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Chromatin regulation by histone chaperone Asf1

by

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For Brad and Chance, my Alberta family.

Abstract

Asf1 is a conserved H3/H4 histone chaperone with multiple functions in chromatin modulation. Using budding yeast as a model, we identify new pathways of Asf1 function, and expand current knowledge regarding the known roles of Asf1. First, we uncover novel genetic interactions between *ASF1* and genes encoding other chromatin regulators. We describe an interaction between *ASF1* and *SET2*, which encodes a histone lysine methyltransferase, and show that Asf1 exists in a pathway to promote Set2-catalyzed H3K36 trimethylation. Second, we present evidence that Asf1 promotes transcriptional derepression of two DNA damage response (DDR) genes (*RNR3* and *HUG1*), which is a hallmark event in the cellular response to replication stress or DNA damage. While Asf1 association with chromatin increases globally during replication stress, we find that direct binding of Asf1 to DDR gene promoters is not needed for their transcriptional derepression. Rather, the contribution of Asf1 is dependent on its ability to stimulate acetylation of H3K56 by the Rtt109 lysine acetyltransferase. This modification occurs in the globular domain of H3, is present on all newly synthesized histones and is lost after H3 incorporation into chromatin. Importantly, DDR gene promoters are occupied by H3K56-acetylated nucleosomes under repressing conditions, and the steady state level of H3K56 promoter acetylation does not change upon derepression. We propose that replication-coupled deposition of K56-acetylated H3 poises newly synthesized DDR genes for derepression. In this model, the known association of transcriptional repressors with DDR gene promoters would ensure that transcription remains minimal under normal conditions. Thirdly, we identify new functions for known Asf1 motifs and

characterize key determinants for the constitutive and inducible association of Asf1 with chromatin. Finally, we identify new genetic, physical, and functional links between *ASF1* and *SNF2*, the catalytic subunit of the SWI/SNF chromatin remodeller. Altogether, the research described in this report is consistent with the idea that Asf1 promotes genome stability through its ability to function in diverse pathways of chromatin regulation.

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List of Abbreviations

Abbreviation	Definition
ARID	AT-rich interaction domain
ATP	adenosine triphosphate
CAF-I	chromatin assembly factor 1
CAN	canavanine
CBP	calmodulin-binding peptide
CBP/p300	CREB binding protein
cDNA	complementary deoxyribonucleic acid
CHD1	chromodomain protein 1
ChIP	chromatin immunoprecipitation
COMPASS	complex of proteins associated with Set1
CPY	carboxypeptidase Y
DDR	DNA damage response
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
FACT	facilitates chromatin transactions
GEN	geneticin
GST	glutathione-S-transferase
HA	hemagglutinin
H3K4	histone H3 lysine 4
H3K36	histone H3 lysine 36
H3K56	histone H3 lysine 56
H3K56ac	K56-acetylated histone H3
HDAC	histone deacetylase
HDA1	histone deacetylase 1
HIR	histone regulator
HU	hydroxyurea
IP	immunoprecipitation
ISWI	imitation switch
KAT	lysine acetyltransferase
MCM	minichromosome maintenance
MIAME	minimum information about a microarray experiment
MMS	methyl methanesulfonate
mRNA	messenger ribonucleic acid
NAT	nourseothricin
NuA3	nucleosome acetyltransferase of histone H3
NuA4	nucleosome acetyltransferase of histone H4
OD	optical density
ORF	open reading frame
PAF	RNA-polymerase associated factor
PCNA	proliferating cell nuclear antigen

PCR	polymerase chain reaction
RFC	replication factor C
RNA	ribonucleic acid
RNR	ribonucleotide reductase
RSC	remodel the structure of chromatin
RT-PCR	real-time polymerase chain reaction
SAGA	Spt-Ada-Gcn5-acetyltransferase
SANT	Swi3/Ada2/NCoR/TFIIIB
SDS-PAGE	sodium dodecyl sulfata polyacrylamide gel electrophoresis
SGA	synthetic genetic array
SWI/SNF	switch/sucrose nonfermentable
TAP	tandem affinity purification
TE	tris ethylenediaminetetraacetic acid
THO	suppressor of the transcriptional defect of Hpr1 by overexpression
TLK	tousled-like kinase
UV	ultraviolet
WCE	whole cell extract
YPD	yeast extract-peptone-dextrose
YPG	yeast extract-peptone-glycerol

Chapter 1

Introduction

Chapter 1

Introduction

Chromatin structure and function. Eukaryotic DNA is packaged into chromatin to allow it to fit into the nucleus. The nucleosome core particle is the basic repeating unit of chromatin, and consists of a histone octamer core wrapped with almost two turns of DNA[1]. The histone octamer itself contains two copies each of histones H2A, H2B, H3 and H4, which exist on the DNA as a histone $(H3/H4)_2$ tetramer and two histone H2A/H2B dimers. Histone proteins contain two distinct domains: a core histone-fold domain involved in histone/histone and histone/DNA interactions, and an N-terminal tail domain, which is the site of covalent post-translational modifications[1].

To ensure a proper supply of histones for DNA replication, histone production is coordinated with DNA synthesis in the S phase of the cell cycle[2]. Hence, histone gene transcription is kept repressed outside of S phase, but is upregulated at the G1/S boundary to allow rapid chromatin assembly to occur behind the replication fork[3]. Newly synthesized histones H3 and H4, containing specific post-translational modifications, are assembled onto DNA as a tetramer, followed by deposition of two H2A-H2B dimers[4,5].

Chromatin must be assembled and disassembled to allow processes that require access to the DNA to occur, including DNA replication, transcription and repair. Defects in chromatin modulation lead to genomic instability[6-8], which is associated with the development of cancer, including aggressive paediatric malignant rhabdoid

tumours[9]. These studies have highlighted the importance of understanding the specific mechanisms by which chromatin is regulated.

Modulation of chromatin structure. Recent research has revealed a number of strategies that the cell employs to modulate chromatin structure. Several highly studied groups of proteins orchestrate this process, including histone chaperones, histone modifiers and chromatin remodelling machineries. Histone chaperones function to deliver histones to the DNA during chromatin assembly, as well as to remove histones from the DNA to facilitate chromatin disassembly. Histone-modifying proteins, such as lysine acetyltransferases (KATs), covalently modify histones, often leading to important transcriptional consequences. For example, histone acetylation is generally associated with active transcription[10]. Another group of proteins that modulate chromatin structure are the ATP-dependent chromatin remodellers, which utilize the energy of ATP hydrolysis to alter the contacts of histones with DNA[11]. Included in this group is the Swi2/Snf2 family of ATPases.

Covalent modification of histones. As mentioned above, one strategy that eukaryotic cells utilize to alter chromatin structure is through the covalent modification of histone proteins. Histones can be modified by acetylation, methylation, phosphorylation, ubiquitination, or sumoylation, all of which are reversible processes. Histone acetylation, deacetylation and methylation have been studied in much detail and are the most relevant to this report. Acetylation of histone lysine residues commonly occurs in the N-terminal tails of histones, and results in the neutralization of positive lysine charges, which is thought to disrupt electrostatic interactions between histones and DNA. All four core histones can be

acetylated at one or more lysine residues in their tails (reviewed in[12], Fig. 1-1; 'a'). More recently, it has been demonstrated that histones can also be acetylated in their globular domains: histone H3 is acetylated on lysine 56[13], and histone H4 on lysine 91[14] (Fig. 1-1).

A number of multisubunit protein complexes capable of catalyzing specific acetylation events have been identified in yeast and higher organisms. For example, the SAGA (Spt-Ada-Gcn5-acetyltransferase) KAT complex preferentially acetylates histone H3 lysines 14 and 18, but can also acetylate lysines 9 and 23 of histone H3, and lysines 11 and 16 of histone H2B[15]. NuA3 (nucleosome acetyltransferase of histone H3) is another KAT complex that catalyzes the acetylation of histone H3 lysine residues[16], whereas Rtt109 specifically acetylates histone H3 lysine 56 (H3K56)[17]. Histone acetylation is in general associated with a more open chromatin configuration, and histone deacetylation with a more repressive chromatin structure[10].

Enzymes that catalyze histone deacetylation are termed histone deacetylases (HDACs). Rpd3 is a well-studied yeast HDAC and functions as the catalytic subunit of two HDAC complexes: Rpd3C(S) (small) and Rpd3C(L) (large)[18,19]. Both complexes contain Rpd3, Sin3, and Ume6; however, each complex has additional unique subunits. In particular, Rpd3C(S) also contains Eaf1 and Rco1, whereas Rpd3C(L) includes Sds3, Cti6/Rxt1, Rxt2, Dep1, Pho23, Sap30, Ash1, Cti1, Rxt3, and Ume6[19]. Although Rpd3 can deacetylate all four histone proteins[15], the small and large complexes appear to differ in their functional roles. Rpd3C(S) can inhibit spurious transcription in coding regions, while Rpd3C(L) functions mainly in gene

repression at promoters[18]. Another HDAC complex, HDA1, is made up of three proteins: Hda1, Hda2, and Hda3[20]. HDA1 preferentially deacetylates histones H3 and H2B at genes that are repressed by Tup1[21], a general transcriptional repressor that regulates a variety of physiological pathways in yeast. Hda2 and Hda3 are both required for the deacetylation activity of the HDA1 complex[20]. Several additional HDAC activities are also found in yeast, including Hst3 and Hst4, which catalyze the deacetylation of H3K56[22].

Histone chaperones in yeast. Histone chaperones make up a family of proteins that physically bind to histones and promote their interactions with other molecules, including DNA, or other protein factors. In budding yeast, several histone chaperones have been known for years[23], but novel proteins exhibiting histone chaperone activities have recently been identified[24,25] (Table. 1-1). It therefore appears that the full complement of histone chaperones in yeast is not yet known and it seems likely that additional chaperones remain to be discovered.

Since most histone chaperones selectively bind histones, they can be generally classified according to which histones they preferentially interact with. Histone chaperones that prefer H3/H4 include Asf1[26-29], CAF-I (chromatin assembly factor 1)[30], HIR (histone regulator)[31], Spt6[32] and Rtt106[24]. FACT (facilitates chromatin transactions) binds preferentially to histones H2A and H2B[33], while Nap1 and Vps75 can associate with all four core histones[34-36]. Although all histone chaperones have certain qualities in common, their cellular roles are diverse.

Spt6 binds histones H3/H4[32] and plays a role in the repression of several genes, including histone-coding genes[37]. Some mutations in *SPT6* sensitize cells to 6-

azauracil[38], a phenotype associated with defects in transcriptional elongation. In addition, Spt6 copurifies with the elongating form of RNA polymerase II, as well as other proteins involved in elongation, such as FACT[39,40].

In yeast, FACT is comprised of two subunits: Spt16 and Pob3, both of which are essential[41]. A third protein, Nhp6, which contains a DNA-binding domain, associates with FACT and provides it with the ability to bind nucleosomes[42,43]. FACT has well-characterized roles in chromatin disassembly and reassembly during transcriptional elongation[44], and also functions in transcriptional initiation (reviewed in[23]). Since subunits of FACT interact with components of the replication machinery, and mutations in FACT result in sensitivity to agents that cause replication stress, FACT has also been implicated in DNA replication[45-49].

Chromatin assembly occurs via replication-coupled and replication-independent pathways. During S phase, replication-coupled chromatin assembly ensures that histones are promptly assembled onto newly replicated DNA to minimize the potential for DNA damage[50]. To facilitate this assembly pathway, chromatin assembly factors, including Asf1, CAF-I and Rtt106, function to deposit histones H3/H4 onto DNA during DNA replication. Asf1 and Rtt106 each stimulate the chromatin assembly activity of, and interact with, CAF-I[24,51-55], a conserved complex comprised of three subunits: Cac1, Cac2 and Cac3[56].

In addition to its roles in replication-coupled chromatin assembly, Asf1 also functions in the replication-independent pathway (Fig. 1-2). This important pathway of chromatin assembly allows for transcription-coupled deposition and histone exchange to occur throughout the cell cycle (recently reviewed in[57]). Asf1 functions

in a replication-independent chromatin assembly pathway in concert with the HIR complex. Genetic studies indicate that Asf1 and HIR are together involved in the same pathway to contribute towards gene silencing, and biochemical analyses have shown that Asf1 stimulates histone deposition by HIR independently of replication[29,58]. In addition, Asf1 and HIR cooperate to exchange histones H3/H4 during transcription[59]. In *S. cerevisiae*, Asf1 copurifies with all four components of HIR (Hir1, Hir2, Hir3 and Hpc2)[29,60]. Interestingly, it appears that Asf1 associates with CAF-I or HIR in a mutually exclusive manner[61]. This finding is consistent with the notion that CAF-I and HIR work in distinct pathways.

Histone chaperone Asf1. *ASF1* was originally identified in *S. cerevisiae* in a screen for genes that result in silencing defects when overexpressed[62]; however, since its discovery, numerous studies have highlighted the fact that the cellular functions of Asf1 are extremely complex. Although Asf1 is not required for viability in *S. cerevisiae*, Asf1 is essential in *S. pombe*, *Drosophila*, chicken and human[63-66]. There are two forms of Asf1 in humans: Asf1a and Asf1b. It appears that the complex functions carried out by yeast Asf1 have been divided among the two human proteins[67].

Structural data has shown that Asf1 binds to a histone H3/H4 heterodimer and makes extensive contacts with both histones. Interestingly, Asf1 interacts with the dimerization surface of histone H3, thereby preventing the formation of the $(H3/H4)_2$ tetramer[27,28,68,69]. Contacts between Asf1 and histone H4 are also important for this interaction[28,70]. Additionally, it is possible that the non-conserved

acidic C-terminal tail of yeast Asf1 may strengthen the interaction between Asf1 and H3/H4[28,71].

While Asf1 is unique in that it acts in both pathways of chromatin assembly, Asf1 also participates in chromatin disassembly[72-77]. It is therefore not surprising that Asf1 plays a key role in the regulation of such processes as DNA replication, transcription and the DNA damage response (DDR) (see below). Multiple studies have also implicated Asf1 in a broad array of other chromatin processes. Importantly, Asf1 is absolutely required for the acetylation of histone H3 lysine 56[17,25,78], and contributes to the acetylation of histone H3 lysine 9[79], two marks associated with newly assembled nucleosomes.

Asf1, DNA replication and the DNA damage response. In wild type cells, the response to DNA damage or replication stress is orchestrated through checkpoint pathways that carry out several important functions to promote cell survival[80]. For example, in response to replication stress, cells arrest in S phase of the cell cycle, and a group of DDR genes is upregulated in a manner that is dependent on key checkpoint protein kinases, including Mec1 and Rad53[81]. In particular, activation of Rad53 via phosphorylation leads to dissociation of the Crt1 repressor from genes such as *RNR3* and *HUG1*, ultimately allowing for their derepression (*RNR3* encodes one of the large subunits of the ribonucleotide reductase complex[82]; however, the function of Hug1 is unknown[83]). Mutants that do not elicit a proper checkpoint response may attempt to proceed through S phase without properly completed DNA replication, often leading to genomic instability and cellular inviability.

Asf1 is one protein that promotes genomic stability and protects against DNA damage or replication stress. In the absence of a functional Asf1 protein that can bind to its histone partners, cells become sensitive to agents that cause DNA damage or replication stress[26,54,62,84]. For example, *asf1*⊿ cells are sensitive to hydroxyurea (HU), an inhibitor of DNA replication[85]. Hydroxyurea inhibits the ribonucleotide reductase (RNR) complex, thereby inhibiting deoxyribonucleotide (dNTP) synthesis[82] and stalling DNA replication.

Recently, the functions of Asf1 in maintaining resistance to DNA damage or replication stress have been mainly attributed to its role in H3K56 acetylation, a histone mark present on all newly synthesized histone H3[86]. Lysine 56 is found in the globular domain of histone H3, near the DNA entry-exit point of the nucleosome[1]. Although H3K56 acetylation does not appear to greatly alter the overall structure of the nucleosome, acetylation at this residue increases the plasticity of nucleosomes, which may facilitate access of necessary protein factors to the DNA[87,88]. H3K56 acetylation is conserved from yeast to human and is catalyzed by the Rtt109 KAT in *S. cerevisiae*[17], and by p300/CBP and/or Gcn5 in humans[8,89]. In yeast, H3K56 acetylation peaks during S phase and is rapidly deacetylated during G2/M by the Hst3 and Hst4 HDACs[22] (Fig. 1-3A).

As mentioned above, Asf1, CAF-I and Rtt106 all participate in replication-coupled chromatin assembly. Interestingly, the functions of these three histone chaperones intersect in the context of H3K56 acetylation. Asf1 physically interacts with Rtt109 and is absolutely required for H3K56 acetylation[17,25,78], whereas CAF-I and Rtt106 together mediate its deposition into chromatin[55] (Fig. 1-3B). However, a

newly identified histone chaperone, Vps75, also interacts with Rtt109 and can promote acetylation of H3K56[25,90]. Interestingly, K56-acetylated H3 is absent in *ASF1* or *RTT109* mutants, but is relatively normal in cells lacking Rtt106, Vps75, or a component of CAF-I[17,78,90].

Asf1, CAF-I and Rtt106 all associate with newly synthesized histones H3 and H4[55]. It also appears that each of these three chaperones can associate with replication forks. CAF-I associates with replication forks through a well-characterized interaction with PCNA[91], a DNA polymerase processivity factor that functions as a clamp to secure the polymerase to its DNA template. Asf1 has been shown to directly interact with origins of replications and can also associate with components of the replisome[92,93]. However, the exact mechanism by which Asf1 is recruited to replication forks in vivo remains to be elucidated. While Rtt106 binding to replication forks has not yet been shown, biochemical evidence implicating Rtt106 in the deposition of K56-acetylated H3 onto replicating DNA predicts this to be the case[55].

In addition to being present at replication forks, Asf1 is thought to maintain the integrity of the replisome through its requirement for H3K56 acetylation. Indeed, in the absence of Asf1 or H3K56 acetylation, components of the replisome are lost upon treatment with HU[92,94]. These findings have led to the model that Asf1 and H3K56 acetylation promote the stability of stalled replication forks, thereby contributing to cellular survival under conditions of replication stress.

Asf1 has also been linked to the DDR. In yeast, Asf1 exists in a dynamic complex with Rad53 (Fig. 1-4). Upon DNA damage or replication stress, Asf1

dissociates from Rad53 and presumably is available to maintain its other chromatin functions[26,95-97]. While this interaction does not appear to be conserved, Asf1 does have a role in the DDR in higher eukaryotes, where a similar mechanism has been postulated. Studies in *Drosophila* and humans have shown that Asf1 binds to inactive (dephosphorylated) tousled-like kinase (TLK), a serine/threonine protein kinase[98,99]. Upon its activation by phosphorylation in S phase, TLK phosphorylates Asf1, thereby allowing it to contribute to chromatin assembly. Human Asf1 is then dephosphorylated in response to replication stress[99], potentially to allow chromatin assembly to stall along with the replication fork.

Asf1 has also been implicated in DNA repair. In particular, recent data has shown that Asf1 and H3K56 acetylation are needed for chromatin reassembly following repair of a double-strand break[100]. Intriguingly, this reassembly of chromatin serves as the signal for the reversal of the DNA damage checkpoint response[100]. Additionally, human Asf1 may have a role in stimulating chromatin assembly by CAF-I during the repair of UV lesions[52], and cells lacking *ASF1* exhibit defects in homologous recombination following replication stress caused by treatment with methyl methanesulfonate (MMS)[101].

Further evidence that Asf1 and H3K56 acetylation contribute to genome stability is that cells lacking *ASF1* show partial constitutive activation of the DNA damage checkpoint[84]. This is thought to be due to elevated levels of spontaneous DNA damage in *asf1*⊥ mutants, including gross chromosomal rearrangements, and increased sister chromatid exchange[6,7,84]. In addition, cells lacking H3K56 acetylation accumulate endogenous DNA damage and are sensitive to agents that cause

replication stress or DNA damage[17,78]. Similarly, mutants that mimic permanent H3K56 acetylation, or are unable to deacetylate H3K56 (*hst3*⊿ *hst4*⊿ cells), exhibit phenotypes consistent with increased spontaneous DNA damage[102]. Since inappropriate levels of H3K56 acetylation, in either direction, can result in genomic instability, careful regulation of the H3K56 acetylation-deacetylation cycle is needed. Moreover, the important finding that Asf1 and H3K56 acetylation levels are elevated in certain types of tumours[8] has linked this histone modification to cancer.

Transcriptional regulation by Asf1. An abundance of research has focussed on uncovering the involvement of Asf1 in transcription. In particular, Asf1 works in many pathways of transcriptional silencing[53,54,58,62,103], and several reports have drawn important links between chromatin assembly/disassembly by Asf1 and transcription. Microarray analysis in G2/M-arrested *asf1*⊿ cells revealed altered expression of 524 genes[104], indicating that Asf1 may regulate transcription globally. In particular, Asf1 has been implicated in transcriptional regulation at the *PHO5* promoter: Asf1 is important for rapid nucleosome disassembly and subsequent gene activation, and contributes to reassembly following gene repression[73-76].

Asf1 is also involved in transcriptional regulation of genes that encode histones[105], genes induced by osmotic stress[106], and the *HO* promoter[107] (*S. cerevisiae*), as well as the Notch target genes[108] (*Drosophila*). In addition, Asf1 was found to mediate histone H3 eviction and deposition during transcriptional elongation[77]. Furthermore, Asf1 has been implicated in transcription-dependent, replication-independent histone H3 exchange at promoters, another process which can deposit K56-acetylated H3[59,109,110].

Consistent with a direct role for Asf1 in transcription, Asf1 has been localized to the promoters and coding regions of transcriptionally active genes[77]. However, due to the presence of Asf1 at multiple genomic loci[29,77,92], it was initially suggested that Asf1 acts as an "opportunist" that waits on the chromatin for signals from transcriptional activator proteins to promote transcription via chromatin disassembly[74]. Although a role for Asf1 in direct nucleosome disassembly has not been ruled out, it has more recently been determined that the contributions of Asf1 towards *PHO5* transcription are mainly dependent on a pathway involving H3K56 acetylation[111]. Whether a similar pathway is responsible for the dependency of other gene promoters on Asf1 remains to be resolved.

Histone methylation and transcriptional elongation. In addition to the possibility of being acetylated on six different lysine residues, histone H3 can also be methylated on lysines 4, 36 and 79 in yeast (Fig. 1-1; 'm'). Methylation of each lysine residue is carried out by distinct histone methyltransferases. Histone H3 lysine 4 methylation is catalyzed by Set1 of the COMPASS multiprotein complex[112], whereas Dot1 methylates histone H3 on lysine 79, which is located in the globular domain[113]. Both histone marks have been linked to telomeric gene silencing[112,113].

Set2, on the other hand, catalyzes the mono-, di- and trimethylation of histone H3 lysine 36 (H3K36)[114]. Initial evidence that Set2 is involved in transcriptional elongation came from the finding that Set2 interacts with the elongating form of RNA polymerase II[115]. Consistent with early studies suggesting that Set2 may act as a transcriptional repressor[115], more recent data has shown that Set2 is involved in the

inhibition of transcription from spurious promoters during transcriptional elongation[18,116].

While the functional consequences of the different states of methylation of H3K36 are not well understood, it is known that dimethylation is adequate to repress spurious intragenic transcription[117,118]. The findings that H3K36 trimethylation requires 1) the interaction of Set2 with RNA polymerase II and 2) the presence of several functional elongation factors, including Spt6, FACT, Bur1/Bur2, and Paf1[117-119], suggest that H3K36 trimethylation functions in transcriptional elongation. Moreover, H3K36 trimethylation correlates with genes that are highly transcribed[10]. In addition, Set2 interaction with particular residues of histones H2A, H3 and H4 is also necessary for proper trimethylation of H3K36[120,121].

Given its role in chromatin modulation, it is perhaps not surprising that Set2 has also been implicated in processes such as DNA replication[122,123] and silencing[124]. Additionally, the importance of proper H3K36 methylation has been highlighted by human studies that implicate Set2 in carcinogenesis[125]. In particular, the gene encoding the human homolog of yeast Set2 is inactivated in certain cancer cells, and H3K36 methylation is reduced in several cancer cell lines[126].

ATP-dependent chromatin remodelling. Genes encoding subunits of the yeast SWI/SNF complex were first identified in screens for mutations that result in defects in sucrose metabolism or mating type switching[127-129]. In the past nearly 30 years, an abundance of other chromatin remodelling complexes have been identified in yeast, and in higher organisms. This has highlighted the evolutionary conservation of chromatin remodelling and led to the classification of ATP-dependent chromatin

remodelling complexes into a number of different subfamilies[11,130]. Most relevant to this study is the SWI2/SNF2 subfamily, which includes yeast SWI/SNF (ySWI/SNF), yeast RSC, the *Drosophila* Brahma complex, and human SWI/SNF (hSWI/SNF) complexes (Table 1-2). Additional remodellers include members of the ISWI, CHD1 and INO80 subfamilies. All chromatin remodelling complexes contain a conserved ATPase subunit along with a multitude of additional core and accessory subunits that convey specific chromatin functions to each remodeller.

The exact mechanisms by which chromatin remodelling complexes catalyze chromatin remodelling to facilitate transcription is unknown; however, the current model suggests that the SWI/SNF and ISWI DNA translocases disrupt contacts between histones and DNA through an intranucleosomal DNA looping mechanism (Fig. 1-5; reviewed in[131]). Recent evidence indicates that the DNA looping mechanism used by SWI/SNF at one nucleosome may result in the complete disassembly of an adjacent nucleosome[132]. SWI/SNF-related chromatin remodelling machines have well-characterized roles in transcriptional activation of a subset of inducible genes. They can facilitate the binding of transcriptional activators to gene promoters, increase the accessibility and mobilization of nucleosomes, and even displace histone octamers (see below). On the other hand, members of the ISWI subfamily have general roles in the establishment of a repressive chromatin structure, sometimes through nucleosome spacing capabilities (for example, to place nucleosomes in a configuration that inhibits transcription), whereas the CHD1 and INO80 chromatin remodelling machines function to reposition nucleosomes along DNA or catalyze histone variant exchange, respectively[131].

The yeast SWI/SNF chromatin remodeller. Yeast SWI/SNF has an apparent molecular mass of 1.14 MDa and contains 12 subunits: Snf2/Swi2, Snf5, Snf6, Snf11, Swi1, Swi3, Swp29/Taf14, Swp73, Swp82, Arp7, Arp9 and Rtt102[133-135]. The Snf2 subunit contains DNA- and nucleosome-dependent ATPase activity[134,136]. While Snf2, Snf5, Swi3, Swp73, Arp7 and Arp9 are all conserved in *Drosophila* and humans, it is thought that Snf2, Snf5 and Swi3 make up a conserved catalytic core[137]. However, a subcomplex of yeast SWI/SNF containing only Snf2, Arp7 and Arp9 exhibited in vitro ATPase and chromatin remodelling activity identical to that of intact SWI/SNF[138]. Arp7 and Arp9, two actin-related proteins, exist as a stable heterodimer, and associate with both SWI/SNF and RSC in yeast[139,140]. Based on genetic and functional studies of RSC, it has been proposed that the Arp7-Arp9 heterodimer, along with other factors, may have a role in achieving proper chromatin architecture at specific promoters[139].

It is likely that other SWI/SNF subunits, especially Snf5 and Swi3, are needed for in vivo SWI/SNF functions and/or complex integrity. Consistent with this idea, the SANT domain of Swi3 is required for normal SWI/SNF assembly and may facilitate interactions between SWI/SNF and the N-terminal tails of histones[138,141]. SWI/SNF assembly is also disrupted in *swi1⊿*, *snf2⊿*, *snf5⊿* and *snf6⊿* cells[142,143]. In addition, *SNF5* mutants are compromised for nucleosome remodelling and transcriptional derepression of SWI/SNF-dependent genes via promoter targeting of SWI/SNF[142].

Whole genome microarray analysis showed that the expression of approximately 6% of the yeast genome is dependent on Snf2 function when cells are grown under normal conditions in nutrient-rich medium[144,145]. However, SWI/SNF is needed for

the transcription of an additional set of genes that are induced under specific environmental conditions. For example, SWI/SNF is required for efficient transcription of *RNR3*, a DNA damage response gene, *SUC2*, a gene induced in the absence of glucose, and *PHO5*, which is transcribed under low phosphate conditions[75,127,146]. Microarray analyses, along with studies of individual gene expression, have revealed that SWI/SNF might contribute to both gene activation and repression. Specifically, when a catalytically inactive version of Snf2 was the only form present in the cell, 126 genes were down-regulated and 203 genes were up-regulated, suggesting that a functional Snf2 protein might be responsible for the repression of these latter genes[145]. Moreover, in response to high or low phosphate, SWI/SNF contributes to the respective rapid reassembly or disassembly of chromatin across the *PHO5* promoter[75,76]. Along with its roles in transcriptional activation and repression it was more recently shown that SWI/SNF can function in transcriptional elongation[147].

Although the roles of SWI/SNF in transcription, especially transcriptional activation, are well-established, the recruitment of this multisubunit remodeller to chromatin is not straightforward. While SWI/SNF has high affinity for DNA, it lacks a sequence-specific DNA binding domain[148]. Interestingly, the Swi1 subunit of SWI/SNF contains an AT-rich interaction domain (ARID domain), which when found in higher eukaryotes normally conveys DNA binding properties; however, the yeast Swi1 ARID domain exhibits only weak DNA binding activity that is probably not physiologically relevant[149]. Nonetheless, an abundance of evidence has demonstrated that SWI/SNF is specifically recruited to DNA. SWI/SNF binding to

promoters can be stabilized through interactions with transcriptional activators or repressors, histone chaperones, or by histone acetylation[74,150-152]. For example, SWI/SNF binding to some promoters can be stimulated by the acetylation of the N-terminal tails of histones H3 or H4[150], or the acetylation of lysine 56 that lies in the globular domain of histone H3[13].

Once bound to promoters, SWI/SNF can contribute to transcriptional activation through a variety of mechanisms. SWI/SNF can facilitate the binding of many different proteins to promoters, including transcriptional activators, general transcription factors, and KATs[146,153]. As mentioned above, SWI/SNF can increase the accessibility of nucleosomal DNA to the necessary protein factors via a DNA looping mechanism (Figure 1-5). This creates large DNA bulges off the nucleosome, thereby allowing regulatory sequences to become exposed[131]. In concert with other factors, or perhaps alone, SWI/SNF action can even result in the displacement of histones from promoter DNA[75,132].

In addition to roles in transcriptional control, SWI/SNF has been implicated in a plethora of other processes. Roles for SWI/SNF in DNA replication, silencing at ribosomal and telomeric DNA, and DNA repair have also been identified[154]. In particular, SWI/SNF contributes to the repair of damaged DNA through the nucleotide excision repair and double-strand break repair pathways[155,156]. Importantly, SWI/SNF functions as a tumour suppressor in humans[157].

Links between perturbations in SWI/SNF and human cancer. In the past decade, a number of studies have linked alterations in SWI/SNF to the initiation of cancer, tumour progression and therapeutic resistance (reviewed in[158]). In 1998,

Versteege et al. first discovered that the most frequently deleted region of chromosome 22 in malignant rhabdoid tumour cell lines encodes the human *SNF5/INI1* gene[9]. Most of the mutations associated with oncogenesis were found in the conserved domain of *SNF5* that mediates its interaction with the hBRM ATPase subunit of SWI/SNF. Further study confirmed that hSNF5 acts as a tumour suppressor mainly through its ability to remodel chromatin and regulate transcription[157].

Alteration of either of the two human ATPase subunits of SWI/SNF, BRG1 and BRM, results in the development and progression of cancer. For example, loss of BRG1 is associated with the development of lung cancer, whereas loss of BRM can lead to prostate, lung or gastric cancers[158]. These results suggest that the ATPase subunits of SWI/SNF have tissue-specific tumour suppressor functions. On the contrary, perturbations in core and accessory SWI/SNF subunits can either promote or inhibit tumour initiation or progression[158]. Overall, recent research suggests that particular combinations of SWI/SNF subunits may have specific roles in contributing to oncogenesis. Given the high degree of conservation of many of these SWI/SNF subunits from yeast to human, there is much to be gained from studying chromatin remodelling processes using yeast as a model system.

Research Overview. Studies of histone chaperone Asf1 have revealed its involvement in multiple pathways of chromatin modulation, including chromatin assembly/disassembly, DNA replication, transcription and the DNA damage response. Our studies not only further the understanding of Asf1 action in known pathways, but also reveal new cellular roles for Asf1, thereby expanding the already

large repertoire of Asf1 functions in the nucleus. **Chapter 3** reports several novel genetic interactions between *ASF1* and genes encoding proteins with roles in chromatin regulation. In particular, we identify genetic and physical interactions between Asf1 and Set2, a histone methyltransferase, leading to the idea that Asf1 contributes to trimethylation of histone H3 lysine 36 by Set2. In **Chapter 4**, we show that Asf1 is important for derepression of *RNR3* and *HUG1*, two DDR genes, in response to replication stress. While Asf1 associates directly with the promoters of these two genes, it appears that Asf1 associates globally with chromatin. However, we determine for the first time that this association is increased during conditions of replication stress. We go on to identify the determinants of Asf1 association with chromatin and find that Asf1 association with the promoters of the DDR genes is not needed for its ability to promote their derepression. Making use of the findings by others that 1) Asf1 is required for the acetylation of H3K56, and 2) H3K56 acetylation is needed for transcriptional induction of *PHO5*, we determine that Asf1 contributes to derepression of the DDR genes through a pathway that depends on H3K56 acetylation. **Chapter 5** follows up a genetic interaction identified in Chapter 3 between *ASF1* and *SNF2*, which encodes the catalytic subunit of the SWI/SNF chromatin remodeller. We demonstrate that *asf1*⊿ *snf2*⊿ cells are slow-growing, highly sensitive to replication stress caused by HU, and partially checkpoint-defective. Asf1 and Snf2 are both important for derepression of DDR genes upon treatment with HU; however, cells lacking both proteins show additive defects, indicating that Asf1 and SWI/SNF likely function in a partially overlapping, redundant pathway. Although Asf1 and Snf2 are each recruited to the

promoters of *HUG1* and *RNR3* during replication stress, we determine that their recruitment does not depend on one another. In addition, we show that Asf1 may also interact physically with Snf2. While it appears that Asf1 and SWI/SNF do not work in a linear pathway to contribute to transcription, our results are consistent with the hypothesis that Asf1 and SWI/SNF may work together in a pathway to promote efficient DNA replication. The implications of these findings are discussed in **Chapter 6**, and ideas for future experiments are presented.

Table 1-1. Histone chaperones in *Saccharomyces cerevisiae*.

Histone Chaperone	Specificity	Cellular Roles
Asf1	H3/H4 dimer	-chromatin assembly[29,54,55] -chromatin disassembly[72] -H3K56 acetylation[78] -DNA replication[92] -transcription[60,73,77,105] -DNA damage response[26,95] -silencing[58,62]
CAF-I	H3/H4	-replication-coupled chromatin assembly[53,55] -silencing[54,56] -DNA repair[56]
Hif1	H3/H4	-telomeric silencing[159]
HIR	H3/H4	-replication-independent chromatin assembly[29,160] -transcriptional repression[161,162] -silencing[58,163]
FACT	H2A/H2B	-transcriptional elongation[44] -DNA replication[45-49]
Nap1	H2A/H2B, H3/H4	-nucleosome assembly[164] -H2A exchange[165,166]
Rtt106	H3/H4	-chromatin assembly[55] -heterochromatin silencing[24] -transcription elongation[167]
Spt6	H3/H4	-transcriptional repression[37] -transcriptional elongation[38-40]
Vps75	H2A/H2B, H3/H4	-H3K56 acetylation[25,90,168] -transcription[36]

Table 1-2. SWI/SNF subunits are conserved in yeast, human and *Drosophila*[*].

S. cerevisiae	Human	*Drosophila*
Swi2/Snf2	BRM, BRG1	Brahma
Swi1	BAF250A	OSA
Swi3	BAF170/BAF155	Moira
Snf5	SNF5/INI1/BAF47	Snr1
Snf12/Swp73	BAF60A/BAF60B	BAP60
Arp7/Swp61	BAF53	BAP55
Arp9/Swp59		
Swp29/Taf30/Anc1/Tfg3		
Swp82		
Snf6		
Snf11		
Rtt102		
	BAF57	Dalao/BAP111
	BAF200	BAP170
	BAF180	Moira/BAP155
	BAF53A	BAP55
	BAF53B	
	BAF60C	
	BAF250B	
	actin	actin

[*]Information is based on the indicated reviews[130,131,169].

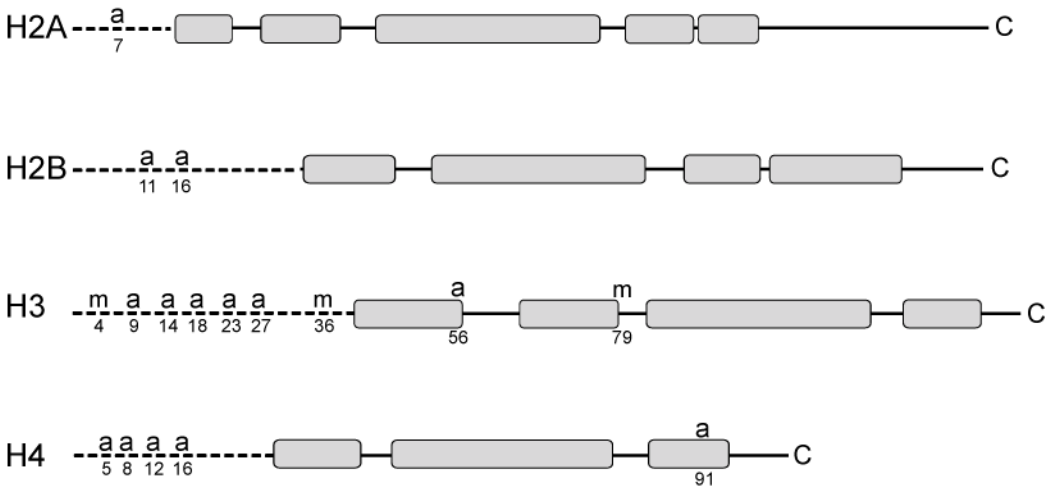

Figure 1-1. Sites of acetylation and methylation on histones H2A, H2B, H3 and H4 in yeast. Acetylation (a) and methylation (m) sites in the N-terminal tails (dashed lines) and globular domains of histones are indicated. Figure adapted from[12].

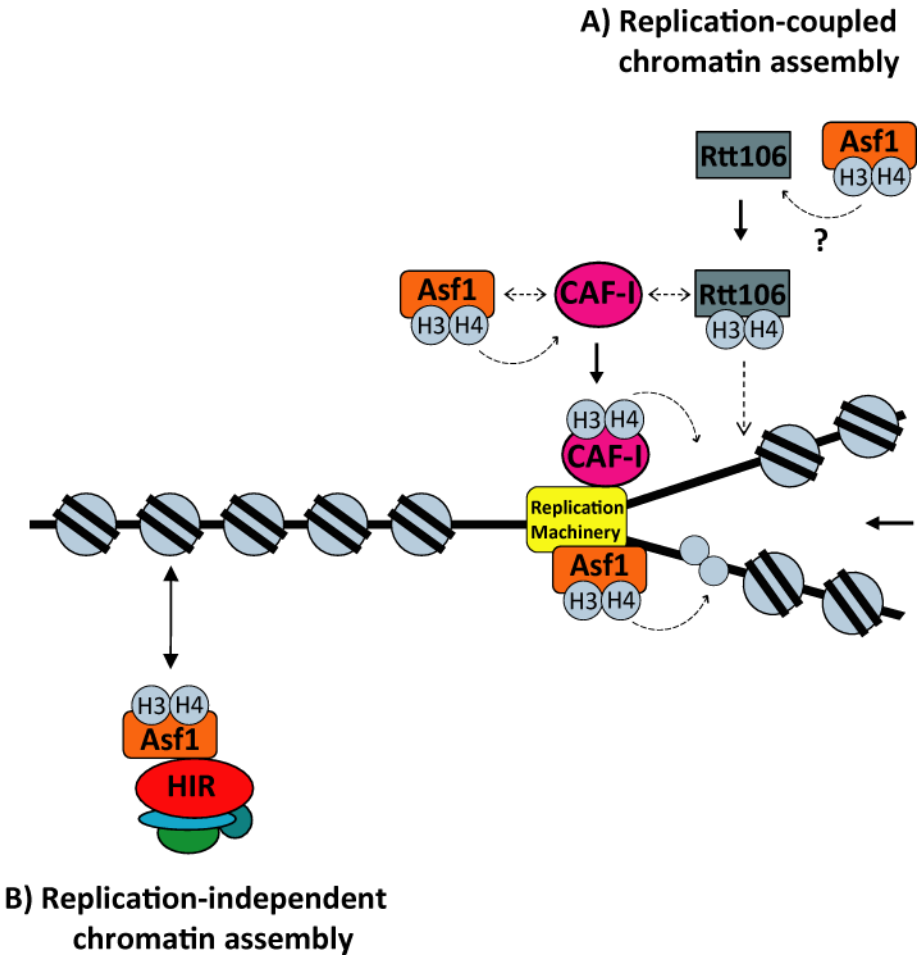

Figure 1-2. Model for Asf1 function in two pathways of chromatin assembly. **A.** During DNA replication, Asf1, Rtt106 and CAF-I all bind histones H3/H4 and contribute to chromatin assembly behind the replication fork. Asf1 and Rtt106 can stimulate chromatin assembly by CAF-I, and each can interact with CAF-I. Rtt106 and Asf1 may deposit histones in the absence of a functional CAF-I complex. Asf1 and CAF-I can each interact with the replication fork, likely through interactions with components of the replication machinery. **B.** Asf1 works in a replication-independent pathway for chromatin assembly with the HIR complex. See references within text. DNA is represented by black lines, and nucleosomal histones by light grey circles.

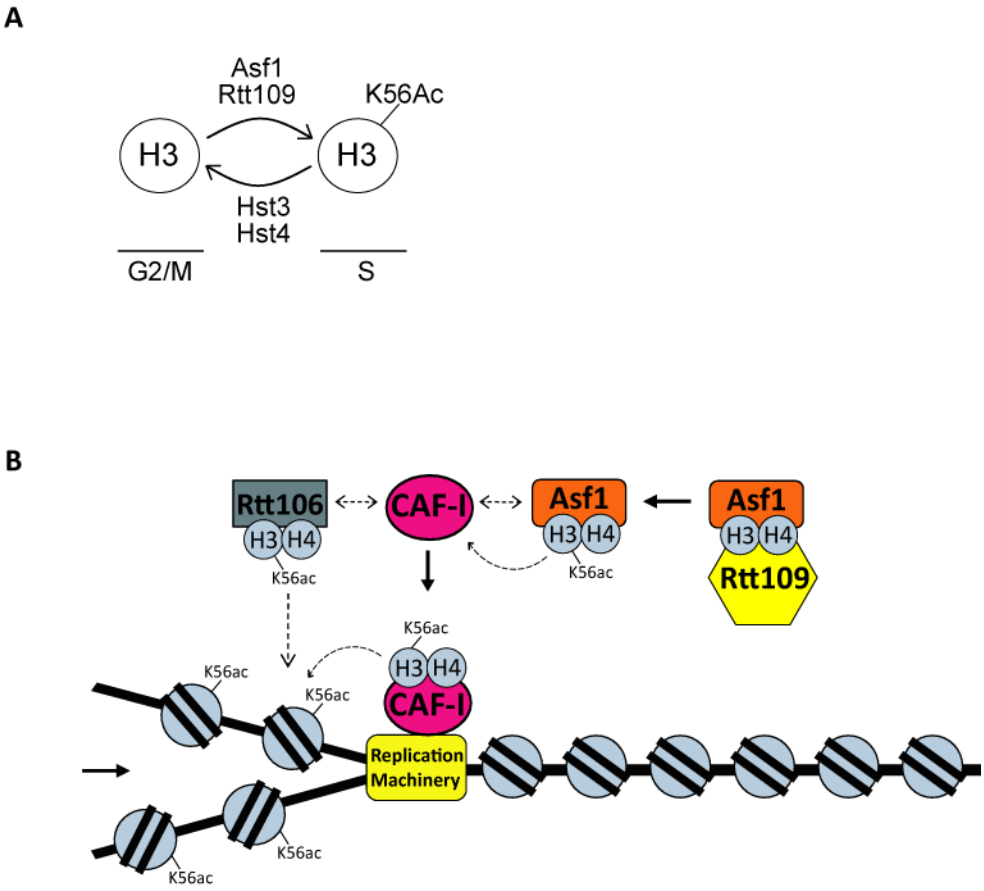

Figure 1-3. Asf1 is required for acetylation of histone H3 lysine 56. A. Histone H3 lysine 56 acetylation/deacetylation pathway in yeast. Rtt109 acetylates H3K56 in S phase. Asf1 is required for this acetylation event. In G2/M, the Hst3/Hst4 HDACs deacetylate H3K56. Proper regulation of H3K56 acetylation is necessary for genomic stability. See text for references. Figure adapted from[170]. **B.** Model for H3K56 acetylation and deposition into chromatin. Asf1 presents newly synthesized H3/H4 to the Rtt109 KAT for acetylation on K56 of H3. Lysine 56-acetylated H3 is then assembled into chromatin by the CAF-I and Rtt106 histone chaperones.

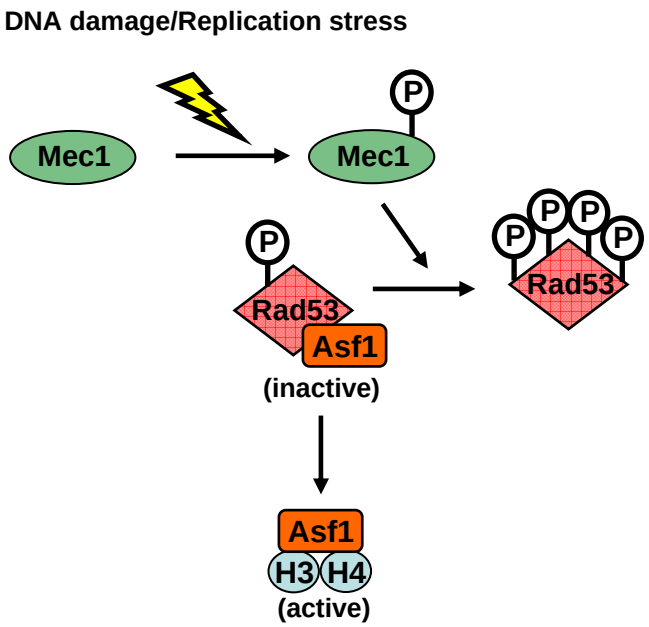

Figure 1-4. Asf1 exists in a dynamic complex with Rad53. In response to DNA damage or replication stress, Mec1 hyperphosphorylates Rad53, thereby disrupting the Asf1/Rad53 interaction. Upon its release from Rad53, Asf1 is competent to associate with histones[171].

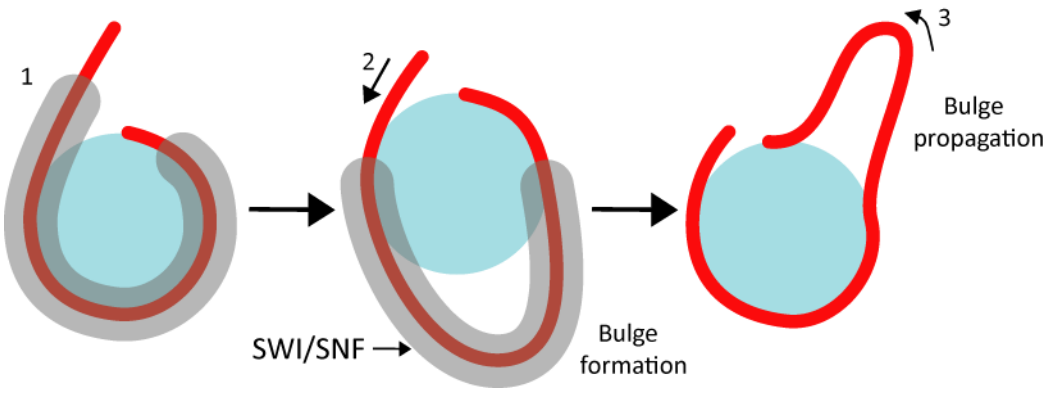

Figure 1-5. Model for mechanism of chromatin remodelling by SWI/SNF via an intranucleosomal DNA looping mechanism. 1) SWI/SNF has extensive interactions with nucleosomal DNA. 2) A large DNA loop is generated on the surface of the histone octamer (circle), which is 3) propagated along the nucleosome surface. Figure adapted from[131] by B. Chisholm.

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Chapter 2

Materials and Methods

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Strains, plasmids and media

Strains used in this study are listed in Table 2-1. All strains used are derived from BY4741[1] unless otherwise specified. Single deletion mutants, from the *S. cerevisiae* haploid nonessential gene deletion library[2], were verified to be correct by PCR using multiple primer sets. Chromosomal mutations were generated by one-step integration using PCR products obtained from previously described plasmids[3-6]. Addition of sequence encoding the 13-Myc epitope tag was verified by PCR using three primer sets: primers flanking the target gene (upstream, downstream) plus primers specific to the Myc epitope, and a primer flanking the target gene plus a primer specific for the *HIS3* selection marker. An *asf1*^{V94R}*-3HA::kanMX* strain, constructed by H. Mewhort, was used to generate *asf1*^{V94R}*-13MYC::HIS3*.

A 2346-bp XhoI-SacI DNA fragment from a pRS314-based plasmid harbouring *ASF1-13MYC* and regulatory sequences[7] was transferred to pRS316 (YCp-*URA3*) and pRS426 (2μ-*URA3*)[6]. The resulting p316-Asf1Myc and p426-Asf1Myc plasmids were transformed into the *asf1*Δ*::kanMX* strain for transcriptional and chromatin immunoprecipitation (ChIP) analyses using RT-PCR (Fig. 4-12). Plasmids p316-Asf1Myc and p316-asf1$^{D37R/E39R}$-Myc were a generous gift from the Cho lab[8] and were used for the ChIP analyses presented in Fig. 4-13. p316-asf1N$^{D37R/E39R}$-Myc was created by D. Hockman using a QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) to generate the D37R and E39R point mutations on a p316-

asf1N-Myc plasmid. All media were prepared as described previously, and standard genetic methods for transformations were used throughout this study[9].

Synthetic Genetic Array analysis

Synthetic genetic array (SGA) analysis was performed in collaboration with the Singer/Johnston laboratory at Dalhousie University according to Tong et al.[10]. An *asf1⊿::NAT* query strain (Y2454 background) was mated to the yeast haploid gene deletion collection (BY4741 background) using standard SGA robotics. To generate the query strain, *ASF1* was replaced with a nourseothricin-resistance cassette[4] in strain Y2454 using homologous recombination. Oligonucleotides bearing sequences flanking the *ASF1* coding region and plasmid pAG25[4] as a template were used to generate the deletion cassette by PCR amplification. Correct replacement of *ASF1* was verified by PCR analysis, and the ability of the *asf1⊿::NAT* query strain to grow was determined to be identical to that of the *asf1⊿::GEN* strain from the deletion collection (*asf1⊿::kanMX*).

Putative synthetic genetic interactions were determined as follows: 1) the interaction had to be present in each of the two SGA screens performed and 2) if a strain was pinned more than once (included in the screen in duplicate or triplicate), the interaction had to be present at least 67% of the time (at least four out of six times, or three out of four times pinned). Growth of viable double mutants was compared to growth of the corresponding single mutant from the deletion collection, and colony size was scored using a subjective rating scale for colony size. If four plusses (++++) equals wild type growth, then a colony scored as +++, ++ or + was slightly, moderately, or severely impaired for growth, respectively. Inviable double

mutants were deemed synthetic lethal. Putative synthetic genetic interactions were then grouped into one of three main categories: 1) synthetic lethal, 2) synthetic lethal/synthetic sick, or 3) synthetic sick. Synthetic lethal interactions were scored as synthetic lethal in 100% of pinnings (note one exception in Table 3-1: *DIA2*). Synthetic lethal/synthetic sick interactions were scored as synthetic lethal in at least one screen, but also as synthetic sick at least once. These interactions were given a numerical score to further describe the strength of the interaction (Table 2-2). Synthetic sick interactions were scored as synthetic sick in each screen, but never as synthetic lethal. Again, numerical scores to describe the strength of each synthetic sick interaction were assigned (Table 2-3).

Tetrad analysis

Diploid yeast strains were obtained from diploid selection SGA plates and grown overnight in pre-sporulation medium (1:1 ratio of YPD and 20% dextrose) at room temperature. 1.5 mL of each diploid culture was spun down, washed in water, resuspended in 10 mL sporulation medium (1% potassium acetate, 0.05% glucose, 20 mg/L leucine, 40 mg/L uracil)[11], and grown for at least three days at room temperature. Sporulation efficiency of cultures was checked using a light microscope. 15 μl of sporulated culture was spun and resuspended in 90 μl 1X TE, pH 8.0. 10 μl of beta-endoglucanase (from K. Robinson) was added to cells for ~5 minutes at 37 °C to allow for digestion of the ascus cell wall. 15 μl of digested cells were immediately plated on a level YPD plate and used for tetrad analysis. Tetrads were dissected using a micromanipulator (provided by D.T. Stuart). Spore genotypes were assessed by streaking each viable spore onto YPD, YPD+GEN, YPD+NAT

and YPD+GEN+NAT solid medium[10]. A synthetic genetic interaction was confirmed if most double mutant colonies were either 1) smaller than the respective single mutant colonies ('synthetic sick') or 2) inviable ('synthetic lethal').

Random spore analysis

Diploid yeast strains were grown in presporulation medium, washed and transferred to sporulation medium as above (see 'Tetrad analysis'). Cells were then inoculated into his-arg- broth, grown to saturation, and appropriate dilutions were plated onto his-arg-CAN, his-arg-CAN+GEN, his-arg-CAN+NAT, and his-arg-CAN+GEN+NAT plates[10]. Normally, 200 μl of culture was diluted 1/350-1/1000X to result in approximately 200-400 viable colonies on the his-arg-CAN plates. A synthetic genetic interaction was confirmed if there were either 1) smaller colonies or 2) less than half the number of colonies present on the double mutant haploid selection plates (his-arg-CAN+GEN+NAT) than on the respective single mutant selection plates (his-arg-CAN+GEN, his-arg-CAN+NAT). Note that in all cases it is expected that there will be half as many viable colonies on the double mutant haploid selection plates compared to the single mutant haploid selection plates. This is due to the ability of both single and double mutants to grow on the single mutant haploid selection plates.

Spotting assays

Cells were grown to early log phase, diluted to 1 x 10^7 cells/mL and 10-fold serial dilutions were spotted onto solid medium as indicated in the relevant figure legends. Five-fold serial dilutions were spotted in Fig. 3-3.

Flow cytometry

Cellular DNA content was determined by flow cytometry as described[12]. Briefly, cells were stained with propidium iodide, sonicated and analyzed using a FACScan flow cytometer (Becton-Dickinson).

Microarray

The role of budding yeast Asf1 in transcription was initially explored by comparing the mRNA expression profile of an *ASF1* deletion mutant to that of its isogenic wild type partner grown in YPD. This experiment was performed by Jessica Williams. Affymetrix S98 arrays were used for this analysis of duplicate biological samples, as outlined in[12]. Our MIAME compliant protocols are available at www.biochem.ualberta.ca/SchultzLab/. An overview of the pattern of mRNA misregulation in *asf1⊿* cells is shown in Fig. 4-1A.

RNA isolation and analysis

Total RNA was isolated by hot phenol extraction[13] from cells grown as described in the relevant figure legends. DNA probes for Northern blotting were prepared by random primed labelling of PCR products obtained with the primers listed in Table 2-4.

cDNA was generated from isolated RNA using Quanta qScript cDNA SuperMix and subjected to RT-PCR on a BioRad iCycler. *RNR3* and *HUG1* expression was normalized to an internal region of *RDN18-1*. Oligonucleotides used were: *RNR3* (+151/+315), *HUG1* (+2/+156) and *RDN18-1* (+756/+866); exact sequences are shown in Table 2-5.

Immunoblotting

Total proteins were prepared by trichloroacetic acid precipitation[12]. Identical cell equivalents of protein were compared between samples (except for the subcellular fractionation experiment, in which all lanes were loaded with the same amount of protein; Fig. 4-4). Antibodies were as follows: α-Rad53 (yC-19, Santa Cruz #sc-6749), α-H3 (Abcam #ab1791), α-penta-acetylated H4 (Upstate #06-946), α-actin (Millipore #MAB1501), α-myc (Millipore #9E10), α-H3K56ac (Upstate #07-677), α-CPY (Millipore #AB1817), α-HA (Roche, #12CA5) and α-CBP (Upstate #07-482).

Chromatin immunoprecipitation

Cells for ChIP were grown at 30 °C in YPD (or CM-ura selection media; Fig. 4-12 and 4-13) to an OD_{600} of 0.4, then grown either in the absence or presence of 0.2 M HU for one hour before crosslinking with formaldehyde. Specifically, after growth in the absence or presence of HU, cells were centrifuged for 4 min, media was removed and cells were resuspended in conditioned medium containing formaldehyde ('slow' HU washout). To decrease cell processing time, a 'fast' HU washout was developed, which involved cell recovery on 0.22 μm polyethersulfone disks by vacuum filtration (30-60 seconds), release of the vacuum, and immediate resuspension in the filter holder using HU-free conditioned medium with formaldehyde. Since we were concerned that resuspension in fresh medium, even when it contains formaldehyde, could give a transient proliferation signal that affects crosslinking, conditioned medium obtained from untreated cells was used for resuspension in both the 'slow' and 'fast' HU washout protocols. The steps after cell

harvesting were performed as described previously[14] with minor changes as outlined below.

Cells were lysed using a Mini-Beadbeater-16 (Biospec products) for 6 cycles of 3 min each at maximum speed with 2 min incubations in an ice-water bath after each 3 min cycle of bead beating. DNA was sheared to an average size of 500 bp (Fig. 4-3A) using a Branson Sonifier 450 for 8 cycles of 15 seconds on 20% constant output. Again, samples were incubated in an ice-water bath for at least 2 min after each cycle of sonication.

Whole cell extracts (WCEs) were divided into 800 µl aliquots and a 5 µl 'input' sample of each WCE was taken prior to immunoprecipitation. 95 µl of ChIP elution buffer[14] was added to the input, which was then boiled for 10 min. Input DNA was purified using the QIAquick PCR purification system. Asf1-Myc proteins were recovered from the remaining 795 µl of WCE using 10 µl of anti-myc antibody, and 10 µl of protein A sepharose beads. Alternatively, no antibody was added ('no antibody' control). Immunoblotting confirmed that a large fraction of the Asf1-Myc present in WCE was immunoprecipitated by the anti-myc antibody (Fig. 4-3B,C). Following immunoprecipitation for 90 min at room temperature, beads were washed for 3 min each with FA lysis buffer/0.15 M NaCl (2 times), FA lysis buffer/0.5 M NaCl, ChIP wash buffer, and finally 1X TE, pH 7.5. Washed beads were incubated at 65 °C for 10 min in ChIP elution buffer and eluted proteins/DNA were boiled for 10 min before DNA purification using the QIAquick PCR purification system. All solutions are described in[14]. Rtt109-Myc, total histone H3 and K56-acetylated H3

ChIPs were performed as above using 10 μl of anti-myc, 5 μg of anti-H3, or 2.5 μl of anti-H3K56ac, respectively.

Purified input and immunoprecipitated DNA was analyzed by semi-quantitative PCR with [α-^{32}P]dCTP as the label, or used for quantitative RT-PCR with SYBR Green (Quanta). RT-PCR reactions were performed in triplicate using a BioRad iCycler and quantitated as described[14] using the equation POWER (1.9, -$^{NET}C_T$). Primer efficiency was tested independently for each set of ChIP primers. RT-PCR was carried out using primer pairs specific to *RNR3*, *HUG1*[15], *SCR1*, *TELV*, *DSE1*, *PHO5* (pUASp2A/B;[16]) and *POL1*[17]. Primer sequences are described in Table 2-6. PCR products range from 90 to approximately 250 base pairs in length. Control experiments revealed that Asf1-Myc immunoprecipitated using anti-myc antibody is enriched approximately 20-fold at the promoters of *RNR3* and *HUG1* compared to a no antibody control (Fig. 4-3E, 4-6A, 4-7, 4-8). Rtt109-Myc was enriched 4- to 6-fold over a no antibody control at tested loci (Fig. 4-9).

Since a region of the genome with which Asf1 does not interact has not been reported, there is no suitable control region that should be used to calculate relative Asf1 binding. Therefore, data is presented as immunoprecipitated/input DNA at each region of the genome tested, with wild type untreated cells set to 1. Graphs represent averages ± standard deviation of at least three independent experiments, show averages of duplicate experiments, or show results from a single experiment, as indicated in the relevant figure legends.

Chromatin fractionation

Cells were grown in the presence or absence of 0.2 M HU for one hour. Chromatin was purified through a sucrose cushion as described in[18] (Fig. 4-4A), or KCl was used at 50 rather than 100 mM in buffer EB (Fig. 4-4B). Ten μg of total protein from each fraction was analyzed by immunoblotting. The experiment was performed three times, and yielded identical results to those shown in Fig. 4-4B.

Tandem Affinity Purification

Tandem affinity purification of protein complexes was performed by K. Lin using extracts from the indicated strains (Fig. 5-2) essentially as described[19].

Statistics and data analysis

Error bars were used to indicate standard deviations from the mean for experiments performed in triplicate. For duplicate experiments, ranges were calculated and are included in the relevant figure legends (the range is the difference between the two values used to calculate the mean).

Table 2-1. Yeast strains used in this study.

Strain	Relevant Genotype	Source
BY4741	*MAT*a *his3Δ1 leuΔ0 met15Δ0 ura3Δ0*	[1]
W303[a]	*MAT*a *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	[20]
Y2454[b]	*MATa mfa1Δ::MFA1pr-HIS3 can1Δ ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*	[21]
asf1Δ	*asf1Δ::kanMX*	[2]
asf1Δ[b]	*asf1Δ::NAT*	This study
sml1Δ	*sml1Δ::kanMX*	[2]
cac2Δ	*cac2Δ::kanMX*	[2]
rtt109Δ	*rtt109Δ::kanMX*	[2]
snf2Δ	*snf2Δ::kanMX*	[2]
snf5Δ	*snf5Δ::kanMX*	[2]
set2Δ	*set2Δ::kanMX*	[2]
sml1Δ rad53Δ	*sml1Δ::kanMX rad53Δ::HIS3*	[22]
sml1Δ asf1Δ	*sml1Δ::kanMX asf1Δ::NAT*	H. Mewhort
sml1Δ rad53Δ asf1Δ	*sml1Δ::kanMX rad53Δ::HIS3 asf1Δ::NAT*	This study
asf1Δ set2Δ	*asf1Δ::kanMX set2Δ::NAT*	[23]
asf1Δ snf2Δ	*asf1Δ::NAT snf2Δ::kanMX*	This study
asf1Δ snf5Δ	*asf1Δ::NAT snf5Δ::kanMX*	This study
ASF1-MYC	*ASF1-13MYC::HIS3*	This study
ASF1-MYC[a]	*ASF1-13MYC::HIS3*	D. Hockman
cac2Δ ASF1-MYC	*cac2Δ::kanMX ASF1-13MYC::HIS3*	This study
rtt109Δ ASF1-MYC	*rtt109Δ::kanMX ASF1-13MYC::HIS3*	This study
sml1Δ ASF1-MYC	*sml1Δ::kanMX ASF1-13MYC::HIS3*	This study
sml1Δ rad53Δ ASF1-MYC	*sml1Δ::kanMX rad53Δ::LEU2 ASF1-13MYC::HIS3*	This study
snf2Δ ASF1-MYC	*snf2Δ::kanMX ASF1-13MYC::HIS3*	This study
asf1N-MYC	*asf1N-13MYC::HIS3*	This study
asf1^{V94R}*-MYC*	*asf1*^{V94R}*-13MYC::HIS3*	This study
asf1N^{V94R}*-MYC*	*asf1N*^{V94R}*-13MYC::HIS3*	This study
ASF1-MYC[c]	*asf1Δ::kanMX [p316-ASF1-13MYC]*	This study[d]
asf1$^{D37R/E39R}$*-MYC*	*asf1Δ::kanMX[p316-asf1*$^{D37R/E39R}$*-13MYC]*	This study[d]
asf1N$^{D37R/E39R}$*-MYC*	*asf1Δ::kanMX [p316-asf1N*$^{D37R/E39R}$*-13MYC]*	This study
asf1Δ[e]	*asf1Δ::kanMX [pRS316]*	This study[f]
ASF1-MYC (low)[e]	*asf1Δ::kanMX [p316-ASF1-13MYC]*	This study
asf1Δ[e]	*asf1Δ::kanMX (pRS426]*	This study[f]
ASF1-MYC (high)[e]	*asf1Δ::kanMX [p426-ASF1-13MYC]*	This study
SY1	*SNF2-13MYC::HIS3*	[24]
asf1Δ SNF2-MYC	*asf1Δ::NAT* in SY1	This study
SNF5-MYC	*SNF5-13MYC::HIS3*	This study
asf1Δ SNF5-MYC	*asf1Δ::kanMX SNF5-13MYC::HIS3*	This study
RTT109-MYC	*RTT109-13MYC::HIS3*	K. Lin

SNF2-HA	*SNF2-3HA::kanMX*	K. Lin
SNF2-HA ASF1-TAP	*SNF2-3HA::kanMX ASF1-TAP::HIS*	K. Lin
HMY133[a]	*hht1-hhf1⊿::LEU2 hht2-hhf2⊿::kanMX3 [YCp22 HHT1 HHF1 TRP1]*	[25]
HMY134[a]	*hht1-hhf1⊿::LEU2 hht2-hhf2⊿::kanMX3 [YCp22 hht1 K56A HHF1 TRP1]*	[25]
HMY135[a]	*hht1-hhf1⊿::LEU2 hht2-hhf2⊿::kanMX3 [YCp22 hht1 K56Q HHF1 TRP1]*	[25]
HMY136[a]	*hht1-hhf1⊿::LEU2 hht2-hhf2⊿::kanMX3 [YCp22 hht1 K56R HHF1 TRP1]*	[25]
H3K56 asf1⊿[a]	*asf1⊿::NAT* in HMY133	This study
H3K56A asf1⊿[a]	*asf1⊿::NAT* in HMY134	D. Hockman
H3K56Q asf1⊿[a]	*asf1⊿::NAT* in HMY135	This study
H3K56R asf1⊿[a]	*asf1⊿::NAT* in HMY136	D. Hockman

[a]W303 strain background
[b]Y2454 strain background
[c]Strain bearing indicated plasmid used only in Fig. 4-13
[d]Plasmid described in[8]
[e]Strain bearing indicated plasmid used only in Fig. 4-12
[f]Plasmid described in[6]

Table 2-2. Synthetic lethal/synthetic sick interaction numerical scores*.

Numerical Score	Possible scores given
1	SL/+
2	SL/+/++
3	SL/++
4	SL/++/+++
5	SL/+++

*for use with Table 3-2
**SL, synthetic lethal
***an 'A' beside any number indicates that one colony was scored as wild type (++++) or better.

Table 2-3. Synthetic sick interaction numerical scores*.

Numerical Score	Possible scores given
1	+
2	+/++
3	+/++/+++
4	+/+++
5	++
6	++/+++

*for use with Table 3-3
**an 'A' beside any number indicates that one colony was scored as wild type (++++) or better.

Table 2-4. Oligonucleotides used for gene expression (Northern blotting).

Oligonucleotide	Sequence
RNR3 F	5'- CTCCCGTATCACCCGTT -3'
RNR3 R	5'- CGATATCGCTACCATGG -3'
RNR2 F	5'- CGCTTCTGACGGTATTGT -3'
RNR2 R	5'- GGCGTCCAAGAAGTATCT -3'
HUG1 F	5'- AAGGCCTTAACCCAAAGC -3'
HUG1 R	5'- CAATGTCAGAAAGACCGC -3'
UBI4 F	5'- GGTTGAATCCTCCGACAC -3'
UBI4 R	5'- GAAGATTCAACCTCTAGGG -3'
ACT1 F	5'- GCTCAATCCAAGAGAGG -3'
ACT1 R	5'- CCAAGGCGACGTAACATAG -3'
SCR1 F	5'- GTGGGATGGGATACGTTGAGA- 3'
SCR1 R	5'- TAAACCGCCGAAGCGATCA -3'

Table 2-5. Oligonucleotides used for gene expression (RT-PCR).

Oligonucleotide	Sequence
RNR3 +151 F	5'- GTTACTACCGTTGAGCTGGAC -3'
RNR3 +315 R	5'- AATCCAGTCGTGTAAATCCTC -3'
HUG1 +2 F	5'- TGACCATGGACCAAGGCCTTA -3'
HUG1 +156 R	5'- GGCAATGATGTTGGCAGAAGG -3'
RDN18-1 F	5'- AATTAGAGTGTTCAAAGCAGG -3'
RDN18-1 R	5'- CTATTAATCATTACGATGGTCC -3'

Table 2-6. Oligonucleotides used for ChIP.

Oligonucleotide	Sequence	Source
RNR3 -179 F	5'- CGTTTTTCGTGTCAGCGT TC -3'	26*
RNR3 +8 R	5'- ACGTACATTTGTGTGGGA G -3'	26*
HUG1 -167 F	5'- AGGCAACTGATTCCCAGCATATA -3'	15
HUG1 +41 R	5'- GGAAGAATTGCTTT GGTTAA -3'	15
SCR1 -151 F	5'- CCCTCGGTATTCTTTTAACA -3'	
SCR1 +11 R	5'- CAGAAAGCCATTACAGCCTA -3'	
TELV F	5'- GGCTGTCAGAATATGGGGCCGTAGTA -3'	
TELV R	5'- CACCCCGAAGCTGCTTTCACAATAC -3'	
PHO5 UASp2-A	5'- GAATAGGCAATCTCTAAATGAATCG -3'	16
PHO5 UASp2-B	5'- GAAAACAGGGACCAGAATCATAAAT -3'	16
DSE1 -118 F	5'- CCAAGCTTTCCTCAAACTAT -3'	
DSE1 +21 R	5'- CGTAGTATTTGGTATCTTGC -3'	
POL1 ORF F	5'- GACAAAATGAAGAAAATGCTGATGCACC -3'	17
POL1 ORF R	5'- TAATAACCTTGGTAAAACACCCTG -3'	17

*primers were designed in this study, but are similar to those used in[26]

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Chapter 3

Synthetic genetic array analysis identifies novel interactions between *ASF1* and multiple other chromatin factors

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Chapter 3

Synthetic genetic array analysis identifies novel interactions between *ASF1* and multiple other chromatin factors

Introduction

Approximately 80% of known or predicted *S. cerevisiae* genes are not essential for viability[1]. To aid in the experimental analysis of this organism, a collection of strains that each lack one of these nonessential genes has been created[2]. This collection of ~4800 single mutants (the 'deletion collection') is crucial to large-scale genetic analyses, such as SGA analysis[3]. In SGA analysis, robotics are employed to mate a query strain (containing the gene mutation/deletion of interest) to the entire deletion collection to generate ~4800 different double mutants. The viability of each double mutant can then be determined and the ability of viable double mutants to grow can be analyzed. If the double mutant is viable, the size of the colony is compared to that of each corresponding single mutant. There is no interaction between the query gene and the appropriate gene from the deletion collection when a double mutant grows similarly to the corresponding single mutants (Fig. 3-1A). A 'synthetic sick' interaction exists when the double mutant colony is more impaired for growth than either single mutant (Fig. 3-1B) and if a double mutant is inviable, a 'synthetic lethal' interaction is said to exist (Fig. 3-1C). The identification of genetic interactions using high-throughput methods, such as SGA analysis, provides an excellent starting point to understand the ways in which a protein, or group of proteins, might work in the cell, and possibly allows new functional roles for a given protein of interest to be discovered.

Although numerous genetic interactions involving *ASF1* have been reported in the literature, we have identified additional novel interactions using SGA analysis. Consistent with the wide assortment of functions Asf1 exhibits in chromatin regulation, we have identified genetic interactions between *ASF1* and genes that encode subunits of chromatin-regulating protein complexes that 1) *ASF1* was not previously known to interact with, and 2) *ASF1* is known to interact with. In many of the latter cases we have extended the number of subunits with which *ASF1* interacts. General discussion of the significance of particular interactions is presented, and further studies involving the genetic interaction between *ASF1* and *SET2* are described.

Results and Discussion

Synthetic Genetic Array analysis identifies novel interactions

Synthetic genetic array analysis was performed using an *asf1*⊿ query strain to identify which nonessential yeast genes *ASF1* interacts with genetically. From the time when we first initiated this SGA screen, other groups have carried out large-scale genetic screens that have identified many genes that interact with *ASF1*[4-7]. In addition to confirming 56 previously identified synthetic genetic interactions, our SGA analysis, performed in duplicate, has identified an additional 540 putative novel synthetic genetic interactions.

In order for a genetic interaction to be deemed 'synthetic' in this study, it had to be identified in each of two independent SGA screens, and strains that were present in duplicate or triplicate in each screen had to be scored as either 'synthetic sick' or 'synthetic lethal' at least 67% of the time overall (i.e., four out of six times, or three

out of four times). It is likely that we identified such a large number of putative interactions because we performed the SGA screen in duplicate as opposed to triplicate, and rather than using computer software to analyze colony size, we analyzed and scored each colony individually by eye, using a subjective rating scale for colony size (see below). While increasing the potential for inconsistencies in scoring, this technique does allow for small differences in colony growth to be identified, including colony size, colour and shape. All putative synthetic interactions (as defined above) obtained from our SGA screens are reported in Tables 3-1, 3-2 and 3-3 (not only those interactions that were confirmed by other analyses). While the more stringent criteria used by others to identify putative interactions can be quite helpful in weeding out false positive interactions, it will undoubtedly result in the dismissal of positive, potentially interesting, interactions. It is certain that even using our base criteria (i.e., synthetic interaction must be present in each independent SGA screen 67-100% of the time) some genetic interactions were missed. For example, our SGA screens did not reliably detect known synthetic growth defects between *ASF1* and components of the CAF-I (*CAC1*, *CAC2*, *CAC3*) histone chaperone complex[4,5,8]. Notably, synthetic genetic interactions between *ASF1* and *CAC1*, *CAC2* and *CAC3* were detected in only one of our two SGA screens.

Scoring and confirmation of novel synthetic interactions

Growth of the double mutant array generated in each SGA screen was compared to growth of the deletion collection. Specifically, each double mutant colony was 'scored' based on size compared to the corresponding single mutant from the deletion collection. Double mutants that grew similarly to the respective single

mutant were scored as 'wild type'; however, double mutant colonies that were smaller than the respective single mutants were given either one, two or three plusses ('+', '++', '+++'), with one plus indicating the most severe growth defect. Double mutants that were absent were deemed 'synthetic lethal'. Each synthetic genetic interaction presented in Table 3-2 or 3-3 was assigned a score (Tables 2-2 and 2-3) to further describe the 'synthetic lethal/synthetic sick' or 'synthetic sick' interaction, respectively (see Materials and Methods for details).

Due to the large-scale nature of SGA analysis, and the limitations of the robotics methodology employed, it is imperative that putative synthetic interactions are confirmed by other means. Therefore, we used tetrad analysis and/or random spore analysis to confirm or exclude several interesting synthetic genetic interactions (Table 3-4, Fig. 3-2, Fig. 3-3A). Although some of these further analyses were inconclusive, most interactions were confirmed. Results from specific tetrad analyses are shown in Fig. 3-2.

ASF1 interacts genetically with *SET2*

One of the highlights of our high-throughput genetic screen was the identification of a synthetic sick interaction between *ASF1* and *SET2*, the gene encoding the sole H3K36 methyltransferase in budding yeast (Table 3-2; this interaction has since been reported by others[5])[9]. We confirmed this interaction using tetrad analysis and random spore analysis (Fig. 3-2, *SET2* panel; 3-3A). We then verified that an *asf1*⊿ *set2*⊿ haploid strain generated by successive one-step gene replacements grew at a much slower rate than either single mutant at 30 °C, and that this phenotype was exacerbated at 37 °C (Fig. 3-3B). Given that the proteins encoded by *ASF1* and

SET2 are both involved in regulating chromatin processes, these results prompted us to examine in more detail the nature of functional interactions between Asf1 and Set2.

We used flow cytometry to assess the impact of individual and combined deletion of *ASF1* and *SET2* on cell cycle progression (Fig. 3-3C). Consistent with previous work[8], we found that *asf1*⊿ cells accumulate with a G2/M content of DNA and take longer than wild type cells to complete S phase. In contrast, the flow cytometry profile of *set2*⊿ cells was very similar to wild type. Furthermore, the cell cycle profile of the *asf1*⊿ mutant was unaffected by deletion of *SET2*. Therefore, Asf1 and Set2 do not perform the same function in parallel pathways of chromatin regulation that affect cell cycle progression under normal culture conditions.

ASF1 and *SET2* do not exist in a shared pathway to protect cells from DNA structure abnormalities

Considering that the regulation of chromatin structure is important for protection against DNA damage, we tested whether Asf1 and Set2 both function in pathways that protect cells from DNA structure abnormalities. As previously reported[8], loss of *ASF1* enhanced the sensitivity of yeast to two genotoxins: HU and MMS (Fig. 3-4A and B). *SET2* null mutants, on the other hand, grew similarly to wild type cells in the presence of these agents. Furthermore, the *asf1*⊿ *set2*⊿ double mutant had only marginally greater sensitivity to HU and MMS than the *asf1*⊿ single mutant. Therefore, Set2 is probably not in a major Asf1-dependent pathway of chromatin metabolism that protects cells from genotoxin-induced DNA structure abnormalities. This conclusion is supported by the observation that *set2*⊿ cells were

more sensitive to UV irradiation than *asf1*⊿ cells, and that the *asf1*⊿ *set2*⊿ double mutant was no more UV-sensitive than the *set2*⊿ single mutant (Fig. 3-4C).

Functional interactions between Asf1 and Set2

The genetic interaction between *ASF1* and *SET2*, coupled with the finding by K. Lin that Asf1 and Set2 also interact physically, prompted us to further explore how these two proteins might interact functionally[9]. This work resulted in several key findings. In particular, Asf1 promotes 1) bulk histone H3K36 trimethylation, 2) Set2 association with the coding region of the highly transcribed *PMA1* gene, and 3) H3K36 trimethylation in the *PMA1* coding region. Importantly, H3K36 dimethylation (bulk and *PMA1*-associated) remained unaffected in *asf1*⊿ cells. These findings led to the conclusion that Asf1 can promote H3K36 trimethylation by Set2. In addition, Asf1 and Set2 can both function to inhibit spurious intragenic transcription from the coding regions of genes.

Genetic interactions between *ASF1* and other genes encoding proteins involved in chromatin processes

In addition to its interaction with *SET2*, *ASF1* also interacted genetically with several other genes that encode proteins with roles in chromatin regulation. Novel genetic interactions uncovered in this study include a synthetic lethal interaction with *HTA1*, synthetic lethal/synthetic sick interactions with *ADA2*, *CPS25*/*SDC1*, *IOC2*, *SNF2* and *YNG1*, and synthetic sick interactions with *ASH1*, *CTI6*, *HDA2*, *HDA3*, *IOC4*, *NGG1*, *NUP2*, *PSH1*, *SDS3*, *SPT3* and *STB2*. These interactions are summarized in Fig. 3-5 using an Osprey map[10]. Each gene is represented by a circle,

and genes that interact are connected by solid lines. This Osprey map includes synthetic genetic interactions identified in this study that have also been reported elsewhere (between *ASF1* and *ARP6*, *BRE2*, *HAT2*, *HHF2*, *HHT2*, *LEO1*, *RCO1*, *RXT2*, *SAP30*, *SAS5*, *SET2*, *SIN3*, *SWD3*, and *SWR1*)[4-6]. Of particular note is the new-found genetic interaction between *ASF1* and *HTA1*, one of two yeast genes encoding histone H2A. Although Asf1 interacts genetically and physically with histones H3 and H4[4,11], no interaction between Asf1 and histone H2A (or H2B) has been previously reported.

ASF1 also interacted genetically with a wide variety of genes that encode proteins that covalently modify histones. As described above, Set2 is one such protein, which methylates histone H3K36. Although genetic interactions between *ASF1* and two subunits (*BRE2*, *SWD3*) of COMPASS, a complex that methylates histone H3 lysine 4 (H3K4), have been previously reported, we have also identified a third genetic interaction between *ASF1* and COMPASS: mutants lacking both *ASF1* and *CPS25/SDC1* were 'synthetic lethal/synthetic sick'. Based on the extensive interactions between *ASF1* and COMPASS, *ASF1* may promote H3K4 methylation in the absence of a fully functional COMPASS complex. Consistent with this idea, H3K4 methylation is reduced, but not absent, in *sdc1*⊿ and *bre2*⊿ mutants[12]. Given that *ASF1* and COMPASS mutants have defects in silencing[12,13], Asf1 and COMPASS may function in partially redundant pathways to control gene silencing.

Asf1 has known roles in histone acetylation (see Chapter 1). Our SGA results confirm previous findings and provide additional evidence for the involvement of Asf1 in histone acetylation as well as histone deacetylation. For example, it is known

that Asf1 promotes histone H3 lysine 9 acetylation by Gcn5, the catalytic subunit of SAGA[14], a multisubunit KAT complex. In this study we have identified new genetic interactions between *ASF1* and three other members of SAGA: *ADA2*, *SPT3* and *NGG1*. We have also uncovered an interaction between Asf1 and NuA3 (*YNG1* gene), another KAT complex that acetylates histone H3 (reviewed in[15]).

With regards to histone deacetylation, we have found synthetic interactions between *ASF1* and members of the Rpd3/Hda1 histone HDAC family. Importantly, we provide evidence for the first time that deletion of *ASF1* is synthetic sick with deletions of *HDA2* and *HDA3*, the protein products of which form a heterodimer that associates with Hda1, the putative catalytic subunit of the complex[16,17]. There are already several known genetic interactions between *ASF1* and Rpd3-containing HDAC complexes[4,5,7,9,18]. In agreement with this data, we have added to the list three synthetic sick interactions between *ASF1* and the Rpd3C(L) complex: *ASH1*, *CTI6*, and *SDS3*[19]. The HDAC activity of Rpd3C(L) can be targeted to specific promoters by its Ash1 and Ume6 subunits[20]. Altogether, these results illustrate the importance of Asf1 in the facilitation of a large network of histone modifications.

In addition to the synthetic genetic interaction identified between *ASF1* and the SWI/SNF chromatin remodelling complex (*SNF2* subunit; see below and Chapter 5), this study has also revealed interactions between *ASF1* and two of the three components of Isw1b (*IOC2* and *IOC4*), an ATP-dependent chromatin remodelling complex involved in the regulation of transcriptional elongation[21]. Other genes whose protein products are involved in transcriptional elongation that

were identified in this screen are *ELP2*, a core subunit of the Elongator complex, *MFT1*, a subunit of the THO complex, *LEO1*, a subunit of PAF, *SUB1*, and *SET2* (see above and Chapter 1). The *ASF1-SUB1*, *ASF1-LEO1* and *ASF1-SET2* interactions have also been reported by others[5,6]. These results are summarized in Fig. 3-6. *ASF1* also interacted with other subunits of Elongator and THO in one of our two SGA screens, but did not meet the base criteria required to be considered 'putative synthetic interactions'; however, a synthetic genetic interaction between *ASF1* and *THP1* of the THO complex has been reported by others[5]. These findings provide support for the notion that Asf1 can contribute to transcriptional elongation[9,22]. It will be important to determine how proteins such as Asf1, Set2, Isw1b, Elongator and the THO complex might cooperate to regulate transcriptional elongation.

Due to the interesting nature of four putative interactions identified in only one of our SGA screens (between *ASF1* and *SNF2*, *PSH1*, *DDC1* and *SET4*) we further tested these interactions using random spore analysis and tetrad analysis even though they did not meet our base criteria to be considered 'synthetic'. *SNF2* is the catalytic subunit of the ATP-dependent SWI/SNF chromatin remodeller, and therefore it would be interesting if *ASF1* and *SNF2* exhibited a synthetic growth defect. The fact that *snf2*⊿ mutants have defects in sporulation[23] is a possible explanation as to why this interaction was only detected in one of the SGA screens. *PSH1* was isolated as a factor that interacts with the FACT chromatin modulator[24], and *DDC1* is a DNA damage checkpoint protein[25]. Given our identification of, and interest in, the *ASF1-SET2* interaction (see above), we also decided to explore the possible

interaction between *ASF1* and *SET4*. *SET4* is a protein of unknown function that contains a SET domain[26]. Overall, tetrad analysis and random spore analysis confirmed that *ASF1* exhibits a 'synthetic lethal/synthetic sick' interaction with *SNF2*, and synthetic sick interactions with *PSH1*, *DDC1* and *SET4* (Table 3-4).

Conclusions

Our SGA analyses have identified 540 novel putative synthetic genetic interactions involving *ASF1* (Table 3-1, 3-2 and 3-3). Several of these interactions were further confirmed using tetrad analysis and random spore analysis (Table 3-4, Fig. 3-2 and 3-3A). We chose to study the interaction between *ASF1* and *SET2* in greater detail because 1) we had also detected a physical interaction between the proteins encoded by these genes[9], and 2) Asf1 and Set2 are both involved in the regulation of chromatin processes. This work was the first to functionally link the mechanism of action of a histone methyltransferase to that of a histone chaperone, showing that Asf1 can contribute to trimethylation of histone H3K36 by Set2.

The SGA analyses performed here also uncovered numerous important genetic interactions between *ASF1* and genes that encode other proteins involved in chromatin metabolism. Of particular interest are the novel interactions between *ASF1* and the genes that encode histone H2A (*HTA1*), Yng1 (of NuA3), subunits of the HDA1 HDAC complex, components of the ISW1b chromatin remodeller, and Snf2 (of SWI/SNF; see Chapter 5). Importantly, we have confirmed many previously identified interactions, in some cases extending the interactions between *ASF1* and genes encoding subunits of chromatin- modifying complexes (COMPASS, SAGA, Rpd3C(L)).

The fact that *ASF1* interacts genetically with so many genes encoding proteins that function in the covalent modification of histones implies that Asf1 may act in redundant pathways to ensure that the proper modification status of chromatin is maintained. Although Asf1 might not be strictly required for a particular methylation/acetylation/deacetylation event, it likely facilitates several of these events through its ability to bind to histones and present them to the necessary histone modifying complexes[27]. When Asf1 is missing from the cell, another histone chaperone might step in to facilitate histone modification. Similarly, when for example an HDAC subunit is missing, deacetylation by another HDAC complex might allow cellular fitness/survival. However, when both Asf1 *and* the HDAC subunit are absent, the cell has two separate problems to deal with, which both contribute to the same overall process: histone deacetylation. Hence, histone deacetylation will be more perturbed in an *asf1*⊿ *hdac*⊿ double mutant than an *asf1*⊿ or *hdac*⊿ single mutant. It will be important to further explore the many pathways of histone modification to which Asf1 contributes.

Given the potential problems with SGA robotics, it is certain that some proportion of the interactions identified in this study are false positive or false negative. To assist in the elimination of some of these false positives/negatives, a third SGA screen could be performed. Other future directions include further testing of additional putative interactions, including determining the biological significance of such interactions, which has the potential for uncovering new cellular roles for Asf1.

Table 3-1. Putative synthetic lethal interactions.

Gene/ORF	Description
ADA2/YDR448W	Transcription coactivator, component of the ADA and SAGA transcriptional adaptor/HAT (histone acetyltransferase) complexes
BEM2/YER155C	Rho GTPase activating protein (RhoGAP) involved in the control of cytoskeleton organization and cellular morphogenesis; required for bud emergence
CIK1/YMR198W*	Kinesin-associated protein required for both karyogamy and mitotic spindle organization
CUP5/YEL027W*	Proteolipid subunit of the vacuolar H(+)-ATPase V0 sector (subunit c; dicyclohexylcarbodiimide binding subunit); required for vacuolar acidification and important for copper and iron metal ion homeostasis
GLY1/YEL046C	Threonine aldolase, catalyzes the cleavage of L-allo-threonine and L-threonine to glycine; involved in glycine biosynthesis
HIS1/YER055C	ATP phosphoribosyltransferase, a hexameric enzyme, catalyzes the first step in histidine biosynthesis
HTA1/YDR225W	Histone H2A, core histone protein required for chromatin assembly and chromosome function; one of two nearly identical subtypes (see also HTA2); DNA damage-dependent phosphorylation by Mec1p facilitates DNA repair; acetylated by Nat4p
MDM20/YOL076W	Non-catalytic subunit of the NatB N-terminal acetyltransferase, which catalyzes N-acetylation of proteins with specific N-terminal sequences; involved in mitochondrial inheritance and actin assembly
NHX1/YDR456W	Endosomal Na+/H+ exchanger, required for intracellular sequestration of Na+; required for osmotolerance to acute hypertonic shock
PLC1/YPL268W	Phospholipase C, hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP2) to generate the signaling molecules inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG); involved in regulating many cellular processes
RPS30B/YOR182C	Protein component of the small (40S) ribosomal subunit
STP22/YCL008C	Component of the ESCRT-I complex, which is involved in ubiquitin-dependent sorting of proteins into the endosome; homologous to the mouse and human Tsg101 tumor susceptibility gene
TFP1/YDL185W	Subunit A of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase
TFP3/YPL234C	Vacuolar ATPase V0 domain subunit c', involved in proton transport activity

UAF30/YOR295W	Subunit of UAF (upstream activation factor), which is an RNA polymerase I specific transcription stimulatory factor composed of Uaf30p, Rrn5p, Rrn9p, Rrn10p, histones H3 and H4; deletion decreases cellular growth rate
VMA7/YGR020C	Subunit F of the eight-subunit V1 peripheral membrane domain of vacuolar H+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system
VPS25/YJR102C*	Component of the ESCRT-II complex, which is involved in ubiquitin-dependent sorting of proteins into the endosome
VPS66/YPR139C	Cytoplasmic protein of unknown function involved in vacuolar protein sorting
YET3/YDL072C	Protein of unknown function; YET3 null mutant decreases the level of secreted invertase; homolog of human BAP31 protein
YGL079W	Putative protein of unknown function
YOR331C	Dubious open reading frame unlikely to encode a protein
DIA2/YOR080W**	Origin-binding F-box protein that forms an SCF ubiquitin ligase complex with Skp1p and Cdc53p; plays a role in DNA replication, involved in invasive and pseudohyphal growth

*Interaction has also been identified by others (see text).
**Strain was 'synthetic lethal' in 3 of 4 pinnings; all other strains were deemed 'synthetic lethal' in all pinnings.

Table 3-2. Putative synthetic lethal/synthetic sick interactions.

Gene/ORF	Score**	Description
BUD20/YLR074C	1	Protein involved in bud-site selection
IME2/YJL106W	1	Serine/threonine protein kinase involved in activation of meiosis
MRE11/YMR224C*	1	Subunit of a complex with Rad50p and Xrs2p (MRX complex) that functions in repair of DNA double-strand breaks and in telomere stability, exhibits nuclease activity that appears to be required for MRX function; widely conserved
MRPL35/YDR322W	1	Mitochondrial ribosomal protein of the large subunit
MTQ2/YDR140W	1	S-adenosylmethionine-dependent methyltransferase of the seven beta-strand family; subunit of complex with Trm112p that methylates translation release factor Sup45p (eRF1) in the ternary complex eRF1-eRF3-GTP
RPA49/YNL248C	1	RNA polymerase I subunit A49
SET2/YJL168C*	1	Histone methyltransferase with a role in transcriptional elongation, methylates a lysine residue of histone H3; associates with the C-terminal domain of Rpo21p; histone methylation activity is regulated by phosphorylation status of Rpo21p
SPT2/YER161C	1	Protein involved in negative regulation of transcription; required for RNA polyadenylation; exhibits regulated interactions with both histones and SWI-SNF components, has similarity to mammalian HMG1 proteins
SSN3/YPL042C	1	Cyclin-dependent protein kinase, component of RNA polymerase II holoenzyme; involved in phosphorylation of the RNA polymerase II C-terminal domain; involved in glucose repression
TSA1/YML028W*	1	Thioredoxin peroxidase
TUP1/YCR084C	1	General repressor of transcription, forms complex with Cyc8p, involved in the establishment of repressive chromatin structure through interactions with histones H3 and H4, appears to enhance expression of some genes
VID22/YLR373C	1	Glycosylated integral membrane protein localized to the plasma membrane
YKE2/YLR200W	1	Subunit of the heterohexameric Gim/prefoldin protein complex involved in the folding of alpha-tubulin, beta-tubulin, and actin
YELO45C	1	Dubious open reading frame unlikely to encode a protein; deletion gives MMS sensitivity

YIL032C	1	Dubious open reading frame unlikely to encode a protein
YLR108C	1	Protein of unknown function
YLR402W	1	Dubious open reading frame unlikely to encode a protein
YNL171C	1	Dubious open reading frame unlikely to encode a functional protein
PHO5/YBR093C	2	Repressible acid phosphatase (1 of 3) that also mediates extracellular nucleotide-derived phosphate hydrolysis; secretory pathway derived cell surface glycoprotein; induced by phosphate starvation and coordinately regulated by PHO4 and PHO2
AAH1/YNL141W	3	Adenine deaminase (adenine aminohydrolase)
AIR1/YIL079C	3	Zinc knuckle protein, involved in nuclear RNA processing and degradation as a component of the TRAMP complex
ARG82/YDR173C***	3	Inositol polyphosphate multikinase (IPMK), sequentially phosphorylates Ins(1,4,5)P3 to form Ins(1,3,4,5,6)P5; also has diphosphoinositol polyphosphate synthase activity; regulates arginine-, phosphate-, and nitrogen-responsive genes
ART10/YLR392C	3	Protein of unknown function that contains 2 PY motifs and is ubiquinated by Rsp5p
ATG33/YLR356W	3	Mitochondrial mitophagy-specific protein
CCC1/YLR220W	3	Putative vacuolar Fe2+/Mn2+ transporter
CHS5/YLR330W	3	Component of the exomer complex
CPR6/YLR216C	3	Peptidyl-prolyl cis-trans isomerase (cyclophilin)
DOA4/YDR069C	3	Ubiquitin isopeptidase, required for recycling ubiquitin from proteasome-bound ubiquitinated intermediates
ECM21/YBL101C	3	Protein involved in regulating the endocytosis of plasma membrane proteins; identified as a substrate for ubiquitination by Rsp5p and deubiquitination by Ubp2p; promoter contains several Gcn4p binding elements
EMP46/YLR080W	3	Integral membrane component of endoplasmic reticulum-derived COPII-coated vesicles
FSH2/YMR222C	3	Putative serine hydrolase that localizes to the cytoplasm; sequence is similar to S. cerevisiae Fsh1p and Fsh3p and the human candidate tumor suppressor OVCA2
GIC1/YHR061C	3	Protein of unknown function involved in initiation of budding and cellular polarization, interacts with Cdc42p via the Cdc42/Rac-interactive binding (CRIB) domain

GZF3/YJL110C	3	GATA zinc finger protein and Dal80p homolog that negatively regulates nitrogen catabolic gene expression by competing with Gat1p for GATA site binding
HPF1/YOL155C	3	Haze-protective mannoprotein that reduces the particle size of aggregated proteins in white wines
HRT3/YLR097C	3	Putative SCF-ubiquitin ligase F-box protein
ICT1/YLR099C	3	Lysophosphatidic acid acyltransferase
IRC9/YJL142C	3	Dubious open reading frame unlikely to encode a protein; null mutant displays increased levels of spontaneous Rad52p foci
ISA2/YPR067W	3	Protein required for maturation of mitochondrial and cytosolic Fe/S proteins
IZH3/YLR023C	3	Membrane protein involved in zinc ion homeostasis
KIN2/YLR096W	3	Serine/threonine protein kinase involved in regulation of exocytosis
PBS2/YJL128C	3	MAP kinase kinase that plays a pivotal role in the osmosensing signal-transduction pathway, activated under severe osmotic stress; plays a role in regulating Ty1 transposition
PEX3/YDR329C	3	Peroxisomal membrane protein (PMP) required for proper localization and stability of PMPs
RAD17/YOR368W	3	Checkpoint protein, involved in the activation of the DNA damage and meiotic pachytene checkpoints; with Mec3p and Ddc1p, forms a clamp that is loaded onto partial duplex DNA; homolog of human and S. pombe Rad1 and U. maydis Rec1 proteins
REG1/YDR028C	3	Regulatory subunit of type 1 protein phosphatase Glc7p, involved in negative regulation of glucose-repressible genes
SDC25/YLL016W	3	Non-essential Ras guanine nucleotide exchange factor (GEF) localized to the membrane; expressed in poor nutrients and on non-fermentable carbon sources; homologous to CDC25; contains a stop codon in S288C; full-length gene includes YLL017W
SNT309/YPR101W	3	Member of the NineTeen Complex (NTC) that contains Prp19p and stabilizes U6 snRNA in catalytic forms of the spliceosome containing U2, U5, and U6 snRNAs
SUL2/YLR092W	3	High affinity sulfate permease
SWR1/YDR334W*	3	Swi2/Snf2-related ATPase that is the structural component of the SWR1 complex, which exchanges histone variant H2AZ (Htz1p) for chromatin-bound histone H2A
TRK2/YKR050W	3	Component of the Trk1p-Trk2p potassium transport system

UBC12/YLR306W	3	Enzyme that mediates the conjugation of Rub1p, a ubiquitin-like protein, to other proteins; related to E2 ubiquitin-conjugating enzymes
VAC14/YLR386W	3	Protein involved in regulated synthesis of PtdIns(3,5)P(2)
VPS63/YLR261C	3	Dubious open reading frame, unlikely to encode a protein; deletion causes a vacuolar protein sorting defect
WSS1/YHR134W	3	Sumoylated protein of unknown function, identified based on genetic interactions with SMT3; UV-sensitive mutant phenotype and genetic interactions suggest a role in the DNA damage response, processing stalled or collapsed replication forks
YNG1/YOR064C	3	Subunit of the NuA3 histone acetyltransferase complex that acetylates histone H3; contains PHD finger domain that interacts with methylated histone H3, has similarity to the human tumor suppressor ING1
YPS3/YLR121C	3	Aspartic protease
YVC1/YOR087W	3	Vacuolar cation channel
YGL046W	3	Merged open reading frame, does not encode a discrete protein
YIL067C	3	Uncharacterized protein of unknown function
YJL147C	3	Mitochondrial protein of unknown function
YLR164W	3	Mitochondrial inner membrane of unknown function
YLR224W	3	F-box protein and component of SCF ubiquitin ligase complexes involved in ubiquitin-dependent protein catabolism; readily monoubiquitinated in vitro by SCF-Ubc4 complexes; YLR224W is not an essential gene
YLR415C	3	Putative protein of unknown function
YPR039W	3	Dubious open reading frame unlikely to encode a protein
HAL5/YJL165C	4	Putative protein kinase
BER1/YLR412W	5	Protein involved in microtubule-related processes
BUD16/YEL029C	5	Putative pyridoxal kinase; involved in bud-site selection
BUD28/YLR062C	5	Dubious open reading frame, unlikely to encode a protein
CAP2/YIL034C	5	Beta subunit of the capping protein (CP) heterodimer (Cap1p and Cap2p) which binds to the barbed ends of actin filaments preventing further polymerization

CRT10/YOL063C	5	Protein involved in transcriptional regulation of RNR2 and RNR3; expression of the gene is induced by DNA damage and null mutations confer increased resistance to hydroxyurea; N-terminal region has a leucine repeat and a WD40 repeat
DFG16/YOR030W	5	Probable multiple transmembrane protein
ECI1/ YLR284C	5	Peroxisomal delta3,delta2-enoyl-CoA isomerase
ELP2/YGR200C	5	Subunit of Elongator complex, which is required for modification of wobble nucleosides in tRNA
GCR2/YNL199C	5	Transcriptional activator of genes involved in glycolysis
GIM3/YNL153C	5	Subunit of the heterohexameric cochaperone prefoldin complex
HPA3/YEL066W	5	D-Amino acid N-acetyltransferase, catalyzes N-acetylation of D-amino acids through ordered bi-bi mechanism in which acetyl-CoA is first substrate bound and CoA is last product liberated; similar to Hpa2p, acetylates histones weakly in vitro
ICE2/YIL090W	5	Integral ER membrane protein with type-III transmembrane domains
IOC2/YLR095C	5	Member of a complex (Isw1b) with Isw1p and Ioc4p that exhibits nucleosome-stimulated ATPase activity and acts within coding regions to coordinate transcription elongation with termination and processing, contains a PHD finger motif
IRC21/YMR073C	5	Putative protein of unknown function; proposed to be involved in resistance to carboplatin and cisplatin; shares similarity to a human cytochrome oxidoreductase; null mutant displays increased levels of spontaneous Rad52p foci
MMS22/YLR320W*	5	Protein that acts with Mms1p in a repair pathway that may be involved in resolving replication intermediates or preventing the damage caused by blocked replication forks; required for accurate meiotic chromosome segregation
MTC2/YKL098W	5	Protein of unknown function; mtc2 is synthetically sick with cdc13-1
NAB6/YML117W	5	Putative RNA-binding protein
NSG1/YHR133C	5	Protein involved in regulation of sterol biosynthesis
PFK2/YMR205C	5	Beta subunit of heterooctameric phosphofructokinase involved in glycolysis
PPM1/YDR435C	5	Carboxyl methyltransferase, methylates the C terminus of the protein phosphatase 2A catalytic subunit (Pph21p or Pph22p)
PPN1/YDR452W	5	Vacuolar endopolyphosphatase with a role in phosphate metabolism

RAS1/YOR101W	5	GTPase involved in G-protein signaling in the adenylate cyclase activating pathway, plays a role in cell proliferation; localized to the plasma membrane; homolog of mammalian RAS proto-oncogenes
ROD1/YOR018W	5	Membrane protein that binds the ubiquitin ligase Rsp5p via its 2 PY motifs
RPS28B/YLR264W	5	Protein component of the small (40S) ribosomal subunit
SAS5/YOR213C*	5	Subunit of the SAS complex (Sas2p, Sas4p, Sas5p), which acetylates free histones and nucleosomes and regulates transcriptional silencing; stimulates Sas2p HAT activity
SCJ1/YMR214W	5	One of several homologs of bacterial chaperone DnaJ, located in the ER lumen where it cooperates with Kar2p to mediate maturation of proteins
SDC1/YDR469W	5	Subunit of the COMPASS (Set1C) complex, which methylates lysine 4 of histone H3 and is required in chromatin silencing at telomeres
SER33/YIL074C	5	3-phosphoglycerate dehydrogenase, catalyzes the first step in serine and glycine biosynthesis; isozyme of Ser3p
SIC1/YLR079W*	5	Inhibitor of Cdc28-Clb kinase complexes that controls G1/S phase transition, preventing premature S phase and ensuring genomic integrity; phosphorylation targets Sic1p for SCF(CDC4)-dependent turnover
SPG3/YDR504C	5	Protein required for survival at high temperature during stationary phase; not required for growth on nonfermentable carbon sources
STD1/YOR047C	5	Protein involved in control of glucose-regulated gene expression; interacts with protein kinase Snf1p, glucose sensors Snf3p and Rgt2p, and TATA-binding protein Spt15p; acts as a regulator of the transcription factor Rgt1p
SWT21/YNL187W	5	Protein involved in mRNA splicing
TRP3/YKL211C	5	Bifunctional enzyme exhibiting both indole-3-glycerol-phosphate synthase and anthranilate synthase activities
UIP5/YKR044W	5	Protein of unknown function that interacts with Ulp1p
URE2/YNL229C	5	Nitrogen catabolite repression transcriptional regulator
VPS21/YOR089C	5	GTPase required for transport during endocytosis and for correct sorting of vacuolar hydrolases
YPK1/YKL126W	5	Serine/threonine protein kinase that phosphorylates and downregulates flippase activator Fpk1p
YIR043C	5	Possible pseudogene in strain S288C

YLR236C	5	Dubious open reading frame unlikely to encode a functional protein
YMR317W	5	Putative protein of unknown function with some similarity to sialidase from Trypanosoma
YOR283W	5	Phosphatase with some similarity to GPM1/YKL152C
YOR292C	5	Putative protein of unknown function
YPL199C	5	Putative protein of unknown function
CNM67/YNL225C	1A	Component of the spindle pole body outer plaque; required for spindle orientation and mitotic nuclear migration
CTK3/YML112W*	1A	Gamma subunit of C-terminal domain kinase I (CTDK-I), which phosphorylates both RNA pol II subunit Rpo21p to affect transcription and pre-mRNA 3' end processing, and ribosomal protein Rps2p to increase translational fidelity
CWH36/YCL007C	1A	Dubious ORF unlikely to encode a protein
LOC1/YFR001W	1A	Nuclear protein involved in asymmetric localization of ASH1 mRNA; binds double-stranded RNA in vitro; constituent of 66S pre-ribosomal particles
RRG1/YDR065W	1A	Protein of unknown function, required for vacuolar acidification and mitochondrial genome maintenance
RSM22/YKL155C	1A	Mitochondrial ribosomal protein of the small subunit
VMA4/YOR332W	1A	Subunit E of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase)
YDR417C	1A	Dubious open reading frame unlikely to encode a protein
YGR219W	1A	Dubious open reading frame unlikely to encode a protein
MRPL31/YKL138C	2A	Mitochondrial ribosomal protein of the large subunit
BUD21/YOR078W	3A	Component of small ribosomal subunit (SSU) processosome that contains U3 snoRNA; originally isolated as bud-site selection mutant that displays a random budding pattern
CTK1/YKL139W*	3A	Catalytic (alpha) subunit of C-terminal domain kinase I (CTDK-I), which phosphorylates both RNA pol II subunit Rpo21p to affect transcription and pre-mRNA 3' end processing, and ribosomal protein Rps2p to increase translational fidelity
KCS1/YDR017C****	3A	Inositol hexakisphosphate (IP6) and inositol heptakisphosphate (IP7) kinase; generation of high energy inositol pyrophosphates by Kcs1p is required for many processes such as vacuolar biogenesis, stress response and telomere maintenance

MRP7/YNL005C	3A	Mitochondrial ribosomal protein of the large subunit
RPP1B/YDL130W	3A	Ribosomal protein P1 beta
SHP1/YBL058W*	3A	UBX (ubiquitin regulatory X) domain-containing protein that regulates Glc7p phosphatase activity and interacts with Cdc48p; interacts with ubiquitylated proteins in vivo
SLX5/YDL013W	3A	Subunit of the Slx5-Slx8 SUMO-targeted ubiquitin ligase (STUbL) complex, stimulated by SUMO-modified substrates; forms SUMO-dependent nuclear foci, including DNA repair centers
UPS1/YLR193C	3A	Mitochondrial intermembrane space protein
IMG2/YCR071C	4A	Mitochondrial ribosomal protein of the large subunit
MSH1/YHR120W	4A	DNA-binding protein of the mitochondria involved in repair of mitochondrial DNA, has ATPase activity and binds to DNA mismatches
OPI9/YLR338W	4A	Dubious open reading frame unlikely to encode a protein
SIN4/YNL236W	4A	Subunit of the RNA polymerase II mediator complex; associates with core polymerase subunits to form the RNA polymerase II holoenzyme; contributes to both postive and negative transcriptional regulation; dispensible for basal transcription
IWR1/YDL115C	5A	Protein involved in transcription from polymerase II promoters; interacts with most of the polymerase II subunits; nucleo-cytoplasmic shuttling protein; deletion causes hypersensitivity to the K1 killer toxin
MRT4/YKL009W	5A	Protein involved in mRNA turnover and ribosome assembly, localizes to the nucleolus
NPL4/YBR170C	5A	Endoplasmic reticulum and nuclear membrane protein, forms a complex with Cdc48p and Ufd1p that recognizes ubiquitinated proteins in the endoplasmic reticulum and delivers them to the proteasome for degradation
PIM1/YBL022C	5A	ATP-dependent Lon protease, involved in degradation of misfolded proteins in mitochondria; required for biogenesis and maintenance of mitochondria
SUV3/YPL029W	5A	ATP-dependent RNA helicase, component of the mitochondrial degradosome along with the RNase Dss1p

*Interaction has also been identified by others (see text).
**For details on scoring, refer to Materials and Methods (Chapter 2).
***_ARG82_ was not deleted in the indicated strain from the deletion collection, as determined by PCR.
****Strain is a diploid, as determined by flow cytometry.

Table 3-3. Putative synthetic sick interactions.

Gene/ORF	Score**	Description
ARP6/YLR085C*	1	Actin-related protein that binds nucleosomes; a component of the SWR1 complex, which exchanges histone variant H2AZ (Htz1p) for chromatin-bound histone H2A
CKA1/YIL035C***	1	Alpha catalytic subunit of casein kinase 2, a Ser/Thr protein kinase with roles in cell growth and proliferation; the holoenzyme also contains CKA2, CKB1 and CKB2, the many substrates include transcription factors and all RNA polymerases
CSM1/YCR086W*	1	Nucleolar protein that forms a complex with Lrs4p and then Mam1p at kinetochores during meiosis I to mediate accurate homolog segregation; required for condensin recruitment to the replication fork barrier site and rDNA repeat segregation
DUS4/YLR405W	1	Dihydrouridine synthase, member of a widespread family of conserved proteins including Smm1p, Dus1p, and Dus3p
ECM18/YDR125C	1	Protein of unknown function, similar to Rlp24p
HUR1/YGL168W*	1	Protein of unknown function; reported null mutant phenotype of hydroxyurea sensitivity may be due to effects on overlapping PMR1 gene
IDS2/YJL146W	1	Protein involved in modulation of Ime2p activity during meiosis, appears to act indirectly to promote Ime2p-mediated late meiotic functions; found in growing cells and degraded during sporulation
NIT2/YJL126W	1	Nit protein, one of two proteins in S. cerevisiae with similarity to the Nit domain of NitFhit from fly and worm and to the mouse and human Nit protein which interacts with the Fhit tumor suppressor; nitrilase superfamily member
RAD27/YKL113C*	1	5' to 3' exonuclease, 5' flap endonuclease, required for Okazaki fragment processing and maturation as well as for long-patch base-excision repair; member of the S. pombe RAD2/FEN1 family
REC102/YLR329W	1	Protein involved in early stages of meiotic recombination; required for chromosome synapsis; forms a complex with Rec104p and Spo11p necessary during the initiation of recombination
RPE1/YJL121C	1	D-ribulose-5-phosphate 3-epimerase, catalyzes a reaction in the non-oxidative part of the pentose-phosphate pathway; mutants are sensitive to oxidative stress
RPS0B/YLR048W	1	Protein component of the small (40S) ribosomal subunit

SRS2/YJL092W*	1	DNA helicase and DNA-dependent ATPase involved in DNA repair, needed for proper timing of commitment to meiotic recombination and transition from Meiosis I to II; blocks trinucleotide repeat expansion; affects genome stability
UBP6/YFR010W*	1	Ubiquitin-specific protease situated in the base subcomplex of the 26S proteasome, releases free ubiquitin from branched polyubiquitin chains; works in opposition to polyubiquitin elongation activity of Hul5p
UMP1/YBR173C	1	Short-lived chaperone required for correct maturation of the 20S proteasome; may inhibit premature dimerization of proteasome half-mers; degraded by proteasome upon completion of its assembly
YPT7/YML001W	1	GTPase; GTP-binding protein of the rab family; required for homotypic fusion event in vacuole inheritance, for endosome-endosome fusion, similar to mammalian Rab7
YJL120W	1	Dubious open reading frame unlikely to encode a protein
ACN9/YDR511W	2	Protein of the mitochondrial intermembrane space, required for acetate utilization and gluconeogenesis
APQ12/YIL040W*	2	Protein required for nuclear envelope morphology, nuclear pore complex localization, mRNA export from the nucleus
ASH1/YKL185W	2	Zinc-finger inhibitor of HO transcription; mRNA is localized and translated in the distal tip of anaphase cells, resulting in accumulation of Ash1p in daughter cell nuclei and inhibition of HO expression; potential Cdc28p substrate
BCH1/YMR237W	2	Member of the ChAPs family (Chs5p-Arf1p-binding proteins)
BNA5/YLR231C	2	Kynureninase, required for the de novo biosynthesis of NAD from tryptophan via kynurenine; expression regulated by Hst1p
BRE2/YLR015W*	2	Subunit of COMPASS (Set1C) complex, which methylates Lys4 of histone H3 and functions in silencing at telomeres
BUB2/YMR055C*	2	Mitotic exit network regulator; blocks cell cycle progression before anaphase in response to spindle and kinetochore damage
BUD2/YKL092C	2	GTPase activating factor for Rsr1p/Bud1p required for both axial and bipolar budding patterns
BUD8/YLR353W	2	Protein involved in bud-site selection; diploid mutants display a unipolar budding pattern instead of the wild-type bipolar pattern
CCS1/YMR038C*	2	Copper chaperone for superoxide dismutase Sod1p, involved in oxidative stress protection
CDA1/YLR307W	2	Chitin deacetylase

CLB2/YPR119W*	2	B-type cyclin involved in cell cycle progression; activates Cdc28p to promote the transition from G2 to M phase; accumulates during G2 and M, then targeted via a destruction box motif for ubiquitin-mediated degradation by the proteasome
CLB4/YLR210W	2	B-type cyclin involved in cell cycle progression; activates Cdc28p to promote the G2/M transition; may be involved in DNA replication and spindle assembly; accumulates during S phase and G2, then targeted for ubiquitin-mediated degradation
COG5/YNL051W	2	Component of the conserved oligomeric Golgi complex
DUS3/YLR401C	2	Dihydrouridine synthase
FMP46/YKR049C	2	Putative redox protein
HHT2/YNL031C*	2	Histone H3, core histone protein required for chromatin assembly, part of heterochromatin-mediated telomeric and HM silencing; one of two identical histone H3 proteins; regulated by acetylation, methylation, and phosphorylation
HRD3/YLR207W	2	Resident protein of the ER membrane that plays a central role in ER-associated protein degradation
LOT5/YKL183W	2	Protein of unknown function
LRP1/YHR081W	2	Nuclear exosome-associated nucleic acid binding protein; involved in RNA processing, surveillance, degradation, tethering, and export
LSM1/YJL124C*	2	Lsm (Like Sm) protein; involved in degradation of cytoplasmic mRNAs
MCA1/YOR197W	2	Putative cysteine protease
MCK1/YNL307C*	2	Protein serine/threonine/tyrosine (dual-specificity) kinase involved in control of chromosome segregation and in regulating entry into meiosis
MET17/YLR303W	2	Methionine and cysteine synthase
MID2/YLR332W	2	O-glycosylated plasma membrane protein that acts as a sensor for cell wall integrity signaling and activates the pathway
MMR1/YLR190W	2	Phosphorylated protein of the mitochondrial outer membrane
MSC3/YLR219W	2	Protein of unknown function; potential Cdc28p substrate
NAS2/YIL007C	2	Proteasome-interacting protein
NCA3/YJL116C	2	Protein that functions with Nca2p to regulate mitochondrial expression of subunits 6 (Atp6p) and 8 (Atp8p) of the Fo-F1 ATP synthase
NDE1/YMR145C	2	Mitochondrial external NADH dehydrogenase

NEJ1/YLR265C	2	Protein involved in regulation of nonhomologous end joining
OAF3/YKR064W	2	Zinc cluster protein; regulates transcription in response to oleate levels
OPI7/YDR360W	2	Dubious open reading frame unlikely to encode a protein
OYE2/YHR179W	2	Widely conserved NADPH oxidoreductase containing flavin mononucleotide (FMN)
PEX1/YKL197C	2	AAA-peroxin that heterodimerizes with AAA-peroxin Pex6p and participates in the recycling of peroxisomal signal receptor Pex5p from the peroxisomal membrane to the cystosol
PEX28/YHR150W	2	Peroxisomal integral membrane peroxin
PGD1/YGL025C	2	Subunit of the RNA polymerase II mediator complex; associates with core polymerase subunits to form the RNA polymerase II holoenzyme; essential for basal and activated transcription; direct target of Cyc8p-Tup1p transcriptional corepressor
PHO86/YJL117W	2	Endoplasmic reticulum (ER) resident protein required for ER exit of the high-affinity phosphate transporter Pho84p, specifically required for packaging of Pho84p into COPII vesicles
PIG1/YLR273C	2	Putative targeting subunit for the type-1 protein phosphatase Glc7p that tethers it to the Gsy2p glycogen synthase
PIP2/YOR363C	2	Autoregulatory oleate-specific transcriptional activator of peroxisome proliferation
PNP1/YLR209C	2	Purine nucleoside phosphorylase
PPH22/YDL188C	2	Catalytic subunit of protein phosphatase 2A (PP2A); involved in signal transduction and regulation of mitosis
PRM10/YJL108C	2	Pheromone-regulated protein, proposed to be involved in mating
PUT1/YLR142W	2	Proline oxidase, nuclear-encoded mitochondrial protein involved in utilization of proline as sole nitrogen source
RAD23/YEL037C	2	Subunit of Nuclear Excision Repair Factor 2 (NEF2) with Rad4p that recognizes and binds damaged DNA
RCO1/YMR075W*	2	Essential subunit of the histone deacetylase Rpd3S complex; interacts with Eaf3p
REC104/YHR157W	2	Protein involved in early stages of meiotic recombination
REX4/YOL080C	2	Putative RNA exonuclease

RIM20/YOR275C	2	Protein involved in proteolytic activation of Rim101p in response to alkaline pH; PalA/AIP1/Alix family member; interaction with the ESCRT-III subunit Snf7p suggests a relationship between pH response and multivesicular body formation
RNH203/YLR154C	2	Ribonuclease H2 subunit, required for RNase H2 activity
RPS28A/YOR167C	2	Protein component of the small (40S) ribosomal subunit
RSA3/YLR221C	2	Protein with a likely role in ribosomal maturation
SDS3/YIL084C	2	Component of the Rpd3p/Sin3p deacetylase complex required for its structural integrity and catalytic activity, involved in transcriptional silencing and required for sporulation
SIR1/YKR101W*	2	Protein involved in repression of transcription at the silent mating-type loci HML and HMR; recruitment to silent chromatin requires interactions with Orc1p and with Sir4p, through a common Sir1p domain; binds to centromeric chromatin
SKG3/YLR187W	2	Protein of unknown function
SRC1/YML034W	2	Inner nuclear membrane protein that functions in regulation of subtelomeric genes and is linked to TREX (transcription export) factors
SWM1/YDR260C	2	Subunit of the anaphase-promoting complex, which is an E3 ubiquitin ligase that regulates the metaphase-anaphase transition and exit from mitosis; required for activation of the daughter-specific gene expression and spore wall maturation
TSA2/YDR453C	2	Stress inducible cytoplasmic thioredoxin peroxidase
UBA4/YHR111W	2	Protein that activates Urm1p before its conjugation to proteins (urmylation)
UBC4/YBR082C*	2	Ubiquitin-conjugating enzyme (E2), mediates degradation of abnormal or excess proteins, including calmodulin and histone H3; interacts with many SCF ubiquitin protein ligases; component of the cellular stress response
UTP30/YKR060W	2	Subunit of U3-containing 90S preribosome complex
VID28/YIL017C	2	Protein involved in proteasome-dependent catabolite degradation of fructose-1,6-bisphosphatase
VTA1/YLR181C	2	Multivesicular body (MVB) protein involved in endosomal protein sorting
YCK1/YHR135C	2	Palmitoylated plasma membrane-bound casein kinase I isoform
YSP1/YHR155W	2	Mitochondrial protein with a potential role in promoting mitochondrial fragmentation during programmed cell death in response to high levels of alpha-factor mating pheromone or the drug amiodarone

YAR043C	2	Deleted ORF
YIL029C	2	Putative protein of unknown function
YIL089W	2	Putative protein of unknown function
YJL045W	2	Minor succinate dehydrogenase isozyme
YJL119C	2	Dubious open reading frame unlikely to encode a functional protein
YJL135W	2	Dubious open reading frame unlikely to encode a protein
YKL187C	2	Putative protein of unknown function
YLR122C	2	Dubious open reading frame unlikely to encode a protein
YLR124W	2	Dubious open reading frame unlikely to encode a protein
YLR126C	2	Putative protein of unknown function; may be involved in copper and iron homeostasis
YLR171W	2	Dubious open reading frame unlikely to encode a functional protein
YLR283W	2	Putative protein of unknown function
YLR400W	2	Dubious open reading frame unlikely to encode a functional protein
YLR416C	2	Dubious open reading frame unlikely to encode a functional protein
YMR158W-B	2	Dubious open reading frame unlikely to encode a protein
YMR306C-A	2	Dubious open reading frame unlikely to encode a functional protein
ARV1/YLR242C	3	Protein functioning in transport of glycosylphosphatidylinositol intermediates into the ER lumen
EAP1/YKL204W	3	eIF4E-associated protein
SNA3/YJL151C	3	Integral membrane protein localized to vacuolar intralumenal vesicles
AAT2/YLR027C	4	Cytosolic aspartate aminotransferase
ACM1/YPL267W	4	Pseudosubstrate inhibitor of the anaphase-promoting complex/cyclosome (APC/C)
AIM19/YIL087C	4	Putative protein of unknown function
ALE1/YOR175C	4	Broad-specificity lysophospholipid acyltransferase

ARO80/YDR421W	4	Zinc finger transcriptional activator of the Zn2Cys6 family
ATG13/YPR185W	4	Regulatory subunit of the Atg1p signaling complex
AVT3/YKL146W	4	Vacuolar transporter
BNA1/YDR428C	4	Formylkynurenine formamidase
BNI1/YNL271C	4	Formin, nucleates the formation of linear actin filaments, involved in cell processes such as budding and mitotic spindle orientation which require the formation of polarized actin cables
BZZ1/YHR114W	4	SH3 domain protein implicated in the regulation of actin polymerization
CBR1/YIL043C	4	Microsomal cytochrome b reductase
CHS6/YJL099W	4	Member of the ChAPs family of proteins (Chs5p-Arf1p-binding proteins)
CMP2/YML057W	4	Calcineurin A; one isoform of the catalytic subunit of calcineurin, a Ca++/calmodulin-regulated protein phosphatase which regulates Crz1p (a stress-response transcription factor)
COS12/YGL263W	4	Protein of unknown function
CRG1/YHR209W	4	Putative S-adenosylmethionine-dependent methyltransferase
CSI2/YOL007C	4	Protein of unknown function
CST6/YIL036W	4	Basic leucine zipper (bZIP) transcription factor of the ATF/CREB family, proposed to be a regulator of oleate responsive genes; involved in utilization of non-optimal carbon sources and chromosome stability
CTI6/YPL181W	4	Protein that relieves transcriptional repression by binding to the Cyc8p-Tup1p corepressor and recruiting the SAGA complex to the repressed promoter; contains a PHD finger domain
DJP1/YIR004W	4	Cytosolic J-domain-containing protein, required for peroxisomal protein import and involved in peroxisome assembly
DMA1/YHR115C	4	Protein involved in ubiquitin ligation; plays a role in regulating spindle position and orientation
DSK2/YMR276W	4	Nuclear-enriched ubiquitin-like polyubiquitin-binding protein, required for spindle pole body (SPB) duplication and for transit through the G2/M phase of the cell cycle, involved in proteolysis, interacts with the proteasome
DYN1/YKR054C	4	Cytoplasmic heavy chain dynein, microtubule motor protein, required for anaphase spindle elongation
ERP5/YHR110W	4	Protein with similarity to Emp24p and Erv25p

EST1/YLR233C	4	TLC1 RNA-associated factor involved in telomere length regulation as the recruitment subunit of the telomerase holoenzyme, has a possible role in activating Est2p-TLC1-RNA bound to the telomere
FRE6/YLL051C	4	Putative ferric reductase
FMP42/YMR221C	4	Putative protein of unknown function
GAS4/YOL132W	4	1,3-beta-glucanosyltransferase
GLC3/YEL011W	4	Glycogen branching enzyme
GSP2/YOR185C	4	GTP binding protein (mammalian Ranp homolog) involved in the maintenance of nuclear organization, RNA processing and transport
HOS4/YIL112W	4	Subunit of the Set3 complex, which is a meiotic-specific repressor of sporulation specific genes that contains deacetylase activity; potential Cdc28p substrate
HSL1/YKL101W	4	Nim1p-related protein kinase that regulates the morphogenesis and septin checkpoints; associates with the assembled septin filament; required along with Hsl7p for bud neck recruitment, phosphorylation, and degradation of Swe1p
HSP150/YJL159W	4	O-mannosylated heat shock protein
HXT4/YHR092C	4	High-affinity glucose transporter
ICS3/YJL077C	4	Protein of unknown function
INP53/YOR109W	4	Polyphosphatidylinositol phosphatase, dephosphorylates multiple phosphatidylinositols
IRC3/YDR332W	4	Putative RNA helicase of the DEAH/D-box family; null mutant displays increased levels of spontaneous Rad52p foci
IRC18/YJL037W	4	Putative protein of unknown function; null mutant displays increased levels of spontaneous Rad52p foci
IRE1/YHR079C	4	Serine-threonine kinase and endoribonuclease
ISU2/YOR226C	4	Conserved protein of the mitochondrial matrix, required for synthesis of mitochondrial and cytosolic iron-sulfur proteins, performs a scaffolding function in mitochondria during Fe/S cluster assembly
KHS1	4	Thermolabile killer toxin
MBR1/YKL093W	4	Protein involved in mitochondrial functions and stress response

MCM21/YDR318W	4	Protein involved in minichromosome maintenance; component of the COMA complex (Ctf19p, Okp1p, Mcm21p, Ame1p) that bridges kinetochore subunits that are in contact with centromeric DNA and the subunits bound to microtubules
MDV1/YJL112W	4	Peripheral protein of the cytosolic face of the mitochondrial outer membrane
MMP1/YLL061W	4	High-affinity S-methylmethionine permease
MRN1/YPL184C	4	RNA-binding protein proposed to be involved in translational regulation
MSN4/YKL062W	4	Transcriptional activator related to Msn2p; activated in stress conditions, which results in translocation from the cytoplasm to the nucleus; binds DNA at stress response elements of responsive genes, inducing gene expression
NCA2/YPR155C	4	Protein involved in regulation of mitochondrial expression of subunits 6 (Atp6p) and 8 (Atp8p) of the Fo-F1 ATP synthase
NMA1/YLR328W	4	Nicotinic acid mononucleotide adenylyltransferase
NTE1/YML059C	4	Serine esterase, homolog of human neuropathy target esterase (NTE); Nte1p-mediated phosphatidylcholine turnover influences transcription factor Opi1p localization, affecting transcriptional regulation of phospholipid biosynthesis genes
OAZ1/YPL052W	4	Regulator of ornithine decarboxylase (Spe1p)
OTU2/YHL013C	4	Protein of unknown function that may interact with ribosomes
PAU2/YEL049W	4	Member of the seripauperin multigene family encoded mainly in subtelomeric regions
PCI8/YIL071C	4	Possible shared subunit of Cop9 signalosome (CSN) and eIF3
PDR5/YOR153W	4	Plasma membrane ATP-binding cassette (ABC) transporter
PIR3/YKL163W	4	O-glycosylated covalently-bound cell wall protein
PMT3/YOR321W	4	Protein O-mannosyltransferase
PPR1/YLR014C	4	Zinc finger transcription factor containing a Zn(2)-Cys(6) binuclear cluster domain, positively regulates transcription of genes involved in uracil biosynthesis; activity may be modulated by interaction with Tup1p
PRM2/YIL037C	4	Pheromone-regulated protein
PRR1/YKL116C	4	Serine/threonine protein kinase that inhibits pheromone induced signalling downstream of MAPK, possibly at the level of the Ste12p transcription factor

PSY3/YLR376C	4	Protein involved in a Rad51p-, Rad54p-dependent pathway for homologous recombination repair; deletion results in a mutator phenotype; deletion increases sensitivity to anticancer drugs oxaliplatin and cisplatin but not mitomycin
PTM1/YKL039W	4	Protein of unknown function
RAS2/YNL098C	4	GTP-binding protein that regulates the nitrogen starvation response, sporulation, and filamentous growth
RPS4B/YHR203C	4	Protein component of the small (40S) ribosomal subunit
SAP30/YMR263W*	4	Subunit of a histone deacetylase complex, along with Rpd3p and Sin3p, that is involved in silencing at telomeres, rDNA, and silent mating-type loci; involved in telomere maintenance
SAP185/YJL098W	4	Protein that forms a complex with the Sit4p protein phosphatase
SIP5/YMR140W	4	Protein of unknown function; interacts with both the Reg1p/Glc7p phosphatase and the Snf1p kinase
SIZ1/YDR409W*	4	SUMO/Smt3 ligase that promotes the attachment of sumo (Smt3p; small ubiquitin-related modifier) to proteins
SNL1/YIL016W	4	Protein of unknown function
SPA2/YLL021W	4	Component of the polarisome, which functions in actin cytoskeletal organization during polarized growth; acts as a scaffold for Mkk1p and Mpk1p cell wall integrity signaling components; potential Cdc28p substrate
SPO22/YIL073C	4	Meiosis-specific protein essential for chromosome synapsis
SPS100/YHR139C	4	Protein required for spore wall maturation
SSA2/YLL024C	4	ATP binding protein involved in protein folding and vacuolar import of proteins
SSP1/YHR184W	4	Protein involved in the control of meiotic nuclear division and coordination of meiosis with spore formation
SWI4/YER111C	4	DNA binding component of the SBF complex (Swi4p-Swi6p), a transcriptional activator that in concert with MBF (Mbp1-Swi6p) regulates late G1-specific transcription of targets including cyclins and genes required for DNA synthesis and repair
TIR4/YOR009W	4	Cell wall mannoprotein
TOK1/YJL093C	4	Outward-rectifier potassium channel of the plasma membrane
TOM6/YOR045W	4	Component of the TOM (translocase of outer membrane) complex responsible for recognition and initial import steps for all mitochondrially directed proteins

TPO1/YLL028W	4	Polyamine transporter that recognizes spermine, putrescine, and spermidine
TPP1/YMR156C	4	DNA 3'-phosphatase that functions in repair of endogenous damage of double-stranded DNA, activity is specific for removal of 3' phosphates at strand breaks
TRM9/YML014W	4	tRNA methyltransferase
TRS33/YOR115C	4	One of 10 subunits of the transport protein particle (TRAPP) complex of the cis-Golgi
UBR2/YLR024C	4	Cytoplasmic ubiquitin-protein ligase (E3); required for ubiquitylation of Rpn4p; mediates formation of a Mub1p-Ubr2p-Rad6p complex
URA4/YLR420W	4	Dihydroorotase, catalyzes the third enzymatic step in the de novo biosynthesis of pyrimidines
VHS2/YIL135C	4	Cytoplasmic protein of unknown function
VPS8/YAL002W*	4	Membrane-associated protein that interacts with Vps21p to facilitate soluble vacuolar protein localization
VPS13/YLL040C	4	Protein of unknown function; involved in sporulation, vacuolar protein sorting and protein-Golgi retention
VPS35/YJL154C	4	Endosomal subunit of membrane-associated retromer complex required for retrograde transport
VPS51/YKR020W	4	Component of the GARP (Golgi-associated retrograde protein) complex
YDC1/YPL087W	4	Alkaline dihydroceramidase, involved in sphingolipid metabolism
YGK3/YOL128C	4	Protein kinase related to mammalian glycogen synthase kinases of the GSK-3 family
YHC3/YJL059W	4	Vacuolar membrane protein involved in the ATP-dependent transport of arginine into the vacuole
YMR1/YJR110W	4	Phosphatidylinositol 3-phosphate (PI3P) phosphatase; involved in various protein sorting pathways
YPF1/YKL100C	4	Putative protein of unknown function
YPS1/YLR120C	4	Aspartic protease
YGL081W	4	Putative protein of unknown function
YHR112C	4	Putative protein of unknown function
YHR113W	4	Cytoplasmic aspartyl aminopeptidase
YHR210C	4	Putative protein of unknown function

YIL014C-A	4	Putative protein of unknown function
YJR111C	4	Putative protein of unknown function
YKL044W	4	Dubious open reading frame unlikely to encode a functional protein
YKL061W	4	Putative protein of unknown function
YKL071W	4	Putative protein of unknown function
YKL075C	4	Putative protein of unknown function
YKL097C	4	Dubious open reading frame, unlikely to encode a protein
YKR015C	4	Putative protein of unknown function
YKR033C	4	Dubious open reading frame unlikely to encode a protein
YKR043C	4	Putative protein of unknown function
YKR045C	4	Putative protein of unknown function
YLR063W	4	Putative S-adenosylmethionine-dependent methyltransferase
YLR211C	4	Putative protein of unknown function
YLR278C	4	Zinc-cluster protein; GFP-fusion protein localizes to the nucleus
YLR290C	4	Putative protein of unknown function
YLR294C	4	Dubious open reading frame unlikely to encode a protein
YML057C-A	4	Dubious open reading frame unlikely to encode a protein
YMR052C-A	4	Dubious open reading frame unlikely to encode a functional protein
YMR144W	4	Putative protein of unknown function
YMR181C	4	Protein of unknown function
YOL046C	4	Dubious open reading frame unlikely to encode a protein
YOR082C	4	Dubious open reading frame unlikely to encode a protein
YOR228C	4	Protein of unknown function
APT1/YLR118C	5	Acyl-protein thioesterase responsible for depalmitoylation of Gpa1p; green fluorescent protein (GFP)-fusion protein localizes to both the cytoplasm and nucleus and is induced in response to the DNA-damaging agent MMS
ATG26/YLR189C	5	UDP-glucose:sterol glucosyltransferase

BUD27/YFL023W*	5	Protein involved in bud-site selection, nutrient signaling, and gene expression controlled by TOR kinase; diploid mutants show a random budding pattern rather than the wild-type bipolar pattern; plays a role in regulating Ty1 transposition
CAP1/YKL007W	5	Alpha subunit of the capping protein (CP) heterodimer (Cap1p and Cap2p) which binds to the barbed ends of actin filaments preventing further polymerization
CKB1/YGL019W*	5	Beta regulatory subunit of casein kinase 2, a Ser/Thr protein kinase with roles in cell growth and proliferation; the holoenzyme also contains CKA1, CKA2 and CKB2, the many substrates include transcription factors and all RNA polymerases
DIA1/YMR316W	5	Protein of unknown function
DYN3/YMR299C*	5	Dynein light intermediate chain
ELM1/YKL048C	5	Serine/threonine protein kinase that regulates cellular morphogenesis, septin behavior, and cytokinesis; required for the regulation of other kinases; forms part of the bud neck ring
ENT2/YLR206W	5	Epsin-like protein required for endocytosis and actin patch assembly
FRE1/YLR214W	5	Ferric reductase and cupric reductase
FRE8/YLR047C	5	Protein with sequence similarity to iron/copper reductases, involved in iron homeostasis
GIS3/YLR094C	5	Protein of unknown function
GYP1/YOR070C	5	Cis-golgi GTPase-activating protein (GAP)
HAT2/YEL056W*	5	Subunit of the Hat1p-Hat2p histone acetyltransferase complex; required for high affinity binding of the complex to free histone H4, thereby enhancing Hat1p activity; similar to human RbAp46 and 48; has a role in telomeric silencing
IRC11/YOR013W	5	Dubious opening reading frame unlikely to encode a protein; null mutant displays increased levels of spontaneous Rad52 foci
IRS4/YKR019C	5	EH domain-containing protein involved in regulating phosphatidylinositol 4,5-bisphosphate levels and autophagy
KKQ8/YKL168C	5	Putative serine/threonine protein kinase with unknown cellular role
LEO1/YOR123C*	5	Component of the Paf1 complex, which associates with RNA polymerase II and is involved in histone methylation; plays a role in regulating Ty1 transposition

LGE1/YPL055C*	5	Protein of unknown function; null mutant forms abnormally large cells, and homozygous diploid null mutant displays delayed premeiotic DNA synthesis and reduced efficiency of meiotic nuclear division
MEU1/YLR017W	5	Methylthioadenosine phosphorylase (MTAP), catalyzes the initial step in the methionine salvage pathway; affects polyamine biosynthesis through regulation of ornithine decarboxylase (Spe1p) activity; regulates ADH2 gene expression
MPC54/YOR177C	5	Component of the meiotic outer plaque; potential Cdc28p substrate
NDL1/YLR254C	5	Homolog of nuclear distribution factor NudE, NUDEL; interacts with Pac1p and regulates dynein targeting to microtubule plus ends
NFI1/YOR156C	5	SUMO ligase, catalyzes the covalent attachment of SUMO (Smt3p) to proteins; involved in maintenance of proper telomere length
NRG1/YDR043C*	5	Transcriptional repressor that recruits the Cyc8p-Tup1p complex to promoters; mediates glucose repression and negatively regulates a variety of processes including filamentous growth and alkaline pH response
NVJ1/YHR195W	5	Nuclear envelope protein
PBA1/YLR199C	5	Protein involved in 20S proteasome assembly
PDC5/YLR134W	5	Minor isoform of pyruvate decarboxylase
PET10/YKR046C	5	Protein of unknown function that co-purifies with lipid particles
PEX18/YHR160C	5	Peroxin required for targeting of peroxisomal matrix proteins containing PTS2
PMR1/YGL167C*	5	High affinity Ca2+/Mn2+ P-type ATPase required for Ca2+ and Mn2+ transport into Golgi
RPL38/YLR325C	5	Protein component of the large (60S) ribosomal subunit
RXT2/YBR095C*	5	Subunit of the histone deacetylase Rpd3L complex; possibly involved in cell fusion and invasive growth
SCS7/YMR272C****	5	Sphingolipid alpha-hydroxylase
SEC22/YLR268W*	5	R-SNARE protein
SLA1/YBL007C	5	Cytoskeletal protein binding protein required for assembly of the cortical actin cytoskeleton
SPO16/YHR153C	5	Meiosis-specific protein involved in synaptonemal complex assembly; implicated in regulation of crossover formation; required for sporulation

SPR1/YOR190W	5	Sporulation-specific exo-1,3-beta-glucanase; contributes to ascospore thermoresistance
SPT3/YDR392W	5	Subunit of the SAGA and SAGA-like transcriptional regulatory complexes, interacts with Spt15p to activate transcription of some RNA polymerase II-dependent genes, also functions to inhibit transcription at some promoters
STM1/YLR150W	5	Protein required for optimal translation under nutrient stress
SUB1/YMR039C*	5	Transcriptional coactivator, facilitates elongation through factors that modify RNAP II; role in peroxide resistance involving Rad2p; role in the hyperosmotic stress response through polymerase recruitment at RNAP II and RNAP III genes
TMA10/YLR327C	5	Protein of unknown function that associates with ribosomes
VHS3/YOR054C	5	Negative regulatory subunit of protein phosphatase 1 Ppz1p
YDR149C	5	Dubious open reading frame unlikely to encode a functional protein
YDR290W	5	Dubious open reading frame unlikely to encode a protein
YGR259C	5	Dubious open reading frame unlikely to encode a protein
YHR078W	5	High osmolarity-regulated gene of unknown function
YKL102C	5	Dubious open reading frame unlikely to encode a functional protein
YKL105C	5	Putative protein of unknown function
YLR225C	5	Putative protein of unknown function
YLR252W	5	Dubious open reading frame unlikely to encode a protein
YLR296W	5	Dubious open reading frame unlikely to encode a protein
YLR312C	5	Putative protein of unknown function
YLR407W	5	Putative protein of unknown function
YML033W	5	Merged open reading frame, does not encode a discrete protein
YML047W-A	5	Dubious open reading frame unlikely to encode a functional protein
YMR141C	5	Dubious open reading frame unlikely to encode a functional protein
YOR304C-A	5	Protein of unknown function
AAP1/YHR047C	6	Arginine/alanine aminopeptidase

ABP140/YOR239W	6	Nonessential protein that binds actin filaments
ABZ2/YMR289W	6	Aminodeoxychorismate lyase
ACE2/YLR131C	6	Transcription factor that activates expression of early G1-specific genes, localizes to daughter cell nuclei after cytokinesis and delays G1 progression in daughters, localization is regulated by phosphorylation; potential Cdc28p substrate
ADH4/YGL256W	6	Alcohol dehydrogenase isoenzyme type IV
ADY4/YLR227C	6	Structural component of the meiotic outer plaque
AIM36/YMR157C	6	Protein of unknown function; null mutant displays reduced respiratory growth and elevated frequency of mitochondrial genome loss
AIM41/YOR215C	6	Putative protein of unknown function
ARO1/YDR127W	6	Pentafunctional arom protein
ARR3/YPR201W	6	Arsenite transporter of the plasma membrane
AVL9/YLR114C	6	Conserved protein involved in exocytic transport from the Golgi
BCK1/YJL095W*	6	Mitogen-activated protein (MAP) kinase kinase kinase acting in the protein kinase C signaling pathway, which controls cell integrity
BUL1/YMR275C	6	Ubiquitin-binding component of the Rsp5p E3-ubiquitin ligase complex
CIN1/YOR349W	6	Tubulin folding factor D involved in beta-tubulin (Tub2p) folding; isolated as mutant with increased chromosome loss and sensitivity to benomyl
CTR1/YPR124W	6	High-affinity copper transporter of the plasma membrane
DSE2/YHR143W	6	Daughter cell-specific secreted protein with similarity to glucanases, degrades cell wall from the daughter side causing daughter to separate from mother; expression is repressed by Camp
FIG4/YNL325C*	6	Phosphatidylinositol 3,5-bisphosphate (PtdIns[3,5]P) phosphatase
GAC1/YOR178C	6	Regulatory subunit for Glc7p type-1 protein phosphatase (PP1)
GDB1/YPR184W	6	Glycogen debranching enzyme
GDE1/YPL110C	6	Glycerophosphocholine (GroPCho) phosphodiesterase
GLO3/YER122C	6	ADP-ribosylation factor GTPase activating protein (ARF GAP), involved in ER-Golgi transport
GPB1/YOR371C	6	Multistep regulator of cAMP-PKA signaling

GUF1/YLR289W	6	Mitochondrial matrix GTPase that associates with mitochondrial ribosomes
KAR3/YPR141C*	6	Minus-end-directed microtubule motor that functions in mitosis and meiosis, localizes to the spindle pole body and localization is dependent on functional Cik1p, required for nuclear fusion during mating; potential Cdc28p substrate
KTR7/YIL085C	6	Putative mannosyltransferase involved in protein glycosylation
HAH1/YGR021W	6	Putative protein of unknown function
HDA3/YPR179C	6	Subunit of a possibly tetrameric trichostatin A-sensitive class II histone deacetylase complex that contains an Hda1p homodimer and an Hda2p-Hda3p heterodimer; required for the activity of the complex; has similarity to Hda2p
HES1/YOR237W	6	Protein implicated in the regulation of ergosterol biosynthesis
HFA1/YMR207C	6	Mitochondrial acetyl-coenzyme A carboxylase
HHF2/YNL030W*	6	Histone H4, core histone protein required for chromatin assembly and chromosome function; one of two identical histone proteins; contributes to telomeric silencing; N-terminal domain involved in maintaining genomic integrity
HHY1/YEL059W	6	Dubious open reading frame unlikely to encode a functional protein
HRT1/YOL133W	6	RING finger containing subunit of Skp1-Cullin-F-box ubiquitin protein ligases (SCF)
HSC82/YMR186W	6	Cytoplasmic chaperone of the Hsp90 family
HXT2/YMR011W	6	High-affinity glucose transporter of the major facilitator superfamily, expression is induced by low levels of glucose and repressed by high levels of glucose
HXT5/YHR096C	6	Hexose transporter with moderate affinity for glucose, induced in the presence of non-fermentable carbon sources
INP1/YMR204C	6	Peripheral membrane protein of peroxisomes involved in peroxisomal inheritance
IOC4/YMR044W	6	Member of a complex (Isw1b) with Isw1p and Ioc2p that exhibits nucleosome-stimulated ATPase activity and acts within coding regions to coordinate transcription elongation with termination and processing
IRC10/YOL015W	6	Putative protein of unknown function; null mutant displays increased levels of spontaneous Rad52p foci
JEN1/YKL217W	6	Lactate transporter, required for uptake of lactate and pyruvate

KSP1/YHR082C	6	Ser/thr protein kinase; nuclear translocation required for haploid filamentous growth
LCL2/YLR104W	6	Putative protein of unknown function
LIN1/YHR156C	6	Non-essential component of U5 snRNP; nuclear protein; physically interacts with Irr1p of cohesin complex; may link together proteins involved in chromosome segregation, mRNA splicing and DNA replication
MAE1/YKL029C	6	Mitochondrial malic enzyme, catalyzes the oxidative decarboxylation of malate to pyruvate
MAM33/YIL070C***	6	Acidic protein of the mitochondrial matrix involved in oxidative phosphorylation
MDM32/YOR147W	6	Mitochondrial inner membrane protein
MFT1/YML062C	6	Subunit of the THO complex that is involved in transcription elongation and mitotic recombination; involved in telomere maintenance
MIC17/YMR002W	6	Mitochondrial intermembrane space protein
MLH1/YMR167W	6	Protein required for mismatch repair in mitosis and meiosis as well as crossing over during meiosis
MNT3/YIL014W	6	Alpha-1,3-mannosyltransferase
MRPL36/YBR122C	6	Mitochondrial ribosomal protein of the large subunit
MRPS17/YMR188C	6	Mitochondrial ribosomal protein of the small subunit
MSS1/YMR023C	6	Mitochondrial protein
MTC6/YHR151C	6	Protein of unknown function
NGG1/YDR176W	6	Transcriptional regulator involved in glucose repression of Gal4p-regulated genes; component of transcriptional adaptor and histone acetyltransferase complexes, the ADA complex, the SAGA complex, and the SLIK complex
NIT3/YLR351C	6	Nit protein, one of two proteins in S. cerevisiae with similarity to the Nit domain of NitFhit from fly and worm and to the mouse and human Nit protein which interacts with the Fhit tumor suppressor; nitrilase superfamily member
NSG2/YNL156C	6	Protein involved in regulation of sterol biosynthesis
NUP2/YLR335W	6	Nucleoporin involved in nucleocytoplasmic transport, binds to either the nucleoplasmic or cytoplasmic faces of the nuclear pore complex depending on Ran-GTP levels; also has a role in chromatin organization

PAC1/YOR269W*	6	Protein involved in nuclear migration, part of the dynein/dynactin pathway
PAC11/YDR488C	6	Dynein intermediate chain, acts in the cytoplasmic dynein pathway
PBY1/YBR094W*	6	Putative tubulin tyrosine ligase associated with P-bodies
PCL7/YIL050W	6	Pho85p cyclin of the Pho80p subfamily, forms a functional kinase complex with Pho85p which phosphorylates Mmr1p and is regulated by Pho81p; involved in glycogen metabolism, expression is cell-cycle regulated
PIN2/YOR104W	6	Protein that induces appearance of [PIN+] prion when overproduced
PLB1/YMR008C	6	Phospholipase B (lysophospholipase) involved in lipid metabolism
PMS1/YNL082W	6	ATP-binding protein required for mismatch repair in mitosis and meiosis; functions as a heterodimer with Mlh1p, binds double- and single-stranded DNA via its N-terminal domain
PPQ1/YPL179W	6	Putative protein serine/threonine phosphatase
PPZ1/YML016C	6	Serine/threonine protein phosphatase Z, isoform of Ppz2p
PRE9/YGR135W	6	Alpha 3 subunit of the 20S proteasome
PSP2/YML017W	6	Asn rich cytoplasmic protein
PTC5/YOR090C	6	Mitochondrial type 2C protein phosphatase
PUS5/YLR165C	6	Pseudouridine synthase
PUT4/YOR348C	6	Proline permease
QCR8/YJL166W	6	Subunit 8 of ubiquinol cytochrome-c reductase complex
RFM1/YOR279C	6	DNA-binding protein required for vegetative repression of middle sporulation genes; specificity factor that directs the Hst1p histone deacetylase to some of the promoters regulated by Sum1p; involved in telomere maintenance
RGM1/YMR182C	6	Putative transcriptional repressor with proline-rich zinc fingers
RNH1/YMR234W	6	Ribonuclease H1; able to bind double-stranded RNAs and RNA-DNA hybrids; associates with RNAse polymerase I
ROM2/YLR371W	6	GDP/GTP exchange protein (GEP) for Rho1p and Rho2p
RPL22A/YLR061W	6	Protein component of the large (60S) ribosomal subunit
RPS25B/YLR333C	6	Protein component of the small (40S) ribosomal subunit
RSN1/YMR266W	6	Membrane protein of unknown function

RVS167/YDR388W*	6	Actin-associated protein
SBE22/YHR103W	6	Protein involved in the transport of cell wall components from the Golgi to the cell surface
SER1/YOR184W	6	3-phosphoserine aminotransferase
SEY1/YOR165W	6	GTPase with a role in ER morphology
SFK1/YKL051W	6	Plasma membrane protein
SLP1/YOR154W	6	Integral membrane protein of unknown function
SNC2/YOR327C	6	Vesicle membrane receptor protein (v-SNARE)
SNO2/YNL334C	6	Protein of unknown function
SNX41/YDR425W	6	Sorting nexin
SPE2/YOL052C	6	S-adenosylmethionine decarboxylase
SRL2/YLR082C	6	Protein of unknown function; overexpression suppresses the lethality caused by a rad53 null mutation
STB2/YMR053C	6	Protein that interacts with Sin3p in a two-hybrid assay and is part of a large protein complex with Sin3p and Stb1p
SWD3/YBR175W*	6	Essential subunit of the COMPASS (Set1C) complex, which methylates histone H3 on lysine 4 and is required in transcriptional silencing near telomeres; WD40 beta propeller superfamily member and ortholog of mammalian WDR5
SYG1/YIL047C	6	Plasma membrane protein of unknown function
TGL2/YDR058C	6	Triacylglycerol lipase that is localized to the mitochondria
TGL4/YKR089C	6	Multifunctional triacylglycerol lipase, steryl ester hydrolase, and Ca2+-independent phospholipase A2; catalyzes acyl-CoA dependent acylation of LPA to PA; required with Tgl3p for timely bud formation; phosphorylated and activated by Cdc28p
TMA46/YOR091W	6	Protein of unknown function that associates with ribosomes
TPK3/YKL166C	6	cAMP-dependent protein kinase catalytic subunit
TUM1/YOR251C	6	Mitochondrial protein
URA1/YKL216W	6	Dihydroorotate dehydrogenase
UTR4/YEL038W*	6	Protein involved in methionine salvage
XYL2/YLR070C	6	Xylitol dehydrogenase

YHK8/YHR048W	6	Presumed antiporter of the DHA1 family of multidrug resistance transporters
YHM2/YMR241W	6	Carrier protein that exports citrate from and imports oxoglutarate into the mitochondrion, causing net export of NADPH reducing equivalents; also associates with mt nucleoids and has a role in replication and segregation of the mt genome
YSP2/YDR326C	6	Protein involved in programmed cell death
YAL056C-A	6	Dubious open reading frame unlikely to encode a protein
YDR282C	6	Putative protein of unknown function
YDR333C	6	Putative protein of unknown function
YDR401W	6	Dubious open reading frame unlikely to encode a functional protein
YER010C	6	Protein of unknown function
YGL214W	6	Dubious open reading frame unlikely to encode a protein
YIL012W	6	Dubious open reading frame unlikely to encode a protein
YIL055C	6	Putative protein of unknown function
YKL070W	6	Putative protein of unknown function
YLR012C	6	Putative protein of unknown function
YLR072W	6	Protein of unknown function
YLR169W	6	Dubious open reading frame unlikely to encode a functional protein
YLR253W	6	Putative protein of unknown function
YLR345W	6	Similar to 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase enzymes responsible for the metabolism of fructoso-2,6-bisphosphate; mRNA expression is repressed by the Rfx1p-Tup1p-Ssn6p repressor complex
YML037C	6	Putative protein of unknown function with some characteristics of a transcriptional activator
YMR010W	6	Putative protein of unknown function
YMR034C	6	Putative transporter
YMR075C-A	6	Dubious open reading frame unlikely to encode a protein
YMR252C	6	Putative protein of unknown function
YMR254C	6	Dubious open reading frame unlikely to encode a protein

YMR304C-A	6	Dubious open reading frame unlikely to encode a protein
YNL058C	6	Putative protein of unknown function
YNL155W	6	Putative protein of unknown function
YNL266W	6	Dubious open reading frame unlikely to encode a protein
YOR015W	6	Dubious open reading frame unlikely to encode a functional protein
YPL025C	6	Dubious open reading frame unlikely to encode a protein
YPL185W	6	Dubious open reading frame unlikely to encode a protein
YPR196W	6	Putative maltose activator
YPR197C	6	Dubious open reading frame unlikely to encode a functional protein
CNM67/YNL225C	1A	Component of the spindle pole body outer plaque; required for spindle orientation and mitotic nuclear migration
RRN10/YBL025W	2A	Protein involved in promoting high level transcription of rDNA, subunit of UAF (upstream activation factor) for RNA polymerase I
SPT21/YMR179W	2A	Protein required for normal transcription at several loci including HTA2-HTB2 and HHF2-HHT2, but not required at the other histone loci; functionally related to Spt10p; involved in telomere maintenance
YVH1/YIR026C	2A	Protein phosphatase involved in vegetative growth at low temperatures, sporulation, and glycogen accumulation
ARO2/YGL148W	3A	Bifunctional chorismate synthase and flavin reductase
AVT6/YER119C	3A	Vacuolar aspartate and glutamate exporter
AMD1/YML035C	4A	AMP deaminase, tetrameric enzyme that catalyzes the deamination of AMP to form IMP and ammonia; may be involved in regulation of intracellular adenine nucleotide pools
DBP7/YKR024C	4A	Putative ATP-dependent RNA helicase of the DEAD-box family involved in ribosomal biogenesis; essential for growth under anaerobic conditions
MEF2/YJL102W	4A	Mitochondrial elongation factor involved in translational elongation
MET18/YIL128W****	4A	DNA repair and TFIIH regulator, required for both nucleotide excision repair (NER) and RNA polymerase II (RNAP II) transcription; involved in telomere maintenance
RPS9B/YBR189W	4A	Protein component of the small (40S) ribosomal subunit

SIN3/YOL004W*	4A	Component of the Sin3p-Rpd3p histone deacetylase complex, involved in transcriptional repression and activation of diverse processes, including mating-type switching and meiosis; involved in the maintenance of chromosomal integrity
SLM6/YBR266C	4A	Protein with a potential role in actin cytoskeleton organization
YDR042C	4A	Putative protein of unknown function
BUD25/YER014C-A	5A	Protein involved in bud-site selection
MGM1/YOR211C*	5A	Mitochondrial GTPase related to dynamin
YOR199W	5A	Dubious open reading frame unlikely to encode a protein
BUD30/YDL151C	6A	Dubious open reading frame, unlikely to encode a protein
CPS1/YJL172W	6A	Vacuolar carboxypeptidase yscS
DSS1/YMR287C	6A	3'-5' exoribonuclease, component of the mitochondrial degradosome
FMP33/YJL161W	6A	Putative protein of unknown function
HAP4/YKL109W	6A	Subunit of the heme-activated, glucose-repressed Hap2p/3p/4p/5p CCAAT-binding complex, a transcriptional activator and global regulator of respiratory gene expression
HDA2/YDR295C	6A	Subunit of a possibly tetrameric trichostatin A-sensitive class II histone deacetylase complex containing an Hda1p homodimer and an Hda2p-Hda3p heterodimer; involved in telomere maintenance
LPD1/YFL018C	6A	Dihydrolipoamide dehydrogenase
MNN9/YPL050C	6A	Subunit of Golgi mannosyltransferase complex
MRPL7/YDR237W	6A	Mitochondrial ribosomal protein of the large subunit
MRPL49/YJL096W	6A	Mitochondrial ribosomal protein of the large subunit
NAT3/YPR131C	6A	Catalytic subunit of the NatB N-terminal acetyltransferase
NSR1/YGR159C	6A	Nucleolar protein that binds nuclear localization sequences, required for pre-rRNA processing and ribosome biogenesis
PET494/YNR045W	6A	Mitochondrial translational activator specific for the COX3 mRNA
YIM2/YMR151W	6A	Dubious open reading frame, unlikely to encode a protein
YJL163C	6A	Putative protein of unknown function
YLL020C	6A	Dubious open reading frame unlikely to encode a protein

YOR364W	6A	Dubious open reading frame unlikely to encode a protein

*Interaction has also been identified by others (see text).
**For details on scoring, refer to Materials and Methods (Chapter 2).
***Physical interaction has been identified between the indicated strain and *ASF1*.
****Phenotypic suppression/enhancement between the indicated strain and *ASF1* has been reported.

Table 3-4. Verification of synthetic genetic interactions using tetrad analysis and random spore analysis.

GENE/ORF	SGA	TETRAD ANALYSIS	RANDOM SPORE	INTERACTION[1]
ADA2/YDR448W	SL	SS	INC	SL/SS
ARG82/YDR173C[2]	SL/SS		SS	SS
ARP6/YLR085C	SS	SS	NO	SS
BEM2/YER155C	SL		INC	INC
BRE2/YLR015W	SS		NO	INC
CIK1/YMR198W	SL		SS	SL/SS
CTK3/YML112W	SL/SS		NO	INC
DDC1/YPL194W	SS[3]	SS	SS/NO	SS
ECM21/YBL101C	SL/SS		NO	INC
HHT2/YNL031C	SS		NO	INC
HPF1/YOL155C	SL/SS		NO	INC
HUR1/YGL168W	SS	SS	SS	SS
ISA2/YPR067W	SL/SS		SS	SS
KCS1/YDR017C[4]	SL/SS		SS	SS
MRE11/YMR224C	SL/SS		SL	SL/SS
PHO5/YBR093C	SL/SS		SS/NO	SS
PLC1/YPL268W	SL		SS	SL/SS
PSH1/YOL054W	SS[3]	INC	SS	SS
PUS1/YPL212C[5]	NO	NO	NO	NO
RAD23/YEL037C	SS		SS	SS
RPP1B/YDL130W	SL/SS		NO	INC
RRG1/YDR065W	SL/SS		SS/NO	SS
SAP30/YMR263W	SS		SS	SS
SAS5/ YOR213C	SL/SS	SS	NO	SS
SDS3/YIL084C	SS		SS	SS
SET2/YJL168C	SL/SS	SS	SS	SS
SET4/YJL105W	SS[3]	INC	SS	SS
SIN3/ YOL004W	SS	SS	INC	SS
SIR1/ YKR101W	SS	NO	NO	NO
SLX5/YDL013W	SL/SS		SS	SS
SNF2/YOR290C	SL/SS[3]	SL/SS	SL/SS	SL/SS
SPT2/ YER161C	SL/SS	SS	SS	SS
SPT21/YMR179W	SS		SS	SS
SRS2/YJL092W	SS		SS	SS
STP22/YCL008C	SL		SS	SL/SS
SWI4/ YER111C	SS	INC	INC	SS
SWR1/YDR334W	SL/SS		NO	INC
TUP1/YCR084C	SL/SS	INC	SL/SS	SL/SS
UAF30/YOR295W	SL	SS	INC	SL/SS
YDR417C	SL/SS		SS	SS

SL, synthetic lethal; SS, synthetic sick; NO, no interaction; INC, inconclusive

[1]Most information is in favour of this outcome

[2]Indicated gene not deleted in the deletion mutant array, as determined by PCR

[3]Interaction did not pass initial tests to be considered a putative synthetic interaction. See text.

[4]Strain is diploid, as analyzed by flow cytometry

[5]Positive control

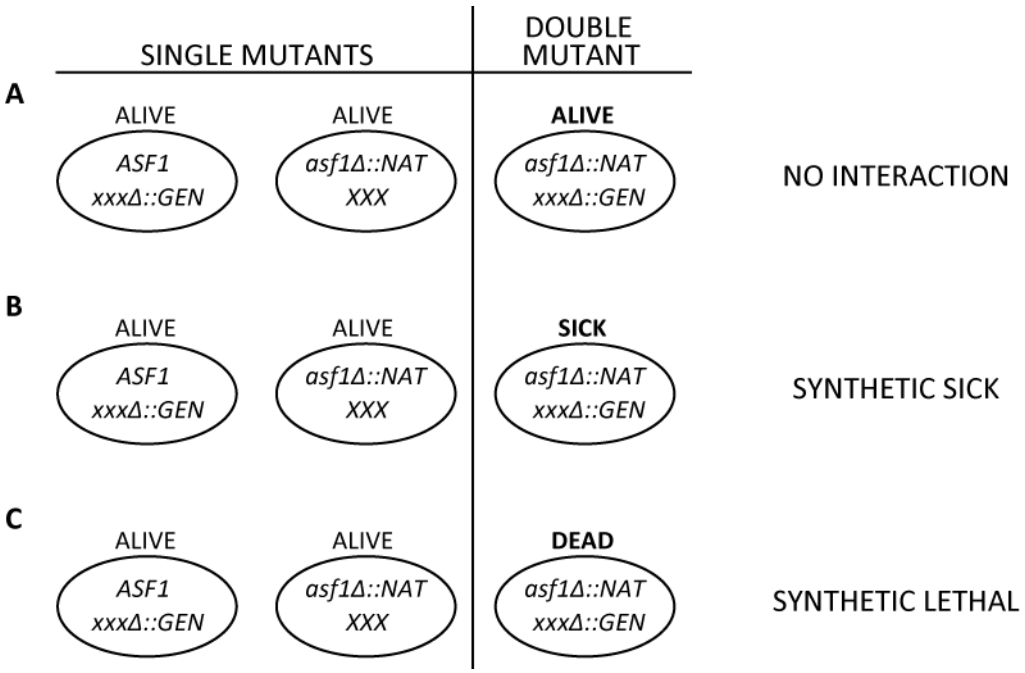

Figure 3-1. Possible outcomes of double mutant growth phenotypes. A. No interaction; double mutant is not impaired for growth compared to single mutants. **B.** Synthetic sick interaction; double mutant is compromised for growth compared to the single mutants. **C.** Synthetic lethal interaction; double mutant is inviable, but each single mutant is viable. *XXX*, gene of interest; *xxx⊿::GEN*, geneticin-marked strain from the deletion collection; *asf1⊿::NAT*, nourseothricin-marked deletion of *ASF1*. Each ellipse represents one haploid yeast cell.

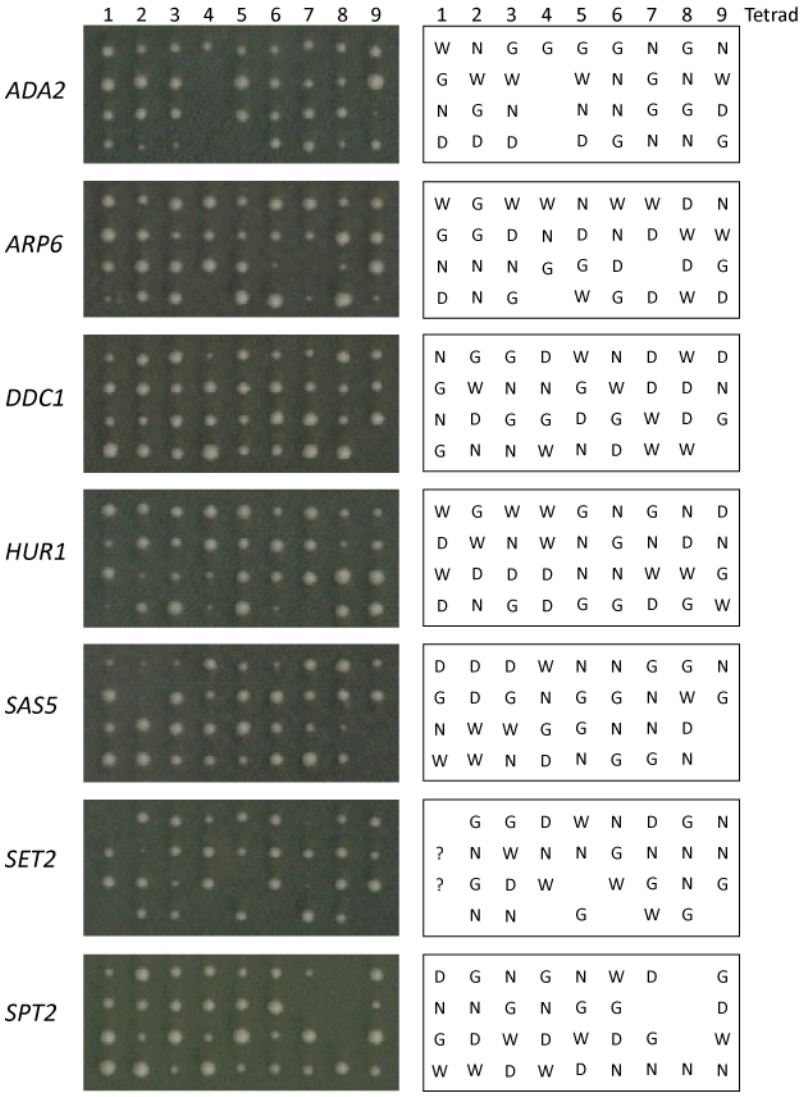

Figure 3-2. Tetrad analysis confirms synthetic genetic interactions. Diploid yeast strains (*ASF1/asf1⊿ XXX/xxx⊿*; *XXX=ADA2/ada2⊿*, *ARP6/arp6⊿*, *DDC1/ddc1⊿*, *HUR1/hur1⊿*, *SAS5/sas5⊿*, *SET2/set2⊿*, or *SPT2/spt2⊿*) were grown in pre-sporulation medium overnight, transferred to sporulation medium for three or more days, and digested using beta-endoglucanase. After digestion, cells were plated onto YPD solid medium and tetrads were dissected using a micromanipulator. Nine independent dissected tetrads are shown for each strain. Genotypes of individual spores were determined and are as indicated: W, wild type; G, geneticin-resistant (*xxx⊿* single mutant from deletion collection); N, nourseothricin-resistant (*asf1⊿* query strain); D, geneticin- and nourseothricin-resistant (*asf1⊿ xxx⊿* double mutant); '?', unidentified genotype; blank, no viable spore.

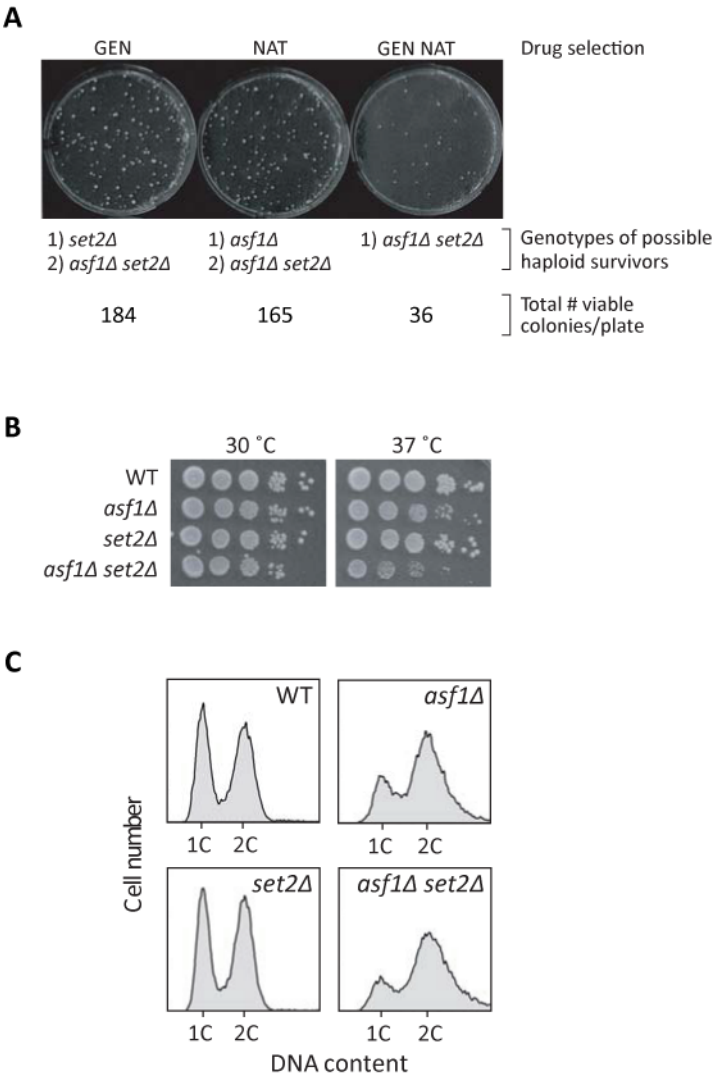

Figure 3-3. *ASF1* and *SET2* interact genetically. A. Random spore analysis was performed on *ASF1/asf1*Δ*::NAT SET2/set2*Δ*::GEN* diploids generated by synthetic genetic array methodology. Diploids were grown in liquid presporulation medium and then transferred to sporulation medium. Sporulated cells were inoculated into his-arg- broth, grown to saturation, and plated onto haploid-specific solid medium containing geneticin (GEN), nourseothricin (NAT) or both GEN and NAT. Plates were photographed after four days growth at 30°C. **B.** Simultaneous deletion of *ASF1* and *SET2* confers a synthetic sick phenotype. Five-fold serial dilutions of cells were spotted onto YPD plates and photographed after three days of growth at 30 and 37 °C. **C.** The DNA content of asynchronous cultures of wild type, *asf1*Δ, *set2*Δ and *asf1*Δ *set2*Δ strains was determined by flow cytometry analysis. (B) and (C) were performed by K. Lin.

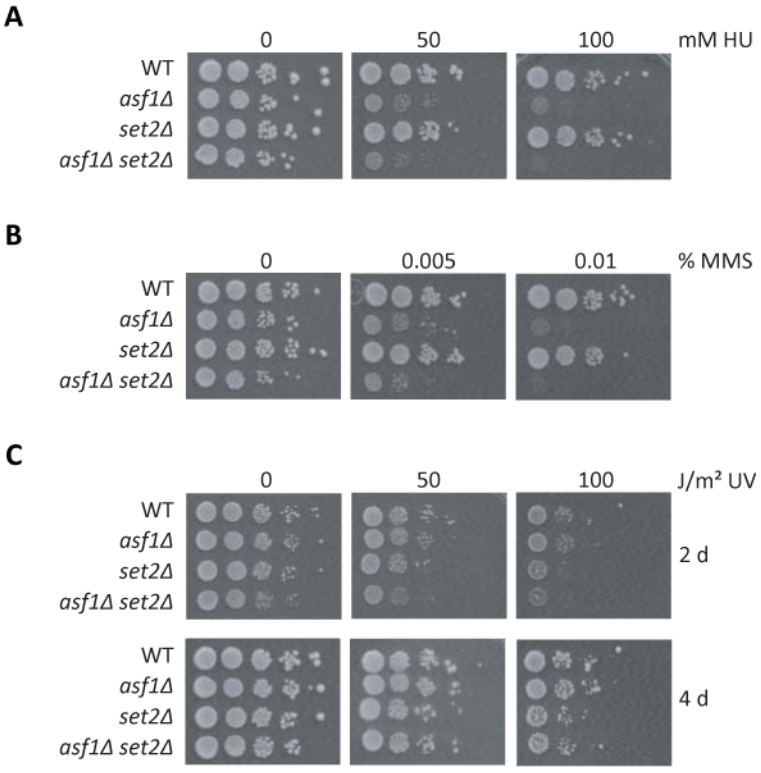

Figure 3-4. *ASF1* and *SET2* do not exist in a shared pathway for responding to replication stress or DNA damage. A. *asf1⊿* mutants are sensitive to HU. Ten-fold serial dilutions of cells were spotted onto rich medium (0 mM HU), and medium containing 50 or 100 mM HU and grown at 30 °C. Photographs were taken after three days. **B.** *asf1⊿* mutants are sensitive to MMS. Ten-fold serial dilutions of cells were spotted onto rich medium (0 % MMS), and medium containing 0.005 or 0.01 % MMS and grown at 30 °C. Photographs were taken after three days. **C.** *set2⊿* mutants are sensitive to UV irradiation. Ten-fold serial dilutions of early log phase cells grown overnight in YPD were diluted to 1 x 10^7 cells/mL, spotted onto rich medium and given 0, 50 or 100 J/m2 of UV irradiation. Plates were incubated in the dark at 30 °C and photographed after two (top panel) or four (bottom panel) days. Experiments in (A) and (B) were done by K. Lin.

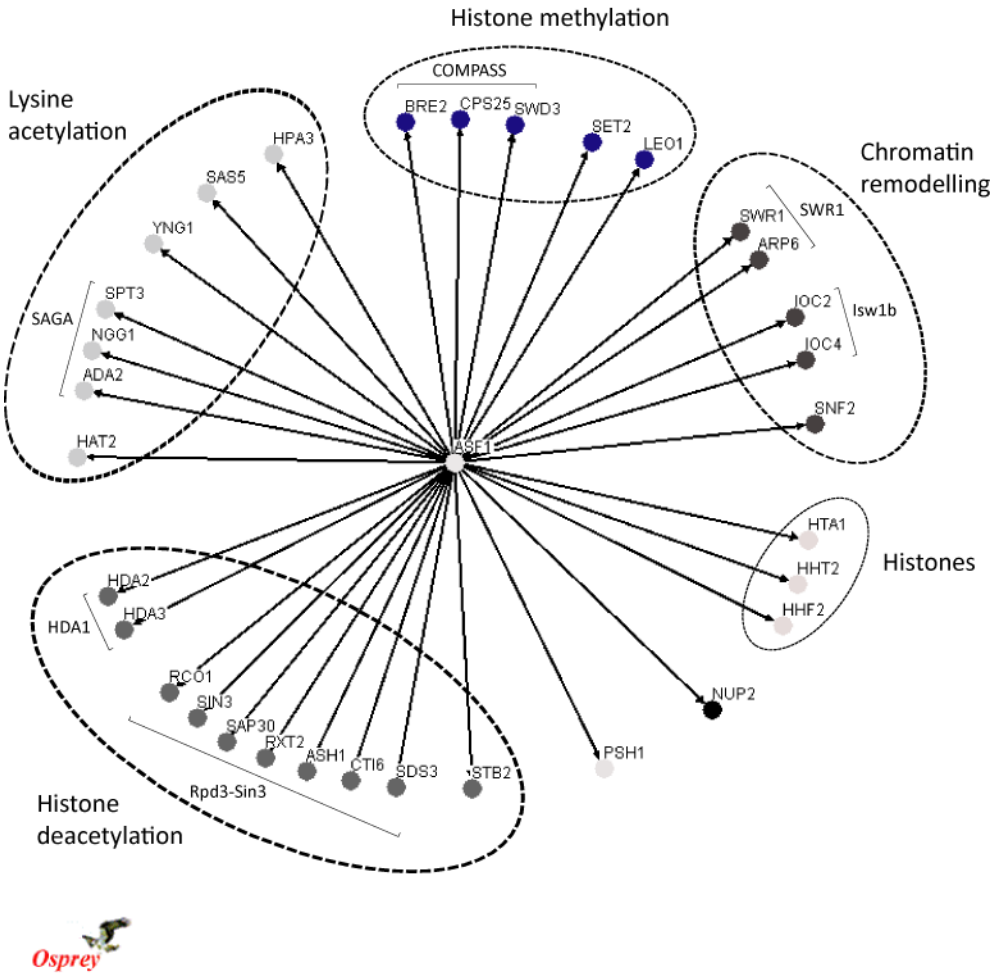

Figure 3-5. Summary of genetic interactions between *ASF1* and genes encoding other chromatin factors. Osprey map[10] showing synthetic genetic interactions identified in this study. *ASF1* interacts with genes encoding subunits of chromatin remodellers (SWI/SNF, SWR1, Isw1b), lysine acetylases (SAGA, NuA3, SAS, Hat1-Hat2), histone deacetylases (HDA1, Rpd3-Sin3), histone methyltransferases (COMPASS, Set2, PAF) as well as histones.

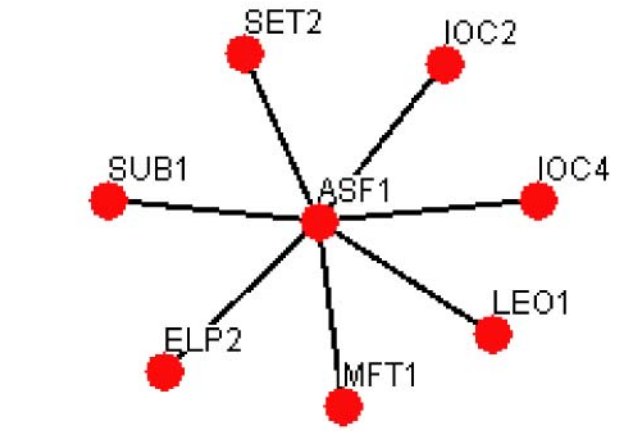

Figure 3-6. Summary of genetic interactions between *ASF1* and genes encoding proteins involved in transcriptional elongation. Osprey map[10] showing synthetic genetic interactions identified in our SGA analysis. Set2 methylates histone H3 at lysine 36. The protein products of *IOC2* and *IOC4* associate with Isw1 to form ISW1b. Elp2 is a core subunit of the Elongator complex. *MFT1* encodes a subunit of THO, Sub1 facilitates transcriptional elongation by RNAPII and RNAPIII and Leo1 is a subunit of PAF.

References

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Chapter 4

Histone chaperone Asf1 stimulates the derepression of DNA damage response genes

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Introduction

The conserved histone H3/H4 chaperone Asf1 has multiple functions in chromatin metabolism. It directly contributes to replication-independent incorporation of H3 and H4 into nucleosome core particles in a pathway involving the HIR proteins[1]. During DNA replication, Asf1 delivers H3/H4 to other chaperones (minimally CAF-I and Rtt106) for incorporation into nucleosomes[2]. In addition to promoting nucleosome assembly, Asf1 can affect the post-translational modification state of histones. It stimulates the activity of KAT's that predominantly modify K9 and K56 of newly synthesized H3, and promotes Set2-dependent trimethylation of H3K36 in chromatin[3-7].

Asf1 contributes to the regulation of transcription by virtue of its effects on chromatin metabolism. Studies in budding yeast suggest that Asf1 destabilizes promoter nucleosomes during periods of high transcription in a way that accommodates initiation and elongation[8-10]. Stimulation of promoter activity by Asf1-dependent chromatin destabilization has been particularly well characterized at *PHO5*, which is induced when cells are starved for phosphate[9,11]. In current models, *PHO5* chromatin is destabilized by Asf1 mainly because: 1) Asf1 is in the supply line which provides K56-acetylated H3 for incorporation into promoter nucleosomes, and 2) H3K56 acetylation may facilitate nucleosome eviction[10,12]. Since phosphate withdrawal does not modulate the capacity of Asf1 to stimulate H3K56 acetylation,

or the availability of newly synthesized H3 for K56 acetylation, Asf1 is seen as having a housekeeping role in *PHO5* induction. That is, Asf1 functions to ensure a constant supply of K56-acetylated H3 under inducing conditions. Incorporation of this H3 into the active *PHO5* promoter by histone turnover drives H3K56 acetylation higher at this location, which favours transcription[10]. In addition to potentiating H3K56 acetylation of chromatin, Asf1 may also partly contribute to *PHO5* induction by directly removing histones from promoter chromatin[10,13,14]. This hypothesis is supported by the observation that Asf1 can dissociate histone H3/H4 dimers from H3/H4 tetramers *in vitro* [10,15]. Furthermore, although not formally shown, the Tyler lab has reported that Asf1 occupies *PHO5* under inducing conditions[16].

It is currently unclear if the mechanisms by which Asf1 stimulates transcription of *PHO5* are important for high transcription of other Asf1-dependent genes. Here we address this issue in work focused on the regulation of two DDR genes in budding yeast: *RNR3* and *HUG1*. Under normal conditions, these genes are kept repressed by a *trans*-acting factor, Crt1[17], which recruits the Ssn6/Tup1 corepressor. By using multiple, redundant mechanism to repress transcription, Ssn6/Tup1 shifts the chromatin in DDR gene promoters towards an inactive state[18,19]. However, in response to DNA damage or replication stress, the DDR genes are derepressed, a step critical in the physiological response of cells to the appearance of abnormal DNA structures in the nucleus. Derepression of the DDR genes involves loss of Crt1 and Ssn6/Tup1 from the promoter, eviction of promoter nucleosomes, and induction of acetylation in the tails of H3 and H4[17,19,20].

While regulation of the DDR genes shares some features in common with the regulation of *PHO5* (for example, involvement of some of the same KAT/HDAC complexes), there are important differences. First, unlike *PHO5*, the DDR genes are kept in the off state by a repressor bound to their promoters[17]. Second, whereas hyperacetylation of the amino-terminal tail of H4 poises *PHO5* promoter chromatin for activation[21], there is no evidence that DDR genes are poised for derepression by enrichment of a histone mark normally linked to high transcription. Finally, in *asf1⊿* cells we observe modest derepression of the DDR genes during normal growth, but no change in *PHO5* expression (see below). Collectively, these results suggest that Asf1 may regulate DDR genes using a novel mechanism. The findings reported in this study support this contention and shed new light on the structure-function relationships of yeast Asf1.

Results

Full derepression of a DNA damage response gene by replication stress signals requires Asf1

Microarrray and Northern blotting experiments revealed abnormally high expression of a subset of DDR genes in *asf1⊿* cells grown under normal conditions (Fig. 4-1A and B). In order to decipher the implications of this finding for possible regulation of DDR genes by Asf1, we studied *RNR3* and *HUG1* in more detail. *RNR3* and *HUG1* encode unrelated proteins (Rnr3 is a subunit of ribonucleotide reductase; the function of Hug1 is unknown[22]). These well-characterized genes[18-20,22-25] have similar promoter structures[20] (Fig. 4-1C). While they differ slightly in some steps of regulation[20,22], *RNR3* and *HUG1* are both controlled by mechanisms which

involve the *trans*-acting factor Crt1 and the co-repressor Ssn6/Tup1[17,22]. Their derepression also involves similar steps of promoter chromatin reconfiguration. Specifically, checkpoint kinase Rad53, which is activated as part of the DNA structure checkpoint signalling pathway, is needed for derepression of both genes[22,26].

Our observation that a subset of DDR genes is derepressed in cells lacking *ASF1* is consistent with the fact that Rad53 is partially activated in unperturbed *asf1⊿* cells[27]. This effect has been attributed to a higher level of spontaneous DNA damage, such as gross chromosomal rearrangements (mainly telomere additions and translocations)[28], in cells lacking *ASF1*. Therefore, we hypothesized that DDR genes are induced in *ASF1* null mutants by a Rad53-dependent pathway. To address this, we measured *RNR3* and *HUG1* mRNA levels in cells lacking both *ASF1* and *RAD53*. Indeed, deletion of *RAD53* suppressed the modest derepression of *RNR3* and *HUG1* in an *asf1⊿* strain (Fig. 4-1D).

Given that Asf1 is involved in facilitating transcription from certain promoters[8,14], we decided to test whether Asf1 is important for the full derepression of *RNR3* and *HUG1*. Hence, we measured the expression of *RNR3* and *HUG1* mRNAs in asynchronous cultures treated with 0.2 M HU to induce replication stress. Despite the fact that both genes were partly derepressed to start with, further *RNR3* induction by HU was delayed in *asf1⊿* cells, and *HUG1* induction was severely dampened (Fig. 4-2A and B). Since Rad53 was activated similarly in wild type and *asf1⊿* cells treated with HU (Fig. 4-2C; compare lanes 2 and 4), the latter dampening is not due to inefficient checkpoint signalling[29]. As mentioned above, Rad53 is partially activated in *asf1⊿* cells grown under normal conditions, consistent with the

fact that the DDR pathway is partly activated in these cells[27] (Fig. 4-2C; compare lanes 1 and 3). Importantly, Rad53 was fully activated in *asf1*Δ cells exposed to prolonged treatment with HU, but *HUG1* transcription was still blocked (Fig. 4-2D and E). Therefore, cells lacking *ASF1* are unable to fully derepress *HUG1* in response to replication stress even though the checkpoint can be fully engaged.

Asf1 association with chromatin is globally induced under conditions of replication stress

It has been proposed that Asf1 can stimulate transcription by disassembling nucleosomes in the course of its untargeted association with chromatin (see Introduction)[10,16]. In addition, Asf1 associates with several loci in normally cycling cells[8,30,31], as well as with, and downstream of, an origin of replication in HU-treated cells[30]. These observations led us to explore the possibility that Asf1 might be present at the promoters of *RNR3* and *HUG1* to directly facilitate transcriptional derepression. To address this, we performed ChIP in cells containing a Myc epitope-tagged version of Asf1. We first optimized a ChIP protocol initially developed in the Struhl lab[32] to ensure that ChIP data obtained using our conditions would be of the highest possible quality. Specifically, sonication conditions were determined to achieve an average DNA fragment size of approximately 500 base pairs (Fig. 4-3A). Next, a variety of immunoprecipitation (IP) conditions were tested to find the optimal IP efficiency of the Asf1-Myc protein. We concluded that a combination of 10 μl each of anti-myc antibody and protein A sepharose beads resulted in the greatest IP efficiency of Asf1-Myc (Fig. 4-3B). No band migrating at the position of Asf1-Myc was detected in protein extracts prepared from wild type cells (Fig. 4-3C).

Consistent with the idea that Asf1 is present at the promoters of DDR genes to directly reconfigure nucleosomes, crosslinking of Asf1 to the promoters of *RNR3* and *HUG1* was readily detected by ChIP in wild type cells grown under normal conditions (Fig. 4-3D shows the raw data from a representative experiment). However, Asf1 also crosslinked to all other tested loci, namely a gene-free region (TELV amplicon), and the promoters of *PHO5*, *DSE1* and *SCR1*. The ChIP results for *RNR3* and *HUG1* are quantitated in Fig. 4-3E: Asf1 was enriched at each promoter by approximately 20-fold over a 'no antibody' control.

Asf1 was also detected in the chromatin fraction obtained from unfixed cells by a conventional biochemical approach (Fig. 4-4A, lane 5; Fig. 4-4B, lane 3). These data support the idea that random association of Asf1 with chromatin in normally cycling cells makes it available for direct reconfiguration of nucleosomes[16] in the course of checkpoint induction of DDR genes.

The fact that Asf1 randomly associates with chromatin under non-inducing conditions does not preclude the possibility that its association with target promoters is responsive to signals which affect transcription. Indeed, there is some evidence that association of Asf1 with the promoters of target genes is regulated by signals that affect transcription. For example, glucose availability more strongly affects Asf1 occupancy of glucose-responsive promoters than a gene-free region (Fig. S2A in[8]).

Therefore, we used ChIP to test whether HU induces Asf1 crosslinking to the promoters of DDR genes. As anticipated[30], Asf1 could be crosslinked to chromatin in HU-treated cells. However, initial experiments suggested that Asf1 crosslinking to *RNR3* and *HUG1* (and indeed all loci tested) was lower in HU-treated cells than in

untreated cells (Fig. 4-5A). We were concerned with this finding for two reasons: 1) we expected Asf1 to have a positive role in transcription of DDR genes and, 2) like lysine in proteins and cytosine in DNA, HU contains a free amine, making it a perfect candidate to react with formaldehyde. Given that a chemical reaction between HU and formaldehyde could potentially outcompete the reaction between formaldehyde and protein/DNA, we suspected that Asf1 crosslinking decreased in HU-treated cells because HU quenched the crosslinker. This possibility of formaldehyde quenching by HU has also been raised by Steve Bell and colleagues, based on the chemistry of formaldehyde crosslinking, the chemical structure of HU, and their own ChIP data[33].

To directly explore this possibility, we tested whether the presence of HU in the medium affects the pattern of Asf1 crosslinking to chromatin. To achieve fixation in the absence of HU, cells were collected by centrifugation after treatment with HU, resuspended in conditioned medium containing formaldehyde, and crosslinking was allowed to occur as per usual. Using this 'HU washout' procedure, we found that that treatment with HU caused a 2- to 3-fold increase in Asf1 crosslinking at *RNR3* and *HUG1* (Fig. 4-5B). Since the HU washout increases cell processing time (because cells are collected by centrifugation, then resuspended), we used vacuum filtration to rapidly collect the cells before immediately resuspending them in the formaldehyde-containing conditioned media ('fast' HU washout; see Materials and Methods). Asf1 crosslinking to *RNR3* and *HUG1* was essentially identical whether the 'slow' or 'fast' HU washout protocol was used (Fig. 4-5C). Therefore, our results strongly suggest that formaldehyde quenching by HU can mask HU induction of

Asf1 recruitment to chromatin. Accordingly, in all subsequent experiments, fixation was performed using the fast HU washout protocol.

The 2- to 3-fold increase in Asf1 association with chromatin upon HU treatment is similar in magnitude to induction of Asf1 crosslinking by other physiological stimuli[8]. Hydroxyurea induction of Asf1 crosslinking to chromatin, however, was not specific to DDR genes (Fig. 4-6A and B): it occurred at a gene-free region (TELV), an Asf1-dependent gene that is not induced under any of the conditions we use (*PHO5*), a gene that is repressed by HU (*DSE1*[34]), and an RNA polymerase III-transcribed gene (*SCR1*). Immunodepletion of Asf1-Myc was essentially identical from whole cell extracts prepared from untreated or HU-treated cells (Fig. 4-6C), suggesting that previous treatment with HU does not influence the ability of Asf1-Myc to be immunoprecipitated from whole cell extracts. Importantly, bulk expression of Asf1-Myc did not change upon treatment with HU (Fig. 4-6D). Therefore, the observed recruitment of Asf1-Myc to DNA during HU treatment is authentic. Furthermore, fixation was not required to detect inducible association of Asf1 with bulk chromatin (Fig. 4-4A, lanes 5 and 6; Fig. 4-4B, lanes 3 and 4). This effect was most readily seen in lysates prepared using a lower concentration of salt (Fig. 4-4B). These results indicate that stimulation of Asf1 association with chromatin, as revealed by ChIP, is due to *de novo* recruitment rather than increased crosslinking of Asf1 molecules that reside permanently on chromatin.

Asf1 association with chromatin was also induced by MMS, another inducer of replication stress that triggers derepression of the DDR genes[35] (Fig. 4-7). Thus, Asf1 is recruited to chromatin when cells experience replication stress caused by HU

or MMS. Given that MMS lacks a free amine group, no washout protocol was necessary before crosslinking with formaldehyde. This finding therefore confirms that the HU-induced increase in Asf1 crosslinking to chromatin following the HU washout protocol is bona fide. Considering our transcriptional data (Fig. 4-2), and the important finding that human Asf1 complexes function in histone regulation during replication stress caused by HU[36], we chose to continue studying the effects of HU, rather than MMS, on Asf1 function in DDR gene regulation.

The experiments outlined above were performed in the BY4741 strain background often used for large-scale genetic analysis in yeast[37]. The results are not restricted to this strain background: HU also induced Asf1 crosslinking in a W303[38] derivative harboring Asf1-Myc (Fig. 4-8A and B). Additionally, although Asf1 recruitment to chromatin during HU treatment seemingly occurs in a nonspecific fashion, this effect is unique to the Asf1 protein. That is, HU does not increase the association of all proteins with chromatin. In particular, chromatin association of the Rtt109 KAT, which is involved in some of the same processes as Asf1, did not increase in response to HU treatment (Fig. 4-9).

Collectively, our transcription and ChIP experiments show that derepression of *RNR3* and *HUG1* occurs concomitantly with increased recruitment of Asf1 to their promoters during replication stress. However, given that HU seems to globally induce the association of Asf1 with chromatin, HU-induced recruitment of Asf1 to the promoters of DDR genes may or may not be required for their transcriptional derepression. These observations present two main possibilities: 1) Asf1 recruitment to the promoters of DDR genes is needed for their subsequent derepression, or 2)

Asf1 recruitment occurs at the same time as, but is dispensable for, gene derepression.

Asf1 association with chromatin: new functions for known Asf1 motifs

In order to directly test whether Asf1 association with chromatin is important for derepression of DDR genes, we first identified Asf1 mutations which dampen its crosslinking to chromatin. Yeast Asf1 consists of two domains (Fig. 4-10A). Amino acids 1-155 comprise its conserved N-terminus ('asf1N') while amino acids 156-279 comprise an acidic C-terminal region, which is restricted to *Saccharomyces* (although phosphorylation might provide a similar negatively charged region in human Asf1)[39]. The conserved N-terminal domain binds tightly to the H3-H4 dimer to form a complex that is possibly stabilized by the C-terminal domain[40,41].

We predicted that association of Asf1 with chromatin would depend primarily on binding of its N-terminal domain to H3 and H4 because mutation of Asf1 valine 94 to arginine nearly abolishes the ability of Asf1 to bind to histones[42]. The V94R mutation confers phenotypes observed in the null mutant, but does not affect overall protein expression[42-44] (Fig. 4-10B). In particular, like *asf1*⊿ cells, asf1^{V94R} mutants are sensitive to HU, and accumulate with a G2/M DNA content when grown under normal conditions (Fig. 4-10C and D). Surprisingly, this mutation had no effect on constitutive or HU induction of Asf1 binding to chromatin. That is, the asf1^{V94R} and wild type Asf1 proteins associated with *HUG1*, *RNR3* and TELV at comparable levels both under normal conditions and during HU treatment (Fig. 4-11A-C). Therefore, Asf1 can associate with chromatin both constitutively and under conditions of replication stress, even when it is unable to robustly bind the H3/H4

dimer. This important finding indicates that binding of H3/H4 by the amino-terminal domain of Asf1 is not 'necessary and sufficient' for HU-induction of Asf1 association with chromatin.

We next tested whether deletion of the C-terminal domain of Asf1 affects its binding to chromatin. asf1N, which lacks the C-terminal domain, was expressed at a slightly higher level than wild type Asf1 during normal growth, and grew similarly to wild type cells in the presence of HU (Fig. 4-10B and C). Nonetheless, asf1N occupancy at *HUG1*, *RNR3* and TELV was on average 60% lower than wild type (Fig. 4-11A-C). The wild type and *asf1N* strains had almost identical cell cycle profiles (Fig. 4-10D), so the low ChIP signal for asf1N is not an indirect consequence of abnormal cycling. We conclude that the C-terminal domain of Asf1, on its own, makes an important contribution to constitutive, untargeted association of Asf1 with chromatin. Even though the total level of asf1N association with chromatin after HU treatment was decreased compared to wild type, binding of asf1N to *HUG1* and *RNR3* was induced by the same fold as wild type when cells are treated with HU (Fig. 4-11A and B). Therefore, the C-terminal domain of Asf1 is dispensable for its further recruitment to the promoters of DDR genes under conditions of replication stress.

Based on the results for strains expressing asf1N and asf1^{V94R}, we hypothesized that histone binding by the core domain of Asf1 accounts for the residual capacity of asf1N to associate with chromatin. Consistent with this hypothesis, asf1N harbouring the V94R mutation had very low chromatin binding activity, even in HU-treated cells (Fig. 4-11A-C; note that this protein is expressed at the wild type level

(Fig. 4-10B)). This effect could not be explained by perturbed cell cycling since the asf1N^{V94R} mutant exhibited the same cell cycle profile as cells expressing asf1^{V94R} (Fig. 4-10D), which crosslinked normally to chromatin (Fig. 4-11A-C). In addition, the asf1N^{V94R} double mutant was not more HU-sensitive than the asf1^{V94R} single mutant (Fig. 4-10C). Given these results, and the fact that the human equivalent of yeast asf1N^{V94R} folds normally[42], we conclude that in the absence of the acidic C-terminal tail, the conserved histone binding domain of Asf1 becomes more important for the ability of Asf1 to associate with chromatin.

The relationship between transcription and association of Asf1 with chromatin

We next determined how the regulation of *HUG1* is affected by the capacity of Asf1 to associate with chromatin. *HUG1* was selected for this analysis because of its strong dependence on Asf1 for derepression (Fig. 4-2B and E). Given that cells expressing asf1^{V94R} phenocopy *asf1⊿* cells, we were not surprised that *HUG1* was modestly derepressed in the *asf1^{V94R}* mutant under normal conditions (Fig. 4-11D; compare to Fig. 4-2B). However, asf1^{V94R} was not more strongly crosslinked to the promoter of *HUG1* than the wild type protein under these conditions (Fig. 4-11A). Deletion of the C-terminal domain of Asf1 strongly inhibits its association with *HUG1*, but has little effect on basal transcription (Fig. 4-11A and D). These results suggest that constitutive association of Asf1 with chromatin, on its own, is not important for *HUG1* transcription under normal conditions. The same is likely true under inducing conditions. The V94R mutation, even in the context of full-length Asf1, severely compromises *HUG1* induction by HU (Fig. 4-11D) without affecting

recruitment upon HU treatment (Fig. 4-11A). It follows that the V94R mutation confers a defect in transcriptional induction that is unrelated to the capacity of Asf1 to bind to chromatin. Importantly, asf1N supports almost normal induction of *HUG1* transcription (Fig. 4-11D), despite the fact that its association with chromatin in HU-treated cells increases only to the baseline observed for wild type protein in unstimulated cells (Fig. 4-11A). Collectively, our studies of Asf1 mutants suggest that Asf1 promotes transcriptional induction of *HUG1* by a mechanism that involves binding of its core domain to H3-H4, but not its inducible association with chromatin.

Targeting of Asf1 to chromatin: Asf1 as an opportunist

Since Asf1 recruitment to chromatin was not necessary for its ability to stimulate transcription at DDR genes, we reasoned that Asf1 must be involved in a different process that requires it to associate with chromatin. To understand this process, we first further characterized the recruitment of Asf1 to chromatin. It has been suggested by the Tyler lab that Asf1 acts as an opportunist that associates non-specifically with chromatin, waiting for signals from transcriptional activators to disassemble chromatin for the purpose of promoting transcription[16]. Given that transcriptional derepression of the DDR genes occurs normally even when Asf1 binding to chromatin is impaired, we have extended this idea to include the possibility that Asf1 may act opportunistically in any number of chromatin-related processes. We hypothesized that when more Asf1 becomes available to the cell, Asf1 association with chromatin would increase if Asf1 truly functions in an opportunistic manner. To test this possibility, Asf1-Myc was produced from a low-

or high-copy plasmid in a chromosomal *asf1*⊿ strain. Asf1-Myc produced from the low- and high-copy plasmids was expressed at lower or higher levels than the endogenous protein, respectively.

During normal proliferation, bulk expression of Asf1 was elevated in the high- compared to the low-copy strain (Fig. 4-12A; lanes 3 and 7); however, this difference was not associated with abnormal cell cycle progression or changed growth rates (Fig. 4-12B and C). Remarkably, Asf1 crosslinking to *HUG1* and *RNR3* under normal growth conditions was approximately 10-fold higher in the overexpressing strain, and was inducible by HU (Fig. 4-12D and E). This finding is consistent with the idea that Asf1 acts as an opportunist, although the fact that *HUG1* and *RNR3* transcription was similar in cells expressing low- or high-copy Asf1 (Fig. 4-12F and G) indicates that the increase in Asf1 association with chromatin is not likely for the purpose of activating transcription. This provides support for the notion that Asf1 may participate opportunistically in a different chromatin process.

Asf1 association with chromatin involves interaction with another histone chaperone

To determine which process might require Asf1 to associate with chromatin, we examined whether protein factors that are known to interact with Asf1 are involved in its recruitment to chromatin. To this end, we monitored the association of wild type Asf1 with chromatin in cells lacking proteins that can interact with Asf1. We first hypothesized that Asf1 may associate with chromatin to carry out an opportunistic role in chromatin assembly, perhaps during DNA replication. Asf1 functions in two different chromatin assembly pathways: 1) a pathway involving

CAF-I during DNA replication and 2) a replication-independent pathway with the HIR histone chaperone complex. Since Asf1 interacts physically with the Cac2 subunit of CAF-I[45], we tested the possibility that Asf1 association with chromatin is dependent on this interaction. However, ChIP analysis showed that Asf1 associated normally with chromatin in cells lacking *CAC2* (Fig. 4-13). To analyze whether the HIR proteins are involved in Asf1 association with chromatin, we next studied an Asf1 mutant[42] that is impaired for binding to the HIR complex, asf1$^{D37R/E39R}$. Residues 37 and 39 are found within the acidic region of Asf1, and are highly conserved[40]. Asf1 exhibits mutually exclusive physical interactions with HIR and CAF-I[46]; therefore, asf1$^{D37R/E39R}$ is unable to bind to either CAF-I or HIR. The association of asf1$^{D37R/E39R}$ with chromatin was markedly reduced under normal conditions (Fig. 4-13). This observation suggests that the HIR complex may play a role in the association of Asf1 with chromatin under normal conditions. The association may depend solely on Asf1 interaction with the HIR complex, or on Asf1 interaction with at least one of CAF-I or HIR. Interestingly, the asf1$^{D37R/E39R}$ mutant protein was still recruited to chromatin following treatment with HU (Fig. 4-13; +HU). This indicates that an interaction between Asf1 and HIR or CAF-I is not needed for inducible association of Asf1 with chromatin.

The chromatin binding phenotypes of the asf1$^{D37R/E39R}$ mutant protein were reminiscent of that of the asf1N truncation mutant: binding of each mutant protein to chromatin was reduced under normal conditions, but inducible under conditions of replication stress (compare Fig. 4-11A and B to Fig. 4-13A and B). This observation presented the possibility that the C-terminal tail of Asf1 may promote its

binding to either HIR or CAF-I, an interaction which might then promote the association of Asf1 with chromatin. To address this, we constructed an asf1N$^{D37R/E39R}$ 'double' mutant and measured its ability to associate with chromatin. asf1N$^{D37R/E39R}$ associated with chromatin similarly to each of the 'single' mutant versions of Asf1 (Fig. 4-13). These findings are consistent with the idea that the C-terminal tail and the CAF-I/HIR binding region of Asf1 function in the same pathway to promote its association with chromatin. However, we have not ruled out the possibility that these two regions of Asf1 may promote its chromatin association independently.

Asf1 association with chromatin does not depend on Rad53 or Rtt109

We next measured the ability of Asf1 to associate with chromatin in cells lacking Rad53. Asf1 exists in a complex with Rad53 during normal growth, but this complex is disrupted under conditions of replication stress, perhaps making Asf1 more available for chromatin regulation[29,47,48]. We therefore hypothesized that in the absence of Rad53, there might be a larger pool of Asf1 available to associate with chromatin. However, constitutive and inducible crosslinking of Asf1 to *HUG1* and *RNR3* was similar in wild type and *rad53*Δ cells (Fig. 4-14). Therefore, Rad53-Asf1 complexes are not reservoirs for Asf1 molecules that can constitutively or inducibly bind to chromatin. This finding is consistent with our previous conclusion that association of Asf1 with chromatin is not related to its ability to promote derepression of the DDR genes because 1) checkpoint signalling is severely compromised in *rad53*Δ mutants[35], which does not allow for induction of the DDR genes (Fig. 4-1D), and 2) Asf1 is still recruited to chromatin in *rad53*Δ cells.

One important function of Asf1 in yeast is to stimulate the catalytic activity of Rtt109, which acetylates K56 in the globular core of histone H3[49-51]. K56-acetylated H3 is then donated to the CAF-I and Rtt106 histone chaperones for deposition onto DNA[2]. Although H3K56 acetylation is thought to occur prior to deposition, we considered the idea that once deposited onto chromatin, K56-acetylated H3 may play a role in Asf1 recruitment. This was plausible because H3K56 acetylation increases under the same conditions in which we observe increased binding of Asf1 to chromatin: replication stress caused by treatment with HU[52]. This prompted us to test whether Asf1 association with chromatin is affected in cells lacking H3K56 acetylation (*rtt109*Δ mutants). We found that Asf1 association with chromatin was somewhat higher in cells lacking *RTT109* (Fig. 4-14A and B), which ruled out the possibility that H3K56 acetylation signals an increase in Asf1 binding to chromatin. Collectively, our results show that while the CAF-I/HIR-binding region and C-terminal tail of Asf1 play a role in Asf1 association with chromatin, the ability of Asf1 to bind chromatin is not influenced by Rad53 or H3K56 acetylation.

H3K56 acetylation is important for derepression of *HUG1* under conditions of replication stress

Although Asf1 association with chromatin is not needed for transcriptional derepression of the DDR genes under conditions of replication stress, cells lacking *ASF1* are strongly impaired for derepression of *HUG1*, and delayed for derepression of *RNR3* (Fig. 4-2A and B). This suggests that Asf1 promotes derepression of the DDR genes via a mechanism that does not require Asf1 to act directly at these promoters. To investigate the mechanism used by Asf1 to promote DDR gene

transcription in response to replication stress, we considered the recent evidence from the Tyler lab that H3K56 acetylation is important for the induction of *PHO5* upon phosphate limitation[10]. Since Asf1 is absolutely required for H3K56 acetylation, and H3K56 acetylation increases the breathability of nucleosomal DNA on/off the histone octamer[12], it seemed likely that Asf1 might promote derepression of DDR genes through its positive effect on the H3K56 acetylation reaction performed by Rtt109.

To explore this possibility, we first compared *HUG1* and *RNR3* mRNA levels in *asf1⊿* and *rtt109⊿* mutants, both of which lack H3K56 acetylation. As in *asf1⊿* cells, *HUG1* and *RNR3* were moderately derepressed under normal conditions in *rtt109⊿* cells (Fig. 4-15A and B). This result was expected since both mutants show chronic checkpoint activation[4,27], as assessed by partial Rad53 activation (Fig. 4-2C). Upon replication stress, *HUG1* derepression was severely compromised in cells lacking *ASF1* or *RTT109*, and *RNR3* derepression was delayed in both mutants. This suggests that H3K56 acetylation may be needed for optimal derepression of *HUG1* and *RNR3*.

We were not surprised to find that *HUG1* and *RNR3* derepressed normally in HU-treated cells lacking *CAC2* (Fig. 4-15A and B) because although CAF-I contributes to deposition of K56-acetylated histone H3 in a pathway involving Rtt106, the total level of H3K56 acetylation is not dramatically reduced in CAF-I mutants[2,52]. It should be noted, however, that Miller et al. report a 35% decrease in chromatin-associated H3K56ac in a *cac1⊿* mutant[53]. Interestingly, both *HUG1* and *RNR3* were partially derepressed in *cac2⊿* cells grown under normal conditions (Fig.

4-15A and B). This phenotype is shared with cells lacking *ASF1*, in which the DDR is constitutively engaged. CAF-I mutants exhibit a slightly higher percentage of DNA damage foci than wild type cells[54], and therefore may also constitutively engage DDR checkpoint signalling (although Rad53 activation is not evident in untreated cells lacking the Cac1 subunit[54,55]). Given that both *asf1*⊿ and *rtt109*⊿ cells are devoid of H3K56 acetylation and impaired for derepression of DDR genes, while *cac2*⊿ mutants exhibit normal H3K56 acetylation and derepression of DDR genes, our results are consistent with a positive role for H3K56 acetylation in the derepression of *RNR3* and *HUG1*.

To more directly explore this possibility, we examined the regulation of DDR genes in H3 K56Q, K56A and K56R mutants. We first confirmed that H3K56 acetylation was absent from each point mutant (Fig. 4-16A). While the K56Q and K56A mutations mimic constitutive acetylation of residue 56, the K56R mutation mimics permanent deacetylation[56]. Under normal conditions, *HUG1* and *RNR3* were partially derepressed in cells harbouring the H3K56Q or H3K56A mutation (Fig. 4-16B and C). This finding is consistent with the observation by Celic et al. that *HUG1* and *RNR3* are upregulated in *hst3*⊿ *hst4*⊿ cells[57], which lack the HDACs responsible for deacetylating H3K56. Therefore, the DDR genes are activated in cells containing or mimicking high levels of H3K56 acetylation. Upon treatment with HU, H3K56Q or H3K56A mutants derepressed *HUG1* and *RNR3* to levels higher than that observed for wild type cells (Fig. 4-16B and C), and induced Rad53 activation normally (Fig. 4-16D and E). Similarly, MMS-induced phosphorylation of Rad53 appears to be normal, or only mildly affected, in H3K56Q mutants[58].

Although Rad53 was also activated in HU-treated cells containing the H3K56R mutation (Fig. 4-16F; see also[58]), whose charge mimics unacetylated K56, transcription of *HUG1* was strongly dampened (Fig. 4-16B). Therefore, muting of *HUG1* derepression in the K56R mutant was not likely to be a consequence of defective checkpoint signalling. We conclude that H3K56 acetylation by Rtt109 is important for derepression of *HUG1* and *RNR3* by replication stress checkpoint signals. Since efficient acetylation of H3K56 by Rtt109 requires Asf1, it follows that Asf1 contributes to derepression of the DDR genes by stimulating H3K56 acetylation. This conclusion is strongly reinforced by our finding that the K56Q and K56A mutations suppress the transcriptional defects of the *ASF1* null strain (Fig. 4-16B and C). Furthermore, the K56R mutation did not produce additive transcriptional defects in combination with a deletion of *ASF1*, indicating that *ASF1* and H3K56 acetylation function in the same pathway to contribute to derepression of DDR genes during replication stress.

H3K56 acetylation at DDR genes during their derepression

Studies of the *PHO5* gene have revealed that increased H3K56 acetylation favours induction of transcription[10]. It was proposed that, under inducing conditions, an increased proportion of K56-acetylated H3 molecules in the promoter facilitiates high transcription because this modification may weaken nucleosomal histone-DNA contacts, thereby promoting chromatin disassembly. Therefore, we next tested whether the correlations between H3K56 acetylation and the genetic requirements for derepression reflect a similar role for H3K56 acetylation in the regulation of the DDR genes.

Initially, we determined how H3 occupancy at the promoters of *RNR3* and *HUG1* is affected under derepressing conditions. The bulk H3 antibody used for this ChIP experiment (Abcam #ab1791) has been widely employed by others (for example,[10,20]), and in my hands on average yielded a 2,250-fold higher ChIP signal than 'no antibody' control immunoprecipitations. At both *RNR3* and *HUG1*, there was a modest trend towards decreased promoter crosslinking of H3 in wild type cells in the presence of HU (Fig. 4-17A and B). This result is consistent with previous evidence that H3 and H4 are lost from the *RNR3* promoter when cells are treated with MMS[20,24]; however, the effect of MMS at *RNR3* was much larger (i.e., histone loss was greater) than we observed using HU. We suspect that this difference may be due to the longer drug treatment times used by others, or to differences between MMS and HU in the way that they affect overall cellular physiology. For example, MMS causes damage to lipids and RNA that HU is incapable of generating[59]. Nevertheless, our results show minimal histone loss from the promoter of *RNR3*, even when it is maximally induced by HU treatment (Fig. 4-17A and 4-2A; 60 minutes). Similarly, on average, less than 20% of histone H3 was lost from the *HUG1* promoter at the time when *HUG1* was fully derepressed by HU (Fig. 4-17C and 4-2E; 4 hours). To our knowledge, histone eviction from the promoter of *HUG1* under derepressing conditions has not been reported by others.

H3K56 acetylation was monitored by ChIP using a commercial H3K56ac antibody extensively validated in the literature (Upstate #07-677). We readily detected K56-acetylated H3 at *RNR3* and *HUG1* under repressing and derepressing conditions. In the absence of HU, the ChIP signal for K56-acetylated H3 was

approximately 10-fold higher at the promoters of *RNR3* and *HUG1* in wild type cells than in *asf1*⊿ cells, which lack H3K56 acetylation. While K56-acetylated H3 was also present at the *RNR3* and *HUG1* promoters in HU-treated cells, its enrichment was lower than in untreated cells (Fig. 4-17D and E; compare fold differences in '–HU' (grey bars) to fold differences in '+HU' (black bars)). We determined that this reduction in enrichment was caused by variations in background binding of the antibody to chromatin. Specifically, we found that background binding of the H3K56ac antibody to chromatin in *asf1*⊿ cells increased approximately 5-fold at *RNR3* and 2-fold at *HUG1* upon HU treatment (Fig. 4-17D and E; '+HU'). This change will dampen real reductions, and artificially inflate real increases, in H3K56ac occupancy after HU treatment. To deal with this problem, in further analyzing the H3K56ac ChIP results, we decided to subtract the background signal calculated for *asf1*⊿ cells (-/+ HU) from the H3K56ac signal obtained for wild type cells (both normalized to bulk H3 crosslinking). Others have also reported that non-specific binding of the H3K56ac antibody to chromatin can vary in *asf1*⊿ cells. For example, in the careful study of Williams et al., the background H3K56ac signal at *PHO5* was found to fluctuate up to 3.7-fold between induction time points (Fig. S3B in[10]). Together, these findings point out the importance of performing all H3K56 acetylation ChIPs not only in the strain of interest, but also in a congenic *ASF1* null strain, or K56R mutant.

When effects on background binding are taken into account, the data reveal a slight increase or slight decrease in the proportion of K56-acetylated H3 at the promoters of *RNR3* and *HUG1*, respectively, upon HU treatment (Fig. 4-17F).

Therefore, derepression of the DDR genes under conditions of replication stress is not associated with a large increase in H3K56 acetylation, despite the fact that overall H3K56 acetylation is induced (by 5-fold after 3 hours in 0.2 M HU[52]). In other words, while *HUG1* strongly requires the pathway which controls H3K56 acetylation for full derepression (Fig. 4-15 and 4-16), its regulation is not like that of *PHO5*, where a transcription-coupled mechanism establishes a higher steady state condition of H3K56 acetylation concomitantly with transcriptional induction. Conversely, H3K56 acetylation increases slightly at the promoter of *RNR3* under derepressing conditions even though H3K56 acetylation plays a minimal role at this promoter (it affects the kinetics of derepression but not the ability to fully derepress transcription). The results for *HUG1* and *RNR3* indicate that in the case of the DDR genes, an increase in the proportion of H3 that is K56-acetylated in the promoter region is not needed for high transcriptional derepression.

Discussion

Here we have shown that histone chaperone Asf1 is important for transcriptional derepression of two DDR genes in budding yeast under conditions of replication stress. In cells lacking *ASF1*, derepression of *HUG1* in response to replication stress caused by treatment with HU is severely compromised while derepression of *RNR3* is delayed (Fig. 4-2). Although Asf1 association with the promoters of *HUG1* and *RNR3* increased under these same conditions, we determined that HU induces Asf1 binding to chromatin non-specifically (Fig. 4-3 to 4-8). That is, Asf1 associates constitutively with all regions of the genome tested thus far, and this association is globally induced by treatment with HU. Importantly, the identification of Asf1

mutants that are compromised in their ability to bind chromatin revealed that Asf1 association with the promoters of the DDR genes was not needed for their transcriptional derepression (Fig. 4-11).

The functional significance of Asf1 association with chromatin in budding yeast remains unknown. Our mapping of the determinants of this association however raises some interesting possibilities. We find that the acidic C-terminal tail of Asf1 is important for its binding to chromatin (Fig. 4-11). This domain stabilizes the interaction of Asf1 with the RFC (replication factor C) complex, which loads PCNA onto DNA[30], and may strengthen the interaction between Asf1 and histones[40,43]. Furthermore, a mutation which virtually eliminates Asf1 binding to the H3/H4 dimer[42] (asf1^{V94R}) abolishes residual chromatin binding by the C-terminal tail mutant. Collectively, these findings are consistent with the notion that chromatin-associated Asf1 has a role in the control of replication[30] that is directly tied to nucleosome metabolism at forks.

The fact that Asf1 promotes transcription of the DDR genes even when its binding to their promoters is impaired led us to hypothesize that Asf1 contributes to derepression of the DDR genes via a pathway other than direct chromatin disassembly by Asf1. Indeed, we show that H3K56 acetylation, which is catalyzed by Rtt109 and requires Asf1 as a cofactor, is important for derepression of the DDR genes upon treatment with HU. Mutations which perturb the H3K56 acetylation reaction (*rtt109⊿*, *asf1⊿*, *asf1^{V94R}*) or mimic the unacetylated state (H3K56R), severely compromise derepression of *HUG1* and cause *RNR3* derepression to be delayed. Conversely, derepression of the DDR genes is normal or better than normal in cells

expressing mutations that mimic constitutive H3K56 acetylation (H3K56Q, H3K56A).

In an important model based on studies of *PHO5*, one step in the pathway of transcriptional induction by H3K56 acetylation is induction of promoter acetylation (relative to H3 occupancy)[10]. However, we find that H3K56 acetylation is only slightly increased at the promoter of *RNR3* and slightly decreased at the promoter of *HUG1* under derepressing conditions. Therefore, regulation of DDR genes does not seem to conform to the model previously described for *PHO5*. That is, the promoters of *RNR3* and *HUG1* do not shift from a state of low to high H3K56 acetylation upon derepression (Fig. 4-17).

This difference between *PHO5* and the DDR genes raises two possibilities. On the one hand, the dependence of *RNR3* and *HUG1* gene derepression on H3K56 acetylation might reflect an indirect effect of H3K56 acetylation on transcriptional regulation of these genes. We have not ruled out all potential mechanisms by which abnormal regulation of H3K56 acetylation might indirectly impact transcriptional control of the DDR genes. However, since Rad53 activation remains quite robust in mutants compromised for H3K56 acetylation and derepression of *HUG1* (Fig. 4-2D and E; Fig. 4-16B and F), it is unlikely that abnormal regulation of H3K56 acetylation has a detrimental effect on DDR gene expression by virtue of interference with checkpoint signalling.

A plausible alternative to indirect regulation of DDR gene derepression by H3K56 acetylation is direct regulation by similar mechanisms that apply at other genes, including *PHO5*. Perhaps most importantly, H3K56 acetylation in the

promoters of DDR genes may increase the plasticity of nucleosomes and therefore the permissiveness of promoter chromatin for transcription[12].

If this scenario is correct, then why is derepression of the DDR genes not associated with increased H3K56 acetylation of promoter chromatin (as observed upon induction of *PHO5*)? A simple answer to this question could be that H3K56 acetylation is maintained at a high level in the promoters of the DDR genes, even in the absence of transcription. Deposition of K56-acetylated H3 molecules during replication-coupled assembly by CAF-I, Rtt106 and other chaperones results in global incorporation of H3K56ac into chromatin[56], which would allow for H3K56ac to be incorporated into the promoters of DDR genes in the S phase of the cell cycle (Fig. 4-18). This high level of H3K56 acetylation in the promoters of DDR genes would facilitate immediate activation of the DDR genes upon dissociation of the Crt1 and Ssn6-Tup1 repressors in response to replication stress in S phase.

The idea that K56-acetylated H3 deposited by replication-coupled chromatin assembly is maintained in the promoters of DDR genes, thereby poising them for derepression, is supported by several facts. First, HU elicits derepression of DDR genes only in cells that are in S phase. *PHO5* induction, however, can occur outside of S phase[60]. Second, overall H3K56 acetylation of chromatin is dramatically induced during S phase and all newly synthesized H3 molecules used for replication-coupled nucleosome assembly are acetylated at K56[56]. Therefore, derepression of the DDR genes occurs at a time when replication-coupled chromatin assembly increases the probability that the promoters of newly replicated DDR genes will harbour one or more H3K56-acetylated nucleosomes. Third, high H3 acetylation in

the promoters of *RNR3* and *HUG1* is insufficient for their derepression[19], indicating that high levels of acetylation can be tolerated at these promoters. Similarly, an H3K56Q mutant that mimics permanent acetylation shows only partial derepression of the DDR genes under normal conditions (Fig. 4-16B,C). We therefore propose that H3K56 acetylation poises newly replicated DDR genes for derepression in the event that replication interference triggers the checkpoint response.

In addition to presenting the possibility that H3K56 acetylation poises DDR genes for derepression, we further speculate that removal of H3K56ac after H3 incorporation into chromatin may be in some way prevented, or rendered unnecessary, in the promoters of DDR genes. For example, H3K56 deacetylation by the Hst3 and Hst4 HDACs could be inhibited. This would result in the local preservation of H3K56 acetylation throughout the cell cycle. Consistent with this idea are the findings that 1) Crt1 and Tup1 can inhibit HDAC binding to *RNR3*[20], and 2) H3K56 can remain in the acetylated state at sites of DNA damage[56]. Further support for this idea comes from our own observation that the level of H3K56 acetylation in the promoters of *RNR3* and *HUG1* is similar in asynchronous cells or HU-treated cells that are synchronized in S phase (Fig. 4-17). Thus, H3K56 may remain in the acetylated state for the duration of the cell cycle at DDR gene promoters.

An inherent risk in maintaining genes in a poised state is an increased likelihood of spurious transcription under repressing conditions. In this regard, poising by H3K56 acetylation might be well tolerated at DDR genes because, unlike *PHO5*, the DDR genes are actively repressed by a *trans*-acting factor (Crt1) during normal

growth[17]. Since *PHO5* transcription is not blocked by binding of a transcriptional repressor to its promoter, H3K56 acetylation at this location may be kept low to prevent spurious transcription during normal growth. Upon phosphate removal, increased incorporation of K56-acetylated H3 into the *PHO5* promoter (via replication-coupled and/or replication-independent histone deposition pathways) would facilitate high levels of *PHO5* transcription. Therefore, previous evidence for dissimilar regulation of *PHO5* and DDR gene transcription (de novo activation versus derepression) may provide an explanation for the difference in regulation of H3K56 acetylation at the DDR genes compared to *PHO5*. To summarize, we propose that the DDR genes are maintained in a poised but repressed state during normal proliferation by the combined action of the replication-coupled chromatin assembly machinery, which reconstitutes K56-acetylated H3 into new nucleosomes, and Crt1, which recruits the Ssn6-Tup1 corepressor complex.

This model predicts that derepression of the DDR genes by HU will be dampened in mutants in which the activity of replication-dependent H3/H4 chaperones is compromised. One key replication-dependent H3/H4 chaperone is CAF-I, which is inactive in the absence of its Cac2 subunit[61]. However, derepression of DDR genes is normal in HU-treated *cac2*⊿ cells (Fig. 4-15). This result is consistent with the fact that deposition of K56-acetylated H3 into nucleosomes remains unaffected in CAF-I single mutants, but is reduced in cells lacking both Rtt106 and a component of CAF-I[2]. Additional histone chaperones are also thought to contribute to replication-coupled H3/H4 deposition into nucleosomes. Once the full complement of replication-coupled H3/H4 chaperones has been identified (this

work is ongoing), it should be feasible to rigorously test whether disruption of replication-coupled H3/H4 assembly into nucleosomes compromises derepression of DDR genes by HU.

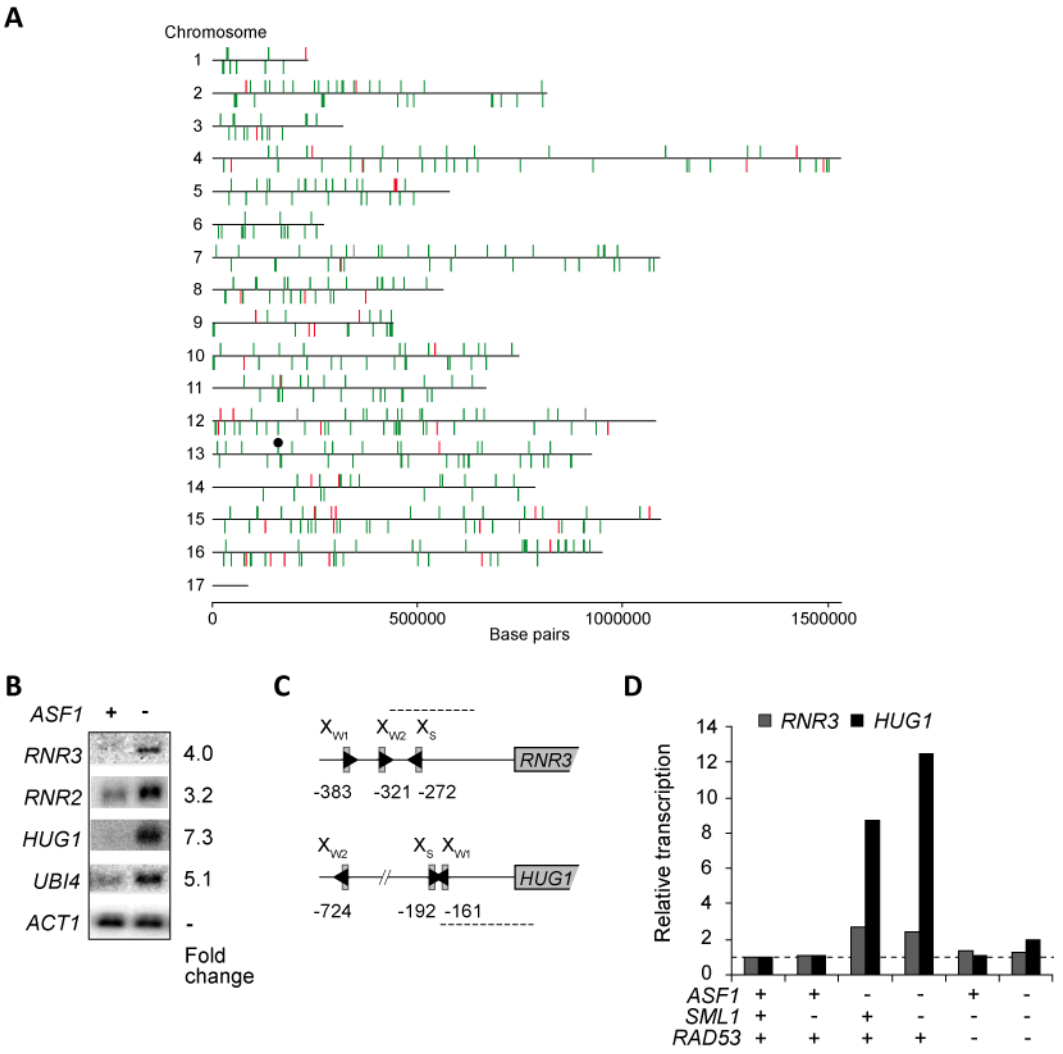

Figure 4-1. DNA damage response genes are misregulated in *asf1Δ* cells.
A. Microarray analysis shows that misregulated genes in *asf1⊿* cells are not clustered in specific chromosomal regions. GeneSpring 5.1 map showing the location of genes which are induced (green) or repressed (red) by 2-fold or more in *asf1⊿* cells compared to wild type. In this representative experiment, 536 genes differed by 2-fold or more in expression between the strains. The position of the *HUG1* gene is indicated by a black dot. Microarray analysis was performed by J. Williams.
B. Northern blotting analysis of selected genes during normal growth. *ACT1* is the loading control. **C.** Promoter structures of *RNR3* and *HUG1*[17,22]. Xs and Xw have strong and weak identity to the X box, which is bound by the sequence-specific trans-acting factor Crt1. The dotted lines represent the ChIP amplicons used in this study. **D.** Graph showing the effects of selected mutations on mRNA levels of *RNR3* and *HUG1*, both of which were normalised to the *SCR1* control. *SML1* is deleted to allow viability of *rad53⊿* cells. mRNAs were detected by Northern blotting. The average mRNA level obtained from three independent *asf1⊿ sml1⊿ rad53⊿* triple mutants is shown (range = 0.73 (*RNR3*) and 1.05 (*HUG1*)).

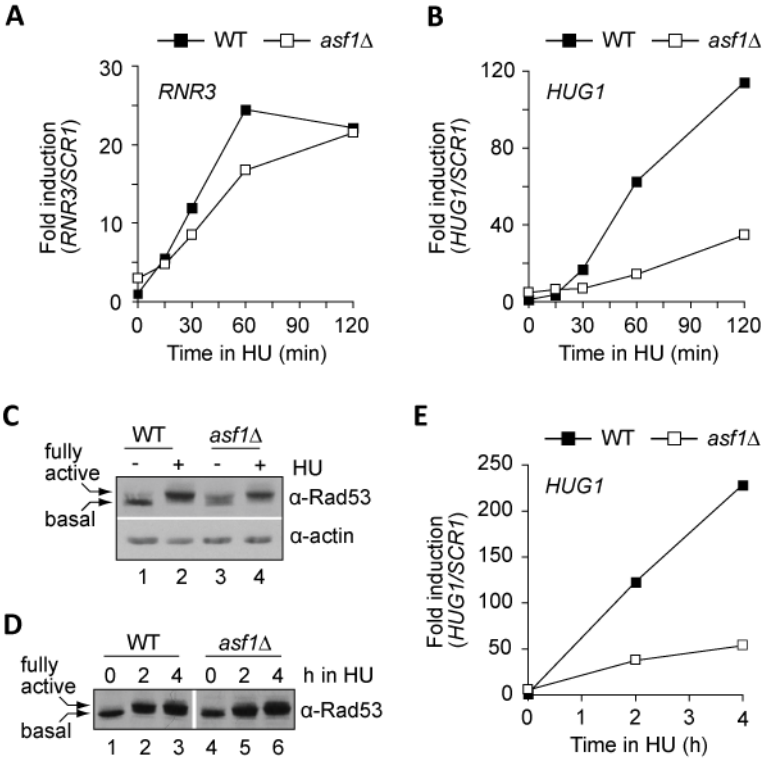

Figure 4-2. Asf1 contributes to the rapid derepression of DNA damage response genes. A. *RNR3* derepression is delayed in *asf1⊿* cells. Early log phase cells were grown in YPD or YPD + 0.2 M HU for 15, 30, 60 or 120 min. RNA was isolated at each time point and Northern analysis was performed using probes specific for *RNR3* or *SCR1* (control). Results were normalised to *SCR1* and quantitated using Phosphorimaging and ImageQuant TL software. Results of a single, representative experiment are shown. **B.** *HUG1* derepression is blocked in *asf1⊿* cells. Quantitation of *HUG1* Northern blot normalised to *SCR1*. Northern analysis was carried out as in (A). **C.** Rad53 activation is similar in wild type and *asf1⊿* cells. Cells grown in YPD (-) or YPD + 0.2 M HU (+) for two hours were collected at each time point and protein was prepared using a standard TCA preparation protocol. SDS-PAGE and Western blotting were done using anti-Rad53 and anti-actin (loading control) antibodies. Fully active (hyperphosphorylated) Rad53 is retarded in its migration compared to basal state Rad53. **D.** *asf1⊿* mutants fully activate Rad53. Early log phase cells were grown in YPD or YPD + 0.2 M HU for 0, 2 or 4 hours. Cells were processed as described in (A). See Fig. 5-5B for the actin loading control. **E.** *HUG1* derepression is blocked in *asf1⊿* cells during prolonged exposure to HU. Quantitation of *HUG1* Northern blot normalised to *SCR1*. Northern analysis was carried out as in (A). Results of a single, representative experiment are shown.

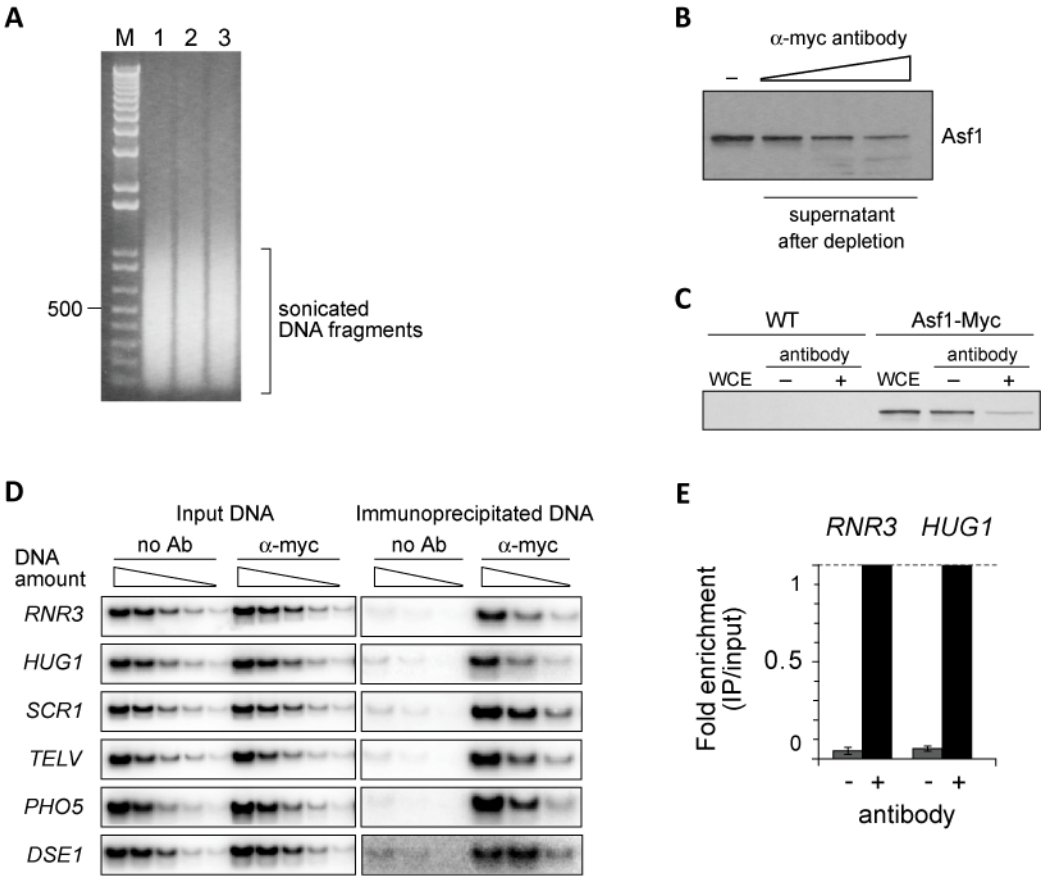

Figure 4-3. Asf1 is present at the promoters of *RNR3* and *HUG1*.
A. Validation of ChIP sonication protocol. Whole cell extracts were prepared according to[32] with minor modifications. After formaldehyde crosslinks were reversed by boiling, purified DNA was loaded on a 1% agarose gel and stained with ethidium bromide. Sonicated DNA fragments are on average 500 base pairs in length. Three independent whole cell extracts were sonicated under the same conditions (lanes 1 to 3). M, DNA Marker. **B.** Antibody titration to establish optimal conditions for immunodepletion of Asf1-Myc in whole cell extracts prepared as in (A). Asf1-Myc was monitored by anti-myc immunoblotting. **C.** Immunoprecipitations with/without anti-myc antibody were carried out using whole cell extracts prepared from wild type or Asf1-Myc cells. Asf1-Myc was detected by anti-myc immunoblotting. WCE, whole cell extract. **D.** Asf1-Myc crosslinking to indicated genomic locations, as detected by ChIP. Asf1-Myc was enriched at each locus compared to a 'no antibody' (-) control. **E.** Quantitative comparison of the Asf1-Myc signal at *HUG1* and *RNR3* from ChIP experiments performed with or without anti-myc antibody. PCR signal from immunoprecipitations that included antibody was set to 1 for each primer pair. Experiment was performed in triplicate; error bars indicate standard deviations.

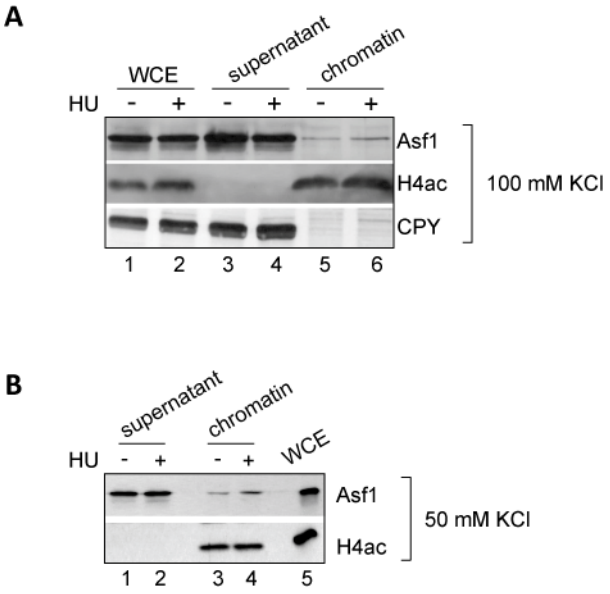

Figure 4-4. Asf1 is associated with chromatin in unfixed cells. Immunoblotting analysis of Asf1-Myc in the chromatin and non-chromatin fractions from unfixed cells grown in the absence (-) or presence (+) of hydroxyurea. **A.** Lysates were prepared using 100 mM KCl. As expected, tail-acetylated H4 (H4ac) is enriched in the chromatin fraction, and carboxypeptidase-Y (CPY) is enriched in the supernatant fraction. **B.** Lysates were prepared using 50 mM KCl by Amelia Walker.

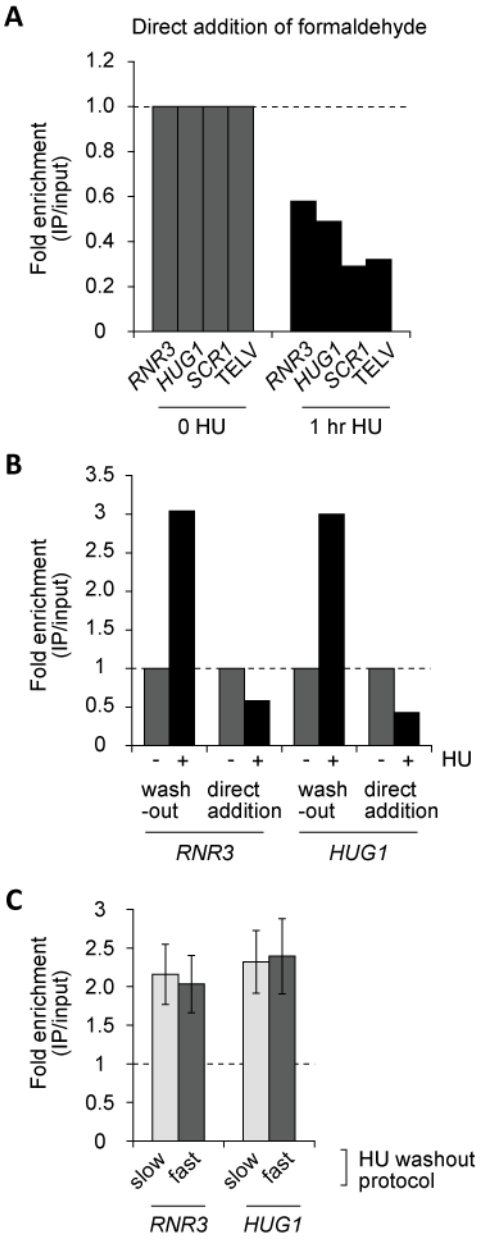

Figure 4-5. A modified ChIP assay for Asf1 association with chromatin in HU-treated cells. A. Chromatin immunoprecipitation analysis of Asf1-Myc crosslinking in cells to which formaldehyde was added without removal of HU. Four loci were analyzed as indicated. Signal from radioactive PCR reactions (IP/input) in untreated cells was set to 1 (quantitated using Phosphorimaging and TL software). These are the results of a single experiment. **B.** Comparison of Asf1-Myc crosslinking when formaldehyde is added to cells in medium containing HU (direct addition), or medium lacking HU (washout). Two loci were analyzed as indicated. These are the results of a single experiment. **C.** Comparison of Asf1-Myc crosslinking to the indicated loci when HU removal was performed by the slow or fast processing protocol. Average of multiple experiments are shown (n=7 for *RNR3*; n=5 for *HUG1*). Error bars represent standard deviations.

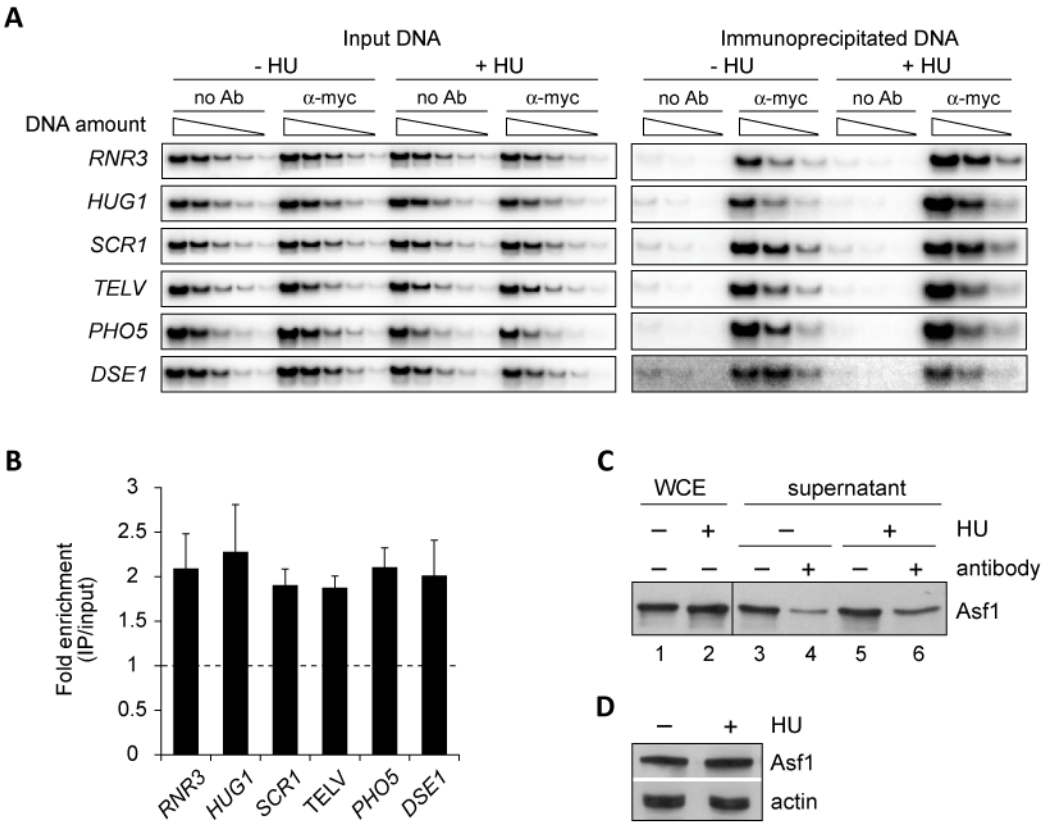

Figure 4-6. Asf1 is recruited to DNA upon treatment with hydroxyurea.
A. Raw PCR data from a representative Asf1-Myc ChIP experiment. The six loci analyzed are indicated on the left. Cells were either untreated, or exposed to 0.2 M HU for 1 hour. The data from Fig. 4-3A are included in this panel. **B.** Quantitation of ChIP analysis of Asf1 occupancy at specific loci in HU-treated cells relative to untreated cells (the latter set to 1). Averages of experiments that were repeated 16 (*RNR3* and *HUG1*), 6 (TELV), 4 (*SCR1* and *DSE1*) or 3 (*PHO5*) independent times are shown. Error bars indicate standard deviations from the mean. **C.** Asf1-Myc is efficiently immunoprecipitated from HU-treated whole cell extracts (WCEs). Representative example of Asf1-Myc immunodepletion monitored by anti-myc immunoblotting. The starting WCE was from cells grown in the absence (-) or presence (+) of HU for 1 hour. **D.** Asf1 protein levels do not change upon treatment with HU, as measured by anti-myc immunoblotting. Early log phase cells were grown in the absence (-) or presence (+) of HU for one hour. Actin is the loading control.

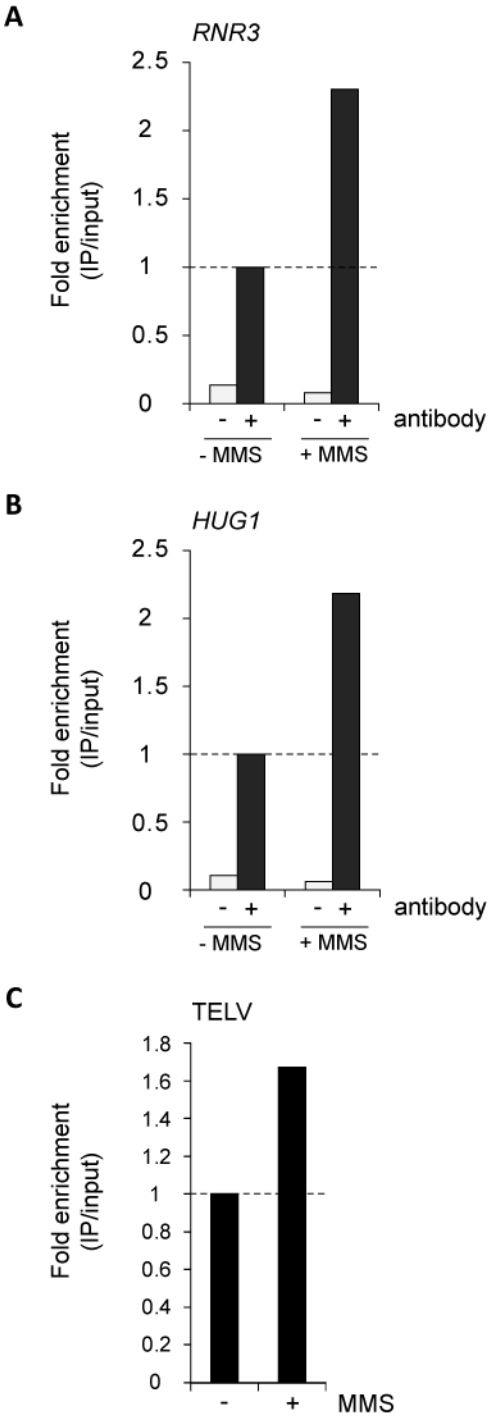

Figure 4-7. Asf1 is recruited to chromatin upon treatment with MMS. Chromatin immunoprecipitation analysis of Asf1-Myc crosslinking in cells treated with 0.05 % MMS for 1 hr (control cells were untreated). PCR primers specific to the promoters of *RNR3* (A) or *HUG1* (B), or to the TELV geneless region (C) were used. These are the results of a single experiment. PCR reactions in (C) were performed by Amelia Walker.

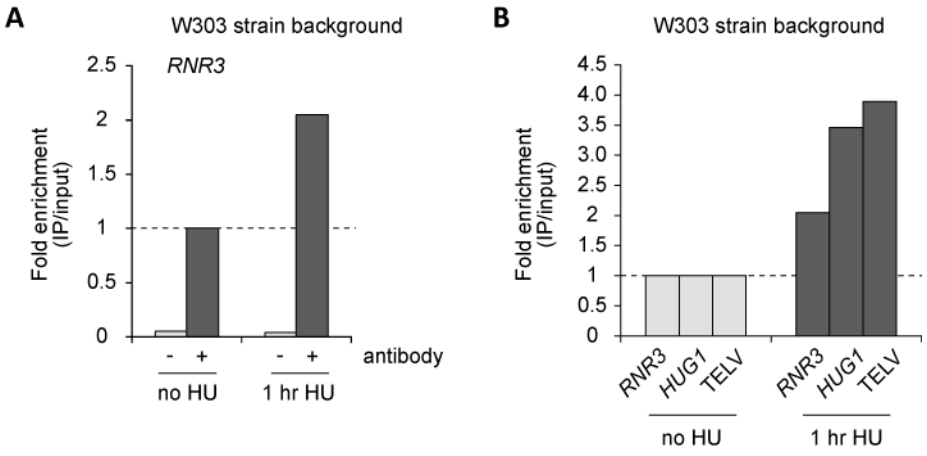

Figure 4-8. Asf1 association with chromatin increases upon HU treatment in W303 cells. A. Chromatin immunoprecipitation analysis of Asf1-Myc in the W303 strain background. This single analysis of crosslinking to *RNR3* included a 'no antibody' control. **B.** Chromatin immunoprecipitation analysis of Asf1-Myc in the W303 strain background. Crosslinking to three loci was analyzed in a single experiment.

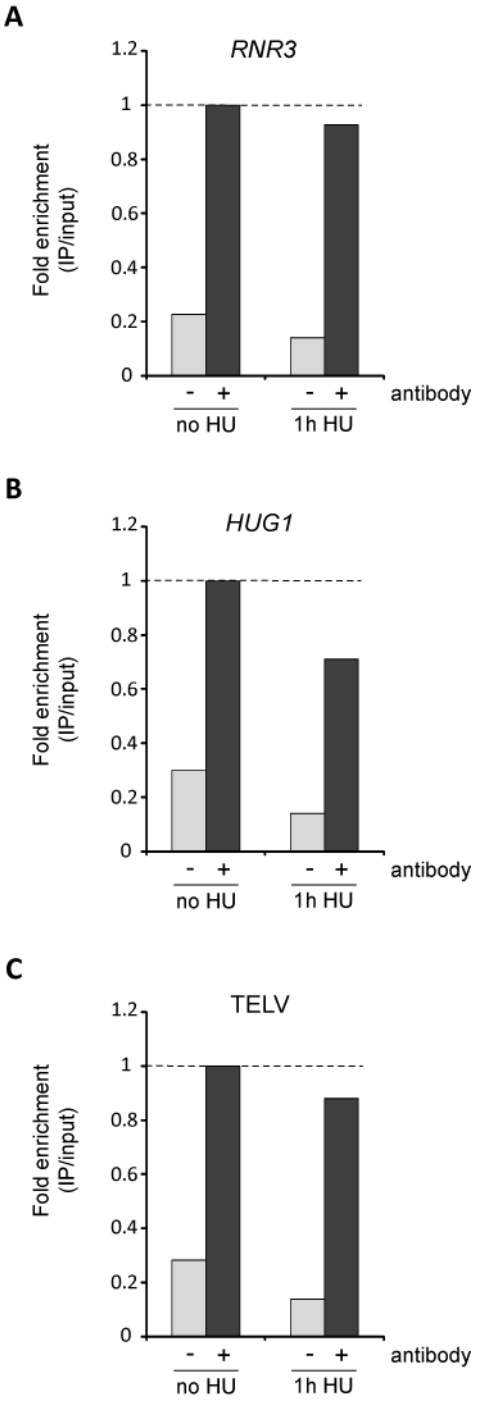

Figure 4-9. Rtt109 is not recruited to chromatin under conditions of replication stress caused by HU. Early log phase cells were grown in the absence (no HU) or presence (1h HU) of HU for one hour prior to formaldehyde crosslinking and ChIP. Rtt109-Myc associates with the promoters of *RNR3* (A) and *HUG1* (B), and with TELV (C), under normal conditions, as detected by ChIP and RT-PCR. Rtt109-Myc was not further recruited to these regions of chromatin upon treatment with HU. These are the results of a single analysis.

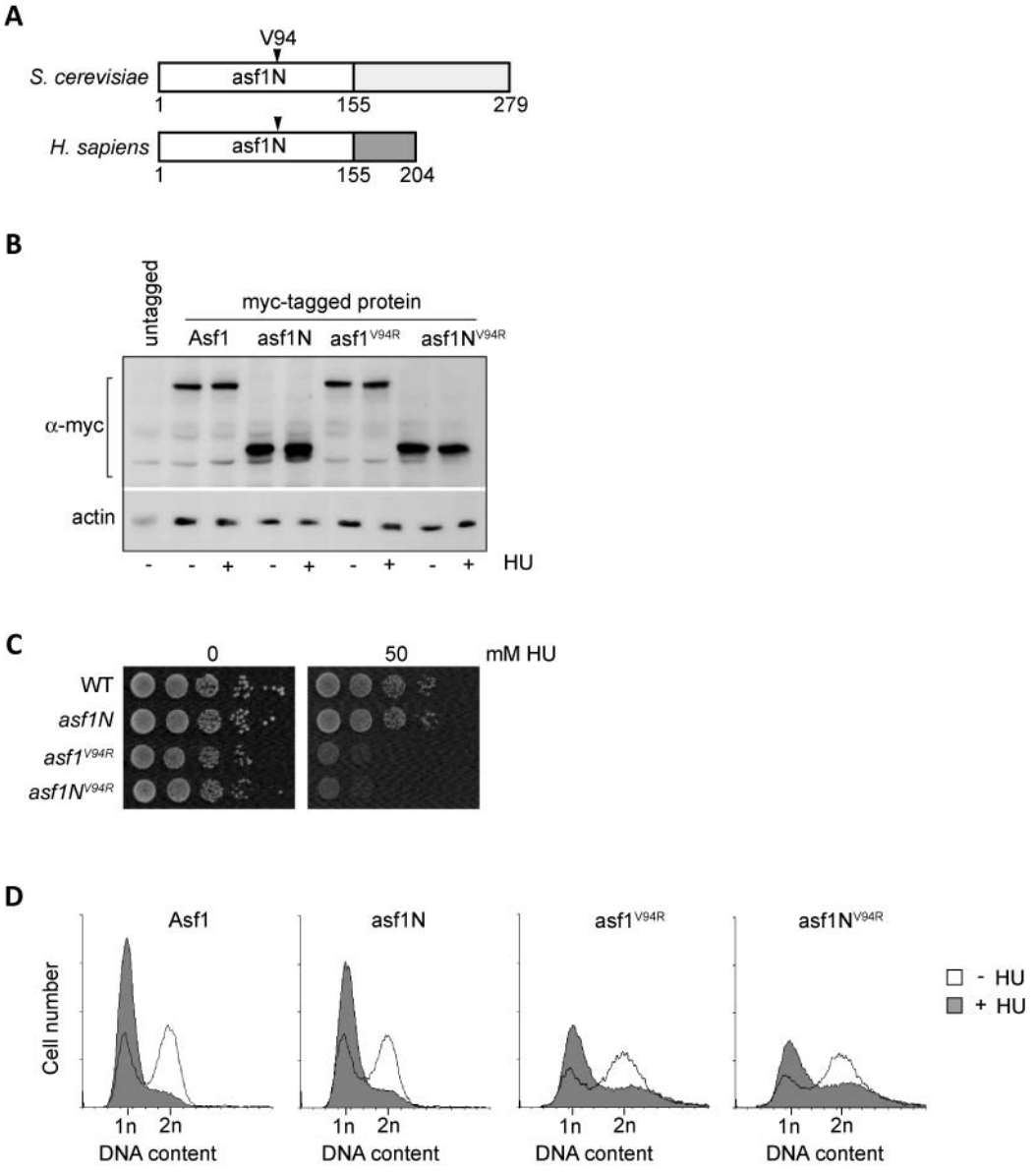

Figure 4-10. Characterization of Asf1 mutants. A. Domain structure of Asf1 from budding yeast and human. **B.** Immunoblotting analysis showing steady-state expression of myc-tagged versions of Asf1, asf1V94R, asf1N and asf1NV94R. Actin is the loading control. **C.** Hydroxyurea sensitivity of wild type and *ASF1* mutant strains. Serial dilutions of early log phase cells were spotted onto rich medium (0 mM HU) or medium containing 50 mM HU. Photographs were taken after two days. **D.** Flow cytometry analysis of DNA content of untreated and HU-treated cells (1 hr).

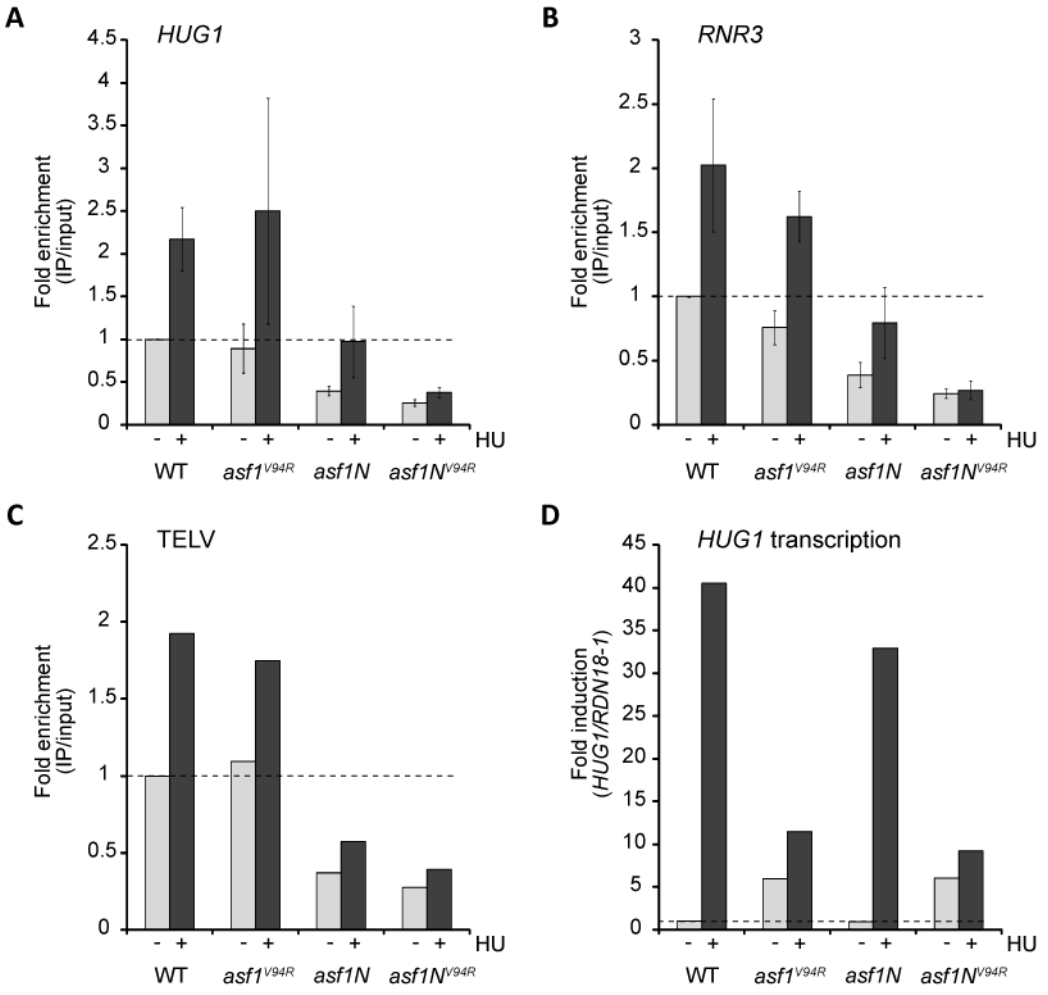

Figure 4-11. Association of Asf1 with chromatin is not needed for its ability to promote derepression of the DNA damage response genes. A. Wild type and mutant versions of Asf1 were assayed for crosslinking to the promoter of *HUG1* by ChIP. Protein occupancy is normalized to the signal for wild type Asf1 in untreated cells (set to 1). Experiment was performed in triplicate. Error bars indicate standard deviation from the mean. **B.** Chromatin immunoprecipitation was performed as in (A) using primers specific to the promoter of *RNR3*. **C.** Chromatin immunoprecipitation was performed using primers specific to the TELV geneless region. These are the results of a single experiment. **D.** Relative transcription of *HUG1* normalized to *RDN18-1*. The RT-PCR signal obtained from untreated wild type cells is set to 1. Bars represent the averages obtained from two independent experiments. Ranges (in order of bars, left to right) are: n/a, 19.43, 2.52, 5.22, 0.36, 23.19, 0.33 and 4.89. n/a = not available.

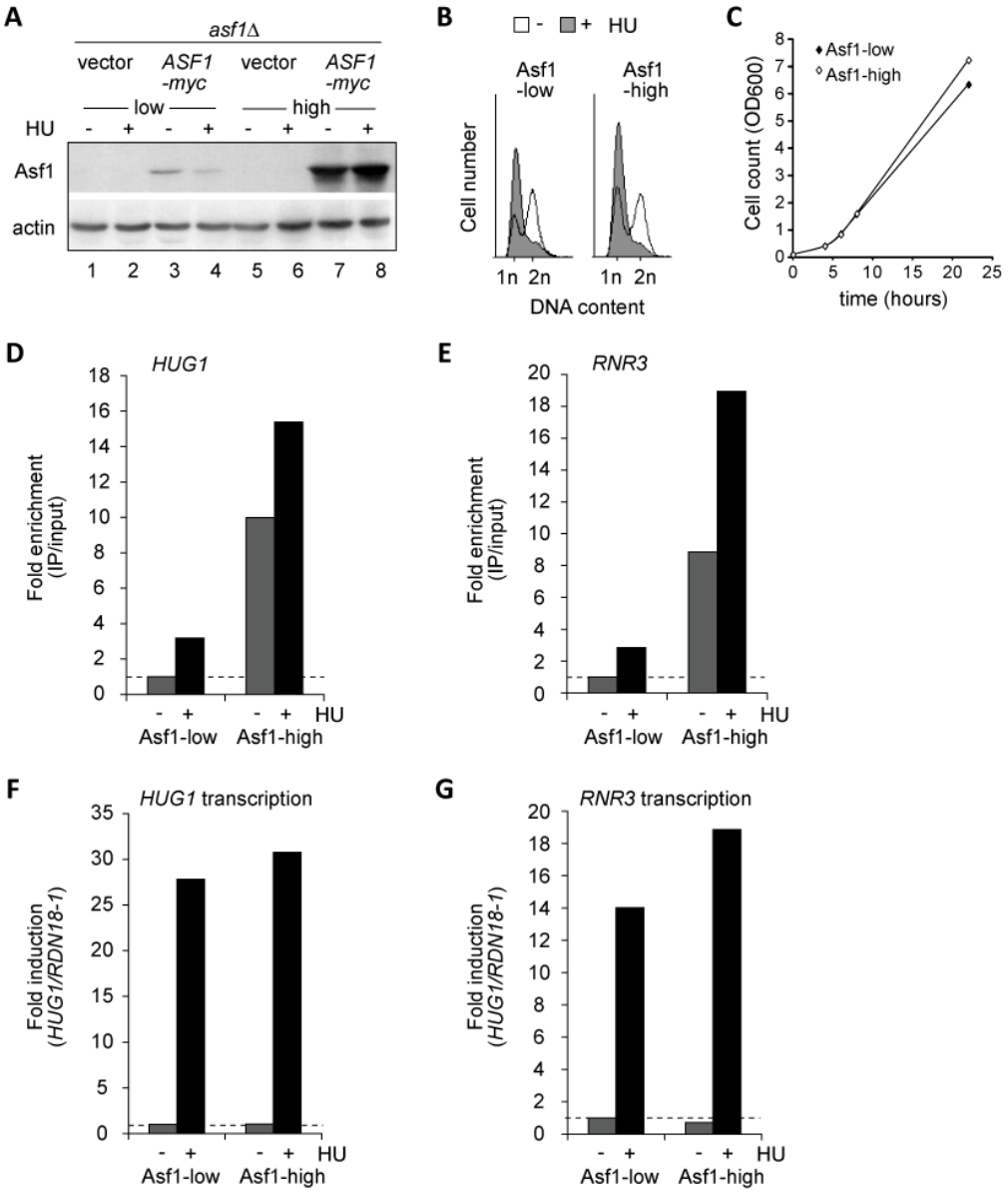

Figure 4-12. Asf1 association with chromatin is increased when Asf1 is overexpressed. **A.** Immunoblotting analysis of Asf1-Myc expression. Actin is the loading control. **B.** Flow cytometry analysis of DNA content of low- and high-expressing Asf1-Myc strains. **C.** Cells expressing low- or high-copy Asf1-Myc grow at similar rates. **D.** Relative Asf1 occupancy at *HUG1* in untreated and HU-treated (1 hr) cells (occupancy in untreated, low-expressing, cells is set to 1) as measured by ChIP. **E.** Analysis of Asf1 occupancy at *RNR3* (as for *HUG1*, D). **F.** Relative transcription of *HUG1/RDN18-1* in low- and high-expressing Asf1-Myc strains -/+ HU treatment (1 hr). RT-PCR signal from untreated Asf1-Myc (low) cells are set to 1. **G.** Analysis of *RNR3* transcription (as for *HUG1*, E). Graphs in D-G represent averages of duplicate experiments. Ranges (in order of bars, left to right) are: (D) n/a, 2.85, 1.07, 0.49; (E) n/a, 0.76, 4.52, 6.04; (F) n/a, 1.29, 0.03, 1.7; (G) n/a, 0.4, 0.17, 0.06. n/a = not available.

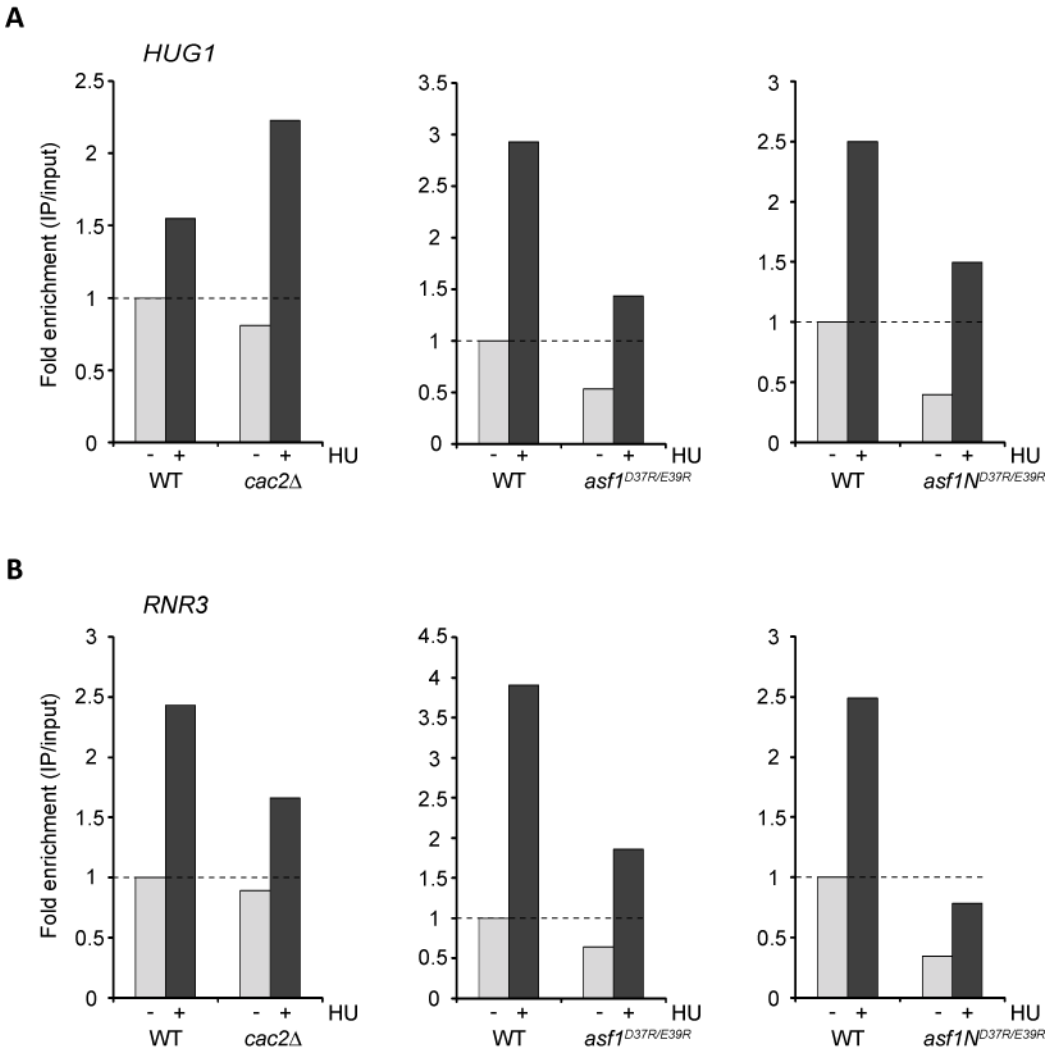

Figure 4-13. Constitutive binding of Asf1 to chromatin is decreased by a mutation in *ASF1* that reduces binding to CAF-I or HIR. A. Relative Asf1 occupancy at *HUG1* in untreated and HU-treated (1 hr) cells was measured by ChIP. Occupancy in wild type, untreated cells is set to 1. **B.** Analysis of Asf1 occupancy at *RNR3* (as for *HUG1*, A). Averages from two independent experiments are shown. Ranges (in order of bars, left to right) are: (A) (left): n/a, 0.04, 0.02, 0.23; (middle): n/a, 1.44, 0.03, 0.17; (right): n/a, 0.58, 0.03, 0.46; (B) (left): n/a, 0.49, 0.21, 0.05; (middle): n/a, 2.46, 0.08, 0.16; (right): n/a, 0.38, 0.01, 0.19. n/a = not available.

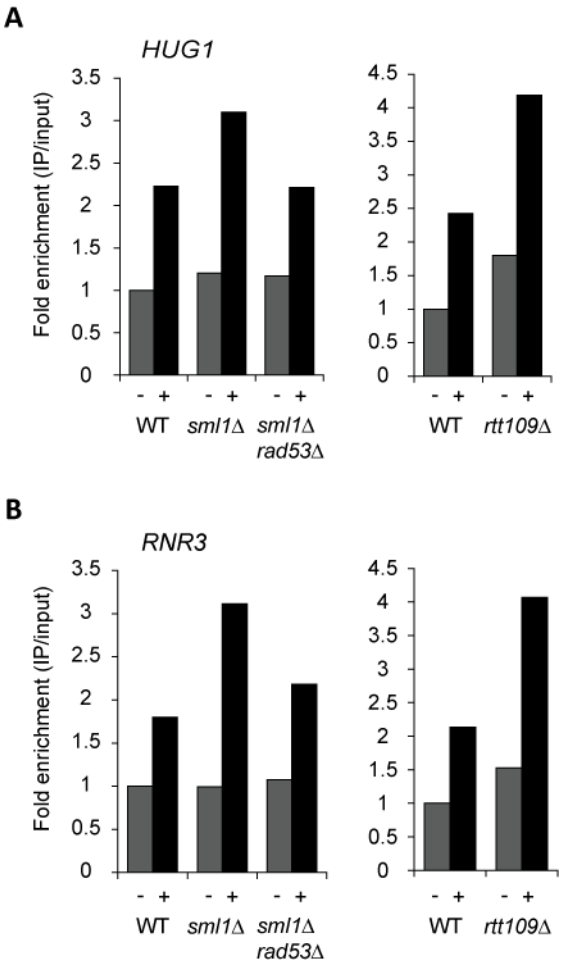

Figure 4-14. Asf1 is recruited to chromatin independently of Rad53 and Rtt109. A. Relative Asf1 occupancy at *HUG1* in untreated and HU-treated (1 hr) cells was measured by ChIP in the indicated strains. *SML1* is deleted in cells lacking RAD53 to allow viability. Occupancy in wild type, untreated cells is set to 1. **B.** Analysis of Asf1 occupancy at *RNR3* (as for *HUG1*, A). Averages from two independent experiments are shown. Ranges (in order of bars, left to right) are: (A) (left): n/a, 0.68, 0.25, 2.39, 0.77, 1.12; (right): n/a, 1.27, 0.5, 1.32; (B) (left): n/a, 0.15, 0.28, 2.35, 0.58, 0.95; (right): n/a, 0.14, 0.22, 2.75. n/a = not available.

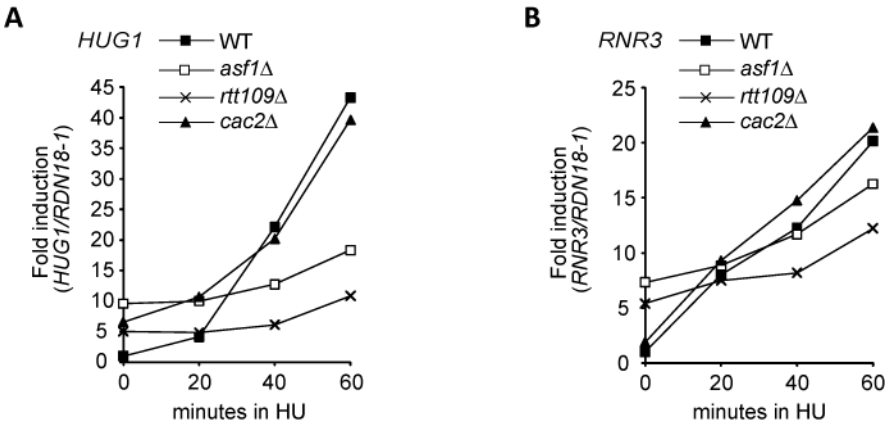

Figure 4-15. Transcription of DNA damage response genes is impaired in cells that lack H3K56 acetylation. A. *HUG1* derepression is compromised in *asf1⊿* and *rtt109⊿* cells. Early log phase cells were grown in YPD or YPD + 0.2 M HU for 20, 40 or 60 min. RNA was isolated from the indicated strains at each time point and quantitative RT-PCR was performed. Relative transcription of *HUG1* was normalized to *RDN18-1*. The RT-PCR signal obtained from untreated wild type cells is set to 1. Time points represent the averages obtained from two independent experiments. Ranges are listed in order of time (0, 20, 40, 60 min) for each strain: WT: n/a, 0.22, 8.03, 5.57; *asf1⊿*: 3.24, 1.07, 1.01, 1.7; *rtt109⊿*: 2.78, 1.85, 2.7, 2.98; *cac2⊿*: 2.8, 0.63, 3.19, 11.07. **B.** *RNR3* derepression is delayed in *asf1⊿* and *rtt109⊿* mutants. Experiments were performed as in (A) using primer pairs specific to the promoter of *RNR3*. Ranges are listed in order of time (0, 20, 40, 60 min) for each strain: WT: n/a, 1.94, 1.82, 2.81; *asf1⊿*: 0.62, 0.68, 2.76, 4.42; *rtt109⊿*: 3.51, 3.16, 2.95, 5.15; *cac2⊿*: 0.51, 1.23, 1.75, 6.84. n/a = not available.

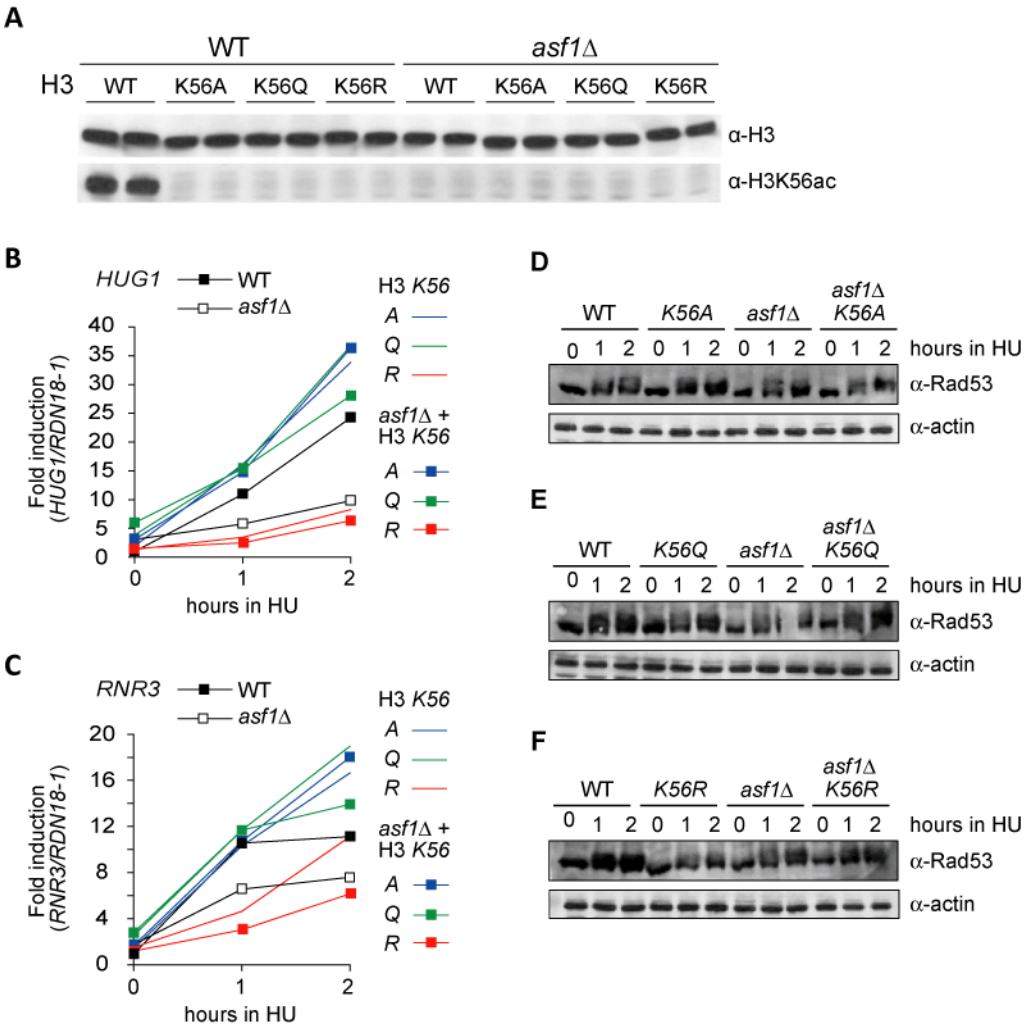

Figure 4-16. H3K56 acetylation by Rtt109 is important for the derepression of DNA damage response genes under conditions of replication stress.
A. Specificity of the H3K56ac antibody. Cells grown to early log phase in YPD were collected and total protein was prepared using a standard TCA preparation protocol. SDS-PAGE and Western blotting was performed using anti-H3K56ac or anti-H3 (control) antibodies. Identical cell equivalents of protein were loaded. **B.** *HUG1* derepression is impaired in H3K56R point mutants. Early log phase cells were grown in YPD or YPD + 0.2 M HU for 0, 1 or 2 hours. RNA was isolated from the indicated strains at each time point and quantitative RT-PCR was performed. Relative transcription of *HUG1* was normalised to *RDN18-1*. The RT-PCR signal obtained from untreated wild type cells is set to 1. These are the results of a single experiment. **C.** *RNR3* derepression is delayed in H3K56R cells. Experiment was performed as in (B) using primer pairs specific to the promoter of *RNR3*. **D-F.** H3K56 point mutants activate Rad53. The indicated strains were grown as in (B) and protein was prepared as in (A). Immunoblotting was done using anti-Rad53 and anti-actin (control) antibodies.

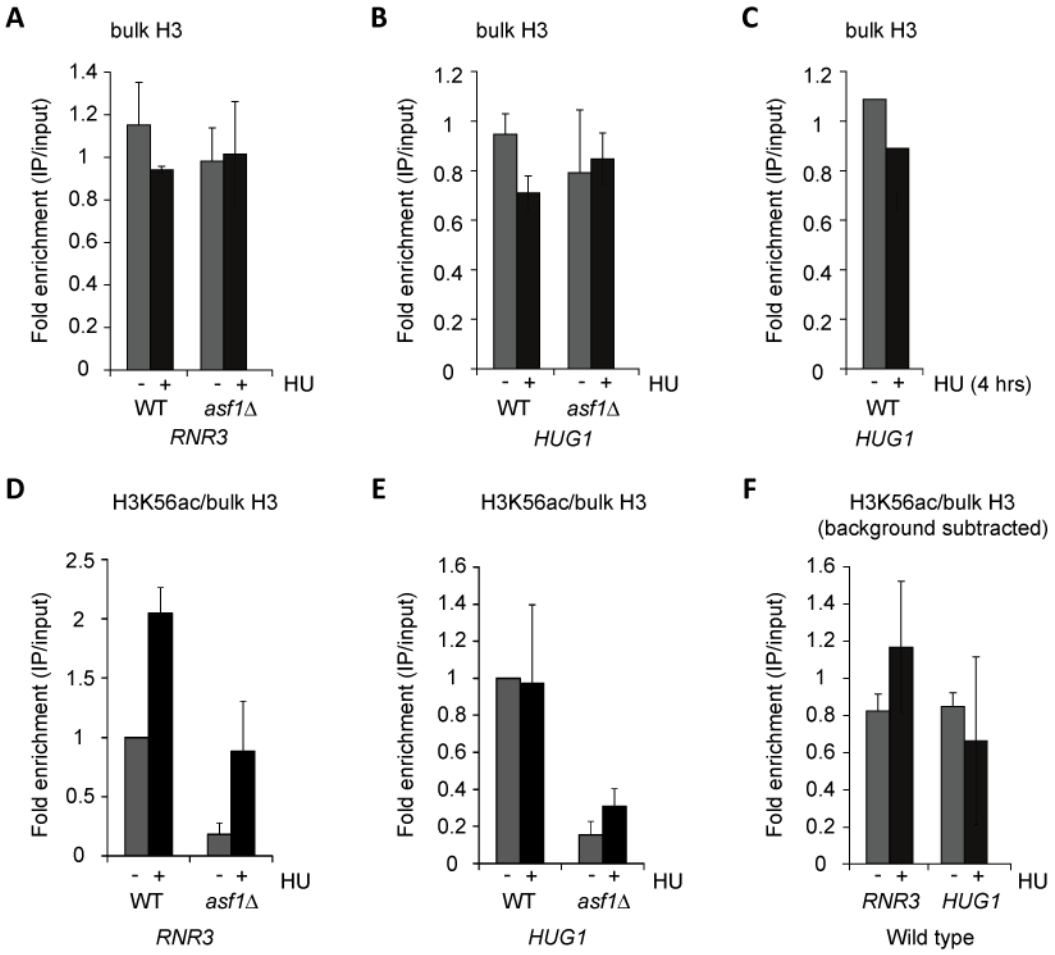

Figure 4-17. H3K56 acetylation at the promoters of DNA damage response genes. A. Wild type and *asf1*⊿ cells were grown in YPD (-HU) or YPD + 0.2 M HU (+HU) for one hour prior to formaldehyde crosslinking and ChIP using α-H3 antibody. Quantitative RT-PCR reactions were performed in triplicate using primer pairs specific to the promoter of *RNR3*. Immunoprecipitated DNA was normalised to input DNA and signal obtained in untreated cells was set to 1. **B.** RT-PCR reactions were performed as in (A) using primer pairs specific for the promoter of *HUG1*. **C.** Wild type cells were grown in YPD (-HU) or YPD + 0.2 M HU (+HU) for four hours and ChIP was carried out as in (A). **D.** Chromatin immunoprecipitation from whole cell extracts in (A) was performed using α-H3K56ac antibody and primer pairs specific to the promoter of *RNR3*. Immunoprecipitated DNA was normalised to input DNA and signal obtained in untreated cells was set to 1. H3K56ac PCR signal was normalised to bulk H3. **E.** Chromatin immunoprecipitation was performed as in (D) using primer pairs specific to the promoter of *HUG1*. **F.** Quantitation of RT-PCR reactions from (D) and (E) after background subtraction: H3K56ac/H3 signal obtained in *asf1*⊿ cells was subtracted from that of WT cells. Experiments were performed in triplicate, except (C), which was performed in duplicate. Error bars indicate standard deviations from the mean.

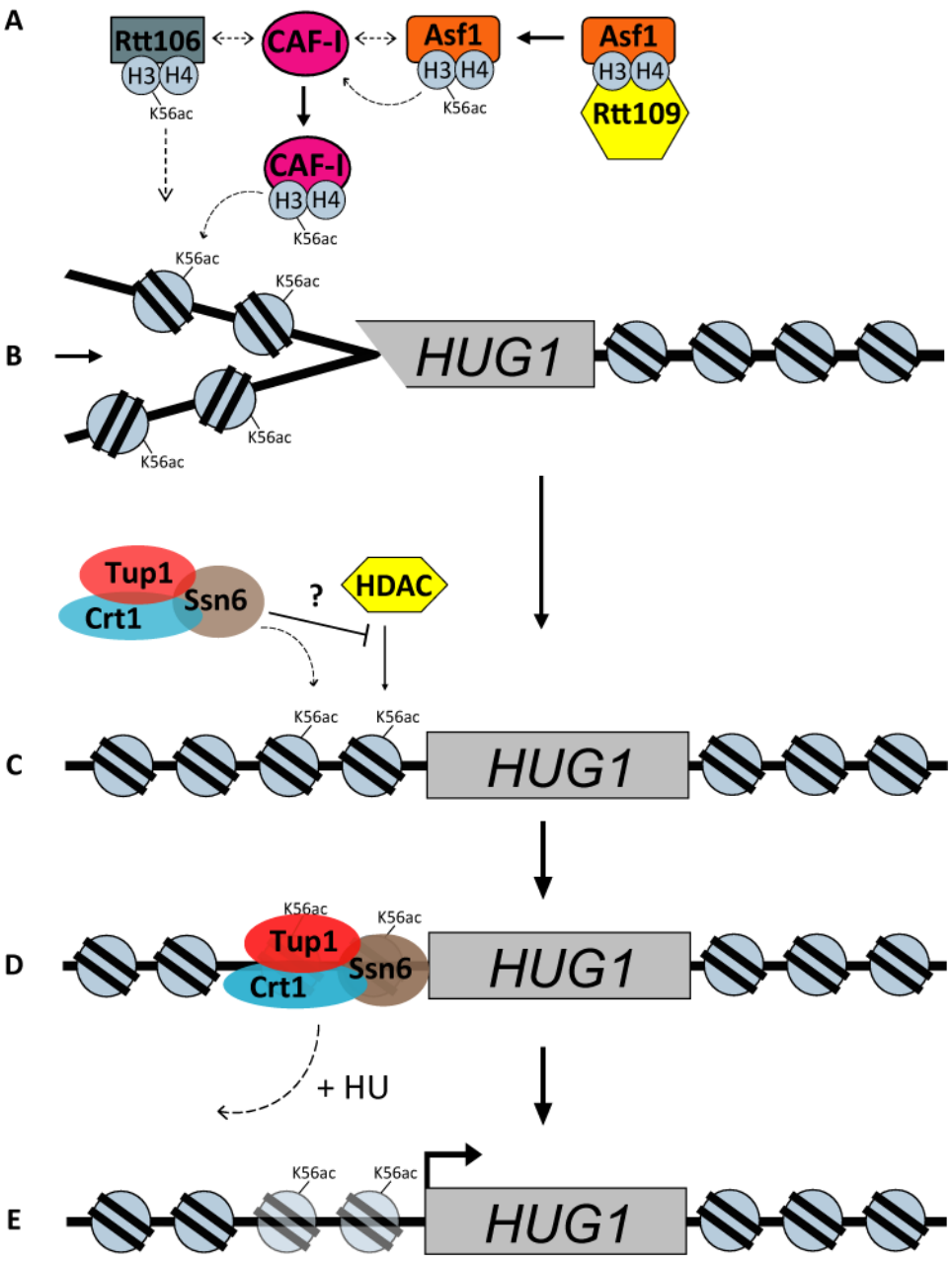

Figure 4-18. Model for the regulation of DDR gene transcription by H3K56 acetylation. A. Asf1 stimulates H3K56 acetylation by the Rtt109 lysine acetylase. **B.** CAF-I, Rtt106 and Asf1 contribute to H3K56ac deposition during DNA replication. **C.** Under normal growth conditions, the Crt1 and Tup1/Ssn6 repressors are recruited to the promoter of *HUG1* and deacetylation of H3K56 in this region is prevented. **D.** *HUG1* transcription is kept repressed under normal conditions, but upon treatment with HU, Crt1 and Tup1/Ssn6 dissociate. **E.** Preserved H3K56-acetylated molecules in the *HUG1* promoter allow rapid *HUG1* derepression upon replication stress.

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Chapter 5

Genetic and functional interactions between histone chaperone Asf1 and the SWI/SNF chromatin remodeller

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Introduction

SWI/SNF is a large multisubunit complex that possesses DNA-stimulated ATPase activity[1,2] and chromatin remodelling functions[3-5]. In yeast, SWI/SNF contains 12 subunits[6,7]: Snf2, Snf5 and Swi3 make up the conserved catalytic core[8], with Snf2 being the ATPase subunit. The expression of approximately 6% of genes is dependent on Snf2 function[9,10]. At these genes, SWI/SNF alters histone-DNA interactions, probably through intranucleosomal DNA looping, which could lead to histone octamer displacement and subsequent gene activation[5,11]. SWI/SNF may also have roles in transcriptional repression[9,10,12] and elongation[13]. In addition, SWI/SNF has been implicated in DNA replication[14], DNA silencing[15], DNA repair[16,17], and is thought to function as a tumour suppressor in human cells[18].

Early evidence indicating that Asf1 and Snf2 might function together came from studies in *Drosophila*. dAsf1 and subunits of the Brahma chromatin remodelling complex, including the Snf2 ATPase homologue, interacted genetically and coimmunoprecipitated in vitro[19]. Additionally, GST pull-down experiments indicated that the physical interaction between dAsf1 and Brahma is likely direct[19]. Microarray studies have revealed significant overlap between the genes affected by deletion of *ASF1* and components of the SWI/SNF remodelling complex[9,20], suggesting that Asf1 and SWI/SNF may have common functions in transcription. Indeed, Asf1 and SWI/SNF both appear to have roles in transcriptional regulation at

the *PHO5* gene[12,21-23]: *asf1*Δ and *snf2*Δ mutants show delayed rates of histone eviction (upon gene induction) and histone deposition (following gene repression) at the *PHO5* promoter, and synthetic effects for histone eviction were seen in the *asf1*Δ *snf2*Δ double mutant. Moreover, chromatin disassembly by an Asf1-dependent mechanism was necessary for SWI/SNF recruitment to the promoter of *PHO5*[22]. In addition to their roles in transcriptional initiation, Asf1 and SWI/SNF may also work together in transcriptional elongation[13,24].

Although several studies have addressed how Asf1 and SWI/SNF might coordinate transcriptional activation of *PHO5*, little is known about the other ways in which these two chromatin modulators might function together in the cell. In this study, we provide evidence that in the course of carrying out its chromatin functions Asf1 can work both together with, and separately from, the SWI/SNF chromatin remodelling complex.

Results

ASF1 interacts genetically with the SWI/SNF chromatin remodelling complex

Our SGA analysis uncovered a potential genetic interaction between *ASF1* and *SNF2*, the catalytic subunit of the SWI/SNF chromatin remodelling complex (see Chapter 3). To confirm this interaction an *asf1*Δ *snf2*Δ double mutant haploid strain was generated using single-step gene replacement and its growth rate was compared to the growth rates of the *asf1*Δ and *snf2*Δ single mutants. The *asf1*Δ and *snf2*Δ single mutants grew at similar rates and both were slower-growing than the congenic wild type strain; however, the *asf1*Δ *snf2*Δ double mutant grew at an even slower rate than either single mutant (Fig. 5-1A). To determine whether *ASF1* also interacts

genetically with other components of the SWI/SNF complex, an *asf1Δ snf5Δ* double mutant strain was created. Snf2 and Snf5, along with Swi3, make up the conserved catalytic core of SWI/SNF[8]. Indeed, the *asf1Δ snf5Δ* double mutant was slower-growing than the corresponding single mutants (Fig. 5-1B). Therefore, combined deletion of *ASF1* and SWI/SNF subunits results in synthetic growth defects.

Consistent with a synthetic sick interaction between *ASF1* and *SNF2*, *asf1Δ snf2Δ* double mutants were more compromised for growth at 37 °C than either the *asf1Δ* or *snf2Δ* single mutants, although *snf2Δ* cells were slightly impaired for growth at 37 °C (Fig. 5-1C). We next tested the ability of wild type, *asf1Δ*, *snf2Δ* and *asf1Δ snf2Δ* cells to grow on solid medium containing either glucose (YPD) or glycerol (YPG) as the sole carbon source. *asf1Δ* cells were only slightly, if at all, impaired for growth on YPG compared to wild type cells (Fig. 5-1D). However, in agreement with early studies reported by Neigeborn and Carlson[25], *snf2Δ* cells were severely compromised for growth on YPG. *asf1Δ snf2Δ* double mutants were unable to grow after five days when glycerol was the sole carbon source. One reason that *asf1Δ snf2Δ* mutants were unable to grow on YPG medium could be that they lack mitochondrial DNA and consequently have impaired cellular respiration. We explored this possibility by confirming the presence of the *COX3* mitochondrial gene in *snf2Δ* and *asf1Δ snf2Δ* mutants using PCR (Fig. 5-1E). *COX3* DNA was present in three independent wild type, *snf2Δ* or *asf1Δ snf2Δ* colonies, but was absent in wild type rho° control cells. Therefore, *asf1Δ snf2Δ* mutants are unable to use glycerol as their only carbon source, but this defect is probably not due to a lack of mitochondrial DNA in these mutants. Collectively, these results show that deletion of both *ASF1* and *SNF2*

results in additive phenotypes with respect to overall growth, suggesting that *ASF1* and *SNF2* may function in partially overlapping, redundant pathways to contribute to normal growth.

Asf1 and Snf2 may interact physically

Preliminary evidence in our lab has suggested for the first time that there may be a physical interaction between yeast Asf1 and Snf2 (Fig. 5-2). Using a standard tandem affinity purification (TAP) protocol[26], we have found that Asf1 and Snf2 copurify in native protein complexes. Specifically, Snf2-HA was enriched in the elution fraction from the *SNF2-HA ASF1-TAP* extract compared to the elution from the *SNF2-HA* control (Fig. 5-2). The reciprocal TAP pulldown remains to be performed; however, the evidence that Asf1 interacts physically with SWI/SNF subunits in *Drosophila* provides support for an interaction between Asf1 and Snf2 that is conserved in higher organisms[19].

Asf1 and SWI/SNF work in partially overlapping pathways to protect against replication stress caused by depletion of dNTPs, but separate pathways for responding to DNA damage caused by covalent modification of DNA

To gain some idea as to how Asf1 and SWI/SNF might be interacting functionally, we looked for additional phenotypes of cells lacking both *ASF1* and a component of the SWI/SNF complex (*SNF2* or *SNF5*). Given that Asf1 and SWI/SNF have both been implicated in DNA replication and the DDR[14,16,17,27-30] we tested the ability of *asf1*∆ *snf2*∆ and *asf1*∆ *snf5*∆ double mutants (and corresponding single mutants) to grow in the presence of agents that cause replication stress or

DNA damage. Wild type cells grow more slowly in the presence of HU, an inhibitor of DNA replication[31], than in media lacking HU (compare Fig. 5-1A and 5-3C). Consistent with shared functions for Asf1 and SWI/SNF in protecting against replication stress, *asf1⊿*, *snf2⊿* and *snf5⊿* single mutants were all more sensitive to HU than wild type cells[17,27] (Fig. 5-3). However, *asf1⊿ snf2⊿* and *asf1⊿ snf5⊿* double mutants were more sensitive to HU than the matching single mutants, which implies that Asf1 and SWI/SNF must also exist in separate pathways to promote cellular resistance to replication stress that is caused by dNTP depletion.

Compared to wild type cells, cells lacking *ASF1* were sensitive to MMS (as previously reported[27] and Fig. 5-4A). On the contrary, loss of *SNF2* is not associated with an enhanced sensitivity to MMS[32] (Fig. 5-4A). *asf1⊿ snf2⊿* cells were more sensitive to MMS than cells lacking only *ASF1*, indicating that *ASF1* and *SNF2* probably do not function in the same pathway for responding to DNA alkylation. Similarly, *asf1⊿ snf2⊿* double mutants were also more sensitive to DNA damage caused by UV irradiation than either single mutant (Fig. 5-4B). In conclusion, *ASF1* and *SNF2* exist in separate, redundant pathways to promote cell survival after DNA damage caused by treatment with MMS or UV irradiation.

The fact that *snf2⊿* single mutants were not sensitive to MMS at the doses used in this study suggests that Snf2 might play a more important role in responding to reduced levels of cellular dNTPs, rather than general replication stress. On the other hand, due to the redundancy in the yeast genome, it is possible that in the absence of *SNF2*, a different chromatin or replication protein can protect against replication stress induced by treatment with MMS. Although HU and MMS both result in

replication stress, there is a handful of examples of yeast gene deletions that result in sensitivity to growth in the presence of MMS, but not HU[33]. These examples include deletions of *CAC2*, a subunit of the CAF-I histone chaperone, and *VID21*, a component of the NuA4 KAT complex. Interestingly, the reverse does not seem to be a common phenomenon; that is, to our knowledge, all reported examples of gene deletions that result in HU sensitivity also cause sensitivity to MMS. However, a *rad53Ha* allele that encodes an HA-tagged version of Rad53 has been identified that conveys resistance to MMS, but HU-sensitivity[34]. Nonetheless, this effect was attributed to lower levels of Rad53-HA protein present in *rad53Ha* cells, which is not the case in cells lacking *SNF2* (Fig. 5-5A). Therefore, *snf2⊿* mutants seem to present a unique case; however, it is still possible that *snf2⊿* cells are sensitive to more lethal doses of MMS than were used in this experiment, or that *SNF2* deletions in other genetic backgrounds might be MMS-sensitive. Interestingly, it has been suggested by Chai et al. that *snf2⊿* cells are indeed sensitive to MMS, even though this claim is not evident from their published results[17]. It seems probable that these results may have been misinterpreted due to the slower-growing phenotype of *snf2⊿* cells under normal conditions.

Asf1 and Snf2 have partially overlapping functions in cell cycle and checkpoint control

Since checkpoint kinase Rad53 is partially activated in normally growing *asf1⊿* cells[30] (Chapter 4), we used SDS-PAGE and western blotting to investigate whether this would also be the case in *asf1⊿ snf2⊿* cells. Indeed, *asf1⊿ snf2⊿* cells growing in rich medium showed a similar slow-migrating form of Rad53 as seen in *asf1⊿* single

mutants (Fig. 5-5A). However, after treatment with HU, Rad53 was only slightly further activated in *asf1*⊿ *snf2*⊿ cells, even though activation was normal in cells lacking only one of *ASF1* or *SNF2*. We hypothesized that Rad53 activation in response to replication stress may be delayed in *asf1*⊿ *snf2*⊿ cells because they are slow-growing (Fig. 5-1A and C; 5-3C). To test this, Rad53 activation was monitored in cells that were treated with HU for up to eight hours. As shown in Fig. 5-5B, after eight hours in HU, Rad53 was activated to near wild type levels in *asf1*⊿ *snf2*⊿ mutants. Therefore, under conditions of replication stress, Rad53 activation is delayed, but not absent, in *asf1*⊿ *snf2*⊿ cells.

Wild type cells treated with HU arrest in S phase because they are unable to replicate their DNA due to a limited supply of dNTPs; however, mutants with checkpoint problems attempt to continue through S phase, but encounter problems with DNA replication, resulting in further mutation or death[35]. Since *asf1*⊿ *snf2*⊿ cells may have problems with checkpoint control (Fig. 5-5A and B), we monitored cell cycle progression in wild type and mutant cells grown in the presence of HU using flow cytometry analysis. After two hours in HU, wild type and *snf2*⊿ cells arrested in early S phase, as did most *asf1*⊿ cells (Fig. 5-5C). However, consistent with problems in checkpoint control, *asf1*⊿ *snf2*⊿ mutants failed to fully arrest in S phase during even an eight-hour HU treatment.

To determine whether these checkpoint control problems cause the *asf1*⊿ *snf2*⊿ mutants to die when grown in HU, cells were grown in the presence of HU for 24 hours and then plated onto rich solid medium. Cells that have arrested growth when cultured in HU should be able to recover from replication stress when grown on

solid medium lacking HU. Indeed, wild type cells and *snf2*⊿ single mutants, both of which arrested growth in early S phase, were able to recover from the lengthy HU treatment (Fig. 5-5D, compare –HU and +HU). In agreement with their cell cycle progression profiles (Fig. 5-5C), *asf1*⊿ single mutants and *asf1*⊿ *snf2*⊿ double mutants were slightly, or severely, compromised in their ability to recover from replication stress, respectively (Fig. 5-5D). We conclude that *asf1*⊿ *snf2*⊿ mutants are unable to recover from extended replication stress and die as a consequence of faulty checkpoint control.

Derepression of two DNA damage response genes depends on Asf1 and SWI/SNF under conditions of replication stress

Since Asf1 and SWI/SNF have both been implicated in transcriptional activation of the *PHO5* gene[23], we tested the idea that Asf1 and Snf2 may function together to promote derepression of the DDR genes. As described in Chapter 4, Asf1 promotes derepression of *HUG1* and *RNR3* upon treatment with HU. Similarly, SWI/SNF has known roles in the derepression of *RNR3* during treatment with MMS[36]. Therefore, we used Northern blotting to measure the ability of cells lacking *ASF1*, *SNF2*, or both, to derepress *HUG1* and *RNR3* (Fig. 5-6A). Compared to wild type cells, *asf1*⊿ and *snf2*⊿ single mutants were partly blocked in their abilities to derepress *HUG1* and *RNR3* in response to treatment with HU (Fig. 5-6B and C). Strikingly, *HUG1* derepression was almost completely blocked in the *asf1*⊿ *snf2*⊿ double mutant, and *RNR3* transcription remained lower in the double mutant than either single mutant. We used an extended HU time course to show that even when given additional time, *asf1*⊿ *snf2*⊿ double mutants were unable to derepress *HUG1*

to the same levels as the single mutants (Fig. 5-6D). At *RNR3*, the double mutant was able to reach a similar level of derepression as the *snf2⊿* single mutant after four hours in HU (Fig. 5-6E). Collectively, our data suggest that Asf1 and SWI/SNF both work to promote the rapid derepression of DDR genes when cells are grown under conditions of replication stress.

Asf1 and SWI/SNF are recruited to the promoters of DNA damage response genes during replication stress

As shown in Chapter 4, Asf1 constitutively and inducibly associates with the promoters of DDR genes (Fig. 4-3 and 4-5 to 4-8): Asf1 is present at the promoters of *RNR3* and *HUG1* under normal conditions, and is further recruited to each promoter after treatment with HU or MMS. Similarly, the Reese group has shown that Snf2 is recruited to the promoter of *RNR3* after treatment with MMS[36]. To determine whether SWI/SNF is also recruited to the promoter of *RNR3* after treatment with HU, we performed ChIP in cells expressing Snf2-Myc or Snf5-Myc. Cells were grown in the presence or absence of 0.2 M HU for one hour prior to crosslinking with formaldehyde in conditioned media that lacks HU (see Chapter 4). Whole cell extracts were prepared and IPs carried out as described[37]. Approximately 3.5-fold more PCR signal was obtained from Snf2-Myc IPs that included anti-myc antibody compared to a 'no antibody' control (Fig. 5-7A; left panel). This indicates that Snf2 is likely present at the promoter of *RNR3* under normal conditions. After HU treatment, Snf2-Myc was enriched 11-fold above background (no antibody), confirming that Snf2-Myc association with the promoter of *RNR3* is increased after replication stress. Interestingly, although these are the results of a single experiment,

Snf5-Myc did not associate with the promoter of *RNR3* above background levels during normal growth (Fig. 5-7A; right panel). Snf5-Myc, however, was recruited to the *RNR3* promoter upon replication stress (Fig. 5-7A; right panel). These results are consistent with the possibility that a functional SWI/SNF complex is not present at the promoters of DDR genes until transcription becomes derepressed.

In ChIP analysis, it is often effective to normalise PCR signal obtained for a given gene/promoter to a negative control region of DNA[37], that is, a region of DNA with which the protein of interest does not associate with. In the case of varying growth/environmental conditions, a negative control region of DNA might be one where the binding of the protein of interest remains constant across treatment conditions. A common negative control region that is sometimes used to evaluate SWI/SNF binding is the *POL1* ORF[38,39]. To determine the specificity of SWI/SNF binding we therefore analyzed Snf2-Myc and Snf5-Myc association with *POL1* using primer pairs specific to the *POL1* ORF[40]. Upon HU treatment, we found little change in Snf2-Myc association with *POL1*; however, Snf5-Myc binding increased 1.7-fold at *POL1* (Fig. 5-7B; left panel). Next, we normalised the PCR signal obtained using *RNR3* primers to that obtained using *POL1* primers to confirm that Snf2-Myc recruitment to *RNR3* is specific and Snf5-Myc recruitment is non-specific (Fig. 5-7B; right panel). While these experiments need to be repeated, this preliminary data suggests that SWI/SNF is recruited to *RNR3* under conditions of replication stress caused by depletion of dNTPs. In addition, Snf5 may have a previously uncharacterized SWI/SNF-independent role in chromatin regulation at the *POL1* ORF when cells are experiencing replication stress. Similarly, since Snf2-

Myc, but not Snf5-Myc, remained associated with the promoter of *RNR3* under normal conditions, it may be true that Snf2 can also function separately from the rest of the SWI/SNF complex. In this case, promoter-bound Snf2 may set the stage for binding of other subunits upon replication stress conditions. Consistent with the idea of a SWI/SNF-independent role for Snf2 is the finding that Snf2, but not Snf5, is needed for the indirect repression of *SER3*, a gene needed for serine biosynthesis[41,42].

Given the putative physical interaction between Asf1 and Snf2 (Fig. 5-2), the synthetic transcriptional effects in the double mutant (Fig. 5-6), and the fact that Asf1 and SWI/SNF subunits are recruited to the promoter of *RNR3* under replication stress conditions (Fig. 4-6 and 5-7A), we hypothesized that the association of these proteins with the *RNR3* promoter might depend on one other. In support of this idea, Asf1 is needed for stable SWI/SNF recruitment to the promoter of *PHO5*[22]. We therefore used ChIP to first ask whether Asf1 is required for the association of Snf2-Myc or Snf5-Myc with the promoter of *RNR3*; however, Snf2-Myc and Snf5-Myc binding remained relatively unchanged in the absence of *ASF1* (Fig. 5-7C). We next tested whether the association of Asf1 with chromatin is altered in cells lacking *SNF2* and found that Asf1 associated normally with the promoters of *RNR3* and *HUG1* in *snf2*⊿ cells (Fig. 5-7D). In summary, Asf1 and SWI/SNF are independently recruited to the promoters of DDR genes during replication stress.

Histone H3 lysine 56 acetylation is not perturbed in cells lacking *SNF2*

Asf1 promotes transcriptional derepression of the DDR genes via an H3K56-dependent pathway (Chapter 4). Asf1 is needed for the in vivo acetylation of H3K56

by Rtt109, and as such, H3K56 acetylation is absent in *asf1*⊿ cells[43]. To determine whether Asf1 and SWI/SNF might work together in a pathway that involves the regulation of H3K56 acetylation, we compared bulk expression of H3K56 acetylation in wild type, *asf1*⊿, *snf2*⊿, and *asf1*⊿ *snf2*⊿ cells grown in rich medium. As expected, H3K56 acetylation was essentially absent from extracts prepared from *asf1*⊿ and *asf1*⊿ *snf2*⊿ cells (Fig.5-8A). Importantly, H3K56 acetylation was similar in wild type and *snf2*⊿ cells. Therefore, under normal conditions, *SNF2* is not required for H3K56 acetylation. However, this analysis did not rule out the idea that chromatin remodelling by SWI/SNF is needed for efficient deposition of K56-acetylated histone H3 into chromatin. To address this, we first performed ChIP using antibodies against bulk histone H3 in wild type cells, or cells lacking one or both of *ASF1* or *SNF2*.

Apart from a slight decrease in histone H3 occupancy at the promoter of *HUG1* in *snf2*⊿ cells (Fig. 5-8B), averaged results from three independent experiments showed that there were no large differences in histone H3 occupancy at the promoters of *HUG1* or *RNR3* in the strains tested under normal or HU conditions (Fig. 5-8B and C). Using bulk H3 occupancy as a control, we were then in a position to examine the state of H3K56ac at the *HUG1* and *RNR3* promoters. As discussed in Chapter 4, H3K56ac PCR signal (IP/input, normalised to bulk H3) obtained from *asf1*⊿ cells was subtracted from wild type cells. Likewise, H3K56ac signal obtained from *asf1*⊿ *snf2*⊿ cells was subtracted from *snf2*⊿ cells. Under normal conditions, there was a small increase in H3K56ac at the promoters of *HUG1* and *RNR3* in *snf2*⊿ cells compared to wild type cells (Fig. 5-8D and E; light grey bars). This effect

could be due to slightly higher levels of total H3K56ac in *snf2⊿* cells (Fig. 5-8A). During replication stress caused by HU, H3K56ac was decreased at the promoter of *HUG1* in wild type cells as well as in cells lacking *SNF2* (Fig. 5-8D). At *RNR3*, H3K56ac was increased upon treatment with HU in *snf2⊿* cells (Fig. 5-8E). Interpretation of these small changes in H3K56ac is hampered by the variability between experiments; however, it appears that there are no major defects in H3K56ac in cells that lack the Snf2 subunit of SWI/SNF. We conclude that Asf1 and SWI/SNF do not participate in a shared pathway to influence H3K56 acetylation.

Discussion

Using a large-scale genetic analysis in yeast, we have identified an interaction between histone chaperone Asf1 and the SWI/SNF complex. Although this interaction has been studied by others[12,19,22,23], we present the first thorough characterization of the interaction in yeast using genetic, physical and functional analyses. Firstly, *ASF1* interacts genetically with *SNF2* and *SNF5*, two subunits of the SWI/SNF chromatin remodelling complex (Fig. 5-1). Secondly, Asf1 and Snf2 copurify in vivo (Fig. 5-2). Thirdly, Asf1 and Snf2 both contribute to cell survival during replication stress conditions caused by depletion of dNTPs (Fig. 5-3). Asf1 also has a known protective role against DNA damage caused by treatment with MMS or UV irradiation, but Snf2 seems only to be needed when *ASF1* is missing, indicating that Asf1 and SWI/SNF participate in separate, redundant pathways to protect against DNA damage caused by covalent modification of DNA (Fig. 5-4). Fourthly, Asf1 and Snf2 exist in partially overlapping pathways to promote normal

cell cycle and checkpoint control (Fig. 5-5). In addition, Asf1 and Snf2 both promote derepression of two DDR genes: *HUG1* and *RNR3* (Fig. 5-6). Although Asf1 and SWI/SNF are both recruited to the promoter of *RNR3* during replication stress, their association with this promoter does not depend on one another (Fig. 5-7). Finally, bulk H3K56 acetylation is normal in *snf2⊿* cells, and there are only small changes in K56-acetylated histone H3 incorporation into the promoters of *HUG1* and *RNR3* in *snf2⊿* cells (Fig. 5-8).

Given that 1) the association of Asf1 with the promoter of DDR gene promoters is not required for its ability to contribute to derepression of these genes (Chapter 4), 2) Asf1 mainly contributes to transcriptional derepression of the DDR genes through an H3K56 acetylation-dependent pathway (Chapter 4), 3) H3K56 acetylation remains unaffected in *snf2⊿* cells, 4) SWI/SNF subunits are recruited to the promoter of *RNR3* normally in the absence of H3K56 acetylation, and 5) *asf1⊿ snf2⊿* double mutants have additive defects in *HUG1* and *RNR3* transcription after HU treatment, it is probable that Asf1 and SWI/SNF do not participate in the same major pathway to control transcriptional derepression of the DDR genes. Rather, we favour a model in which Asf1 and SWI/SNF have shared functions in DNA replication. Consistent with this notion, Asf1 and SWI/SNF have both been implicated in the process of DNA replication[14,44], and there is evidence that Asf1 and SWI/SNF interact physically (this study and[19]). We hypothesize that Asf1 and SWI/SNF associate with chromatin to carry out their respective histone chaperone and chromatin remodelling abilities to together facilitate an aspect of DNA replication.

Alternatively, the association of SWI/SNF with the promoters of DDR genes may be due solely to its role in facilitating transcriptional derepression of these genes. This idea is plausible because there is currently a lack of evidence supporting a link between SWI/SNF and replication fork stability and/or progression. However, given the repressive nature of chromatin and the fact that SWI/SNF can contribute to in vivo minichromosome maintenance[14], it does seem logical that SWI/SNF could play a role, along with Asf1, in facilitating DNA replication. In this view, under normal conditions, SWI/SNF may assist in the removal of histones ahead of the replication fork via chromatin remodelling functions, perhaps donating excess histones to Asf1 (Fig.5-9A). In the same way, under replication stress conditions, SWI/SNF could remodel chromatin at or near stalled replication forks, again supplying Asf1 with a surplus of histones that would be kept in close proximity to the stalled fork, thereby allowing its efficient resumption after replication stress removal (Fig. 5-9B). Consistent with this idea, other chromatin remodelling machineries have been implicated in the DNA replication process, and Asf1 binds to excess soluble histones in human cells[45,46]. This would explain the putative physical interaction between Asf1 and SWI/SNF, and the exacerbated phenotypes observed when *asf1⊿ snf2⊿* double mutants are grown in the presence of HU, in particular, the inability of the double mutant to recover from replication stress. Due to redundancy in the yeast genome, this process can remain partially functional in the absence of either Asf1 or SWI/SNF, in which case a second chromatin remodeller or histone chaperone may be able to interact with Asf1 or SWI/SNF, respectively. Since *snf2⊿*

cells are not sensitive to MMS, it seems likely that Asf1 and SWI/SNF might be specifically involved in a pathway that responds to the depletion of dNTPs.

In conclusion, we propose a model in which Asf1 and SWI/SNF exist in a shared, partially redundant pathway for responding to replication stress that is caused by a reduction in the concentration of dNTPs available to the cell for replication of the genome. Further research into the mechanism by which Asf1 and SWI/SNF might function together to respond to replication stress will be of the utmost importance. In particular, do Asf1 and SWI/SNF exist in a pathway that contributes to the stability of stalled replication forks? Franco et al. have elegantly shown that Asf1 is involved in replisome stability[44] and perhaps SWI/SNF too is involved in this process. To address this, ChIP analyses of replisome components in *ASF1* and *SNF2* mutants, coupled with additional studies probing the physical interaction between Asf1 and the SWI/SNF complex, should be performed.

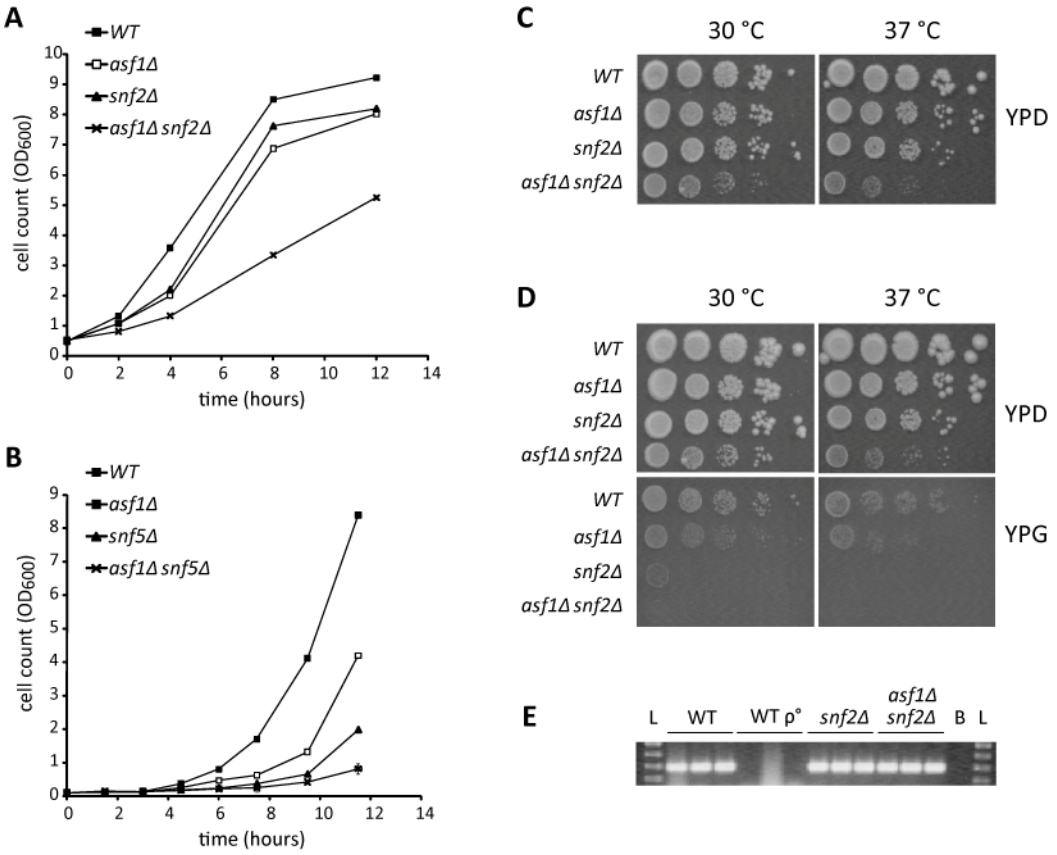

Figure 5-1. *ASF1* shows synthetic sick interactions with components of the SWI/SNF complex. A. *asf1Δ snf2Δ* double mutants are slower-growing than either single mutant. Cells were grown to an OD_{600} of 0.5 in YPD medium overnight and cell densities were measured using a spectrophotometer after 2, 4, 8 and 12 hours in culture. **B.** *asf1Δ snf5Δ* double mutants are slower-growing than either single mutant. Cells were grown in YPD medium overnight, diluted to an OD_{600} of 0.1, and cell densities were measured using a spectrophotometer at the indicated timepoints. Averages of three independent *asf1Δ snf5Δ* isolates are shown and error bars are indicated. **C.** *asf1Δ snf2Δ* double mutants are slightly temperature-sensitive. Ten-fold serial dilutions of early log phase cells diluted to 1 x 10^7 cells/mL were spotted onto rich medium (YPD) and grown at 30 or 37 °C. Photographs were taken after 3 days. **D.** *asf1Δ snf2Δ* double mutants are unable to grow when glycerol is the sole carbon source. Cells from (C) were spotted onto rich medium (YPD) or medium containing glycerol (YPG) and grown at 30 or 37 °C. Photographs were taken after 5 days. **E.** Mitochondrial DNA is present in *snf2Δ* and *asf1Δ snf2Δ* cells. Three wild type rho+, wild type rho°, *snf2Δ* and *asf1Δ snf2Δ* colonies were subjected to colony PCR using primer pairs specific to the *COX3* mitochondrial gene. L, ladder; B, blank (no template control PCR reaction).

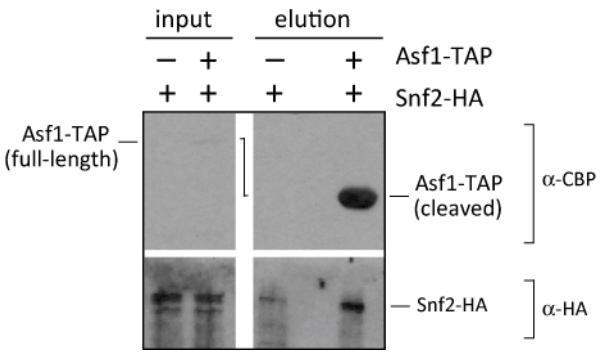

Figure 5-2. Snf2 copurifies with Asf1. *ASF1-TAP SNF2-HA* and *SNF2-HA* lysates were used for standard tandem affinity purification. Inputs were obtained before binding to the first affinity column. Final eluates were resolved by SDS-PAGE and probed with either anti-CBP (top) or anti-HA (bottom) antibodies to detect Asf1 or Snf2, respectively. Experiment performed by Kathy Lin.

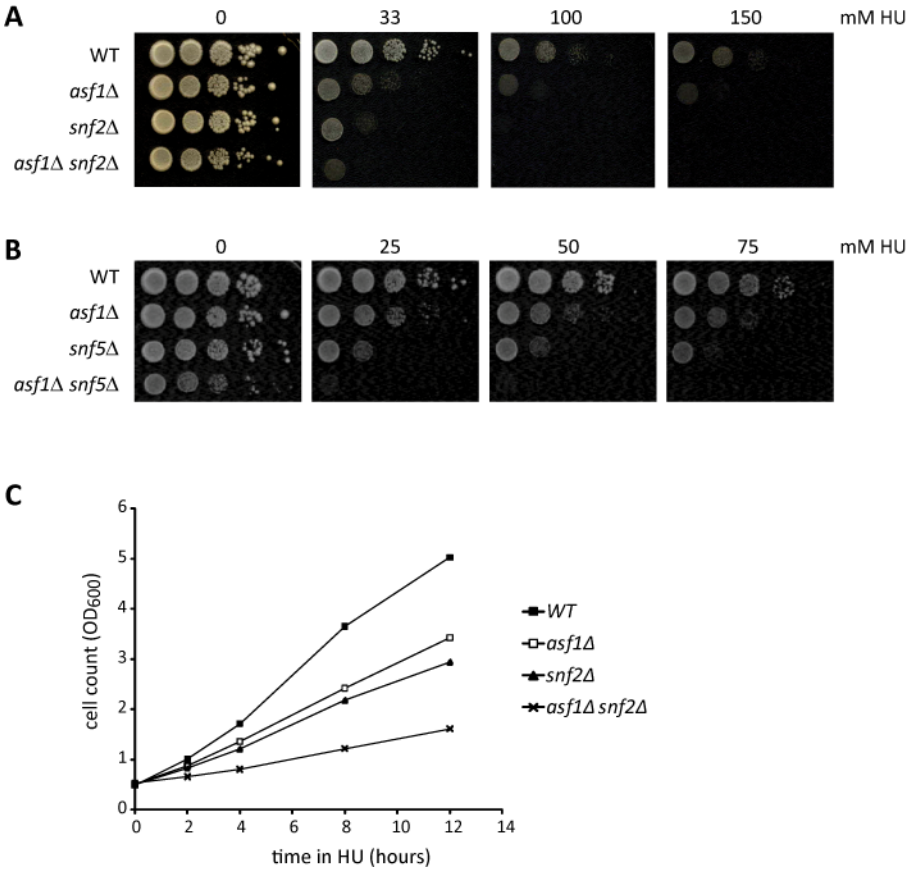

Figure 5-3. Asf1 and SWI/SNF contribute to cell survival during hydroxyurea treatment. A. *asf1⊿ snf2⊿* double mutants are sensitive to hydroxyurea. Ten-fold serial dilutions of early log phase cells diluted to 1 x 10^7 cells/mL were spotted onto rich medium (0 mM HU), and medium containing 33, 100 or 150 mM HU and grown at 30 °C. Photographs were taken after 4 days. **B.** *asf1⊿ snf5⊿* double mutants are sensitive to HU. Cells diluted as described in (B) were spotted onto rich medium (0 mM HU), and medium containing 25, 50 or 75 mM HU and grown at 30 °C. Photographs were taken after 4 days. **C.** *asf1⊿ snf2⊿* double mutants are slow-growing in medium containing HU. Cells from Fig. 5-1A were grown to early log phase in rich medium and HU was added at a final concentration of 200 mM.

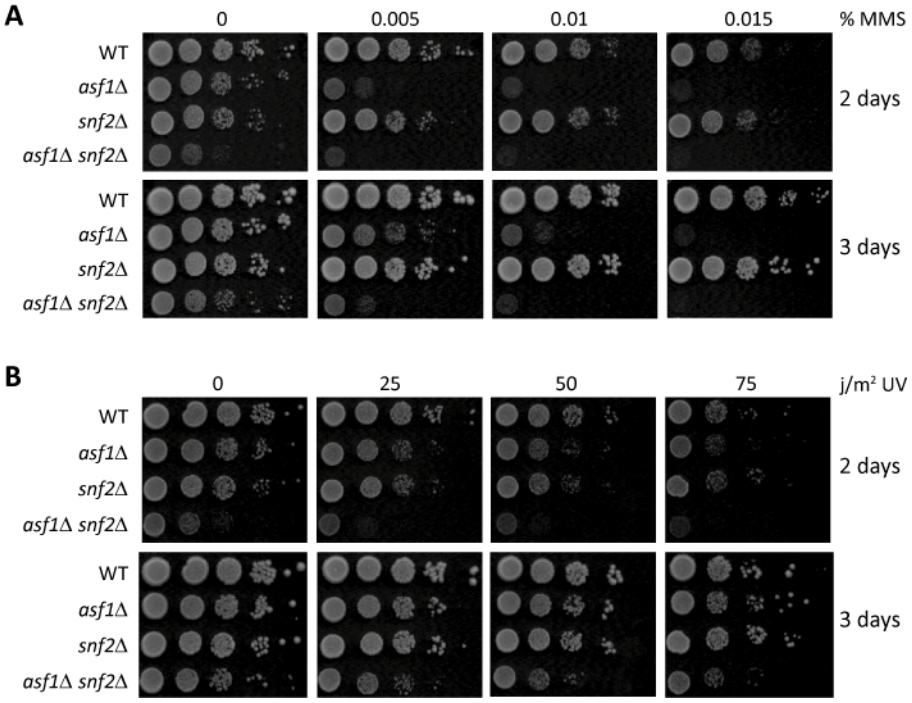

Figure 5-4. *asf1Δ snf2Δ* mutants are sensitive to DNA-damaging agents.
A. *asf1⊿ snf2⊿* double mutants are sensitive to MMS. Ten-fold serial dilutions of early log phase cells diluted to 1 x 10^7 cells/mL were spotted onto rich medium (0 % MMS), and medium containing 0.005, 0.01 or 0.015 % MMS and grown at 30 °C. Photographs were taken after 2 (top panel) and 3 (bottom panel) days. **B.** *asf1⊿ snf2⊿* double mutants are sensitive to UV irradiation. Ten-fold serial dilutions of cells grown overnight in YPD were diluted to 1 x 10^7 cells/mL, spotted onto rich medium and given 0, 25, 50 or 75 J/m^2 of UV irradiation. Plates were incubated in the dark at 30 °C and photographed as in (A).

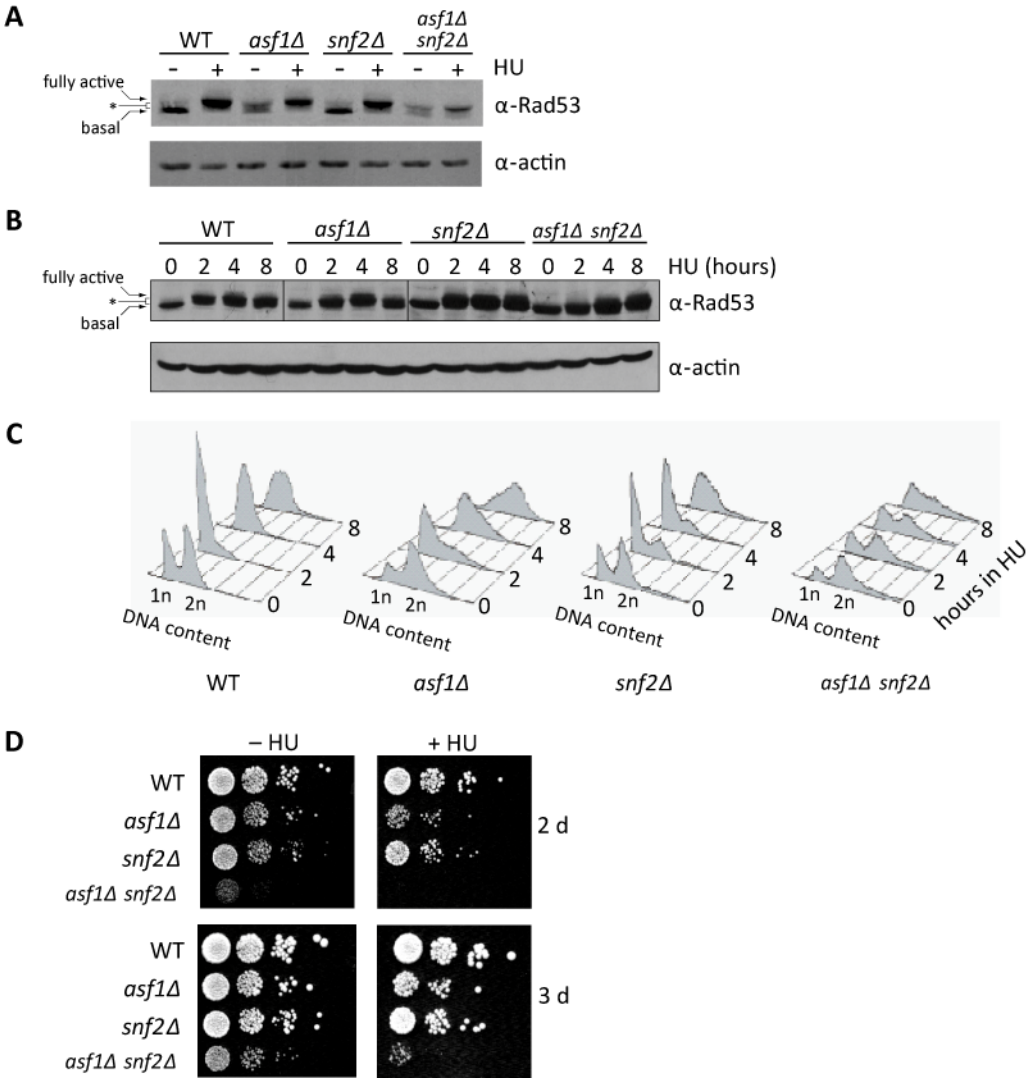

Figure 5-5. Asf1 and Snf2 have partially overlapping functions in cell cycle and checkpoint control. A. Rad53 activation is impaired in *asf1⊿ snf2⊿* double mutants. Cells grown in YPD (-) or YPD + 0.2 M HU (+) for two hours were collected at each time point and protein was prepared using a standard TCA preparation protocol. SDS-PAGE and Western blotting were done using anti-Rad53 and anti-actin (control) antibodies. Asterisk indicates intermediate level of Rad53 phosphorylation. **B.** *asf1⊿ snf2⊿* double mutants are delayed for activation of Rad53. Early log phase cells were grown in YPD or YPD + 0.2 M HU for 0, 2, 4 or 8 hours. Cells were processed as described in (A). 6 (WT and *asf1⊿*) or 10 µl (*snf2⊿* and *asf1⊿ snf2⊿*) of protein cell equivalents were loaded (Rad53 blot). **C.** *asf1⊿ snf2⊿* mutants fail to fully arrest in S phase in response to HU. Cells from Fig. 5-3C were collected at each time point and flow cytometry analysis was conducted. **D.** Recovery from replication stress is compromised in *asf1⊿ snf2⊿* mutants. Ten-fold serial dilutions of early log phase cells grown in YPD or YPD + 0.2 M HU for 24 hours were diluted to 1 x 10^6 cells/mL and spotted onto rich solid medium. Cells were grown at 30 °C and photographs were taken after 2 and 3 days.

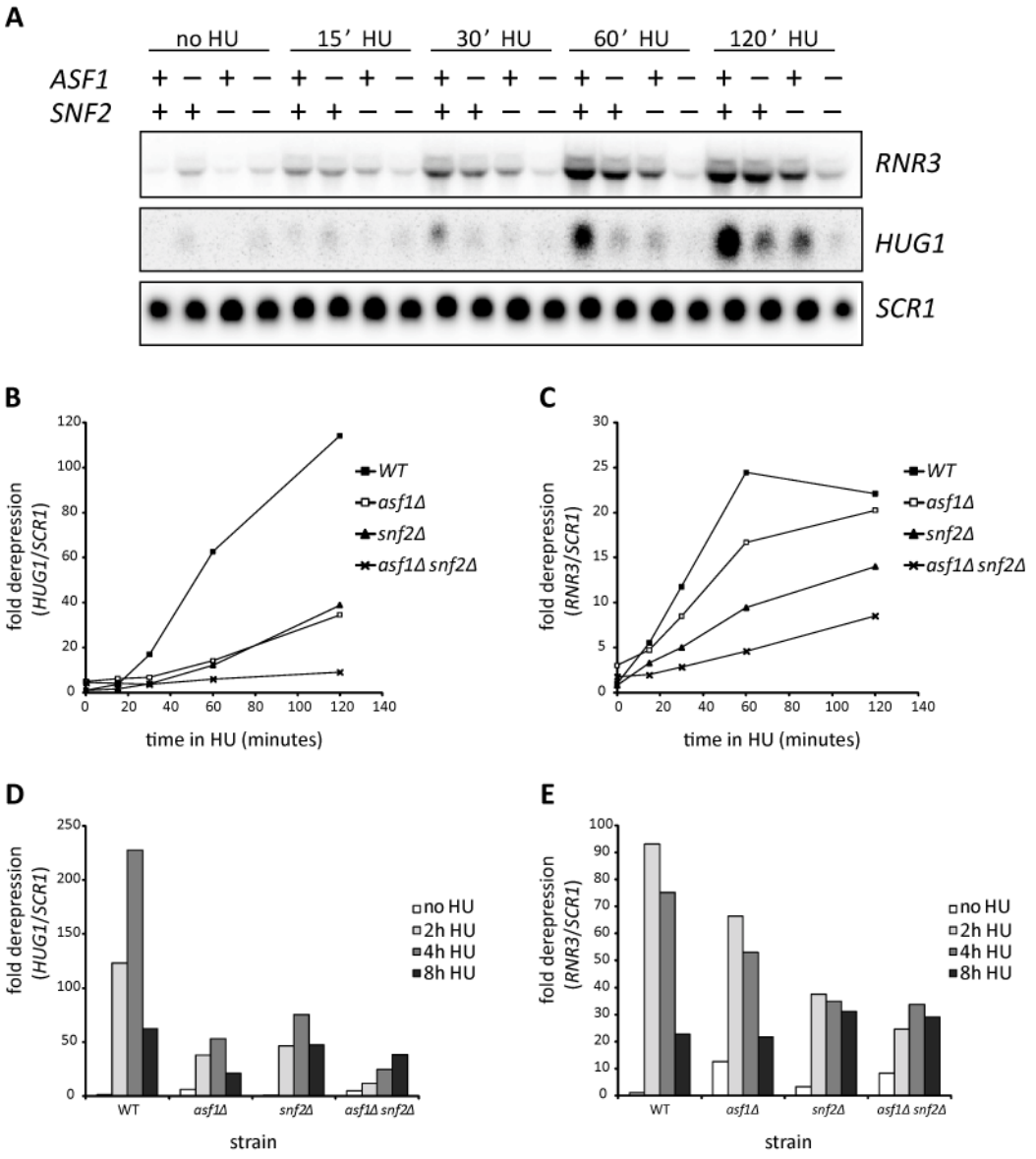

Figure 5-6. Asf1 and SWI/SNF both promote derepression of the DNA damage response genes. A. Early log phase cells were grown in YPD (no HU) or YPD + 0.2 M HU for 15, 30, 60 or 120 min. RNA was isolated at each time point and Northern analysis was performed using probes specific for *HUG1*, *RNR3*, or *SCR1* (control). Probes were labelled by random priming. Results were normalised to *SCR1* and quantitated using Phosphorimaging and ImageQuant TL software. Cells are the same as used in Fig. 5-5A. **B.** Quantitation of *HUG1* Northern blot normalised to *SCR1*. **C.** Quantitation of *RNR3* Northern blot normalised to *SCR1*. **D.** RNA isolated from cells from Fig. 5-1A and 5-3C was used for Northern blotting as described in (A). *HUG1* expression was normalised to *SCR1*. **E.** *RNR3* expression was normalised to *SCR1*. Details are described in (D). These are the results of a single analysis; however, comparable results were obtained in similar experiments. Cells are the same as used for experiments in Fig. 4-2.

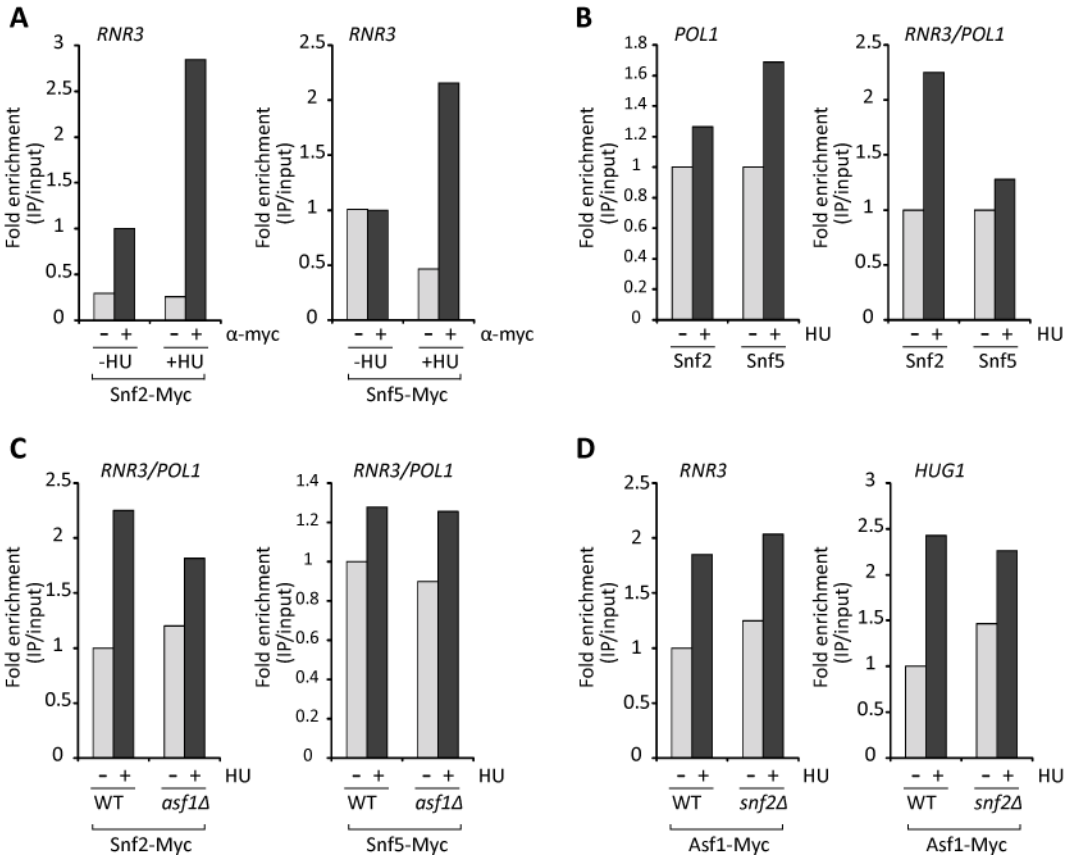

Figure 5-7. Asf1 and SWI/SNF are both recruited to the promoters of DNA damage response genes during replication stress. A. Snf2 and Snf5 are recruited to the promoter of *RNR3* after HU treatment. *SNF2-MYC* or *SNF5-MYC* cells were grown in YPD (-HU) or YPD + 0.2 M HU (+HU) for one hour prior to formaldehyde crosslinking and ChIP using 0 or 10 µl of α-myc antibody. Quantitative RT-PCR reactions were performed in triplicate using primer pairs specific to the promoter of *RNR3*. Immunoprecipitated DNA was normalised to input DNA and signal obtained in untreated (+ antibody) cells was set to 1. **B.** Snf2 is specifically recruited to *RNR3*. RT-PCR reactions were performed in triplicate as above or using primer pairs specific for *POL1*. **C.** SWI/SNF recruitment does not depend on Asf1. Chromatin immunoprecipitation was performed using the indicated strains as described in (A). Experiments in (A) to (C) are the results of a single analysis. **D.** Asf1 association with the promoters of DNA damage response genes does not depend on SWI/SNF. Chromatin immunoprecipitation was performed as described in (A) using the indicated strains and primer pairs designed to specifically amplify the promoter region of *RNR3* or *HUG1*. The average of duplicate experiments is shown. Ranges (in order of bars, left to right) are: n/a, 0.02, 0.02, 0.16 (*RNR3*); n/a, 0.15, 0.29, 0.11 (*HUG1*). n/a = not available.

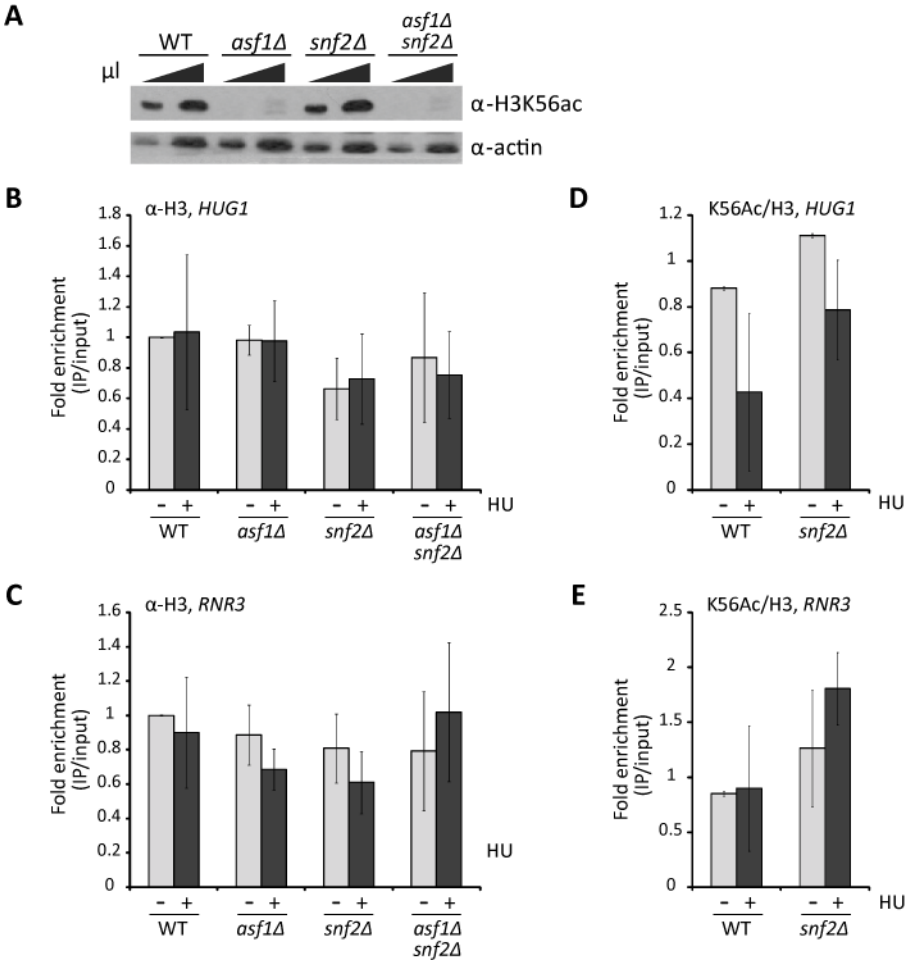

Figure 5-8. Regulation of histone H3 lysine 56 acetylation is similar in wild type and *snf2Δ* cells. A. Cells grown to early log phase in YPD were collected and total protein was prepared using a standard TCA preparation protocol. SDS-PAGE and Western blotting were done using anti-H3K56ac and anti-actin (control) antibodies. Identical cell equivalents of protein (either 3 or 9 µl) were loaded. **B.** Indicated strains were grown in YPD (-HU) or YPD + 0.2 M HU (+HU) for one hour prior to formaldehyde crosslinking and ChIP using 5 µg of anti-H3 antibody. Quantitative RT-PCR reactions were performed in triplicate using primer pairs specific to the promoter of *HUG1*. Immunoprecipitated DNA was normalised to input DNA and signal obtained in untreated cells was set to 1. **C.** RT-PCR reactions were performed as in (B) using primer pairs specific for the promoter of *RNR3*. **D.** Chromatin immunoprecipitation was performed on whole cell extracts from (B) using 2.5 µl of anti-H3K56ac antibody and primer pairs specific to the promoter of *HUG1*. Immunoprecipitated DNA was normalised to input DNA and signal obtained in untreated cells was set to 1. H3K56ac PCR signal was normalised to bulk H3 and signal obtained in *asf1⊿* and *asf1⊿ snf2⊿* cells was subtracted from WT and *snf2⊿* cells, respectively. **E.** Chromatin immunoprecipitation was performed as in (D) using primer pairs specific to the promoter of *RNR3*. Error bars show standard deviation between three biological replicates.

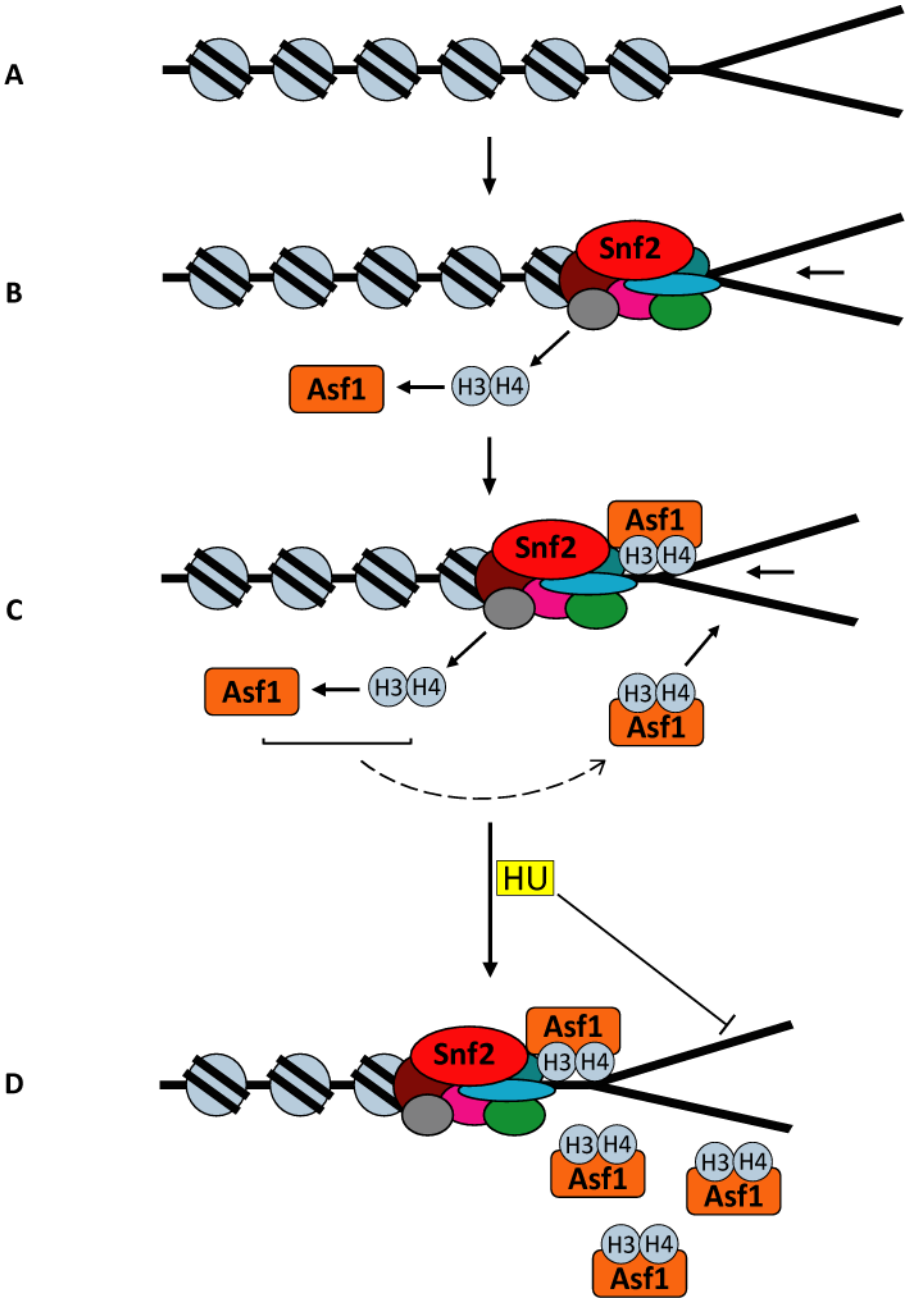

Figure 5-9. Model for Asf1 and SWI/SNF function in DNA replication.
A. Replication fork. DNA is represented by black lines, and nucleosomal histones by blue circles. **B.** During DNA replication, SWI/SNF could remodel chromatin ahead of the replication fork to facilitate fork progression. Displaced histones could be bound by Asf1. **C.** Following fork passage, histones bound to Asf1 are in close proximity to the DNA, allowing efficient reassembly of chromatin. Asf1 and SWI/SNF may interact physically. **D.** Under conditions of replication stress caused by HU, dNTPs are depleted leading to replication fork stalling. SWI/SNF and Asf1 may contribute to replication fork stability.

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Chapter 6

General Discussion and Conclusions

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General Discussion & Conclusions

The research presented here has revealed novel pathways in which Asf1 acts, as well as provided new insight into previously known roles of Asf1 in the cell. This chapter describes our major findings and conclusions, and discusses the implications of specific results. We present preferred speculative models and examine possible alternative explanations in the context of our data alongside the work of others. Future experiments designed to help discern between competing models are mentioned. Lastly, we touch on the significance of chromatin research in yeast for higher organisms, and discuss important ways in which recent studies on Asf1 have advanced the field.

Implications of the *ASF1-SET2* interaction

As described in Chapter 3, we took advantage of synthetic genetic array technology to identify genes that interact with *ASF1*. This large-scale screen led to the discovery of multiple new genetic interactions, including many between *ASF1* and genes that encode other proteins involved in chromatin regulation. In particular, we found an interaction between *ASF1* and *SET2*, which encodes the histone methyltransferase responsible for mono-, di- and trimethylation of H3K36. This genetic interaction was especially intriguing because we (K. Lin) also identified a physical interaction between Asf1 and Set2[1]. Although *asf1⊿ set2⊿* double mutants were slow-growing compared to *asf1⊿* and *set2⊿* single mutants, the single mutants did not demonstrate similar sensitivities to agents that cause replication stress or

DNA damage. Our overall phenotypic analysis indicated that Asf1 and Set2 do not likely function in a shared pathway to respond to DNA damage or replication stress.

Further experiments by K. Lin revealed that 1) Asf1 and Set2 contribute to the suppression of spurious intragenic transcription in separate, functionally overlapping pathways, and 2) Asf1 promotes bulk and *PMA1*-associated trimethylation of H3K36 by Set2[1]. First, Asf1 and Set2 do not function in a linear pathway to suppress spurious intragenic transcription; rather, the *asf1⊿ set2⊿* double mutant shows additive defects. Therefore, it is possible that the slow growth phenotype of *asf1⊿ set2⊿* cells may be partly due to the fact that this double mutant is more compromised in its ability to inhibit spurious intragenic transcription. This is plausible since cell growth and proliferation may proceed abnormally in cells that accumulate high levels of cryptic transcripts. Second, given that Asf1 is important for H3K36 trimethylation, a histone mark associated with transcriptional elongation, it seems probable that the Asf1-Set2 interaction plays a currently unknown role during this process. Of special note is our observation that Asf1 must be able to tightly bind H3/H4 to stimulate H3K36 trimethylation, and the recent findings by others[2,3] that Set2 interaction with histone H4 is needed for its ability to catalyze di- and trimethylation of H3K36. Given that Asf1 is not needed for H3K36 dimethylation[1] and that Asf1 is viewed as capable of presenting histones to other chromatin-regulating complexes[4,5], we propose a model in which Asf1 transiently presents an H3/H4 dimer that is dimethylated at H3K36 to Set2 for its subsequent trimethylation. Previous dimethylation of H3K36 may be facilitated by a different

histone H3/H4 chaperone involved in transcriptional elongation, such as Rtt106. Consistent with this idea, Rtt106 has been localized to the coding region of *PMA1*[6].

Future experiments in this area will likely be aimed at determining the specific mechanism by which histone chaperone Asf1 is able to stimulate H3K36 trimethylation. Given this new functional link between a histone chaperone and a histone methyltransferase, the possible involvement of other known histone chaperones in H3K36 methylation should be studied, including Rtt106 and FACT, an H2A/H2B chaperone. Finally, determining whether Asf1 plays a role in promoting methylation of other histone H3 residues will help establish the specificity of this interaction, and may lead to the identification of additional pathways in which Asf1 functions.

Future studies to understand the association of Asf1 with chromatin

We have presented evidence that Asf1 associates constitutively with chromatin. Given its multiple roles in chromatin regulation, this is perhaps not surprising. However, we have also determined that the apparent global association of Asf1 with chromatin is inducible by agents that cause replication stress, such as HU. Even though Asf1 was required for the derepression of DDR genes under replication stress conditions and Asf1 was recruited to the promoters of two DDR genes under these same conditions, its recruitment was not needed for their concomitant derepression. This finding led to two central questions. First, what is the biological significance of the association of Asf1 with chromatin? For instance, *why* is Asf1 recruited to chromatin? Second, *how* does replication stress signal the recruitment of Asf1 to chromatin?

Although our current understanding of Asf1 binding to chromatin is limited, we have identified some of the key determinants for Asf1 association with chromatin. Unexpectedly, we find that while the conserved histone-binding region of Asf1 is not needed for its chromatin association, the acidic C-terminal tail region makes a substantial contribution to the ability of Asf1 to constitutively bind chromatin. In other words, in *asf1N* cells, which lack the C-terminal tail, Asf1 association with chromatin is markedly reduced under normal conditions. This suggests that the acidic tail of Asf1 promotes its chromatin association. However, asf1N can still be recruited to chromatin under conditions of replication stress, indicating that factors other than the C-terminal tail of Asf1 may be necessary for its inducible association with chromatin.

Interestingly, we find that when the C-terminal tail is missing, the conserved histone-binding region of Asf1 becomes more important for its ability to bind chromatin. That is, cells lacking the C-terminal tail that also contain a mutation in the conserved histone-binding region (V94R) show extremely low levels of Asf1 chromatin association under both constitutive and inducible conditions. This important result indicates that these two regions of Asf1 may separately promote normal association of Asf1 with chromatin. In this view, Asf1 would use multiple mechanisms to bind to chromatin, and such mechanisms may depend in part on other proteins.

Future studies will focus on determining the exact complement of proteins that facilitate Asf1 association with chromatin. Although we have ruled out a requirement for SWI/SNF, Rad53, Rtt109 or CAF-I, there are many other proteins

that could carry out this function. A screen of new candidates should be implemented by performing Asf1 ChIPs in cells lacking proteins that are predicted to be involved in processes regulated by Asf1. This task may be complicated by the redundant nature of chromatin processes in yeast. For example, perhaps in the absence of one protein, another protein can substitute and facilitate Asf1 association with chromatin. Evidence for this idea comes from our observation that Asf1 associates with chromatin normally in cells lacking a component of CAF-I, but not when cells contain a mutation in Asf1 that compromises its binding to CAF-I or HIR. The finding by others that Asf1 binding to *PMA1* was similar in wild type and *hir1*⊿ cells[7] provides support for the hypothesis that Asf1 can associate normally with chromatin as long as it has the ability to bind to one of CAF-I or HIR. Therefore, construction of strains lacking two or more candidate proteins may be necessary for the eventual identification of the proteins that promote association of Asf1 with chromatin. Indeed, current work in our lab is investigating the ability of Asf1 to associate with chromatin in cells lacking a component of CAF-I and a component of HIR.

Under conditions of replication stress, we hypothesize that one or more components of the replication machinery may be needed for Asf1 recruitment to chromatin. This is based on previous work implicating Asf1 directly in chromatin regulation at the replication fork, which also showed that Asf1 contributes to integrity of the replisome at stalled replication forks[8]. Given that an interaction between Asf1 and RFC has been reported[8], we are primarily interested in monitoring Asf1 recruitment in RFC mutants. Since treatment with HU leads to 1) stalled

replication forks, and 2) recruitment of Asf1 to chromatin, it may be that RFC targets Asf1 to stalled forks through a direct interaction. Alternatively, a different component of the replisome may interact with Asf1 and facilitate its recruitment to chromatin (see below).

As there may not be a stalled replication fork at each region of the genome that Asf1 is recruited to upon HU treatment, processes other than DNA replication may target Asf1 to chromatin. For example, DNA damage produced during HU treatment may signal a global increase in Asf1 binding to chromatin. In this case, Asf1 would then be in a position to carry out opportunistic functions to promote proper chromatin regulation, thereby increasing the stability of the genome. Present studies in our lab are aimed at distinguishing between these possibilities.

In our current model, we speculate that Asf1 association with chromatin under normal conditions is promoted by its physical interaction with another histone chaperone, possibly through its C-terminal tail (Fig. 6-1A). As mentioned above, Asf1 binding to either CAF-I or HIR is needed for Asf1 to bind chromatin at wild type levels. However, even when Asf1 cannot bind to either of these two chaperones, Asf1 can still associate with chromatin, albeit at levels much lower than wild type. Therefore, the defect in Asf1 association with chromatin is partial, and it seems likely that a third histone chaperone may be involved. An obvious candidate that remains to be tested is Rtt106. In addition, we theorize that Asf1 recruitment to chromatin during conditions of replication stress may be through an interaction with a component of the replication machinery (Fig. 6-1B). Perhaps direct recruitment of Asf1 to stalled replication forks allows it to carry out a role in maintaining the

stability of the replisome[8], thereby promoting resumption of DNA replication rather than fork collapse.

On the other hand, Asf1 association with chromatin may not depend on another protein factor. In this view, Asf1 must possess all of the necessary features to associate with, and be recruited to, chromatin. However, given that 1) Asf1 does not directly bind to DNA, and 2) a mutant version of Asf1 that is essentially unable to bind to histones can still associate with chromatin, we favour the idea that another protein is involved.

It is probable that uncovering the specific mechanism by which Asf1 is recruited to chromatin will ultimately result in a better understanding of the significance of Asf1 association with chromatin. For example, if Asf1 is recruited to chromatin through an interaction with a component of the replication machinery, then it seems almost certain that Asf1 associates with chromatin to participate in an aspect of DNA replication.

Poising of DDR genes by H3K56 acetylation

We determined that Asf1 is important for transcriptional derepression of *HUG1* in response to treatment with HU. Significantly, the requirement for Asf1 does not depend on its ability to associate with the promoter of *HUG1*. Rather, Asf1 promotes derepression through a pathway that depends on H3K56 acetylation. This idea is supported by a series of experiments using mutants that are defective in proper H3K56 acetylation (i.e., *asf1*⊿, *asf1*^{V94R}, *rtt109*⊿, *K56Q*, *K56A*, *K56*R, and various double mutants). Altogether, these experiments confirmed that derepression of *HUG1* is severely compromised in cells lacking H3K56 acetylation.

To assess whether H3K56 acetylation levels increase at the promoters of DDR genes to facilitate transcription, as is the case at *PHO5*[9], we measured H3K56ac occupancy at the *RNR3* and *HUG1* promoters using ChIP. However, we found that the proportion of promoter-bound H3 that is K56-acetylated did not change dramatically upon full derepression of these DDR genes. This discovery led to our proposal that the deposition of H3K56-acetylated molecules onto newly replicated DNA at the promoters of DDR genes poises them for derepression (Fig. 4-18). This idea is conceivable given that transcriptional repressors inhibit expression of the DDR genes in the absence of checkpoint signals that promote their derepression, even under conditions of high acetylation. We also consider the possibility that H3K56 acetylation could be preserved in the promoters of the DDR genes throughout the cell cycle, maybe via a mechanism that inhibits HDAC activity.

Nevertheless, at our current level of understanding, other possibilities could explain our findings. An alternative explanation for the seemingly unchanged levels of K56-acetylated H3 in DDR gene promoters upon derepression is that these promoters constantly undergo rapid histone turnover[10]. In this case, in addition to replication-coupled deposition (Fig. 6-2A), the dynamic nature of nucleosomes in the DDR gene promoters would allow replication-independent deposition (Fig. 6-2B) of H3K56-acetylated molecules. Support for this idea comes from studies showing that H3 deposited into promoters of inactive genes can be acetylated on K56 outside of S phase[11]. Therefore, the exact mechanism by which H3K56 acetylation promotes rapid derepression of DDR genes is not yet known, and additional work is needed to further examine the different possibilities.

One obvious experiment is to test whether transcriptional derepression of the DDR genes during treatment with HU is limited in cells lacking a component of CAF-I and Rtt106. Given that these two histone chaperones cooperate during replication-coupled chromatin assembly to deposit K56-acetylated H3 onto chromatin[12], it is possible that there will be less H3K56ac incorporated into the promoters of DDR genes, and therefore a lower level of derepression. However, since *cac1⊿ rtt106⊿* cells are only partially compromised for H3K56ac deposition (~50% reduction compared to wild type[12]), it seems almost certain that additional histone chaperones are involved in this process. Therefore, until the full complement of proteins needed for H3K56ac deposition during S phase has been identified, the results obtained from the described experiment may be difficult to interpret. Another complicating factor is that even though H3K56ac may be reduced at the DDR gene promoters under normal conditions in these mutants, there may be other mechanisms to ensure that H3K56ac is rapidly deposited when cells experience replication stress.

To distinguish whether H3K56ac is preserved at DDR gene promoters throughout the cell cycle, synchronization experiments should be performed. Specifically, synchronizing cells and monitoring H3K56ac occupancy throughout the cell cycle will establish if the acetylation state of lysine 56 can be maintained at *HUG1* and *RNR3* outside of S phase.

Common roles for Asf1 and SWI/SNF, a chromatin remodeller

In the course of our SGA analysis, we came across a potential genetic interaction between *ASF1* and *SNF2*, which encodes the catalytic subunit of the SWI/SNF

chromatin remodeller. We began our characterization of *asf1⊿ snf2⊿* double mutants by verifying their slow-growth phenotype, and further determined that they are sensitive to agents that cause replication stress or DNA damage, defective in cell cycle control and checkpoint regulation, and compromised for derepression of two DDR genes.

Preliminary evidence suggested that Asf1 and Snf2 copurify in yeast, a finding consistent with studies in *Drosophila* indicating that the fly counterparts of Asf1 and Snf2 interact physically[13]. However, further study is required to fully verify this interaction in yeast. Presuming that the Asf1-SWI/SNF interaction is bona fide, a number of exciting possibilities can be envisaged. Initially, we thought that Asf1 and Snf2 may function together transiently in a complex to promote transcriptional derepression of the DDR genes, but a number of experiments indicate that this may not be the case. For example, Asf1 association with the promoters of DDR genes is not needed for its role in derepression; instead, Asf1 contributes via an H3K56 acetylation-dependent pathway. Since *asf1⊿ snf2⊿* double mutants show additive defects in derepression, it is likely that Asf1 and Snf2 function in overlapping pathways, rather than in a linear pathway, to promote DDR gene derepression. In addition, SWI/SNF is recruited to DDR genes in the absence of H3K56 acetylation, as is Asf1, which led us to hypothesize that Asf1 and SWI/SNF may interact transiently to carry out a different chromatin function.

It is tempting to speculate that Asf1 and SWI/SNF may cooperate in some way to facilitate DNA replication. However, Asf1 and SWI/SNF have also both been implicated in the DDR (see references within Chapters 1, 4 and 5). Future

experiments will aim to address the significance of the Asf1-SWI/SNF interaction. In human cells, chromatin-associated Asf1 can interact with the MCM (minichromosome maintenance) replicative helicase by an H3/H4 bridging mechanism[14]. Upon replication stress caused by HU, Asf1 is trapped in a complex with MCM and histones H3/H4 on chromatin[15]. Given that an H3/H4-binding mutant of Asf1 is recruited normally to chromatin during replication stress in yeast, and that human Asf1 only associates with MCM through an interaction with histones[15], it would seem that Asf1 binding to MCM is not needed for its chromatin association. However, it is possible that the C-terminal tail of yeast Asf1 can stabilize the interaction of Asf1 with MCM, perhaps especially when the conserved histone-binding domain is mutated. This notion is supported by our finding that the *asf1N*^{V94R} double mutant is most severely compromised for Asf1 association with chromatin. One possible way to explain the physical interaction between Asf1 and SWI/SNF is that a SWI/SNF-Asf1 complex could associate with chromatin via binding to MCM. In this view, SWI/SNF would be anchored to chromatin ahead of the replication fork and in a perfect position to facilitate resumption of fork movement via chromatin remodelling upon removal of replication stress. Further support for this idea comes from our observations that Asf1 and SWI/SNF can both be recruited to chromatin during treatment with HU. However, it remains to be seen whether these proteins are directly recruited to stalled replication forks, and if so, whether recruitment is dependent on the MCM complex. An alternative idea is that Asf1 and SWI/SNF may physically interact only transiently as they both promote two different aspects of DNA replication (histone deposition and remodelling,

respectively), or in passing as Asf1 contributes to replication or transcription while SWI/SNF promotes transcription in the same chromatin vicinity.

Significance of chromatin research in yeast: links to cancer

Often, what is first discovered in model organisms is later found to be conserved in higher eukaryotes. Our area of research has been no exception: Asf1, Set2, SWI/SNF and H3K56 acetylation, all identified in yeast, were subsequently shown to be conserved in humans. While some differences between yeast and human systems undoubtedly exist, much can be gained from initially studying complicated processes in the simpler organism.

As described in Chapter 1, components of the SWI/SNF chromatin remodelling complex were first identified in yeast[16-18], followed by nearly 30 years of research that revealed a diverse group of protein complexes involved in chromatin remodelling in yeast and higher organisms. These studies have revealed that ATP-dependent chromatin remodelling is evolutionarily conserved, and have led to the intense investigation of the involvement of chromatin remodelling processes in cancer. Significantly, alterations in SWI/SNF have been intimately connected to the development and progression of human cancer[19,20]. Similarly, the Set2 histone methyltransferase, originally identified in *S. cerevisiae*[21], has very recently been implicated in carcinogenesis in humans[22,23]. However, it has not yet been determined if the human homologs of Asf1 and Set2 or SWI/SNF can interact, what the cellular consequences of such interactions might mean in humans, and whether these proteins can collaborate to defend against cancer.

The finding in 2005 that yeast histone H3 can be acetylated in the globular domain[24], on lysine 56, has also had a huge impact on human studies. Last year, the first evidence that H3 can be K56-acetylated in humans was presented[25], only four years after its original discovery in yeast. While determining the significance of H3K56 acetylation in humans is in its early stages, it has already been shown that this modification colocalizes with sites of DNA repair[26], is critical for genomic stability[27], and is involved in transcription[25]. In addition, H3K56 acetylation, along with the protein level of Asf1, is increased in certain types of tumours[28]. Whether the regulation of H3K56 acetylation is comparable in yeast and humans is controversial[15,25]; however, some evidence has suggested that similarities do exist[26-28]. Importantly, two reports have shown that, as in yeast, Asf1 is needed for H3K56 acetylation in humans[27,28].

It seems almost certain that ongoing investigations in yeast, including our research presented here, will accelerate a more complete understanding of chromatin modulation in humans. Specifically, further characterization of our observed interactions between Asf1 and SWI/SNF or Set2 may provide new ideas to explain how these proteins work in human cells. In addition, a better comprehension of the significance of Asf1 association with chromatin in yeast might help to unveil the ways in which Asf1 and SWI/SNF or Set2 function together. Finally, the dependence of DDR gene transcription on H3K56 acetylation may be conserved in humans, and may play a role in the maintenance of genomic stability.

Final Comments

While much about the precise ways in which Asf1 functions remains to be determined, studies of Asf1 over the past decade have dramatically increased our understanding of chromatin regulation. In particular, during the course of this work, two major discoveries have completely changed the way in which chromatin regulation has been viewed. First, structural studies of Asf1 showed that Asf1 associates with an H3/H4 dimer[4,29-31]. This exciting finding transformed current views on chromatin assembly and indicated that H3/H4 can exist as a dimer rather than an $(H3/H4)_2$ tetramer, as previously thought. This indicated that two H3/H4 dimers may be deposited independently of one another during chromatin assembly, and along with other data[14], might provide a mechanism for the cellular inheritance of epigenetic chromatin marks.

The second key finding showed that Asf1 is absolutely required for H3K56 acetylation. Subsequent studies revealed that many of the previously identified functions of Asf1 can be attributed to its ability to stimulate H3K56 acetylation. Likewise, most phenotypes of *asf1⊿* cells can be ascribed to a lack of H3K56 acetylation in these mutants. Significantly, the once popular notion that Asf1 promotes transcription by directly disassembling nucleosomes[32], is gradually being overturned in favour of the idea that H3K56 acetylation is what is important for transcriptional activation.

Although the research presented here describes a broad array of Asf1 functions in chromatin regulation, a few recurring themes are unveiled. Significantly, this work is consistent with the notion that Asf1 can act as a 'presenter' of histones[4,5] to other

complexes involved in chromatin modulation. For example, we have evidence to suggest that Asf1 may present dimethylated H3K36 to Set2 for trimethylation. It is also thought that Asf1 presents newly synthesized histones to Rtt109 for H3K56 acetylation[5], and Asf1 may then donate acetylated histones to other chaperones in the course of replication-coupled and/or replication-independent chromatin assembly[12]. In addition, the genetic interactions outlined in this report also suggest the existence of new partners to which Asf1 might donate histones.

Our research also falls in line with the hypothesis that Asf1 acts as an opportunistic regulator of chromatin[32]. While previous studies speculated an opportunistic role for Asf1 in transcription, we expand this idea to include the possibility that Asf1 can act opportunistically in additional chromatin processes, such as DNA replication and the DDR. Opportunistic or not, it is clear that Asf1 is a multifunctional protein involved in a vast number of pathways of chromatin regulation. As the involvement of newly identified or thus far unknown chromatin regulators becomes apparent, it will not be surprising to learn that Asf1 also works in additional as yet unidentified pathways of action. Given the current level of excitement surrounding Asf1 and H3K56 acetylation in the chromatin field, we predict that research within the next five years will reveal novel roles for Asf1 in the nucleus, and lead to a more complete understanding of the specific ways in which Asf1 modulates chromatin.

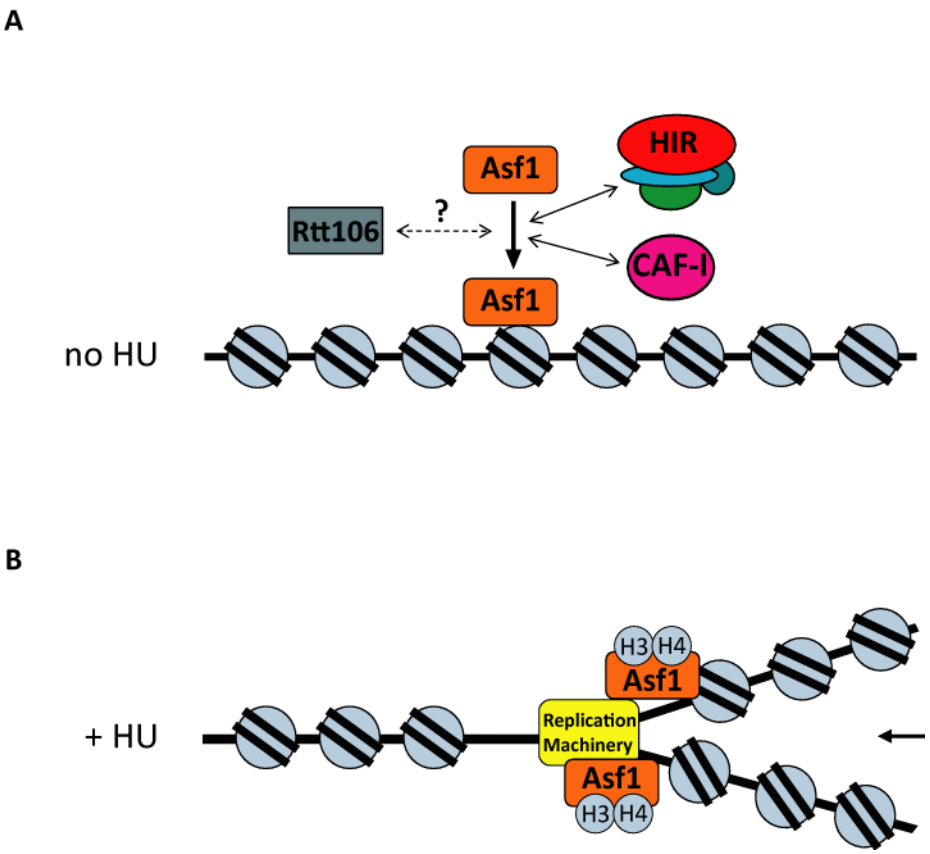

Figure 6-1. Model for constitutive and inducible Asf1 binding to chromatin. **A.** Constitutive binding of Asf1 to chromatin is partially dependent on its interaction with CAF-I or HIR. Rtt106 may also play a role in the association of Asf1 with chromatin. **B.** An interaction between Asf1 and a component of the replication machinery, such as RFC, could facilitate recruitment of Asf1 to chromatin during replication stress.

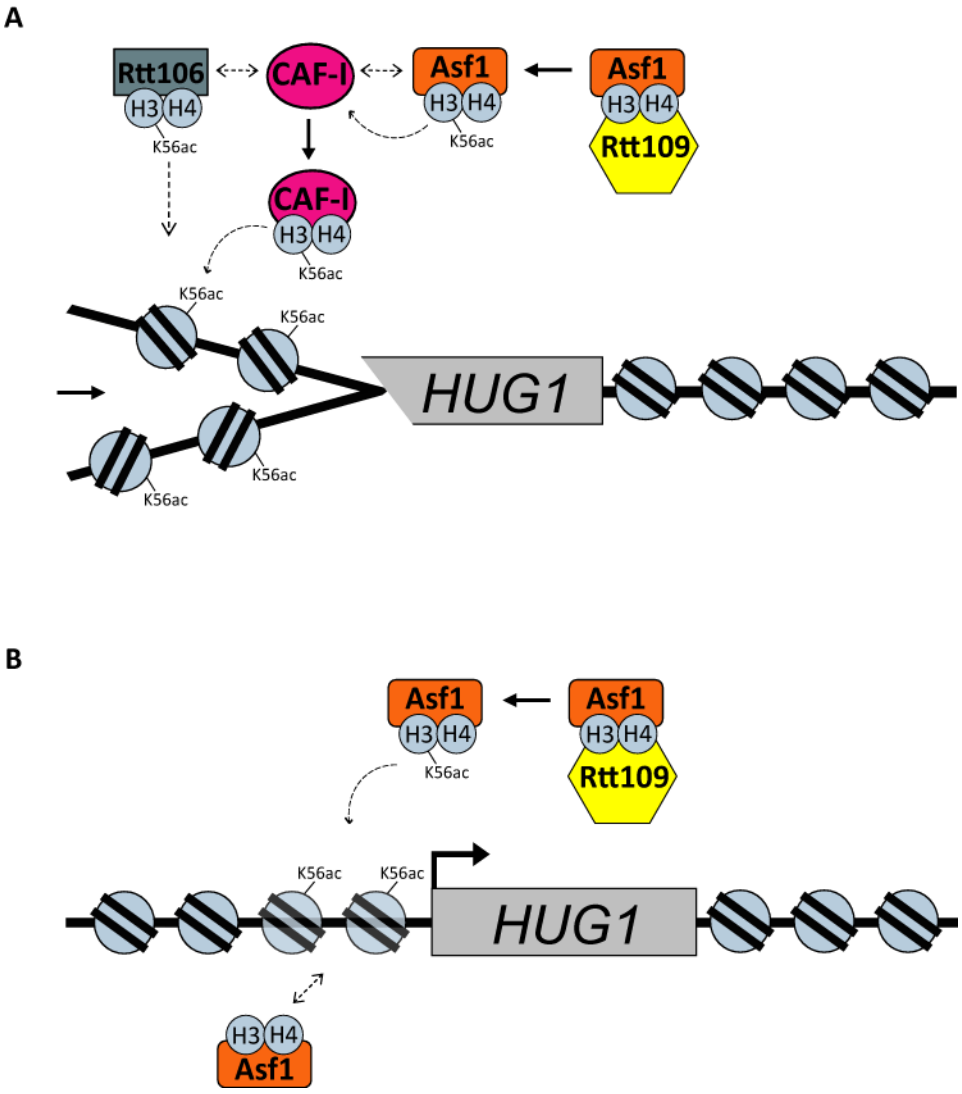

Figure 6-2. Models for replication-coupled and replication-independent deposition of H3K56ac into the promoters of DDR genes. A. Replication-coupled deposition of H3K56ac poises *HUG1* for derepression. Asf1 promotes H3K56 acetylation by the Rtt109 lysine acetylase. CAF-I, Rtt106 and Asf1 contribute to H3K56ac deposition during DNA replication. **B.** Replication-independent deposition of H3K56ac may promote *HUG1* derepression. High turnover of histone proteins in DDR gene promoters may facilitate derepression independently of replication. These two models are not mutually exclusive.

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