University of Alberta

New chromatin regulators contributing to the transcriptional control of *HUG1*

by

Amelia Clare Walker

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science
Department of Biochemistry

Fall 2012
Edmonton, Alberta

Abstract:

Chromatin structure is important aspect of transcriptional regulation. Replication-coupled (RC) nucleosome assembly is the process of depositing newly synthesized H3-H4 onto nascent DNA behind the replication fork, mediated by the histone chaperones Asf1, CAF-1, and Rtt106. The experiments described in thesis test our hypothesis that the RC chaperones contribute to the regulation of *HUG1* due to the important role they play in RC nucleosome assembly and therefore chromatin structure. Collectively, research in this thesis provides evidence that CAF-1 and Rtt106 contribute to the repression of *HUG1* in a way that is unrelated to its normal regulation. Interestingly, this repression does not involve Asf1, even though Asf1 functions upstream of these chaperones during RC nucleosome assembly. These results suggest divergent functions of the RC chaperones that differently affect the regulation of *HUG1*. These divergent functions of the RC chaperones also have opposing roles in promoting survival during prolonged replication stress.

Acknowledgements:

I would first like to thank my supervisor Mike Schultz for helping me develop lab skills and teaching me how to be a successful researcher. Many thanks to Darren for showing me how to do pretty much everything in the lab, and to Laura for introducing me to her project and helping me form a project of my own. Thanks to Kathy, Bret and Magnus for helping me decipher results and making the lab a fun place to work.

I need to thank Laura, Gina and Stacey for forcing me to join many intramural teams. I am really going to miss playing hockey! Thanks to all my wonderful friends and coworkers who made my time in Edmonton a lot of fun, especially Kyle, Sarah, Mike, Laura, Marco and Nick. A special thanks to Shawn for listening to me talk about my project even though he never understood half the words I was saying.

Table of Contents:

List of tables:

List of figures:

Abbreviations:

Act1	Actin
Asf1	Anti-silencing factor 1
ATP	Adenosine triphosphate
CAF-1	Chromatin assembly factor 1
cDNA	complementary DNA
ChIP	Chromatin immunoprecipitation
Cin8	Chromosome instability
CPY	Carboxypeptidase Y
Ddc2	DNA damage checkpoint 2
DDR	DNA damage response
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DSB	Double strand break
EDTA	Etylenediaminetetraacetic acid
H3K56Ac	Acetylation of lysine 56 on histone H3
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HIR	Histone regulation
Hst3&4	Homolog of Sir two
HU	Hydroxyurea
Hug1	HU, UV and gamma radiation induced

ISW2	Imitation switch
Kb	Kilobase
kDa	Kilodalton
MCM helicase	Mini chromosome maintenance helicase
MIG3	Multicopy inhibitor of growth
MMS	Methyl methanesulfonate
MNase	Micrococcal nuclease
mRNA	messenger RNA
NAM	nicotinamide
OD	Optical density
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
Pho5	Phosphate metabolism
PMSF	Phenylmethylsulfonyl fluoride
Rad52	Radiation sensitive
RC chaperones	Replciation-coupled chaperones
RC	Replication-coupled
RFC	Replication factor C
RI	Replication-independent
RNA	Ribonucleic acid
RNAPII	RNA polymerase II
RNR	Ribonucleotide reductase
RPA	Replication protein A

RT-PCR	real-time PCR
Rtt106	Regulator of Ty1 Transposition
SAC	Spindle assembly checkpoint
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIR proteins	Silent information regulator
SWI/SNF	Switch/Sucrose nonfermentable
TFIID	Transcription factor II D
Tris	Tris-(hydroxymethyl) aminomethane
TUP1	Deoxythymidine monophosphate uptake
UV	Ultraviolet
Vps75	Vacuolar protein sorting
v/v	volume per unit volume
Wt	Wild type
w/v	weight per unit volume
YPD	Yeast-peptone-dextrose

Chapter 1:

Introduction

1-1: Introduction

Chromatin

Eukaryote DNA is highly condensed into chromatin. The first level of folding is the wrapping of 146bp of DNA around a histone octamer [1, 2], forming the nucleosome core particle, which is the basic repeating unit of chromatin. The histone octamer is composed of two H2A-H2B dimers and one H3-H4 tetramer [2,3]. Histones are abundant, highly conserved proteins of small molecular weights. They are basic proteins with an overall positive charge, allowing them to easily interact with negatively charged DNA [2]. The packaging of DNA into nucleosomes presents a barrier to transcription machinery, as accessibility to the DNA is reduced [1]. In this way the presence of nucleosomes contribute to the regulation of chromatin. Chromatin regulation determines whether the chromatin is structured to promote or inhibit transcription by facilitating or impeding transcription machinery.

Two domains are present within histone proteins; a globular core domain and an N-terminal tail domain [4]. The globular core is structured and associates with the globular cores of other histones forming the octamer, around which DNA is wrapped. The N-terminal tails of histones are not structured and protrude from the nucleosome core particle [4]. Both domains can be post-translationally modified by histone modifiers, thus altering the structure and regulation of chromatin. This is another layer of chromatin regulation by nucleosomes.

The post-translational modifications of histones include acetylation, methylation, sumoylation, phosphorylation and ubiquitination [5]. Specific modifications can promote or impede cellular processes, and can be indicative of highly transcribed regions, regions of silent chromatin, newly replicated DNA or regions that have been recently repaired [5, 1,6,7,2].

DNA is replicated in the synthesis (S) phase of the cell cycle, during which histones associate with newly replicated DNA. The transcription of genes encoding the histone proteins is tightly regulated, to ensure an appropriate supply of histones for incorporation onto newly synthesized DNA [8,9]. In addition to duplicating parental DNA into two daughter DNA strands, parental chromatin structure is also duplicated during replication. There are multiple methods to alter chromatin structure, which are accomplished by histone chaperones, ATP-dependent chromatin remodelers and histone modifiers [10,11]. Histone chaperones facilitate nucleosome assembly and disassembly by escorting histones to and from DNA. Nucleosome assembly occurs during replication, repair, and transcription. Therefore, nucleosome assembly is just as prominent outside of S phase as it is during DNA replication, when the entire genome is copied and reassembled into chromatin [12]. Once DNA has been assembled into chromatin it remains dynamic, as histone exchange occurs continuously throughout the cell cycle [12]. ATP-dependent chromatin remodelers alter nucleosome positioning by disrupting and reforming histone-DNA interactions [11,13,14]. As a result, DNA sequences can be exposed to promote the binding of regulatory factors and transcription machinery. Histones modifiers post-translationally modify histones to alter chromatin structure. As previously described, post-translational modifications are a major contributor to chromatin regulation since they can promote or impede transcription machinery. Duplication of chromatin state, and thus the phenotypes associated with it, during DNA replication is termed epigenetic inheritance [15]. Epigenetic inheritance is especially important in higher eukaryotes in which it ensures the continuation of a cells gene expression profile, which is important to prevent developmental disorders or disease [16]. The mechanisms for how epigenetic information is transferred from parental to daughter DNA is unclear, but it is suspected to be associated with nucleosome assembly that occurs on newly synthesized DNA in the wake of the replication fork [16]. This is termed

replication-coupled (RC) nucleosome assembly and understanding the mechanism and proteins involved in this specific type of nucleosome assembly may further our understanding of epigenetic inheritance.

Chromatin structure and transcription

Chromatin structure is an important aspect of transcriptional regulation [7,17,18.] As previously described, the contributions of three processes are central to the regulation of chromatin structure: nucleosome assembly and disassembly by histone chaperones, the alteration of nucleosome placement by ATP-dependent chromatin remodelers, and post-translational modifications to the histones. Each contribution provides another layer of chromatin regulation.

In yeast, nucleosome assembly and disassembly during transcriptional repression and activation has been extensively studied at *PHO5*, a gene which is induced in low phosphate conditions [19,20,21, 22]. Nucleosome disassembly permits access of transcriptional machinery to the promoter to initiate transcription. Likewise, nucleosome assembly corresponds to transcriptional repression [20]. In addition, chromatin remodeling and post-translational modifications are important for the regulation of *PHO5* [23,22,24,25].

This thesis concerns the role of histone chaperones in transcriptional regulation of the so-called DNA damage response (DDR) genes of budding yeast. These genes are induced when cells experience genotoxic stress. Nucleosome assembly and disassembly has not been extensively studied at the DDR genes. However, contributions of ATP-dependent chromatin remodelers and histone modifiers are important for DDR gene transcription [26,27,28]. Based on this information we suspected that histone chaperones mediating nucleosome assembly and disassembly could also play a role in the regulation of DDR gene transcription. RC nucleosome assembly is a major contributor to histone

deposition in a cell. We therefore predicted that the action of RC chaperones to assemble nucleosomes during replication contributes to gene regulation.

Histone chaperones

Histone chaperones are a family of proteins that associate with histones. They can bind to histones and escort them to sites of chromatin assembly. Histone chaperones catalyze nucleosome assembly without being part of the final product [10]. They are specific for histones H3-H4, H2A-H2B, or both. Asf1, CAF-1 and Rtt106 are the chaperones that are the focus of this thesis. They are H3-H4 chaperones which mediate histone deposition during RC nucleosome assembly [29]. The homology and functions of each are summarized in Table 1-1.

Histone chaperones: CAF-1 (Chromatin Assembly Factor 1)

CAF-1, an H3-H4 histone chaperone, is a key player in RC nucleosome assembly. It is highly conserved, consisting of three different subunits. These subunits are p150, p60, p48 in humans and Cac1, Cac2, Cac3 in yeast (Table 1-1). The removal of any subunit renders CAF-1 non-functional [30]. The experiments described in this thesis utilize a *cac1*Δ strain, so it is important to note that CAF-1 in this strain is not functional even though *CAC2* and *CAC3* are still present.

CAF-1 is essential in humans and when it is depleted by siRNA no RC nucleosome assembly is observed and cells arrest in S-phase [31]. Yeast cells lacking any or all of the CAF-1 subunits are viable, however they are sensitive to genotoxins [32]. During RC nucleosome assembly, the replication processivity factor proliferating cell nuclear antigen (PCNA) recruits CAF-1 to sites of replication by interacting with Cac1 [33]. This positions CAF-1 to deposit H3-H4 onto newly synthesized DNA behind the replication fork. During RC nucleosome assembly, Asf1 first transfers newly synthesized H3-H4 to CAF-1, presumably through an interaction

with the Cac2 subunit [34] (Figure 1-1). CAF-1 preferentially binds to newly synthesized H3-H4 as opposed to parental H3-H4 because of an interaction with the acetylated lysine 56 in newly synthesized H3 [6].

In addition to its role in RC nucleosome assembly, CAF-1 is also involved in centromere segregation [35]. Both CAF-1 and another histone chaperone, the histone regulation (HIR) complex, localize to centromeric DNA. Moreover, when a subunit of CAF-1 is deleted along with a subunit of HIR, centromeres are miss-segregated [35]. Not only does this effect indicate other roles for histone chaperones besides assembling nucleosomes, it also suggests that CAF-1 specifically has a broader function than originally suspected.

CAF-1 is important for a cells recovery from DNA damage. Studies have demonstrated that in response to double strand breaks (DSBs), CAF-1 is important for signalling the completion of repair so the DNA damage checkpoint returns to its inactive state and the cells can progress through the cell cycle [36]. Moreover, CAF-1 is recruited to chromatin upon UV treatment to sites of DNA damage. A recent study has suggested that CAF-1 functions with Asf1 in assembling nucleosomes during repair, and that the K56Ac mark on the newly incorporated histone H3 which specifically signals the completion of repair [37].

Heterochromatin-like silencing involves the orderly binding of silencing information regulator (SIR) proteins, which spread over a specific region to be silenced and ultimately result in a chromatin structure that is inhibitory to transcription [38]. Studies of transcriptional silencing provide hints that CAF-1 may play a role in transcriptional regulation. This is because itself along with Rtt106, Asf1, and HIR have been shown to be required for heterochromatin-like silencing at the silent mating type loci and telomeres [39, 30]. Asf1 and HIR play a role in transcriptional regulation, so the involvement of CAF-1 and Rtt106 in similar processes as Asf1 and HIR may suggest that they are also involved in transcriptional regulation.

Rtt106 interacts with silencing proteins Sir3 and Sir4, and CAF-1 along with Rtt106 are required for the binding and spreading of these Sir proteins along regions to be silenced [30].

Due to the wide variety of cellular processes CAF-1 is involved in, it is likely that inactivation of CAF-1 results in phenotypes not yet discovered. A recent study has investigated the possible role of CAF-1 in transcription, and it was demonstrated that CAF-1, like Asf1 and HIR , is recruited to actively transcribed genes [40]. This suggests that CAF-1 may be involved in transcriptional regulation. The studies described in this thesis also suggest that CAF-1 plays a role in transcriptional regulation.

Histone chaperones: Rtt106 (Regulator of Ty1 Transposition)

Rtt106 is a 51.6 kDa single subunit histone chaperone which specifically targets histones H3-H4. Rtt106 was only recently discovered by Li *et al.* to be a key player in RC nucleosome assembly [6]. Rtt106 functions in parallel with CAF-1 to deposit newly synthesized H3-H4 onto nascent DNA immediately behind the replication fork (Figure 1-1). Like CAF-1, Rtt106 preferentially binds newly synthesized H3-H4 as opposed to parental H3-H4, because of the H3K56Ac mark.

Rtt106 also plays a role in replication-independent (RI) nucleosome assembly, which is a term used to describe nucleosome assembly that is not coupled to replication. This was discovered because histone H3 is decreased at coding regions of actively transcribed genes in *rtt106*Δ cells [41]. In addition, Rtt106 has been found to interact with several transcription elongation factors and repress spurious transcription from cryptic promoters [41]. This is presumably through its role in RI nucleosome assembly during transcription. The fact that Rtt106 interacts with transcription elongation factors suggests that it may have a role in transcriptional regulation beyond preventing spurious transcription from cryptic promoters. Because CAF-1 functions in parallel with Rtt106 during

RC nucleosome assembly, perhaps CAF-1 is also involved in preventing transcription from cryptic promoters and maybe transcriptional regulation.

Other functions of Rtt106 include its role downstream of Asf1 and HIR to repress the histone genes. In the absence of Rtt106, histone gene expression increases[42]. More recently, it was found that Rtt106 binds to and recruits SWI/SNF and RSC to histone genes. SWI/SNF and RSC are the chromatin remodelers that control the cell cycle regulation of the histone genes and consistent with recruitment by Rtt106, the histone gene *HTA1* was constitutively expressed throughout the cell cycle in *rtt106*Δ cells [43]. As previously described, Rtt106 also has an important role in transcriptional silencing. Along with CAF-1, Rtt106 is important for the recruitment and spreading of SIR proteins along the region to be silenced [39,44, 30].

While thus far Rtt106 seems to be fungal specific, the newly discovered mammalian histone chaperone DAXX may be a homolog [45].

Histone chaperones: Asf1 (Anti Silencing Factor 1) & H3K56Ac

Asf1 is a single subunit, H3-H4 histone chaperone that is 31.6 kDa. It is a well conserved protein and humans contain Asf1a and Asf1b, which together make up similar functions to Asf1 in yeast (Table 1-1) [46]. Yeast Asf1 plays a central role in RC nucleosome assembly, which is to transfer newly synthesized H3-H4 dimers to CAF-1 or Rtt106. These chaperones subsequently deposit H3-H4 onto nascent DNA behind the replication fork (Figure 1-1). Asf1 is also required for RC nucleosome assembly in vertebrate and human cells [8,46]. Recent evidence suggests that Asf1 in higher organisms may also disrupt nucleosomes ahead of the replication fork and shift parental H3-H4 to the nascent DNA behind the replication fork [8].

In addition to RC nucleosome assembly, Asf1 plays an important role in RI nucleosome assembly, which occurs during repair and

transcription [47,48,49]. During transcription, Asf1 interacts with all four proteins making up the HIR complex and together they deposit histones onto DNA [50]. Asf1 not only associates with actively transcribed gene promoters and coding regions, it also promotes nucleosome disassembly and reassembly during RNAP II elongation [48]. Moreover, Asf1, HIR, and Rtt106 repress transcription from cryptic promoters within coding regions of genes [51, 48]. DNA repair mechanisms require nucleosome assembly, and both Asf1 and CAF-1 are involved in this process [3,52]. Since the recent discovery of the H3K56Ac post-translational modification, it has been suggested that H3K56Ac on newly assembled chromatin is critical for signalling completion of repair. Thus the importance of Asf1 in promoting repair is a consequence of its requirement for H3K56Ac [37,36].

Transcription of histone genes is stringently regulated to ensure proper amounts of histone protein during DNA replication. It was discovered that Asf1 and HIR together promote a chromatin structure that represses transcription of the histone genes outside of S phase [53]. This repression functions upstream of histone gene regulation by Rtt106.

Like Rtt106 and CAF-1, Asf1 is also involved in heterochromatin-like silencing. Cells lacking Asf1 have defective silencing at the silent mating type locus and at telomeres, likely through its role in chromatin assembly [54,55].

Although Asf1 is important for chromatin assembly, DNA from cells lacking *ASF1* is less sensitive to micrococcal nuclease (MNase) digestion than DNA from wild type cells [32]. Because MNase digests linker DNA not protected by nucleosome core particles, decreased MNase digestion indicates increased nucleosome density. This suggests that Asf1 is also important for chromatin disassembly. It has been demonstrated that Asf1 is important for nucleosome disassembly during transcriptional activation [21, 56]. The ability of Asf1 to disassemble nucleosomes may be through its ability to bind H3-H4 at the tetramerization interface, which disrupts H3-H4

tetramers into dimers [57]. Recent evidence however, demonstrates that Asf1 cannot disassemble nucleosomes itself, so other factors bay be required [58].

Because Asf1 is involved in such a wide variety of cellular processes, it is curious that yeast cells lacking Asf1 are viable. They are however, more sensitive to genotoxins [47]. Moreover, the lack of Asf1 results in a less stable genome, thus activating the DNA damage checkpoint [59, 36].

As noted above, Asf1 is essential for the acetylation of lysine 56 on histone H3. Acetylation of lysine residues results in the removal of a positive charge. When histone proteins are acetylated their interaction with negatively charged DNA is weakened, promoting transcriptional initiation. Thus histone acetylation is indicative of a chromatin structure promoting transcription while histone deacetylation is indicative of a chromatin structure inhibiting transcription. Likewise, highly expressed regions of DNA are commonly hyperacetylated while silenced regions are hypoacetylated [7, 60].

Acetylation of specific residues can be identified on newly synthesized histones incorporated onto DNA during replication. H3K56Ac occurs in the globular core domain and is a well-conserved post-translational modification among eukaryotes [61,25]. This modification is present on all newly synthesized H3-H4, and thus is suggested to act as a mark so RC chaperones can distinguish between newly synthesized histones and parental histones during replication [8]. Moreover, H3K56Ac increases the affinity of CAF-1 and Rtt106 for newly synthesized H3-H4 containing this modification [6].

The incorporation of an acetyl group onto a lysine residue is achieved by a class of enzymes called lysine acetyl transferases (KATs), and removal of an acetyl group by histone deacetylases (HDACs). In

budding yeast, the KAT Rtt109 acetylates H3K56, whereas the histone chaperones Asf1 and Vps75 are required to stimulate this reaction [62]. H3K56Ac is of intense interest because it has been recently demonstrated to play an important role in recovery from DNA damage [37]. This is because newly synthesized histones with the H3K56Ac modification are assembled into nucleosomes during repair, signalling that repair is complete and relieving the cell from the DNA damage checkpoint [37].

Interestingly, the KAT activity of the acetyltransferase Gcn5 may be important for RC nucleosome assembly, like that of Rtt109. Gcn5 acetylates multiple lysine residues on the N-terminal tail domain of histone H3. These modifications can increase the affinity of newly synthesized H3 for CAF-1 but not for Rtt106 [63]. These findings may suggest divergent functions for CAF-1 and Rtt106 in RC nucleosome assembly.

Replication-Coupled (RC) nucleosome assembly

A highly accurate duplication of DNA sequence and epigenetic information must be ensured during DNA replication. RC nucleosome assembly is the packaging of newly replicated DNA into nucleosomes behind the replication fork. This process is mediated by three H3-H4-specific RC chaperones; Asf1, CAF-1 and Rtt106. These chaperones are recruited to replication forks through interactions with the replication machinery [64,65,33]. They ensure a steady supply of histones to the replication fork and assemble nucleosomes on the nascent DNA strands.

RC nucleosome assembly is an essential process which occurs when DNA is replicated during S phase of the cell cycle [66]. To facilitate replication, nucleosomes ahead of the replication fork must be disassembled to accommodate passage of the replication machinery. On the nascent DNA behind the replication fork nucleosomes are immediately assembled to re-establish chromatin structure. The histones used in the assembly of these nucleosomes come from two possible sources: newly

synthesized histones which have not previously been incorporated into DNA, and parental histones which existed in nucleosomes on the parental DNA and have been shifted behind the replication fork to the nascent DNA strands [66]. Because there are two sources of histone proteins there are also two pathways of histone deposition mediated by the RC chaperones.

Parental histones are shifted from DNA ahead of the replication fork and randomly deposited onto the nascent DNA strands. As such, each daughter strand contains on average 50% parental histones. This recycling of parental histones presents a possible method to accurately re-establish parental chromatin structure onto daughter DNA, which is central to epigenetic inheritance [67]. It is yet to be determined how histones are shifted onto the nascent DNA strands behind the replication fork, however there is evidence suggesting that Asf1 is involved in this process in mammals [8]. Another histone chaperone, facilitates chromatin transcription (FACT), is also involved in parental histone recycling [68]. FACT is recruited to replication forks by the MCM helicase, disassembles nucleosomes ahead of the replication fork by interacting with histones H2A-H2B and shifts them to the nascent DNA behind the replication fork [69].

Newly synthesized histones are deposited through a pathway called *de novo* nucleosome assembly, which is better understood than parental histone recycling. *De novo* nucleosome assembly involves a sequential series of events. Newly synthesized histones contain characteristic modifications, H3K56Ac is one of particular importance [6]. Asf1, CAF-1, and Rtt106 coordinate *de novo* nucleosome assembly, however the exact mechanisms remain unclear. Current data suggest a model which begins with Asf1. Asf1 binds to H3-H4 dimers and promotes acetylation of K56 on histone H3 by Rtt109 [70]. Asf1 then transfers H3-H4 with the H3K56 post-translational modification to CAF-1 or Rtt106 (Figure 1-1). The mechanism of this transfer has yet to be determined. However, it is clear that Asf1 can only bind H3-H4 dimers since it binds the tetramerization interface [10].

Once H3-H4 dimers are transferred to CAF-1 or Rtt106, they are directly deposited onto nascent DNA behind the replication fork (Figure 1-1). Detailed information about the mechanism of H3-H4 deposition behind the replication fork is not available. Whether CAF-1 and Rtt106 deposit H3-H4 dimers or tetramers onto DNA remains unclear [71]. Moreover any functional differences between CAF-1 and Rtt106 in RC nucleosome assembly are yet to be determined.

The interaction of RC chaperones with specific post-translationally modified histones is central to the role of RC chaperones in DNA replication. Specific modifications are present on newly synthesized H3-H4, which enhance binding to the RC chaperones and differentiates between newly synthesized histones and parental histones [6,63,8]. H3K56Ac is an example of such a modification. Other characteristic modifications to newly synthesized histones are acetylation of lysine 5, 12 [72], and 91 [73] on H4 and acetylation of lysine 9, 27 [74,63], and 56 [75] on H3.

Replication independent (RI) nucleosome assembly

Nucleosome assembly is not just coupled to DNA replication. It also occurs during DNA repair or transcription in a process called replication-independent (RI) nucleosome assembly. RI nucleosome assembly utilizes different histone chaperones than RC nucleosome assembly, however Asf1 and Rtt106 function in both processes.

Genotoxic stress

DNA replication occurs during S phase of the cell cycle and is a particularly important time for cells because they must ensure the accurate duplication of DNA and epigenetic information. Genotoxic stress is a term used to describe a situation where DNA replication or genome integrity is threatened [76]. Genotoxins are substances which cause DNA damage or replication stress and thus threaten genome integrity. Genotoxins used for experiments in this thesis include hydroxyurea (HU), and methyl

methanesulfonate (MMS). HU reversibly inhibits the enzyme ribonucleotide reductase, thus preventing production of deoxyribonucleotide triphosphates (dNTPs) [77]. As a result of depleted dNTP levels, replication forks stall and the replication checkpoint is activated. MMS is commonly used to generate chromosome lesions and study cellular responses to DNA damage [78]. It is a methylating agent which directly adds a methyl group to DNA bases, resulting in single and double strand breaks[79, 80]. Nicotinamide (NAM) is also used, however its genotoxicity is due to effects on H3K56Ac. NAM noncompetitively inhibits Sir2, a protein involved in heterochromatin-like silencing [81,82]. Sir2 also has sirtuin deacetylase activity, as does the Sir2-related Hst3 and Hst4 histone deacetylases. Hst3 and Hst4 are important for the deacetylation of H3K56 following S phase of the cell cycle. NAM is an inhibitor of sirtuin deacetylase activity, thus treatment with NAM results in impaired deacetylation of H3K56 [83,84].

DNA structure checkpoints

Replication fork progression is not only impeded by nucleosomes, but also by chromosomal lesions caused by genotoxins, and by dNTP depletion caused by HU [85]. Surveillance systems present in the cell detect DNA damage or replication stress, which can threaten genome integrity. These are part of a cellular response known as checkpoints, and include the DNA damage checkpoint which responds to DNA damage (such as double strand breaks), and the replication checkpoint which responds to compromised DNA replication [86]. Collectively these checkpoints are known as the DNA structure checkpoints [87]. Both checkpoints utilize many of the same sensor, transducer and effector proteins involved in the complex signalling cascade that occurs upon checkpoint activation. Therefore the DNA damage and replication checkpoints elicit similar responses upon activation [86]. One major difference between these checkpoints is the phase of the cell cycle in which the checkpoint is active. The replication

checkpoint is activated during S phase in response to replication stress while the DNA damage checkpoint is activated anytime when chromosome lesions are recognized [87].

The DDR genes *HUG1* and *RNR3* are induced by activation of either DNA structure checkpoint [88]. Because HU is used to study *HUG1* and *RNR3* dynamics in this thesis, and HU treatment induces replication stress, only the replication checkpoint will be described in detail.

Replication checkpoint

There are three important components to checkpoints; sensors to sense stalled replication forks, transducers to perpetuate the signal, and effectors which have specific targets to elicit a response [86]. Central to the replication checkpoint is the sensing of defective replication by a protein kinase called mitotic entry checkpoint 1 (Mec1). Single stranded DNA at replication forks is bound by replication protein A (RPA). When replication forks stall, a protein called DNA damage checkpoint 2 (Ddc2) binds RPA and recruits Mec1 [89]. This “sensing” of defective replication is the first step in a complex signalling cascade. Figure 1-2 (adapted from Labib and De Piccoli (2011) [85]) illustrates a simplified replication checkpoint pathway in *S. cerevisiae*. Radiation sensitive 53 (Rad53) is an effector kinase which propagates the replication stress signal by activating relevant downstream targets. The replication checkpoint and DNA damage checkpoint both rely on Rad53, however each checkpoint utilizes different factors for sensing and mediating signal propagation [85]. Rad53 is phosphorylated at many sites, and it is suspected that differential phosphorylation directs activation of various checkpoints [90]. For example, arresting cells by treating with a drug called nocodazole does not illicit a DNA damage response, however Rad53 becomes phosphorylated [91]. As will be described later, nocodazole disrupts microtubule polymerization resulting in arrest in G2/M phase. In terms of the replication checkpoint, Rad53 becomes hyperphosphorylated by autophosphorylation in response to replication stress [88]. Rad53

phosphorylation is commonly used as a marker for activation of the DNA damage checkpoint [92].

One important downstream target of Rad53 relevant to this thesis is DNA damage uninducible 1 (Dun1), which is phosphorylated and activated by Rad53 [88]. Once activated, Dun1 phosphorylates and inhibits the primary repressor for the DDR genes *HUG1* and *RNR3*, constitutive RNR transcription 1 (Crt1). Induction of these DDR genes is important for maintaining dNTP levels [86]. Rad53 has downstream targets other than Dun1, which are important for cell survival during DNA damage and replication stress [93]. In addition to the induction of *HUG1* and *RNR3*, the replication checkpoint also serves to ensure replication fork stability, slows S phase progression and inhibits late firing of replication origins [77, 94].

Structure and regulation of *HUG1*

HU, UV and gamma radiation induced (Hug1) is a small protein of a molecular weight of 7.5 kDa. It is expressed when the DNA structure checkpoints are activated by DNA damage or replication stress [95]. Hug1 is so strongly induced under these conditions that it is suggested to be a better candidate for a GFP-fusion DNA damage detection system than Rad52, Rad54, or Rnr2, which are currently used [96]. Despite the strong expression of *HUG1* by DNA damage or replication stress, *hug1*Δ cells are not particularly sensitive to any genotoxin [97].

Although the function of Hug1 is unknown, it is suspected to regulate RNR activity due to its sequence similarity with two proteins that regulate RNR activity, Dif1 and Sml1. Further evidence for the role of Hug1 in regulating RNR activity is the elevated dNTP levels in *hug1*Δ cells [97].

HUG1 transcription is regulated by a repressor called constitutive *RNR* transcription (Crt1) which binds to three sites at its promoter. A simplified illustration of *HUG1* regulation is presented in Figure 1-3. Crt1

functions downstream of the Mec1-Rad53 signal transduction pathway that is activated by DNA structure checkpoint activation. In response to checkpoint activation, Dun1 hyperphosphorylates Crt1, resulting in its removal from the *HUG1* promoter [88]. Recent evidence suggests that *HUG1* is regulated by another repressor in addition to Crt1[98]. This repressor is Mig3, which primarily functions with Mig1 and Mig2 to regulate the expression of a protein involved in sucrose hydrolysis [99,98]. Mig3 binding at the *HUG1* promoter is reduced during *HUG1* induction during ultraviolet (UV) radiation [98]. While this study by Wade *et al.* (2009) provides a first insight into a potential alternate regulation of *HUG1* by Mig3, it does not investigate the role of Rad53 in this regulation. This is important because Crt1 is a strong repressor of HUG1 and its regulation is dependent on Rad53. Therefore, it is important to understand how the DNA structure checkpoint signalling cascade contributes to Mig3 regulation of *HUG1.*

Structure and regulation of *RNR3*

RNR3 is a gene that is also induced in response to DNA structure checkpoint activation. It is cell cycle regulated to be expressed in S phase [100]. Rnr3 is one subunit in ribonucleotide reductase (RNR), which is an enzyme complex that catalyzes the formation of dNTPs. This reaction is finely tuned to ensure a specific level of dNTPs during DNA replication [101]. Like *HUG1, RNR3* is also regulated by Crt1. In response to DNA structure checkpoint activation, Crt1 is hyperphosphorylated and no longer binds the *RNR3* promoter [88]. More recent data has revealed that Rox1 and Mot3 are also repressors of *RNR3* [102]. Rox1 directly binds Ssn6, which is part of the Ssn6-Tup1 global corepressor complex. Collectively, Klinkenbeg *et al.* (2006) have discovered that Crt1, Rox1 and Mot3 function synergistically to regulate *RNR3* transcription. This provides the cell with more intricate control of *RNR3* transcription, depending on the stresses it experiences.

The binding of Rox1 and Mot3 to the *RNR3* promoter was not investigated in this thesis because we primarily focused on the regulation of *HUG1*.

Histone acetylases (HATs) and deacetylases (HDACs) can aid in the activation and repression of genes by acetylating and deacetylating histones, respectively [7]. Rpd3 and Hos2 are HDACs which are important for the activation of *HUG1* and *RNR3*. It is proposed that these HDACs reset the acetylation balance at active promoters of these DDR genes which promotes multiple rounds of transcription [28].

Crt1 repressor and Ssn6-Tup1 corepressors

The regulation of *HUG1* and *RNR3* is dependent on the repressor Crt1, which represses several genes including *RNR4, RNR3, RNR2, HUG1,* and itself [103]. Other novel Crt1 regulated genes have been identified based on the presence of a consensus sequence for the repressor in their promoters [103]. Crt1 was extensively studied by Huang and Zhou, (1998) [88]. In summary, they found that Crt1 functions downstream of Dun1 in the Mec1-Rad53 signalling pathway following DNA structure checkpoint activation. Crt1 is a homolog of the mammalian RFX DNA binding proteins and binds to similar DNA sequences called X-box like consensus sequences that can be strongly (Xs) or weakly (Xw) conserved. In response to DNA damage, Crt1 is hyperphosphorylated by Dun1 and it no longer binds to its consensus sequence (for example at the *HUG1* promoter). Moreover, the level of Crt1 phosphorylation is dependent on the type and extent of DNA damage. Importantly, Crt1 is self regulated and thus its transcription is induced by DNA damage or replication stress. Crt1 therefore is part of a negative feedback pathway in which DDR genes can be quickly repressed following inactivation of the DNA structure checkpoints.

Crt1 represses the transcription of *HUG1, RNR3,* and other genes it regulates by recruiting the global corepressors Ssn6-Tup1 [88,104,105]. Ssn6-

Tup1 do not directly bind DNA, but are specifically recruited to target genes by DNA binding proteins. Ssn6-Tup1 repress target genes by three mechanisms. First, they influence nucleosome positioning by the ISW2 chromatin remodeling complex to generate repressive chromatin structure [106]. Tup1 also interacts with the N-terminal domain of H3 and H4 to position nucleosomes. Second, they recruit histone deacetylases (such as HDA1), which results in hypoacetylated promoter histones, promoting repressive chromatin structure. HDA1 deacetylates lysine 9 and 14 on histone H3 at *RNR3*. Third, Ssn6-Tup1 interfere with the binding of general transcription factors such as Mediator [104]. As Zhang and Reese (2004) [104] demonstrated, *RNR3* and *HUG1* are significantly derepressed in cells lacking ISW2 and HDA1, even though Tup1 is still bound to the promoters. Thus Ssn6-Tup1 require chromatin remodeling and histone deacetylation to repress these DDR genes.

Although Crt1 is critical for the repression of *HUG1* and *RNR3,* it also plays a role in the activation of these genes. The N-terminal domain of Crt1 is the repression domain which recruits Ssn6-Tup1. Also at the N-terminal domain, but separate from the repression domain, is a region that recruits TFIID and SWI/SNF, which are important factors in DDR gene induction [107]. SWI/SNF is a chromatin remodeler important for the activation of *HUG1* and RNR3 [26,108], and TFIID is important for transcriptional initiation. Therefore, Crt1 functions primarily as a repressor, but also an activator.

The necessity of nucleosome positioning in repression by Ssn6-Tup1 has been demonstrated at *RNR3*. By depleting promoter nucleosomes, *RNR3* was constitutively transcribed during unperturbed growth [108]. This regulation has not yet been demonstrated at *HUG1*. However, due to the similarities in the structure and regulation of these DDR genes, it is possible that depletion of promoter nucleosomes at *HUG1* is sufficient to promote transcription.

1-2: Research overview

Nucleosome assembly is an essential process that is mediated by histone chaperones. Histone chaperones are widely studied because of their involvement in replication, repair, and transcription. Replication utilizes a specific set of histone chaperones including Asf1, Rtt106 and CAF-1, which specifically mediate deposition of newly synthesized H3-H4. Understanding the details of RC nucleosome assembly is of great interest because the accurate duplication of DNA sequence and epigenetic information is central to preventing the accumulation of mutations and disease. The RC chaperones are involved in heterochromatin-like silencing at telomeres and the silent mating loci in budding yeast [39,54]. Asf1 is also involved in transcriptional regulation of *ARG1, PHO5, PHO8, HUG1*, and *RNR3* genes [21,109,110], and Rtt106 is important for preventing transcription from cryptic promoters [41]. Based on this information we predicted that CAF-1 and Rtt106 could also contribute to transcriptional regulation.

Specifically, we hypothesized that the RC chaperones contribute to the transcriptional regulation of *HUG1* due to the important role they play in RC nucleosome assembly and therefore chromatin structure. Collectively, the research in this thesis provides evidence that CAF-1 and Rtt106 contribute to the repression of *HUG1* in a way that is unrelated to its normal repression requiring Crt1. Interestingly, this repression does not involve Asf1, even though Asf1 functions upstream of these chaperones during RC nucleosome assembly. This suggests divergent functions of the RC chaperones that differently affect the regulation of *HUG1*. These divergent functions of the RC chaperones also have opposing roles in promoting survival during prolonged replication stress.

Table 1-1: Histone chaperones involved in RC nucleosome assembly

S. cerevisiae	***H. sapiens***	**Function**
Asf1	Asf1a, Asf1b	RC and RI Nucleosome assembly, nucleosome disassembly, silencing, H3K56Ac
CAF-1 (Cac1, Cac2, Cac3)	p150, p60, p48	RC nucleosome assembly, silencing
Rtt106	DAXX?	RC and RI Nucleosome assembly, silencing

Table 1-2: Proteins involved in the replication checkpoint

S. cerevisiae	***H. sapiens***	**Function**
Mec1	ATR	Senses DNA damage and replication stress
Mrc1	Claspin	Adapter in replication stress signal
Rad53	Chk2	Effector kinase in checkpoint signal transduction
Dun1		Protein kinase activated by hyperphosphorylated Rad53
Crt1	RFX1	Repressor for *RNR3* and *HUG1,* inhibited by active Dun1

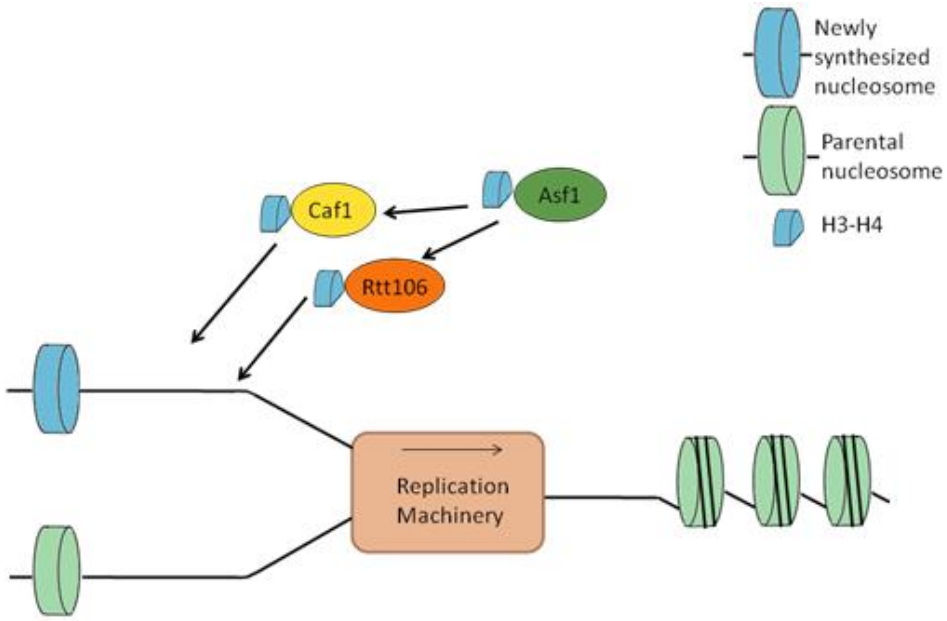


Figure 1-1: Order of newly synthesized H3-H4 deposition in replication-coupled nucleosome assembly. H3-H4 is transferred from Asf1 to CAF-1 or Rtt106, which subsequently deposit H3-H4 directly onto nascent DNA behind the replication fork.

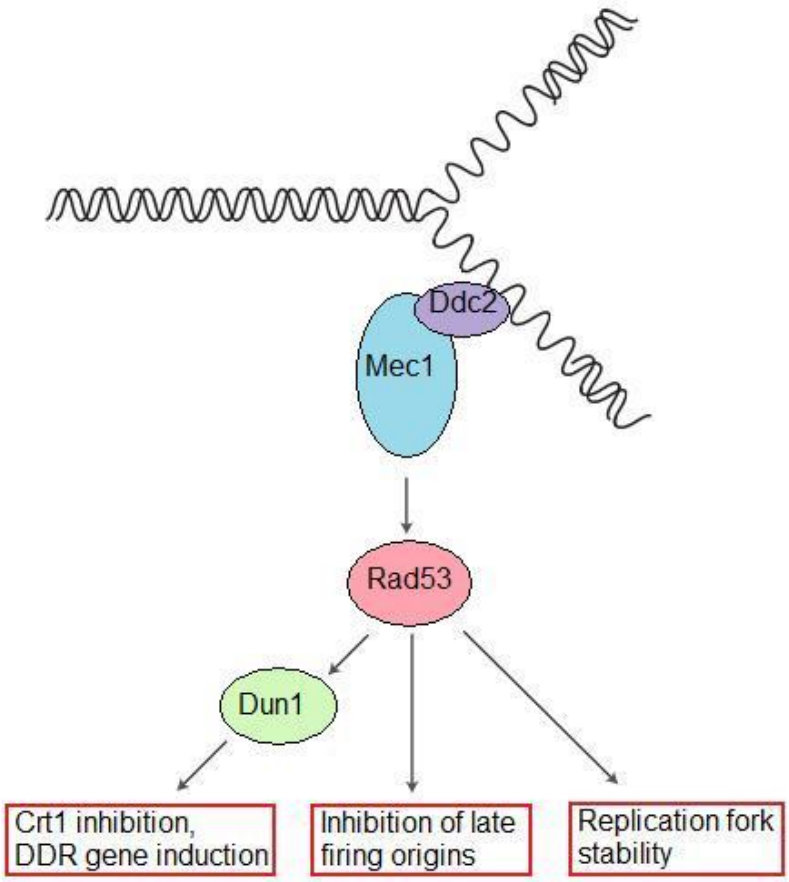


Figure 1-2: Replication checkpoint pathway in *S. cerevisiae.* Replication stress is sensed by Mec1 which is recruited to single stranded DNA by Ddc2. Mec1 phosphorylates Rad53, which results in the phosphorylation of Dun1 and Crt1. Outcomes of replication checkpoint activation are the induction of DDR genes, inhibition of late replication firing origins and stabilization of the replication fork. This figure is modified from Labib and De Piccoli (2011) [85].

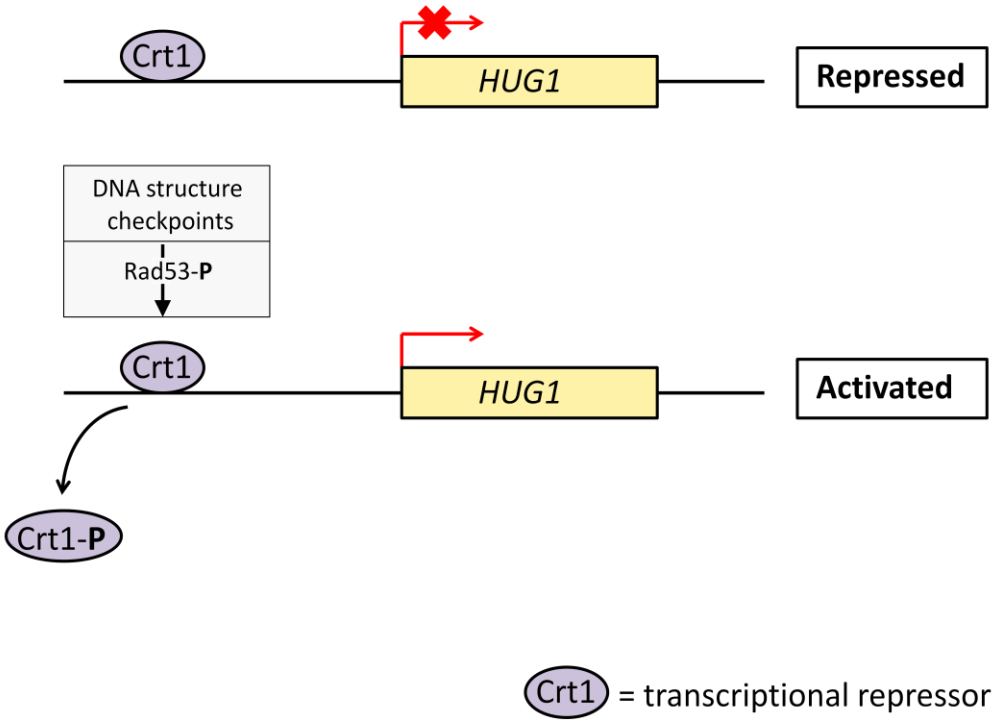


Figure 1-3: Regulation of *HUG1*. Under repressive conditions Crt1 is bound to the *HUG1* promoter. Activation of the DNA structure checkpoints results in the hyperphosphorylation of Rad53. Crt1 is phosphorylated and no longer binds the *HUG1* promoter resulting in the derepression of *HUG1.*

Chapter 2:

Materials and methods

Chemicals and equipment

Unless otherwise specified, all chemicals were obtained from Fisher Scientific, Invitrogen or Sigma. For Chromatin Immunoprecipitation experiments, cells were lysed using a mini-beadbeater-16 from Biospec Products and DNA was sheared using a Branson Sonifier. Real-Time PCR analysis was carried out using a BioRad iCycler. An Image Quant 300 was used to visualize ethidium-bromide stained PCR products.

Yeast strains, cloning and media

The *S. cerevisiae* strains used in this study were derived from W303-1A and BY4741, and are described in Table 2-1. The following strains were received from Dr. Z. Zhang[6]: W303-1A, *cac1*Δ, *rtt106*Δ, *cac1*Δ *rtt106*Δ. Gene deletions and epitope tagging were accomplished by the one-step integration of a PCR product derived from previously described plasmids[111]. Briefly, cells grown to mid-log phase were washed with sterile water and resuspended in 1x TE/LiAc. Fish sperm carrier DNA (freshly boiled and cooled on ice), approximately 15µg of the PCR product and PEG were added to the cells and samples incubated at 30^{o}C for 2-5 hours. Following the incubation 10% (v/v) DMSO was added and cells heat-shocked at 42^{o}C for 15 minutes. Cells were allowed to recover in YPD for 8 hours at room temperature and then plated on agar medium containing appropriate selective markers to select for transformants. Potential transformants were verified by PCR using multiple primer sets followed by agarose gel electrophoresis. Protein tagging and gene deletions were also verified by Western blot (whenever possible).

All strains were grown in YPD (2% (w/v) yeast extract, 1% (w/v) peptone, 95% (v/v) water, 5% (v/v) 2% dextrose). Media, and reagents added to media are described in relevant figure legends.

Agarose gel electrophoresis

1% agarose gels were made using Ultrapure Electrophoresis Grade Agarose in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and ethidium bromide. Samples were prepared in 6X Orange DNA Loading Dye (Fermentas) and run alongside a GeneRuler 1kb Plus DNA Ladder (Fermentas) to determine PCR product sizes. For cloning, appropriate DNA bands were excised and purified using a QIAquick gel extraction kit (Qiagen).

Spot assay for measuring sensitivity to replication stress inducers and DNA damaging agents

Cells were grown to log phase and diluted to an OD_{600} of 0.1. From this, 10X serial dilutions were made and spotted onto YPD agar medium containing no additional chemical, HU, MMS, or NAM at concentrations indicated in the appropriate figures legends.

For the HU recovery experiment (Figure 4-4), cells were diluted to OD_{600} 0.5 in YPD containing 0 mM, 50 mM or 200 mM HU and grown at 30^{o} for 24 hours. Cells were then harvested by centrifugation, diluted to OD_{600} of 0.1 in sterile water and spotted onto YPD agar medium in 10X serial dilutions.

Growth curves

Cells were grown to mid-log phase and diluted to OD_{600} 0.5 in YPD containing 0 mM or 200 mM HU and grown at $30^{o}C$. OD_{600} was taken every 2 hours for 12 consecutive hours. Experiments were repeated in duplicate.

Nocodazole and HU treatments

Unless otherwise noted, cells treated with HU were treated at 200 mM concentrations. HU treatment for the growth curves and spot assays was accomplished by pre-dissolving HU into the media, while all other HU treatments were accomplished by adding the appropriate amount of solid

HU directly to the cell culture to yield the desired final HU concentration.

Nocodazole stock was prepared in DMSO at a concentration of 5 mg/ml and stored at -20^{o}C. Cells were treated with nocodazole by adding the appropriate volume of nocodazole stock directly to the cell culture to yield a final concentration of 10 µg/ml. As a control, identical cell cultures were treated with the same volume of DMSO.

Western Blot

To isolate total protein using the TCA precipitation method, cells were grown to OD_{600} 0.5-1.0 then pelleted by centrifugation and frozen in liquid nitrogen. Treatments to cells (if any) are indicated in the appropriate figure legends. Cells were disrupted using β-mercaptoethanol/NaOH and total protein precipitated using TCA. The resulting protein pellet was washed with acetone and resuspended in SDS-PAGE sample buffer. Cell equivalents were loaded onto SDS-PAGE gels (unless otherwise noted), and run alongside BioRad molecular weight markers. SDS-PAGE was carried out in 6% (Rad53), 12% (Asf1-MYC, Crt1-HA and CPY), or 15% (H3, H3K56Ac, and penta-Ac-H4) polyacrylamide to effectively resolve the proteins of interest. Following electrophoresis proteins were transferred to nitrocellulose membrane using a semi-dry transfer apparatus. The membranes were then blocked for 1-4 hours at room temperature using 4% (w/v) BSA in TBST (10 mM Tris, 140 mM NaCl, 0.1% (v/v) Tween20). After blocking, membranes were incubated overnight at 4^{o} C in primary antibody diluted to concentrations recommended in manufacturer's instructions with 4% BSA in TBST. Membranes were then washed in TBST and incubated for 1-2 hours at room temperature with the corresponding secondary antibody conjugated to HRP. After a final washing in TBST, protein-antibody complexes were visualized by rinsing membranes in enhanced chemiluminescent agent (ECL) from GE Healthcare and exposing to photo-sensitive film.

Primary antibodies used were as follows: α-Rad53 (yC-19, Santa Cruz #sc-6749), α-H3 (Abcam #ab1791), α-actin (Millipore #MAB1501), α-myc (Millipore #9E10), α-H3K56Ac (ActivMotif), α-CPY (Millipore #AB1817), α-HA (Roche, #12CA5), α-penta-acetylated H4 (Upstate #06-946).

Chromatin fractionation

Performed as described by Parnas *et al.* (2009)[112], except that potassium chloride concentration in all solutions was lowered to 50 mM. Briefly, mid-log phase cells were harvested by centrifugation and treated with a 0.1% sodium azide solution for 10 minutes. Treatments (if any) prior to cell harvest are described in appropriate figure legends. Cells were then pelleted and resuspended in a solution containing 50 mM potassium phosphate and 0.6M sorbitol. Cells were spheroplasted using zymolase T-100 then lysed using triton X-100, yielding the whole cell extract (WCE). The WCE was separated into soluble (SUP) and chromatin-associated (CHR) fractions by centrifugation over a 60% sucrose layer. Following centrifugation the SUP fraction remains above the sucrose layer while the CHR fraction is pelleted below the sucrose layer.

Following the fractionation, protein concentration was determined using a Protein Assay dye reagent from Bio-Rad, after which samples were diluted to 2 µg/µl and equal volumes were loaded onto SDS-PAGE gel lanes. Proteins in each fraction were visualized by Western blot. Penta-Ac-H4 is only found in the chromatin fraction and Carboxypeptidase Y only in the supernatant fraction [113], thus each serve as controls to ensure the fractionation was successful.

Total RNA isolation

Total RNA was isolated using a hot phenol extraction method described in Friis *et al.* (2009) [114]. Briefly, cells were pelleted by

centrifugation and resuspended in AE buffer (50 mM sodium acetate, 10 mM EDTA). 10% SDS was added along with AE-buffered phenol and cells were incubated at 65^{o}C for 10 minutes with periodic vortexing. This was followed by flash freezing in a dry ice/ethanol bath and centrifugation at maximum speed. The upper aqueous phase was transferred to a new tube with AE buffered phenol, chloroform and isoamyl alcohol (24:24:1). The mixture was again centrifuged at maximum speed and the upper aqueous layer was transferred to a new tube along with 100% ethanol and 3M sodium acetate. RNA was precipitated at -20^{o}C over night. RNA was then pelleted by centrifugation at maximum speed, washed with 70% ethanol, air dried for 2 hours and finally resuspended in DEPC-treated water.

Total RNA in each sample was measured by reading the optical density at OD_{260} and 0.2 µg/µl dilutions were made. cDNA was generated using qScript cDNA Supermix (Quanta) according to manufacturer's instructions, using 0.5 µg RNA in each 10 µl reaction. Following PCR, 10 µl reactions were diluted with 55 µl water and subsequently analyzed using RT-PCR. Details of PCR program are described in the "RT-PCR and analysis" section.

Expression of all genes was normalized to an internal region of *RDN18-1*, as described in the RT-PCR and analysis section. Primer sequences used in quantitative RT-PCR to analyse gene expression are shown in Table 2-2.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described by Aparicio *et al.* (2009) [115] with a few exceptions. Without pre-treating the cells with HU, formaldehyde was added to 200 ml cells (Crt1-HA ChIPs) to a final concentration of 1%, and cells cross-linked at room temperature for 20 minutes with constant rotation. 2.5 M glycine was then added and reactions incubated for 8 minutes at room temperature with constant rotation. Cells were harvested

by vacuum filtration over 0.22 µm polyethersulfone disks and washed twice with Tris buffered saline (TBS) (20 mM Tris, 150 mM NaCl), once with FA lysis buffer (FLB) (50 mM HEPES, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) and resulting cell pellets were frozen in liquid nitrogen and stored at -80^{o}C. For histone H3 and RNAP II ChIPs only 100 ml cells were harvested so volumes of formaldehyde, glycine, TBS and FLB were halved accordingly. When cells were pre-treated with HU or nocodazole, cells were recovered on the polyethersulfone disks by vacuum filtration and immediately resuspended in pre-conditioned media containing 1% formaldehyde (as described in[110]). Crosslinking and other steps in cell harvest were carried out as described above.

Cells were lysed using a mini bead-beater and silica-zirconia beads for 6X 3 minute cycles. DNA was sheared by sonication to an average fragment size of 500 bp. Resulting cell extracts were diluted in 4ml FLB and frozen in liquid nitrogen in 850 µl aliquots.

Immunoprecipitation conditions and antibody volumes are described in Table 2-4. Alternatively, no antibody was added (no antibody control) while all other conditions remained the same. Following immunoprecipitation, protein A-Sepharose beads were washed twice with FLB/0.15M NaCl, and once with each of FLB/0.5M NaCl, ChIP wash buffer, and 1X TE buffer pH 7.5. To elute proteins crosslinked to DNA, beads were resuspended in ChIP elution buffer and incubated at 65^{o}C for 10 minutes. Resulting eluate was then boiled for 10 minutes to reverse crosslinks and DNA purified using a QIAquick PCR Purification system (Qiagen), resuspended in a final volume of 50µl EB, and subsequently analysed by quantitative RT-PCR. Results were normalized to the total DNA prior to IP (termed ‘input’) in each respective sample, as described in the RT-PCR and analysis section. Primer sequences used for ChIP analysis are shown in Table 2-3.

RT-PCR and analysis

DNA was analyzed by quantitative RT-PCR using PerfeCT SYBR Green mix (Quanta) and all reactions performed in triplicate. Thermal cycling parameters are as follows:

1X 10min 95^{o}C

32X 30sec 95^{o}C, 30sec 55^{o}C, 1min 72^{o}C

1X 4min 72^{o}C

As previously described[115] results were analyzed in Microsoft Excel using the equation:

POWER(1.9, -C_t), where C_t is the net value of:

$C_{t\ experimental} - C_{t\ input}$ (ChIP), or

$C_{t\ experimental} - C_{t\ \textit{RDN18-1}}$ (RNA analysis)

All primers used for quantitative RT-PCR are described in Tables 2-2 and 2-3, and have been either previously published or tested to ensure an efficiency of 90-110%. The latter was achieved by amplifying (by quantitative RT-PCR) a 100x dilution series of known DNA concentration.

Most figures show averages of at least three independent experiments and error bars represent the standard deviation. In such cases, p values were calculated using Student's one- or two-tailed t test. In relevant figures, (*) indicates significance at $p<0.05$ compared to Wt control. Throughout the thesis p values were calculated using Student's two-tailed t test, except the nocodazole arrest and release experiments in Figures 3-6 and 3-7, in which a Student's one-tailed t test was used. As noted in the figure legends, some figures show averages of two independent experiments and error bars represent the range.

Flow cytometry

Cellular DNA content was determined as described by[116]. Briefly, cells were fixed using 70% methanol. Pre-treatment to cells (if any) is

described in relevant figure legends. After fixation, cells were treated with RNase and pepsin, and finally stained with propridium iodide. Cells were sonicated prior to being analyzed by a FACScan flow cytometer (Becton-Dickinson). Data files were analyzed and displayed in relevant figures using Cyflogic software.

Table 2-1: Strains used in this study

Strain	Genotype	Source
W303-1A	MATa *ade2-1 ura3-1 his3-11 15, trp1-1 leu2-3 112, can1-100*	[6]
*cac1*Δ	*cac1*Δ*::LEU2*	[6]
*rtt106*Δ	*rtt106*Δ*::kanMX6*	[6]
*cac1*Δ rtt106Δ	*cac1*Δ*::LEU2 rtt106*Δ*::kanMX6*	[6]
*asf1*Δ	*asf1*Δ*::kanMX6*	This study
*cac1*Δ rtt106Δ asf1Δ	*cac1*Δ*::LEU2* rtt106Δ::kanMX6 asf1Δ*::HIS3MX6*	This study
CRT1-HA	*CRT1-3HA::TRP1*	This study
*cac1*Δ *rtt106*Δ *CRT1-HA*	*cac1*Δ*::LEU2 rtt106*Δ*::kanMX6 CRT1-3HA::TRP1*	This study
*asf1*Δ *CRT1-HA*	*asf1*Δ*::kanMX6 CRT1-3HA::TRP1*	This study
ASF1-MYC	*ASF1-13MYC::HIS3*	L. Minard
*hir1*Δ	*hir1*Δ*::HIS3MX6*	This study
*hir2*Δ	*hir2*Δ*::HIS3MX6*	This study
*cac1*Δ *rtt106*Δ *hir1*Δ	*cac1*Δ*::LEU2 rtt106*Δ*::kanMX6 hir1*Δ*::HIS3MX6*	This study
*cac1*Δ *rtt106*Δ *hir2*Δ	*cac1*Δ*::LEU2 rtt106*Δ*::kanMX6 hir2*Δ*::HIS3MX6*	This study.

Table 2-2: Oligonucleotides used for gene expression

Oligonucleotide	Sequence	Source
HUG1 +2 F	5'- TGACCATGGACCAAGGCCTTA -3'	L. Minard
HUG1 +156 R	5'- GGCAATGATGTTGGCAGAAGG -3'	L. Minard
RNR3 +151 F	5'- GTTACTACCGTTGAGCTGGAC -3'	L. Minard
RNR3 +315 R	5'- AATCCAGTCGTGTAAATCCTC -3'	L. Minard
RDN18-1 F	5'- AATTAGAGTGTTCAAAGCAGG -3'	L. Minard
RDN18-1 R	5'- CTATTAATCATTACGATGGTCC -3'	L. Minard
ACT1 F	5'- TGTCCTTGTACTCTTCCGGT -3'	[119]
ACT1 R	5'- CCGGCCAAATCGATTCTCAA -3'	[119]
PHO5 F	5'- GTTTAAATCTGTTGTTTATTCA -3'	[119]
PHO5 R	5'- CCAATCTTGTCGACATCGGCTA -3'	[119]
CIN8 F	5'- GGCAACTCTCAGGTTGGATGTC -3'	This study
CIN8 R	5'- GTATCTCCCGTCGTGTTAATGG -3'	This study

Table 2-3: Oligonucleotides used for ChIP

Oligonucleotide	Sequence	Source
HUG1 -167 F	5'- AGGCAACTGATTCCCAGCATATA -3'	L. Minard
HUG1 +41 R	5'- GGAAGAATTGCTTTGGGTTAA -3'	L. Minard
RNR3 -179 F	5'- CGTTTTTCGTGTCAGCGTTC -3'	L. Minard
RNR3 +8 R	5'- ACGTACATTTGTGTGGGAG -3'	L. Minard
TELV F	5'- GGCTGTCAGAATATGGGGCCGTAGTA -3'	L. Minard
TELV R	5'- CACCCCGAAGCTGCTTTCACAATAC -3'	L. Minard
HUG1-promoter F	5'- TCCACAGCAACGAGAAACGA -3'	This study
HUG1-promoter R	5'- AGAACAAGAAAGGAAAGGAATG -3'	This study
RNR3-promoter F	5'- AGCAAGCCCTCGTTCTTGGCT -3'	This study
RNR3-promoter R	5'- TTTCCCATCTTGTCGTTTGGTCC -3'	This study
HUG1 +2 F	5'- TGACCATGGACCAAGGCCTTA -3'	L. Minard
HUG1 +156 R	5'- GGCAATGATGTTGGCAGAAGG -3'	L. Minard
PHO5 UASp2-A	5'- GAATAGGCAATCTCTAAATGAATCG -3'	[120]
PHO5 UASp2-B	5'- GAAAACAGGGACCAGAATCATAAAT -3'	Korber et al., 2006)

Table 2-4: Immunoprecipitation conditions

Protein of interest	Antibody	Volume of sheared DNA	Length of incubation
Crt1-HA (Roche)	12µl α-HA	800µl	Overnight 4^{o}C
H3 (Abcam 1791)	5µl α-H3	400µl DNA + 400µl FLB	90min RT
H3K56Ac (ActivMotif)	5µl α-H3K56Ac	400µl DNA + 400µl FLB	90min RT
RNAP II (8WG16	3µl α-RNAP II	800µl	Overnight 4^{o}C

Chapter 3:

Replication-Coupled chaperones contributing to the control of *HUG1* transcription

3-1: Introduction

Replication-Coupled (RC) nucleosome assembly is an essential process that functions to deposit histones onto nascent DNA strands immediately following the replication fork. Histone deposition, nucleosome position, and modifications to histones are central to transcriptional regulation. Therefore, nascent chromatin behind the replication fork is structured by these characteristics to mimic the parental chromatin. This duplication of chromatin structure is suggested to contribute to epigenetic inheritance [121]. How information regarding parental chromatin structure is propagated to daughter chromatin structure is unknown. However, this propagation of information is suspected to revolve around RC nucleosome assembly [67,16]. Because chromatin structure is a major contributor to the transcriptional regulation process, we predicted that the RC chaperones may play a role in transcriptional regulation. The RC chaperones Asf1, CAF-1, and Rtt106 have already been implicated in heterochromatin-like silencing at telomeres and the silent mating loci in budding yeast [39,54]. Asf1 is also involved in transcriptional regulation of the *PHO5, RNR3, HUG1* and *ARG1* genes [21,109,110], and Rtt106 is important for preventing transcription from cryptic promoters [41]. Because the three RC chaperones work together during RC nucleosome assembly, we predicted they may also work together in transcriptional regulation.

It was previously demonstrated in our lab that Asf1 is important for induction of the DDR genes *HUG1* and *RNR3* [110]. The current model of RC nucleosome assembly proposes that Asf1 transfers newly synthesized H3-H4 marked with H3K56Ac to CAF-1 or Rtt106. These chaperones function redundantly to deposit H3-H4 onto the daughter DNA strands (see Figure 1-1). Based on this model, we predicted that chromatin (and thus transcriptional) changes at *HUG1* should be additive in *cac1*Δ and *rtt106*Δ cells to equal that of *cac1*Δ *rtt106*Δ cells. Additionally, the *HUG1*

transcription phenotypes of *cac1*Δ *rtt106*Δ cells should be very similar to *asf1*Δ cells; RC nucleosome assembly should be nearly abolished in both strains. The fact that both strains are viable suggests residual RC nucleosome assembly even in the absence of the RC chaperones known to mediate this process. The data presented in this chapter support the prediction that each RC chaperone contributes to *HUG1* regulation. However regulation by CAF-1 and Rtt106 is through a novel pathway independent of the DNA structure checkpoints. *HUG1* is ordinarily regulated by Crt1, which is a repressor that binds to *HUG1* and recruits Ssn6-Tup1. These co-repressors recruit chromatin remodelers and histone deacetylases, to promote a repressive chromatin structure at *HUG1* that is inhibitory to transcription [104]. Upon activation of the DNA structure checkpoints, Crt1 is hyperphosphorylated in a Rad-53 dependent manner and no longer binds to the *HUG1* promoter, thus relieving the repressive chromatin structure and promoting transcription [88]. This chapter describes how Asf1 regulates *HUG1* by preventing DNA damage checkpoint activation, while CAF-1 and Rtt106 regulate *HUG1* in a way that does not involve the DNA damage checkpoint. This is the first evidence that RC chaperones can directly contribute to the regulation of an inducible gene.

To investigate how CAF-1 and Rtt106 repress *HUG1,* ChIP experiments were performed to measure Crt1 and histone H3 occupancy at the *HUG1* promoter. We found that derepression of *HUG1* in *cac1*Δ *rtt106*Δ does not follow the conventional model of Crt1-dependent *HUG1* regulation. Furthermore, H3 occupancy is decreased at the *HUG1* promoter in *cac1*Δ *rtt106*Δ, and is unexpectedly high in *asf1*Δ. This decrease in H3 occupancy may be the cause of *HUG1* derepression in *cac1*Δ *rtt106*Δ cells.

Overall, we found that CAF-1 and Rtt106 contribute to the repression of *HUG1*. Interestingly, this repression does not seem to

involve Asf1, even though Asf1 functions upstream of these chaperones during RC nucleosome assembly. This indicates that nucleosome metabolism involving the RC chaperones is more complex than the current model suggests. Future work is required to fully understand the mechanism of this regulation.

3-2: Results

HUG1 is derepressed in the absence of *CAC1* and *RTT106*

It has been previously demonstrated that *HUG1* is derepressed in the absence of its repressor Crt1, or its co-repressors Ssn6-Tup1 [104]. Asf1 has recently been found to regulate several DDR genes including *HUG1*: the absence of *ASF1* results in the derepression of *HUG1* by ultimately reducing repressor and corepressor binding. This is because the DDR is partially active in *asf1*Δ cells. This partial activation of the DNA damage checkpoint has been demonstrated in Minard *et al.* (2011) [110], Ramey *et al.* (2004) [59], and also in Figure 3-3 as a band shift in SDS-PAGE that corresponds to this activating phosphorylation of Rad53 in the absence of *ASF1*. The transcription data presented in Figure 3-1 (A&B) shows that deletion of *ASF1* results in an approximate 7 fold derepression of *HUG1.* Interestingly, these data also reveals that *HUG1* is derepressed about 7-fold in the absence of *CAC1* or *RTT106.* When these two genes are deleted together to form a double mutant, *HUG1* derepression is doubled. Aside from *asf1*Δ cells, this result is predicted by the current model of RC nucleosome assembly as chromatin defects resulting from *CAC1* deletion contribute the same amount to *HUG1* derepression as the deletion of *RTT106.* CAF-1 and Rtt106 function in parallel. Therefore deletion of both *CAC1* and *RTT106* results in a doubling of *HUG1* derepression, as both pathways of RC nucleosome assembly are abolished.

HUG1 derepression in *cac1*Δ *rtt106*Δ cells is higher compared to the *asf1*Δ strain. Because an *ASF1* deletion activates the DNA damage checkpoint which induces *HUG1,* we wanted to test whether checkpoint activation in the absence of Asf1 masks any checkpoint-independent derepression that may occur because of disrupted RC nucleosome assembly. Deleting *ASF1* in addition to *CAC1* and *RTT106* in a *cac1*Δ *rtt106*Δ *asf1*Δ triple mutant slightly increased *HUG1* derepression from the

level seen in the *cac1*Δ *rtt106*Δ double mutant (Figure 3-1 (B)). This suggests that mechanisms resulting in *HUG1* derepression in *asf1*Δ cells are independent of the mechanisms resulting in *HUG1* derepression in *cac1*Δ *rtt106*Δ cells. Also that activation of the checkpoint does not affect the *HUG1* derepression due to RC nucleosome assembly disruption. Evidence will be presented later in this chapter to demonstrate that *HUG1* derepression in the absence of CAF-1 and Rtt106 is not due to partial activation of the DDR as it is in *asf1*Δ cells.

To test whether the increased *HUG1* mRNA observed in the *cac1*Δ *rtt106*Δ double and *cac1*Δ *rtt106*Δ *asf1*Δ triple mutants was a result of increased transcription, the occupancy of RNA polymerase II (RNAPII) at *HUG1* was measured by ChIP (Figure 3-1 (C)). These results indicate that the level of RNAPII occupancy correlates with the level of *HUG1* derepression observed in each strain. In other words, RNAPII occupancy is slightly increased in *asf1*Δ and considerably increased in *cac1*Δ *rtt106*Δ cells. The approximate 4 fold increase in RNAPII occupancy at *HUG1* in *cac1*Δ *rtt106*Δ compared to wild type is similar to published results at *RNR3*, whereby removal of a promoter nucleosome resulted in Rad53-independent *RNR3* derepression [108]. Surprisingly, RNAPII occupancy at *HUG1* in *cac1*Δ *rtt106*Δ *asf1*Δ is closer to *asf1*Δ levels than *cac1*Δ *rtt106*Δ levels (Figure 3-1 (C)). Because *HUG1* derepression in the triple mutant is the same as in the double mutant, it was expected that RNAPII occupancy would be equally high in both strains.

RNR3 derepression was also measured in the RC chaperone mutants and results presented in Figure 3-2 (B). These data indicate that *RNR3* transcription is higher in *asf1*Δ cells compared to wild type. This is expected since the DNA damage checkpoint is partially active in this strain. Unlike *HUG1* however, expression in each single mutant is the same as wild type and *RNR3* derepression in the *cac1*Δ *rtt106*Δ double mutant is equal to *asf1*Δ (Figure 3-2 (A&B)). Why derepression in the

double mutant is not higher than *asf1*Δ cells (as was observed at *HUG1)* is unknown. The difference between *HUG1* and *RNR3* expression in dependence on histone chaperones may relate to their inducibility. Typically *HUG1* is induced about 100 fold by genotoxic stress, whereas *RNR3* is only induced about 60 fold [110]. Therefore effects observed at *HUG1* in the RC chaperone mutants may be less apparent at *RNR3*.

HUG1 derepression in *cac1*Δ *rtt106*Δ cells is not a result of DNA structure checkpoint activation

It is extremely interesting that the deletion of CAF-1 and Rtt106 results in the derepression of a DDR gene because this is the first evidence that they contribute to the control of an inducible gene. In the case of *ASF1*, deletion of this gene results in the partial activation of the DNA damage checkpoint as demonstrated by the hyperphosphorylation of Rad53 [59, 110].

A common way to detect DNA structure checkpoint activation is to analyze Rad53 phosphorylation by Western blot. Figure 3-3 shows total protein isolated from various cycling RC chaperone mutants run on a gel for detection of Rad53. Because of the low bis-acrylamide content in the gel (6%), band shifts due to hyperphosphorylation can be visualized. It is well established in the literature that all known band shifts of Rad53 are due to phosphorylation [90,122]. Figure 3-3 (A) shows that Rad53 in *cac1*Δ*, rtt106*Δ*,* and *cac1*Δ *rtt106*Δ cells is identical to Rad53 in wild type cells in that there is no band shift caused by hyperphosphorylation. Figures 3-3 (B&C) show a Rad53 band shift in strains where *ASF1* is deleted. This is expected as the DNA damage checkpoint is partially active in these strains. Additionally, the amount of shifted Rad53 appears to be equal in *asf1*Δ and *cac1*Δ *rtt106*Δ *asf1*Δ cells, suggesting that it is the deletion of *ASF1* which results in activation of the DNA damage checkpoint.

Crt1 occupancy at *HUG1* is influenced by Asf1, but not CAF-1 or Rtt106

The regulation of *HUG1* involves the repressor Crt1, which recruits the global co-repressors Ssn6-Tup1. Because *HUG1* is derepressed in the absence of *CAC1* and *RTT106* and this derepression is not due to DNA structure checkpoint activation, we investigated if the derepression could be a result of decreased Crt1 binding. In the RC chaperone mutants, chromatin may no longer be poised for efficient recognition by Crt1 at *HUG1* because RC nucleosome assembly is disrupted. This would result in a decreased Crt1 presence at *HUG1,* less Ssn6-Tup1 recruitment, and a gene that is not as efficiently repressed as it normally would be. To test this theory, we performed ChIP experiments in wild type, *asf1*Δ*,* and *cac1*Δ *rtt106*Δ cells, each harbouring an epitope-tagged version of Crt1. In this experiment Crt1-HA occupancy was measured at *HUG1* and *RNR3* in cycling cells.

Crt1-HA occupancy is noticeably lower in *asf1*Δ cells at *HUG1* compared to wild type cells (Figure 3-4 (A)). This is expected since the DNA damage checkpoint is partially active in *asf1*Δ cells, so Crt1 is hyperphosphorylated and no longer binds to the *HUG1* promoter [59]. Crt1-HA occupancy in *cac1*Δ *rtt106*Δ cells is higher than in *asf1*Δ cells, and not as high as wild type cells. However, there is a large overlap of error bars to both *asf1*Δ and wild type cells. Nevertheless, statistical analysis indicates that Crt1-HA occupancy in the double mutant is not significantly different than wild type, suggesting that *HUG1* derepression in the double mutant is not due to a decrease in Crt1 occupancy. Moreover, *HUG1* is more derepressed in the double mutant than in *asf1*Δ cells, and higher derepression should be associated with lower Crt1 occupancy [88]. It is possible that the level of *HUG1* transcription does not correlate linearly with Crt1 occupancy, however published results do not suggest that this is the case [88].

Figure 3-4 (B) is the same Crt1-HA ChIP experiment as in Figure 3-4 (A), however Crt1-HA occupancy is analyzed at *RNR3.* Each strain appears to have the same Crt1-HA occupancy at *RNR3*. It has been previously shown that upon treatment with MMS, Crt1 occupancy at *HUG1* and *RNR3* decreases approximately 5-fold [107, 28]. Crt1-HA occupancy was only measured at *HUG1* and *RNR3* in untreated cells, and at *HUG1* there is an observable decrease of the repressor at *HUG1* in *asf1*Δ cells. At *RNR3* there is no observable decrease of Crt1-HA at *HUG1* in *asf1*Δ cells, even though it is partially induced. This may be because of the different levels of inducibility between *HUG1* and *RNR3*.

Overall, I found that Crt1-HA occupancy is similar in wild type and *cac1*Δ *rtt106*Δ cells; and thus it cannot explain the derepression of *HUG1* in this double mutant. This reveals a novel pathway of *HUG1* regulation that is independent of Rad53 and the DNA structure checkpoints. In addition, the data provides further evidence that the DNA structure checkpoints are not active in *cac1*Δ *rtt106*Δ cells, as this would result in the loss of Crt1-HA from the *HUG1* promoter as observed in the *asf1*Δ strain.

CAF-1 and Rtt106 influence histone H3 occupancy at *HUG1* and *RNR3*

The structure of chromatin can affect gene transcription. Chromatin structure is determined in part by nucleosome density and the post-translational state of the histones. Highly transcribed genes tend to have lower histone occupancy at their promoters [124]. Transcriptional induction or repression has also been correlated with low- or high-histone occupancy, respectively [20,125]. Because *HUG1* is derepressed in the absence of *CAC1* and *RTT106* and these chaperones are central players in RC nucleosome assembly, we predicted that the compromised pathway of histone deposition in this double mutant leads to the derepression of *HUG1.*

To test this possibility, histone H3 occupancy was measured by ChIP in cycling cells at various locations in the genome (Figure 3-5). At the genes *HUG1* and *RNR3,* H3 occupancy was measured over the promoter region. *PHO5* is highly induced in conditions of low phosphate. Cells in all experiments in this thesis were grown in low phosphate conditions. *PHO5* is also slightly induced during M phase of the cell cycle [23]. H3 was measured at the upstream activating sequence (UAS) at *PHO5. TELV* is a telomere which is gene free.

H3 occupancy at *HUG1* is lower in *cac1*Δ *rtt106*Δ and *cac1*Δ *rtt106*Δ *asf1*Δ cells than wild type or *asf1*Δ cells (Figure 3-5 (A)). This is interesting for several reasons. First, when *HUG1* is fully induced (through activation of the DNA damage structure checkpoints), H3 occupancy only slightly decreases in wild type cells, albeit of a BY4741 background [110]. However the double and triple mutants shown in Figure 3-5 (A) have lower H3 occupancy than wild type cells, even though *HUG1* is only modestly derepressed (Figure 3-1). Secondly, *HUG1* is partially induced (due to partial DNA damage checkpoint activation) in *asf1*Δ cells, yet H3 occupancy in this strain is higher than wild type. These data suggest that there is not a perfect correlation between H3 occupancy and transcription. It has been reported that Asf1 may be involved in nucleosome disassembly. When *ASF1* is deleted, there is a higher histone occupancy observed on DNA, and DNA is less sensitive to digestion by MNase [56]. This may explain why at *HUG1, RNR3, PHO5,* and *TELV* (Figure 3-5 (A-D) respectively) there is a higher H3 occupancy in *asf1*Δ cells than wild type cells.

Histone H3 occupancy at *PHO5* and *TELV* in *cac1*Δ *rtt106*Δ cells was very similar to wild type cells (Figure 3-5 (C&D)). Based on the results at *HUG1,* we expected to see a similar decrease in H3 occupancy in *cac1*Δ *rtt106*Δ cells. We may not see the expected decrease because *PHO5* and *TELV* are regulated differently than the DDR genes. *PHO5* is

induced when cells are in low phosphate conditions and requires an activator to induce transcription [23]. *TELV* is a gene free region and thus not under the control of transcription factor proteins.

In summary, *asf1*Δ cells have increased H3 occupancy at *HUG1, RNR3, PHO5* and *TELV* compared to wild type. *cac1*Δ *rtt106*Δ cells have decreased H3 at *HUG1,* and similar H3 occupancy at *RNR3, PHO5,* and *TELV.* This data suggests that *HUG1,* a gene normally repressed by the combination of nucleosome positioning and histone modifications, is affected differently by RC chaperone mutations than other regions of the genome, which are regulated differently.

In contrast to the previous strains discussed, H3 occupancy in *cac1*Δ *rtt106*Δ *asf1*Δ cells is quite intriguing. H3 occupancy in the triple mutant closely resembles that of the double mutant at *HUG1,* despite also lacking *ASF1* (Figure 3-5 (A)). Therefore, even though nucleosome disassembly may be impaired due to the lack of *ASF1* in the triple mutant, the histone deposition defects in the *cac1*Δ *rtt106*Δ double mutant have a dominant effect on H3 occupancy than the disassembly defects in cells lacking *ASF1*. At *PHO5* and *TELV* however, H3 occupancy in the triple mutant closely resembles that of the *asf1*Δ single mutant (Figure 3-5 (C&D). Therefore unlike at *HUG1,* the lack of *CAC1* and *RTT106* in the triple mutant does not affect H3 occupancy at these regions of the genome. Moreover, the possible impaired chromatin disassembly due to the lack of *ASF1* in the triple mutant seems to be the dominant factor in determining H3 occupancy at these regions of the genome.

HUG1 is cell cycle regulated

As demonstrated above, the transcriptional regulation of *HUG1* is dependent on the RC chaperones. *HUG1* is derepressed independently of DNA structure checkpoint activation in the absence of *CAC1* and *RTT106.*

This derepression was measured in cycling cells (Figure 3-1), thus the population analyzed contained cells in all stages of the cell cycle. To investigate whether the derepression of *HUG1* in *cac1*Δ *rtt106*Δ cells was consistent throughout the cell cycle, *HUG1* transcription was measured while cells were arrested in G2/M phase by nocodazole, and while cells progressed into S phase following release into preconditioned medium (Figure 3-6).

Prior to analysing transcription, we performed flow cytometry to confirm that cells successfully arrested in nocodazole and progressed into S phase (Figure 3-6 (D)). 1N and 2N refer to the number of cell equivalents of DNA in each cell, so a peak at 2N represents cells in G2/M phase which have replicated their DNA but have not yet undergone mitosis. These data clearly show that cycling *asf1*Δ cells have a noticeably different profile than cycling wild type and *cac1*Δ *rtt106*Δ cells, as the 2N DNA peak is much broader. This provides an indication that the DNA damage checkpoint is not active in *cac1*Δ *rtt106*Δ cells as it is in *asf1*Δ cells, since a higher percentage of *asf1*Δ cells accumulate in G2/M phase [59].

The flow cytometry data shown in Figure 3-6 (D) indicate successful G2/M arrest by nocodazole treatment in all strains. These data also show an increase in DNA content beyond the 2N DNA peak during nocodazole arrest in all strains. This is likely due to increased mitochondrial DNA, which is replicated throughout the cell cycle and continues to replicate while cells are arrested in G2/M [126], [127]. In addition to a successful G2/M arrest, the flow cytometry data shown here also indicates that approximately 80 minutes following release into preconditioned media cells progress through S phase and there is an enrichment of cells in S phase compared to cycling cells.

Nocodazole disrupts microtubule polymerization and therefore activates the spindle assembly checkpoint (SAC) and arrests cells in G2/M phase since mitosis cannot be completed[128]. A characteristic of SAC activation is the change in phosphorylation state of Rad53, although this is not the primary response to SAC activation [91,129]. This change in Rad53 phosphorylation in response to nocodazole arrest can be observed in the Western blot in Figure 3-6 (E). Although Rad53 is a major component in DNA structure checkpoint activation, its phosphorylation in response to SAC activation is unrelated to the DNA structure checkpoints [91]. Therefore, the change in phosphorylation of Rad53 in response to nocodazole arrest should in no way affect the interpretation of the *HUG1* and *RNR3* transcription results in shown in Figure 3-6, and instead provides assurance that the cells are responding correctly to nocodazole treatment.

Figure 3-6 (E) shows that once nocodazole is washed out and cells progress into S phase, Rad53 only remains hyperphosphorylated in *asf1*Δ cells. DNA is replicated in S phase. Chromosomal lesions impeding replication may therefore be more apparent at this time, thus activation of the DNA structure checkpoints (characterized by Rad53 hyperphosphorylation) may be more apparent in synchronized cells progressing through S phase than in asynchronous cells. Because Rad53 hyperphosphorylation is not observed in synchronized wild type or *cac1*Δ *rtt106*Δ cells progressing through S phase (Figure 3-6 (E)), this is evidence to support our previous finding that the DNA structure checkpoints are not active in these strains.

To my knowledge there had been no data published on the specific cell cycle regulation of *HUG1.* Therefore, I first investigated if *HUG1* is cell cycle regulated by measuring its transcription in wild type cells while they are arrested in G2/M phase, and comparing it to the transcription level while cells are progressing into S phase (Figure 3-6 (A)). From this

experiment, I observed an increase in *HUG1* transcription in wild type cells as they progressed through S phase. Although this increase in transcription is small compared to the transcription level when *HUG1* is fully induced, there is a reproducible increase in transcription when cells are in S phase. It is not surprising to find that *HUG1* transcription increases when cells are in S phase. This is because another DDR gene, *RNR3*, is cell cycle regulated and also has increased transcription when cells are in S phase [130]. Figure 3-6 (B) also shows the increase in *RNR3* transcription as cells progress into S phase. In addition to wild type, each RC chaperone mutant also demonstrates a likewise increase in *RNR3* transcription as cells progress through S phase.

Figure 3-6 (C) shows *HUG1* transcription data in RC chaperone mutants as they progress from G2/M into S phase. It is evident that *HUG1* transcription increases in the RC chaperone mutants similar to wild type cells. Moreover, the comparative levels of *HUG1* derepression between each strain remain the same in G2/M and S phase. However, the overall levels of *HUG1* transcription in each strain increase in S phase (Figure 3-6 (C)). More investigation is required to find out how *HUG1* is specifically cell cycle regulated. Data presented here suggests that it is not dependent on the RC chaperones. This is because despite *HUG1* misregulation in the RC chaperone mutants, transcription still increases as cells progress into S phase.

Figure 3-7 (A-C) shows *ACT1* (A), *PHO5* (B) and *CIN8* (C) gene expression in the various RC chaperone mutants while cells are arrested in G2/M phase and released into S phase. These genes were investigated as controls for analyzing cell cycle regulated gene expression during nocodazole arrest and release. *ACT1* is routinely used for normalizing gene expression data as it is constitutively expressed [131]. Although *ACT1* has been shown to have fluctuations in gene expression, these are primarily during sporulation or when cells are in stationary phase, not

during log phase growth [131]. As seen in Figure 3-7 (A), *ACT1* expression does not change as cells shift from G2/M towards S phase. This provides assurance that the increase in *HUG1* expression as cells progress into S phase is due to cell cycle regulation, and not an unrelated effect of nocodazole treatment. The DMSO control data in Figure 3-7 (F) also shows that the DMSO component of nocodazole treatment does not affect gene expression of *ACT1.*

PHO5 gene expression (shown in Figure 3-7 (B)) decreases as cells shift from G2/M towards S phase, which is expected as *PHO5* is slightly mitotically expressed [23]. Interestingly, the DMSO control data measuring *PHO5* expression also shows a decrease in expression as cells are released into preconditioned medium following DMSO treatment (Figure 3-7 (G)).

CIN8 expression is shown in Figure 3-7 (C). In each strain there is an increase in *CIN8* expression as the cells shift from G2/M into S phase, which is expected since *CIN8* expression increases in S phase [132]. Interestingly, ***asf1***Δ cells do not show a significant increase in *CIN8* expression. This observation along with the flow cytometry data in Figure 3-6 (D) suggest that ***asf1***Δ cells were not enriched in S phase thus we cannot draw any conclusions about the involvement of Asf1 in cell cycle regulation of *HUG1*. The failure to release ***asf1***Δ cells from nocodazole and re-enter the cell cycle has been previously demonstrated after treatment with MMS and bleomycin [47]. This suggests that ***asf1***Δ cells may have difficulty deactivating the SAC or DNA structure checkpoints. The DMSO control data presented in Figure 3-7 (H) shows no change in *CIN8* expression. This indicates that treatment with DMSO does not affect *CIN8* expression.

The comparative expression levels of *HUG1, RNR3, ACT1, PHO5* and *CIN8* differ between wild type and the RC chaperone mutants. This

observation was not explored further, however may provide further information on the role of the RC chaperones in gene regulation.

Collectively the data in Figure 3-6 and 3-7 indicate that *HUG1* is cell cycle regulated and this regulation is not dependent on the RC chaperones CAF-1 and Rtt106. More research is required to determine if Asf1 plays a role in the cell cycle regulation of *HUG1*.

3-3: Discussion

Data presented in this chapter suggest that the RC chaperones CAF-1, Asf1 and Rtt106 are all involved in the transcriptional regulation of *HUG1*. Moreover, these RC chaperones have divergent functions in this regulation.

HUG1 is derepressed in the RC chaperone mutants during unperturbed growth (Figure 3-1). The reason for *HUG1* derepression in *asf1*Δ cells is because the DNA damage checkpoint is partially active due to genomic instability and an increased rate of chromosomal lesions [59]. Figure 3-3 indicates that *HUG1* derepression in *cac1*Δ *rtt106*Δ cells is not due to DNA damage checkpoint activation since Rad53 is not hyperphosphorylated in these mutants. Although the extent of Rad53 phosphorylation in the *cac1*Δ *rtt106*Δ *asf1*Δ triple mutant and *asf1*Δ single mutant is similar, *HUG1* derepression in the triple mutant closely resembles that of *cac1*Δ *rtt106*Δ cells (Figure 3-1 (B)). Therefore, while Asf1 contributes to *HUG1* regulation through the typical DNA structure checkpoint-dependent pathway, CAF-1 and Rtt106 contribute to *HUG1* regulation through a novel DNA structure checkpoint-independent pathway.

It is important to mention that the observations described above concerning Rad53 phosphorylation in *cac1*Δ *rtt106*Δ cells is directly countered in a very recent paper by Clemente-Ruiz *et al.*[123]. In this study the authors detected increased Rad53 phosphorylation in *cac1*Δ *rtt106*Δ*, asf1*Δ*,* and *rtt109*Δ cells by Western blotting analysis using an antibody directed towards Rad53. They attribute this activation of the DNA damage checkpoint in each of these strains to increased recombination.

Interestingly Clemente-Ruiz *et al.* (2011) also make the observation that accumulation of recombinogenic DNA damage and DNA damage

checkpoint activation are not genetically linked, as deletion of *RAD52* in combination with *ASF1* does not increase the level of Rad53 phosphorylation. Rad52 is protein involved in recombination and cells lacking Rad52 have increased Rad53 phosphorylation [123]. Based on these findings the increased recombination frequency in *cac1*Δ *rtt106*Δ cells should not be the cause of the increased Rad53 phosphorylation this group observed. Additionally, our *cac1*Δ *rtt106*Δ strain has the expected phenotype of the inability to efficiently arrest in α-factor because of silencing defects at the silent mating loci [39, 6]. α-factor is a mating pheromone which signals the presence of a cell of the opposite mating type. Treatment with α-factor typically arrests cells in G1 phase. Experiments performed in the study by Clemente-Ruiz *et al.* (2011) [123] utilize α-factor to successfully arrest all strains in G1 phase, however *cac1*Δ *rtt106*Δ cells should have had difficulty arresting in α-factor. This leads into the final point that strains used in the study by Clemente-Ruiz *et al.* (2011) [123] are of a BY4741 genetic background. Since all experiments in this thesis use cells with a W303-1A background, it is possible that the difference in observations regarding Rad53 phosphorylation are a result of variations in genetic background.

Overall, we find that our W303-1A background strains (originally generated and characterized by Dr. Z. Zhang and described in Li *et al.* (2008) [6] have the expected phenotype of not efficiently arresting in α-factor. Moreover, Clemente-Ruiz *et al.* (2011)[123] did not show any flow cytometry data in cycling cells, which may have provided clues into DNA damage checkpoint activation by analyzing the DNA content in wild type versus *cac1*Δ *rtt106*Δ cells. Activation of the DNA damage checkpoint results in the accumulation of cells in G2/M phase, a phenotype that can be observed by measuring DNA content by flow cytometry [59]. Our *cac1*Δ *rtt106*Δ strain does not appear to have more cells in G2/M phase then wild type cells, indicating that there is no activated checkpoint.

Crt1-HA occupancy at *HUG1* is significantly lower in *asf1*Δ cells compared to wild type cells (Figure 3-4 (A)). Crt1-HA occupancy in *cac1*Δ *rtt106*Δ cells is, however, not significantly different than wild type. Crt1 represses the DDR genes by recruiting the co-repressors Ssn6 and Tup1. One option to ensure that Crt1 in *cac1*Δ *rtt106*Δ cells is efficiently repressing *HUG1* is to measure Ssn6 or Tup1 occupancy at *HUG1* by ChIP in each strain. Based on the data presented in this thesis we would expect that Ssn6 and Tup1 occupancy in the double mutant will be the same as in wild type because there is no checkpoint activation, and Ssn6 and Tup1 occupancy will be lower in *asf1*Δ cells.

One aspect to be aware of is that the known regulation of *HUG1* may be more complicated than published data suggests. For example, it was only recently discovered that in addition to Crt1, Rox1 and Mot3 are also repressors of *RNR3* [102]. There has been recent evidence suggesting that *HUG1* is also regulated by another repressor, Mig3 [98]. Mig3 primarily functions with Mig1 and Mig2 to regulate the expression of a protein involved in sucrose hydrolysis [99,98]. Because a detailed mechanism describing Mig3 regulation of *HUG1* is unavailable, and Crt1 is a strong repressor of *HUG1,* we did not consider Mig3 to interfere with any conclusions drawn from experiments in this thesis.

Further evidence of the divergent functions of the RC chaperones in *HUG1* regulation is demonstrated by the occupancy of histone H3 at the *HUG1* promoter. Cells lacking *CAC1* and *RTT106* have less H3 at the *HUG1* promoter than wild type cells, and this decrease in H3 is unaffected by the presence or absence of *ASF1* (Figure 3-5 (A)). This suggests that the functions of the RC chaperones may be more diverse and complex than what is expected based on the current model of RC nucleosome assembly. Importantly, we find that the way RC chaperones effect histone occupancy is likely dependent on the region of the genome.

The decrease in H3 occupancy in *cac1*Δ *rtt106*Δ cells correlates with the increase in *HUG1* derepression observed in this strain (Figure 3-5 (A&B), and Figure 3-1 (A)). The removal of promoter histones has been shown to promote transcription [20], and highly transcribed genes typically have lower histone occupancy in promoter regions [124]. *HUG1* and *RNR3* are regulated by Crt1 and the Ssn6-Tup1 co-repressors therefore, regional histone deacetylation and nucleosome positioning are important for repressing these genes. In fact, *RNR3* and *HUG1* are significantly derepressed in cells lacking ISW2 and HDA1, which are involved in chromatin remodeling and histone deacetylation [104]. Promoter nucleosome positioning is important for repression at *RNR3*, and depleting these promoter nucleosomes results in *RNR3* derepression [108]. Removal of CAF-1 and Rtt106, the redundant RC chaperones involved in RC nucleosome assembly, likely results in inefficient histone deposition during replication. This may explain the low H3 occupancy observed at *HUG1,* which may result in derepression of this gene. Histone occupancy in wild type cells has been shown to slightly decrease at *HUG1* after induction with HU [110]. Confirmation of this finding in the W303-1A strains would suggest that specific nucleosome positioning is important for repression. Depleting H3 by disrupting RC nucleosome assembly may prevent the specific nucleosome positioning important for repression of *HUG1* and *RNR3*. Thus the repressive chromatin structure established by Ssn6-Tup1 may be impaired in *cac1*Δ *rtt106*Δ cells, resulting in *HUG1* derepression.

The cell cycle regulation of *HUG1* has yet to be specifically studied. *RNR3* transcription increases when cells are in S phase and decreases following the completion of DNA replication [133]. Figure 3-6 confirms that *RNR3* transcription increases as cells progress into S phase, and reveals that *HUG1* is cell cycle regulated in a similar fashion. Interestingly, the derepression of *HUG1* in cells lacking *CAC1* and *RTT106* remains, whether the cells are arrested in G2/M or released into S phase, however

the cell-cycle mediated increase in transcription is still apparent as cells progress into S phase. This suggests that the cell cycle regulation of *HUG1* occurs independently of CAF-1 and Rtt106. At this time it is not clear if Asf1 is important for this cell cycle regulation.

Overall, I found that the RC chaperones contribute to *HUG1* regulation. However, the contributions from CAF-1 and Rtt106 differ from that of Asf1. Asf1 regulates *HUG1* by preventing DNA structure checkpoint activation, while CAF-1 and Rtt106 regulate *HUG1* in a way that does not involve the DNA structure checkpoints (Figure 3-8). The cell cycle specific regulation of *HUG1* appears to be independent of these RC chaperones. Future research is required to fully understand the mechanism of *HUG1* regulation by CAF-1 and Rtt106. However, Figure 3-9 illustrates a possible explanation: we propose that the compromised RC nucleosome assembly in *cac1*Δ *rtt106*Δ cells results in decreased H3 occupancy at *HUG1,* which likely causes its derepression. This is a novel pathway of *HUG1* regulation as Crt1 remains bound to the promoter. A detailed mechanism of RC nucleosome assembly would greatly assist in understanding how, and to what extent, RC nucleosome assembly may regulate gene transcription.

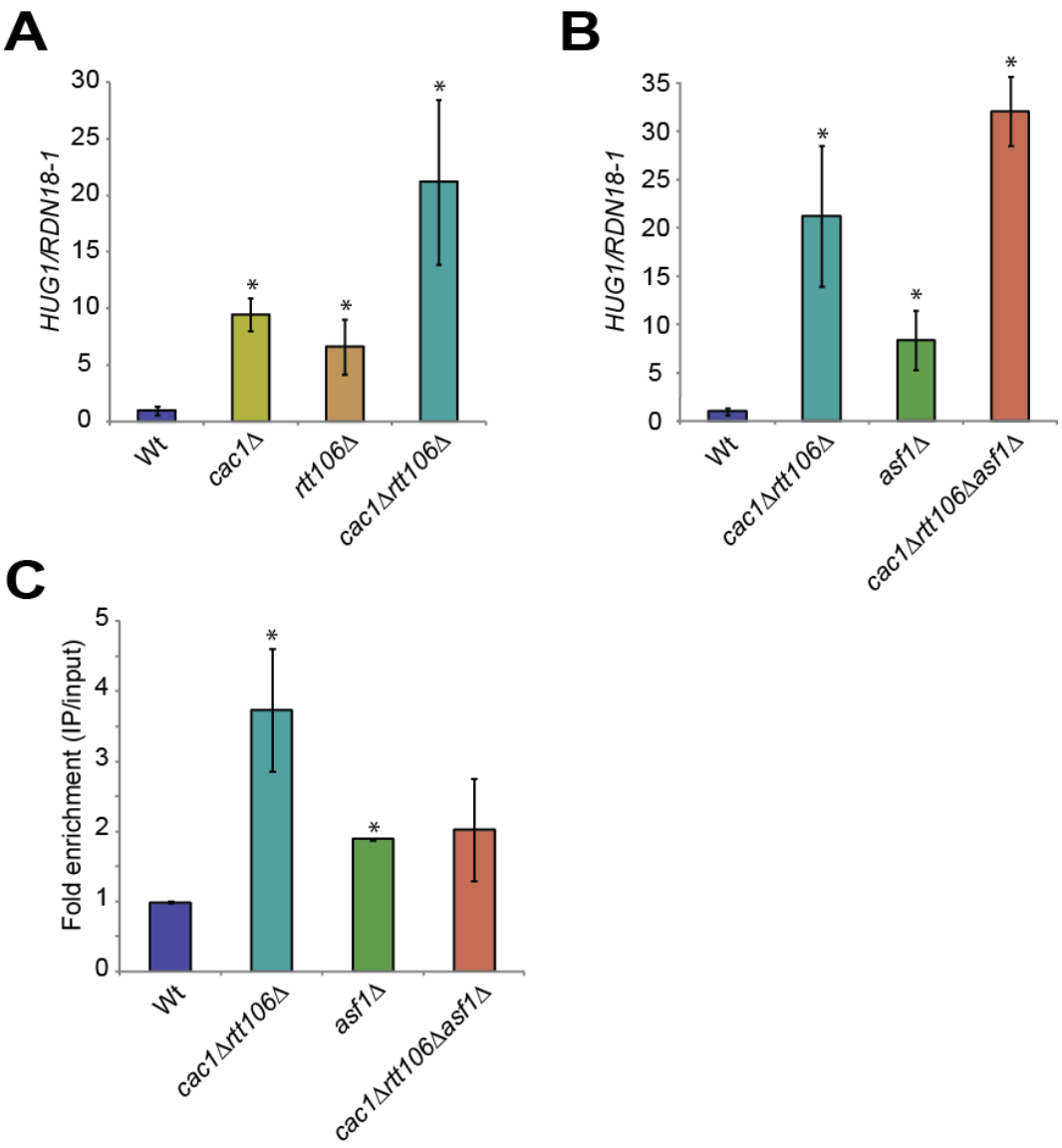


Figure 3-1: *HUG1* is derepressed in replication-coupled chaperone mutants. A&B) RNA was isolated from early log phase cells and subjected to quantitative RT-PCR using primers specific for *HUG1.* Transcription is normalized to *RDN18-1* and error bars represent standard deviation of three independent experiments. **C)** RNAPII occupancy at *HUG1*. Cells were grown to early log phase and ChIP analysis performed using an antibody directed towards RNAPII. RNAPII occupancy is normalized to *TELV* and the average of two independent experiments is shown. Error bars represent the range of the two experiments. (*) indicates significance at $p<0.05$ compared to Wt control. Throughout this thesis, p values were calculated using Student's two-tailed t-test.

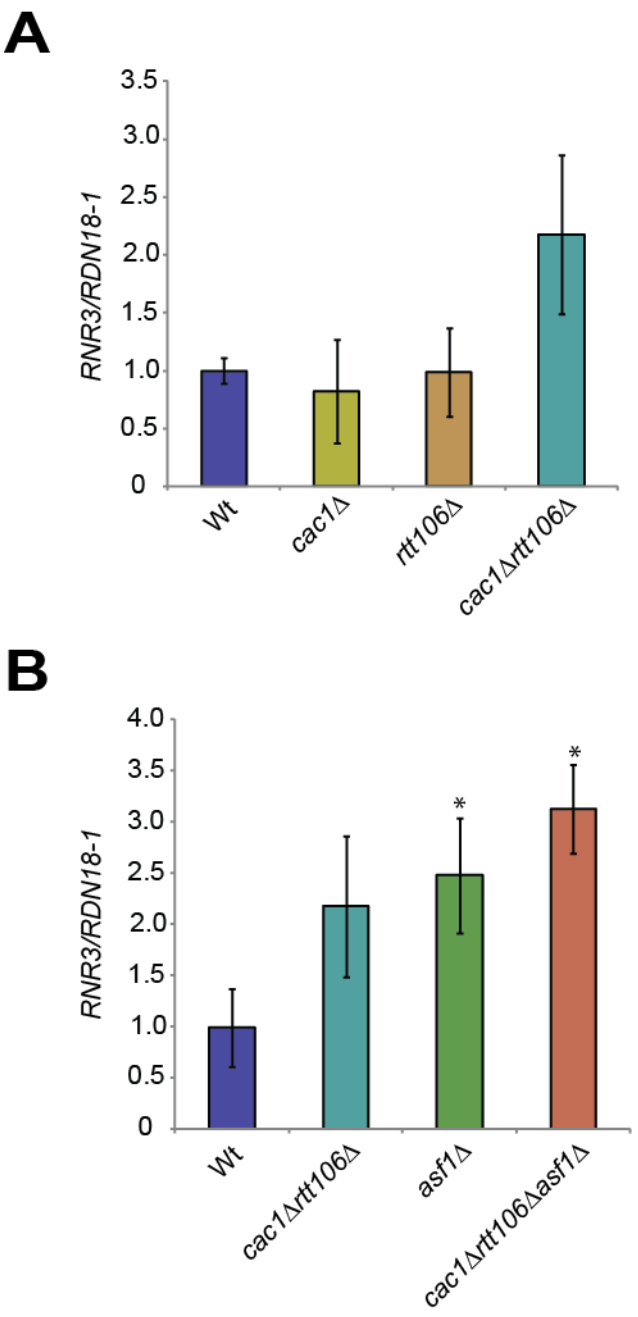


Figure 3-2: *RNR3* is derepressed in replication-coupled chaperone mutants. A&B) RNA was isolated from early log phase cells and subjected to quantitative RT-PCR using primers specific for *RNR3*. Transcription is normalized to *RDN18-1* and error bars represent the standard deviation of three independent experiments. (*) indicates significance at $p<0.05$ compared to Wt control.

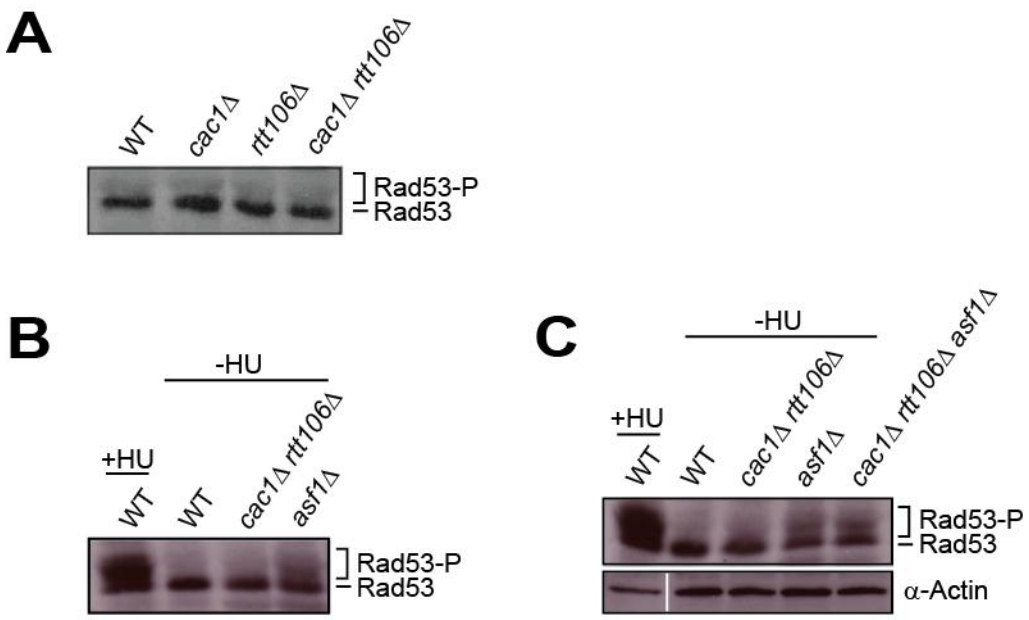


Figure 3-3: DNA damage checkpoint is partially activated as a result of deleting *ASF1*, but not *CAC1* or *RTT106*. Cells were grown to log phase and total protein isolated. Total protein was subjected to Western blot analysis using antibodies directed towards Rad53 and Actin. Results shown are representative of multiple experiments. **A)** Rad53-P in cycling cells. **B&C)** Rad53-P in cells treated with 0 mM HU (-HU) or 200 mM HU (+HU) for 1h. Actin serves as a loading control in **(C)**.

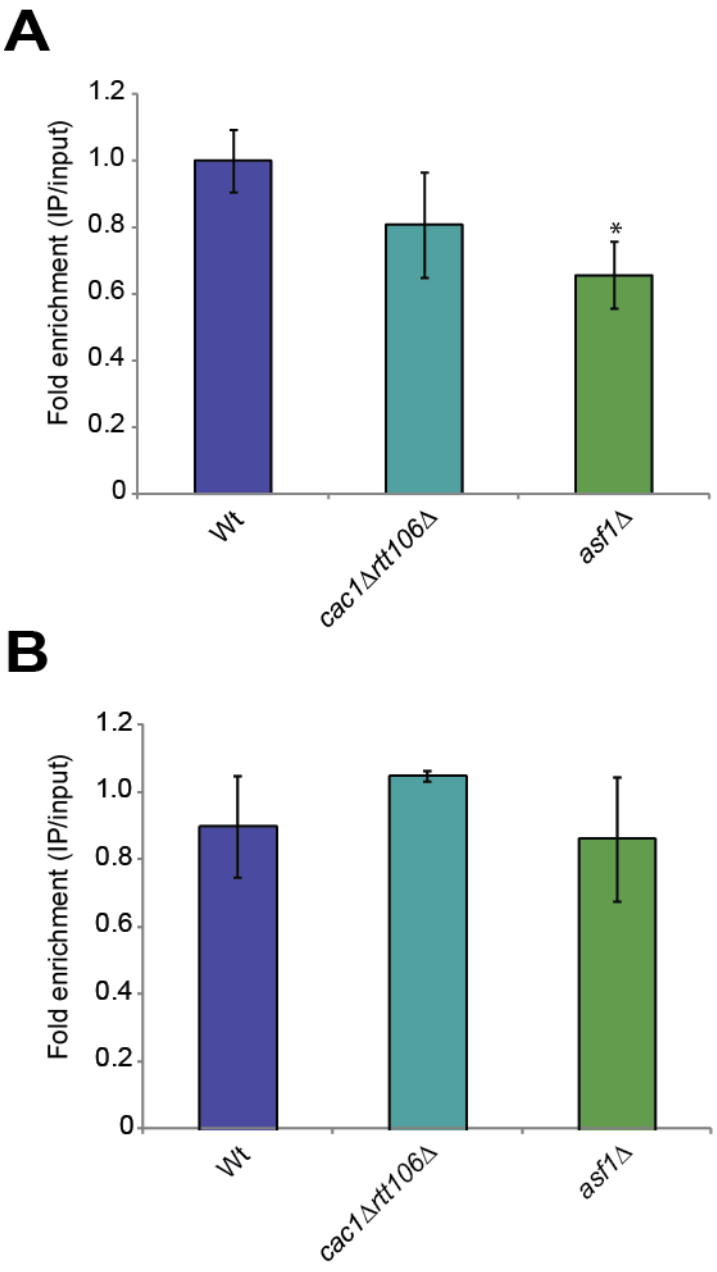


Figure 3-4: Crt1-HA occupancy is influenced by the deletion of *ASF1*, but not *CAC1* and *RTT106*. Cells were grown to early log phase in YPD and ChIP analysis performed using an antibody directed towards an HA epitope tag. Quantitative RT-PCR primers are specific towards *HUG1* **(A)** or *RNR3* **(B)** and span the binding sites of Crt1. Error bars represent the standard deviation of at least three independent experiments. (*) indicates significance at $p<0.05$ compared to Wt control.

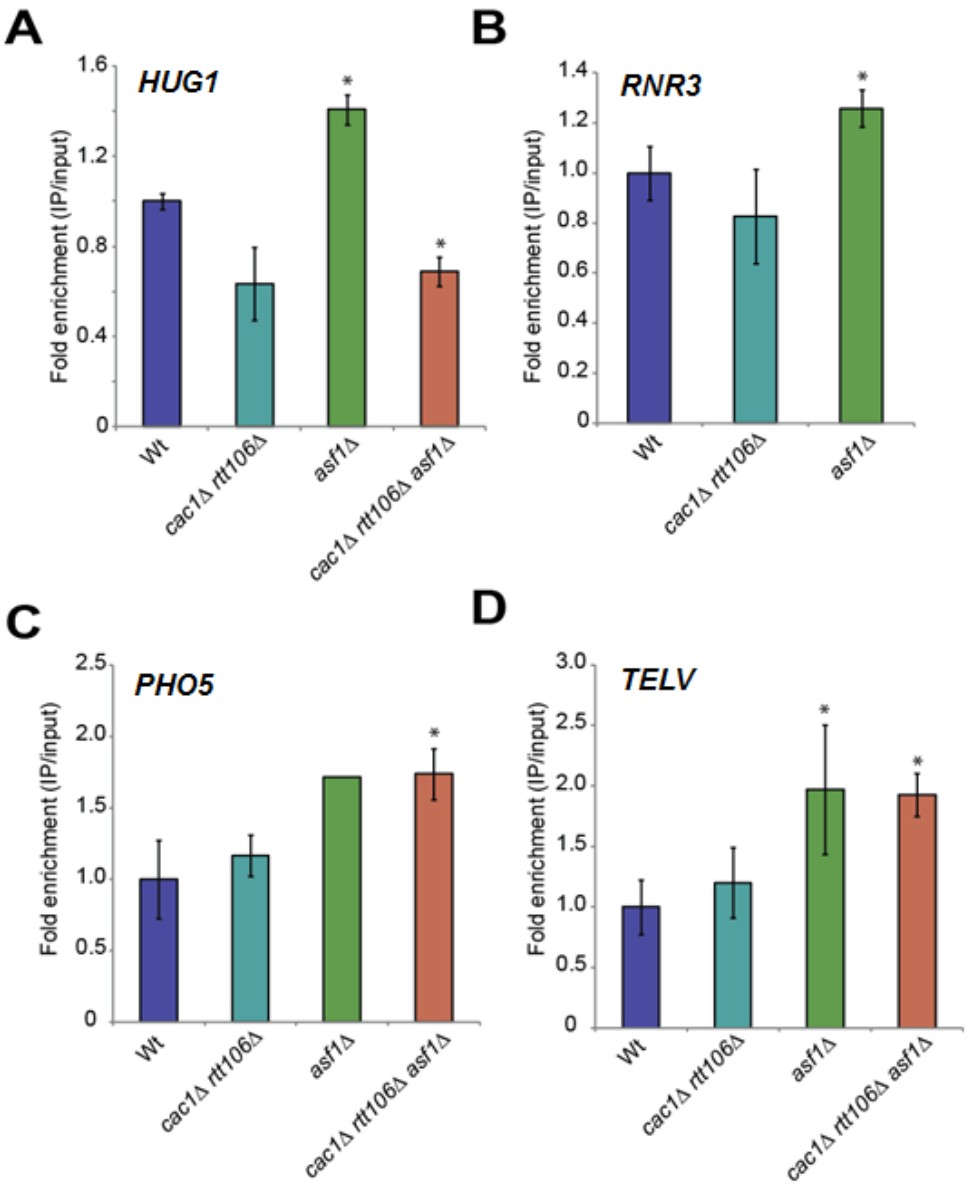


Figure 3-5: Histone H3 occupancy at various locations is influenced by the RC chaperones. Cells were grown to early log phase and ChIP analysis was performed using an antibody directed towards histone H3. Quantitative RT-PCR primers are specific towards *HUG1* **(A)**, *RNR3* **(B)**, *PHO5* **(C)** and *TELV* **(D)**. (*) indicates significance at $p<0.05$ compared to the Wt control.

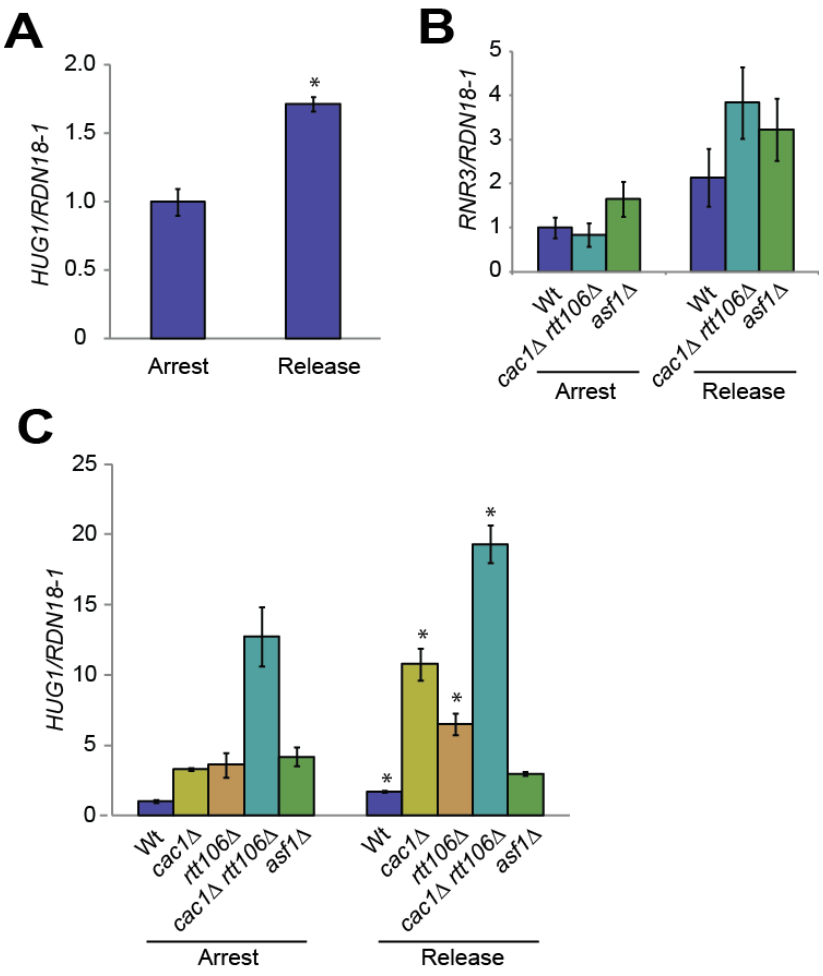


Figure 3-6: *HUG1* is cell cycle regulated and this regulation is unaffected by various deletions of the RC chaperones. Arrest and release experiment where cells were treated with 10 µg/ml nocodazole for 3.5 hours then resuspended in preconditioned media. RNA was isolated when cells were arrested in nocodazole (G2/M phase) and 75 minutes after release into preconditioned media (S phase). **A)** *HUG1* transcription in Wt cells in G2/M phase and S phase. **B&C)** *RNR3* **(B)** and *HUG1* **(C)** transcription in various RC chaperone mutants in G2/M phase and S phase. (*) indicates significance at $p<0.05$ between arrested versus released transcription levels. A Student's one-tailed t test was used.

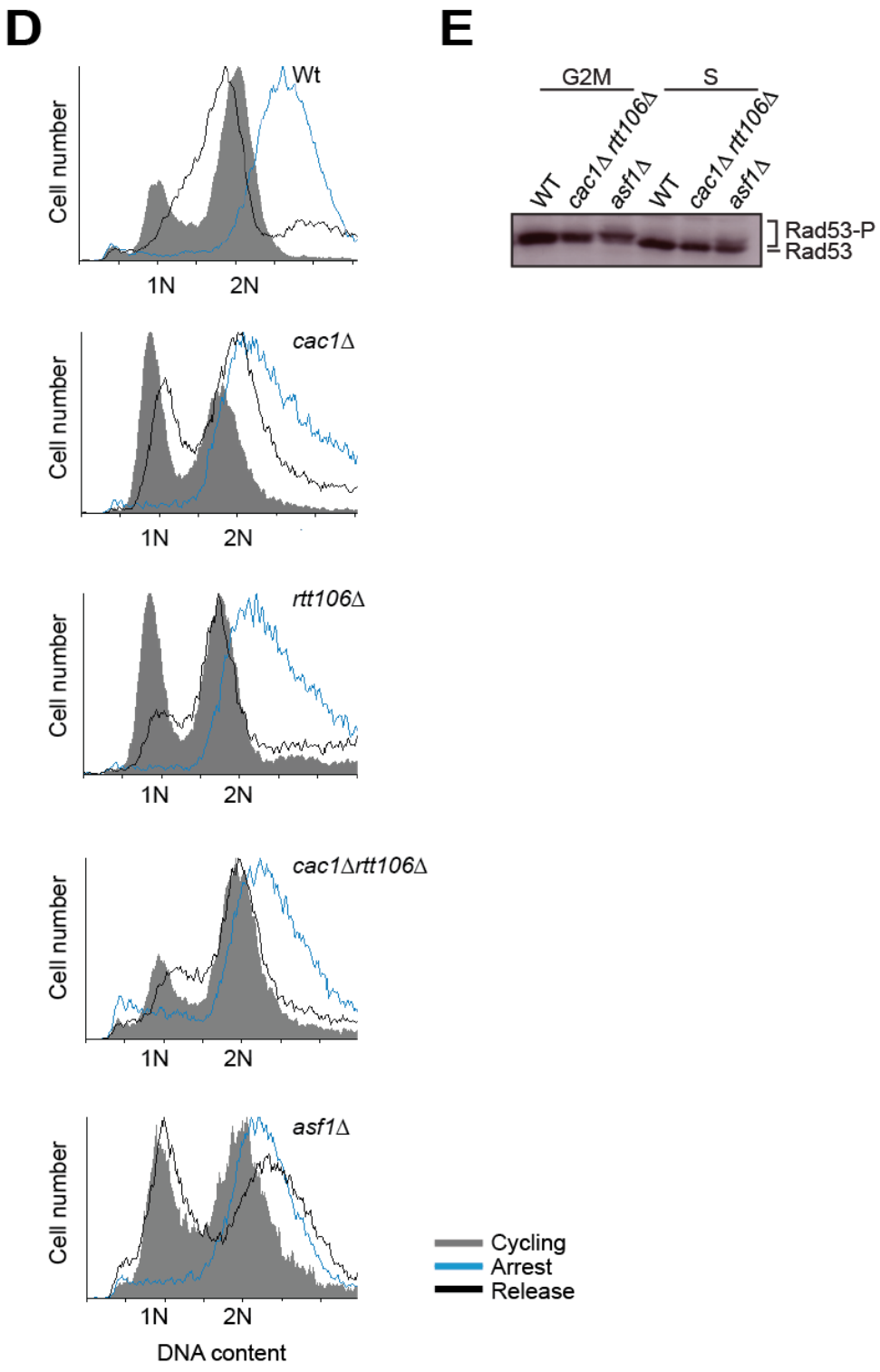


Figure 3-6 (continued): D) Flow cytometry analysis of DNA content in cells used in Figures 3-6 & 3-7. **E)** Western blot analysis of Rad53 phosphorylation in cells used in Figures 3-6 & 3-7.

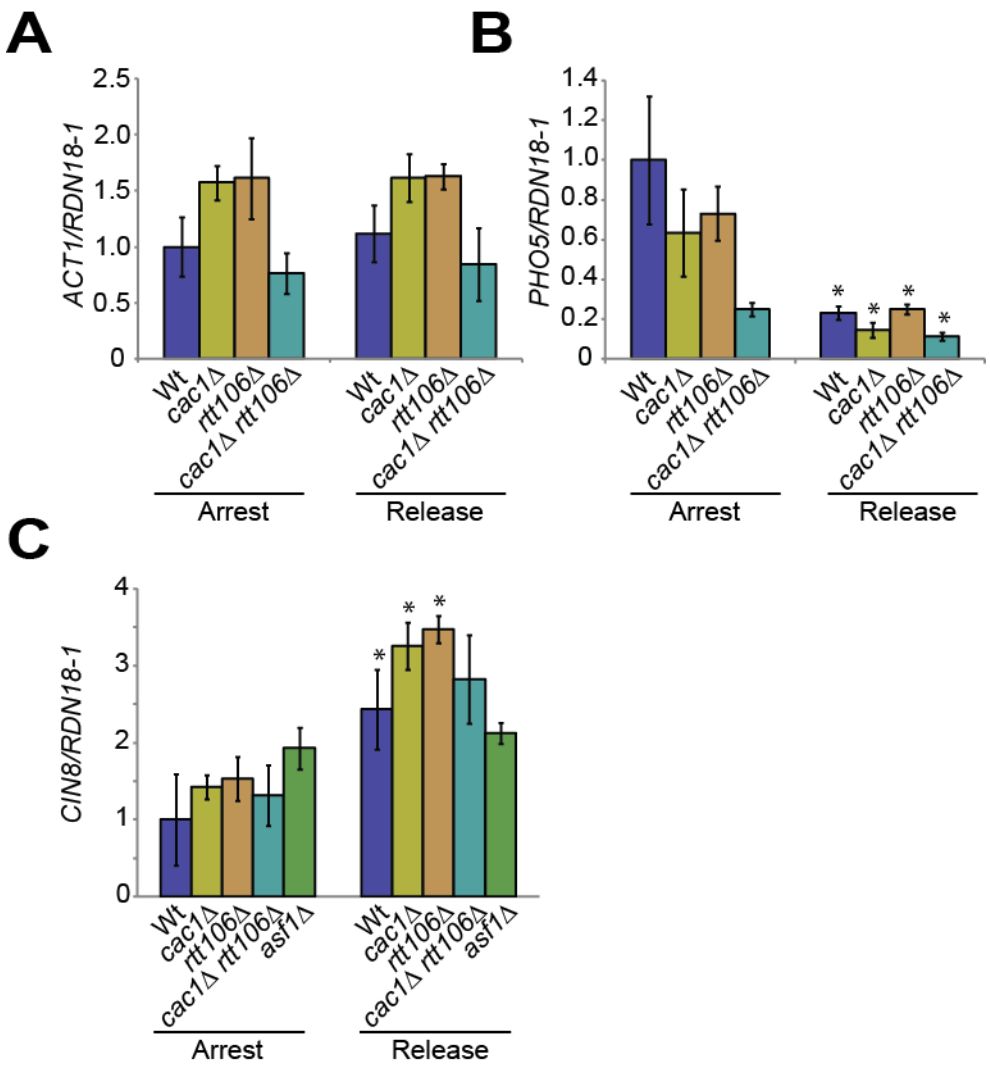


Figure 3-7: Transcription of control loci upon nocodazole arrest and release. *ACT1* **(A)**, *PHO5* **(B)** and *CIN8* **(C)** transcription in various replication-coupled chaperone mutants in G2/M phase and progressing into S phase. RNA used for this quantitative RT-PCR analysis is the same as in Figure 3-6. (*) indicates significance at $p<0.05$ between arrested versus released transcription levels. A student's one-tailed t test was used.

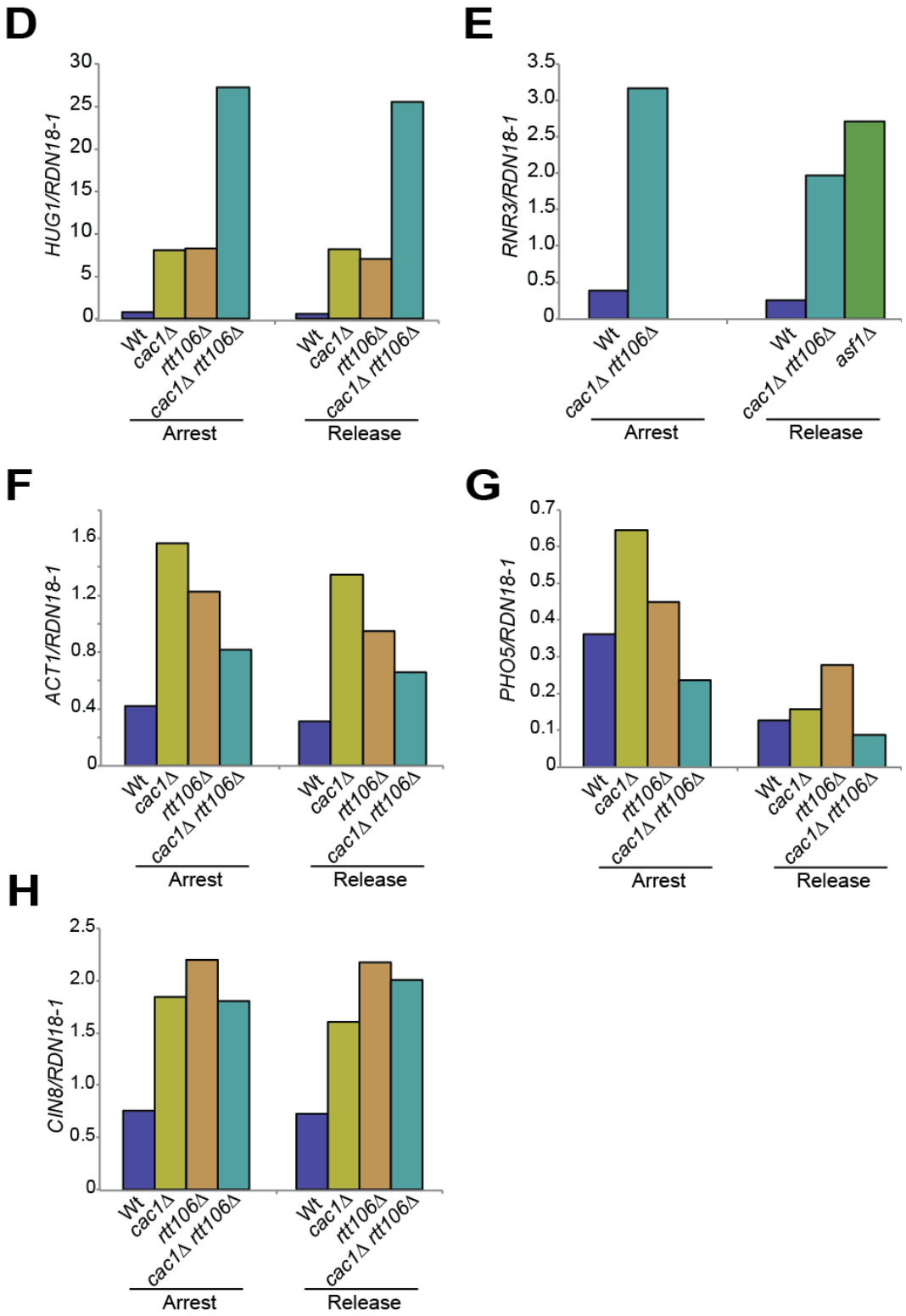


Figure 3-7 (continued): *HUG1* **(D)**, *RNR3* **(E)**, *ACT1* **(F)**, *PHO5* **(G)** and *CIN8* **(H)** transcription in cells treated with DMSO for 3.5 h (arrest) then resuspended in preconditioned medium (release). Performed once.

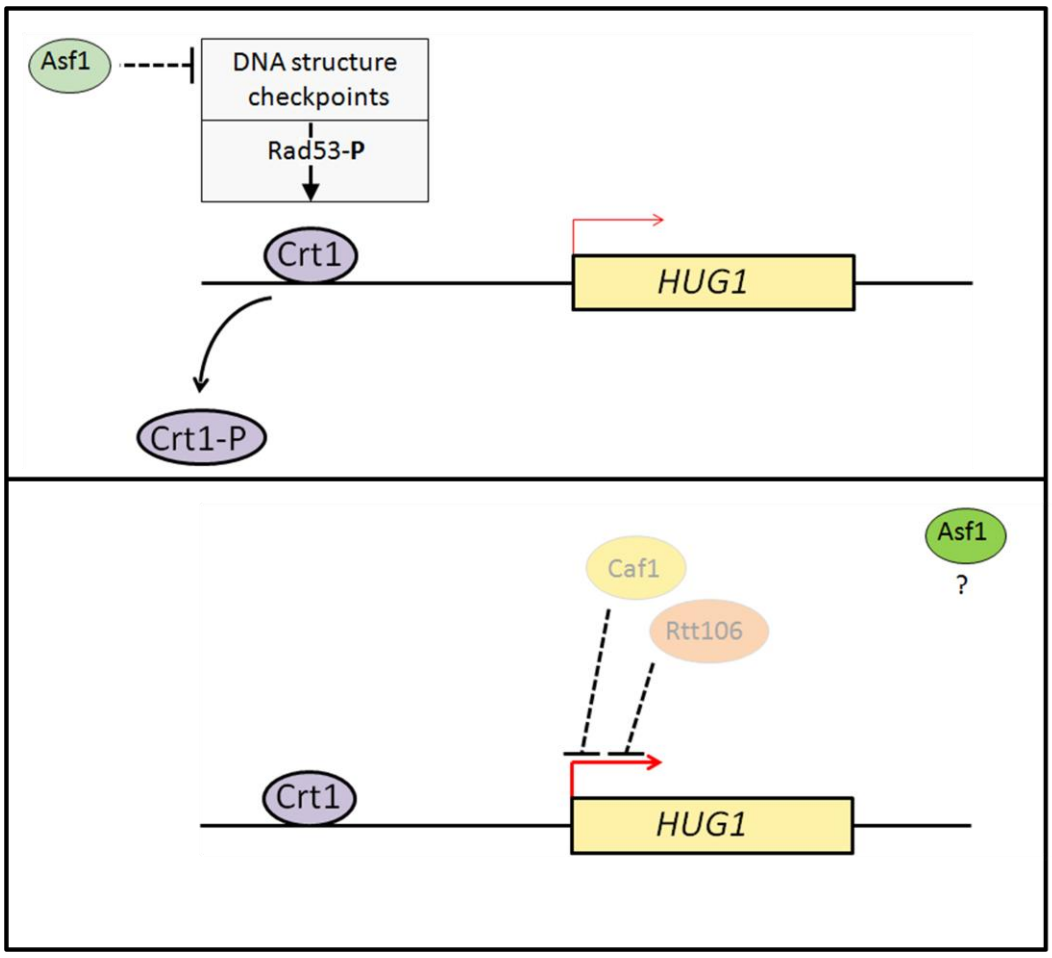


Figure 3-8: Transcriptional regulation of *HUG1* involving the RC chaperones. Asf1 regulates *HUG1* transcription by preventing DNA structure checkpoint activation. **Upper panel)** When *ASF1* is deleted, the DNA damage checkpoint is active, resulting in Rad-53 hyperphosphorylation followed by the hyperphosphorylation and loss of Crt1 from the *HUG1* promoter and the subsequent derepression of *HUG1*. **Lower panel)** CAF-1 and Rtt106 regulate *HUG1* in a way independent of the DNA structure checkpoints. The role of Asf1 in this regulation is yet to be determined.

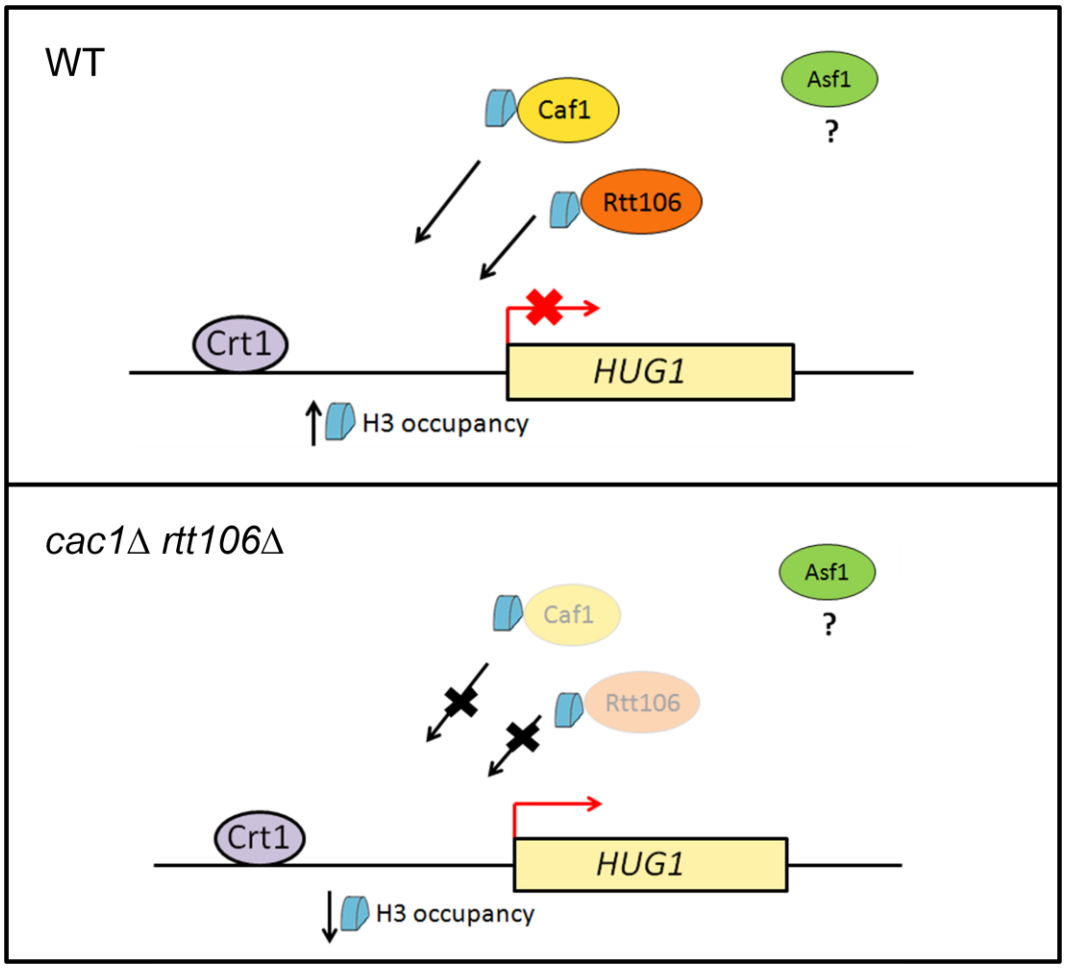


Figure 3-9: Proposed model of *HUG1* repression by CAF-1 and Rtt106 during unperturbed growth. Wt) CAF-1 and Rtt106 function in RC nucleosome assembly which is important for proper H3 deposition at *HUG1*. ***cac1*Δ *rtt106*Δ)** In the absence of *CAC1* and *RTT106*, histone deposition is compromised at *HUG1* resulting in decreased H3 occupancy. This results in *HUG1* derepression even though the Crt1 repressor remains bound to the promoter.

Chapter 4:

Divergent functions of CAF-1, Rtt106 and Asf1 have opposing effects on cell survival during prolonged replication stress

4-1: Introduction

This chapter centers around our discovery that *cac1*Δ *rtt106*Δ *asf1*Δ cells are more viable than *asf1*Δ cells on YPD plates supplemented with hydroxyurea (HU). This discovery suggested that the divergent functions of CAF-1, Rtt106, and Asf1 have opposing effects on cell survival during prolonged replication stress. Following this discovery we performed various experiments to characterize and attempt to understand the mechanism for this sensitivity rescue. HU reversibly inhibits RNR thus preventing production of dNTPs [77]. As a result of depleted dNTP levels, replication forks stall and the replication checkpoint is activated. This ultimately results in induction of DDR genes, stabilization of stalled forks, increased level of dNTPs and the maintenance of genome integrity [86]. Progression through S phase also slows because initiation of replication origins is significantly delayed [77,94]. Central to this checkpoint is the hyperphosphorylation of Rad53 [93]. The involvement of the RC chaperones in the replication checkpoint may help understand why the co-deletion of *CAC1* and *RTT106* rescues the HU sensitivity of *asf1*Δ cells.

We have shown in the previous chapter that the RC chaperones control the regulation of a DDR gene, *HUG1.* Moreover, the chaperones contribute to *HUG1* control in different ways: Asf1 prevents DNA damage checkpoint activation, whereas CAF-1 and Rtt106 maintain proper histone H3 occupancy, presumably through RC nucleosome assembly. To attempt to understand the mechanism of HU sensitivity rescue in *asf1*Δ cells by *CAC1* and *RTT106* deletions we measured the induction of *HUG1* and *RNR3* during HU treatment. Because Asf1 is required for *HUG1* induction [110], we predicted that *HUG1* would be induced to wild type levels in the triple mutant. Contrary to our predictions, the *HUG1* transcription results suggest that it is not the induction of *HUG1* or *RNR3* which rescues HU sensitivity of *asf1*Δ cells. Treating RC chaperone mutants with HU and

observing their recovery by growth on YPD plates indicated that HU sensitivity rescue in *asf1*Δ cells is not due to more successful HU recovery. Utilizing a chromatin fractionation procedure we found that *asf1*Δ cells have increased amounts of H3 associated with chromatin whereas *cac1*Δ *rtt106*Δ *asf1*Δ cells have wild type levels. Importantly, flow cytometry analysis of DNA content in RC chaperone mutants during HU treatment suggested that replication checkpoint activation may be delayed in *asf1*Δ cells but not *cac1*Δ *rtt106*Δ *asf1*Δ cells. Finally, we also studied the responses of each strain to other genotoxins by observing growth on YPD plates supplemented with Nicotinamide (NAM) or methyl methanesulphonate (MMS). NAM inhibits two HDACs, the silencing protein Sir2 and Hst3. Hst3 is important for deacetylating H3K56Ac. MMS is a methylating agent which creates chromosomal lesions resulting in single strand or double strand breaks.

Further research is required for our understanding of the mechanism behind the HU sensitivity rescue in *cac1*Δ *rtt106*Δ cells and identifying the opposing contributions of Asf1 and CAF-1/Rtt106 to cell viability. The misregulation of *HUG1* in cells lacking Cac1 and Rtt106 may play a role. Furthermore, we suspect that the activation of the replication checkpoint is impaired in *asf1*Δ*,* yet may be more efficient in *cac1*Δ *rtt106*Δ *asf1*Δ cells, thus enhancing survival during prolonged HU treatment.

4-2: Results

Deleting *CAC1* and *RTT106* in addition to *ASF1* rescues the HU sensitivity of an *asf1*Δ strain

In human cells, CAF-1 is an essential protein. Cells are not viable if they are missing any of the subunits making up CAF-1 because CAF-1 is the primary chaperone involved in RC nucleosome assembly [31]. In budding yeast, CAF-1, Rtt106 and Asf1 function together to assembly nucleosomes during DNA replication. Although each individual single mutant is viable, it was still quite surprising to find that a *cac1*Δ *rtt106*Δ *asf1*Δ triple mutant is viable as no other H3-H4 chaperones have yet been discovered to play a role in RC nucleosome assembly.

To gain a better insight of the phenotype of this triple mutant, the RC chaperone mutants were plated in 10X serial dilutions onto YPD plates containing 0 mM or 50 mM HU and grown at various temperatures (Figure 4-1). Cells lacking *CAC1* and *RTT106* have impaired histone deposition during RC nucleosome assembly (Chapter 3) and grow slower than Wt cells in 0 mM and 50 mM HU (Figure 4-1). Cells lacking *ASF1* are quite sensitive to genotoxins due to genome instability and a higher spontaneous rate of DNA damage [59]. We therefore expected to see that *cac1*Δ *rtt106*Δ *asf1*Δ cells grow extremely slowly in the presence of HU. Surprisingly, we observed that *cac1*Δ *rtt106*Δ *asf1*Δ cells grow more efficiently on 50 mM HU at 30^{o}C than the *asf1*Δ single mutant (Figure 4-1 (A)). This growth rescue only occurs in the presence of HU since *cac1*Δ *rtt106*Δ *asf1*Δ cells grow noticeably slower than all other strains on YPD plates containing no HU.

To further investigate this finding, three colonies each of *asf1*Δ and *cac1*Δ *rtt106*Δ *asf1*Δ cells were plated in 10X serial dilutions on YPD plates containing 0 mM or 50 mM HU and grown at room temperature

(Figure 4-1 (B)). The triple mutant appears to grow more efficiently in the presence (rather than the absence) of HU at room temperature. When cells are grown in 10X serial dilutions at 37^{o}C, growth of *cac1*Δ *rtt106*Δ *asf1*Δ cells is severely impaired in 0 mM HU and no growth occurs in 50 mM HU (Figure 4-1 (C)). These results suggest that the co-deletion of *CAC1* and *RTT106* rescues the HU sensitive phenotype of *asf1*Δ cells. Moreover, all RC mutants appear to be mildly heat sensitive, especially *cac1*Δ *rtt106*Δ *asf1*Δ at 37^{o}C.

HU sensitivity rescue in *cac1*Δ *rtt106*Δ *asf1*Δ cells decreases as HU concentration increases

We next investigated the range of HU concentrations over which the HU sensitive rescue by the co-deletion of *CAC1* and *RTT106* in *asf1*Δ cells occurs. RC chaperone mutants were spotted in 10X serial dilutions on YPD containing varying concentrations of HU and grown at 30^{o}C (Figure 4-1 (A) & Figure 4-2). At concentrations of 0 mM and 5 mM HU, all strains grew similarly except for *cac1*Δ *rtt106*Δ *asf1*Δ cells which grew slightly slower than the other strains. At 25 mM HU, growth of *asf1*Δ cells was severely compromised while growth of *cac1*Δ *rtt106*Δ *asf1*Δ cells was only slightly slower than Wt cells. As Figure 4-1 indicates, viability of *asf1*Δ cells in 50 mM HU is almost completely lost and no growth is observed at all by 75 mM HU (Figure 4-2). Wild type and *cac1*Δ *rtt106*Δ cells at these HU concentrations of HU appear to grow as efficiently as they do on YPD with no HU, while growth of *cac1*Δ *rtt106*Δ *asf1*Δ cells is almost completely abolished. This data indicates that the co-deletion of *CAC1* and *RTT106* together with *ASF1* rescues the HU sensitivity of *asf1*Δ cells up to approximately 50 mM HU concentrations, at which point growth becomes severely compromised.

Deleting *CAC1* and *RTT106* in addition to *ASF1* increases growth in liquid media compared to an *asf1*Δ strain

In addition to the spotting assays illustrated in Figures 4-1 & 4-2, the growth of RC chaperone mutants was also measured in liquid media containing 0 mM or 200 mM HU (Figure 4-3). It is evident from these data that growth in liquid media largely reflects the growth in solid media. However, *cac1*Δ *rtt106*Δ cells grow noticeably slower than Wt in 0 mM and 200 mM HU, whereas on YPD plates containing varying concentrations of HU growth of Wt and the double mutant appeared equal (Figure 4-2). The growth curve of *cac1*Δ *rtt106*Δ *asf1*Δ cells in 200 mM HU becomes noticeably steeper than *asf1*Δ cells after approximately 8 hours, while growth in these strains is equal in YPD containing no HU (Figure 4-3).

The observations from the growth curves in liquid media and spotting assays on agar plates indicate that the HU sensitive phenotype of *asf1*Δ cells can be rescued by the additional deletion of the other two RC chaperones, *CAC1* and *RTT106.* The remainder of this chapter focuses on exploring this finding to determine possible mechanisms of this HU sensitivity rescue.

_cac1_Δ _rtt106_Δ _asf1_Δ cells do not recover from HU treatment more efficiently than _asf1_Δ cells

To better understand why *cac1*Δ *rtt106*Δ *asf1*Δ cells are less sensitive to HU than *asf1*Δ cells, we analyzed the ability of each strain to recover from HU by measuring growth on YPD plates following HU treatment (Figure 4-4). Recovery from the DNA structure checkpoint is important for resuming cell cycle progression. Although Asf1 is important for a cells' recovery from DNA damage [36,37], perhaps the additional deletions of *CAC1* and *RTT106* enhance the cells' ability to recover from checkpoint activation. We predicted that following a 24 hour treatment with HU, *cac1*Δ *rtt106*Δ *asf1*Δ cells would show faster growth compared to *asf1*Δ cells on YPD plates, indicating more efficient checkpoint recovery. Figure 4-4 (A) shows cells treated with 0 mM or 200 mM HU for 24 hours, then washed and spotted onto YPD plates in 10X serial dilutions. Growth

of cells treated with 0 mM HU is the same as previously observed (Figure 4-1); *cac1*Δ *rtt106*Δ *asf1*Δ cells grow extremely slowly. Surprisingly, growth of cells pre-treated with 200 mM HU was identical the growth of cells pre-treated with 0 mM HU (Figure 4-4 (A)). *cac1*Δ *rtt106*Δ *asf1*Δ cells still grew extremely slowly. This indicates that the triple mutant does not recover from 200 mM HU more efficiently than *asf1*Δ cells.

Reflecting back on cell growth in varying concentrations of HU (Figure 4-2), the HU sensitivity rescue in *cac1*Δ *rtt106*Δ *asf1*Δ cells only occurs up to a maximum concentration of 50 mM HU. We therefore predicted that pre-treating the cells with a lower concentration of HU within the range of successful sensitivity rescue would allow *cac1*Δ *rtt106*Δ *asf1*Δ cells to recover more efficiently than *asf1*Δ cells. Figure 4-2 (B) shows the growth of cells pre-treated for 24 hours with 0 mM, 50 mM and 200 mM HU then washed and plated on YPD plates. We were again surprised to find that even pre-treatment with a lower HU concentration did not change the relative viabilities of each strain; *cac1*Δ *rtt106*Δ *asf1*Δ cells had the slowest growth indicating they again did not recover from HU as well as the other strains. It is evident from these observations that *cac1*Δ *rtt106*Δ *asf1*Δ cells do not recover from extended replication stress more efficiently than *asf1*Δ cells, and thus is not the reason for the decreased HU sensitivity observed in this strain.

HUG1 induction is impaired in both *caf1*Δ *rtt106*Δ *asf1*Δ and *asf1*Δ cells

We next predicted that the induction of *HUG1,* while impaired in *asf1*Δ cells [110], is induced similar to wild type levels in *cac1*Δ *rtt106*Δ *asf1*Δ cells. *HUG1* induction is a hallmark of DNA structure checkpoint activation and although its function is unknown, *HUG1* is suspected to aid in dNTP metabolism [97]. Cells in which *HUG1* has been deleted are only mildly sensitivity to various genotoxins. However, it is also one of the strongest inducible genes in response to DDR activation [97], suggesting that it is an

important component of the DDR. To investigate if *cac1*Δ *rtt106*Δ *asf1*Δ cells are more efficient at inducing *HUG1* than *asf1*Δ cells, I evaluated *HUG1* induction in response to HU (Figure 4-5 (A)). This was achieved by generating cDNA then using quantitative RT-PCR with primers within the *HUG1* coding region to determine the relative levels of transcript in each strain during unchallenged growth. Results of this experiment reveal that after 2 hours in HU, *HUG1* is induced to the same extent in Wt, *cac1*Δ, *rtt106*Δ and *cac1*Δ *rtt106*Δ cells. There is very little induction of *HUG1* in *asf1*Δ and *cac1*Δ *rtt106*Δ *asf1*Δ cells. Nonetheless, the level of *HUG1* expression is higher in the triple mutant than in the *asf1*Δ single mutant in cycling cells (Figure 3-1 and 4-5 (A)).

There is no induction of *RNR3* in *asf1*Δ or *cac1*Δ *rtt106*Δ *asf1*Δ cells either. However, in contrast to *HUG1*, the overall level of *RNR3* expression between these two strains is equal (Figure 4-5 (B)). As previously described, similar expression of *RNR3* in *asf1*Δ and *cac1*Δ *rtt106*Δ *asf1*Δ cells may be due to the inducibility of this gene. *RNR3* is not induced to the same level as *HUG1* in response to DNA structure checkpoint activation, thus any misregulation caused by disrupted RC nucleosome assembly in the RC chaperone mutants may not be apparent.

Flow cytometry was performed to measure DNA content in each strain during HU induction (Figure 4-5 (C)). Focusing first on the cycling profiles of each strain (shown in solid grey), the profiles of Wt, *cac1*Δ, *rtt106*Δ, and *cac1*Δ *rtt106*Δ cells are very similar. The cycling profile of *asf1*Δ cells, however, shows a higher proportion of cells in late S and G2/M phase. The cycling profile of *cac1*Δ *rtt106*Δ *asf1*Δ cells shows a large number of cells in G2/M phase and very few cells in G1 phase. A larger proportion of cells in G2/M phase is consistent with DNA damage checkpoint activation which occurs in *cac1*Δ *rtt106*Δ *asf1*Δ and *asf1*Δ cells [59]. It is surprising that *asf1*Δ cells have a much smaller 2N peak representing cells in G2/M phase, however, since Rad53 appears to be

equally phosphorylated in *asf1*Δ and the triple mutant during unperturbed cycling and Rad53 hyperphosphorylation is characteristic of DNA structure checkpoint activation (Figure 3-3 (C)).

*cac1*Δ *rtt106*Δ *asf1*Δ cells also have a distinct sub-G1 population. These cells have less than one cell equivalent (1N) of DNA demonstrated by the sub-1N DNA peak indicated by the arrow in Figure 4-5 (C). Often during flow cytometry analysis the sub-G1 population of cells is gated out so no peak is visible. In this case, gating out the sub-G1 population would be inappropriate because it is very apparent in the triple mutant and almost non-existent in wild type cells. A sub-G1 peak is indicative of cells undergoing apoptosis [135, 136]. During apoptosis DNA is fragmented, and intact cells with fragmented DNA may have less than one cell equivalent of DNA thus forming a sub-G1 peak. The sub-G1 peak can also indicate cells that are mechanically damaged or cells with abnormal chromatin structure [137]. The large difference in cell cycle profile and overall slow unchallenged growth in the triple mutant hints at the sick status of the strain. In view of these phenotypes during unchallenged growth it is remarkable that *cac1*Δ *rtt106*Δ *asf1*Δ cells are less sensitive to HU than *asf1*Δ cells.

Comparing the cell cycle profiles during HU treatment in Figure 4-5 (C) reveals another intriguing phenotype of *cac1*Δ *rtt106*Δ *asf1*Δ cells; they appear to arrest more efficiently in response to HU treatment. As HU treatment causes cell cycle arrest in early S phase, observing the accumulation of cells in early S phase during HU treatment provides information on the efficiency of HU arrest. wild type, *cac1*Δ, *rtt106*Δ, and *cac1*Δ *rtt106*Δ cells arrest very efficiently during HU treatment. This is indicated by the quick accumulation of cells in early S phase, characterized by an increase in the 1N DNA content peak and corresponding decrease in the 2N DNA content peak. *asf1*Δ cells on the other hand do not arrest well in HU. Although there is a small increase in the number of cells in early S phase, a clear G2/M peak still exists. This

may suggest that activation of the replication checkpoint is impaired in *asf1*Δ cells, resulting in inefficient arrest in early S in response to replication fork stalling.

Interestingly, the poor HU arrest we observed in *asf1*Δ cells (Figure 4-5 (C)) was not observed in *cac1*Δ *rtt106*Δ *asf1*Δ cells. The triple mutant showed a clear decrease in the number of cells in G2/M phase upon treatment with HU, which is characteristic of wild type cells. This data may suggest that replication checkpoint activation in *cac1*Δ *rtt106*Δ *asf1*Δ cells is more efficient than *asf1*Δ cells in HU, resulting in a quicker arrest in early S phase. While in HU, *cac1*Δ *rtt106*Δ *asf1*Δ cells not only show a decrease in G2/M cells, but the sub-G1 population grows. The 1N DNA peak shifts towards a sub-G1 peak after 120 minutes in HU, and this effect is not observed in any other strain. At this time, we are unsure what the significance of this sub-G1 peak is, or why it becomes more pronounced after 2 hours of HU treatment.

Total histone H3 is decreased in the absence of Cac1 and Rtt106 in the whole cell extract (WCE) but not in the chromatin (CHR) fraction of cell lysates

As described in the introduction, H3K56Ac is a post-translational modification occurring on histone H3. The presence of this mark has been shown to be important for signalling the completion of DNA repair, and the subsequent resumption of the cell cycle [37]. Histone gene transcription is stringently regulated as histone levels strongly affect genome stability and sensitivity to genotoxins [140]. Asf1 is essential for stimulating H3K56Ac by Rtt109. Asf1 also functions with CAF-1 and Rtt106 to deposit histones bearing the H3K56Ac mark onto DNA in RC nucleosome assembly. Because these chaperones are involved in this essential process, we utilized a chromatin fractionation procedure to determine the levels of H3 and H3K56Ac globally associated with chromatin. We predicted based on the current model of RC nucleosome assembly, that compromised histone

deposition as a result of *CAC1* and *RTT106* deletions would cause a global reduction in H3 levels associated with chromatin. The possibility of impaired nucleosome disassembly due to the absence of *ASF1* [21] in *asf1*Δ and *cac1*Δ *rtt106*Δ *asf1*Δ would result in a global increase in H3 levels on chromatin.

A chromatin fractionation was performed on asynchronous cycling cells. WCE and CHR fractions were analyzed by immunoblotting (Figure 4-6). Carboxypeptidase Y (CPY), used as a control, is a vacuolar protein which does not associate with chromatin. CPY is not present in CHR fractions following fractionation [113]. As expected, the CPY protein band is only visible in the WCEs and not in the chromatin fractions, indicating that our chromatin fractionation was successful.

The chromatin fractionation results for H3K56Ac (Figure 4-6) complement the ChIP results in the literature: CAF-1, Rtt106, and Asf1 are major players in RC nucleosome assembly, and CAF-1 and Rtt106 preferentially bind H3-H4 marked with H3K56Ac. Moreover, decreased deposition of $(H3\text{-}H4)^{H3K56Ac}$ behind the replication fork has been observed by ChIP in *cac1*Δ *rtt106*Δ cells [6]. In the CHR fractions in Figure 4-6, H3K56Ac is prominent in wild type cells, non-existent in *asf1*Δ and *cac1*Δ *rtt106*Δ *asf1*Δ cells due to the lack of *ASF1,* and extremely decreased in *cac1*Δ *rtt106*Δ cells. Because the double mutant can still acetylate H3K56, histones bearing this mark can associate with chromatin through replication-independent methods. Thus the decrease, but not complete loss of H3K56Ac in the chromatin fraction of *cac1*Δ *rtt106*Δ cells is expected. Surprisingly however, H3K56Ac in the WCE fraction is also decreased in *cac1*Δ *rtt106*Δ cells even though *ASF1* and *RTT109* are still present to acetylate H3K56. One explanation for this observation may be found in our chromatin fractionation results for histone H3. H3 in the WCEs of each strain varies depending on the RC chaperones present. Wild type and *asf1*Δ cells have equal levels of H3, while *cac1*Δ *rtt106*Δ and

*cac1*Δ *rtt106*Δ *asf1*Δ cells have decreased levels of H3. Therefore the ratio of H3K56Ac:H3 may be the same in the WCEs of wild type and *cac1*Δ *rtt106*Δ cells, but because there is less H3 in the WCE of the double mutant there is also less H3K56Ac.

The decrease in total H3 in the absence of CAC1 and *RTT106* is interesting because Rtt106 participates in histone gene regulation [42,43]. Therefore if we were expecting any change in H3 levels in the absence of *CAC1* and *RTT106* it would have been an increase.

HUG1 repression during recovery from HU treatment is delayed in the absence of Cac1 and Rtt106

We further investigated the dynamics of *HUG1* regulation in the RC chaperone mutants by measuring *HUG1* repression following removal of HU. This experiment serves two purposes; first, it will further explore the role of CAF-1 and Rtt106 in the proper regulation if *HUG1.* Second, comparing *HUG1* repression between *asf1*Δ and *cac1*Δ *rtt106*Δ *asf1*Δ cells may provide insight into the significance of *HUG1* regulation in survival during replication stress.

HUG1 transcription was measured in various RC chaperone mutants during unperturbed cycling, 1 hour of HU treatment, 30 minutes following HU washout and release into preconditioned media, and 90 minutes following HU washout and release into preconditioned media (Figure 4-7 (A)). There is clear repression of *HUG1* after HU removal n wild type and *asf1*Δ cells between 30 and 120 minutes following resuspension of cells in medium without HU. Moreover, *HUG1* was almost fully repressed 120 minutes after HU was removed in these strains (although there is significant overlap of the error bars in *asf1*Δ cells at all time points). On the other hand, unlike wild type and *asf1*Δ cells, *cac1*Δ *rtt106*Δ and *cac1*Δ *rtt106*Δ *asf1*Δ cells appear to have difficulty repressing

HUG1 following HU removal since no decrease in *HUG1* transcription is observed.

A curious observation is that in all strains, *HUG1* and *RNR3* continue to be induced at least 30 minutes after HU is removed (Figure 4-7 (A&B)). We did not focus on this result because it occurs in wild type cells, thus must not involve the RC chaperones or RC nucleosome assembly.

All strains appear to repress *RNR3* transcription normally following induction in HU (Figure 4-7 (B)). *RNR3* transcription was not measured in *cac1*Δ *rtt106*Δ *asf1*Δ cells. However, based on previous results (Figure 4-5 (B)), *RNR3* regulation is not drastically different between *asf1*Δ and *cac1*Δ *rtt106*Δ *asf1*Δ cells. Therefore, measuring *RNR3* transcription will likely not provide any insight into why *cac1*Δ *rtt106*Δ *asf1*Δ cells are less sensitive to prolonged HU treatment than *asf1*Δ cells.

Wild type and *cac1*Δ *rtt106*Δ cells showed an efficient early S phase arrest after 1 hour of HU treatment (Figure 4-7 (C)). 30 minutes after release from HU, there was a slight shift as cells entered S phase, and cells were in G2/M phase at 90 minutes after release. *asf1*Δ cells only moderately arrested in HU, as previously observed in Figure 4-5(C). However, *cac1*Δ *rtt106*Δ *asf1*Δ cells arrested slightly more efficiently in HU than *asf1*Δ cells. 30 minutes after HU washout, the cells remained arrested and cells were synchronized in G2/M phase after 90 minutes in HU. These data show that in terms of cellular responses to HU, *cac1*Δ *rtt106*Δ *asf1*Δ cells more closely resemble wild type cells than *asf1*Δ cells.

Deleting *CAC1* and *RTT106* in addition to *ASF1* rescues the *asf1*Δ sensitivity to HU and NAM but not MMS

HU depletes dNTP levels thus causing replication forks to stall. We discovered that *cac1*Δ *rtt106*Δ *asf1*Δ cells are more viable than *asf1*Δ cells during prolonged HU treatment (Figures 4-1 and 4-2). This unexpected

finding prompted us to explore how cells would respond to prolonged exposure to other genotoxins. Specifically, we investigated how cells would respond to MMS and NAM. NAM is indirectly a genotoxin because it inhibits the H3K56 deacetylases, Hst3 and Hst4.

We investigated the sensitivities of each strain to 50 mM HU, 25 mM and 50 mM NAM, and 0.005% MMS (Figure 4-8). Cells were spotted in 10X serial dilutions onto YPD plates containing the indicated genotoxin and grown at 30^{o}C (A) or 37^{o}C (B) for three days. On YPD plates (no genotoxin) at either temperature, *asf1*Δ and especially *cac1*Δ *rtt106*Δ *asf1*Δ cells have slow growth. All of the RC mutants appear to be heat sensitive as viability decreases from 30^{o}C to 37^{o}C. However, the comparative viabilities between each strain remains the same. The heat sensitivity of these mutants was previously observed (Figure 4-1). Note that the decreased HU sensitivity of *cac1*Δ *rtt106*Δ *asf1*Δ cells is lost at 37^{o}C because *cac1*Δ *rtt106*Δ *asf1*Δ and *asf1*Δ cells cannot grow at this temperature.

Nicotinamide (NAM) noncompetitively inhibits Sir2, which is required for silencing [81,82]. NAM also inhibits Hst3, which is a deacetylase of H3K56Ac. During treatment with NAM the frequency of H3K56Ac should therefore be increased. The sensitivities of each strain to 25 mM NAM at 30^{o}C are very puzzling. *cac1*Δ *rtt106*Δ and *cac1*Δ *rtt106*Δ *asf1*Δ cells appear to be more viable than *asf1*Δ and even wild type cells. At 50 mM NAM, *cac1*Δ *rtt106*Δ *asf1*Δ cells lose much of their viability such that they grow similarly to wild type and *asf1*Δ cells. This result is puzzling because the deletions of RC chaperones which noticeably results in decreased growth on YPD somehow enhance growth on NAM. At 37^{o}C the heat sensitive phenotype of RC chaperone mutants is again apparent as wild type cells have the most growth in 25 mM NAM (Figure 4-8 (B)), as opposed to *cac1*Δ *rtt106*Δ cells which have the most growth at 30^{o}C. At 37^{o}C in 50 mM NAM there is no growth of wild type or *asf1*Δ cells,

however *cac1*Δ *rtt106*Δ and *cac1*Δ *rtt106*Δ *asf1*Δ cells can still manage minimal growth.

4-3: Discussion

In the previous chapter it was demonstrated that the RC chaperones have divergent functions in the transcriptional control of *HUG1.* The data presented in this chapter also suggests that these chaperones have divergent functions, this time contributing to cell survival during prolonged HU treatment. We were surprised to find that *cac1*Δ *rtt106*Δ *asf1*Δ cells are more viable than *asf1*Δ cells on YPD plates supplemented with HU. This indicates that the co-deletion of *CAC1* and *RTT106* rescues the HU sensitivity of *asf1*Δ cells. *cac1*Δ *rtt106*Δ *asf1*Δ cells have a slow growing phenotype during unperturbed growth; noticeably slower than *asf1*Δ cells. It was therefore very interesting to find that they were less sensitive to HU than *asf1*Δ cells. The spotting assays in Figures 4-1 & 4-2 indicate that this sensitivity rescue is temperature and HU concentration dependent, as the viability of *cac1*Δ *rtt106*Δ *asf1*Δ cells decreases by 37^{o}C and 75 mM HU. Moreover, the advantage *cac1*Δ *rtt106*Δ *asf1*Δ cells have over *asf1*Δ cells during HU treatment cannot increase viability to wild type or *cac1*Δ *rtt106*Δ levels.

The observations presented in Figures 4-1 & 4-2 suggest that the functions of CAF-1 and Rtt106 have an opposing role as Asf1 in supporting viability during HU exposure. In other words, when RC nucleosome assembly is disrupted by the absence of Asf1, the sensitivity to HU that this deletion causes is rescued by additionally deleting *CAC1* and RTT106. Somehow the presence of Cac1 and Rtt106 in cells lacking Asf1 is more detrimental to a cells' survival in prolonged HU treatment than in the absence of Cac1 and Rtt106.

Although we have not yet constructed *cac1*Δ *asf1*Δ and *rtt106*Δ *asf1*Δ double mutants, published results indicate that an *cac1*Δ *asf1*Δ double mutant grows less efficiently than either single mutant on YPD or media containing HU [134]. This was tested in cells of W303-1A background,

which is the same genetic background as the strains used in this thesis. It would be very interesting to analyze the HU sensitivity of a *rtt106*Δ *asf1*Δ double mutant to investigate if it is the co-deletion of *CAC1* and *RTT106* or the single Rtt106 deletion that rescues the HU sensitivity of *asf1*Δ cells. Determining the specific gene deletion (*CAC1, RTT106,* or both) that rescues the HU sensitivity of *asf1*Δ cells will be important to better understand this mechanism. If it is simply the deletion of *RTT106* which rescues *asf1*Δ cells, this may suggest further divergent functions of the RC chaperones in which Rtt106 can contribute negatively to cell survival during prolonged replication stress.

Asf1 is essential for H3K56Ac, a mark which increases the binding affinity of CAF-1 and Rtt106 to H3-H4. Without Asf1 (and thus H3K56Ac), the binding affinity of histones to the remaining RC chaperones is decreased but still functional [6]. Thus, nucleosome assembly during replication and repair may be less efficient. In the absence of all three RC chaperones, perhaps other chaperones not normally involved in RC nucleosome assembly can substitute. If these chaperones do not prefer interaction with $(H3\text{-}H4)^{H3K56Ac}$ over H3-H4, then they may be slightly more efficient at nucleosome assembly during replication then CAF-1 and Rtt106 which preferentially bind $(H3\text{-}H4)^{H3K56Ac}$. Utilizing an H3K56Q or H3K56R mutant lacking *CAC1, RTT106,* and *ASF1* may clarify if H3K56Ac plays a role in HU sensitivity rescue. This is because a lysine to glutamine (K56Q) mutation mimics permanent acetylation while a lysine to arginine (K56R) mimics permanent deacetylation.

Because of the interesting observation that *cac1*Δ *rtt106*Δ *asf1*Δ cells are less sensitive to prolonged HU treatment than *asf1*Δ cells, we performed various experiments to attempt to understand this mechanism.

We initially predicted that *cac1*Δ *rtt106*Δ *asf1*Δ cells can recover from extended HU treatment more efficiently than *asf1*Δ cells. Figure 4-4 indicates that this is not the case, as growth following a 24 hour HU

treatment is severely compromised, especially in *cac1*Δ *rtt106*Δ *asf1*Δ cells. We next investigated if the altered dynamics of *HUG1* regulation in cells lacking *ASF1* are relieved in *cac1*Δ *rtt106*Δ *asf1*Δ cells. *HUG1* induction is severely compromised in *asf1*Δ cells [110] (Figure 4-5 (A)). Although *cac1*Δ *rtt106*Δ *asf1*Δ cells have the same difficulty in inducing *HUG1* as *asf1*Δ cells (Figure 4-5 (A)), *HUG1* is expressed at a higher level under normal unchallenged growth conditions in the triple mutant. This increased *HUG1* derepression in the triple mutant (compared to the *asf1*Δ single mutant) may provide a fitness advantage leading to increased viability during prolonged replication stress. However, *hug1*Δ cells are not particularly sensitive to 100 mM HU treatment [97]. Therefore it is likely there are other reasons for the HU sensitivity rescue of *cac1*Δ *rtt106*Δ *asf1*Δ cells. Although it would be simple to delete *HUG1,* analysis of the phenotypes of a *hug1*Δ might not be straightforward to interpret. Alterations in RNR activity in *hug1*Δ cells could have diverse effects on cells that are unrelated to the mechanisms by which CAF-1 and Rtt106 repress *HUG1* [97].

Another possible reason for the HU sensitivity rescue of *cac1*Δ *rtt106*Δ *asf1*Δ cells is hinted by the fact that *asf1*Δ cells accumulate in early S phase quite slowly during HU treatment. On the other hand, *cac1*Δ *rtt106*Δ *asf1*Δ cells, although still missing *ASF1*, appear to accumulate in early S phase more efficiently (Figure 4-5 (C), Figure 4-7 (C)). It has been previously demonstrated that slow accumulation in early S phase during HU treatment correlates with a delayed activation of the replication checkpoint, characterized by a delayed hyperphosphorylation of Rad53 [26]. We therefore predict that Rad53 activation in response to HU in our *asf1*Δ cells of a W303 background will be delayed, however will not be noticeably delayed in *cac1*Δ *rtt106*Δ *asf1*Δ cells. DNA structure checkpoint activation is extremely important for cell survival, as demonstrated by the sensitivity of cells lacking *RAD53* to genotoxins and the well conserved surveillance mechanisms to protect against DNA damage [138]. Upon activation, origin

firing that normally occurs in late S phase is delayed, stability and integrity of stalled replication forks is maintained, and entrance into mitosis is prevented until replication and repair is properly completed [85]. *cac1*Δ *rtt106*Δ *asf1*Δ cells may have more efficient replication checkpoint activation, based on the flow cytometry analysis in Figure 4-5 (C), and thus are better able to survive in HU. To investigate checkpoint activation efficiencies in each strain, Rad53 phosphorylation during HU treatment can be analyzed in a similar experiment to that performed in Minard *et al.* (2011) [26]. In such an experiment we predict that Rad53 hyperphosphorylation will be delayed in *asf1*Δ cells, indicating a delay in checkpoint activation. The speed of Rad53 hyperphosphorylation in *cac1*Δ *rtt106*Δ *asf1*Δ cells however will closer resemble that of wild type cells.

It is important to note that in the literature *asf1*Δ cells are not reported to have difficulty arresting in HU. However, combining the deletion of *ASF1* with another gene often results in very noticeable impaired checkpoint activation [26,47, 139, 53]. Moreover, yeast strains used in this thesis have a W303-1A genetic background. It is therefore possible that the impaired arrest in HU in cells lacking Asf1 is apparent in W303-1A strains, but not strains of other genetic backgrounds.

A particularly intriguing observation is that both mutants lacking Cac1 and Rtt106 had the same amount of H3 associated with chromatin as wild type cells. In contrast, *asf1*Δ cells had slightly more H3 associated with its chromatin (Figure 4-6). This could be because of the role Asf1 may play in nucleosome disassembly. In the absence of *ASF1,* nucleosome disassembly is somehow impaired, and thus more H3 is associated with chromatin. Interestingly, *cac1*Δ *rtt106*Δ *asf1*Δ cells do not share this increase in chromatin-associated H3 despite lacking *ASF1*. Although we do not yet have an explanation for the varying H3 levels in the WCE and CHR fractions of each strain, these results may provide a hint as to why *cac1*Δ *rtt106*Δ *asf1*Δ cells are less sensitive to prolonged HU treatment

than *asf1*Δ cells. As H3 levels are stringently regulated and can have drastic consequences if levels are abnormal [140], the wild type level of H3 associated with chromatin in *cac1*Δ *rtt106*Δ *asf1*Δ cells may provide an advantage over *asf1*Δ cells which have increased H3 associated with chromatin. Specifically, the wild type levels of H3 associated with chromatin in *cac1*Δ *rtt106*Δ *asf1*Δ cells may allow them to respond more normally to HU treatment, resulting in efficient arrest in HU. This is in contrast to *asf1*Δ cells which have difficulty arresting in HU Figure 4-5 (C), Figure 4-7 (C)). To better interpret these results, the chromatin fractionation should be repeated and WCE, CHR and supernatant (SUP) fractions analyzed in each strain. It has been demonstrated that the RC chaperones are involved in histone gene repression [53,43], thus it is unexpected that in the absence of these chaperones total H3 is decreased. Performing a quantitative Western to measure total histone H3 levels in each strain will provide more information on the possible differing levels of H3 in cells lacking Cac1 and Rtt106. Measuring H3 levels in the SUP fraction of a chromatin fractionation would also help clarify the possible differing levels of H3 in these cells Measuring the protein levels of histones H4, H2A and H2B along with H3 would also provide more detail on histone deposition in the RC chaperone mutants.

Collectively, these data in Chapter 4 suggest that CAF-1 and Rtt106 contribute to *HUG1* repression following induction, and Asf1 contributes to *HUG1* induction. Because *HUG1* dynamics are different in *cac1*Δ *rtt106*Δ *asf1*Δ cells than *asf1*Δ cells, these data also suggest that *HUG1* may play a role in cell viability during prolonged HU treatment. The differential *HUG1* dynamics in the RC chaperone mutants is due to the roles of each RC chaperone in nucleosome assembly and disassembly.

To investigate how *HUG1* regulation differs between *cac1*Δ *rtt106*Δ and *cac1*Δ *rtt106*Δ *asf1*Δ cells, *HUG1* transcription should be measured over a prolonged period of HU treatment in the RC chaperone. Based on

the HU recovery results in Figure 4-7, we predict that *HUG1* would remain induced in *cac1*Δ *rtt106*Δ and *cac1*Δ *rtt106*Δ *asf1*Δ cells even though *HUG1* in wild type cells is eventually repressed while cells are still in HU (Dr. Laura Minard, unpublished data).

Finally, we investigated the sensitivities of each strain to NAM and MMS (Figure 4-8). We found that *cac1*Δ *rtt106*Δ and *cac1*Δ *rtt106*Δ *asf1*Δ cells had increased growth compared to wild type and *asf1*Δ cells on plates supplemented with NAM. It is difficult to understand why cells lacking *CAC1* and *RTT106* have a growth advantage in NAM. As with prolonged HU treatment, it is possible that the derepression of *HUG1* in cells lacking *CAC1* and *RTT106* is sufficient to enhance cell viability. The decreased sensitivity of cells lacking *CAC1* and *RTT106* to NAM likely does not involve H3K56Ac because *asf1*Δ and *cac1*Δ *rtt106*Δ *asf1*Δ cells do not have any H3K56Ac, yet *cac1*Δ *rtt106*Δ *asf1*Δ cells are much less sensitive to NAM than *asf1*Δ cells. To get a better understanding why cells lacking *CAC1* and *RTT106* are less sensitive to NAM than wild type cells, it is important to investigate if treating cells with NAM activates the DNA structure checkpoints by analyzing Rad53 phosphorylation by immunoblotting or DDR gene induction.

The comparative viabilities of each strain on YPD were similar to the comparative viabilities on 0.005% MMS; wild type cells were the most viable, followed by *cac1*Δ *rtt106*Δ*,* then *asf1*Δ and finally *cac1*Δ *rtt106*Δ *asf1*Δ cells which had almost no growth. A distinctive difference with growth on MMS compared to growth on YPD is that all RC chaperone mutants have much poorer growth on MMS. MMS treatment results in single and double strand breaks[79, 80]. Because the sensitivity of *asf1*Δ cells to 0.005% MMS is not rescued by the co-deletion of *CAC1* and *RTT106*, this suggests that the mechanism which rescues the sensitivity of *asf1*Δ cells to prolonged replication stress is not involved in double strand break detection or repair. Treating cells with UV irradiation also generates also

double strand breaks [141], thus it would be interesting if the co-deletion of *CAC1* and *RTT106* can rescue the sensitivity of *asf1*Δ cells to this form of DNA damage, or if results will be similar to those observed with MMS treated cells.

A final model describing the details of HU sensitivity rescue in *asf1*Δ cells by the co-deletion of *CAC1* and *RTT106* has not been provided as further research is required to understand the mechanism. However, we speculate that in addition to increased *HUG1* transcription (due to compromised histone deposition), *cac1*Δ *rtt106*Δ *asf1*Δ cells can more efficiently activate the replication checkpoint in response to replication stress.

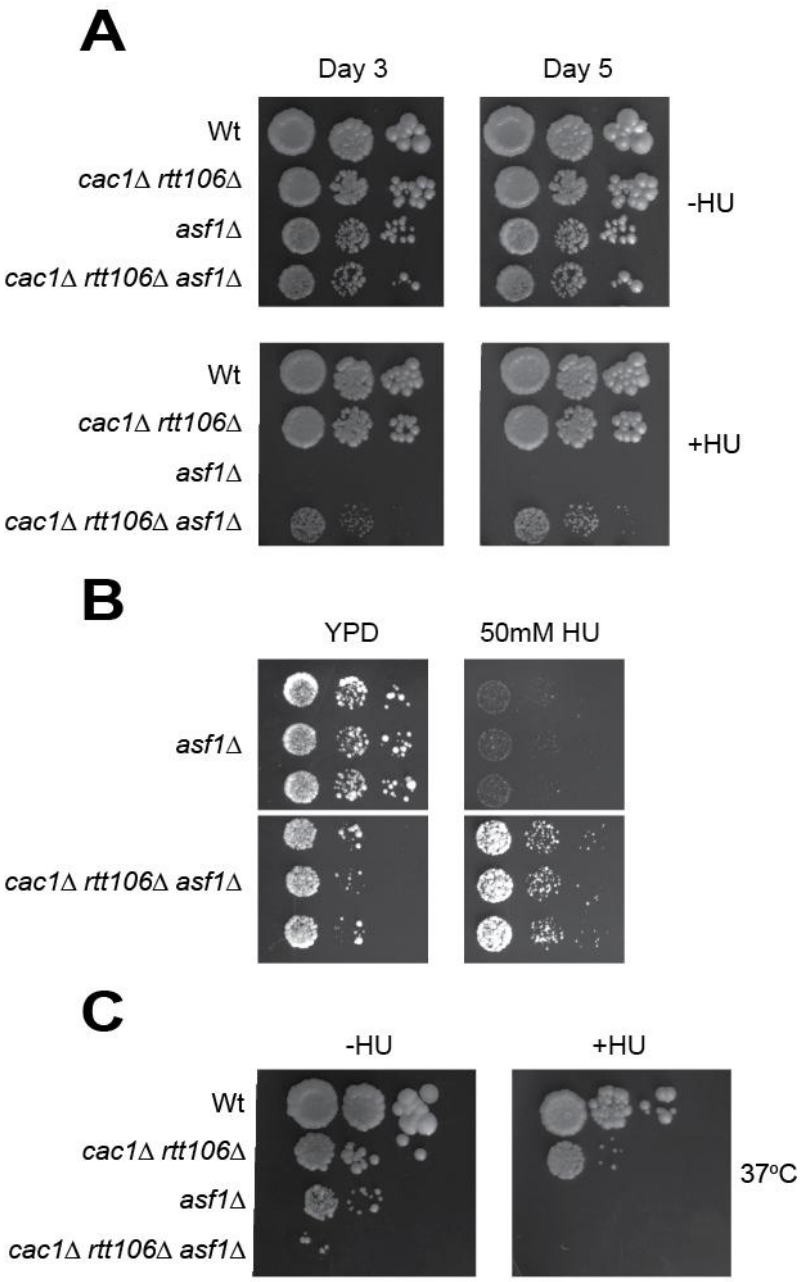


Figure 4-1: Deleting *CAC1* and *RTT106* in addition to *ASF1* rescues the HU sensitivity of an *asf1*Δ strain. A) Cells were spotted on YPD plates containing 0 mM (-HU) or 50 mM HU (+HU) and grown at 30°C for the indicated times. **B)** Three colonies each of *asf1*Δ and *cac1*Δ *rtt106*Δ *asf1*Δ were grown in liquid culture and cells were spotted in 10X serial dilutions onto YPD plates containing 0 mM (YPD) or 50 mM HU and grown at RT for 6 days. **C)** Cells were spotted in 10X serial dilutions onto YPD plates containing 0 mM (-HU) or 50 mM HU (+HU), and grown for 3 days at 37°C.

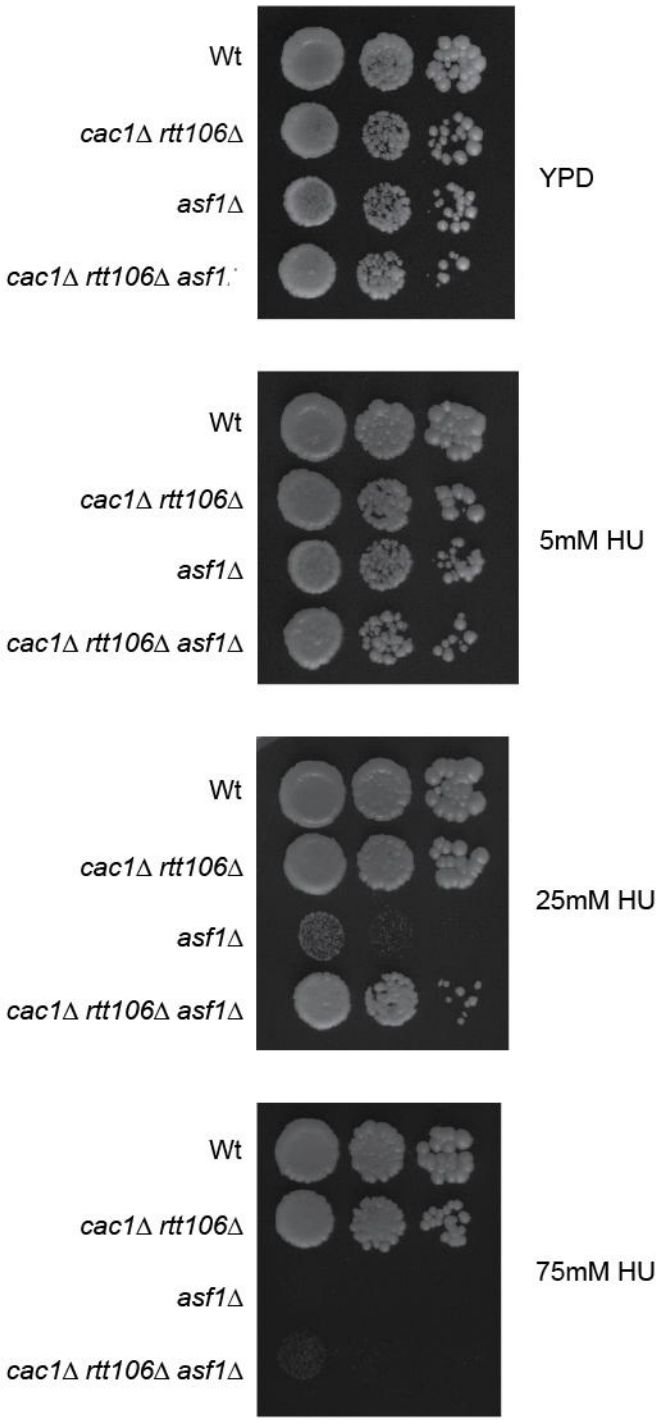


Figure 4-2: Sensitivity rescue in *cac1*Δ *rtt106*Δ *asf1*Δ cells decreases as HU concentration increases. Cells were spotted in 10X serial dilutions onto YPD plates containing the indicated concentrations of HU and grown for 3 days (YPD) or 4 days at 30^{o}C.

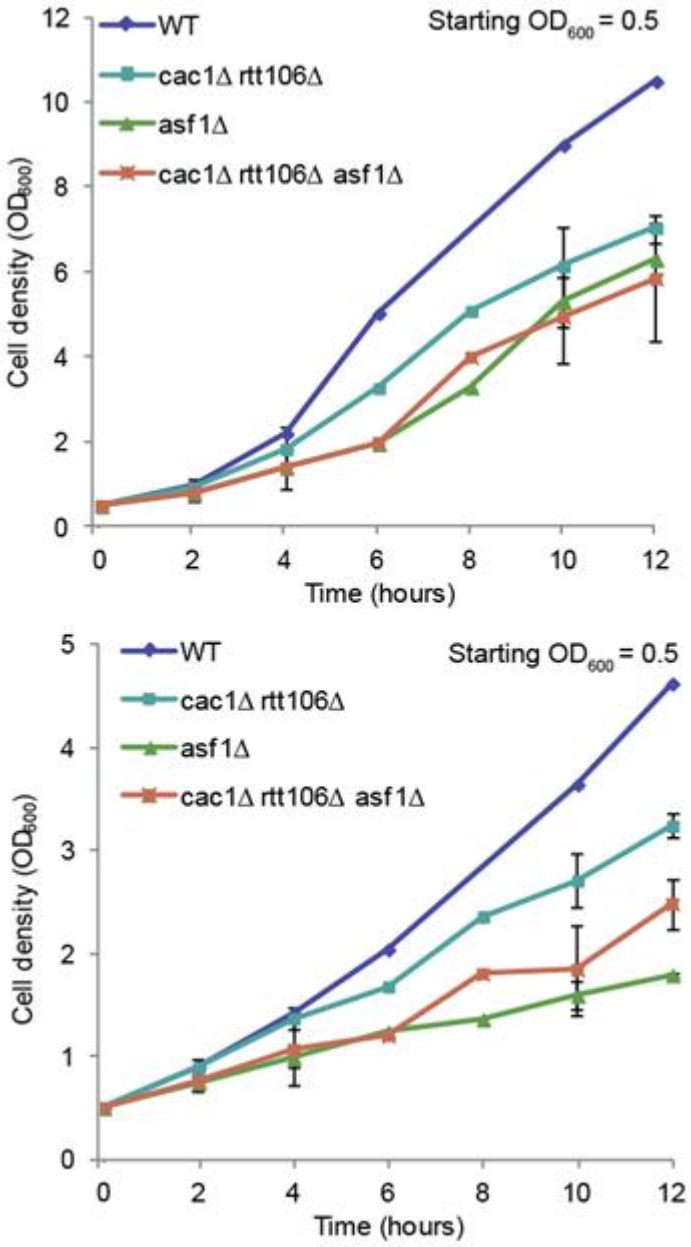


Figure 4-3: Deleting *CAC1* and *RTT106* in addition to *ASF1* increases growth in liquid media compared to an *asf1*Δ strain. Cells were diluted to OD_{600} 0.5 in YPD containing 0 mM (upper graph) or 200 mM (lower graph) HU and grown for 12 hours. OD_{600} was measured every 2 hours. Single experiments (Wt) or an average of 2 experiments are shown. Error bars indicate the range.

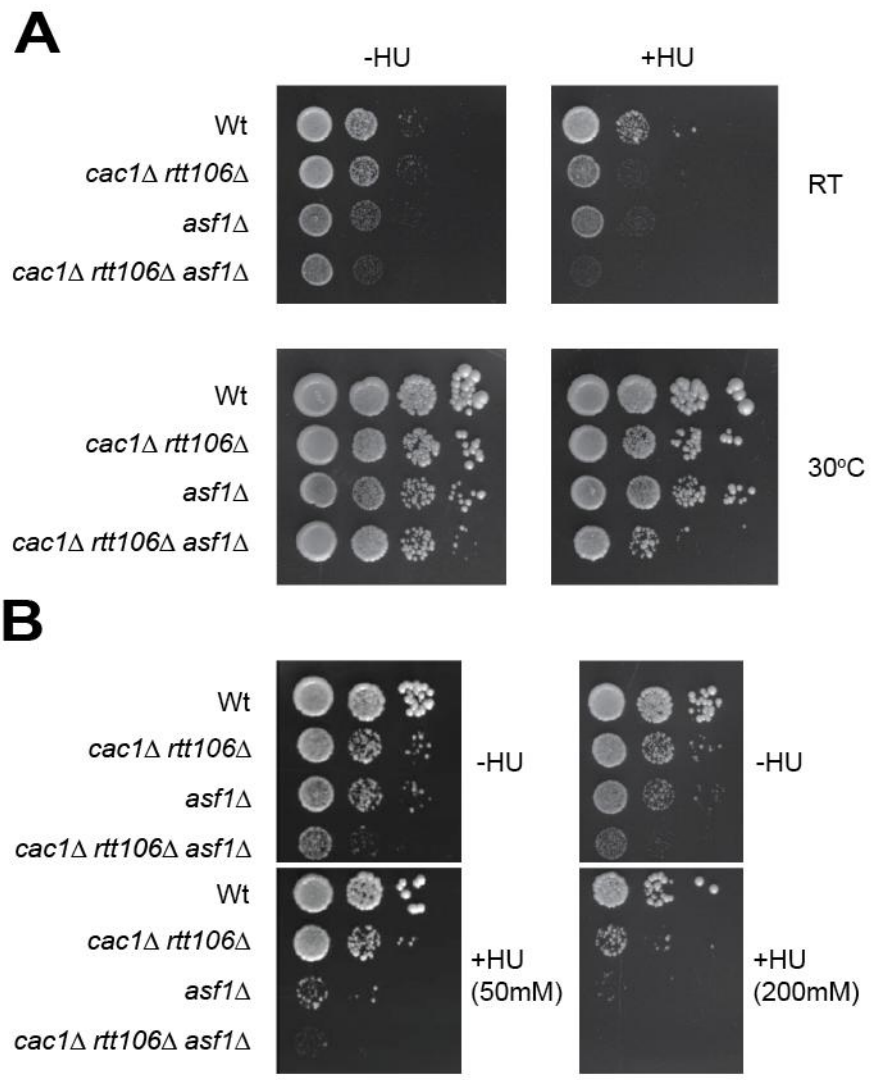


Figure 4-4: *cac1*Δ *rtt106*Δ *asf1*Δ cells do not recover from HU treatment more efficiently than *asf1*Δ cells. A) Cells were treated with 0 mM HU (-HU) or 200 mM HU (+HU) for 24 hours. Cells were then washed in water, spotted in 10X serial dilutions on YPD plates and grown for 4 days at RT or 30^{o}C. **B)** Cells were treated with 0 mM (-HU), 50 mM or 200 mM HU for 24 hours. Cells were than washed in water, spotted in 10X serial dilutions on YPD plates and grown for 2 days at 30^{o}C. 0 mM and 50 mM HU treatments are shown in the left column and 0 mM and 200 mM HU treatments are shown in the right column.

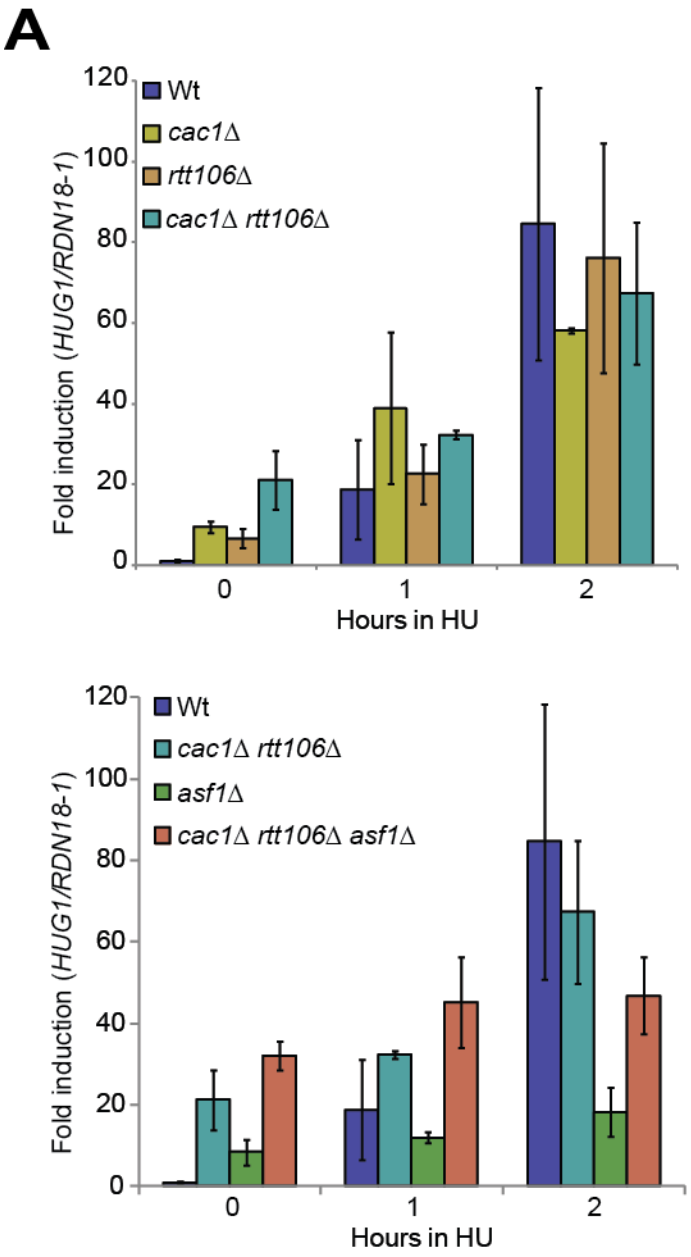
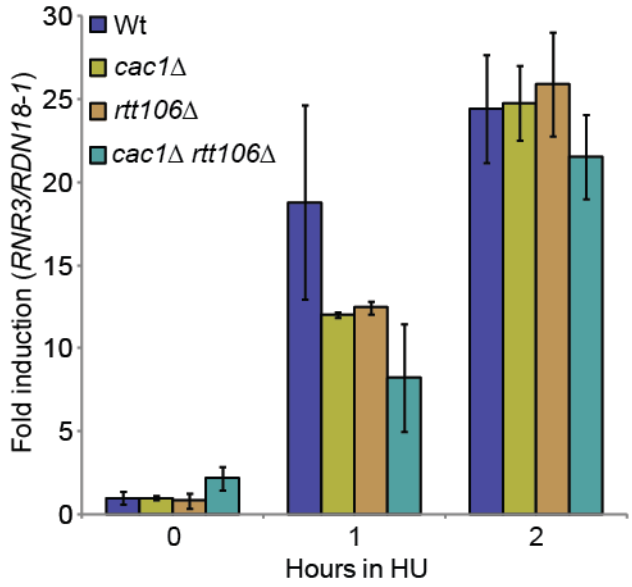


Figure 4-5: A&B) *HUG1* is induced to a greater degree in *caf1*Δ *rtt106*Δ *asf1*Δ cells than *asf1*Δ cells. Cells were treated with 200 mM HU for 2 hours and *HUG1* (A) or *RNR3* (B) induction measured after each hour. Transcription is normalized to *RDN18-1*.

B



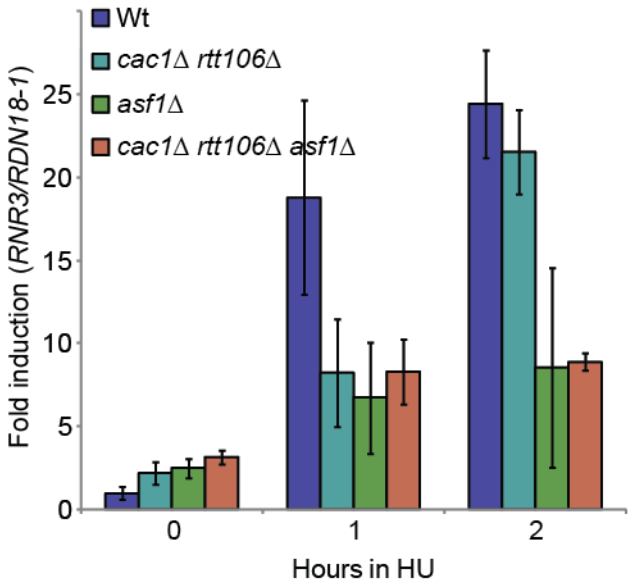


Figure 4-5 (continued): A&B) Cells were treated with 200 mM HU for 2 hours and *HUG1* (A) or *RNR3* (B) induction measured after each hour. Transcription is normalized to *RDN18-1*.

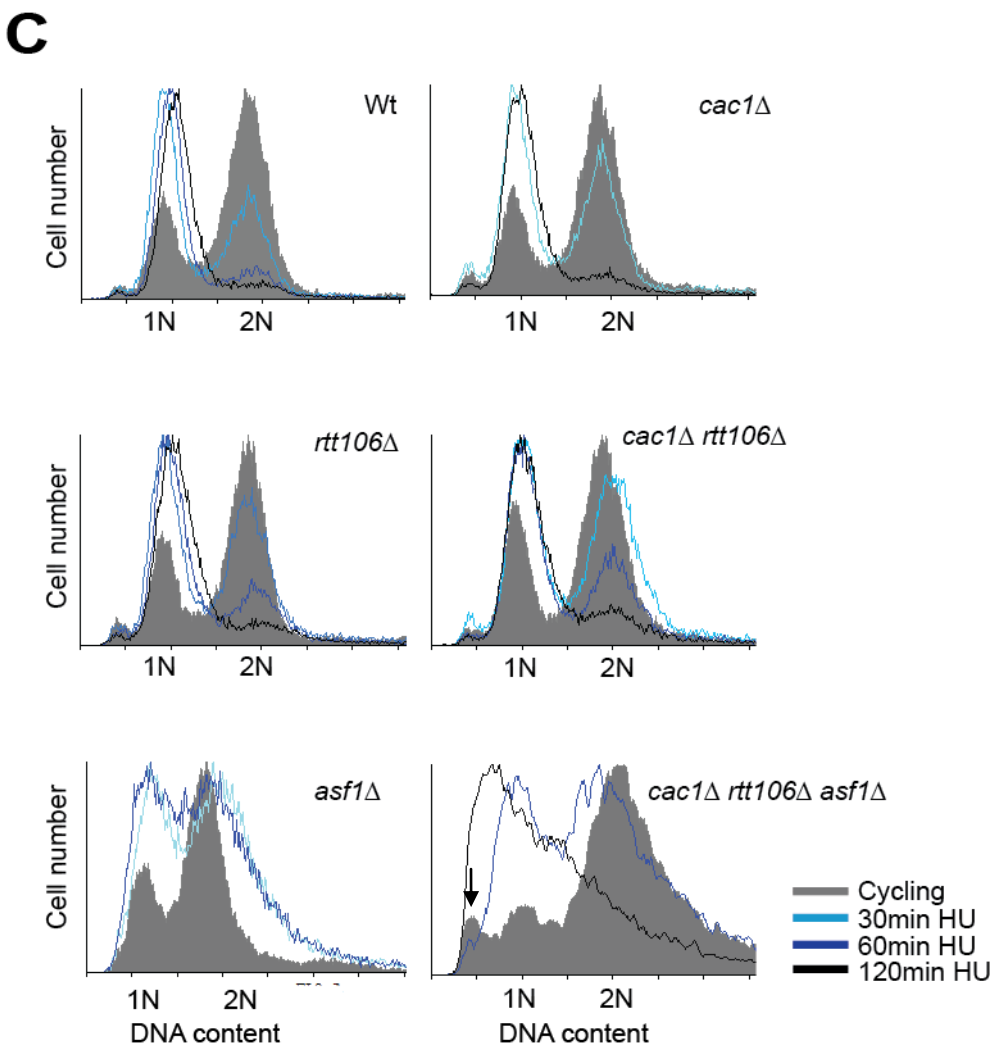


Figure 4-5 (continued): C) Flow cytometry analysis of cells in A and B. Arrow indicates sub-G1 population in *cac1*Δ *rtt106*Δ *asf1*Δ cells. Results shown here are representative of three independent experiments.

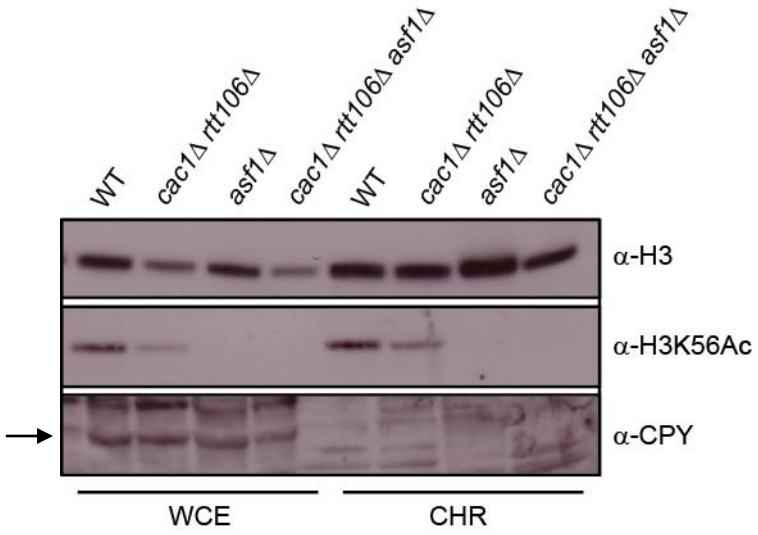


Figure 4-6: Histone H3 is decreased in the absence of Cac1 and Rtt106 in the WCE but not the CHR fraction of cell lysates. Western blot analysis of H3, H3K56Ac and CPY control in lysates from log-phase cycling cells subjected to a chromatin fractionation. Samples were diluted to 2µg/µl and equal amounts of protein loaded. Arrow denotes CPY band. Experiment performed once.

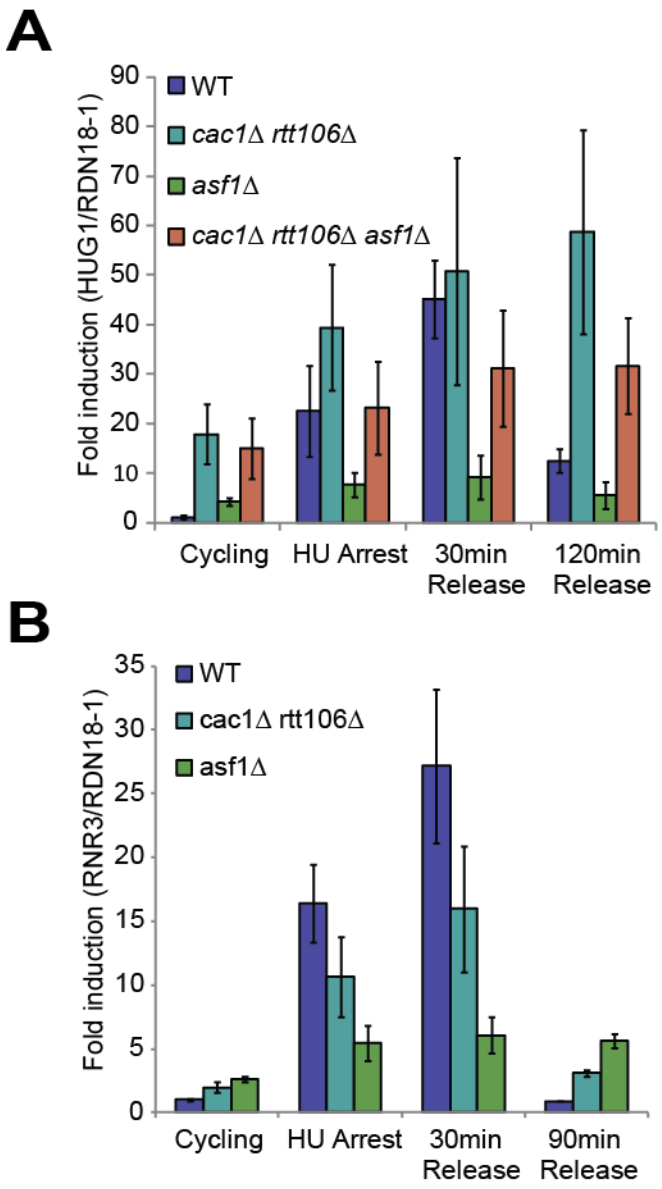


Figure 4-7: *HUG1* repression during recovery from HU is delayed in the absence of Cac1 and Rtt106. Cells were treated with 200 mM HU for 1h (HU Arrest) then released into pre-conditioned media. RNA was isolated from samples collected at 30, 90 and 120min after release. *HUG1* **(A)** or *RNR3* **(B)** transcription was measured by quantitative RT-PCR and normalized to *RDN18-1*.

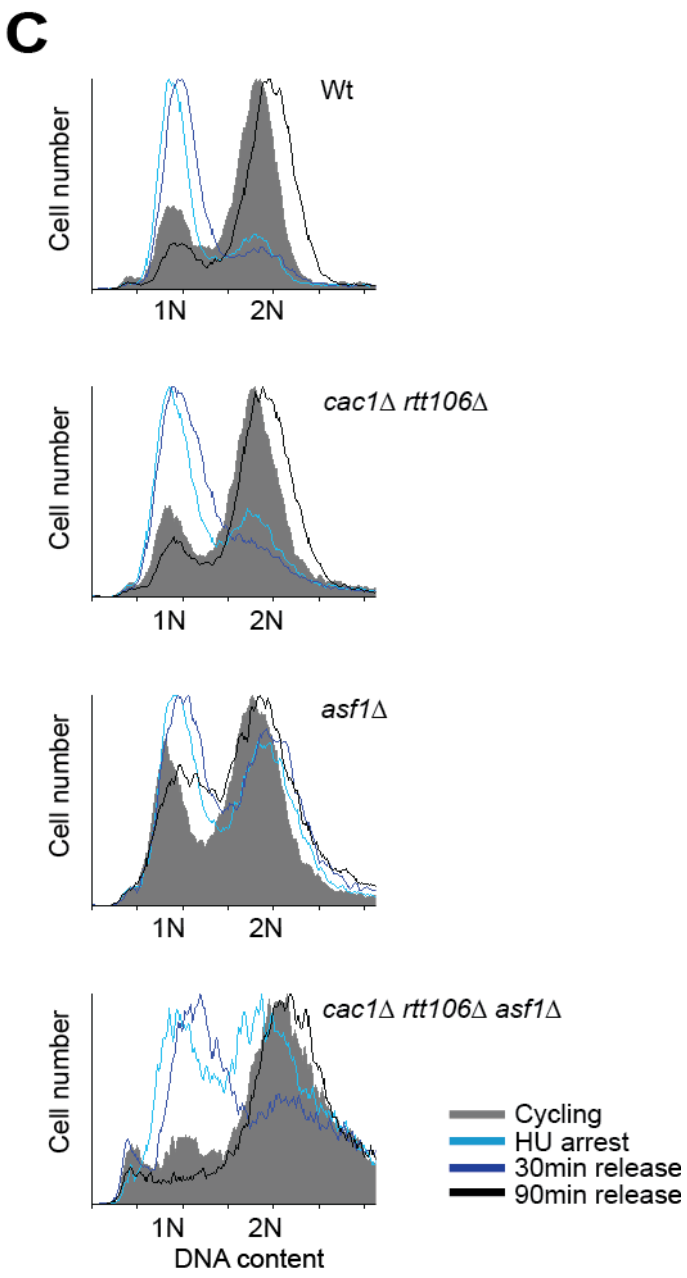


Figure 4-7 (continued): C) Flow cytometry analysis of cells in A&B. Results shown here are representative of three independent experiments.

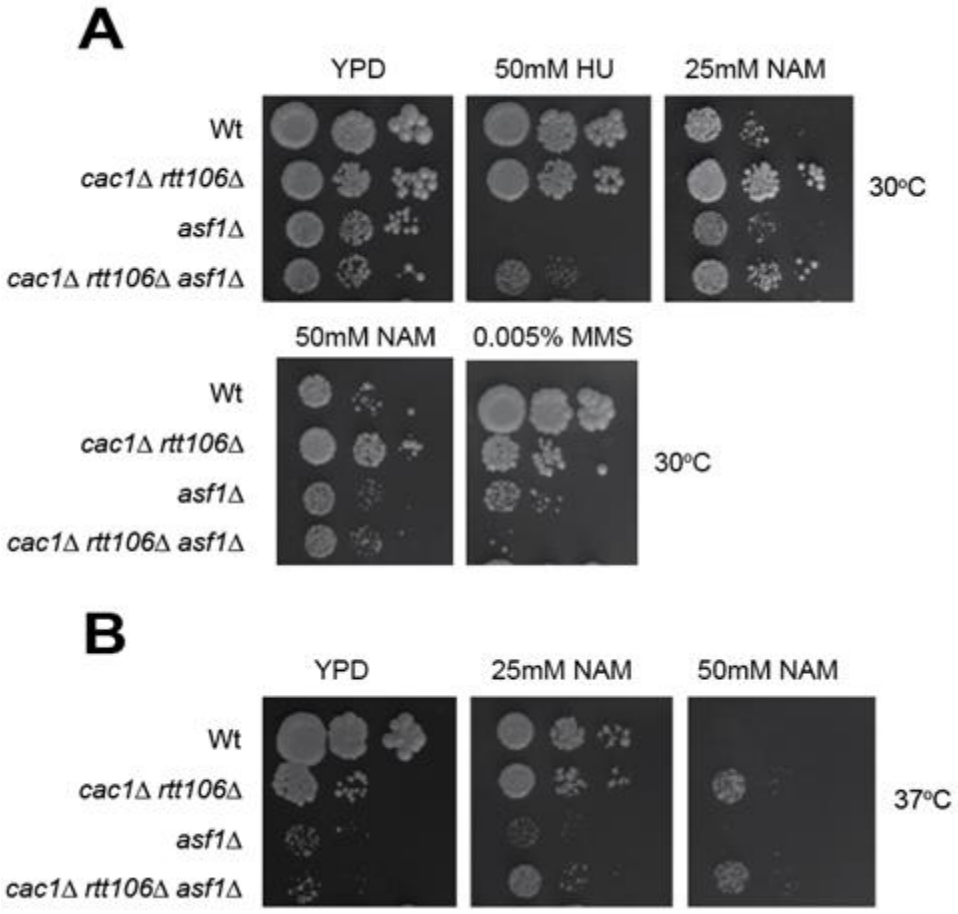


Figure 4-8: Deleting *CAC1* and *RTT106* in addition to *ASF1* rescues *asf1*Δ sensitivity to HU and NAM but not MMS. Cells were spotted onto YPD plates containing the indicated genotoxins in 10X serial dilutions and grown at 30°C (A) or 37°C (B) for 3 days. Results shown are representative of results from at least 2 independent experiments.

Chapter 5:

Conclusions

5-1: Conclusions

Replication coupled (RC) nucleosome assembly is an essential process which helps to establish parental chromatin structure on daughter DNA following DNA replication. The mechanisms and factors involved in RC nucleosome assembly are not yet well understood. During DNA replication, DNA sequence and epigenetic information must be accurately duplicated to prevent the propagation of errors and maintain the same gene expression profile in future generations. It is suspected that during the process of RC nucleosome assembly, the establishment of parental chromatin structure occurs on daughter DNA by duplication of epigenetic information. This is one reason why there is ongoing interest in the RC nucleosome assembly and the chaperones that mediate this process.

The RC chaperones CAF-1, Rtt106 and Asf1 mediate a sequential process to deposit newly synthesized H3-H4 onto nascent DNA immediately following the replication fork. CAF-1 and Asf1 have individually been extensively studied and are well conserved among eukaryotes, while Rtt106 has only recently been discovered and non-fungal homologues are yet to be confirmed [45]. Asf1 and CAF-1 are both involved in RC and RI nucleosome assembly. Specifically, CAF-1 is involved in DNA repair and Asf1 in DNA repair and transcription. Asf1 is essential for the post-translational modification H3K56Ac. Rtt106 is involved in RC and RI nucleosome assembly. Specifically Rtt106 is important for preventing aberrant transcription from cryptic promoters [41]. The three RC chaperones are also each important for heterochromatin-like silencing at the silent mating type loci and telomeres [39],[54]. Collectively this information suggests a possible role for the CAF-1 and Rtt106 in transcription, however this is yet to be studied.

Utilizing various RC chaperone mutants we measured the expression of *HUG1*. We used ChIP to measure Crt1 and histone H3 occupancy, and Rad53 immunoblotting in order to study the regulation of

HUG1 in the RC chaperone mutants. The objective of this work was to investigate a possible role for the RC chaperones in the transcriptional control of *HUG1*. These studies revealed that *HUG1* is derepressed in the absence of *CAC1* and *RTT106*. It is also derepressed to a lesser extent in cells lacking *ASF1.* The *HUG1* derepression in *asf1*Δ cells is due to the constitutive activation of the DNA damage checkpoint [59]. Thus *HUG1* is induced through its normal regulation involving the Crt1 repressor. *HUG1* derepression in *cac1*Δ *rtt106*Δ cells, in contrast, is independent of DNA damage checkpoint activation. Rad53 hyperphosphorylation could not be detected by Western blot analysis in this double mutant. Interestingly, there is a decrease in H3 occupancy at *HUG1* in *cac1*Δ *rtt106*Δ cells, which may be the reason for its derepression in this strain. Our results also suggest that effects the RC chaperones have on histone occupancy are likely dependent on the region of the genome. Measuring *HUG1* expression in cells in G2/M phase and progressing into S phase suggested that *HUG1,* like other DDR genes, is cell cycle regulated. This is a novel discovery since the cell cycle regulation of *HUG1* is a previously unstudied area. *HUG1* expression in the RC chaperone mutants suggested that the cell cycle regulation of *HUG1* is independent of the mechanisms of *HUG1* derepression in cells lacking *CAC1* or *RTT106*.

Overall, we found that CAF-1 and Rtt106 contribute to the repression of *HUG1*. Interestingly, this repression does not seem to involve Asf1, even though Asf1 functions upstream of these chaperones during RC nucleosome assembly. This indicates that the RC chaperones have varying functions that contribute differently to *HUG1* transcriptional regulation. Moreover, nucleosome assembly involving the RC chaperones may be more complex than the current model suggests.

Future experiments are necessary to better understand exactly how CAF-1 and Rtt106 contribute to the transcriptional control of *HUG1*. We have demonstrated that H3 occupancy at *HUG1* is decreased in cells

lacking *CAC1* and *RTT106*. To truly understand the significance of this decrease, H3 occupancy at *HUG1* should be measured by ChIP in wild type and the RC chaperone mutants prior to, and after induction by HU treatment. *HUG1* is regulated by the Ssn6-Tup1 corepressors which repress genes using a combination of chromatin remodeling, histone deacetylation and interfering with the binding of transcription machinery [104]. Depleting promoter nucleosomes by deleting *CAC1* and *RTT106* may therefore play an important part of the increased *HUG1* transcription in this strain. Ssn6-Tup1 may no longer control the nucleosome remodeling aspect required for full repression of *HUG1*. Because the corepressors are still able to promote histone deacetylation and prevent binding of transcription machinery, however, the level of derepression is finite, and *HUG1* can be induced further in response to checkpoint activation.

At this time it is not clear if and how *HUG1* derepression resulting from the co-deletion of *CAC1* and *RTT106* contributes to the increased viability of *asf1*Δ cells in HU. Various experiments in this thesis were performed in an attempt to characterize and understand the mechanism for this HU sensitivity rescue.

Investigating recovery from prolonged HU treatment by observing growth on YPD plates indicated that *cac1*Δ *rtt106*Δ *asf1*Δ cells recover no better than *asf1*Δ cells. Induction of *HUG1* during HU treatment is equally impaired in *asf1*Δ and *cac1*Δ *rtt106*Δ *asf1*Δ cells. This result agrees with the finding that Asf1 is important for the induction of *HUG1* [110]. While *HUG1* induction is impaired in *cac1*Δ *rtt106*Δ *asf1*Δ cells, the level of *HUG1* expression is higher in this triple mutant than in *asf1*Δ cells. This increased level of *HUG1* transcription may provide the advantage required to increase viability during prolonged replication stress. Deleting *HUG1* to test if its derepression results in the HU sensitivity rescue in the triple mutant may result in diverse cellular responses unrelated to HU sensitivity rescue. This approach therefore may not be the appropriate experiment to

investigate the mechanism of HU sensitivity rescue. Moreover, studies in *hug1*Δ cells demonstrate that a loss of *HUG1* does not significantly sensitize them to genotoxins [97]. Therefore we focus on other phenotypes of *cac1*Δ *rtt106*Δ *asf1*Δ cells to attempt to explain the HU sensitivity rescue in this strain.

A chromatin fractionation experiment revealed that *cac1*Δ *rtt106*Δ *asf1*Δ cells have wild type levels of H3 associated with chromatin while *asf1*Δ cells have increased H3 levels associated with chromatin. The wild type level of H3 associated with chromatin may provide the triple mutant with the advantage required for increased viability during prolonged HU treatment. Flow cytometry to analyze DNA content over a period of HU treatment revealed that *cac1*Δ *rtt106*Δ *asf1*Δ cells have a clear decrease in G2/M phase cells, which is characteristic of the wild type response to HU treatment. *asf1*Δ cells do not appear to arrest as efficiently in HU, however, because the G2/M peak does not decrease to the same extent. Appropriate activation of the replication checkpoint, characterized by Rad53 hyperphosphorylation and early S phase arrest, may also provide *cac1*Δ *rtt106*Δ *asf1*Δ cells with an advantage over *asf1*Δ cells resulting in the observed decrease in HU sensitivity.

Further experiments are needed to understand the mechanism of HU sensitivity rescue in *asf1*Δ cells by the deletion of *CAC1* and *RTT106*. Rad53 hyperphosphorylation should be measured over a period of HU treatment to observe replication checkpoint activation in each strain. This would indicate if *cac1*Δ *rtt106*Δ *asf1*Δ cells can activate the checkpoint more efficiently than *asf1*Δ cells. If this is the case, understanding the involvement of the RC chaperones in the replication checkpoint may help understand why the co-deletion of *CAC1* and *RTT106* rescues the HU sensitivity of *asf1*Δ cells. It is difficult to speculate how the RC chaperones may contribute to replication checkpoint activation because they are not

directly involved in the sensing of replication stress or the signalling cascade following activation.

Collectively, results in this chapter suggest divergent functions for the RC chaperones in regulating the transcription of an inducible gene. They also suggest that RC nucleosome assembly may be more complex than the current model predicts. Moreover, the divergent functions of the RC chaperones have opposing effects on cell survival during prolonged replication stress.

Bibliography

1. Richmond, R.K., Sargent, D.F., Richmond, T.J., Luger, K. & Ma, A.W. Crystal structure of the nucleosome core particle at 2 . 8 A˚ resolution. *Nature* **7**, 251-260 (1997).

2. Wu, J. & Grunstein, M. 25 Years After the Nucleosome Model: Chromatin Modifications. *Trends in biochemical sciences* **25**, 619-623 (2000).

3. Ransom, M., Dennehey, B.K. & Tyler, J.K. Chaperoning histones during DNA replication and repair. *Cell* **140**, 183-195 (2010).

4. Mersfelder, E.L. & Parthun, M.R. The tale beyond the tail: histone core domain modifications and the regulation of chromatin structure. *Nucleic acids research* **34**, 2653-2662 (2006).

5. Strahl, B.D. & Allis, C.D. The language of covalent histone modifications. *Nature* **403**, 41-45 (2000).

6. Li, Q. *et al.* Acetylation of histone H3 lysine 56 regulates replication-coupled nucleosome assembly. *Cell* **134**, 244-255 (2008).

7. Kurdistani, S.K. & Grunstein, M. Histone acetylation and deacetylation in yeast. *Nature reviews. Molecular cell biology* **4**, 276-284 (2003).

8. Groth, A. *et al.* Regulation of replication fork progression through histone supply and demand. *Science (New York, N.Y.)* **318**, 1928-1931 (2007).

9. Spector, M.S., Raff, A., Silva, H.D.E., Lee, K. & Osley, M.A.N.N. Hir1p and Hir2p function as transcriptional corepressors to regulate histone gene transcription in the Saccharomyces cerevisiae cell cycle . *Microbiology* 545-552 (1997).

10. Avvakumov, N., Nourani, A. & Côté, J. Histone chaperones: modulators of chromatin marks. *Molecular cell* **41**, 502-514 (2011).

11. Niederacher, G., Klopf, E. & Schüller, C. Interplay of dynamic transcription and chromatin remodeling: lessons from yeast. *International journal of molecular sciences* **12**, 4758-4769 (2011).

12. Linger, J. & Tyler, J.K. Global replication-independent histone H4 exchange in budding yeast. *Eukaryotic cell* **5**, 1780-1787 (2006).

13. Tyler, J.K. & Kadonaga, J.T. The " Dark Side " of Chromatin Remodeling : Repressive Effects on Transcription. **99**, 443-446 (1999).

14. Varga-Weisz, P. ATP-dependent chromatin remodeling factors: nucleosome shufflers with many missions. *Oncogene* **20**, 3076-3085 (2001).

15. Radman-Livaja, M. *et al.* Patterns and mechanisms of ancestral histone protein inheritance in budding yeast. *PLoS biology* **9** (2011).

16. Xu, M. & Zhu, B. Nucleosome assembly and epigenetic inheritance. *Protein & cell* **1**, 820-829 (2010).

17. Yu, Y., Eriksson, P. & Stillman, D.J. Architectural transcription factors and the SAGA complex function in parallel pathways to activate transcription. *Molecular and cellular biology* **20**, 2350-2357 (2000).

18. Paranjape ,SM. Role of chromatin structure in the regulation of transcription by RNA polymerase II. *Structure* **63** 265-297 (1994).

19. Boeger, H., Griesenbeck, J., Strattan, J.S. & Kornberg, R.D. Removal of Promoter Nucleosomes by Short Article Disassembly Rather Than Sliding In Vivo. **14**, 667-673 (2004).

20. Williams, S.K. & Tyler, J.K. Transcriptional regulation by chromatin disassembly and reassembly. *Current opinion in genetics & development* **17**, 88-93 (2007).

21. Adkins, M.W., Howar, S.R. & Tyler, J.K. Chromatin Disassembly Mediated by the Histone Chaperone Asf1 Is Essential for Transcriptional Activation of the Yeast PHO5 and PHO8 Genes. **14**, 657-666 (2004).

22. Adkins, M.W. & Tyler, J.K. Transcriptional activators are dispensable for transcription in the absence of Spt6-mediated chromatin reassembly of promoter regions. *Molecular cell* **21**, 405-416 (2006).

23. Neef, D.W. & Kladde, M.P. Polyphosphate Loss Promotes SNF / SWI- and Gcn5-Dependent Mitotic Induction of PHO5. *Society* **23**, 3788-3797 (2003).

24. Bai, L. & Morozov, A.V. Gene regulation by nucleosome positioning. *Trends in genetics* **26**, 476-483 (2010).

25. Williams, S.K., Truong, D. & Tyler, J.K. Acetylation in the globular core of histone H3 on lysine-56 promotes chromatin disassembly during transcriptional activation. *Proceedings of the National Academy of Sciences of the United States of America* **105** 9000-9005 (2008).

26. Minard, L.V., Lin, L.-J. & Schultz, M.C. SWI/SNF and Asf1 Independently Promote Derepression of the DNA Damage Response Genes under Conditions of Replication Stress. *PloS one* **6**, (2011).

27. Li, B. & Reese, J.C. Ssn6-Tup1 regulates RNR3 by positioning nucleosomes and affecting the chromatin structure at the upstream repression sequence. *The Journal of biological chemistry* **276**, 33788-33797 (2001).

28. Sharma, V.M., Tomar, R.S., Dempsey, A.E. & Reese, J.C. Histone deacetylases RPD3 and HOS2 regulate the transcriptional activation of DNA damage-inducible genes. *Molecular and cellular biology* **27**, 3199-3210 (2007).

29. Li, Q., Burgess, R. & Zhang, Z. All roads lead to chromatin: Multiple pathways for histone deposition. *Biochimica et biophysica acta* 1-9 (2011).

30. Ridgway, P. & Almouzni, G. CAF-1 and the inheritance of chromatin states: at the crossroads of DNA replication and repair. *Journal of cell science* **113** 2647-2658 (2000).

31. Hoek, M. & Stillman, B. Chromatin assembly factor 1 is essential and couples chromatin assembly to DNA replication in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 12183-12188 (2003).

32. Linger, J. & Tyler, J.K. The yeast histone chaperone chromatin assembly factor 1 protects against double-strand DNA-damaging agents. *Genetics* **171**, 1513-1522 (2005).

33. Shibahara, K. & Stillman, B. Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin. *Cell* **96**, 575-585 (1999).

34. Harkness, T.A.A. *et al.* Contribution of CAF-I to Anaphase-Promoting-Complex-Mediated Mitotic Chromatin Assembly in Saccharomyces cerevisiae. *Society* **4**, 673-684 (2005).

35. Sharp, J. a, Franco, A. a, Osley, M.A. & Kaufman, P.D. Chromatin assembly factor I and Hir proteins contribute to building functional kinetochores in S. cerevisiae. *Genes & development* **16**, 85-100 (2002).

36. Kim, J.-A. & Haber, J.E. Chromatin assembly factors Asf1 and CAF-1 have overlapping roles in deactivating the DNA damage checkpoint when DNA repair is complete. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 1151-1156 (2009).

37. Chen, C.-C. *et al.* Acetylated lysine 56 on histone H3 drives chromatin assembly after repair and signals for the completion of repair. *Cell* **134**, 231-243 (2008).

38. Lustig, a J. Mechanisms of silencing in Saccharomyces cerevisiae. *Current opinion in genetics & development* **8**, 233-239 (1998).

39. Huang, S., Zhou, H., Tarara, J. & Zhang, Z. A novel role for histone chaperones CAF-1 and Rtt106p in heterochromatin silencing. *The EMBO journal* **26**, 2274-2283 (2007).

40. Kim, H.-J., Seol, J.-H. & Cho, E.-J. Potential role of the histone chaperone, CAF-1, in transcription. *BMB reports* **42**, 227-231 (2009).

41. Imbeault, D., Gamar, L., Rufiange, A., Paquet, E. & Nourani, A. The Rtt106 histone chaperone is functionally linked to transcription elongation and is involved in the regulation of spurious transcription from cryptic promoters in yeast. *The Journal of biological chemistry* **283**, 27350-27354 (2008).

42. Fillingham, J. *et al.* Two-color cell array screen reveals interdependent roles for histone chaperones and a chromatin boundary regulator in histone gene repression. *Molecular cell* **35**, 340-351 (2009).

43. Ferreira, M.E., Flaherty, K. & Prochasson, P. The Saccharomyces cerevisiae Histone Chaperone Rtt106 Mediates the Cell Cycle Recruitment of SWI/SNF and RSC to the HIR-Dependent Histone Genes. *PloS one* **6**, (2011).

44. Huang, S. *et al.* Rtt106p is a histone chaperone involved in heterochromatin-mediated silencing. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 13410-13415 (2005).

45. Ouararhni, K., Depaux, A., Shuaib, M. & Hamiche, A. The death-associated protein DAXX is a novel histone chaperone involved in the replication-independent deposition of H3 . 3. *Genes & Development* **1**, 1253-1265 (2010).

46. Sanematsu, F. *et al.* Asf1 is required for viability and chromatin assembly during DNA replication in vertebrate cells. *The Journal of biological chemistry* **281**, 13817-13827 (2006).

47. Tyler, J.K. *et al.* The RCAF complex mediates chromatin assembly during DNA replication and repair. *Nature* **402**, 555-560 (1999).

48. Schwabish, M. a & Struhl, K. Asf1 mediates histone eviction and deposition during elongation by RNA polymerase II. *Molecular cell* **22**, 415-422 (2006).

49. Mousson, F., Ochsenbein, F. & Mann, C. The histone chaperone Asf1 at the crossroads of chromatin and DNA checkpoint pathways. *Chromosoma* **116**, 79-93 (2007).

50. Green, E.M. *et al.* Replication-Independent Histone Deposition by the HIR Complex and Asf1. **15**, 1-11 (2010).

51. Silva, A.C. *et al.* The replication-independent H3-H4 chaperones HIR, ASF1 and RTT106 co-operate to maintain promoter fidelity. *The Journal of biological chemistry* **287**, 1709-1718 (2011).

52. Mello, J. a *et al.* Human Asf1 and CAF-1 interact and synergize in a repair-coupled nucleosome assembly pathway. *EMBO reports* **3**, 329-334 (2002).

53. Sutton, a, Bucaria, J., Osley, M. a & Sternglanz, R. Yeast ASF1 protein is required for cell cycle regulation of histone gene transcription. *Genetics* **158**, 587-596 (2001).

54. Sharp, J. a, Fouts, E.T., Krawitz, D.C. & Kaufman, P.D. Yeast histone deposition protein Asf1p requires Hir proteins and PCNA for heterochromatic silencing. *Current biology* **11**, 463-473 (2001).

55. Miele, A., Bystricky, K. & Dekker, J. Yeast silent mating type loci form heterochromatic clusters through silencer protein-dependent long-range interactions. *PLoS genetics* **5**, (2009).

56. Adkins, M.W. & Tyler, J.K. The histone chaperone Asf1p mediates global chromatin disassembly in vivo. *The Journal of biological chemistry* **279**, 52069-52074 (2004).

57. English, C.M., Maluf, N.K., Tripet, B., Churchill, M.E. a & Tyler, J.K. ASF1 binds to a heterodimer of histones H3 and H4: a two-step mechanism for the assembly of the H3-H4 heterotetramer on DNA. *Biochemistry* **44**, 13673-13682 (2005).

58. Donham, D.C., Scorgie, J.K. & Churchill, M.E. a The activity of the histone chaperone yeast Asf1 in the assembly and disassembly of histone H3/H4-DNA complexes. *Nucleic acids research* **39**, 5449-5458 (2011).

59. Ramey, C.J. *et al.* Activation of the DNA Damage Checkpoint in Yeast Lacking the Histone Chaperone Anti-Silencing Function 1 Activation of the DNA Damage Checkpoint in Yeast Lacking the Histone Chaperone Anti-Silencing Function 1. *Society* 10313-10327c(2004).

60. Monson, E.K., de Bruin, D. & Zakian, V. a The yeast Cac1 protein is required for the stable inheritance of transcriptionally repressed chromatin at telomeres. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 13081-13086 (1997).

61. Yuan, J., Pu, M., Zhang, Z. & Lou, Z. Histone H3-K56 acetylation is important for genomic stability in mammals. *Cell cycle* **8**, 1747-1753 (2009).

62. Schneider, J., Bajwa, P., Johnson, F.C., Bhaumik, S.R. & Shilatifard, A. Rtt109 is required for proper H3K56 acetylation: a chromatin mark associated with the elongating RNA polymerase II. *The Journal of biological chemistry* **281**, 37270-37274 (2006).

63. Burgess, R.J. & Zhang, Z. Roles for Gcn5 in promoting nucleosome assembly and maintaining genome integrity. *Cell cycle* **9**, 2979-2985 (2010).

64. Franco, A. a, Lam, W.M., Burgers, P.M. & Kaufman, P.D. Histone deposition protein Asf1 maintains DNA replisome integrity and interacts with replication factor C. *Genes & development* **19**, 1365-1375 (2005).

65. Groth, A. *et al.* Regulation of replication fork progression through histone supply and demand. *Science (New York, N.Y.)* **318**, 1928-1931 (2007).

66. Verreault, a, Kaufman, P.D., Kobayashi, R. & Stillman, B. Nucleosome assembly by a complex of CAF-1 and acetylated histones H3/H4. *Cell* **87**, 95-104 (1996).

67. Li, Q. & Zhang, Z. Linking DNA replication to heterochromatin silencing and epigenetic inheritance. *Acta Biochimica et Biophysica Hungarica* **44**, 3-13 (2012).

68. Stuwe, T. *et al.* The FACT Spt16 “peptidase” domain is a histone H3-H4 binding module. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 8884-8889 (2008).

69. Kohn, K.W., Aladjem, M.I., Weinstein, J.N. & Pommier, Y. Chromatin Challenges during DNA Replication : A Systems Representation. *Molecular Biology of the Cell* **19**, 1-7 (2008).

70. Kaplan, T. *et al.* Cell cycle- and chaperone-mediated regulation of H3K56ac incorporation in yeast. *PLoS genetics* **4** (2008).

71. Das, C., Tyler, J.K. & Churchill, M.E. a The histone shuffle: histone chaperones in an energetic dance. *Trends in biochemical sciences* **35**, 476-489 (2010).

72. Ai, X. & Parthun, M.R. The nuclear Hat1p/Hat2p complex: a molecular link between type B histone acetyltransferases and chromatin assembly. *Molecular cell* **14**, 195-205 (2004).

73. Ye, J. *et al.* Histone H4 Lysine 91 Acetylation: A Core Domain Modification Associated with Chromatin Assembly. **18**, 123-130 (2010).

74. Fillingham, J. *et al.* Chaperone control of the activity and specificity of the histone H3 acetyltransferase Rtt109. *Molecular and cellular biology* **28**, 4342-4353 (2008).

75. Driscoll, R., Hudson, A. & Jackson, S.P. Yeast Rtt109 promotes genome stability by acetylating histone H3 on lysine 56. *Science* **315**, 649-652 (2007).

76. Bartek, J. & Lukas, J. DNA damage checkpoints: from initiation to recovery or adaptation. *Current opinion in cell biology* **19**, 238-245 (2007).

77. Alvino, G.M. *et al.* Replication in hydroxyurea: it’s a matter of time. *Molecular and cellular biology* **27**, 6396-6406 (2007).

78. Lundin, C. *et al.* Methyl methanesulfonate (MMS) produces heat-labile DNA damage but no detectable in vivo DNA double-strand breaks. *Nucleic acids research* **33**, 3799-3811 (2005).

79. Kaina, B. Mechanisms and consequences of methylating agent-induced SCEs and chromosomal aberrations: a long road traveled and still a far way to go. *Cytogenetic and genome research* **104**, 77-86 (2004).

80. Wyatt, M.D. & Pittman, D.L. Methylating agents and DNA repair responses: Methylated bases and sources of strand breaks. *Chem Res Toxicol.* **19**, 1580-1594 (2006).

81. Bitterman, K.J., Anderson, R.M., Cohen, H.Y., Latorre-Esteves, M. & Sinclair, D. a Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. *The Journal of biological chemistry* **277**, 45099-45107 (2002).

82. Sinclair, D.A. *et al.* Nicotinamide and PNC1 govern lifespan extension by calorie restriction in Saccharomyces cerevisiae. *Nature* **378**, 181-185 (2003).

83. Avalos, J.L., Bever, K.M. & Wolberger, C. Mechanism of sirtuin inhibition by nicotinamide: altering the NAD(+) cosubstrate specificity of a Sir2 enzyme. *Molecular cell* **17**, 855-868 (2005).

84. Thaminy, S. *et al.* Hst3 is regulated by Mec1-dependent proteolysis and controls the S phase checkpoint and sister chromatid cohesion by deacetylating histone H3 at lysine 56. *The Journal of biological chemistry* **282**, 37805-37814 (2007).

85. Labib, K. & De Piccoli, G. Surviving chromosome replication: the many roles of the S-phase checkpoint pathway. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **366**, 3554-3561 (2011).

86. Nyberg, K. a, Michelson, R.J., Putnam, C.W. & Weinert, T. a Toward maintaining the genome: DNA damage and replication checkpoints. *Annual review of genetics* **36**, 617-656 (2002).

87. de Bruin, R. a M. & Wittenberg, C. All eukaryotes: before turning off G1-S transcription, please check your DNA. *Cell cycle* **8** 214-217 (2009).

88. Huang, M., Zhou, Z. & Elledge, S.J. The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. *Cell* **94**, 595-605 (1998).

89. Branzei, D. & Foiani, M. The checkpoint response to replication stress. *DNA repair* **8**, 1038-1046 (2009).

90. Lee, S.-jung, Schwartz, M.F., Duong, J.K. & Stern, D.F. Rad53 Phosphorylation Site Clusters Are Important for Rad53 Regulation and Signaling. *Society* **23**, 6300-6314 (2003).

91. Clémenson, C. & Marsolier-Kergoat, M.-C. The spindle assembly checkpoint regulates the phosphorylation state of a subset of DNA checkpoint proteins in Saccharomyces cerevisiae. *Molecular and cellular biology* **26**, 9149-9161 (2006).

92. Putnam, C.D., Jaehnig, E.J. & Kolodner, R.D. Perspectives on the DNA damage and replication checkpoint responses in Saccharomyces cerevisiae. *DNA Repair* **8**, 974-982 (2010).

93. Boddy, M.N. & Russell, P. DNA replication checkpoint. *Current biology* **11** (2001).

94. Rhind, N. An intrinsic checkpoint model for regulation of replication origins**.** *Cell* **7** 2619-2620 (2010).

95. Basrai, M.A., Velculescu, V.E., Kinzler, K.W. & Hieter, P. NORF5 / HUG1 Is a Component of the MEC1 -Mediated Checkpoint Response to DNA Damage and Replication Arrest in Saccharomyces cerevisiae *Society* (1999).

96. Benton, M.G., Glasser, N.R. & Palecek, S.P. The utilization of a Saccharomyces cerevisiae HUG1P-GFP promoter-reporter construct for the selective detection of DNA damage. *Mutation research* **633** 21-34 (2007).

97. Kim, E. & Siede, W. Phenotypes associated with Saccharomyces cerevisiae Hug1 protein, a putative negative regulator of dNTP Levels, reveal similarities and differences with sequence-related Dif1. *Journal of microbiology* **49** 78-85 (2011).

98. Wade, S.L., Poorey, K., Bekiranov, S. & Auble, D.T. The Snf1 kinase and proteasome-associated Rad23 regulate UV-responsive gene expression. *The EMBO journal* **28** 2919-2931 (2009).

99. Westholm, J.O. *et al.* Combinatorial control of gene expression by the three yeast repressors Mig1, Mig2 and Mig3. *BMC genomics* **9** 601 (2008).

100. Elledge, S.J. & Davis, R.W. Two genes differentially regulated in the cell cycle and by DNA-damaging agents encode alternative regulatory subunits of ribonucleotide reductase. *Genes & Development* **4**, 740-751 (1990).

101. Chabes, A. & Thelander, L. DNA Building Blocks at the Foundation of Better Survival. *Cell Cycle* **2**, 172-173 (2003).

102. Klinkenberg, L.G., Webb, T. & Zitomer, R.S. Synergy among differentially regulated repressors of the ribonucleotide diphosphate reductase genes of Saccharomyces cerevisiae. *Eukaryotic cell* **5**, 1007-1017 (2006).

103. Zaim, J., Speina, E. & Kierzek, A.M. Identification of new genes regulated by the Crt1 transcription factor, an effector of the DNA damage checkpoint pathway in Saccharomyces cerevisiae. *The Journal of biological chemistry* **280**, 28-37 (2005).

104. Zhang, Z. & Reese, J.C. Redundant mechanisms are used by Ssn6-Tup1 in repressing chromosomal gene transcription in Saccharomyces cerevisiae. *The Journal of biological chemistry* **279**, 39240-39250 (2004).

105. Smith, R.L. & Johnson, a D. Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes. *Trends in biochemical sciences* **25**, 325-330 (2000).

106. Zhang, Z. & Reese, J.C. Ssn6-Tup1 requires the ISW2 complex to position nucleosomes in Saccharomyces cerevisiae. *The EMBO journal* **23**, 2246-2257 (2004).

107. Zhang, Z. & Reese, J.C. Molecular Genetic Analysis of the Yeast Repressor Rfx1 / Crt1 Reveals a Novel Two-Step Regulatory Mechanism. *Society* **25**, 7399-7411 (2005).

108. Zhang, H. & Reese, J.C. Exposing the core promoter is sufficient to activate transcription and alter coactivator requirement at RNR3. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 8833-8838 (2007).

109. Lin, L.-J. & Schultz, M.C. Promoter regulation by distinct mechanisms of functional interplay between lysine acetylase Rtt109 and histone chaperone Asf1. *Proceedings of the National Academy of Sciences of the United States of America* (2011).

110. Minard, L.V., Williams, J.S., Walker, A.C. & Schultz, M.C. Transcriptional regulation by Asf1: new mechanistic insights from studies of the DNA damage response to replication stress. *The Journal of biological chemistry* **286**, 7082-7092 (2011).

111. Longtine, M.S. *et al.* Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. *Yeast* **14**, 953-961 (1998).

112. Parnas, O. *et al.* The ELG1 clamp loader plays a role in sister chromatid cohesion. *PloS one* **4**, (2009).

113. Marcusson, E.G., Horazdovsky, B.F. & Cereghino, J.L. The Sorting Receptor for Yeast Vacuolar Carboxypeptidase Y Is Encoded by the VFW0 Gene. *Cell* **77**, 579-586 (1994).

114. Friis, R.M.N. *et al.* A glycolytic burst drives glucose induction of global histone acetylation by picNuA4 and SAGA. *Nucleic acids research* **37**, 3969-3980 (2009).

115. Aparicio, O. *et al.* Chromatin immunoprecipitation for determining the association of proteins with specific genomic sequences in vivo. *Current protocols in molecular biology* **Chapter 21**, (2005).

116. Ramaswamy, V., Williams, J.S., Robinson, K.M., Sopko, R.L. & Schultz, M.C. Global Control of Histone Modification by the Anaphase-Promoting Complex Global Control of Histone Modification by the Anaphase-Promoting Complex. *Society* **23**, 9136-9149 (2003).

117. Brachmann, C.B., Davies, A., Cost, G.J. & Caputo, E. Designer Deletion Strains derived from Saccharomyces cerevisiae S288C : a Useful set of Strains and Plasmids for PCR-mediated Gene Disruption and Other Applications. *Yeast* **132**, 115-132 (1998).

118. Masumoto, H., Hawke, D., Kobayashi, R. & Verreault, A. A role for cell-cycle-regulated histone H3 lysine 56 acetylation in the DNA damage response. *Nature* **436**, 294-298 (2005).

119. Wang, S., Zhou, B.O. & Zhou, J. Histone H3 lysine 4 hypermethylation prevents aberrant nucleosome remodeling at the PHO5 promoter. *Molecular and cellular biology* **31**, 3171-3181 (2011).

120. Korber, P. *et al.* The histone chaperone Asf1 increases the rate of histone eviction at the yeast PHO5 and PHO8 promoters. *The Journal of biological chemistry* **281**, 5539-5545 (2006).

121. Ohsawa, R., Adkins, M. & Tyler, J.K. Epigenetic inheritance of an inducibly nucleosome-depleted promoter and its associated transcriptional state in the apparent absence of transcriptional activators. *Epigenetics & chromatin* **2**, 11 (2009).

122. Smolka, M.B. *et al.* Dynamic changes in protein-protein interaction and protein phosphorylation probed with amine-reactive isotope tag. *Molecular & cellular proteomics* **4**, 1358-1369 (2005).

123. Clemente-Ruiz, M., González-Prieto, R. & Prado, F. Histone H3K56 Acetylation, CAF1, and Rtt106 Coordinate Nucleosome Assembly and Stability of Advancing Replication Forks. *PLoS Genetics* **7**, (2011).

124. Sekinger, E. a, Moqtaderi, Z. & Struhl, K. Intrinsic histone-DNA interactions and low nucleosome density are important for preferential accessibility of promoter regions in yeast. *Molecular cell* **18**, 735-748 (2005).

125. Schwabish, M.A. & Struhl, K. Evidence for Eviction and Rapid Deposition of Histones upon Transcriptional Elongation by RNA Polymerase II. *Society* **24**, 10111-10117 (2004).

126. Lebedeva, M.A. & Shadel, G.S. Cell Cycle- and Ribonucleotide Reductase-Driven Changes in mtDNA Copy Number Influence mtDNA Inheritance Without Compromising Mitochondrial Gene Expression *Cell Cycle* **6,** 2048-2057 (2008).

127. Hwang, L.H. Budding Yeast Cdc20: A Target of the Spindle Checkpoint. *Science* **279**, 1041-1044 (1998).

128. Luca, F.C. & Winey, M. MOB1, an essential yeast gene required for completion of mitosis and maintenance of ploidy. *Molecular biology of the cell* **9**, 29-46 (1998).

129. Silva, P. *et al.* Monitoring the fidelity of mitotic chromosome segregation by the spindle assembly checkpoint. *Cell proliferation* **44**, 391-400 (2011).

130. Domkin, V., Thelander, L. & Chabes, A. Yeast DNA damage-inducible Rnr3 has a very low catalytic activity strongly stimulated after the formation of a cross-talking Rnr1/Rnr3 complex. *The Journal of biological chemistry* **277**, 18574-18578 (2002).

131. Dolinski, K. & Botstein, D. Changing perspectives in yeast research nearly a decade after the genome sequence. *Genome research* **15**, 1611-1619 (2005).

132. Pramila, T., Wu, W., Miles, S., Noble, W.S. & Breeden, L.L. The Forkhead transcription factor Hcm1 regulates chromosome segregation genes and fills the S-phase gap in the transcriptional circuitry of the cell cycle. *Genes & development* **20**, 2266-2278 (2006).

133. Elledge, S.J. & Davis, R.W. Two genes differentially regulated in the cell cycle and by DNA-damaging agents encode alternative regulatory subunits of ribonucleotide reductase. *Genes & Development* **4**, 740-751 (1990).

134. Tamburini, B. a, Carson, J.J., Linger, J.G. & Tyler, J.K. Dominant mutants of the Saccharomyces cerevisiae ASF1 histone chaperone bypass the need for CAF-1 in transcriptional silencing by altering histone and Sir protein recruitment. *Genetics* **173**, 599-610 (2006).

135. Kajstura M, Halicka HD, Pryjma J, Darzynkiewicz Z. Discontinuous fragmentation of nuclear DNA during apoptosis revealed by discrete "sub-G1" peaks on DNA content histograms. *Cytometry* **71** 125-131 (2007)

136. Aouida, M., Mekid, H., Belhadj, O., Mir, L.M. & Tounekti, O. Mitochondria-independent morphological and biochemical apoptotic alterations promoted by the anti-tumor agent bleomycin in Saccharomyces cerevisiae. *Cell* **55**, 49-55 (2007).

137. Camplejohn, R.S. *et al.* A possible screening test for inherited p53-related defects based on the apoptotic response of peripheral blood lymphocytes to DNA damage. *British journal of cancer* **72**, 654-662 (1995).

138. Cordón-Preciado, V., Ufano, S. & Bueno, A. Limiting amounts of budding yeast Rad53 S-phase checkpoint activity results in increased resistance to DNA alkylation damage. *Nucleic acids research* **34**, 5852-5862 (2006).

139. Franco, A. a, Lam, W.M., Burgers, P.M. & Kaufman, P.D. Histone deposition protein Asf1 maintains DNA replisome integrity and interacts with replication factor C. *Genes & development* **19**, 1365-1375 (2005).

140. Gunjan, A. & Verreault, A. A Rad53 Kinase-Dependent Surveillance Mechanism that Regulates Histone Protein Levels in S . cerevisiae. **115**, 537-549 (2003).

141. Hanway, D. *et al.* Previously uncharacterized genes in the UV- and MMS-induced DNA damage response in yeast. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 10605-10610 (2002).