionally overlap with Gcn5, as *gcn5*Δ *sas3*Δ double mutants are lethal (Howe *et al.*, 2001; Rosaleny *et al.*, 2007). Given the similarities, it is possible that Sas3 levels may also be cell cycle regulated in an APC-dependent manner. Sas2 was the only HAT found to improve *apc5*^{CA} growth when deleted. As heterochromatin spreads out from telomeric and mating-type loci when *SAS2* is deleted it may be that genes that contribute to the *apc5*^{CA} temperature sensitive phenotype become silenced. In fact, Sas2 was shown to bind to non-intensely transcribed genes including those involved in double-strand break repair and ubiquitin specific protease activity (Rosaleny *et al.*, 2007). Targeted deletion of genes found in these sub-telomeric regions may result in alteration of the *apc5*^{CA} phenotype.

The APC is an evolutionarily conserved complex and has been shown to interact with the ribosome in both yeast and humans (Koloteva-Levine *et al.*, 2004; Costanzo *et al.*, 2010). The finding that genes that encode the HDACs Hos1, Hos2 and Hos3 each genetically interact with the APC in the same manner further highlights this conservation (Figure 3.2A). Hos2 preferentially targets ribosomal protein-encoding genes while Hos1 and Hos3 each target the rDNA locus (Robyr *et al.*, 2002). The APC has been shown to physically associate with the poly(A) binding protein (PABP), which inhibits the translation of specific mRNAs in humans (Koloteva-Levine *et al.*, 2004). A recent synthetic genetic array screen in yeast also showed the *apc5*^{CA} allele to genetically interact with ribosomal subunits, as well as proteins involved in the decay, nuclear export and quality control of mRNA (Table 3.1; Costanzo et al., 2010). It will be of interest to determine if the interaction of the APC and the *HOS* genes is limited to the expression of ribosomal components or due to additional functions. The study by Koloteva-Levine and colleagues (2004) demonstrated that the entire APC inhibited mRNA translation by binding to PABP but it was the Apc5 subunit that physically bound to the ribosome in a complex independent manner. As all of the genetic screens presented in this thesis involved the *apc5*^{CA} allele it will be of interest to determine if the altered growth effects are due to impaired activity of the entire complex or just the individual Apc5 subunit.

ELP3 or *GCN5* single mutants have decreased H3K9 and H3K14 acetylation with the double HAT mutant showing even further decreased levels of H3K9 acetylation. Acetylation of H3K9 and H3K14 was previously shown to be decreased in *elp3*Δ *gcn5*Δ cells, but a decrease of total H3 was not demonstrated (Kristjuhan *et al.*, 2002). Even though global levels were decreased, Co-IPs of 20 randomly selected genes demonstrated that H3K14 was not as severely hypoacetylated in the double mutant as was H3K9. This suggests that Elp3 and Gcn5 may not act in a global manner to control

gene transcription, but may target specific genes. In our hands, the double HAT mutant also showed decreased levels of total H3 (Figure 3.5A). This difference may be due to a difference in background or technique, as the S288c yeast genetic background was used in this study and equalized total protein load was compared, while Kristjuhan and colleagues (2002) used the W303 background and compared equalized levels of total histone. More work will need to be done to clarify this issue. Nevertheless, the finding that total histone levels are decreased in the double mutant may signify that the loss of acetylation may, at least partially, be due to loss of total histone.

The finding of an epistatic relationship between the APC and the two HATs, Elp3 and Gcn5, leads to the question of whether these proteins are upstream or downstream of the APC. The hypothesis is that Elp3 and Gcn5 are downstream and therefore their absence should have no effect on APC activity. If either Elp3 or Gcn5 were upstream of the APC, one would expect to see an effect on Clb2 levels when these HATs are deleted. Unexpectedly, Clb2 levels in *elp3*Δ, *gcn5*Δ, *elp3*Δ *apc5*^{CA} and *gcn5*Δ *apc5*^{CA} cells were less than in *apc5*^{CA} mutants. The two exceptions were *elp3*Δ and *apc5*^{CA} *gcn5*Δ cells. This finding suggests that cells lacking *ELP3* and *GCN5* degrade Clb2 but still progress through the cell cycle very slowly. It may be that the slow progression through the cell cycle gives a compromised APC the time needed to properly target Clb2 for degradation. Interestingly, cells lacking both *ELP3* and *GCN5* exhibited even further decreases in Clb2. This may be attributed to these mutants spending a prolonged time in G1, a phase of the cell cycle where levels of Clb2 are low but still targeted by the APC (Yeong *et al.*, 2000). Alternatively, Elp3 and Gcn5 may act as upstream inhibitors of the APC, as Clb2 instability is increased in *elp3*Δ *gcn5*Δ mutants. Nevertheless, the observation that Clb2 is still unstable in the HAT mutants suggests that Elp3 and Gcn5 are not upstream activators of the APC.

The inability of galactose driven overexpression of *ELP3* or *GCN5* to rescue the temperature sensitivity of the *apc5*^{CA} allele could be due to increased histone levels. Increased expression of *ELP3* resulted in an increase in histone H3 levels in APC mutants as well as in wild-type cells grown in galactose-supplemented media (Figures 3.7A and 3.8A). Excess histones are toxic so free unincorporated histones are targeted for degradation (Meeks-Wagner & Hartwell, 1986; Gunjan & Verreault, 2003). As *GCN5* overexpression is toxic to both wild-type and *apc5*^{CA} cells histone H3 levels may be even further elevated. However, expression of the histones H3 and H4 from the *GAL1* promoter did rescue the *apc5*^{CA} temperature sensitive growth phenotype, presumably by increasing the rate of histone deposition. Thus, it is more likely that the toxic effect of *ELP3* (and *GCN5*) overexpression is due to a block in cell cycle progression in G1 rather than an overabundance of histones.

6.3 Gcn5 is targeted for degradation by the APC to allow progression through S-phase

Elp3 and Gcn5 are required for G1 progression but must be removed to exit G1 (Figures 3.5B and 3.7C). The results presented in this thesis show that with increased expression of the HATs *ELP3*, *GCN5* or *RTT109*, the temperature sensitivity of the *apc5*^{CA} allele can be rescued (Figures 3.7A and 3.11). One explanation for this is that these HATs act upstream of the APC by promoting the transcription of genes required for APC activity. Given that the APC target Clb2 was still degraded in *elp3*Δ *gcn5*Δ mutants (Figure 3.6) it is unlikely that Elp3 and Gcn5 are upstream activators of APC activity. An alternate possibility is that these HATs act in a pathway redundant to the APC to promote progression through mitosis and G1. Increased expression, but not overexpression, of *ELP3* or *GCN5* rescued the *apc5*^{CA} temperature sensitive phenotype possibly due to the

cells spending a prolonged period in G1. The delayed progression through G1 may allow a compromised APC the extra time necessary to complete G1-dependent functions, such as maintaining low levels of Clb2. Interestingly, the *apc5*^{CA} temperature sensitive phenotype was not rescued when either *ELP3* or *GCN5* was overexpressed using galactose. This indicates that there is a threshold after which the cell can no longer tolerate excess protein levels. Even wild-type cell growth was hindered by overexpression of *GCN5*. Increased expression of *GCN5* was able to counteract the toxic effects of Apc5 overexpression, suggesting Gcn5 abundance is low due to overactive APC. In fact, analysis of the amino acid sequence of Gcn5 disclosed the presence of two putative D boxes. These data support the hypothesis that Elp3 and Gcn5 may be targeted by the APC for degradation. Indeed, both Elp3 and Gcn5 were observed to be unstable during G1 in wild-type cells (Figures 4.3A and 4.6A). This instability is associated with APC activity as Gcn5 was stabilized in APC mutants while modification patterns of Elp3 were dependent on components of the ubiquitin pathway (*apc5*^{CA}, *apc10*Δ, *ubc1*Δ) (Figures 4.3A, 4.6B and 4.6C). Further research is warranted to determine if other E2s, Ubc4 for example, also affect the modification pattern of Elp3 and if Gcn5 is affected in a similar manner. Taken together, these data suggest that Elp3 and Gcn5 have independent functions during mitosis but act redundantly to pass through G1.

Gcn5 has recently been linked to cell cycle passage in budding yeast as *gcn5*Δ mutants exhibit defective interactions between centromeres and kinetochores, resulting in delayed G2 progression, spindle elongation, defective nuclear division and chromosomal loss (Vernarecci *et al.*, 2008). Gcn5 has also been shown to cycle in human cells as Gcn5 levels peak at early S-phase and decrease by mid-S-phase (Paolinelli *et al.*, 2009). In early S-phase human Gcn5 acetylates the nuclear Cdc6, which is required for

the formation of pre-replication complexes during G1. Once acetylated, Cdc6 is phosphorylated and exported to the cytosol (Paolinelli, Mendoza-Maldonado, Cereseto, & Giacca, 2009). Overexpression of Gcn5 resulted in the majority of Cdc6 localizing to the cytosol while treatment with *GCN5* RNAi resulted in Cdc6 remaining nuclear, suggesting that a tight level of control is needed for the cell cycle to progress efficiently. Thus, APC-dependent turnover of Gcn5 observed in this study may be an evolutionarily conserved function.

6.4 The APC's role in chromatin maintenance and stability

Our proposal that the APC is required for histone metabolism in actively growing cells fits well with current literature on chromatin structure being linked to genomic instability and cancer (Myung *et al.*, 2003; Ye *et al.*, 2003; Nabatiyan & Krude, 2004; Prado *et al.*, 2004; Kops *et al.*, 2005). Histone metabolism plays an important role in chromatin structure; furthermore, many cancers have been shown to exhibit altered chromatin structure (Zhu *et al.*, 2004; Kops *et al.*, 2005). New targets of the APC continue to be identified. Many APC substrates required for cell cycle progression exist for only a brief period in the cell as they are transcribed in mitosis and then rapidly degraded (Seki & Fang, 2007; Zhao *et al.*, 2008). One recently identified target of the APC in humans is Rcs1 (Zhao *et al.*, 2008). Rcs1 interacts with the NuRD chromatin remodeling complex and physically associates with HDAC1 and HDAC2. This provides evidence that the APC is involved in regulating chromatin-modifying activities, at least in humans. Given that the APC is evolutionarily conserved it is likely that its involvement in chromatin modification is also evolutionarily conserved.

6.5 Chromatin Assembly

Throughout this thesis the overexpression of genes encoding proteins involved in chromatin assembly (Asf1, Gcn5, histones H3 and H4, Rtt109, and possibly Elp3) has been shown to rescue the *apc5*^{CA} temperature sensitive phenotype (Figures 3.7A, 3.8B, 3.11, 5.2B). This overexpression may allow increased chromatin assembly to occur. The *apc5*^{CA} chromatin assembly defects can be rescued by the overexpression of *MSI1* or *CAC1,* while wild-type assembly is increased by the addition of excess histones H3 and H4 to cellular extracts (Harkness *et al.*, 2005). It would be of interest to determine if increased amounts of the other components of the assembly pathway are also capable of rescuing the chromatin assembly defect of *apc5*^{CA} and other APC mutants.

Cancer is tightly associated with chromatin metabolism as defects can lead to genomic instability, a hallmark occurrence in many cancers (Myung *et al.*, 2003; Prado *et al.*, 2004; Zhu *et al.*, 2004; Kops *et al.*, 2005). The observation that histone levels are decreased in many APC mutants may be related to the increased temperature sensitivity of those mutants. Decreased histone levels may result in compromised transcription, increased DNA damage and genomic instability. In fact, a recent paper has linked decreased histone levels with loss of silencing and the loosening of chromatin (Feser *et al.*, 2010). The decrease in histone levels in APC mutants may not be due to decreased histone transcription as histone H3 mRNA levels were increased in APC mutants exhibiting decreased histone H3 protein levels (Figure 3.1B); it may be due to decreased translation or increased degradation. We favor the latter as global decreases in general protein levels were not observed in the APC mutants used in this study as determined by Ponceau S staining (data not shown). Our laboratory has demonstrated that the APC genetically interacts with genes that encode the histone chaperones Asf1, CAF-I, Hir1 and Hir2 and physically associates with Asf1 and CAF-I (Figure 5.4; Harkness *et al.*,

2005). It is possible that the defect in chromatin assembly in APC mutant cells may lead to a decrease in histones, as the cell targets excess histones for degradation (Harkness *et al.*, 2002; Gunjan & Verreault, 2003).

6.6 Scaffolding

One of the ways the APC may help to facilitate chromatin assembly is to act as a scaffold in which the HATs and chromatin assembly factors needed are brought into close contact with each other. Reports demonstrating Apc5 to bind proteins other than APC subunits support this possibility (Koloteva-Levine *et al.*, 2004; Turnell *et al.*, 2005; Herzog *et al.*, 2009). Mutation to the APC may hinder the binding of one or more components of this pathway. This may explain why *apc5*^{CA} cells are still viable at low temperatures, while deletion of *APC5* is lethal (Zachariae *et al.*, 1998b). The majority of the proteins involved are still able to interact, albeit at a reduced rate. A similar scenario has been demonstrated with the mating-pheromone-response pathway scaffold protein Ste5 (Park *et al.*, 2003).

The main role of Ste5 appears to be to bring members of the mating pheromone kinase response, Ste11-Ste7-Fus3, into proximity with each other to facilitate their interaction. Park and colleagues (2003) performed an elegant experiment using a mutated Ste5 unable to effectively bind to the involved kinases. Ste5 and Ste11 were artificially bound to each other using prosthetic binding domains and cells were exposed to mating pheromones. Exposure did result in activation of Fus3, but at a dramatically reduced rate compared to cells containing wild-type Ste5.

A role of a scaffold may also be as an insulator, or to focus a protein's activities to a specific pathway by preventing its association with another scaffold or pathway. This was exemplified by tethering two different pathway scaffolds together, the mating pheromone pathway scaffold, Ste5, and the osmosensing pathway scaffold, Pbs2, (which involves Ste11-Pbs2-Hog1). Ste11 is the MAPKKK for both pathways. The osmosensing pathway allows survival in high salt conditions. Exposure of cells containing these tethered scaffolds to mating pheromone resulted in phosphorylation of Hog1 and activation of the osmosensing pathway (Park *et al.*, 2003). It may be that the APC allows the activities of the HATs involved to be focused on mitotic chromatin assembly rather than transcriptional activation.

6.7 Future Directions

The findings of this thesis raise several questions pertaining to the role of the APC in chromatin assembly and histone modification. First, does the APC actually act as a scaffold to bring together the proteins involved in chromatin assembly? While the data in this thesis suggest that the APC does physically associate with at least some of the proteins involved, it does not explain exactly how these interactions take place. For example, does the APC bind to each protein or just one that happens to bind others? An example of this problem is Cac2. Cac2 has been shown in the literature to bind to Asf1 (Tyler *et al.*, 2001; Krawitz *et al.*, 2002; Mello *et al.*, 2002). APC physically interacted with both Cac2 and Asf1; however, Cac2 still interacted with the APC in the absence of Asf1. This raises the question of whether Asf1's interaction with the APC is direct or if it is mediated through Cac2. Additionally, we do not know which APC subunit(s) these proteins associate with; the Co-IPs in this thesis were performed in cells containing an intact and complete APC. Further studies should be done to determine which subunits

are responsible for these various interactions. Apc5 would be a likely candidate as it has been shown to physically bind to the ribosome as well as to the transcription factor CBP but it is possible that other subunits are also involved (Koloteva-Levine *et al.*, 2004; Turnell *et al.*, 2005).

This thesis demonstrated Elp3 and Gcn5 to be unstable in G1; however Gcn5 was also observed to be unstable in cells arrested in S-phase. There are two major E3 ligases in the cell active during G1, the APC and SCF, but only the SCF is active during S-phase. Therefore, it would be prudent to confirm that Gcn5, and possibly Elp3, are in fact targeted by the APC and not the SCF. The finding that Gcn5 levels in asynchronous cells are not altered in SCF mutants does suggest that the SCF does not target Gcn5, but additional work must be done on G1 and S-phase arrested cells. For example, the instability of Gcn5 and the effect of APC mutation were only apparent after arrest in G1 or S-phase. Therefore, the degradation assays used in the study should also be performed in SCF mutants.

An extension of confirming the APC as being responsible for targeting Gcn5, and possibly Elp3, for destruction would be to actually confirm the presence of poly-ubiquitin ladders associated with Elp3 and Gcn5. Unfortunately, due to the transient nature of ubiquitin attachments this modification has been difficult to detect. While two putative D boxes have been detected in the amino acid sequence of Gcn5, so APC degradation targets were detected in Elp3 (Figure 4.1D and data not shown). New degradation sites have been detected since the discovery of the original D and KEN boxes so it is possible that Elp3 contains a novel site. Using the *ubc1*Δ mutant, an E2 associated with the APC, as well as various APC mutants, alteration of the Elp3 modification patterns was demonstrated, suggesting that the APC-ubiquitin pathway does indeed play a role. A

laddering effect on Elp3-HA and Gcn5-HA in *rpn10*Δ mutants was also observed. Rpn10 is one component of the proteasome that is responsible for recognizing poly-ubiquitin chains so this suggests that Elp3 and Gcn5 are indeed targeted to the proteasome. Further mutation to the ubiquitin-proteasome system may further support this possibility, as would mutation of the lysines within Elp3 and Gcn5 believed to be candidates for ubiquitination. By systematically mutating each lysine, both singly and multiply, Elp3 and Gcn5 may be effectively rendered stable by preventing targeting to the proteasome. This would confirm that Elp3 and Gcn5 are ubiquitinated and targeted to the proteasome for destruction.

6.8 Conclusions

The APC promotes genomic stability and its activity is compromised in many cancers. This effect on genomic stability may be linked to the APC interacting with multiple HATs and HDACs. It is likely that the APC requires a specific transcriptional profile in order to promote mitotic exit and G1 progression. Decreased histone acetylation may impair reestablishment of this transcriptional profile as cells progress through mitosis and G1. The APC is also involved in mitotic chromatin assembly, which when impaired can lead to genomic instability. Hyperacetylation of histones during mitosis has been shown to induce genomic instability (Cimini *et al.*, 2003). One of the ways the APC may maintain genomic stability is to bring together the proteins required for histone acetylation and chromatin assembly. It is possible that, once bound together, the APC will target excess HATs for degradation to prevent detrimental hyperacetylation, or may regulate the cell cycle specificities of these enzymes. Given that each of the proteins involved are present in a wide range of organisms, from yeast to humans, the results found in yeast should be directly applicable to research in humans.

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