CHARACTERIZATION OF ASF1 AND GCN5 IN THE CILIATED PROTOZOAN TETRAHYMENA THERMOPHILA

By

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Characterization of Asf1 and Gcn5 in the ciliated Protozoan *Tetrahymena thermophila*

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Abstract

One method of regulating accessibility of DNA is chromatin remodelling via histone post-translational modifications (PTM). Adding an acetyl group to the lysine residues (K) on the core histone H3 is one of these chemical modifications. Acetylation of H3 on lysine 56 (H3K56ac) is an important histone alteration that is conserved among most if not all eukaryotes including humans. Several histone acetyl transferases (HAT) have been shown to be responsible for H3K56ac in different organisms including Gcn5 and p300/CPB in human cells and Rtt109 in fungi including the yeast *Saccharomyces cerevisiae*. In addition the histone chaperone Asf1, is also required for these modifications in yeast and human cells. The ciliated protozoan *Tetrahymena thermophila* is an effective model organism for studying the function of histone PTMs in certain processes including meiosis and RNA interference. Here, I show that tGcn5 has H3 acetylation activity and that tAsf1 binds Histone H3.

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List of abbreviations:

Ac: Acetylated/acetylation

Asf1: Anti-Silencing factor 1

BLAST: Basic Local Alignment Search Tool

Caf1: Chromatin Assembly Factor1

DNA: Deoxyribo-Nucleic Acid

ds: double stranded

dd: Double distilled

GCN5: General Control of Amino acid Synthesis 5

HAT: Histone Acetyl Transferase

H3: Histone H3

H4:Histone H4

NLS: Nuclear Localization Signal

rpm: round per minute

Rtt109: Regulator Of Ty1 Transposition Protein 109

K:Lysine

PTM: Post-Translational Modification

WCE: Whole Cell Extract

WT: Wild Type

Chapter 1: Introduction

1.1 Chromatin and the nucleosome

Chromatin is a thread-like structure that is composed of DNA and proteins to package DNA in the nucleus of the cell. Its functions are to package DNA into a smaller volume so that it can fit in the nucleus, to provide the physical scaffolds for DNA that makes segregation of chromosomes during mitosis and meiosis possible, to prevent DNA damage, and to control gene expression and DNA replication. Chromatin can be generally classified into two types which consist of euchromatin and heterochromatin. Euchromatin contains DNA coding active genes and is structurally “loose” to allow access to DNA polymerase that replicates DNA and RNA polymerases that transcribe RNA from DNA. Euchromatin is generally associated with the interphase of the cell cycle. The second type is heterochromatin that is tightly packaged and is associated with structural proteins and generally contains transcriptionally inactive genes (Aunuziato, 2008).

There are three levels of DNA packaging. First DNA wraps around histone proteins forming nucleosomes which are repeating structures and also is defined as a "beads on a string" structure. Afterwards, multiple nucleosomes wrap into a 30 nm wide fibre consisting of nucleosome arrays. Finally there is higher level packaging of the 30 nm fibres in the metaphase chromosome which occurs during chromosome segregation in mitosis and meiosis. (Fischle et al., 1999).

The structural unit of chromatin is the nucleosome. The nucleosome is composed of a short stretch of DNA and a histone octamer. It achieves packaging of the DNA by wrapping around it 147 base pairs (bp) of DNA. In the nucleus, histones are arranged as an octamer. The histone octamer is made of two dimers of histone H2A and H2B (H2A-H2B) which are arranged

on either side of a tetramer of histone $(H3\text{-}H4)_2$ (Luger et al., 1997 & 2003). Nucleosomes are regularly spaced along DNA forming the beads-on-a-string structure. Histone H1 plays a role in the 30 nm fibre by interacting with DNA at the entry and exit of each nucleosome (Travers, 1999).

Histones have a globular domain within the nucleosome that functions to interact with the DNA and a N-terminal tail protruding on the outside of the nucleosome which is thought to have in post translational modifications. Histones goes through several biological and chemical processes such as chromatin remodelling, assembly/disassembly and post translational modifications (PTM) (Luger et al., 1997).

1.2 Chromatin remodelling

Despite the compact packaging of chromatin, DNA must be accessible to proteins involved with processes such as transcription, replication, recombination, and repair. This accessibility is carried out by several mechanisms, including ATP-dependent chromatin remodelling (Tang et al., 2010), exchange of histone variants (Draker et al., 2009), and post-translational chemical modification (PTM) of histones (Barth et al., 2010). Enzymes involved in PTM pathways chemically modify mostly the N-terminal tails or very rarely at the junction between the N-terminal tail and the globular domain of histone proteins. They perform this to alter the structure of chromatin and provide recognizable binding sites for other regulatory proteins. Many chromatin associated proteins contain domains within them that bind these chemical groups such as the bromodomain, which recognizes acetylated lysine residues (Zentner et al., 2010). Through direct interactions with histone modifications, other proteins are targeted to specific post translational modification sites on chromatin, like transcriptionally active regions

that are abundant in H3K4me3 marks, or repressed regions which are tri-methylated at H3K27 (Schuettengruber et al., 2007).

1.2.1 ATP dependent chromatin remodelling

ATP dependent remodelling is achieved by shuffling core histone particles along the DNA strand revealing other regulatory elements such as transcription factor binding sites. This process is carried out by ATP dependent protein complexes (Tang et al., 2010). Many of these enzymes are required for diverse and specific embryonic development of different cell types including pluripotency (Gao et al., 2008), cardiac development (Lickert et al., 2004), dendritic morphogenesis and self-renewal of neural stem cells (Yoo et al., 2009). It has been demonstrated that deletion or mutation of these proteins often leads to apoptosis or tumorigenesis as a consequence of dys-regulation of cell cycle control (Vissers et al., 2004 and Layman et al., 2009).

The first chromatin remodelling proteins that were identified are the SWI/SNF (Switching defective/Sucrose nonfermenting) family of proteins (Hirschhorn et al., 1992). A member of this family is RSC (Remodel the Structure of Chromatin) complex which was discovered based on homology of its ATPase subunit STH1 and shared Arp7 and Arp9 subunit with ySWI/SNF (Cairns et al., 1996). Both SWI/SNF and RSC complexes appear to regulate gene expression (Sudarsanam et al., 2000). Other families of chromatin ATP dependent chromatin remodelers are the INO80, the ISWI and the CHD family of proteins that function within large macromolecular structures to affect chromatin dynamics and thus regulate transcription (Hargreaves and Crabtree, 2011).

1.2.2 Post Translational modification

Another class of chromatin remodelling is ATP independent post-translational modification (PTM) which is crucial for regulating the functions of chromatin. PTMs involves serine, threonine and tyrosine phosphorylation, lysine ubiquitination, sumoylation, methylation and acetylation, arginine methylation, and proline isomerization. The lysine residue is a target of different PTMs (Kouzarides, 2007 and Ruthenburg et al., 2007).

1.2.3 The Histone code

It has been hypothesized that histone post-translational modifications act as a "code" which is read by other proteins to silence or to transcribe certain genes (Strahl and Allis, 2000). An examples of a "reader" complex that silences genes is the SIR (Silent information regulators) complex in *S. cerevisiae* which requires H4 amino acid residues 16 to 29 for Sir3 binding (Hecht etal, 1995). Histone H4K16 acetylation dominantly prevents Sir mediated heterochromatin spreading (Kimura et al., 2002). In telomeric heterochromatin formation, the double-stranded telomeric DNA-binding protein Rap1 physically interacts with Sir4 and recruits the whole Sir complex to the telomeres (Lue et al., 2002). Once recruited to telomeres, Sir2 deacetylates the H4K16ac marks and hence silences the genes contained in that chromatin region (Imai et al., 2000).

Example of "reader" proteins that activate transcription are the ones that contain a Chromodomain. A class of such proteins is the chromodomain helicase DNA-binding (CHD) family of ATP-dependent chromatin remodelers. Proteins in this family have emerged as important regulators of cellular differentiation (Zentner et al., 2010). CHD proteins function in the nucleus by binding to the nucleosome and regulating gene transcription (Zentner et al.,

2010). It has been shown that CHD7, a member of the CHD family, tracks H3K4me and promotes transcription (Shnetz et al., 2009). CHD7 is mutated in human CHARGE (Coloboma of the eye, Heart defects, Atresia of the choanae, severe Retardation of growth and development, Genital abnormalities, and Ear abnormalities) syndrome, a multiple organ development disorder that affects several body functions such as hearing, vision, cardiac, craniofacial, and nervous system development (Vissers et al., 2004). There are several lines of evidences that CHD7 binds thousands of sites in the genome and thus is a redundant protein involved in regulation of gene expression (Shnetz et al., 2009).

1.3 Chromatin assembly

The tight compaction of nuclear DNA profoundly influences DNA replication, transcription, recombination, and repair. The interactions between the basic lysine and arginine residues of histones with the acidic DNA make the nucleosomal unit of chromatin a highly stable structure. For the cellular machinery to access DNA, chromatin must be disassembled so that DNA is freely available. Afterwards, the DNA has to be reassembled into chromatin (Ransom et al., 2010). The reassembly process is carried out by a class of proteins called histone chaperones. (Groth et al., 2007)

1.4 Histone chaperones

Histone chaperones (HC) bind histones after synthesis in the cytoplasm and then escort them into the nucleus. They also aid in their deposition into nucleosomes during different processes such as DNA replication, repair, and transcription. Furthermore, HC have a crucial role, directly or indirectly, in PTMs (Avvakumov et al., 2011). The functions of HC are often

linked to ATP-dependent chromatin remodeler complexes that use the energy produced from ATP hydrolysis to break histone-DNA interactions during nucleosome disassembly and to redeposit histones during reassembly (Haushalter and Kadonaga, 2003).

Histone chaperones are a diverse network of proteins that have little sequence similarity and are relatively acidic which facilitates their interaction with the basic histones. Many of them have a hydrophobic β-sheet motif which is also involved in interacting with histones (English et al., 2006). Some HCs that lack the β-sheet motif have other structures that allow them to bind histones. For example, Chz1 has an irregular chain as well as α-helices that mediate the interaction with H2B dimers and the histone variant H2A.Z (Zhou et al., 2008).

1.5 ASF1

The histone chaperone Anti-silencing function 1 (Asf1) is a highly conserved HC among eukaryotes both structurally and functionally. It is found in two isoforms in human cells, Asf1a and Asf1b (Das et al., 2009). The N-terminal 155 residues of Asf1 form a globular core that consists of an immunoglobulin-like fold (Daganzo et al., 2003; Mousson et al., 2005). Asf1 that contains only this globular core (Asf1N) has been shown to function as the full length Asf1 including binding to Hir protein that is required for gene silencing (Daganzo et al., 2003). It has been demonstrated that Asf1 binds to a heterodimer of histones H3 and H4 (English et al., 2005). More specifically, the C-terminus of histone H3 is one of the interfaces that interacts with Asf1 (Munakata et al., 2000). Structural mapping have shown interactions of the C-terminal end of H3 peptide (residues 122-135) with a highly conserved and acidic amino acid sequence on the concave surface of Asf1 (Mousson et al., 2005). This histone H3 peptide is of 3 helixes, which is a crucial part of a four-helix bundle that forms the H3:H3 dimerization site in the nucleosome

(Luger et al., 1997). Furthermore, a mutation in the middle of the H3-interacting region of Asf1 that replaces Valine 94 with an Arginine residue (V94R) abolished the interaction with histone H3 (Mousson et al., 2005). This data indicates that the region that mediates H3:H3 dimerization in the H3/H4 heterotetramer is also the region of H3 that binds to Asf1 and thus explains why Asf1 binds a dimer of H3-H4 instead of a tetramer as found in the nucleosome (Mousson et al., 2005).

In the nucleus, the association of H3-H4 with Asf1 is required for H3K56ac by an acetyltransferase (Collins et al., 2007; Driscoll et al., 2007; Han et al., 2007; Recht et al., 2006; Fillingham et al., 2008). Additionally, Asf1 can recruit other acetyltransferases for acetylation of H4 (Fillingham et al., 2008). Asf1 is also involved in transcription activation (Adkins et al., 2004) and chromatin disassembly or reassembly during the process of transcriptional elongation (Schwabish and Struhl, 2006). Furthermore, it has been found that most non DNA-associated histones are bound to Asf1 (Groth et al., 2005; Tagami et al., 2004), which underscore its importance in the role as an important histone chaperone in eukaryotes. Asf1 is the only HC that has a role in both replication-dependent and replication-independent chromatin assembly which will be talked about in more details in the next section (Nakatani et al., 2004).

1.6 Replication dependent versus Replication independent chromatin assembly

During the S phase of the cell cycle where the genome is duplicated, there is also high level of histone synthesis to assemble the nascent strands of DNA. HCs function to assemble histones into new nucleosomes. In DNA replication, existing nucleosomes are disassembled and removed to facilitate access for the DNA replication fork. Newly synthesized DNA is then re-packaged into nucleosomes. The re-assembled nucleosome is comprised of both parental and

newly synthesized histones. This process is called replication coupled (RC) nucleosome assembly and it plays a major role in the inheritance of epigenetic information (Groth et al., 2007; Morrison and shen, 2009). During progression of DNA replication, the hexameric MCM2-7 helicase complex advances ahead of the replication fork and unwinds the dsDNA to facilitate DNA polymerase passage, and in doing so it disrupts existing histone-DNA binding and releases the parental histones (Gambus et al., 2006). A histone chaperone such as Asf1 then forms an Asf1-H3-H4 complex (Groth et al., 2007). Asf1 also binds to newly synthesized H3-H4 dimers during DNA replication and after directing their acetylation presents all bound acetylated H3-H4 to Caf1, which in turn assembles the dimers into replicating DNA by physically interacting with proliferating cell nuclear antigen (PCNA) (Stillman, 1986) (Figure 1).

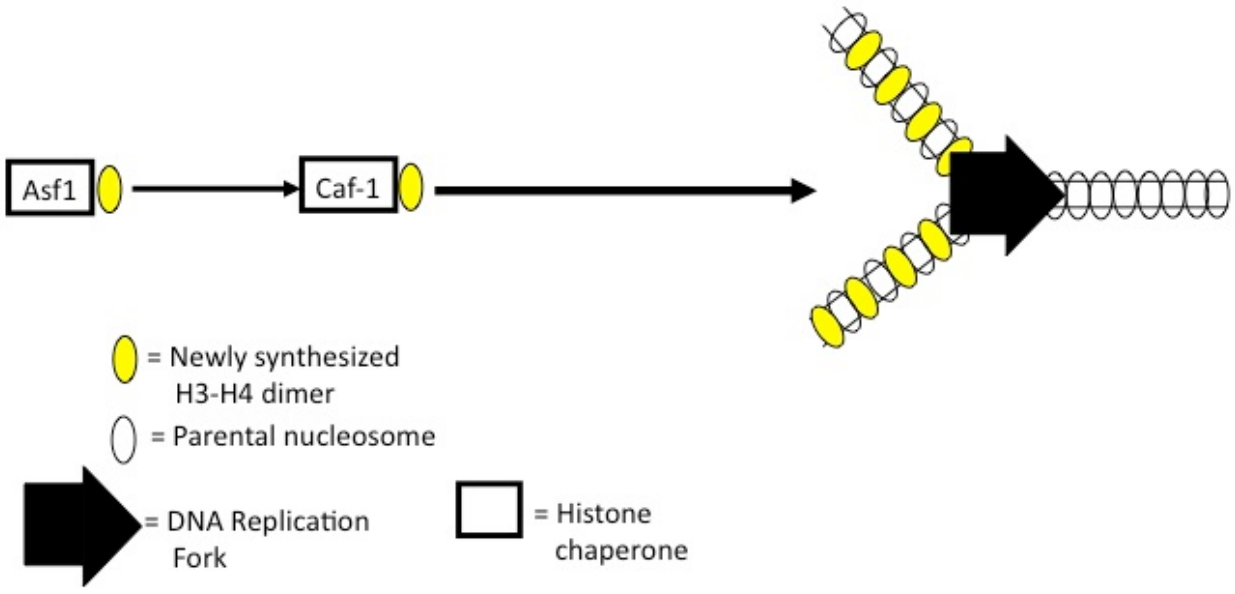

Figure 1: Replication dependent chromatin assembly: In this model Asf1 binds to H3-H4 dimers and present them to Caf-1 which in turn assembles them.

Replication independent chromatin assembly involves nucleosomes disassembly and reassembly during DNA repair or transcription (Avvakumov et al., 2011). During DNA repair, the process is referred to as Access-Repair-Restore; the process begins with chromatin

disassembly to permit the repair machinery access to the site of damage, followed by repair of the DNA lesion and the final step involves re-establishing the pre-existing chromatin structure, including PTMs (Smerdon, 1991).

During DNA damage repair the chromatin structure around the break site is altered or completely disassembled to make DNA accessible. The type of modifications involved in this process varies with severity of damage and also the method of repair. In homologous recombination repair (HRR) of double strand breaks (DSBs), removal of nucleosomes and resection of DNA to generate single stranded free DNA overhangs is needed for recombination of the damaged DNA with homologous sequences (Rossetto et al., 2010). After repair is complete, chromatin returns to its original state. This process is aided by Asf1 and subsequently CAF1 (Rocha and Verreault, 2008). Nucleosomal involvement in non-homologous end joining repair (NHEJ) is still unclear but is being extensively investigated (Kanikarla-Marie et al., 2011)

In transcription, chromatin is disassembled to facilitate transcription. Nucleosomes are very stable protein-DNA complexes that create significant obstacles in the way of the transcription machinery by blocking the production of transcripts (elongation) (Williams and Tyler, 2007). At the start of transcription HCs work with ATP-dependent chromatin remodelers and histone modifiers to alter or remove nucleosomes that occlude the promoter DNA sequences (Li et al., 2007a). After transcription initiation, same or different HCs work together with histone-modifying and chromatin-remodeling enzymes to allow the progression of RNA polymerase II (RNAPII) along the genes' coding regions. A model exists which predict that histones are modified in front of the RNAPII (mainly by acetylation) as it progresses, promoting the dissociation of histone-DNA interaction and thus the disassembly of nucleosomes with the

aid of different HCs, including FACT, Nap1, Asf1, HIR/HPC, and Spt6. Subsequently, the same HCs mediate nucleosome re-assembly after RNAPII has passed through (Li et al., 2007).

1.7 The histone code of chromatin assembly

Newly-synthesized histone H3-H4 dimers are acetylated before being assembled into nucleosomes (Roth et al., 2001). Histone H4 acetylation at lysines 5 and 12 is conserved from yeast to humans (Ai and Parthun, 2004; Kleff et al., 1995; Sobel et al., 1995). Patterns of acetylation on newly-synthesized H3s are not as conserved; In HeLa cells, acetylation of newly synthesized histone H3 is barely detectable, while in *Tetrahymena* new H3 is acetylated at H3K9 and H3K14 but in *Drosophila* it is acetylated at H3K14 and H3K23 (Benson et al., 2006; Sobel et al., 1995). In yeast cells, newly synthesized H3 is mainly acetylated at lysine 56 (H3K56ac) (Masumoto et al., 2005).

In addition to H3K56ac, in yeast, newly synthesized H3 is acetylated at H3K9 and H3K27 (Kuo et al., 1996). Evidence shows that several lysine residues in the N-terminus of H3 (K9, K14, K18, K23, and K27) are important for nucleosome assembly (Li et al., 2008; Ma et al., 1998; Qin and Parthun, 2002).

It has been shown that histones carrying the H3K56ac co-purify with the histone chaperone Caf-1 and that H3K56ac promotes chromatin assembly during the S phase of the cell cycle. This PTM is believed to enhance the ability of the HCs CAF-1 and Rtt106 to bind histone H3-H4 and then assemble them into nucleosomes. In *S. cerevisiae* deletion of either ASF1 or RTT109 as well as a mutation in H3K56 highly diminishes the co-purification of CAF-1 and Rtt106 (a histone chaperone) with H3. This suggests that H3K56ac is required for their binding. It was observed that Rtt106 and Cac1 (the large subunit of CAF-1) have stronger binding affinity

to H3-H4 when they were hyper-acetylated *in vitro*. They bound unmodified H3-H4 to a lower extent H3-H4 which indicates direct interaction with H3K56ac. This data suggested that H3K56ac is required for H3-H4 assembly into chromatin by CAF-1 and Rtt106 (Chen et al., 2008 and Li et al., 2008). It has been postulated that the specific recognition of H3K56ac by CAF-1 and Rtt106 extends the "histone code" hypothesis (Strahl and Allis, 2000) to include nucleosome assembly (Fillingham and Greenblatt, 2008).

1.8 H3K56

Several groups have reported histone H3 Lysine 56 as a site for acetylation that controls gene expression in the budding yeast *S. cerevisiae* (Masumoto et al., 2005; Ozdemir et al., 2005, and Xu et al., 2005). This modification has been also reported in the fission yeast, *Schizosaccharomyces pombe* (Haldar and Kamakaka, 2008), in *Drosophila* (Xu et al., 2005) and in humans (Das et al., 2009). What makes Lysine 56 in H3 unique is its location in the nucleosome; It is the last amino acid residue of the α-N-helix terminal tail, which precedes the globular domain of histone H3. Furthermore, it has been shown that histone-DNA interaction at this entry and exit points (H3K56) in the nucleosome is weakened by H3K56 acetylation (Masamuto et al., 2005). H3K56ac is thought to promote transcription (Rufiange et al., 2007) and it is also involved in the response to DNA damage during nucleosome reassembly following repair and replication of DNA that is a result of DNA insults caused by DNA damaging agents (Miller et al., 2006, Haldar and Kamakaka, 2008). H3K56ac is found in the newly synthesized H3 until it is incorporated into the nucleosome and it persists until the G_2/M where it is removed by the histone deacetylases Hst3 and Hst4 (Celic et al., 2006 and Masumuto et al., 2005).

It has been shown that the binding of H3 with Rtt106 is barely detectable in cells that lack H3K56ac, whereas the association of H3 with CAF-1 is only reduced in cells lacking this modification suggesting that while H3K56ac is the only site for binding with Rtt106 other modifications on H3 may also regulate the binding of H3 with CAF-1 (Li et al., 2008). It was also shown that the function of H3K56ac in nucleosome assembly appears to be conserved in mammalian cells (Das et al., 2009).

H3K56ac is also present in, the protozoan *T. themrophilla* (Shahbazian et al., 2007). Recent mass spectrometry results have shown abundant H3K56ac on newly synthesized histones in *Tetrahymena* (Cosgrove, 2007). Initial Western blot (WB) data also shows localization of H3K56ac in the macronucleus of *T. themrophilla* (Garg and Pearlman, unpublished).

H3K56ac has shown to be a promising target for clinical applications. It has been found that the inhibition of the histone deacetylase enzymes induces hyperacetylation of histones, which causes cell cycle arrest and cell death in cancer cells but not in normal cells (Santini et al., 2007). In addition, yeast cells that lack or are deficient in H3K56ac age more rapidly and have shorter life spans (Dang et al., 2009). Furthermore, normal yeast cells have shown decreasing levels of H3K56ac as they age (Dang et al., 2009). It has also been shown that decreased H3K56ac levels lessens longevity in yeast (Feser et al., 2010).

Interestingly, the enzymology behind H3K56ac biology appears to be an effective target for anti fungal treatment; it has been shown that reduced levels of H3K56ac sensitize *Candida albicans* to genotoxic and antifungal agents. Targeted pharmacological alterations in Rtt109 (a fungal specific enzyme that catalyses the transfer of acetyl group to H3K56) resulted in reduced H3K56ac levels and thus reduced virulence in a mouse model with *C. albicans* infection. This could be applied to other yeast infections (Wurtele et al., 2010).

1.9 Enzymology behind H3K56

The enzymatic reaction of acetylation is carried out by a histone acetyl transferase (HAT), which transfers an acetyl group from acetyl CoA to the target lysine residue. The acetyl residues are removed by a class of enzymes called Histone Deacetylases (HDACs) (Roth et al., 2001) HATs and HDACs function in multi-protein complexes that can posses different functions, including PTM and nucleosome remodeling activities, as well as interactions with gene activators and transcription factors (Naar et al., 2001). Furthermore, HATs can have directed activity against non-nucleosmal (free) or nucleosomal histones (Ohba et al., 1999).

1.9.1 HATs and HDACs

Several HATs have been identified among species. There are three major groups of HATs: GNATs (Gcn5-related N-acetyltransferases), p300 (E1A-associated protein of 300 kDa)/CBP (CREB-binding protein), and MYST proteins (Allis et al., 2007; Lee and Workman, 2007). The p300/CBP group is unique to metazoans such as humans (Goodman and Smolik, 2000). In humans H3K56 acetylation is mediated by CBP/p300 (Das et al.,2009), P/CAF (Nagy et al., 2007) and Gcn5 (Tjeertes et al., 2009).

HDACs are divided into the Rpd3/Hda1 and the Sirtuin families. In humans, the Sirtuins contains HDAC1, 2, 3 and 8 (class I is similar to yeast Rpd3), HDAC4, 5, 6, 7, 9 and 10 (class II is a homolog of yeast Hda1), and HDAC11 (class IV) (Yang and Seto, 2008). In the Sirtuin family, there are seven members in humans (SIRT1-7 which is related to yeast Sir2; also known as class III HDACs) (Haigis and Guarente, 2006). Both HATs and HDACs are catalytic subunit of multi protein complexes (Lee and Workman, 2007).

1.9.2 Rtt109

In *S. cerevisiae*, H3K56ac is catalyzed by Rtt109 (Collins et al., 2007; Driscoll et al., 2007; Han et al., 2007) and is dependent upon the histone chaperone Asf1 (Recht et al., 2006) (Figure 2). It has been suggested that Rtt109 specificity is determined *in vivo* by the histone chaperones it is using; H3-H4 or Rtt109 is altered by the chaperone in a manner that leads to a specific acetylation on H3K9 or H3K56 (Fillingham et al., 2008). It has been suggested that the K56 residue is exposed when H3 is bound to Asf1 (Antczak et al., 2006). Rtt109 itself could also be promoting its specificity supported by the fact that H3K9 and H3K56 are the only lysine residues in H3 to be followed by ST residues which could explain Rtt109 specificity of these sites and it can be explained why Rtt109 alone cannot acetylate H3K9 when bound to Asf1 by that Asf1 could be having an inhibitory effect on Rtt109 (Fillingham et al., 2008). It has also been demonstrated that *S. cerevisiae* Rtt109 promotes genome stability and resistance to DNA-damaging agents, and does that by functionally cooperating with Asf1 to maintain normal chromatin structure (Driscoll et al., 2007, Han et al., 2007)

1.9.3 Vps75

Vps75 is an H3-H4 histone chaperone that belongs to the Nap1 family (Selth et al., 2007). It has also been shown that Vps75 co-purfies with Rtt109. Vps75 is involved in stabilizing the HAT and is essential for H3K9ac by Rtt109 *in vivo* in *S. cerevisiae* (Fillingham et al., 2008) (Figure 2).

Deletion of VPS75 results in very slight decrease on H3K56ac levels during the S phase of the cell cycle and does not alter DNA sensitivity to genotoxic agents as deletion of ASF1 or RTT19 does (Han et al., 2007b) suggesting that the Rtt109-Vps75 complex functions

independently from Asf1-dependent H3K56ac (Han et al., 2007 and Tsubota et al., 2007). Biochemical analyses of Vps75 has shown that it supplies Rtt109 with histone substrates and stimulate its HAT activity (Berndsen et al., 2008 and Park et al., 2008). Vps75 significantly enhances Rtt109 activity to acetylate H3K9, H3K23, and H3K27 (Burgess et al., 2010 and Fillingham et al., 2008). In the current understanding, Rtt109 requires both Asf1 and Vps75 to acetylate H3K9, whereas it only needs Asf1 to bind to the H3-H4 dimer to acetylate H3K56 (Fillingham et al., 2008).

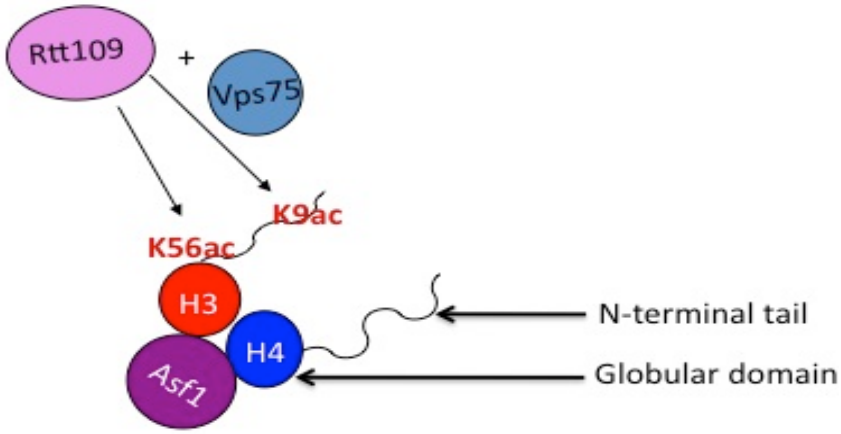

Figure 2: Model for Rtt109 and Asf1 in H3 acetylation in *S. Cerevisiae:* Rtt109 acetylate both H3K9 and H3K56. It needs Asf1 to bind to H3-H4 dimer for both and needs Vps75 for H3K9ac only.

Vps75 also has Rtt109-unrelated functions in chromatin dynamics that are related to transcription; analysis of epistatic miniarray profiles showed that VPS75 genetically interacts with factors involved in transcription regulation and additional genetic and biochemical experiments revealed a close relationship between Vps75 and RNAPII. In addition, Vps75 is recruited to activated genes in an Rtt109-independent manner. Furthermore, it has been shown that Vps75 might have a role in histone H2A/H2B eviction/deposition during transcription. (Park et al., 2008 and Selth et al., 2009)

1.9.4 Hat1

Hat1 is a member of the GNAT superfamily of HATs and is an evolutionary conserved protein. In *S. cerevisiae* it interacts with Asf1 and is part of a protein complex that includes Hat2 and Hif1 (Ai and Parthun, 2004 and Poveda et al., 2004). Hat1 is recruited by Asf1 to carry out H4K5/12ac, two marks that are associated with histone deposition into nucleosomes (Fillingham et al., 2008).

Homologs of Hat1 have been isolated from chicken, maize, *Xenopus laevis*, yeast and humans (Eberharter et al., 1996; Parthun et al., 1996 and Ahmad et al., 2000). Despite the fact that Hat1 is highly conserved among eukaryotes, it is not essential for the viability of yeast and vertebrate cells, as are the acetylation marks at H4K5/12 (Ai and Parthun, 2004; Barman et al., 2006). Experiments have revealed that Hat1 also plays a role in telomeric silencing which needs both the catalytic activity of the enzyme and its sub-cellular localization (Kelly et al., 2000; Parthun, 2007). Interestingly, Hat1 has been shown to bind H3-H4 in the cytoplasm and then localize to the nucleus (Ejlassi-Lassallette et al., 2011) Furthermore, Hat1 is now known to bind in yeast nuclei to Hif1 which is a histone chaperone that interacts specifically with H3-H4 (Ai and Parthun, 2004).

1.9.5 Gcn5

The first transcription-related HAT to be identified was the *Tetrahymena* p55 protein which is a homologue of the *S. cerevisiae* Gcn5 (Brownell et al., 1996). Gcn5 was originally identified through genetic screens as a transcriptional co-activator in yeast (Grant et al., 1998)

and before that in *Tetrahymena* which linked histone acetylation and transcriptional stimulation (Brownell et al., 1996).

Gcn5 is a subunit of several transcription regulatory complexes including SAGA, SLIK and ADA in yeast. All of these Gcn5-containing complexes regulate transcription. In *S. Cerevisiae,* Gcn5 has been shown to catalyze nucleosomal H3K9ac (Grant et al., 1999).

In vitro studies have shown that the activity and specificity of Gcn5 is regulated by its associated complexes; recombinant yeast Gcn5 acetylates core histones on H3K9/18 but shows no activity on nuclesomal H3 (Kue et al., 1996), As a subunit of the SAGA complex however, Gcn5 can acetylate both free and nucleosomal H3K9/18 (Grant et al., 1999). On the other hand, the SAGA and ADA complexes acetylate both free and nucleosomal H3. While ADA acetylates K14 and K18 of nucleosomal H3, SAGA acetylates both nucleosmal K14 and K18 to a lesser degree as well as nucleosomal K23 and K9 (Grant et al., 1999). Cells lacking Gcn5 are sensitive to DNA damaging agents, which strongly suggests that Gcn5, in addition to its role in gene transcription, also has a role in DNA replication and DNA repair through acetylation (Choy and Kron, 2002; Tamburini and Tyler, 2005). It has been shown that cells lacking GCN5 are more sensitive to DNA damaging agents probably due to the loss of enzymatic activities of Gcn5 (Burgess et al., 2010). It has also been demonstrated that GCN5 genetically interacts with genes that are involved in DNA replication, DNA damage response, and nucleosome assembly. In addition, cells that have had GCN5 deleted or have been mutated at five lysine residues in the N-terminus of histone H3 (K9, K14, K18, K23, and K27) show decreased deposition of newly synthesized H3 into replicating DNA which indicates reduced binding of H3 to CAF-1. These results show that Gcn5 promotes nucleosome assembly by acetylating H3 lysine residues at the N-terminus which in turn contributes to the H3 binding with CAF-1 (Burgess et al., 2010). It has

also been shown that Rtt109 and Gcn5 are in parallel pathways for H3K9ac (Fillingham et al., 2008, Burgess et al., 2010). *S. cerevisiae* PTM enzymology is summarized in Table 1.

Modification	**H3K56ac**	**H3K18ac**	**H3K9ac**
Enzymes involved	Rtt109+Asf1	Gcn5	Rtt109-Vps75+Asf1 and Gcn5

Table 1: PTM enzymology: PTM are shown with the enzymes required for acetylation in *S. cerevisiae*.

1.10 *T. thermophila* as a model:

Tetrahymena thermophila is a ciliated unicellular protozoan. This model organism is convenient for the molecular genetic study of several processes including telomere replication (Blackburn et al., 2006), RNA interference (Mochizuki K, 2010), and large scale genome rearrangements (Yao et al., 2005). In addition, over the years *T. thermophila* has been a vital model organism for chromatin research. As discussed before, p55 was identified as the protein responsible for HAT activity in a biochemical assay made possible by *Tetrahymena's* unique biology (Brownell et al., 1995). The p55 gene was then cloned and became the first gene identified encoding a HAT in any organism (Brownell et al., 1996). As stated earlier, the p55 gene was found by comparative sequence analysis to be homologous to yeast GCN5. As discussed earlier, the finding of a HAT by sequence comparison with known transcriptional activators linked histone acetylation to transcriptional co-activation (Brownell et al., 1996).

Tetrahymena thermophila has a dimorphic nuclei. It contains a transcriptionally active somatic macronucleus, which makes it a rich source of acetylated chromatin. The macronucleus is not inherited. *T.thermophila* also has a micronucleus that is inherited sexually and sexual

reproduction can be induced by starvation (Elliot, 1973), thus making it a genetically and biochemically tractable model for several biological processes including meiosis and RNAi. (Yao and Chao, 2005)

Tetrahymena thermophila has abundant H3K56ac modification (Shahbazian et al., 2007) but comparative sequence analysis indicates that no gene exists in its sequenced genome homologous to either yeast RTT109, metazoan CBP/p300 or P/CAF (Karsou and Fillingham, unpublished). As discussed above, *T. thermophila* encodes p55/GCN5, a gene homologous to human Gcn5 which can catalyze H3K56ac in human cells (Tjeertes et al., 2009). In addition, Gcn5 catalyzes H3K9ac, a modification also catalyzed by Rtt109 in *S.cerevisiae* (Fillingham et al., 2008) and *S. pombe* (Radovani and Fillingham, unpublished). Gcn5 is highly conserved among eukaryotes, which suggests an important role for it in H3 acetylation.

1.11 Research question and approach:

The goal of my thesis research involves trying to characterize tGcn5 activity *in vitro* and more specifically if it has H3K56ac activity, my main hypothesis is that it does. The enzymology behind H3K56ac has not been well characterized in any other organism other than yeast and humans.

To accomplish this I will determine if tGcn5 can catalyze H3K56ac *in vitro* in the presence or absence of either tAsf1 or yAsf1. In addition, I am using molecular genetics to assess the degree to which tGcn5 or tAsf1 can restore wild type functions of the respective mutant backgrounds in yeast. The goal of this research is to provide a basis to understand the phenotypic role of Gcn5 and Asf1 in yeast which will lead to further downstream genetic studies designed to assess the function of these important genes in *Tetrahymena* itself. If we find the H3K56 HAT in

Tetrahymena we can start experiments to see if it has a role in DNA rearrangements, RNAi, or meiosis.

Chapter 2: Materials and methods

2.1 Media used:

The composition of all growth media used is listed in table 2. All solutions are listed in table 3. Unless otherwise noted, all competent cells, enzymes, DNA ladders and reagents were acquired from New England Biolabs. Catalogue numbers are in parentheses. All small scale centrifugation (1.5 ml or less) were performed using a Sorvall MC 12V microcentrifuge. Large scale centrifugations (2 ml to 50ml) was performed using IEC Centra CL3 centrifuge.

Table 2. The composition of media used for culturing *E. coli.*

Media	**Contents**
SOB pH 7.0 (1L)	20 g Bacto-tryptone
SOC (100ml)	97ml SOB 1 ml 2M glucose 1 ml 1M $MgCl_2$ 1 ml 1M $MgSO_4$
YT (1L)	10 g Bacto-tryptone 5 g yeast exctract 5 g NaCl 15 g agar for plates 1 L ddH_2O
YT+ Ampicillin (402 ml)	400 ml YT 2 ml Ampicillin (10mg/ml)
YT+ Kanamycin (402 ml)	400 ml YT 2 ml Kanamycin (10mg/ml)

Table 3: The composition of all solutions used.

Method	Solution	Contents
Agarose Gel Analysis	0.8 % Agarose Gel (50 ml)	0.4 g agarose in 50 ml 1x TEB (Microwave for 2-3 minutes), 5µl of Ethidium Bromide 5µg/ml
	10x TEB buffer pH 8.0 (4L)	10 g TRIS base 5.5 g Boric acid 0.93 EDTA 4 L H_2O Add HCl until pH is 8.0
Miniprep	Miniprep Solution 1 (400 ml)	10 ml 1M TRIS pH 8.0 8 ml 0.5 M EDTA 9 ml 40 % glucose 373 ml H_2O
	Miniprep Solution 2 (10 ml) (prepare fresh)	1 ml 10 % SDS 2 ml 1N NaOH 7 ml H_2O
	Miniprep Solution 3 (500 ml)	147.2 g 3M Potassium Acetate 120.1 g Acetic Acid 500 ml H_2O

Western Blotting	5% Blotto (100ml)	5 g skim milk Powder 100 ml TBS
	5% Stacking gel (5ml)	3.5 ml H_2O 0.625 ml 1M TRIS pH 6.8 0.95 ml Acrylamide 29:1 0.05 ml 10% SDS 3.75 µl TEMED 31.25 µl 10% APS
	10% Running Gel (15 ml)	4.14 ml H_2O 5.6 ml 1M TRIS pH 9.0 4.99 ml Acrylamide 29:1 0.15 ml 10% SDS 3.75 µl TEMED 112 µl 10% APS
Continued Western Blotting	10% APS (1 ml)	0.1 g ammonium persulfate 1 ml H_2O
	2X SDS sample buffer (100 ml)	3 g SDS 5ml betamercaptoethanol 10 ml 100% glycerol 6ml 2M TRIS HCL pH 6.8 50 mg Bromophenol Blue H_2O to 100 ml
	5x TBS (Tris-Buffered	48.44 g Tris

	Saline)	584.4 NaCl 4L dd H_2O Add HCl to pH 7.5
Continued Western Blotting	Running Buffer (4X) (4L)	230.4 g glycine 48 g TRIS 16 g SDS H_2O to 4 L
	Transfer Buffer (2L)	400 ml methanol 6.05 g TRIS base 28.84 g glycine H_2O to 2 L

2.2 Sequence alignments:

Protein sequences in FASTA format were obtained for all species from NCBI protein (http://www.ncbi.nlm.nih.gov/) and sequence alignment were run using CLASTALW (http://www.genome.jp/tools/clustalw/). The ALU format for the sequence alignments was then taken for the similar sequences and shaded using BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). Nuclear localization sequences were predicted using Expasy's ELM tool (http://elm.eu.org/).

2.3 *E. coli* transformation:

DNA was transformed into DH5-alpha high efficiency competent cells (Lot#C2987H). 20 µl of DNA from ligation reaction, 5 µl of pure DNA from supplier, or minipreps, were added to 50 µl of competent cells. In all the cases the DNA concentration was around 1µg/dl. The cells were incubated for thirty minutes on ice, followed by a 60 seconds heat shock at 42 ºC. After an additional five minutes on ice, 950 µl of SOC medium was added and the competent cells were shaken for one hour at 37 ºC to allow for initial expression of the antibiotic resistance marker. One hundred µl of competent cells were plated on YT+ antibiotic at thirty seven degrees overnight. To screen for positive colonies, single colonies were picked from the plate and were grown overnight in YT+ antibiotic broths at thirty 37 ºC for DNA analysis.

2.4 DNA Isolation from *E.coli* (Alkaline Lysis Miniprep Method):

Twox1.5 ml *E.coli* cultures were centrifuged for one minute at 12,000 rpm. The supernatant was removed and 100 µl of miniprep solution 1 was added to the cell pellet. Following vortexing, the mixture was incubated at room temperature for five minutes, 200µl of miniprep solution 2 was added followed by a five minute incubation on ice, and next 200µl of miniprep solution 3 added. After another five minute incubation on ice, the mixture was centrifuged at 12,000 rpm for ten minutes to remove cellular debris. The supernatant was transferred to another Eppendorf tube and 95% ethanol was added to precipitate the DNA. After a ten minutes incubation at room temperature, the mix was centrifuged at 12,000 rpm for ten minutes. The supernatant was aspirated and the pellet was washed with 70% alcohol by adding and aspirating. The pellet is then dried out in a desiccator to dry any extra ethanol. The DNA was

resuspended in 50 µl H20. To remove RNA, 1 µl of DNAase free RNAase (10mg/ml) was added and then the mix was incubated for two hours at thirty seven degrees.

2.5 DNA isolation from *E. coli* for sequencing:

For small scale minipreps DNA was isolated from three ml cultures of *E. coli* using the Sigma GenElute Plasmid Miniprep Kit (PLN-701KT) according to the supplier's instructions.

2.6 Enzymatic restriction digest:

Plasmid DNA and insert DNA were digested with one unit of restriction enzyme/s per µg of DNA. For small scale digestions a total volume of 10 µl was digested for one to two hours. Large scale digestions were done on a volume of 400 µl and digested overnight. All restrictions reactions were incubated at the temperature specified by the supplier (37 ºC for *BamH1* and *Xho1* or 25 ºC for *Sma1*).

2.7 Gel electrophoresis:

About 0.8 to 1.0% agarose gel were electrophoresed 100-120 volts in TBE buffer. Gels were stained by adding Ethidium Bromide (0.1 % of the total gel volume) and DNA bands were visualized using an Alpha Innotech FlourChem system in the Core Molecular Facility at York University.

2.8 Ligation:

Ligation reactions were performed using 1 µl (400 units) of T4 DNA Ligase (NEB#M0202L) in a total volume of 20 µl reaction. Equal molar amounts of both DNA

fragments and plasmid DNA to be ligated were added to the mix and the reactions were incubated at 16 ºC overnight. The next morning the ligation mix was used to transform DH5 alpha cells.

2.9 PCR "Polymerase Chain Reactions" :

PCR reactions were done in 50 µl volumes with 0.5 µl (2.5 units) of Phusion Polymerase (NEB, F-530L). Forward and Reverse primers were obtained from Invitrogen. The sequences for primers used are mentioned in the supplemental information.

2.10 Purification of DNA:

PCR products as well as DNA from enzymatic reactions "Digestions" were purified using Sigma GenElute PCR Clean-up kit (NA1020-1KIT). DNA extraction from agarose gels were performed using Sigma GenElute Gel Extraction Kit (NA1111-1KIT).

2.11 Genomic DNA extraction from *S. cerevisiae:*

Approximately 1.5 ml of dense overnight culture was centrifuged and the supernatant was aspirated off. The pellets were washed in two hundred µl ddH_2O. The cells were then resuspended in 200 µl of solution A (1 M Sorbitol, 100 mM Na citrate pH 7.0, 60 mM EDTA, 0.1 M β-mercaptoethanol, and 1 mg/ml zymolase) and incubated for 30 minutes at 37 ºC to break down the cell wall. 700 µl of solution B (0.5 % SDS, 100 mM Tris-HCL pH 8.0 and 50 mM EDTA) was then added to break down the cells. The mixture was then vortexed briefly and incubated at 70 ºC for 20 minutes and then allowed to cool down at room temperature. 200 µl of fresh 5 M Pottassium acetate was then added to precipitate all proteins and was centrifuged at

12,000 rpm. The supernatant was then treated in 2:1 of ice-cold 100% ethanol. Next the tube was incubated at -80 ºC for 30 minutes to precipitate nucleic acid. The DNA was then pelleted on at 12,000 rpm for 15 minutes and washed with 750 µl of ice cold 70 % ethanol. Next the DNA was vacuum dried for 15 minutes. The DNA was then treated with 500 µl of Phenol/chloroform/isoamyl alcohol 25:24:1. Next the tube was pelleted and the upper aqueous phase was obtained. This step was repeated twice. 10 % of the volume was then added with 3M Na-acetate and two times of 100 % ice cold ethanol and incubated for 30 minutes in -80 ºC. Next the tube was centrifuged and the precipitated DNA was vacuum dried for 15 minutes. The DNA was then resuspended in 200 µl of ddH2O.

2.12 Yeast transformation:

Cells were grown overnight to saturation. The next morning they were diluted 1/20 and grown to about 1 OD_{600}. Approximately 50 ml were pelleted, washed in 10 ml ddH2O, and then washed in 1 ml of 0.1 M Lithium acetate before being resuspended in 400 µl of 0.1 M Lithium acetate.

50 µl of the mixture is then taken for transformation and pelleted. To the pellet the following is added: 240µl of Poly-Etheleneglycol PEG (50%), 36µl of 1M LiOAc pH7.2, 50µl of Salmon Sperm (pre boiled and diluted 1:5) 45µl of sterile ddH2O 5µl (1 µg) of plasmid DNA. The tube is vortexed for 1 minutes and placed at 30°C for 20min. After the incubation 40µl of 10% DMSO is added and the cells are incubated at 42 ºC for 10 min. After second incubation cells are spinned down for 10 seconds at 12,000 rpm. The supernatant is removed and cells re-suspended in 250 µl of sterile ddH2O. The whole volume of the tube is then spread plated onto -LEU plates.

2.13 Spot tests:

Yeast cells were grown overnight to saturation. The next morning they were diluted to 0.1 OD_{600} for fast growing cells (+yASF1, yASF1N, +yGCN5, +RTT109, single mutants and wild type) and 0.25 OD for slow growing cells (+tASF1, +tASF1N, tGCN5 and double mutants). Cells were then grown to 0.5 OD_{600}. Serial dilutions of 1/5, 1/25 and 1/125 were made in –LEU media. For each strain 5 µl of undiluted as well as diluted culture was inoculated in the plate, dried and incubated at 30 ºC for 48 hours and 72 hours.

2.14 Yeast harvesting and whole cell extract:

Cells were grown overnight at 30 ºC. In the morning the cells were diluted 1:20 and grown to mid log phase, approximately 0.5 OD_{600}. Equal OD_{600} were then collected and centrifuged. The cells were re-suspended in 10 ml 20 % TCA, pelleted, and flash frozen in dry ice or liquid nitrogen and kept at -80 °C for later use. The cells were then re-suspended in 250 µl of 20% TCA and 350 µl of glass beads were added. Next the cells were lysed by hand-vortexting for 1 minute, 4 times with a 1 minute break on ice in between. The whole cell extract was then collected by centrifugation and the beads were washed one more time with 5% TCA. The precipitated protein is then resuspended in 250 µl SDS-PAGE loading buffer and 50 µl un-phed Tris buffer.

2.15 Western Blotting:

The whole cell extract of yeast was resuspended in a volume of 2x SDS and was then boiled for 5 minutes. 20 µl of sample was electrophoresed through an SDS polyacrylamide gel

(SDS-PAGE), transferred to a nitrocellulose membrane overnight, and blocked in 5% Blotto. The blots were incubated in primary antibody (diluted according to manufacturer data) in 10 ml of Blotto for one hour at room temperature. Next the blots were washed three time in TBS and at room temperature with 5 minutes for each wash. Next the blots were incubated with horseradish peroxidase-conjugated secondary antibody in PBS for one hour at room temperature. The blots were then washed three times in TBS, again at room temperature with five minutes for each wash. Finally, the blots were visualized using Denville Scientific's HyGLO Chemiluminescent HRP AntiBody detection Kit (E2500) as per the manufacturer's instructions.

2.16 Protein expression induction:

Colonies that contained clones of the desired genes were confirmed by gel electrophoresis after restriction digestion. The colonies that contained the inserts were grown in LB+Kanamycin overnight. Then next morning a new culture was inoculated in LB + KAN media to 0.05 OD_{600} by diluting the overnight culture 1:100. The culture was then grown to 0.5 OD. At 0.5 OD the time zero (t0) control sample was obtained, by centrifuging and freezing the cell pellet at -80 ºC for next processing. IPTG was then added to a final concentration of 1mM (by using 100mM stock solution) to induce expression. Samples were obtained at 4h after induction.

For all samples: 1.5ml of media was pipetted in Eppenndorf tubes, spun for 1min to pellet cells. The supernatant was then aspirated, the pellet was frozen on dry ice and then stored -80 for further processing (SDS-PAGE or Western Blotting). Note: It was attempted to obtain the same amount of cells in every tube i.e. for t0, 1.5ml at OD_{600} 0.5 = 0.75 total OD_{600}. The OD was measured at each point of collection. For example at time 4h if the OD is 0.75 only 1 ml will be taken to obtain a total of 0.75 OD_{600}.

When ready to run gel (10% SDS-PAGE) pellets were thawed briefly on ice. Next, 100 µl of 1x SDS-PAGE buffer (made fresh) was added and pipetted up and down on ice to resuspend pellet. The samples were boiled for 10 minutes and loaded on the gel.

2.17 Nickel agarose affinity chromatography (protein purification):

Purification was done under native conditions. Transformed bacterial cells (BL21 *E. coli*) were grown overnight to saturation in terrific broth (Sigma). Cells were then diluted 1:20 in the same media and grown to 1.0 OD_{600} and then IPTG added to 1.0 mM. Cells were then incubated at 30 ºC for 2 hours, then 24 ºC and then for 4 hours and at 16 ºC overnight. The next morning the cells were harvested by centrifugation and re-suspended in 40 ml of binding buffer (5 mM imidazole, 500mM NaCl, 50 mM Tris-HCl pH 8.0, 0.5 mM phenylmethylsulfonyl fluoride and 1mM benzamadine). The suspension was then sonicated 12 times with 8 seconds on, 15 seconds off intervals on ice. The mixture was then centrifuged and the supernatant was added to 5 ml of packed nickel beads equilibrated in binding buffer and rotated at 4 ºC for 30 minutes. The mixture was then passed through a column. The remaining beads were washed once with binding buffer and once with wash buffer (25 mM imidazole, 500mM NaCl, 50 mM Tris-HCl pH 8.0, 0.5 mM). Next beads were eluted with elution buffer (250mM imidazole, 500mM NaCl, 50 mM tris-HCL pH 8.0, 0.5 mM phenylmethylsulfonyl fluoride and 1mM benzamadine) until Bradford analysis of the elute was almost negative. The elute was then dialyzed for six hours in dialysis buffer (10 mM Tris-HCl pH 7.9, 0.1% Triton X-100, 0.5 mM DTT, 0.2 mM EDTA, 20% glycerol, 100 mM NaCl).

2.18 HAT assays:

HAT assays were performed using either chicken core histones or *T.thermophila* histones. HAT assays were done by incubating for 30 minutes at 30°C in a 30 µl volume containing 3 µg core histones as substrate and 5µl (2 µg) aliquots of nickel affinity-purified recombinant protein in HAT assay buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 ug acetyl-CoA and 1 mM phenylmethylsulfonyl fluoride). Reactions were stopped by addition of 30 µl 2x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading dye and boiled. Aliquots (20% of total volume) were run on several 15% SDS-PAGE gels. One gel was fixed and stained with Coomassie blue, whereas the other gels were transferred to nitrocellulose for Western blots analysis.

2.19 Macronuclei isolation from *T. thermophila*:

This method was adopted from Gorovsky et al., (1975) *T. thermophila* cells are grown to about $2x10^5$/ml and then starved in 10 mM tris-HCL buffer pH:7.5 overnight. The cells are then harvested by centrifugation. Next the cells were re-suspended in 10 ml ddH2O and then washed with 100 ml of nuclei wash solution (1mM $MgCl_2$, 0.25 M sucrose, 10mM Tris-HCl pH 7.5, 3mM $CaCl_2$). Protease inhibitors were added at this step (0.1mM AEBSF, 10 uM leupeptin, 1 uM Pepstatin). The cells were then re-suspended in nuclei isolation medium A (2 mM $MgCl_2$, 0.1 M Sucrose, 10 mM Tris-HCl pH 6.75, 4% Gum Arabic). Protease inhibitors were added as above. The cells were then put into a Warring blender and Octanol was added to 1%. The cells were blended 3 times for 30 seconds and chilled for 20 seconds on ice between each blend. EDTA was then added to the mixture to a final concentration of 8 mM. The mixture was then

blended again for another 20 seconds and the yield was checked using methyl green stain. Next the mixture was centrifuged and the supernatant discarded. The pelleted macronuclei were re-suspend in 5 ml of medium A with no octanol.

2.20 Histone purification from *T. thermophila*:

Core histones were purified from *T. thermophila* macronuclei (Macs). The Macs isolation is described later. Purified Macs ($1x10^6$) were re-suspended to 0.2M H_2SO_4 (or 0.4N) in about 1 ml and rotated at 4 degrees overnight. In the morning the tube was centrifuged at 13500 rpm at 4 ºC for 15 minutes. The supernatant was transferred to a new tube; the core histones are part of the H_2SO_4 acid soluble fraction now. 100% trichloroacetic acid was added to 20% final concentration (100ul 100% TCA per 400ul H2SO4 acid supernatant), the tube is then mixed by inversion, and then chilled on ice for 2 hours. Next the tube is spun at 13500 rpm at 4 ºC for 15 minutes; core histones are now part of the pellet so the supernatant was removed and the pellet was then washed with 500 µl cold, acidified acetone (0.024M HCL in acetone) to get rid of the TCA. The tube was then spun in 13500 rpm at 4 ºC for 15 minutes and the supernatant was removed. The pellet was then washed once with 500 µl cold acetone to get rid of the HCL. Next the tube was spun as above and the supernatant was completely removed. The remaining acetone was evaporated in a vacuum desiccator and the histone pellet was resupended in 200 µl ddH2O and analysed by SDS-PAGE and WB.

2.21 Histone Binding assay:

In Vitro histone binding was done using chicken core histones as well as *T.thermophila* core histones. The enzymes were incubated with histones in assay buffer (50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride and 1mM benzamadine). Approximately 50 µg of histone and 100 µg of enzyme were added to a total of 750 µl. The mixture was then rotated at 30 ºC for 30 minutes. Then 50 µl was obtained for input and 20 µl of packed nickel beads were added to the remaining mixture followed by a 15 minutes rotation at 4 ºC. Next, the beads were washed four times with the assay buffer. The sample was then eluted by boiling the beads in 0.1 M EDTA SDS loading buffer. The eluant was run on 15 % SDS-PAGE and then stained with Commassie blue, protein silver stain or transferred to Nitrocellulose for western blots as needed.

Chapter 3: Results

3.1 Conservation of Asf1 and Gcn5 between humans, yeast and *T. thermophila* :

Rtt109 is the fungal H3K56 HAT (Fillingham, 2008). In humans p300/CBP (Das et al., 2009) and Gcn5 (Tjeertes et al., 2009) are H3K56 HATs. Since *T.thermophila* appears to encode a homologue only of Gcn5 and not Rtt109 or p300/CBP (Karsou and Fillingham, unpublished) we began our analysis with tGcn5. I obtained the Gcn5 amino acid sequences of several species from the NCBI protein database and performed comparative sequence alignments as outlined in materials and methods. Gcn5 is very well conserved among eukaryotes (Figure 3). Using the BLASTP function from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and by comparing tGcn5 with Gcn5 of several species I have calculated the similarity of Gcn5 of examined species with the entire region of tGcn5 (Table 4). I also used Expasy's ELM tool (http://elm.eu.org/) to predict the nuclear localization signal of each sequence.

Species	**Percentage of similarity with entire region of tGcn5**
T. thermophila	100%
H.sapiens	85%
G.gallus	85%
M.musculus	85%
D.melanogaster	84%
S.cerevisiae	83%
S.pombe	88%
P.tetraurelia	88%

Table 4: Similarity of Gcn5 of indicated species with the entire region of tGcn5: FASTA amino acid residue sequences were obtained from NCBI protein database and comparative sequence analyses were performed using BLASTP from NCBI.

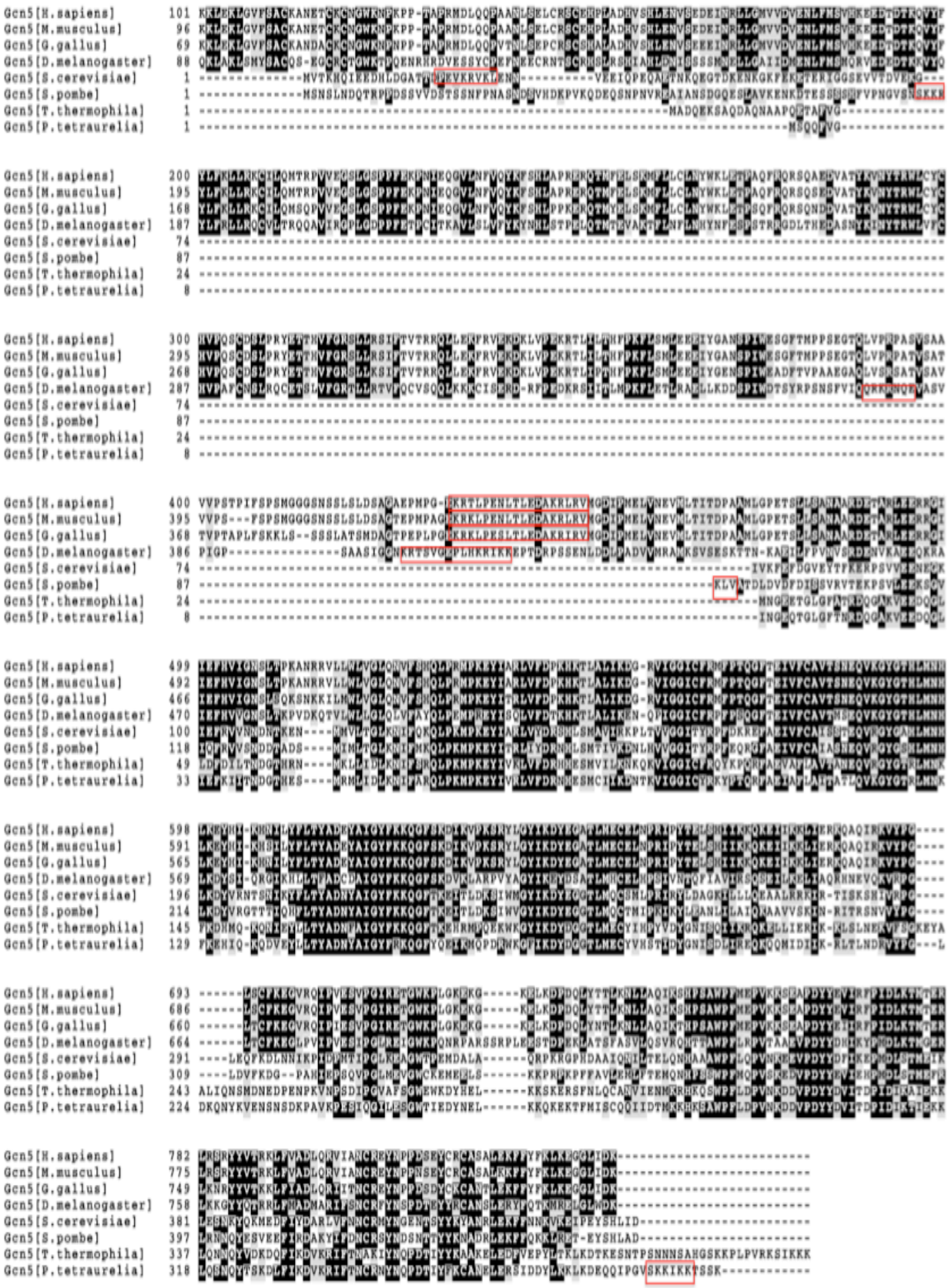

Figure 3:. Gcn5 structure is well conserved among eukaryotes: Continues on next page.

Figure 3: Gcn5 primary structure is well conserved among eukaryotes: Gcn5 amino acid sequences were obtained for the species shown and sequence alignment is obtained using CLASTALW (http://www.genome.jp/tools/clustalw/). Aligned amino acid residues are shaded using BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html) Black: identical amino acid residues, Grey: Similar amino acid residues. NLSs are predicted using Expasy's ELM tool (http://elm.eu.org/) and are within red rectangles.

Yeast Asf1 functions with the HAT Rtt109 to acetylate H3K56 (Fillingham et al., 2008). In order to discover if tAsf1 has a similar role, I similarly obtained Asf1 amino acid sequences and performed comparative sequence alignments using both the entire region and N-terminal domains (Figure 4). I have found that tAsf1 is very well conserved at its N-terminal domain but not in the C-terminal domain (Table 5), similar to what is known for other organisms. Sequence alignment and domains and of Asf1 for several species as wells as yeast H3-H4 binding sites are shown in Figure 4. I also used Expasy's ELM tool (http://elm.eu.org/) to predict the nuclear localization signal of each sequence.

Species	**Percentage of similarity with entire region of tAsf1**	**Percentage of similarity within N-terminal domain of tAsf1 (tAsf1N)**
T. Thermophila	100%	100%
H.sapiens Asf1a	85%	99%
H.sapiens Asf1b	75%	99%
G.gallus	85%	99%
M.musculus	85%	99%
D.melanogaster	70%	99%
S.cerevisiae	56%	99%
S.pombe	60%	99%
P.tetraurelia	76%	100%

Table 5: Similarity of the entire region and N-terminal domain of indicated species with tAsf1: FASTA amino acid sequences were obtained from NCBI protein database and comparative sequence analyses were performed using BLASTP from NCBI.

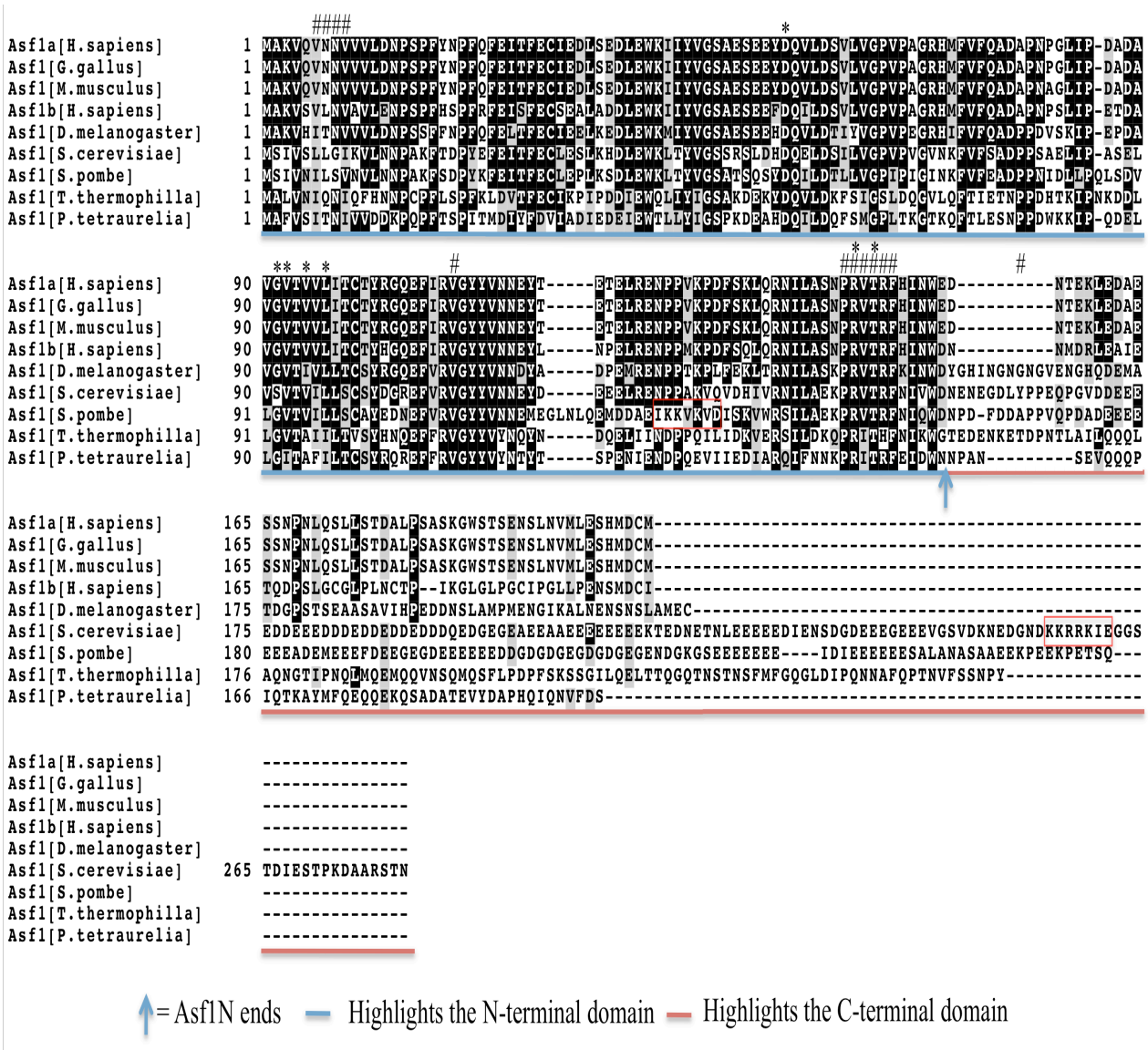

Figure 4: Asf1 is very well conserved at its N-terminal domain: Asf1 amino acid sequences were obtained for the species shown and sequence alignment is obtained using CLASTALW (http://www.genome.jp/tools/clustalw/). Aligned amino acid residues are shaded using BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html) Black: identical amino acid residues, Grey: Similar amino acid residues. NLS's are predicted using Expasy's ELM tool (http://elm.eu.org/) and are within red rectangles. Asf1N ends are shown in blue arrows. N-terminal highly conserved domain is shown underscored in blue. C-terminal domain is underscored in red. *Residues of yAsf1 involved in interaction with *S. cerevisiae* H3. # Residues involved in interaction with *S. cerevisiae* H4. (Reference: Antczak et al., 2006; English et al., 2006)

3.2 Construction of *T. thermophila* synthetic genes (tGCN5,tASF1):

We constructed synthetic genes for tASF1 and tGCN5. It was previously shown that *T. thermophila* has a slightly different genetic code than the universal genetic code (Horowitz and Gorovsky, 1985). In *T. thermophila* the TAA and TAG codons specify for glutamic acid (Q), while in the universal genetic code they specify stop codons. Therefore, in order to express *T. thermophila* proteins in bacteria or yeast TAA has to be changed to CAA and TAG changed to CAG. One method to do this is site directed mutagenesis. However, with the 18 codons to change in tASF1 (Figure 5, top sequence) and 21 codons to change in tGCN5 (Figure 6, top sequence) it was easier and cheaper to construct synthetic genes by sending changed cDNA sequences for tASF1 and tGCN5 to GENESCRIPT. Predicted cDNA gene sequences for tASF1 and tGCN5 were obtained from the *Tetrahymena* genome database (www.cilliate.org) and then modified *in silico* to produce cDNA sequence that *E.coli* and *Saccharomyces cerevisiae* are able to translate (Figure 5 and 6, bottom sequences).

Figure 5: Modifications made to tASF1 to correspond to universal genetic code: Modified TAA and TAG codons in tASF1 are indicated by blue arrows. tASF1 gene (1st sequence) and modified gene (2nd sequence) and their amino acid sequence translation. tAsf1N end is shown by an arrow at amino acid residue 155.

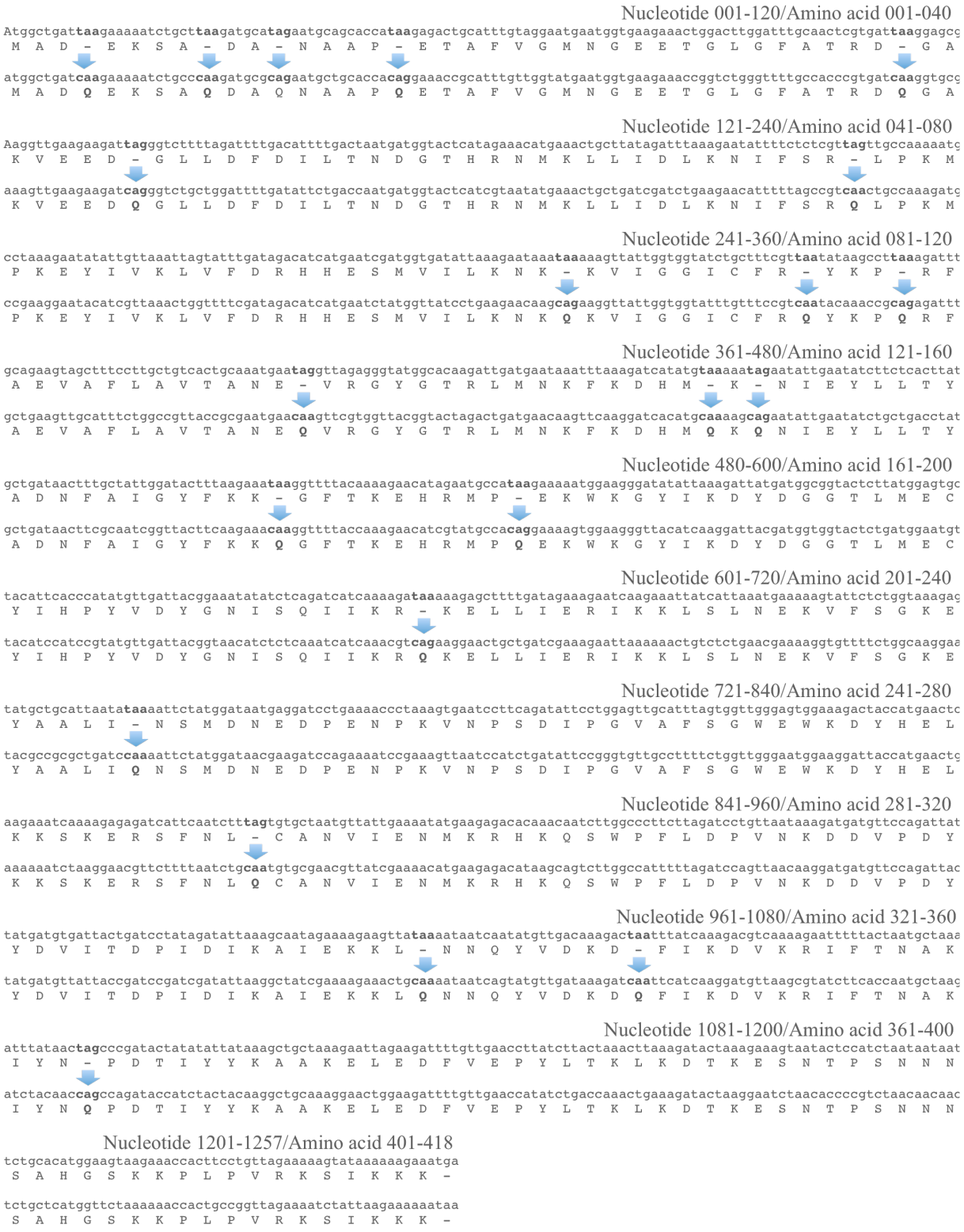

Figure 6: Modifications made to tGCN5 to correspond to universal genetic code: Modified TAA and TAG codons in tGCN5 are indicated by blue arrows. tGCN5 gene (1st sequence) and modified gene (2nd sequence) and their amino acid sequence translation.

3.3 Preparation of template DNA for amplification of yeast *ASF1*, *ASF1N*, *RTT109* and *GCN5*:

In order to have a template to amplify several yeast genes used in this study, I prepared genomic DNA from *S. cerevisiae* (see materials and methods). Figure 7 shows 1µg of yeast genomic DNA run out on a 0.8% agarose gel. The DNA was intact and thus appropriate for use as a template for PCR.

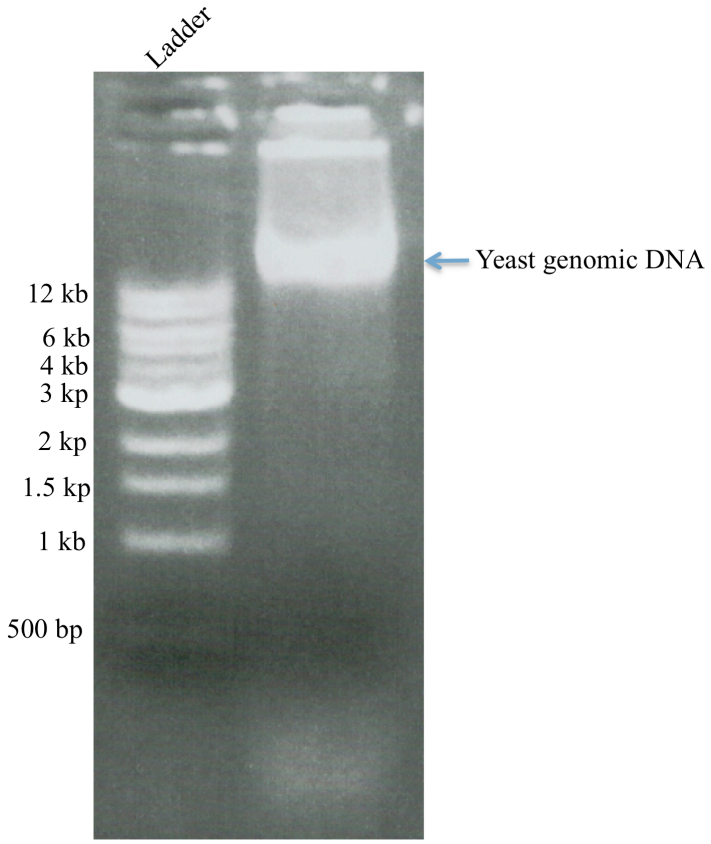

Figure 7: Purified yeast genomic DNA: A 0.8% agarose gel made in 1x TBE buffer, embedded with ethidium bromide and visualized by Alpha Innotech FlourChem system. Around 1 µg of genomic DNA was loaded. DNA ladder was obtained from New England Biolab (NEB).

3.4 Amplification and molecular cloning of *T. thermophila* and yeast ASF1, ASF1N and GCN5:

The *T.thermophila* and yeast genes were amplified by PCR using either yeast genomic DNA or *T. thermophila* synthetic genes provided by GENESCRIPT in plasmid pUC57 then cloned into pET28a (Novagen), a bacterial expression vector that contains DNA sequence that will give them an N-terminal 6-histidine tag (6xHIS). Primers were designed with appropriate restriction site on their 5\ ends (*BamH1* for all except *Sma1* for yGCN5) and their 3\ end (*Xho1*) for cloning purposes. Ligated plasmids were then transformed into *E.coli* DH5α before being re-purified and digested with appropriate restriction enzymes and those with inserts of correct size were sequenced (Lee Wong, York University) to ensure 100% match to template.

Plasmids were then transformed into BL21 (DE3) *E.coli* for expression. Plasmid pET28a encodes a lac repressor that control gene expression and is antagonized by IPTG. Thus IPTG is used to induce expression of the recombinant proteins. The pET28a plasmid has a T7 promoter and BL21 (DE3) *E. coli* cells has a T7 RNA polymerase, which makes expression of proteins in those cells feasible.

The same set of genes were amplified and cloned into pRB415-12myc, a CEN-based plasmid that contains DNA sequence encoding an N-terminal 12-MYC epitope tag and the yeast ADH1 promoter for expression of cloned genes in *S. cerevisiae.* The plasmids that were obtained were digested with appropriate restriction endonucleases and sequenced as above to make sure that there were no alterations that might have happened during the ligation or PCR. Figure 8 shows the restriction enzyme digestion that confirms successful cloning of tASF1 and tGCN5 into pET28a.

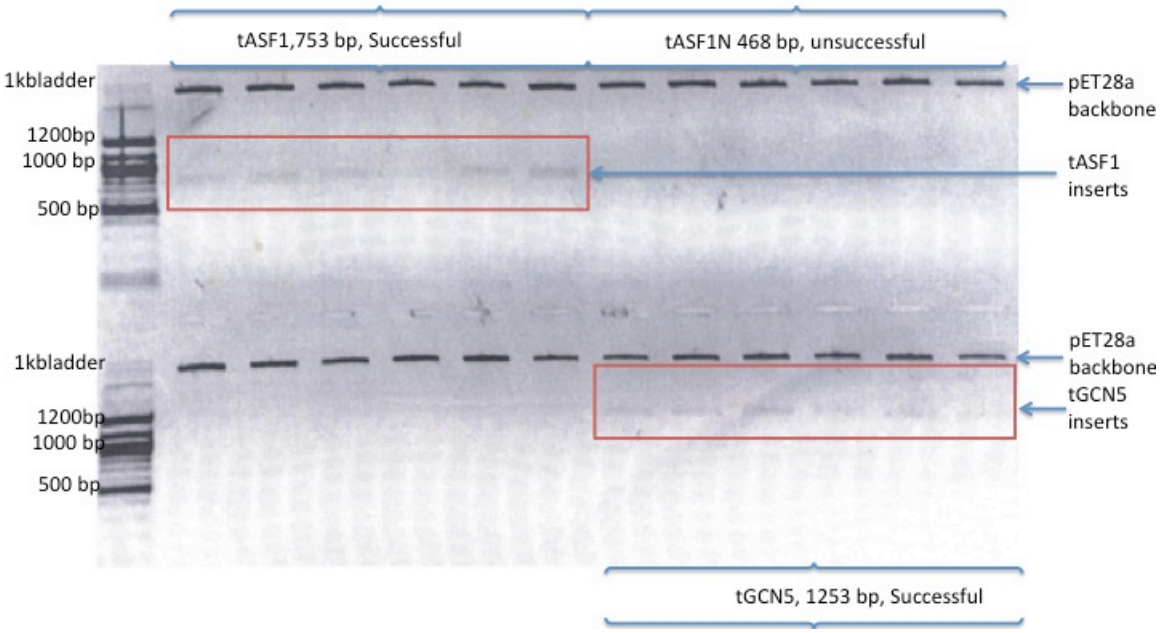

Figure 8: Restriction enzyme digestion for verification of molecular cloning: A 0.8% agarose gel embedded with ethidium bromide and visualized by Alpha Innotech FlourChem system. 0.25 µg of DNA digested with *BamH1* and *Xho1* were loaded on each well and arrows shows the inserts of correct size for corresponding genes. 100 bp DNA ladder was used. This particular experiment shows successful cloning of tASF1 and tGCN5 into pET28a in that digestion released a fragment of the expected size.

3.5 Yeast transformation:

pRB415-12MYC-based plasmids were used in an *in vivo* assay to determine if tAsf1 or tGcn5 could rescue the requirement in yeast of either Asf1 or Rtt109 for H3K56ac. Plasmids were transformed into either *asf1*/*gcn5* or *rtt109*/*gcn5* double mutants. As mentioned in the introduction, in addition to their role in H3K56ac, Rtt109 and Asf1 together in yeast also acetylate H3K9 in a parallel pathway to Gcn5. To be clear, in yeast Rtt109 and Asf1 collaborate together to acetylate all H3K56 and about half H3K9. In yeast Gcn5 acetylates all H3K18 and about half H3K9a. Therefore, the two double mutants are completely null for H3K56ac, H3K9ac and H3K18ac and therefore even partial or weak rescue can be easily assessed. The

double mutants were made and characterized in Fillingham et al. (2008) (Summarized in Table 6 and 7).

Empty plasmid pRB415-12myc was transformed into both of the double mutants (Δ*asf1/gcn5* and Δ*rtt109/gcn5*) as well as the single mutants (Δ*asf1,* Δ*gcn5* and Δ*rtt109*). *yASF1*, *yASF1N*, *tASF1*, and *tASF1N* genes cloned into pRB415-12myc were transformed into a yeast strain that lacks *asf1* and *gcn5*. *yGCN5*, *tGCN5* and *RTT109* cloned into the same plasmid were transformed into yeast strains lacking *gcn5* and *rtt109*.

Modification	**Δ*asf1***	**Δ*gcn5***	**Δ*asf1/gcn5***
H3K56ac	Null	Normal	Null
H3K18ac	Normal	Null	Null
H3K9ac	Decreased	Decreased	Null

Table 6: Characterization of Δ*asf1* and Δ*gcn5* single and double mutants: The effect of the respective gene deletion on the shown PTM's is described. Data is summarized from Fillingham et al. (2008).

Modification	**Δ*rtt109***	**Δ*gcn5***	**Δ*rtt109/gcn5***
H3K56ac	Null	Normal	Null
H3K18ac	Normal	Null	Null
H3K9ac	Decreased	Decreased	Null

Table 7: Characterization of Δ*rtt109* and Δ*gcn5* single and double mutants: The effect of the respective gene deletion on the shown PTM's is described. Data is summarized from Fillingham et al. (2008).

3.6 tASF1 and tGCN5 do not rescue the growth defect of Δ*asf1* and Δ*gcn5* in yeast:

I examined possible interplay between *tGCN5*, *yGCN5* and *RTT109* by determining whether I could rescue Δ*rtt109*/*gcn5* double mutant yeast strains by transforming them with *tGCN5* (along with positive controls *RTT109* and *yGCN5*) and determining whether it could rescue the mutants slow growth phenotype. I asked a similar question by determining whether *tASF1*, *tASF1N*, *yASF1* and *yASF1N* cloned in pRB4151-2MYC could rescue the Δ*asf1*/*gcn5* double mutant.

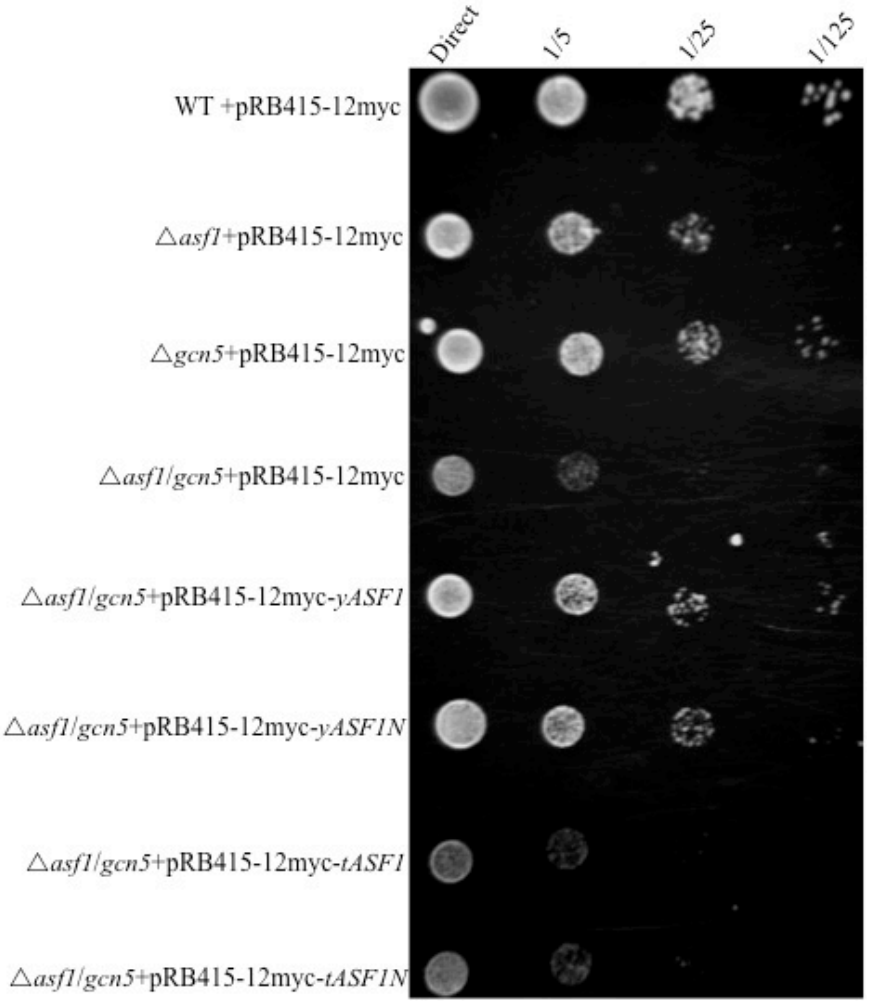

Figure 9: Growth spot tests, Growth comparison for *tASF1* and *tASF1N*: Media lacking the Leucine amino acid (-Leu) plates are inoculated with 5 µl of culture (direct) plus its serial dilution of 1/5, 1/25 and 1/125. Growth is compared with indicated strains against wild type (WT) growth.

The △*asf1*/*gcn5* strain shows very weak growth compared to that of the wild type (WT) (Figure 9), similar to the growth defect observed on rich media in Fillingham et al. (2008). The △*asf1* and △*gcn5* single mutants strains showed intermediate growth between wild type and the △*asf1*/*gcn5* double mutant. The △*asf1*/*gcn5* strain transformed with pRB415-12myc-*yASF1* and pRB415-12myc-*yASF1N* shows growth similar to that of △*gcn5* single mutant, indicating the assay is working and there is rescue of the slow growth defect by pRB415-12myc-*yASF1* and pRB415-12myc-*yASF1N* as expected (Daganzo et al., 2003). △*asf1*/*gcn5* showed no significant change in growth rate after being transformed with pRB415-12myc-*tASF1* or pRB415-12myc-*tASF1N* (Figure 9).

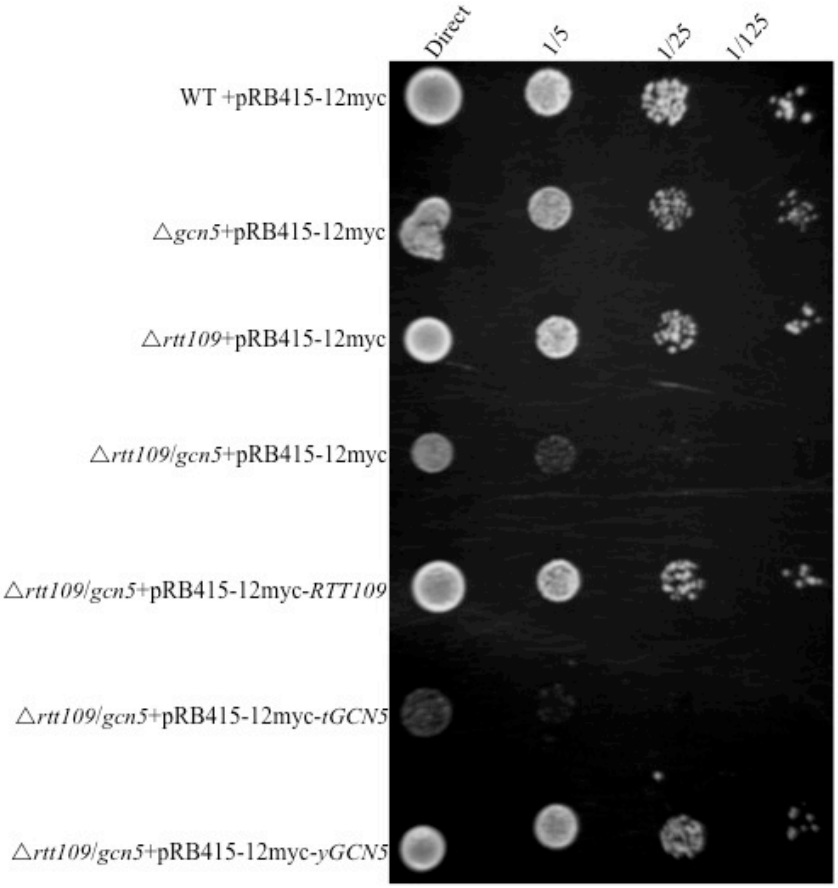

Figure 10: Growth spot tests, Growth comparison for *tGCN5*: -Leu plates are inoculated with 5 µl of culture (direct) plus its serial dilution of 1/5, 1/25 and 1/125. Growth is compared with indicated strains against wild type (WT) growth.

The △*rtt109*/*gcn5* strain showed very weak growth compared with WT (Figure 11), as also seen on rich media in Fillingham et al. (2008). The △*rtt109* and △*gcn5* single mutants strains shows intermediate growth compared with WT and the △*rtt109*/*gcn5* double mutant, while the △*rtt109*/*gcn5* strain transformed with either pRB415-12myc-*yGCN5* or pRB415-12myc- *RTT109* shows growth similar to either the △*rtt109* or △*gcn5* single mutants respectively (Figure 11) showing the assay is working. By contrast the △*rtt109*/*gcn5* double mutant showed no change in growth after being transformed with pRB415-12myc- *tGCN5* (Figure 11).

Together these data suggest the following:

1. *tGCN5* does not rescue the slow growth phenotype of Δ*rtt109*/*gcn5* double mutant and therefore does not substitute for either.
2. *tASF1* or *tASF1N* does not rescue the slow growth phenotype of Δ*asf1*/*gcn5* double mutant and therefore does not substitute for either.

Thus, despite the large homology between yAsf1 and tAsf1 (Figure 4) on one hand, and yGcn5 and tGcn5 on the other hand (Figure 3), tAsf1 was unable to rescue the slow growth defect of Δ*asf1*, and tGcn5 was unable to rescue the slow growth defect of Δ*gcn5*. To further investigate whether any rescue was occurring, we next assessed histone acetylation levels in these strains.

3.7 *In Vivo* acetylation assays on yeast transformed mutants:

To assess levels of H3K9ac, H3K18ac and H3K56ac in transformed strains, I made whole cell extracts and used Western Blotting (WB). In *S. cerevisiae,* Rtt109 and Asf1 are required for H3K56ac (Fillingham et al., 2008), and Gcn5 for H3K18ac (Grant et al., 1997). In addition, both are required for full H3K9ac (Fillingham et al., 2008) (Table 1).

In this experiment, I also blotted with an antibody that recognizes unmodified histone H3 to ensure I was comparing roughly equal amount of whole cell extracts (WCE). I also blotted with an antibody that recognizes the N-terminal 12xmyc tag to ensure the genes cloned into pRB415-12myc are actually being expressed in *S. cerevisiae,* and that the lack of rescue was not due to a lack of expression. Table 8 lists expected results for the positive controls for this experiment based on Fillingham et al. (2008).

Plasmid Transformed into Δ*rtt109/gcn5* or Δ*asf1/gcn5*	**α-H3K56ac**	**α-H3K18ac**	**α-H3K9ac**
pRB415-12myc	Negative	Negative	Negative
pRB415-12myc-*RTT109*	Positive	Negative	Positive
pRB415-12myc- *yGCN5*	Negative	Positive	Positive
pRB415-12myc- *yASF1*	Positive	-	Positive

Table 8: *In vivo* histone assay controls: Expected acetylation results for the indicated plasmids transformed into either Δ*asf1/gcn5* or Δ*rtt109/gcn5*.

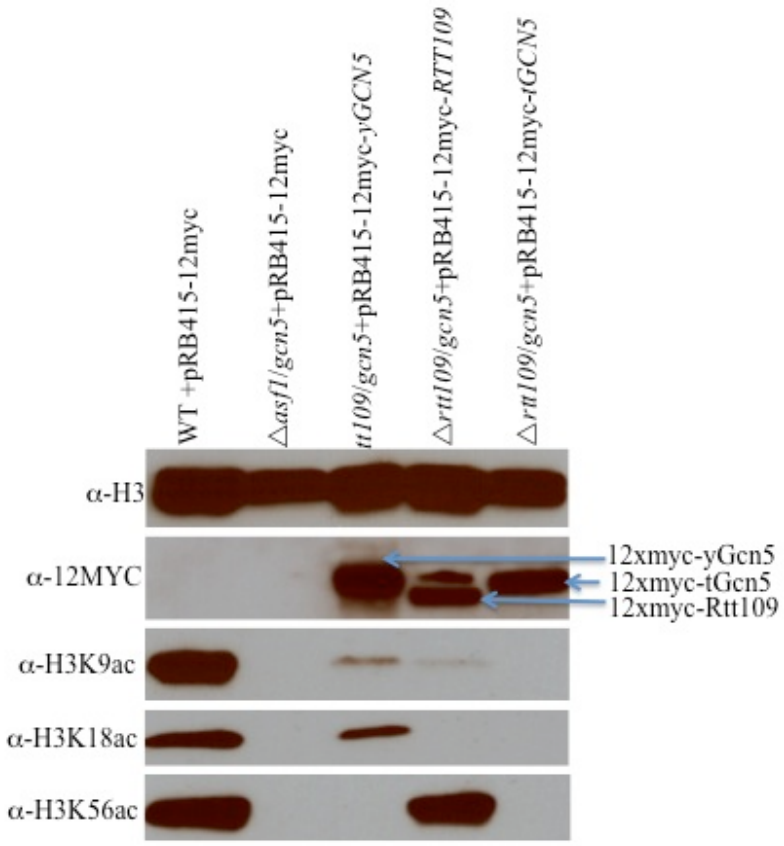

Figure 11: *In vivo* histone acetylation experiments for *tGCN5*: WB comparison of WT, pRB415-12myc-*yGCN5*, and pRB415-12myc-*RTT109* with pRB415-12myc-*tGCN5*. TCA WCE of indicated strains separated in 15% SDS-PAGE gel, transferred into nitrocellulose and blotted against indicated antibodies

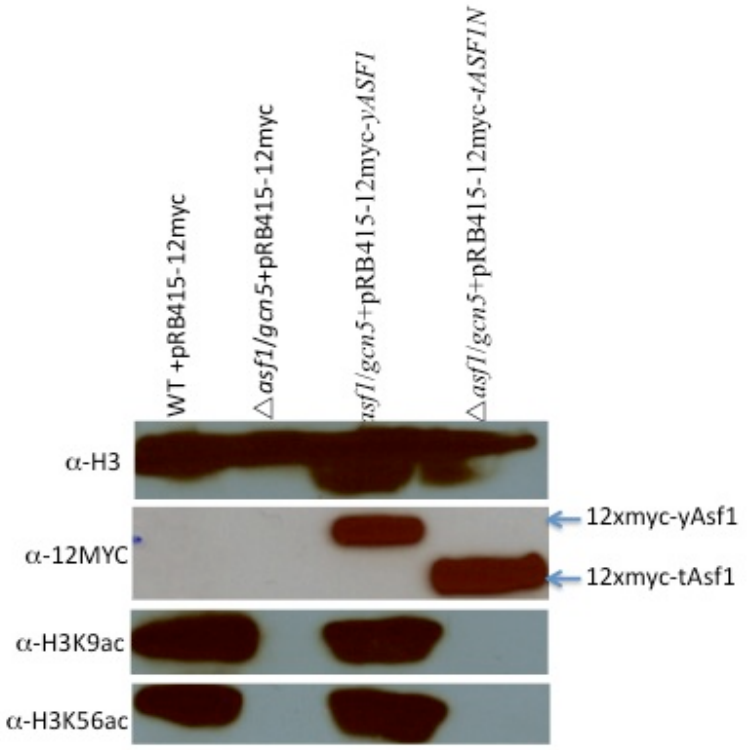

Figure 12: *In vivo* histone acetylation experiments for *tASF1*: WB comparison of WT and pRB415-12myc- *yASF1*, with pRB415-12myc-*tASF1*. TCA WCE of indicated strains separated in 15% SDS-PAGE gel, transferred into nitrocellulose and blotted against indicated antibodies

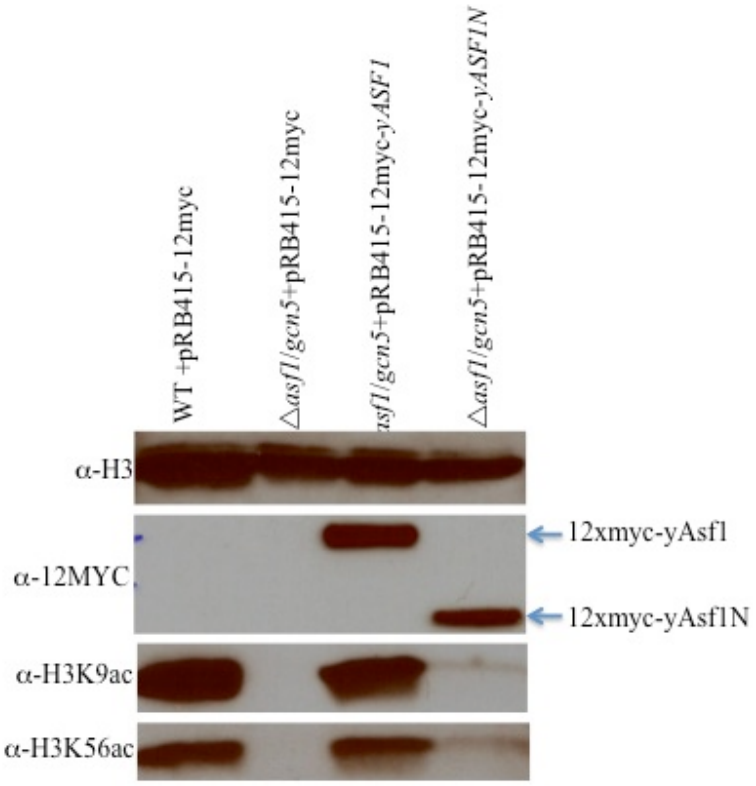

Figure 13: *In vivo* histone acetylation experiments for *yASF1N*: WB comparison of WT and pRB415-12myc-*yASF1*, with pRB415-12myc-*yASF1N*. TCA WCE of indicated strains separated in 15% SDS-PAGE gel, transferred into nitrocellulose and blotted against indicated antibodies

WCE of WT are positive for all three of H3K9ac, H3K18ac, and H3K56ac (Figure 11,12, and 13). WCE from △*asf1*/*gcn5* △*rtt109*/*gcn5* are all negative for the three modifications as expected from Fillingham et al. (2008) (Figure 12,13, and 14). The △*rtt109*/*gcn5* double mutant transformed with pRB415-12myc-*yGCN5* acted as positive control for H3K9ac and H3K18ac and was negative at H3K56ac as expected (Figure 11). The △*rtt109*/*gcn5* double mutant transformed with pRB415-12myc- *RTT109* was positive for H3K9ac & H3K56ac & negative for

H3K18ac as expected (Figure 11). The △*asf1*/*gcn5* double mutant transformed with pRB415-12myc-*yASF1* was positive for H3K9ac &H3K56ac as expected (Figure 12 and 13).

The first set of Western blots showed that *tGCN5* expressed in yeast does not rescue the H3K56ac deficiency of the △*rtt109*/*gcn5* double mutant phenotype (Figure 11) which is consistent with the spot tests results showing failure to rescue growth (Figure 10). tASF1 did not show any acetylation at H3K9 or H3K56 (Figure 12). Interestingly, *yASF1N* shows quite reduced levels of H3K9ac and H3K56ac (Figure 13) despite rescuing the slow growth phenotype (Figure 9). The significance of this is still unclear.

Together these data suggest the following:

1. *yGCN5* and *RTT109* partially rescues H3K9ac in Δ*rtt109*/*gcn5* double mutants:

It has been demonstrated that yGcn5 and Rtt109 act in parallel pathways to fully acetylate global H3K9 in budding yeast (Fillingham et al., 2008. Burgess et al., 2010). The partially acetylated state of H3K9 when either yGcn5 (Figure 11) or Rtt109 (Figure 11) is expressed is consistent with this.

2. *tGCN5* does not rescue H3K9/18/56ac in Δ*rtt109*/*gcn5* double mutants:

Despite the fact that yGcn5, tGcn5 and hGcn5 share extensive amino acid sequence homology (Figure 3), *tGCN5* did not rescue the acetylation defect of the Δ*rtt109*/*gcn5* double mutant. This does not refute my hypothesis that tGcn5 is an H3K56ac HAT since this could be due to the inability of tGcn5 to interact with yeast H3-H4, or its inability to integrate within the SAGA complex, or even a failure to localise to the nucleus.

3. *yASF1N* only partially rescues H3K9/56ac in Δ*asf1*/*gcn5* double mutants:

Despite the fact from the growth tests that shows *yASF1* and *yASF1N* rescue the Δ*asf1* defect of the double mutant to the same degree, there appears to be a clear difference between these proteins in the ability to rescue H3K9/56ac (Figure 13). Daganzo et al., (2003) has published data indicating that yAsf1N functions fully, at least in the context of what was known then. I will talk about the significance of this later in the discussion.

4. *tASF1* doesn't rescue H3K9/56ac in Δ*asf1*/*gcn5* double mutants:

Consistent with its inability to rescue the slow growth defect observed in the spot tests (Figure 9) *tASF1* does not rescue the H3K9/56ac defect of the Δ*asf1*/*gcn5* double mutants (Figure 12). yAsf1 was shown to interact with H3 at Aspartic acid (D) 54, Serine (S) 91, Valine (V) 92, Valine (V) 94, Leucine (L) 96, Arginine (R) 145 and Threonine (T) 147(Antczak et al., 2006; English et al., 2006). All these amino acid residues are conserved between *S. cerevisiae* and *T. thermophila* except S91, V94 and L96 (Figure 4). One possible explanation of this result is that tAsf1 may not bind yeast histones like yAsf1. Therefore, I moved to a set of *in vitro* experiments designed to assess histone binding, as well as ability to synergize with Rtt109, by tAsf1, and histone acetylation activity of tGcn5.

3.8 Expression of recombinant tAsf1, tAsf1N, yGcn5 and tGcn5:

To produce recombinant proteins, *E. coli* BL21 (DE3) transformed with the appropriate plasmids (Table 9) were induced using 1mM IPTG, at 30 ºC for 4 hours before being lysed by suspending cell pellets in SDS sample buffer and boiling for 8 minutes and then checked to see if they express the protein by separating the whole cell lysate on SDS-PAGE and staining the gel

with Coomassie-Blue stain and comparing it to un-induced state (Figure 14). The results obtained indicate that the strains express proteins of appropriate expected molecular size, tAsf1=27 kDa, tAsf1N=20 kDa, tGcn5= 52 kDa. I have also performed the same experiment for yGcn5 which have confirmed that the strain is expressing the protein of expected molecular size, yGcn5=55 kDa. Table 9 summarizes what I have cloned, transformed, expressed, and purified.

Gene	**tASF1**	**tASF1N**	**tGCN5**	**yASF1**	**yASF1N**	**yGCN5**	**RTT109**
Cloned/ Bacterial expression	✔	✔	✔	Fillingham et al., 2008	Fillingham et al., 2008	✔	Fillingham et al., 2008
Expressed	✔	✔	✔	Fillingham et al., 2008	Fillingham et al., 2008	✔	Fillingham et al., 2008
Purified	✔	✔	✔	Fillingham et al., 2008	Fillingham et al., 2008	✔	Fillingham et al., 2008
Cloned/ Rescue experiment	✔	✔	✔	✔	✔	✔	Radovani et al., un-published

Table 9: A summary of the expression of clones used in this study. The check mark indicates work specifically constructed in this study.

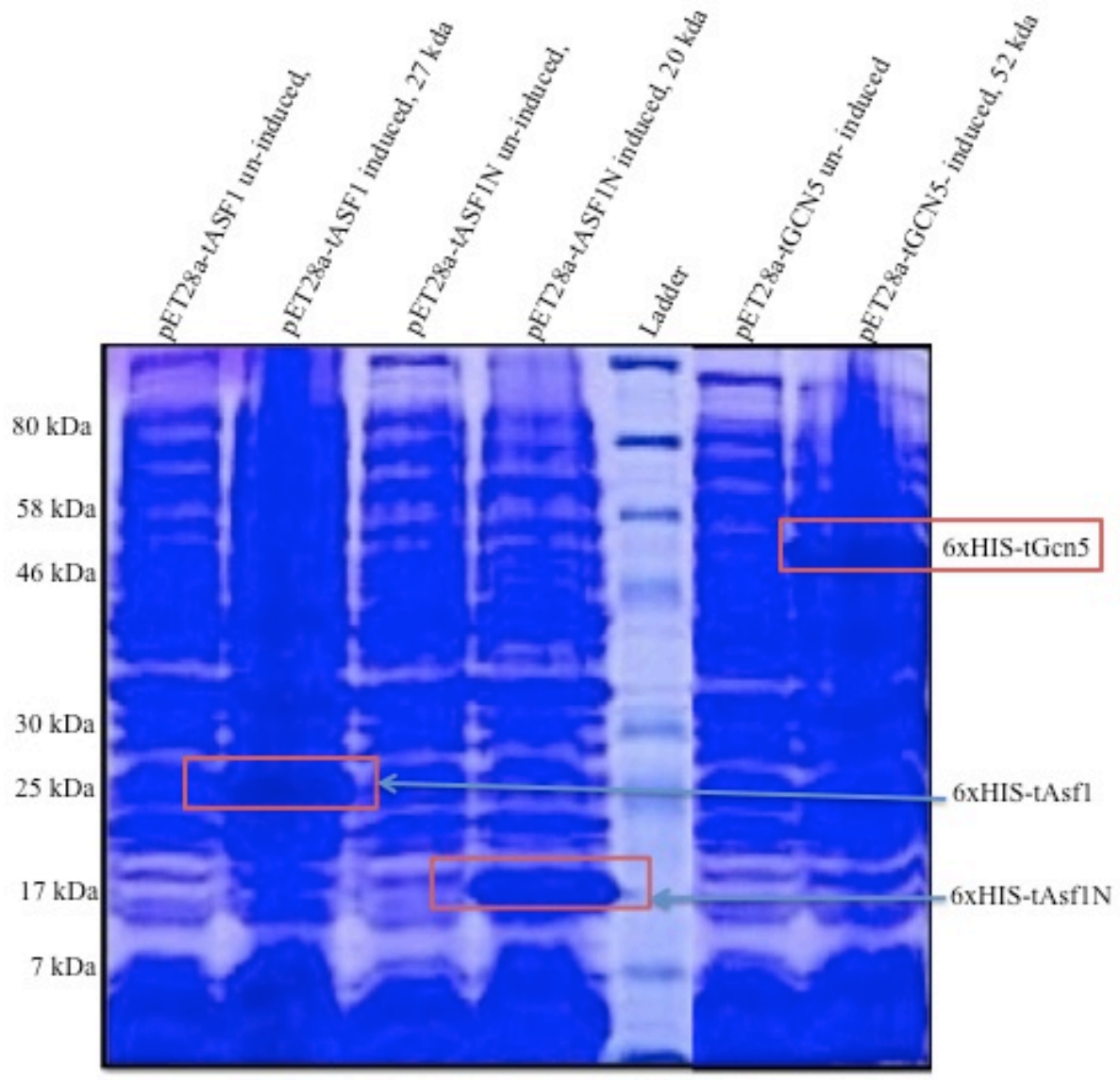

Figure 14: Coomassie-Blue stained SDS-PAGE of whole cell lysates from BL21 (DE3) *E. coli* transformed with indicated plasmids: 10% SDS-PAGE showing separation of whole cell lysate and 2 µl ladder from Invitrogen.

After it was confirmed that the strains expressed the proteins of predicted molecular weight, a series of experiments to test the optimum expression temperature and IPTG concentration were conducted (Figure 15 and Figure 16). Different conditions were analysed using SDS-PAGE to observe if an increase or a decrease of expression resulted from the change in condition. I have found from these data that a temperature of 30 ºC is the most optimum

among the ones tested (16, 24, 30°C) (Figure 15), and that different concentrations of IPTG (0.4, 0.8, 1.2 mM) have little effect after growing them at 30 ºC (Figure 16). The rationale for doing these experiments is to optimize the protein expression and solubility. These conditions were used for affinity purification of recombinant proteins in subsequent experiments.

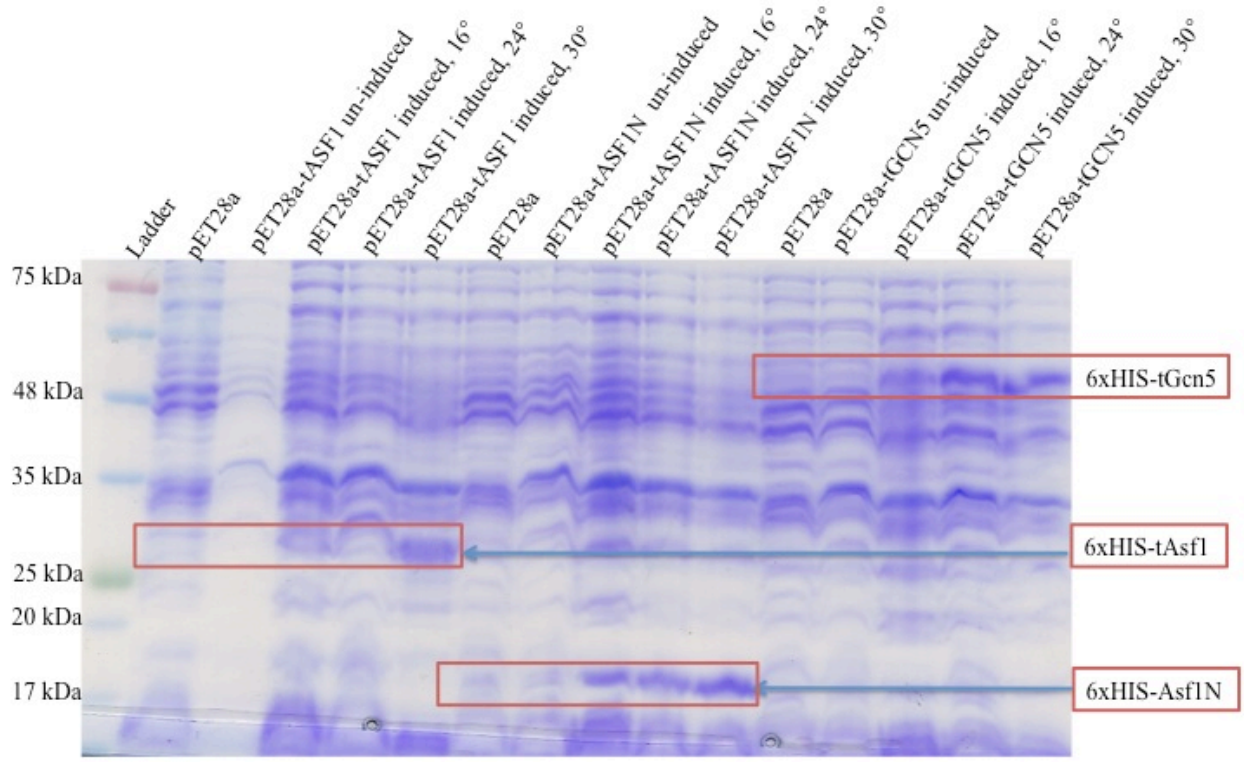

Figure 15: Coomassie-Blue stained SDS-PAGE of whole cell extract of BL21 (DE3) *E. coli* transformed with indicated plasmids expressed in different temperatures for 4 hours at 1 mM IPTG. 10% SDS-PAGE gel showing separation of whole cells lysate and 2 µl ladder from Invitrogen.

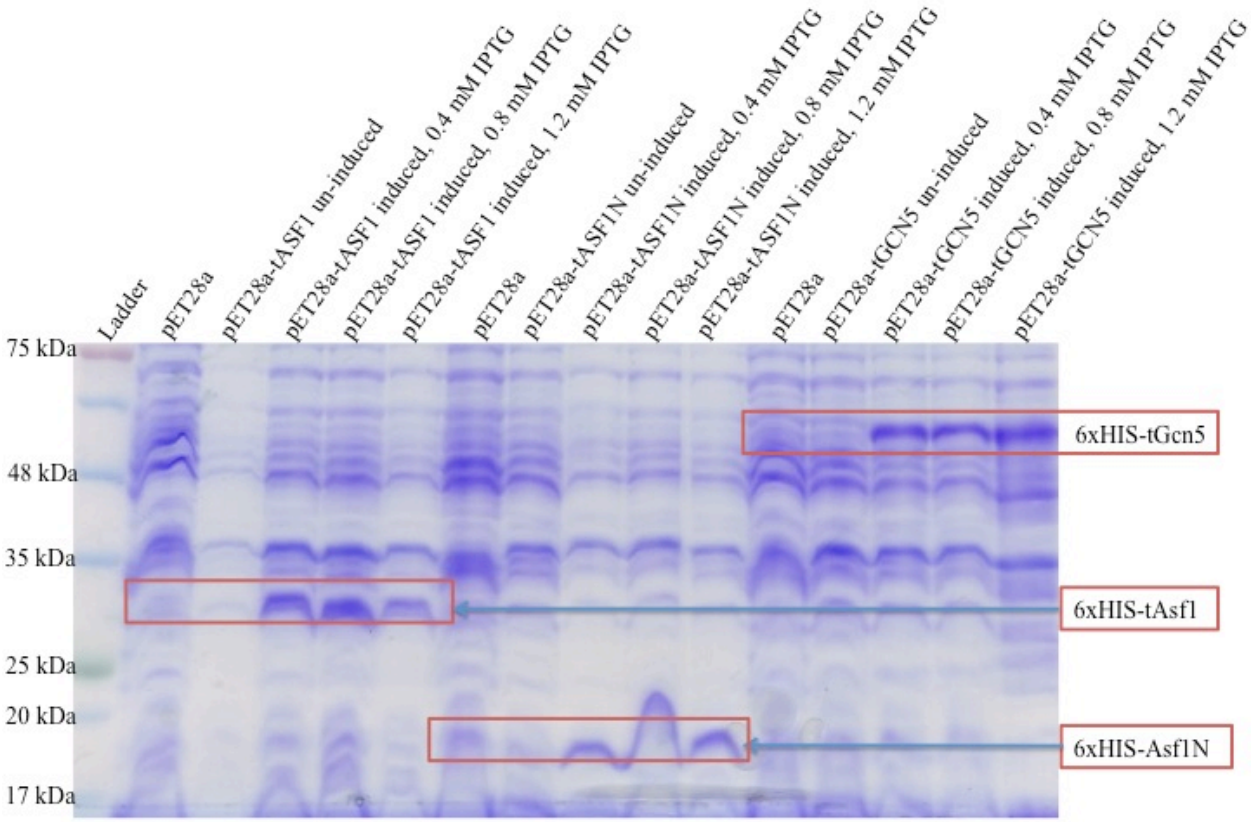

Figure 16: Coomassie-Blue stained SDS-PAGE of whole cell lysate of BL21 (DE3) *E. coli* transformed with indicated plasmids and expressed in several IPTG concentrations for 4 hours at 30°C. 10% SDS-PAGE showing separation of whole cell lysate and 2 µl ladder from Invitrogen.

3.9 Purification of recombinant proteins expressed in *E. coli*:

After I optimized protein expression, I moved on to affinity purification of the recombinant protein. For preparative expression, I used Terrific Broth (TB) instead of LB which I used for small scale expression. Growth in TB optimizes expression and purification by using glycerol as the sole energy source for bacteria thus lowering the amount of toxic metabolic by-products (Dr. Vivian Saridaskis, York University Department of Biology, personal communication).

In the initial affinity purification experiment, 6xHIS-tAsf1 was purified. However the 6xHIS-tAsf1N and 6xHIS-tGcn5 were lost despite being well-expressed (Figure 17). Therefore in order to optimize 6xHIS-based purifications, in subsequent experiments I adjusted the pH of the whole cell lysate. In the original protocol, the lysate is mixed with binding buffer that is at pH 8.0, and thus the nickel bead mixing with the cell lysate also should have a pH 8.0. However, when I checked the pH of the lysate I found it to be around 6.5 for 6xHIS-tGcn5. This lower pH would cause the 6xHIS tag to be protonated and thus prevent it from binding to the nickel on the beads. I resolved this issue by adjusting the pH of the mixture by adding Tris-HCl buffer pH 9.0 until the lysate was back to pH 8.0. This has shown to be an effective step to purify 6xHIS-tGcn5, 6xHIS-yGcn5 and 6xHIS-tAsf1N (compare Figure 18 and 19 with Figure 24).

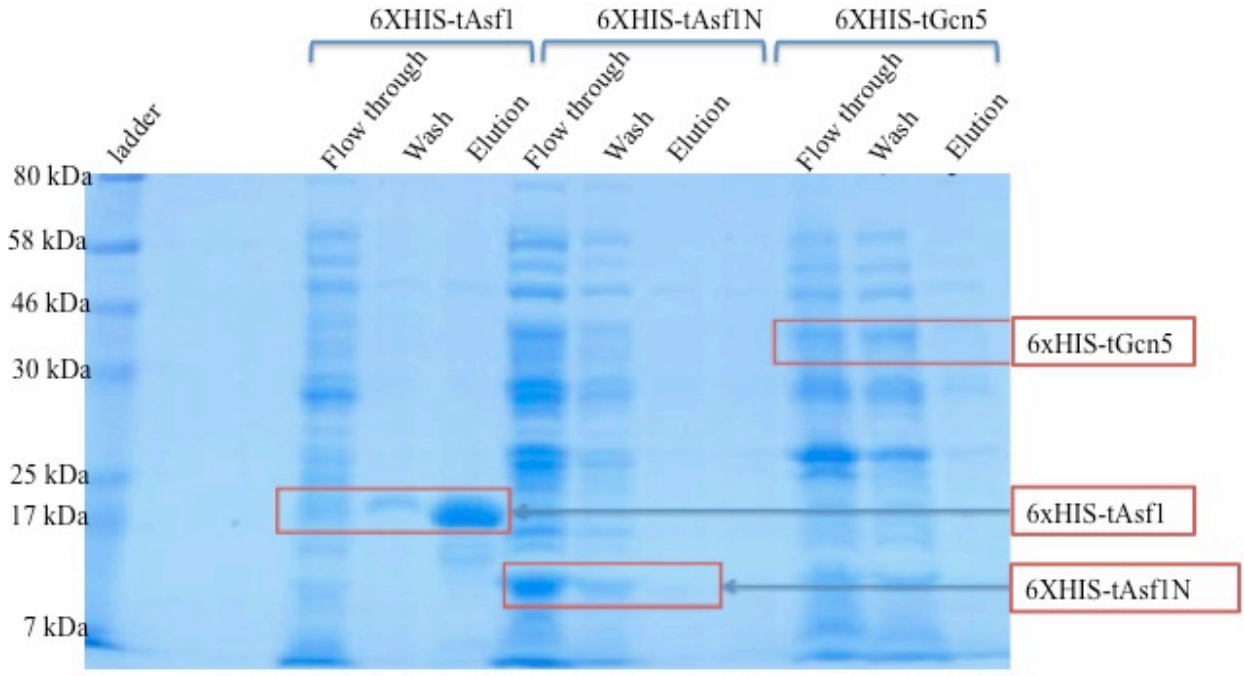

Figure 17: Coomassie-Blue stained SDS-PAGE of different aliquots from the purification of tASF1, tASF1N, tGCN5 at pH 6.5: tASF1N and tGCN5 were not recovered. pH of the cell lysate was not checked as it was not part of the original protocol. 10% SDS-PAGE showing separation of indicated aliquots and 2 µl ladder from Invitrogen.

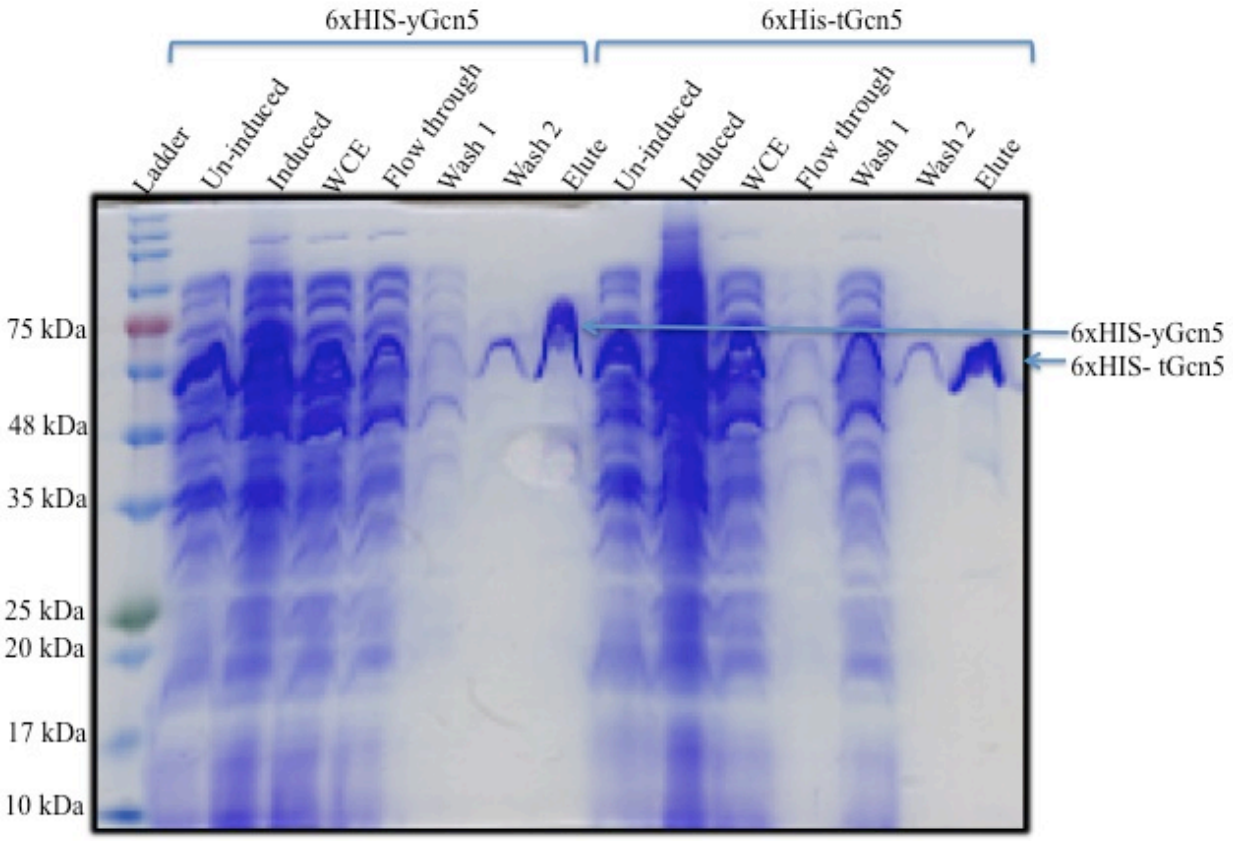

Figure 18: Coomassie-Blue stained SDS-PAGE of different aliquots from the purification of yGcn5 and tGcn5 with adjusted buffer pH: 10% SDS-PAGE showing separation of indicated aliquots and 2 µl ladder from Invitrogen.

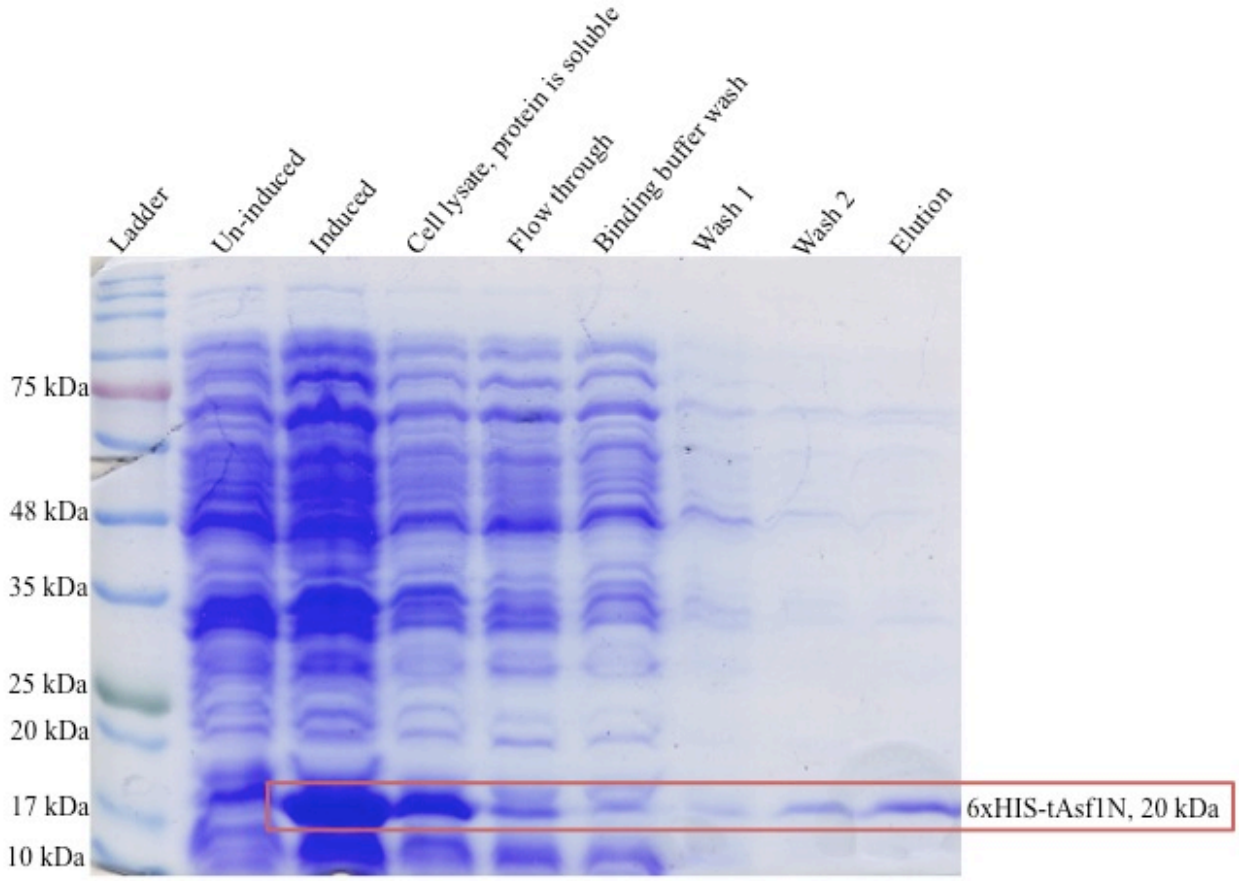

Figure 19: Coomassie-Blue stained SDS-PAGE of different aliquots from the purification of tAsf1N with adjusted buffer pH: 10% SDS-PAGE showing separation of indicated aliquots and 2 µl ladder from Invitrogen.

3.10 *In Vitro* results:

I expressed recombinant proteins in order to test their function in (A) histone acetylation assays and (B) histone binding assays.

The idea behind the histone acetylation assay is to measure the acetyl group transfer from the acetyl group donor (acetyl-CoA) to the lysine residues on histone H3. In this assay, prospective candidate enzymes (tGcn5, yGcn5 or Rtt109 with or without either tAsf1/Asf1N or yAsf1/Asf1N) will be incubated with chicken or *T. thermophila* core histone proteins as substrate (Figure 27). Using WB, I examined the presence of H3K9ac, H3K18ac and H3K56ac marks. H3K18ac is a positive control for yGcn5 activity. H3K56ac is positive by Rtt109 with yAsf1 *in vitro.*

The idea behind the histone-binding assays is to measure the histone chaperone tAsf1 ability to bind H3-H4. 6xHIS-tAsf1 or 6xHIS-tAsf1N is incubated with chicken or *T. thermophila* core histone and then the mixture is purified using nickel bead. The elute is then checked using SDS-PAGE to determine if H3-H4 from either source co-purify with the histone chaperone. 6xHIS-yAsf1 was also tested as a positive control.

3.11 HAT assay results:

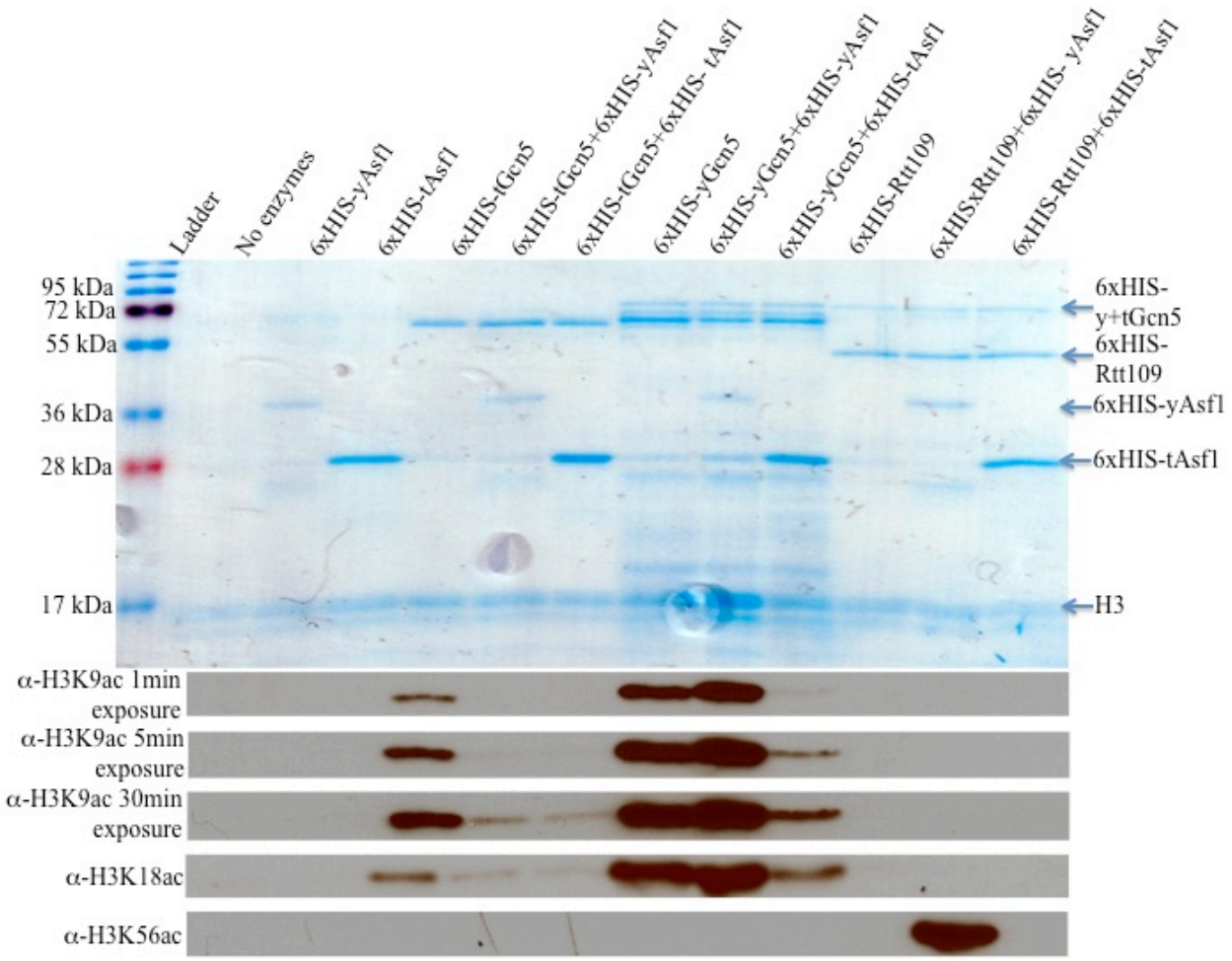

Figure 20. *In vitro* HAT assays with chicken core histones: recombinant enzymes and chicken core histones are mixed as indicated. 10 µl from the reaction is separated on several 15% SDS-PAGE stained with Coomassie-Blue and transferred to nitrocellulose and blotted against indicated antibodies for PTM marks detection.

As expected from previous work, 6xHIS-Rtt109 acetylated H3K56 in presence of 6xHIS-yAsf1 (Figure 20) (Fillingham et al., 2008) but not by itself, nor did it acetylate H3K9 or H3K18 (Figure 20). The lack of H3K18ac is expected since yGcn5 does this in yeast. The lack of *in vitro* H3K9ac by Rtt109-yAsf1 is a consequence of the additional requirement of Vps75 (Fillingham et al., 2008). tAsf1 failed to substitute the role of yAsf1 in H3K56ac in that it did not synergize with Rtt109 (Figure 20). yGcn5 was able to acetylate H3K9 and H3K18 by itself

but not H3K56. yAsf1 seems to have no effect on yGcn5 while tAsf1 appears to greatly inhibit it (Figure 20). Interestingly tGcn5 *in vitro* performs the same functions as yGcn5 in H3K9ac and H3K18ac and does not do H3K56ac, however its *in vitro* activity is almost abolished by the presence of either yAsf1 or tAsf1 (Figure 20).

This experiment can be summarized as follows:

1. tGcn5 acetylate chicken H3K9 and H3K18 *in vitro:*

In the HAT assay, tGcn5 has abundant activity on chicken H3K9/18 acetylation (Figure 20). Therefore, my recombinant tGcn5 is active and able to acetylate histones.

2. tGcn5 does not acetylate chicken H3K56 *in vitro:*

In the HAT assay, tGcn5 does not have any activity on H3K56, with or without both tAsf1 or yAsf1 (Figure 20). The *in vitro* data is consistent with the *in vivo* phenotype of tGcn5 expressed in yeast (Figure 9 to 13).

3. tAsf1 may inhibit tGcn5 acetylation of chicken H3K9/18 *in vitro*:

tAsf1 appears to inhibit the activity of tGcn5 on chicken core H3 in that levels of H3K9ac and H3K18ac were significantly decreased when tGcn5 was incubated in presence of tAsf1 (Figure 20). This could be due to competitive binding from tAsf1 that blocks the function of tGcn5, suggesting that tAsf1 is functional and can bind chicken H3.

4. yAsf1 may inhibit tGcn5 acetylation of chicken histone H3 *in vitro*:

Similar to the presence of tAsf1, I observed that yAsf1 inhibited the activity of tGcn5 on chicken core H3. Both H3K9 and H3K18 are present within close proximity of each other in the N-terminal tail of H3. This suggests that yAsf1 and tAsf1 could occlude or sequester the N-terminus of H3 away from tGcn5 when it binds histones. Protection of N-terminal tails from spurious post-translational modification has previously been proposed to be a function of histone chaperones (Recht et al., 2006; Fillingham et al., 2008).

5. yAsf1 with tGcn5 does not acetylate chicken H3K56 *in vitro*:

In yeast yAsf1 is believed to bind to H3-H4 and adjust its structure in such a manner that it exposes H3K56 for acetylation by Rtt109 (Antczak et al., 2006). Even though yAsf1 binds to chicken H3-H4 and in doing this it aid Rtt109 to acetylate H3K56 both *in vivo* (Figure 12) and *in vitro* (Figure 20), it has failed to synergize with tGcn5 with respect to H3K56ac (Figure 20). This could be due to tGcn5 not being a HAT for H3K56ac, or the physical interaction with chicken histone being different than that of *T. thermophila* histones.

6. tAsf1 doesn't substitute the role of yAsf1 in aiding Rtt109 in the acetylation of chicken H3K9/56 *in vitro*:

As discussed above and in the introduction, yAsf1 binds H3 and H4 and exposes H3K56 to Rtt109 for acetylation. tAsf1 failed to substitute for yAsf1 in that H3K56 acetylation was not observed when tAsf1 was incubated with Rtt109 (Figure 20). This could be due to differences in the pattern of binding of tAsf1 to H3-H4 than that of yAsf1, or differences in the manner Rtt109 is recruited by Asf1 to acetylate the H3-H4 dimer.

7. Rtt109 does not acetylate chicken H3K9 *in vitro* without Vps75:

Rtt109 needs Vps75 to acetylate H3K9 *in vitro* and *in vivo* (Fillingham et al., 2008). The *in vivo* requirement of Asf1 in the Rtt109 likely reflects Asf1 binding to the H3-H4 dimer and presenting them to a Rtt109-Vps75 protein complex for acetylation (Fillingham et al., 2008). Hence the result that Rtt109 does not acetylate H3K9 in the *in vitro* HAT assay without the presence of Vps75, although it clearly requires it *in vivo* (Figure 12) (Fillingham et al., 2008).

3.12 *T. thermophila* histones purified:

One possible reason that tGcn5 does not acetylate H3K56 *in vivo* in yeast or *in vitro* using chicken core histones, is that it requires bona fide *T. thermophila* histones. Similarly, one reason that tAsf1 does not rescue H3K56ac defect of yeast Δ*asf1* is that it won't synergize with Rtt109 *in vitro* because it can only bind *T. thermophila* H3. Because of what was discussed earlier that yAsf1 was shown to interact with H3 at Aspartic acid (D) 54, Glycine (G) 91, Valine (V) 92, Valine (V) 94, Leucine (L) 96, Arginine (R) 145 and Threonine (T) 147 (Antczak et al., 2006; English et al., 2006) and that all these amino acid residues are conserved between *S. cerevisiae* and *T. thermophila* except V94 (Figure 4). Therefore, in order to perform HAT assay on *T. thermophila* core histones, as well as to perform the histone binding assay, core histone from the macronuclei of *T. thermophila* cells was purified (Figure 21). And WB analysis to characterize the prepared histones was performed (Figure 21 B). Although the antibody that was used against H3 did not give any signal, it was shown that it does not work against histones prepared from starved cells (Pearlman Lab, unpublished communication).

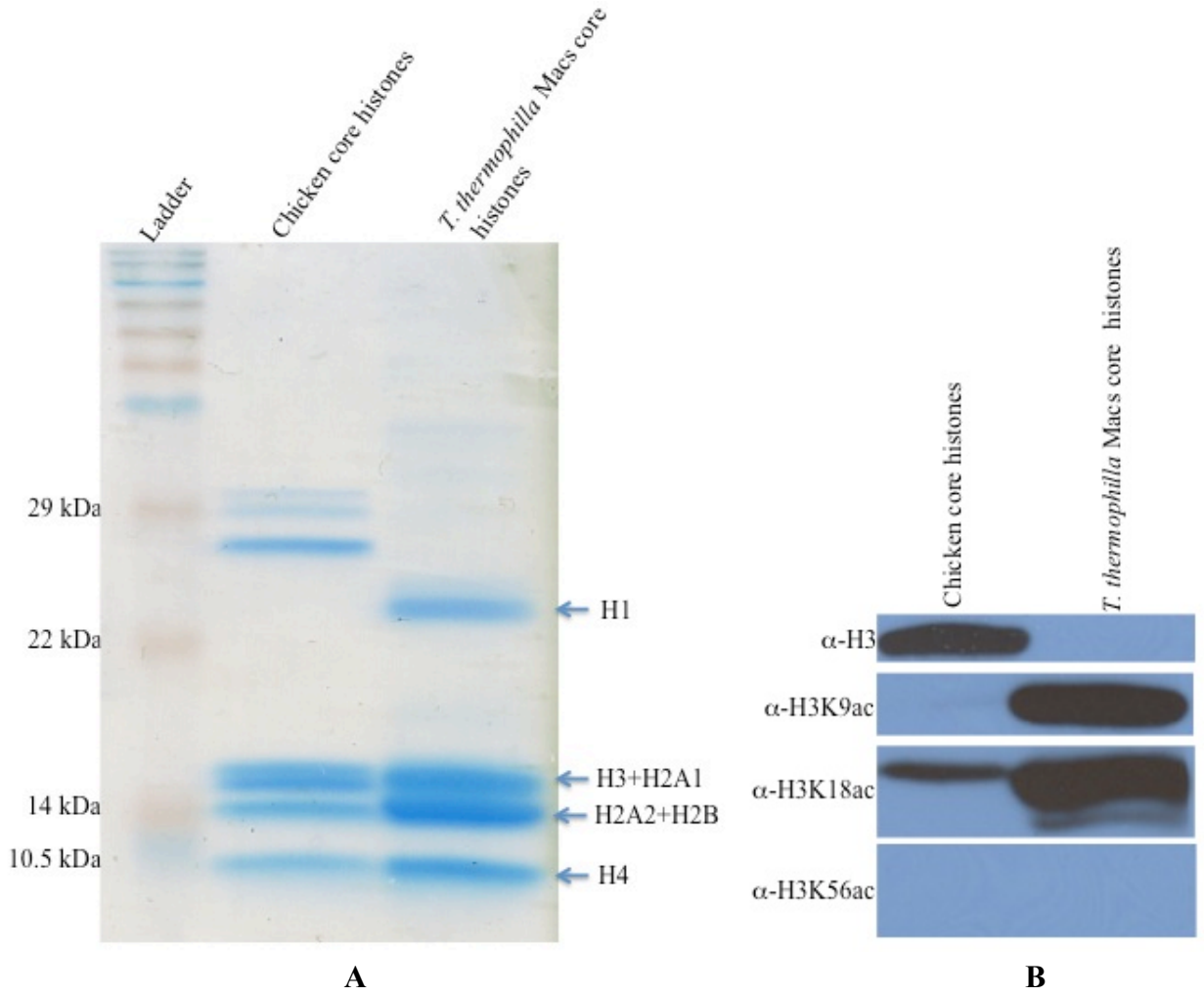

Figure 21: Purified *T. thermophila* histones: A:15% SDS-PAGE separation of purchased chicken core histone (MilliPore) and core histones prepared form *T. thermophila* macronuclei (Macs). *T. thermophila* histones are labelled based on molecular weight; H1=18 kDa, H2A1 or H3=15kDa, H2A2=14 kDa, H2B=13kDa and H4=11 kDa. 5 µl for each sample and 2 µl ladder from Fermnetas is used. **B:** WB blot analysis of purchased chicken histones and prepared *T. thermophila* hisotones with indicated antibodies for the PTM's.

3.13 *In Vitro* Histone binding assay results:

An experiment was designed where either 6xHIS tagged tAsf1, tAsf1N or yAsf1N is incubated with chicken or *T. thermophila* histones at 30 °C for 30 minutes. The mixture was then incubated with nickel beads for 15 minutes and after several high stringency washes, the beads were treated to elute bound protein and separated by SDS-PAGE. The gel was then either stained with Coomassie, silver stain, or Western blotted as required to see what histones co-purified with the respective enzyme

To ensure that the experiment conditions were appropriate, I used yAsf1N as a positive control as it has been shown to bind H3-H4 *in vitro* (Daganazo et al., 2003). The experiment was proven to be working by showing that yAsf1N binds to H3-H4 (Figure 22 and Figure 23).

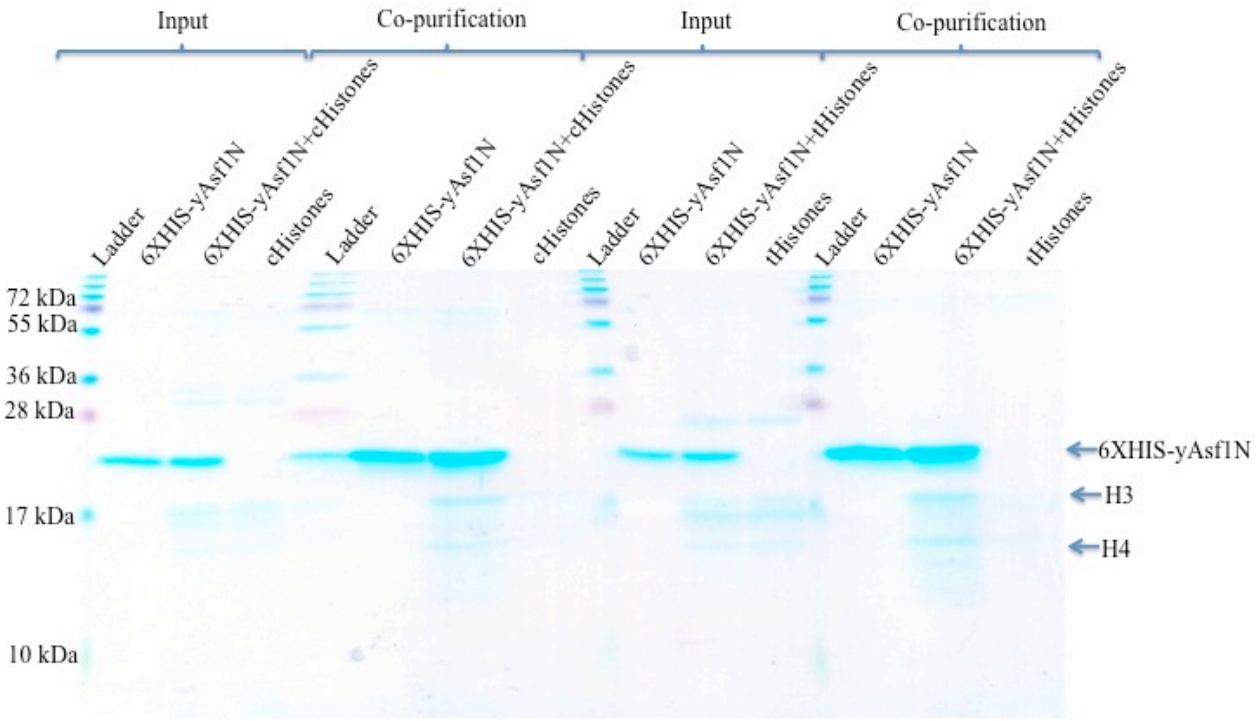

Figure 22: yAsf1N histone binding Assay. Chicken histone or *T.thermophila* histones and 6xhis-yAsf1N are incubated together or alone as indicated and 10 µl is separated for input and the mixture is then purified using nickel beads and the elute is separated to check if histone co-purify with yAsf1N. Coomassie stain of 15% SDS-PAGE gel. Input and co-purification were separated to allow for different development times depending on protein concentration.

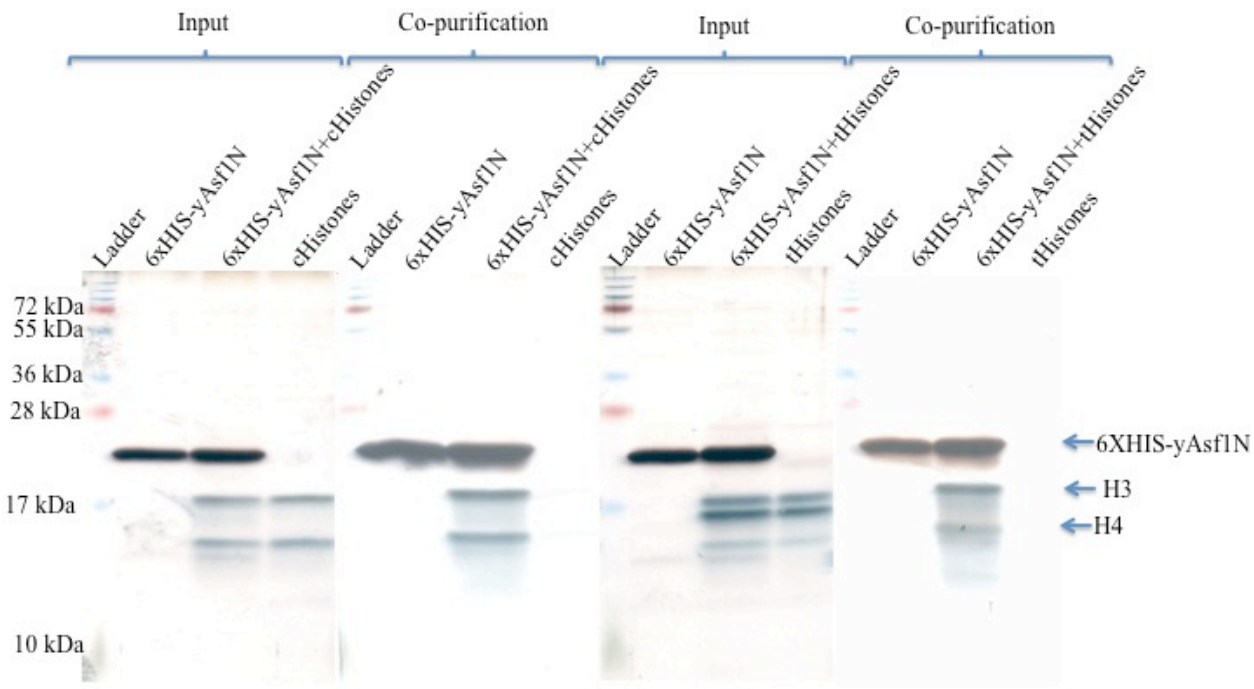

Figure 23: yAsf1N histone binding Assay silver stain. Chicken histone or *T.thermophila* histones and 6xhis-yAsf1N are incubated together or alone as indicated and 10 µl is separated for input and the mixture is then purified using nickel beads and the elute is separated to check if histone co-purify with yAsf1N. Silver stain of 15% SDS-PAGE gel. Input and co-purification were separated to allow for different development times depending on protein concentration.

3.13.1 tAsf1 binds both chicken and *T. thermophila* H3-H4 *in vitro*:

After performing the histone binding assay on 6xHIS-tAsf1 I have shown that it does bind to H3-H4 (Figure 24), in that 6xHIS-tAsf1 co-purifies with H3-H4. Therefore, the lack of ability of tAsf1 to synergize with Rtt109 *in vitro* (Figure 20), or in vivo (Figure 12) is not due to lack of H3-H4 binding.

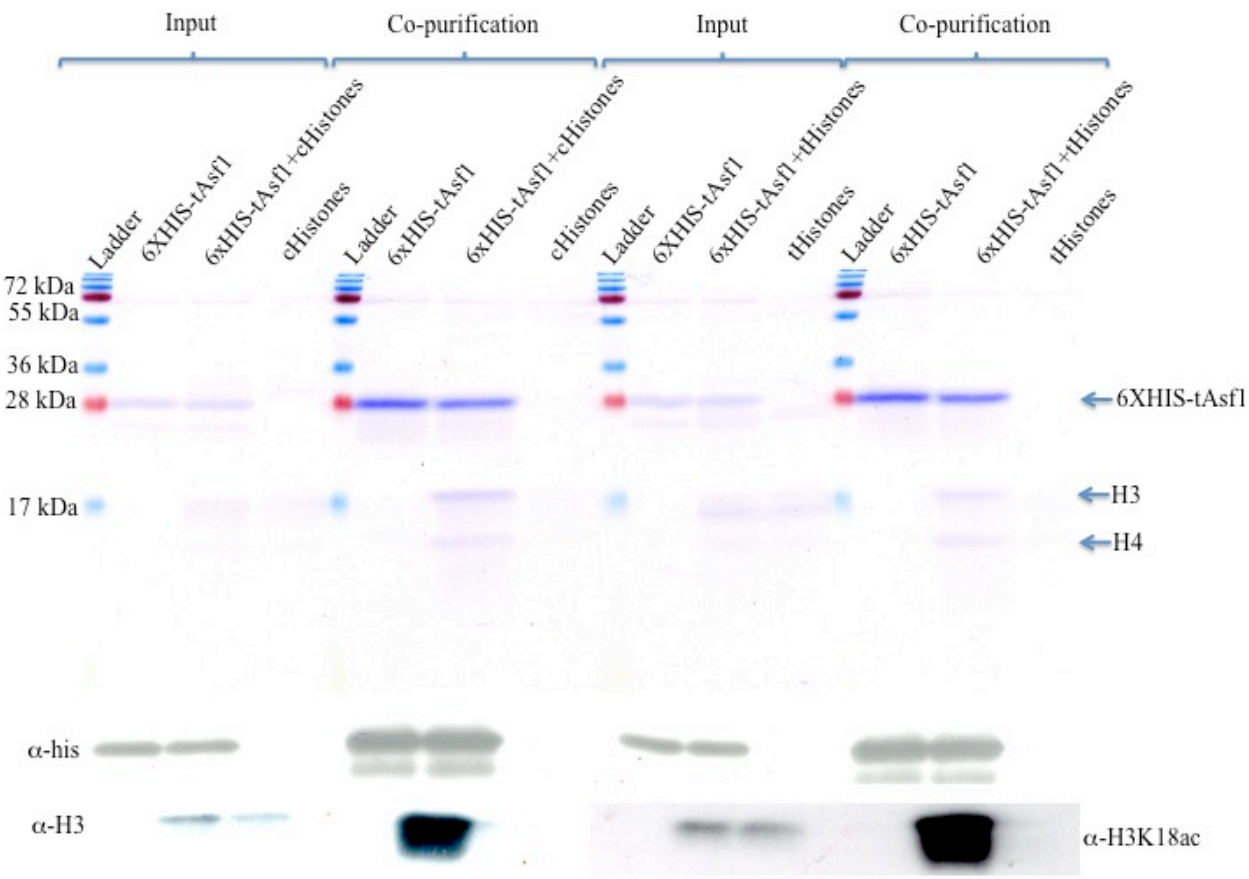

Figure 24: tAsf1 histone binding Assay: Chicken histone or *T.thermophila* histones and 6xhis-tAsf1 are incubated together or alone as indicated and 10 µl is separated for input and the mixture is then purified using nickel beads and the elute is separated to check if histone co-purify with 6xhis-tAsf1**.** Several 15% SDS-PAGE gels are used and stained with coomassie and blotted for indicated antibodies.

3.13.2 tAsf1N binds both chicken and *Tetrahymena* H3-H4 *in vitro*:

Since there are differences in the C-terminal domain of tAsf1 and yAsf1 (Figure 4). I was interested to see if loss of the C-terminal domain in tAsf1N would affect its binding to H3-H4. I have shown here that it still binds both chicken and *T. thermophila* H3-H4 (Figure 25), in that affinity purified 6xHIS-tAsf1N co-purifies with H3-H4. It was confirmed by this experiment that tAsf1 binds H3-H4 at its highly conserved N-terminal domain.

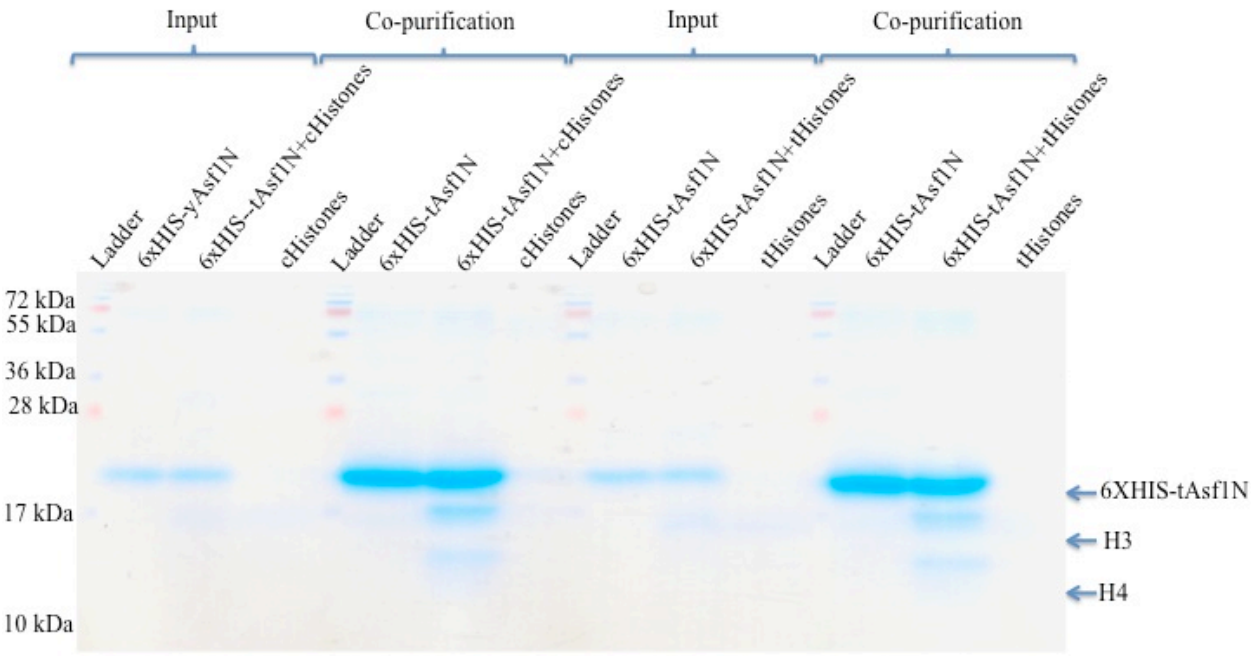

Figure 25: tAsf1N histone binding Assay: Chicken histone or *T.thermophila* histones and tAsf1N are incubated together or alone as indicated and 10 µl is run for input and the mixture is then purified using nickel beads and the elute is separated to check if histone co-purify with tAsf1N. Showing Coomassie stain of 15% SDS-PAGE gel.

Chapter 4: Discussion

4.1 Rtt109 and yGcn5 have two separate pathways for H3K9 acetylation

As discussed in the introduction, it has been shown that Rtt109 and yGcn5 have two distinct pathways for H3K9ac (Fillingham et al., 2008 and Burgess et al., 2010); when *yASF1* was knocked out, the yeast mutants lost some H3K9ac activity by Rtt109 but not from Gcn5 (Fillingham et al., 2008). My data suggest the same conclusion; when Δ*rtt109*/*gcn5* was transformed with either *RTT109* or *yGCN5* some but not all of the H3K9ac activity was restored compared to wild type. Thus either yGcn5 or Rtt109 can acetylate H3K9 by themselves without the existence of the other but not to the same extent when both of them are present.

4.2 tGcn5 has HAT activity on H3 *in vitro*

In the *in vitro* analysis, tGcn5 showed strong H3K9ac and H3K18ac activity but failed to perform the same functions *in vivo* in *S. cerevisiae.* In the sequence alignment analysis I have shown that tGcn5 is the only Gcn5 homolog that lacks a predicted nuclear localization signal (NLS) (Figure 3). One possible explanation for the lack of acetylation *in vivo* is that tGcn5 does not localize to the nucleus where H3 is in *S. cerevisiae* although this needs to be confirmed using GFP analysis (in progress). This could also suggest that nuclear localization may occur differently in *T.thermophila* than yeast.

4.3 tAsf1 does not synergize with Rtt109

In the HAT assay tAsf1 failed to substitute yAsf1 role in aiding Rtt109 to acetylate H3K56 *in vitro* (Figure 20). Despite the fact that tAsf1 does bind to H3-H4 (Figure 24), this

binding is probably not performing the same function in exposing H3K56 for aceytelation by Rtt109 as does yAsf1 .

4.4 tAsf1 binds to H3 in a way that does not resemble yAsf1

In the HAT assay tAsf1 but not yAsf1 showed an inhibitory effect on H3K18ac activity by tGcn5 *in vitro* (Figure 20). This could be explained by the Asf1 amino acid sequence alignment that shows that some of the H3 binding sites (S91, V94 and L96) of *S. cerevisiae* Asf1 are not conserved in *T. thermophila* and thus the binding of tAsf1 with H3 is different than that of yAsf1.

4.5 tAsf1 binds H3-H4 through its highly conserved N-terminal domain

From the histone binding assays. tAsf1, tAsf1N and yAsf1N were all able to bind H3-H4. It is safe to conclude from this experiment that *S. cerevisiae* and *T.thermophila* Asf1 bind H3-H4 through the highly conserved N-terminal domain and that the C-terminus, deleted in both yAsf1N and tAsf1N, probably plays no role in this interaction.

4.6 tGcn5 have a different structural mechanism for interacting with H3 than yGcn5

As shown by the *in vitro* HAT assays, tGcn5 can acetylate both H3K9 and H3K18. Interestingly yAsf1 inhibits tGcn5 activity but not yGcn5. This suggest a difference in the structural interaction of tGcn5 than yGcn5.

4.7 Future experiments

In the future, work will be done to assess the localization of tAsf1, tAsf1N, tGcn5 and yAsf1N within yeast cells. This will be achieved using GFP tagging to examine if these proteins localize within the nucleus or the cytoplasm. The questions that will be asked here are, is tGcn5 able to localize to the nucleus of *S. cerevisiae* as yGcn5 does and does any of yAsf1N, tAsf1 and tAsf1N localize to the nucleus of *S. cerevisiae* as does yAsf1. I expect that tGcn5, tAsf1 and yAsf1N will fail to localize in the nucleus of *S.cerevisiae* as predicted by the sequence alignments that shows a lack of predicted NLSs in tGcn5, tAsf1 and yAsf1N (Figure 3 and Figure 4).

Another experiment that will be done is the knock out of the tGCN5 gene in *T.thermophila* in order to study the effect on H3K9/18/56 acetylation *in vivo.* I expect that all these marks will be substantially if not completely abolished. tAsf1 is being knocked out in *T.thermophila* to study its role in aiding the acetylation of H3K9/18/56 *in vivo.*

After doing all these experiments, the next question would be is what if tGcn5 is not an H3K56 HAT?. and then what is?. Other candidates could be Hat1 which was discussed in the introduction or 3 of the EsaI family ESA1 is an essential HAT which has been discovered to acetylate primarily histone H4 (Smith et al., 1998 and Clarke et al., 1999).

Another candidate enzyme is MEC-17 that has shown to be an alpha-tubulin acetyltransferase in *T. thermophila* and *C. elegans* (Akella et al., 2010).

Chapter 5: Conclusions

These analyses by *in vivo* and *in vitro* methods characterized the roles of yAsf1N, tGcn5 & tAsf1 in the acetylation of H3K9, H3K18 & H3K56. tGcn5 was able to acetylate H3K9 and H3K18 *in vitro* but not *in vivo* in *S. cerevisiae,* and was unable to acetylate H3K56 in either case. tGcn5 was also incapable of rescuing the △*rtt109*/*gcn5 S. cerevisiae* mutant phenotype. The activity of yAsf1N was greatly decreased *in vivo* probably due to it is lack of the amino acid sequence ^{255}KKRRKIE^{261} which is a predicted NLS that could be important for the localization of the protein in the nucleus. tAsf1 failed to substitute the role of yAsf1 in the acetylation of H3K56 by Rtt109, and even had an inhibitory effect on yGcn5 with regards to H3K9 and H3K18 acetylation. tAsf1 was also unable to rescue the △*asf1*/*gcn5 S. cerevisiae* mutant phenotype.

Using database searches I found that *T. thermophila* is the only organism that encodes a Gcn5 without a predicted NLS. This can explain the inability of tGcn5 to acetylate H3K9 and H3K18 in *S.cerevisiae* while being capable to acetylate those lysines *in vitro,* although this needs to be demonstrated. In *T.thermophila* Gcn5 could bind to another protein to localize to the nucleus or even by itself and carry out the acetylation of H3K9 & H3K18.

Chapter 6: Appendices

Appendix 1: The sequences for the *Tetrahymena thermophila* synthetic genes and all primers

tASF1: 753bp

ATGGCCTTAGTCAACATCCAAAACATCCAATTTCATAACAACCCTTGCCCTTTTTTGA
GTCCCTTTAAACTTGACGTAACCTTCGAATGTATCAAACCTATACCTGATGACATTG
AATGGCAATTAATTTATATTGGTTCTGCTAAAGATGAGAAATACGATCAAGTCCTTG
ATAAATTCAGTATTGGGTCATTAGATCAAGGAGTGCTTCAATTTACCATTGAAACAA
ATCCACCTGATCACACTAAGATTCCAAACAAAGATGATTTACTTGGTGTAACTGCTA
TAATTCTAACTGTTTCATACCACAATCAGGAGTTTTTCCGTGTTGGATACTATGTCTA
CAATCAATACAATGATCAGGAACTTATTATTAATGATCCTCCTCAAATTCTAATTGA
TAAGGTTGAGCGTAGTATCCTTGATAAACAGCCTCGTATCACTCATTTCAACATAAA
ATGGGGTACTGAGGATGAAAATAAAGAAACTGACCCTAATACTTTAGCTATCCTCCA
ACAGCAATTAGCTCAAAATGGAACCATTCCTAATCAACTCATGCAAGAAATGCAAC
AAGTGAATAGCCAAATGCAATCTTTCTTACCTGATCCTTTTTCTAAGTCATCAGGAAT
ACTCCAAGAGTTAACAACACAAGGACAGACTAACAGCACAAATAGTTTCATGTTTG
GTCAAGGGCTAGACATACCTCAAAATAATGCATTCCAACCTACAAATGTATTCAGTT
CTAATCCTTACTAA

tASF1N, need to be produced by PCR, 468 bp:

ATGGCCTTAGTCAACATCCAAAACATCCAATTTCATAACAACCCTTGCCCTTTTTTGA
GTCCCTTTAAACTTGACGTAACCTTCGAATGTATCAAACCTATACCTGATGACATTG
AATGGCAATTAATTTATATTGGTTCTGCTAAAGATGAGAAATACGATCAAGTCCTTG

ATAAATTCAGTATTGGGTCATTAGATCAAGGAGTGCTTCAATTTACCATTGAAACAA
ATCCACCTGATCACACTAAGATTCCAAACAAAGATGATTTACTTGGTGTAACTGCTA
TAATTCTAACTGTTTCATACCACAATCAGGAGTTTTTCCGTGTTGGATACTATGTCTA
CAATCAATACAATGATCAGGAACTTATTATTAATGATCCTCCTCAAATTCTAATTGA
TAAGGTTGAGCGTAGTATCCTTGATAAACAGCCTCGTATCACTCATTTCAACATAAA
ATGGGGTACT

tGCN5, 1257 bp:

ATGGCTGATCAAGAAAAATCTGCCCAAGATGCGCAGAATGCTGCACCACAGGAAAC
CGCATTTGTTGGTATGAATGGTGAAGAAACCGGTCTGGGTTTTGCCACCCGTGATCA
AGGTGCGAAAGTTGAAGAAGATCAGGGTCTGCTGGATTTTGATATTCTGACCAATGA
TGGTACTCATCGTAATATGAAACTGCTGATCGATCTGAAGAACATTTTTAGCCGTCA
ACTGCCAAAGATGCCGAAGGAATACATCGTTAAACTGGTTTTCGATAGACATCATGA
ATCTATGGTTATCCTGAAGAACAAGCAGAAGGTTATTGGTGGTATTTGTTTCCGTCA
ATACAAACCGCAGAGATTTGCTGAAGTTGCATTTCTGGCCGTTACCGCGAATGAACA
AGTTCGTGGTTACGGTACTAGACTGATGAACAAGTTCAAGGATCACATGCAAAAGC
AGAATATTGAATATCTGCTGACCTATGCTGATAACTTCGCAATCGGTTACTTCAAGA
AACAAGGTTTTACCAAAGAACATCGTATGCCACAGGAAAAGTGGAAGGGTTACATC
AAGGATTACGATGGTGGTACTCTGATGGAATGTTACATCCATCCGTATGTTGATTAC
GGTAACATCTCTCAAATCATCAAACGTCAGAAGGAACTGCTGATCGAAAGAATTAA
AAAACTGTCTCTGAACGAAAAGGTGTTTTCTGGCAAGGAATACGCCGCGCTGATCCA
AAATTCTATGGATAACGAAGATCCAGAAAATCCGAAAGTTAATCCATCTGATATTCC
GGGTGTTGCCTTTTCTGGTTGGGAATGGAAGGATTACCATGAACTGAAAAAATCTAA

GGAACGTTCTTTTAATCTGCAATGTGCGAACGTTATCGAAAACATGAAGAGACATAA
GCAGTCTTGGCCATTTTTAGATCCAGTTAACAAGGATGATGTTCCAGATTACTATGA
TGTTATTACCGATCCGATCGATATTAAGGCTATCGAAAAGAAACTGCAAAATAATCA
GTATGTTGATAAAGATCAATTCATCAAGGATGTTAAGCGTATCTTCACCAATGCTAA
GATCTACAACCAGCCAGATACCATCTACTACAAGGCTGCAAAGGAACTGGAAGATT
TTGTTGAACCATATCTGACCAAACTGAAAGATACTAAGGAATCTAACACCCCGTCTA
ACAACAACTCTGCTCATGGTTCTAAAAAACCACTGCCGGTTAGAAAATCTATTAAGA
AAAAATAA

the primers are:

tAsf1F Sequence:

5'- CCC GGA TCC ATG GCC TTA GTC AAC ATC CAA AAC ATC CAA -3'

tAsf1R Sequence:

5'- CCC CTC GAG TTA GTA AGG ATT AGA ACT GAA TAC ATT TGT -3'

tAsf1-N-R Sequence:

5`-CCC CTC GAG TTA AGT ACC CCA TTT TAT GTT GAA -3'

tGcn5F Sequence:

5'- CCC GGA TCC ATG GCT GAT CAA GAA AAA TCT GCC CAA GAT -3'

tGcn5R Sequence:

5'- CCC CTC GAG TTA TTT TTT CTT AAT AGA TTT TCT AAC CGG -3'

yAsf1F Sequence:

5'- CCC GGA TCC ATG TCA ATT GTT TCA CTG TTA GGC ATC AAA -3'

yAsf1R Sequence:

5'- CCC CTC GAG TTA ATT CGT TGA ACG TGC CGC ATC CTT TGG-3'

yAsf1-N-R Sequence:

5`-CCC CTC GAG TTA ATC CCA AAC AAT GTT AAA CCT TGT TAC -3'

yGcn5F Sequence:

5'- CCC GGG CCC ATG GTC ACA AAA CAT CAG ATT GAA GAG GAT -3'

yGcn5R Sequence:

5'- CCC CTC GAG TTA ATC AAT AAG GTG AGA ATA TTC AGG TAT -3'

Appendix 2: Yeast strains used in this study:

Strain	Genotype	Source
BY4741	MATa, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0	(Winzler et al., 1999)
BY4741-1	BY4741+ pRB415-12myc [LEU$^+$]	This work
SC217	BY4741, *asf1*Δ::KAN	(Winzler et al., 1999)
SC217-1	SC217+pRB415-12myc [LEU$^+$]	This work
SC218	BY4741, *rtt109*Δ::KAN	(Winzler et al., 1999)
SC218-1	SC218+pRB415-12myc [LEU$^+$]	This work
JF81	BY4741, *gcn5*Δ::KAN	(Fillingham et al., 2008)
JF81-1	JF81+ pRB415-12myc [LEU$^+$]	This work
JF101	BY4741, *rtt109*Δ::NAT	(Fillingham et al., 2008)
JF101-1	JF101+pRB415-12myc [LEU$^+$]	This work
JF103	BY4741, *gcn5*Δ::KAN, rtt109Δ::NAT	(Fillingham et al., 2008)
JF103-1	JF103+pRB415-12myc [LEU$^+$]	This work
JF103-2	JF103+pRB415-12myc [LEU$^+$]-*yGCN5*	This work
JF103-3	JF103+pRB415-12myc [LEU$^+$]-*tGCN5*	This work
JF103-4	JF103+pRB415-12myc [LEU$^+$]-*RTT109*	This work
JF105	BY4741, *asf1*Δ::NAT	(Fillingham et al., 2008)
JF105-1	BY4741, *asf1*Δ::NAT+pRB415-12myc [LEU$^+$]	This work
JF109	BY4741, *gcn5*Δ::KAN, *asf1*Δ::NAT	(Fillingham et al., 2008)
JF109-1	JF109+ pRB415-12myc [LEU$^+$]	This work
JF109-2	JF109+ pRB415-12myc [LEU$^+$]-*yASF1*	This work
JF109-3	JF109+ pRB415-12myc [LEU$^+$]-*yASF1N*	This work

JF109-4	JF109+ pRB415-12myc [LEU$^+$]-*tASF1*	This work
JF109-5	JF109+ pRB415-12myc [LEU$^+$]-*tASF1N*	This work

Chapter 7. References:

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