A synthetic acetylation substrate to study Gcn5 targeting and function in yeast.

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Authorization

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Abstract

Acetylation was previously thought to occur exclusively on histones, but recent high-throughput screens have identified thousands of non-histone substrates. Despite the identification of these sites, little is known about how these acetyltransferase enzymes target their substrates. Gcn5 is the catalytic acetyltransferase found within the highly conserved SAGA complex. Recently, a member of this complex, Ada2, was found to impact Gcn5 substrate selection. In the yeast model organism *Saccharomyces cerevisiae*, a synthetic substrate developed from a proposed Gcn5-specific consensus sequence is used to identify regulators of Gcn5 substrate selection. This work is the first to demonstrate that addition of a consensus sequence is enough to confer acetylation of a non-substrate. With this method, Ada3 was identified as a key regulator, and acetylome profiling identified novel targets for Gcn5 dependent acetylation specifically regulated by Ada3. This system could be adapted for other acetyltransferases to identify regulators of substrate selection.

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List of abbreviations

AcK	Acetyllysine
ATAC	Ada2a-containing complex
Blastp	protein-protein Basic local alignment search tool
BSA	Bovine Serum Albumin
DMSO	Dimethyl sulfoxide
DTT	Dithioreitol
EDTA	Ethylenediaminetetraacetic acid
GFP	Green Fluorescent Protein
HRP	Horseradish peroxidase
IP	Immunoprecipitation
IP'ed	Immunoprecipitated
NAD^+	Nicotinamide adenine dinucleotide
NAM	Nicotinamide
PCAF	P300/CBP-associated factor
PCR	Polymerase chain reaction
PTM	Post translational modification
RAP	Rapamycin
RSC	Chromatin structure remodeling
SAGA	Spt-Ada-Gcn5-acetyltransferase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLIK	SAGA-like
TBP	TATA binding protein

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1. Introduction

1.1 Acetylation is a critical post-translational modification

Maintenance of cellular function relies heavily on a network of dynamic proteins; whose functions typically depend on post translational modifications (PTMs). The number of different PTMs that exist continues to increase as our understanding of protein dynamics grows. Typically, these modifications are responsible for activities such as protein stability, protein-protein interactions, activation of enzymatic function, or protein localization. Phosphorylation, ubiquitylation, sumoylation, methylation and acetylation have all been studied on a broad scale in diverse model systems (Knorre 2009). One of the best studied PTMs to date is acetylation, and this PTM is the focus of this work.

Acetylation as a PTM can fall into two main categories: N-terminal acetylation and lysine acetylation. The former refers to a non-reversible permanent modification that occurs at the α-N-terminus of proteins during translation, catalyzed by N-terminal acetyltransferases (NATs) coupled to the ribosomes (Strous et al. 1974). There are however six NATs in humans, termed NatA through NatF (and five in yeast, excluding NatF) (Arnesen et al. 2009; Osberg et al. 2016), which all function slightly different to each other and serve to target different sequences (Arnesen 2011). Thus, each NAT has its own specific subset of substrates (Polevoda et al. 2009). Originally, the function of N-terminal acetylation was thought to increase protein stability (Persson et al. 1985; Jornvall 1975), recent work has highlighted exceptions to this where the acetylation will instead trigger a signaling cascade leading to its degradation (Hwang et al. 2010). As N-terminal acetylation is a non-reversible modification, studying downstream effects has proven challenging, and as such, the ultimate function of N-terminal acetylation is still poorly

understood. Recent evidence has been found to support roles in regulation of protein stability, interactions and localization (Arnesen 2011; Forte et al. 2011; Linster & Wirtz 2018).

Lysine acetylation however consists of the transfer of an acetyl moiety to the ε-amino group of lysine residues elsewhere in protein sequences. This function is catalyzed by a group of enzymes known as histone acetyltransferases (HATs) and reversed by histone deacetylases (HDACs). Lysine acetylation was originally characterized as a histone specific modification (Allfrey & Mirsky 1964; Allfrey et al. 1964), wherein the N-terminal tails of histones H3 and H4 are acetylated (Shahbazian & Grunstein 2007), and thus neutralizes their positive charge weakening their interactions with the DNA that surrounds them (Eberharter & Becker 2002; Struhl 1998). This results in the restructuring of chromatin to a more open and relaxed state, going from heterochromatin which is more tightly packed relative to euchromatin (Suka et al. 2001; Guillemette et al. 2011). Furthermore, acetylation can act as a competitive modification on certain sites such as at H3K9, which can otherwise be methylated, a PTM traditionally resulting in heterochromatin structure formation and gene silencing (Stewart et al. 2005). In addition to directly affecting chromatin structure, acetylated histone tails can act to provide recognition sites for other recruitment of chromatin modifying complexes (Verdone et al. 2005). These findings are, in part, what led researchers to believe that the role of acetylation as a PTM was to control transcription events.

1.2 Acetyltransferases

As previously mentioned, lysine acetylation is catalyzed by HATs, though more recently the term lysine acetyltransferase (KAT) is also used to describe these same enzymes. In yeast there are currently 10 confirmed HAT enzymes (**Table 1**) (Downey & Baetz 2015; Lee & Workman 2007; Sampath et al. 2013), and even more in humans, however these are divided

into two main classes of enzymes : Type A and Type B (Workman & Kingston 1998). Type A enzymes are those who exert their function on nucleosomal histones once already bound to chromatin, whereas type B enzymes acetylated newly histones once they have been newly synthesized (Workman & Kingston 1998). These enzymes are also organized into subfamilies based on their structure and function (Allis et al. 2007). The first is Gcn5-related histone N-acetyltransferases (GNAT) which include enzymes such as Gcn5, PCAF, Hat1 and Elp3 (Kimura et al. 2005; Marmorstein & Zhou 2014; Dyda et al. 2000). These are classified based on their sequence homology with Gcn5, which is discussed in the next section. The next subfamily is known as the MYST family of enzymes which is named after members MOZ, Yfb2/Sas3, Sas2, TIP60 (Esa1 in yeast) (Marmorstein & Zhou 2014; Utley & Cote 2003; Kimura et al. 2005). These are the two largest subfamilies however there are other smaller groups which include the metazoan specific p300/CBP subfamily of proteins, as well as the fungal specific Rtt109-like subfamily of enzymes (Tang et al. 2008; Marmorstein & Zhou 2014).

One of the characteristics which separate the enzymes into such a broad range of subfamilies is their structure. Crystal structures that were resolved in the past showed overlap between members of the GNAT subfamily throughout various liganded forms (Dutnall et al. 1998; Poux & Marmorstein 2003; Rojas et al. 1999; Trievel et al. 1999). Interestingly, crystal structures of liganded and unliganded forms of MYST family members were also able to superimpose with the structures identified for the GNAT enzymes (Yuan et al. 2012; Yan et al. 2000), establishing a conserved HAT domain, however also highlighting distinct and divergent N- and C-terminal regions (Holbert et al. 2007). Not only are the subfamilies different in terms of structure, but also in terms of their function. For example, in regard to DNA damage/repair, the GNAT enzymes are typically recruited to sites of nucleotide excision repair in response to specific DNA damage events (Brand et al. 2001; Yu & Waters 2005; Yu et al. 2011), whereas the

MYST subfamily of enzymes are more often recruited to sites of double strand break repair (Bird et al. 2002; Ikura et al. 2000).

What is shared across the different subfamilies of enzymes however is the inclusion of chromatin-binding domains. In order for these enzymes to target their substrates, they usually have specific domains that are used for recruitment to their modified sites. Examples of these include Bromo, Chromo, WD40 and SANT domains among others (Lee & Workman 2007). Each of these domains are used to recognize and bind to different structures or already present modifications exposed on targets such as histones. The bromodomain is a small conserved domain that is able to bind acetylated lysines (Dhalluin et al. 1999). Interestingly, despite being conserved, there appears to be only minor structural diversity between bromodomains from different proteins, indicating a range of effectiveness toward different acetylated sites/peptides (Filippakopoulos et al. 2012). This is likely due to the fact that only the structure of these domains is highly conserved, as aside from important residues which directly bind the acetyl-lysine, sequence identity of the bromodomain varies greatly from protein to protein (Jeanmougin et al. 1997). For example, the PCAF bromodomain is comprised of the canonical four helices with specific folds that are present in only certain other proteins (Presnell & Cohen 1989; Dhalluin et al. 1999).

Table 1. List of yeast acetyltransferase enzymes.

Yeast acetyltransferase enzymes	Yeast acetyltransferase complexes
Gcn5	SAGA, SLIK, ADA
Esa1	NuA4, Piccolo NuA4
Eco1	
Hat1	Hat1-Hat2
Elp3	Elongator
Rtt109	
Sas2	SAS
Sas3	NuA3
Hpa2	Hpa2
Hpa3	

Adapted from Downey & Baetz 2015

Not every HAT enzyme can contain each of these same domains. This in part explains differences in substrate targeting for each enzyme. However, to combat this, these enzymes are typically found in the context of larger, multi-subunit complexes (Lee & Workman 2007; Downey & Baetz 2015). The other members of the complexes can then bring these elements to regulate the HAT's catalytic activity toward specific substrates. For example, the yeast HAT Esa1 contains a chromodomain which can be used for recognizing methylated lysine (Selleck et al. 2005; Huang & Tan 2013). Additionally, is commonly found within the NuA4 complex, alongside 12 other members. Of these, members such as Eaf1, Yaf9 and Yng2 have other chromatin-binding domains such as SANT, YEATS and PHD domains respectively (Auger et al. 2008; Wang et al. 2009; Loewith et al. 2000).

1.3 Gcn5 is a conserved acetyltransferase

One of the best studied HAT enzymes in yeast is Gcn5. It was originally thought to act as a transcription factor alongside other proteins such as Gcn4, Ada2 and Ada3 (GA et al. 1994; Georgakopoulos & Thireos 1992). Soon thereafter, an acetyltransferase discovered in *Tetrahymena thermophila* was found to have significant homology to Gcn5 in yeast (Brownell et al. 1996; Brownell & Allis 1995), where its catalytic activity was then shown in yeast. As such, we know it today as highly conserved across eukaryotes from yeast to humans. In fact, the HAT domain of yeast Gcn5 has up to 67% sequence homology with humans (Wang et al. 1998), and can be functionally replaced in either organism (Wang et al. 1997). Interestingly, Gcn5 in yeast is not necessary to its viability, and mutants can still grow despite their slow speed. However, in other organisms such as Drosophila and mice, loss of Gcn5 leads to severe developmental issues and lethality (Carre et al. 2005; Xu et al. 2000).

Type A HATs are usually incorporated into larger, multi-subunit complexes. Gcn5 is one of these and can be found in at least 3 distinct complexes in yeast: SAGA, SLIK/SALSA and ADA (Pray-Grant et al. 2002; Eberharter et al. 1999), which will be discussed further in the following section. Common to all three of these complexes, is the inclusion of the proteins Ada2 and Ada3. Even prior to the discovery of its catalytic activity, Gcn5 was reported to interact with these two in a distinct complex then dubbed ADA (Horiuchi et al. 1995). It was then shown that Gcn5 requires both Ada2/3 to form a ternary complex in order to properly acetylate nucleosomal histones to the same degree as when included in larger complexes (Sendra et al. 2000; Balasubramanian et al. 2002). Alone, Ada2 has been thought to potentiate Gcn5's activity towards its substrates (Syntichaki & Thireos 1998; Candau et al. 1997), but more recently it appears as though Ada2 acts to increase Gcn5's ability to bind Acetyl-CoA prior to its catalytic activity (Sun et al. 2018). As for Ada3, the leading theory is that it can expand the lysine specificity of Gcn5 on nucleosomal histones (Balasubramanian et al. 2002).

According to its traditional/canonical role, Gcn5's primary targets are chromosomal bound histones. More specifically, on its own, Gcn5's main target is H3K14 and cannot efficiently acetylate any other nucleosomal histones (Kuo et al. 1996). Once incorporated into the complexes listed above, its activity is extended to target a broader range of sites across H3 and even few on H4 and H2B (Grant et al. 1997; Grant et al. 1999; Galdieri et al. 2014; Cieniewicz et al. 2014). One of the ways Gcn5 is able to recognize its targets is through its bromodomain (Ornaghi et al. 1999; Cieniewicz et al. 2014; Li & Shogren-Knaak 2009; Hassan et al. 2007). The bromodomain, as described previously, is a small domain that can bind with acetylated lysines (Dhalluin et al. 1999; Hudson et al. 2000), and when mutated, Gcn5 dependent transcription events decrease (Li & Shogren-Knaak 2009). However as mentioned

before, incorporation into complexes permits enzymes such as Gcn5 to broaden its potential targets to an even greater degree (Grant et al. 1999; Cieniewicz et al. 2014).

1.4 The SAGA complex

Like other type A HATs in yeast, Gcn5 is most commonly found in the context of larger, multi-subunit protein complexes. One of these is the Spt-Ada-Gcn5 acetyltransferase (SAGA) complex (**Figure1**). This coactivator complex is comprised of approximately 20 subunits (Han et al. 2014; Sterner et al. 1999; Setiaputra et al. 2015). These subunits are divided into submodules within the complex, depending on their function. The most notable of these is the HAT (acetyltransferase) submodule, were Gcn5 is found, along with Ada2, Ada3 and Sgf29 (Grant et al. 1997; SL et al. 2002). There is also the DUB (deubiquitinase) module which contains the catalytic deubiquitinase Ubp8, Sgf11, Sgf73 and Sus1 (KW et al. 2003; SL et al. 2002; McMahon et al. 2005; Rodríguez-Navarro et al. 2004). Following these there is the SPT (recruiting and structural) module which contains Spt8, Spt20, Spt7, Spt3, Ada1 and Tra1, subunits responsible for complex assembly and structure, as well as recruitment of the TATA binding protein (TBP) (Grant et al. 1998; Grant et al. 1997). Finally, the TAF (coactivator) module, which contains the remaining Taf5, Taf6, Taf9, Taf10 and Taf12, and is the core of the SAGA complex largely responsible for complex assembly (Han et al. 2014; Grant et al. 1998). This complex plays important roles in the context of gene transcription, regulating up to 10% of the yeast genome, mostly related to stress responses (Huisinga & Pugh 2004). Though its main function is transcription activation, the SAGA complex also has roles in DNA replication and repair, mRNA export and histone deubiquitination (Baker & Grant 2007; Köhler et al. 2008).

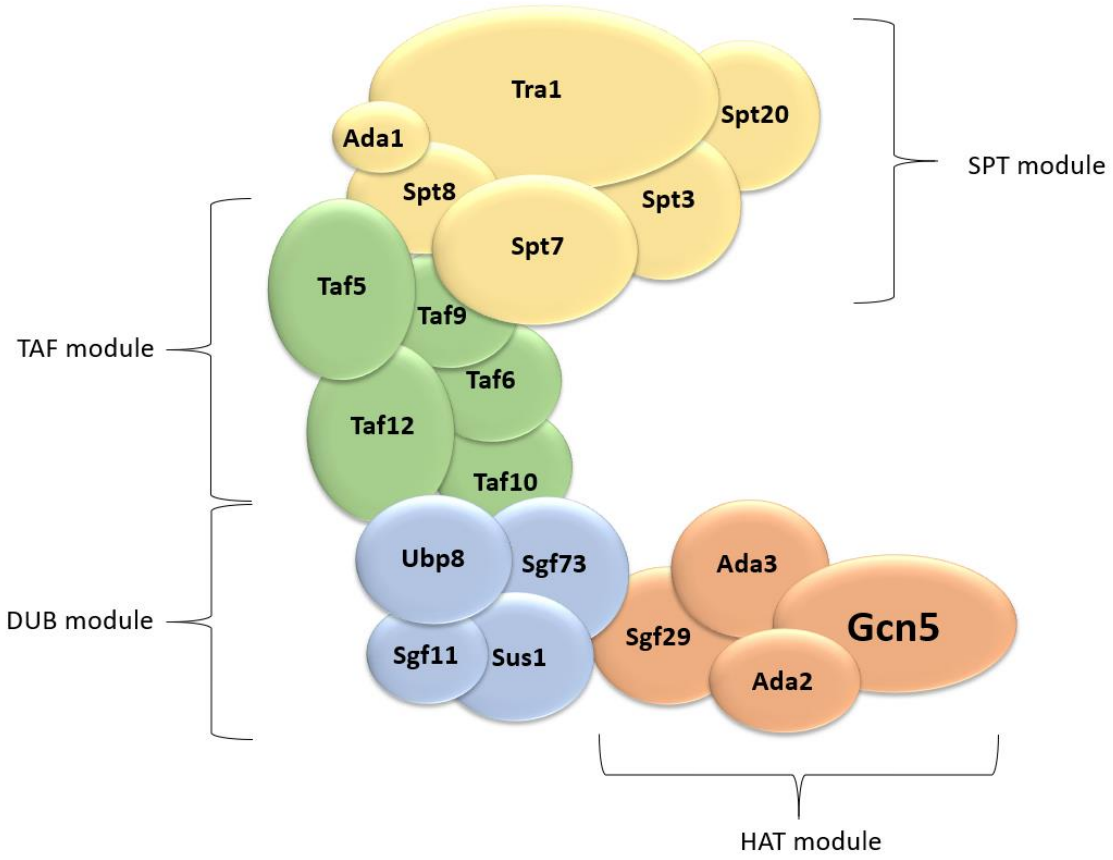

Figure 1. Representative localization of SAGA submodules. Yeast SAGA complex model representing known subunits organized in their respective submodules. Sizes and exact location of proteins are not exact and for demonstrative purposes only.

As mentioned previously, Gcn5 resides in complexes other than SAGA, including SLIK and the ADA complex (Eberharter et al. 1999; Pray-Grant et al. 2002). These are variations of the SAGA complex which contain slight differences to their composition but ultimately retain similar functions in transcription activation, albeit in response to different triggers. Just like Gcn5, the complexes in which it is found, such as SAGA, are highly conserved through eukaryotes and found all the way up to humans (Nagy & Tora 2007), albeit in multiple forms. Again, like in yeast, hGCN5 can exist in other "SAGA-like" complexes such as Ada2a-containing complex (ATAC) (Wang et al. 2008). In the example of SAGA, almost all the members are conserved with human homologs and serve largely the same purpose, save for Spt8 which is not present (Nagy et al. 2009). Interestingly, two forms of the SAGA complex exist in humans, one containing hGCN5 as its catalytic HAT enzyme, and another containing p300/CBP-associated factor (PCAF), a separate HAT enzyme approximately 73% identical to hGCN5 (Yang et al. 1996) though providing somewhat differing function (Jin et al. 2011; Nagy & Tora 2007). hGCN5 is essential to embryonic development in mice whereas PCAF is most commonly found in the tissue of adult mice, indicating a different set of transcriptional targets (Roth et al. 2001; Nagy & Tora 2007; Yamauchi et al. 2000).

Aside from regulating important transcription events, disruptions in the SAGA complex can lead to other diseases (Baker & Grant 2007; Wang & Dent 2014). One example of this is the progression of the neurodegenerative disorder spinocerebellar ataxia type 7, which is the direct result of the expansion of a polyglutamine (polyQ) tract within ATXN7, the human ortholog of Sgf73. Due to the high degree of homology, a humanized yeast background expressing the mutant form of ATXN7 instead of Sgf73 was developed to study the effects of this disorder (McMahon et al. 2005; Burke et al. 2013). These studies have found that the mutant polyQ expanded ATXN7 results in a decrease in Gcn5 HAT activity despite an increase of its localization

to its target genes (Burke et al. 2013). Interestingly, these same effects were seen in transgenic mice (Palhan et al. 2005; Helmlinger et al. 2006) supporting the thought that the polyQ-ATXN7 acts in a dominant-negative way on transcription events regulated by SAGA and Gcn5.

1.5 Sirtuins

One of the key features of lysine acetylation as a PTM versus N-terminal acetylation is that it is a non-permanent and reversible modification (Glozak et al. 2005). Whereas HAT enzymes catalyze the addition of acetyl moieties, it is the role of HDACs to remove the acetylation from their targets. The balance between these actions is very delicate. HDACs are traditionally considered to be negative transcriptional regulators, leading to gene repression and silencing (de Ruijter et al. 2003). Just like with HATs, these enzymes are divided into functional classes based on their similarity to confirmed yeast HDACs. In humans, there are 18 confirmed HDACs, whereas in yeast there are only 10 (Seto & Yoshida 2014; Downey & Baetz 2015). The functional classes are organized according to four of the yeast proteins identified: class I enzymes are those who are similar to yeast Rpd3 (Yang & Seto 2008); class II are similar to yeast Hda1 (Seto & Yoshida 2014; Haberland et al. 2009); class III enzymes are those similar to yeast Sir2 (Schwer & Verdin 2008); and finally there remains HDAC11, the only class IV enzyme in mammals, which is distinct as it has similarity to both class I and II enzymes (Gao et al. 2002; Chen et al. 2016). Importantly, these classes are then organized again into two superfamilies: the arginase/deacetylase superfamily which contains the traditional HDAC enzymes (classes I, II and IV), and the deoxyhypusine synthase-like NAD/FAD-binding domain superfamily which contains the sirtuin (and sirtuin-like; class III) enzymes (Gregoretti et al. 2004).

One such family of these HDAC enzymes is the nicotinamide adenine dinucleotide (NAD^+) dependent sirtuins, and just like Gcn5 and the rest of the SAGA complex, sirtuins are highly conserved between yeast and humans (Brachmann et al. 1995; Smith et al. 2000; Landry et al. 2000). In yeast, there are 5 sirtuins, Sir2 (for which the family is named) and Hst1-Hst4. Sir2 was first characterized as a gene silencer forming part of the silent information regulator (SIR) complex (Moazed et al. 1997) and is now studied alongside Hst1 as transcriptional regulators of specific genes through their HDAC function typically on H3K4, K9, K14 and H4K16 (Froyd & Rusche 2011; Mead et al. 2007). Sir2 is found in two separate complexes: the SIR complex as mentioned, which is responsible for the repression of the silent alternate mating-type loci in yeast; it is also found within the RENT complex, which is primarily responsible for rDNA silencing (Huang & Moazed 2003; Kasulke et al. 2002). Similarly, Hst1 can also be found within two histone deacetylase complexes, the SET3 complex and the Sum1/Rfm1/Hst1 complex (WW et al. 2001; Weber et al. 2008). Hst2 is the only cytoplasmic sirtuin in yeast and as such its functions remain poorly characterized (Perrod et al. 2001; Wilson et al. 2006), however in mammals, its homolog Sirt2 has been shown to target H4K16 during mitosis (Vaquero et al. 2006). Finally, Hst3 and Hst4 are best characterized for their function in DNA repair via deacetylation of H3K56 (Celic et al. 2008; Celic et al. 2006; Che et al. 2015; Masumoto et al. 2005). Hst4 has also been shown to localize to the mitochondria in response to biotin starvation to regulate acetylations on mitochondrial substrates (Madsen et al. 2015).

In mammals there are 7 sirtuin enzymes, SIRT1-SIRT7 (North et al. 2003; Haigis et al. 2006). SIRT1 is the best studied member in mammals, homologous to yeast Sir2 and Hst1. First described to deacetylate histones, it has since been found to target other substrates related to transcription activation like FOXO and p53 in cancer (Haigis & Sinclair 2010; Vaziri et al. 2001;

Brunet et al. 2004). SIRT2 is the only cytoplasmic sirtuin in mammals like Hst2 in yeast and its targets include proteins such as tubulin and H4K16 during mitosis (Vaquero et al. 2006; North et al. 2003). SIRT3 is located primarily in the mitochondria and acts in fatty acid metabolism and protection against reactive oxygen species (Lombard et al. 2007; Someya et al. 2010). SIRT4 and SIRT5 are also mitochondrial and have targets involved in metabolism (Haigis et al. 2006; Nakagawa et al. 2009). SIRT6 has a role in DNA stability and repair, functioning similarly to Hst3 & Hst4 to target H3K56, but also has been reported to have roles in metabolic diseases through glycolysis activation (Zhong et al. 2010; Mostoslavsky et al. 2006). Finally, SIRT7 functions to activate RNA pol I and regulates rRNA transcription (Ford et al. 2006), however its role in disease is still being elucidated (Barber et al. 2012). Interestingly, SIRT4-SIRT6 have functions which classify them as deacylases and not just deacetylases (Bheda et al. 2016).

Studying the roles of sirtuins, as with most proteins, is accomplished through observing downstream effects following inhibition of their function. Nicotinamide, a product of NAD^+ cleavage by sirtuins, is commonly used to inhibit sirtuin deacetylase function and acts both through a negative feedback loop (Bitterman et al. 2002; Jackson et al. 2003) as well as through competitive binding of the NAD^+ catalysis site (Yuan & Marmorstein 2012; Avalos et al. 2005). On a genetic level, these sirtuins have been linked to wide array of cellular processes such as transcription, ageing/cell cycle and stress responses (Houtkooper et al. 2012;Guarente 2007;Sebastiań et al. 2012). They have also been shown to play important roles in multiple diseases such as cancer (Liu et al. 2009) and Alzheimer's disease (Donmez et al. 2010). More recently, Sir2 was shown to interact with the DUB submodule of SAGA which could potentially contribute to the progression of neurodegenerative diseases (McCormick et al. 2014). In this proximity, to the HAT module, Sir2 may serve to deacetylate Gcn5 targets.

1.6 Non-histone substrates

The SAGA complex can regulate transcription in 3 potential ways: the first being through recruitment of transcription machinery, such as TBP, to gene promoters; a second, and more direct way is through the acetylation of histone tails by Gcn5; A third mechanism in which SAGA and other HAT complexes can regulate transcription is through the acetylation of non-histone substrates (Glozak et al. 2005; Downey & Baetz 2015). Though traditionally viewed as a transcription activator due to its activity at histone tails, acetylation is now regarded as a global PTM with multiple roles spanning from gene transcription and silencing, DNA repair and stability as expected, but also now to regulation non-histone substrates (Glozak et al. 2005; Drazic et al. 2016; Downey & Baetz 2015).

This newly revised description of acetylation is in largely due to advancements in proteomic technologies such as high throughput screens coupled with mass spectrometry (Zhang et al. 2009). Thus far, thousands of sites on non-histone substrates have been identified in large scale screens, and some were even able to attribute the HAT enzyme responsible (Downey et al. 2015). These assays have been performed in multiple model organisms, from yeast to humans (Henriksen et al. 2012; Weinert et al. 2011; Choudhary et al. 2009; Downey & Baetz 2015). This method allows for the identification of many sites at once but does not tell of any functional role. A major obstacle in finding the relevance of these sites is that acetylation as a PTM appears to be somewhat unpredictable, in that the function of these marks appear to have different effects on a site per site basis. Assembling these lists do permit however to generate prioritized lists for the characterization of these sites based on the regular function of the proteins themselves. So far, the majority of the few characterized sites in yeast are those on proteins with functions in transcription and chromatin modifications, and in each confer a slightly different role (Downey & Baetz 2015).

An example of this is the Gcn5 dependent acetylation of Rsc4, a member of the chromatin structure remodeling (RSC) complex. The RSC complex is responsible for regulation of transcription, DNA repair and chromosome cohesion (Baetz et al. 2004; Chai et al. 2005; Angus-Hill et al. 2001). To accomplish these functions, the complex consists of more than half of the proteins containing bromodomains in yeast (Cairns et al. 1996). Like the one on Gcn5, bromodomains are used for binding to acetylated lysines (Dhalluin et al. 1999; Hudson et al. 2000), including those on histone tails. Rsc4 has 2 independent bromodomains integral for recognition of H3K14ac (Kasten et al. 2004). However, Rsc4 can be acetylated on K25 by Gcn5 which directly results in its inhibition. This is due to the first bromodomain of Rsc4 binding the acetylation on K25 instead of histones, leading to autoinhibition of the whole RSC complex functions (VanDemark et al. 2007; Charles et al. 2011).

Another similar example would be with acetylation of Snf2 also by Gcn5. Just like the previous example, Snf2 is a member of the SWI/SNF chromatin remodeling complex (CL & JW 1995). Normally, the complex is recruited to gene promoters and alters nucleosome structure to activate transcription of approximately 5% of genes in yeast (Peterson & Workman 2000). The complex is normally recruited by DNA-binding transcription factors that interact with members of the complex (Prochasson et al. 2003). Additionally, Snf2, a member of the complex, contains two AT hook domains and a bromodomain which are used to recruit the complex to DNA (Bourachot et al. 1999; Hassan et al. 2006). Just like in the previous example, Gcn5 can acetylate two sites on Snf2 at K1493 and K1497, leading to autoinhibition by its own bromodomain (Kim et al. 2010). This in turn decreases the complex's interaction with DNA, and thus giving another role to Gcn5 in regulating chromatin structure.

1.7 Substrate targeting

While the Gcn5 bromodomain may also contribute to the recognition and targeting of substrates (Syntlchakl et al. 2000; Li & Shogren-Knaak 2009), as mentioned previously the activity of Gcn5 on its own is severely limited (Grant et al. 1997; Grant et al. 1999; Galdieri et al. 2014; Cieniewicz et al. 2014) and it is therefore assumed again that the other members of the SAGA complex have important roles in substrate selection. It has been well documented how certain subunits are responsible for transcription regulation at specific loci, for example Spt3 and Spt8 being required for the assembly of the preinitiation complex at genes such as *VTC3, PHO84* (Bhaumik & Green 2002; Mohibullah & Hahn 2008; Warfield et al. 2004). Thus, it is expected that it also drives non-histone substrate selection. One example of this is with the transcription factor Ifh1, which is essential for ribosomal protein expression. Ifh1 was determined to be a non-histone target of Gcn5, whose acetylation was dependent on Spt7 (Downey et al. 2013), what was previously thought to act as a structural component of the SAGA complex. Interestingly, Spt7 also contains a weak bromodomain which was determined as not being important for histone substrate recognition, and instead for transcription factor binding (Hassan et al. 2007).

While high throughput screens have been done to identify sites regulated by specific HATs and HDACs in yeast (Downey et al. 2015), other work has gone out to do the same in other organisms. In humans, peptide arrays have been performed to better understand how sirtuins selectively choose their targets for deacetylation (Rauh et al. 2013; Smith et al. 2011). Data from these screens produced patterns in substrate selectivity for each of the human sirtuins. This knowledge can then be applied to identify novel sites regulated by these enzymes. Similarly, acetylome profiling in Drosophila was used to compare findings in other organisms through conservation. In this case, the data was used to identify important signaling cascades

related to the acetylation of an E2 ubiquitin-conjugating enzyme that were conserved from Drosophila to the human homolog UBE2D3 (Weinert et al. 2011).

1.8 Rationale

It is well known that HATs, including Gcn5, don't just target histones, and instead have an ever-evolving catalog of substrates. As mentioned, large scale screens have identified a multitude of acetylation sites on non-histone substrates. However, despite this knowledge it remains largely unknown how these HATs are targeting these substrates in the first place. What is even more interesting is how previous acetylome profiling screens have shown that other non-catalytic enzymes are also responsible for HAT dependent acetylation on certain substrates. All in all, this begs the question of how HATs choose their substrates. It is already known that in large complexes such as SAGA, other members can contribute to site recognition; an example being how Ada2 can drive Gcn5 dependent acetylation on specific substrates (Downey et al. 2015). The question that remains is how important of a role the rest of the complex has in non-histone substrate targeting.

An ideal candidate to investigate this would be a generic target of Gcn5 which has equal chance to be regulated by any of the remaining members of the SAGA complex. A synthetic substrate targeted specifically by Gcn5 could be used to assay non-catalytic subunit contributions by observing relative amounts of acetylation. Consensus sequence-based targeting by Gcn5 has been suggested in the past. Using the *Tetrahymena* Gcn5 bound to a histone H3 based peptide, a consensus motif of G-K-X-P was proposed (Rojas et al. 1999), though this was shown to be histone specific. Further analysis of high confidence Gcn5 substrates from a mass spectrometry-based approach showed a preferred amino acid motif based on the frequency of the residues surrounding the central acetylated lysine, proposing a

consensus sequence of S-X-K(ac)-K/R-P (Downey et al. 2015). This motif is distinct from that which was previously suggested (Rojas et al. 1999), and even showed significant overlap with one that was proposed for sirtuin HDACs. If proven true to being specifically targeted by Gcn5, this consensus sequence can be used to predict new substrates for Gcn5 and potentially other enzymes such as sirtuins. It could lead to a better understanding of how HATs and HDACs target their substrates. This consensus sequence and novel substrates can then be used as a tool to identify whether the SAGA complex acts as a regulator of non-histone substrate selection.

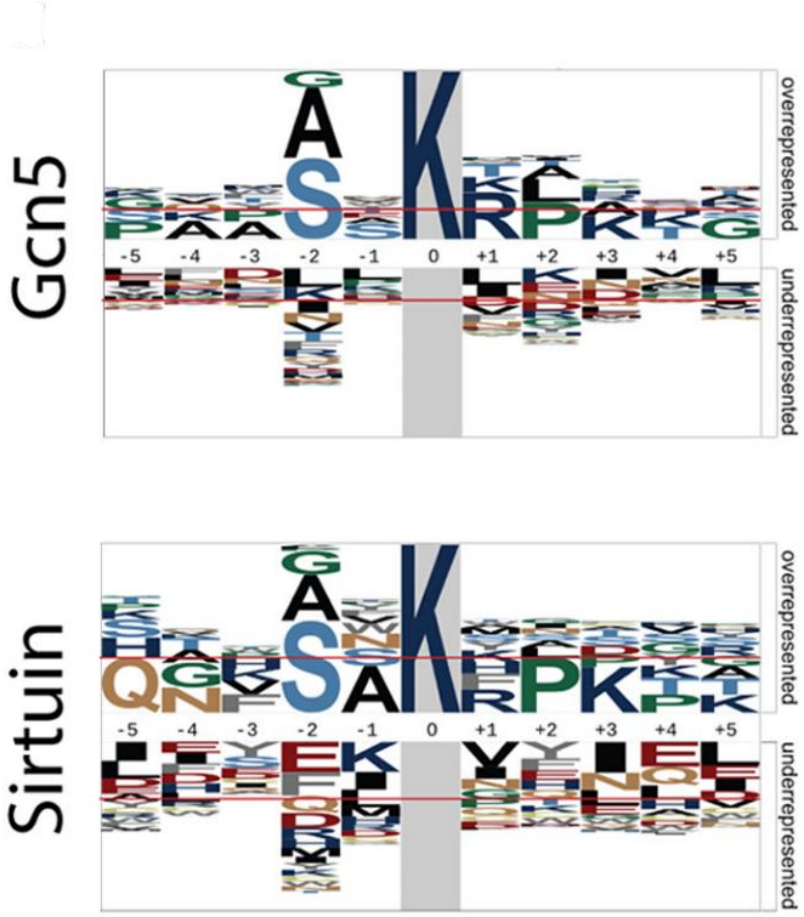

Figure 2. Proposed consensus sequences for Gcn5 and sirtuins. Frequencies of amino acid residues surrounding acetylated lysines of high confidence targets for either Gcn5 or sirtuins. Height of each residue is representative of its frequency. This research was originally published in *Molecular & Cellular Proteomics*. Downey, M *et al*. Acetylome profiling reveals overlap in the regulation of diverse processes by sirtuins, gcn5, and esa1. Mol Cell Proteomics. 2015; 14:162-76. © the American Society for Biochemistry and Molecular Biology *or* © the Author(s).

1.8 Hypothesis & objectives

I hypothesize that a consensus sequence recognition is required for Gcn5 directed acetylation. I also hypothesize that Gcn5 targeting of non-histone substrates is mediated by other members of the SAGA complex.

To determine whether the SAGA complex affects Gcn5 substrate selection, my main objectives will be to:

1. Test the importance of the consensus sequence to Gcn5 substrate selection.
2. Determine the function of non-catalytic SAGA subunits in non-histone substrate selection

If the findings of my first objective demonstrate that a consensus sequence is targeted by Gcn5, this will allow me to generate a system to identify which non-catalytic subunits of the SAGA complex act as regulators of Gcn5 targeting. This system will consist of a synthetic substrate whose acetylation is dependent on Gcn5 and will be used to assay the other members of the SAGA complex to observe their effect on the substrates relative acetylation. Following this, identified regulators will be confirmed via mass spectrometry-based analysis, providing a list of specific sites regulated by each non-catalytic subunit.

2. Materials and Methods

Methods are as described in my latest article available in preprint (Rossl et al. 2018).

2.1 Yeast strain generation

All yeast strains created and used are included in **Table 5**. All strains used are in the S288c background. All deletions were confirmed using PCR and western blotting, as well as colony purification and drug/auxotrophic resistance markers (Rössl et al. 2016).

2.2 Plasmid generation

2.2.1 Synthetic substrate

The vector backbone containing the synthetic substrate and GFP control was created by inserting the multi-cloning site from pRS316 into pRS406 containing the ADH1 promoter and CYC1 terminator. To do so, both vectors were first double digested using *Kpn*I and *Sac*I. The products were then run on a 1% agarose gel to separate the fragments, followed by gel purification (BioBasic) and ligation of the fragments using T4 DNA ligase. GFP control or synthetic substrate inserts were generated by amplifying via PCR from pFA6-GFP-His3MX using PAGE purified primers with or without desired consensus peptide repeats on the 3' end, followed by an *Eco*RI restriction site. Primers also included a *Hind*III restriction site on the 5' end. Vector and inserts were double digested using *Hind*III and *Eco*RI in NEBuffer 3.1 for 1.5 hrs at 37°C, and vector was then treated with Calf-intestinal alkaline phosphatase for an additional hour. Digested fragments ere then ligated at either 16°C or 25°C for times varying between 20 minutes and 12 hours. Ligation products were then transformed into chemically competent DH5α cells and subsequently harvested via plasmid miniprep protocol. Recovered plasmids were then re-digested and ran on a 1% agarose gel to confirm fragment sizes. Plasmids were

then verified by sequencing (Genome Quebec) using primers found in the ADH1 promoter region, within GFP and a reverse primer found in the CYC1 terminator region.

2.2.2 SPT2-GFP

pRS316 was first digested using *Hind*III-HF and *Eco*RI-HF for 15 min at 37°C. Using Gibson cloning technique, *SPT2*-GFP was amplified using primers providing homology with pRS316. All products were run on 1% agarose gel, followed by gel purification. These fragments were then combined in Gibson assembly mix and incubated at 50°C for 15 min. The product was then transformed into chemically competent cells and recovered via miniprep. Recovered plasmid was then sent for verification via sequencing. To generate the *spt2*-K166R-GFP mutant plasmid, site directed mutagenesis was done using overlapping primers introducing 2 separate mutations: the first resulting in the lysine to arginine mutation, and the second introducing a new *Xba*I restriction site. Following amplification, *Dpn*I was used to digest original template plasmid. The reaction mixture was then transformed into chemically competent cells and recovered via plasmid miniprep. Plasmids were confirmed both by digestion using *Xba*I and by Sanger sequencing (Genome Quebec).

2.3 Whole cell extract and immunoprecipitation

Cells were grown in liquid media overnight before being diluted into a larger volume. Cells were let to grow for a minimum of 2 doublings before harvesting (60 OD_{600} equivalents). Cell pellets were either used immediately or flash frozen on dry ice/ liquid nitrogen. Cell pellets were thawed on ice and then resuspended in cold lysis buffer (50 mM Tris-HCl pH8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP40) with inhibitors (10 mM Glycerol-2-phosphate, 5 mM NaF, 10 mM sodium butyrate, 10 mM NAM, 1.75mM PMSF, 1 mM DTT, complete protease inhibitor tablet (without EDTA; Roche)) in 2mL screw cap tubes. Acid washed glass beads were added to

the top of the tube and then samples were lysed in the Bead beater for 8 cycles of 1.5 minutes, with 2 minutes rest on ice in between each cycle. Following lysis, tubes were punctured with 18-gauge needle before being placed inside FACS tube, and centrifuged at ~2K RPM at 4°C. Lysates were transferred to 1.5mL microfuge tubes and centrifuged 5 minutes at 17000g, also 4°C. Supernatants were transferred to new 1.5 mL tubes and centrifuged a second time, transferring the supernatants once more to new 1.5mL tubes. 20 µL cell extract was combined 1:1 with 1X SDS sample buffer, boiled, and saved for inputs. Remaining extract was incubated end over end with 0.5 µL anti-GFP antibody (Abcam, ab290) for 2 hours at 4°C. 20 µL washed "Surebeads" Protein A-coupled magnetic beads (Biorad, 1614013) were added and incubated an additional hour. Samples were then placed on magnetic Dynarack and washed 3 times in IP lysis buffer, followed by elution in 1-2X SDS sample buffer (with final 100 mM DTT) at 65°C for 10 minutes with intermittent flicking/resuspension. Eluates were then placed back on magnetic rack before transferring to new tubes. All samples were boiled at 100°C before loading.

2.4 Immunoblotting

List of antibodies and dilutions used are included in **Table 6**. 8-12% SDS-PAGE (37.5:1 acrylamide:bisacrylamide) were used. Proteins were transferred to PVDF at 75V for 1hr. Membranes were subsequently washed in an antibody specific blocking solution (5%, see **Table 6**) for a minimum of 30 minutes. All antibodies were incubated overnight at 4°C (unless otherwise mentioned) on a rocking platform. Membranes were washed a minimum of 3 times with TBST for at least 10 minutes before incubating with an HRP-conjugated anti-mouse or anti-rabbit secondary antibody (1:10000 in 5% milk or BSA). Membranes were washed again as before, incubated in ECL detection reagents (Millipore) and then exposed to autoradiography film in dark room. Membranes to be re-probed with a new antibody were washed for 1 hour in

0.01% Sodium azide-TBST solution before incubation with antibody. Membranes with whole cell extracts or inputs were stained with Ponceau S and scanned digitally.

2.5 *In vitro* acetylation assay

2.5.1 Recombinant Gcn5 purification

Recombinant enzymes were purified as previous (Downey et al. 2013) with minor changes. Briefly, BL21 DE3 LysS cells were transformed with 6His-TRX-Gcn5 or 6His-TRX-Gcn5-E173Q to produce either wild type or a catalytic deficient mutant respectively. 25 mL cell culture in 2XYT media with 34 µg/mL chloramphenicol and 100 µg/mL ampicillin was grown at 28°C to an OD_{600} of 0.5 and then induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM for 4 hours.

2.5.2 HAT assay

GFP control and synthetic substrate were obtained from *gcn5Δ* cells following IP protocol above, however prior to elution, were instead washed twice with IP buffer and twice again with 1X HAT buffer (see 2X HAT buffer below). Proteins were then resuspended in 25 µL 2X HAT buffer (100 mM Tris-HCl pH 8.0, 100 mM NaCl, 10% glycerol, 0.2 mM EDTA, 2 mM sodium butyrate and 2 mM DTT). 50 µL final reaction mixture was then reached by adding recombinant His tagged Gcn5 or catalytic deficient mutant (approximately 3 µg), water and Acetyl-CoA (800 µM). Tubes were incubated at 30°C for 1 hour with intermittent "flicking" to resuspend beads. Reactions were stopped by addition 50 µL of 3X SDS sample buffer (with final 100mM DTT) and eluting substrates from beads. Samples were boiled prior to loading.

2.6 Acetylome profiling

2.6.1 Whole cell extract and Immunoprecipitation

Paired wild type/mutant differently labelled cells pellets were resuspended and combined in filtered 8M urea buffer (with 150 mM NaCl, 100 mM Tris-HCl pH 8.0, complete protease inhibitor tablet without EDTA, supplemented with 10mM NAM and sodium butyrate). Cells were then lysed using acid washed glass beads in conjunction with a Bead beater for 14 cycles of 1.5 minutes, with 2 minutes rest in between each cycle. Following lysis, tubes were punctured with 18-gauge needle before being placed inside FACS tube and centrifuged at ~2K RPM. Extracts were then quantified and treated with final 4 mM tris(2-carboxyethyl)phosphine, 10 mM iodoacetamide and 10 mM DTT for 30 minutes each, prior to digestion with trypsin (90 µg per sample) overnight. Samples were then adjusted to below pH 3 with 10% trifluoroacetic acid prior to desalting using Sep-pak C18 cartridges. After lyophilizing, samples were then IP'ed using αAcK agarose beads (Immunechem) before a final desalting and speed vac drying with no heat.

2.6.2 Acetylated peptide identification

Samples were analyzed using the Q Exactive mass spectrometer (ThermoFisher Scientific) for high performance liquid chromatography electrospray ionization tandem mass spectrometry. Methods are as described elsewhere (Zhang et al. 2017).

2.6.3 Database searches

Data was acquired using Xcalibur software (ThermoFisher Scientific) and searched against a *Saccharomyces cerevisiae* database (downloaded from Uniprot) using MaxQuant software (v. 1.5.3.30)(Cox et al. 2009). For gel excision and analysis of 3X consensus construct, GFP fusion sequence was added to the database. Parameters used were in line with experimental procedures. Importantly, these include detection of Lys8 heavy labeling, a maximum of 2

missed trypsin induced cleavages on peptides no smaller than 7 amino acids, with a modifications focus on acetyllysine (N-terminal acetylation, cysteine alkylation and methionine oxidation included) (Rossl et al. 2018).

2.6.4 Bioinformatics analyses

DAVID (v6.8, david.ncifcrf.gov) was used to determine GO-term enrichments using *Saccharomyces cerevisiae* database as background. Analyses were redone using total protein recovery as background. Default settings were used as dictated by protocols (Huang et al. 2009; Huang et al. 2007).

3. Results

3.1 Using the consensus sequence to identify non-histone acetylation substrates

3.1.1 Blastp identifies native proteins containing our selected consensus sequence

Having discovered a potential consensus sequence for Gcn5, based on high confidence targets, I wanted to determine whether this consensus sequence is important to substrate selection. First, I inputted the sequence into the online protein-protein Basic local alignment search tool (BLASTp). Focusing on the S-S-K(ac)-R-P motif, which represents the most central and frequent amino acid residues surrounding the target lysine, 4 proteins were identified in the yeast *Saccharomyces cerevisiae* (taxid:4932) model organism database that contain the S-S-K-R-P sequence: Spt2, Far10, Afr1 and Ydr249c (**Figure 3A**). Delving into the roles of these proteins *in vivo* show that Spt2 is a transcriptional regulator which physically interacts with the SWI/SNF complex (Pérez-Martín & Johnson 1998; Winston et al. 1984); Far10 is a member of the Far complex which is involved in cell cycle arrest, Torc2-dependent signaling and mitophagy control (Kemp & Sprague 2003; Furukawa et al. 2018; Pracheil & Liu 2013); Afr1 is involved in maintaining the structural integrity of mating projections (Bharucha et al. 2008); Ydr249c remains an uncharacterized protein (Cherry et al. 2012).

3.1.2 Spt2 is acetylated *in vivo* on site K166

To best determine whether these consensus motif-containing proteins were being targeted by Gcn5 at the S-S-K-R-P motif, GFP fusion strains were developed, starting with *SPT2* considering its role in transcriptional regulation. *SPT2*-GFP cell strains were developed in a wild type and *Δgcn5* background coupled with nicotinamide (NAM) treatment, then assayed using IP/Western blot analysis. The goal here is to treat the GFP as an epitope tag to easily

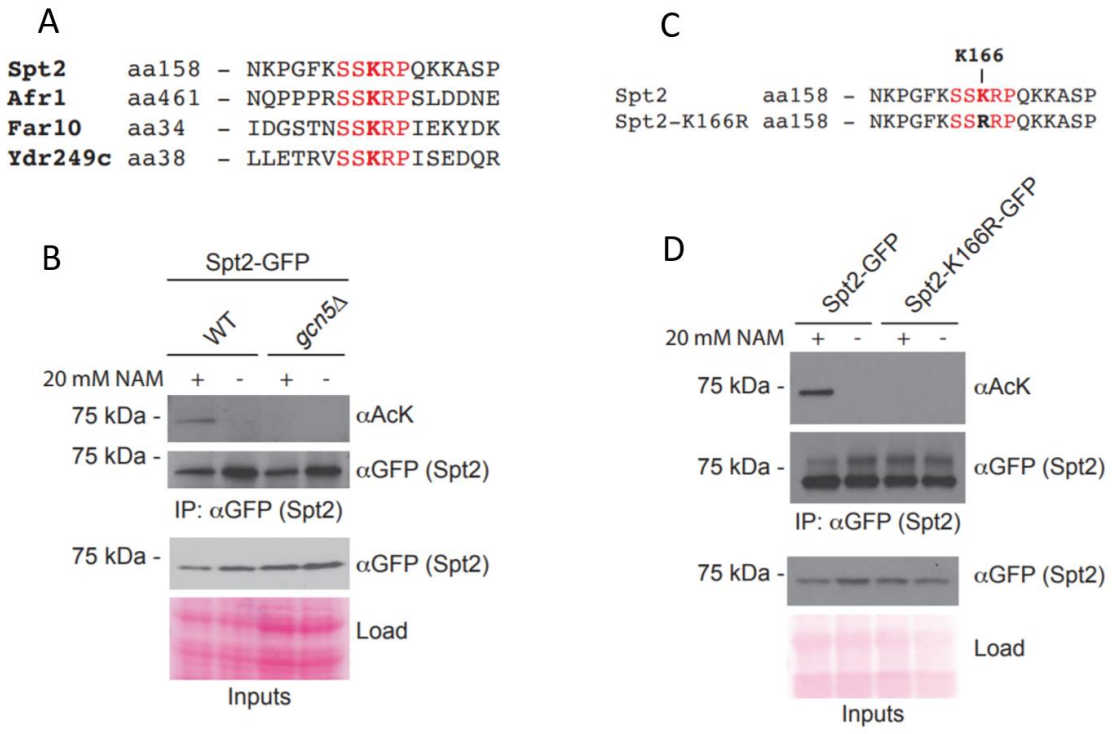

Figure 3. Acetylation of Spt2 at K166 is dependent on Gcn5 and regulated by sirtuins. A) Blastp identifies 4 native yeast proteins in the *S. cerevisiae* (taxid:4932) database which contain the proposed consensus motif when searching "SSKRP" as the query. Protein sequences are aligned according to location of consensus motif. **B)** Spt2-GFP was expressed endogenously in either wild type or *gcn5Δ* strain backgrounds and grown with or without nicotinamide (NAM, 20 mM). Cells were lysed and extracts were immunoprecipitated using anti-GFP. Samples were separated by SDS-PAGE, transferred to PVDF, blocked and then probed using a site-specific anti-acetyllysine and anti-GFP antibodies. Top panels are IP'ed samples and bottom are inputs. **C)** K166 is the central lysine of the consensus motif in Spt2. K166R point mutant cannot be acetylated. **D)** Spt2-GFP and Spt2-GFP-K166R plasmids were expressed in a *spt2Δ* strain backgrounds and grown with or without nicotinamide (20 mM). Cells were lysed and extracts were immunoprecipitated using anti-GFP. Samples were separated by SDS-PAGE, transferred to PVDF, blocked and then probed using a site-specific anti-acetyllysine and anti-GFP antibodies. Top panels are IP'ed samples and bottom are inputs.

immunoprecipitate (IP) the proteins with one same antibody. Once immunoprecipitated (IP'ed), the proteins were separated on a gel and transferred to PVDF where the membrane was incubated overnight with a site-specific antibody. This antibody was created in collaboration with BioLegend, to specifically recognize the acetylated form of our consensus motif (Rossl et al. 2018). When probing the IP'ed Spt2-GFP with the site specific anti-acetyllysine antibody, a distinct band was found in the wild type but not the *gcn5Δ* background when treated with NAM (**Figure 3B**).

The central lysine of the proposed consensus motif in Spt2 is residue K166. To test whether the acetylation is occurring on the suspected lysine residue, a set of plasmids expressing either *SPT2*-GFP or a point mutant with a lysine to arginine mutation at site K166, *spt2*-K166R-GFP (**Figure 3C**), were transformed into a *Δspt2* strain. This mutation retains the same charge as lysine, however cannot be acetylated. These strains were then assayed via IP/Western following NAM treatment. IP'ed Spt2-GFP was detected by the site-specific anti-acetyllysine antibody when treated with NAM. The mutant Spt2-K166R-GFP was not detected by the antibody and did not produce a band regardless of NAM treatment (**Figure 3D**). Altogether, these data are consistent with Gcn5 and sirtuins positively and negatively regulating (respectively) the acetylation of Spt2 on K166, the central lysine of the proposed consensus motif. They also confirm the specificity of the site-specific antibody, which would not recognize the lysine to arginine mutant.

3.1.3 Far10 and Ydr249c are not acetylated

Following the Spt2 result above, a set of GFP tagged strains were developed for the remaining 3 proteins identified by Blastp. Unable to successfully create an *AFR1*-GFP strain, the focus turned to *FAR10*-GFP and *YDR249C*-GFP. When expressed endogenously, not enough

protein was recovered in order to observe acetylation signal when IP'ed. In this case, the inducible GAL1 promoter was introduced upstream of each tagged gene (in separate strains) (Longtine et al. 1998). Cells were then grown in the presence of galactose to activate the new promoter system and overexpress the genes. Even after NAM treatment, no acetylation could be observed via IP/Western blotting (**Figure 4**). One possibility to explain this would be that there is no significant interaction between Gcn5 and either Far10 or Ydr249c, which will be further explained in the discussion.

3.1.4 Prioritized "loose" consensus targets are not acetylated *in vivo*

Returning to Blastp, variations of the consensus motif were used to identify a broader list of potential proteins regulated by Gcn5. The variations were created by changing out individual residues of the motif with the other amino acids found to frequent the respective positions according to Downey *et al.* (2015). Once this new list was generated, a search to determine whether the proteins were already shown to be regulated by either Gcn5 or sirtuins was made to prioritize novel findings, as seen in **Table 2**.

To maximize the efficiency of the new screen, the yeast GFP tag collection was used to obtain a series of strains in which the target genes are already tagged. After confirming the strains via PCR and Western blotting, the strains were grown in the presence or absence of NAM, followed by IP/Western analysis. No change in their acetylation was detected however (data not shown). Following the null result, the core S-S-K(ac)-R-P was cemented as the most likely targeted sequence and was next used to develop a new tool to assay Gcn5 activity.

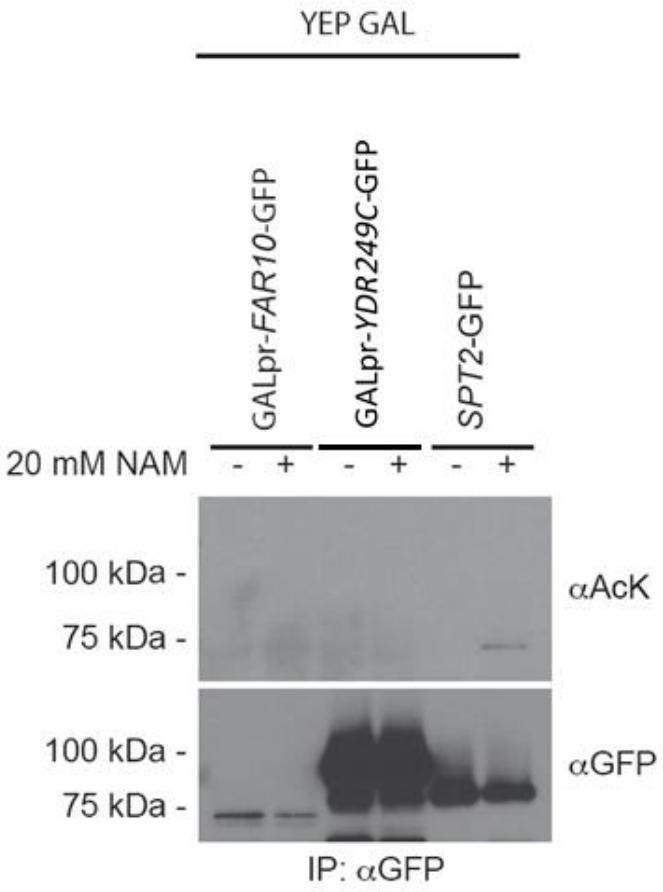

Figure 4. Far10 and Ydr249c are not acetylated at their consensus motif. Independent endogenously GFP tagged strains were generated, then Gal1 promoter was inserted upstream of each gene. Protein expression was induced by growing in culture containing 2% galactose as carbon source, with or without nicotinamide (NAM, 20 mM). Cells were harvested and lysed, then protein were immunoprecipitated using anti-GFP. Samples were separated by SDS-PAGE, transferred to PVDF, blocked and then probed using a site-specific anti-acetyllysine and anti-GFP antibodies.

Table 2. “Loose” consensus motif containing proteins.

Consensus motif	Protein	Regulated by Gcn5 or sirtuins?
SAKRP	Hmg1	
	Hsl7	
	Nab3	Both
	Rrb1	Gcn5
SGKRP	Smc6	
SSKKP	Bud3	
	Rom2	Both
	Dcp2	Both
	Gyp1	
	Hpc2	Both
	Ppq1	
	Cam1	
	Alg14	
	Ykl023c	

3.2 Validating the synthetic substrate

3.2.1 The synthetic substrate is regulated by Gcn5 and sirtuins

Having demonstrated that Gcn5 can acetylate proteins that express the S-S-K(ac)-R-P motif *in vivo*, I next wanted to determine whether the addition of this consensus motif to any protein can confer its acetylation. To continue in testing my first hypothesis, I decided to generate a synthetic substrate to use as a measure of Gcn5's dependence on sequence specificity when it comes to substrate selection. This construct consisted of a GFP fusion protein with a short peptide tail containing 3 repeats of our consensus motif. GFP was chosen as the candidate protein due to fact that it can translocate to the nucleus (Bohm et al. 2006; Seibel et al. 2007), as well as the already optimized protocols that can be used for its manipulation in yeast. In addition to this, GFP was also chosen because it is not known to be targeted by Gcn5, nor sirtuins, and as such, any regulation seen during analysis would be due to the inclusion of the consensus peptide tail. The decision to use 3 repeats of the consensus motif was to maximize the chances of observing the acetyllysine signal on this known non-substrate, and this decision is discussed further below. Once created, this construct was assayed via IP/Western blot to determine if it is targeted by Gcn5 and/or sirtuins, as well as to determine whether the site-specific acetyllysine antibody will detect it.

Using the same IP/Western blot analysis technique as with the tagged Spt2, when expressed in a wild type cell background, our synthetic substrate is detected by the site-specific acetyllysine antibody, and to ensure the antibody is detecting our consensus motif peptide tail and not any other acetyl marks on GFP, a GFP-only control without the consensus motif was separated alongside, and is not detected by the antibody (**Figure 5A**). However, as with Spt2-GFP, when expressed in a *gcn5*Δ background, the synthetic substrate is no longer detected by

the antibody, but is still being IP'ed as seen in the GFP loading (**Figure 5A**). The shift in molecular weight observed between the GFP control and the synthetic substrate is due to the addition of the consensus sequence peptide tail, and served to differentiate the two constructs.

When the consensus motif was first proposed, it was expected to have significant overlap with one designed against sirtuin enzymes (Downey et al. 2015). This is in line with previous findings of significant overlap in targets of Gcn5 and sirtuins (Downey et al. 2013). Following our results with Spt2 where we saw that the consensus motif is inversely regulated by sirtuins, I set out to determine if this was also true for our synthetic substrate. To determine whether the consensus peptide is inversely regulated/deacetylated by sirtuins, a nicotinamide treatment assay was performed, by growing wild type cells transformed with either the synthetic substrate or the GFP control in the presence of varying concentrations of nicotinamide. Cells were then subjected to IP/Western blot analysis. At 0mM NAM, the synthetic substrate is detected as acetylated by the site-specific antibody (**Figure 5B**). However, as the concentration of NAM increases to 5mM, and then 20mM, there is a dramatic increase in acetylation signal with each step, despite no change in recovered protein (**Figure 5B**, lanes 1-3). In contrast, the anti-acetyllysine antibody did not react with the GFP control, regardless of NAM concentration (**Figure 5B**, lanes 4-6).

To confirm these results and to narrow down which sirtuin is responsible for the dramatic increase in acetylation observed, the synthetic substrate was transformed into cell strains deleted for the sirtuins used to uncover the consensus motif. Both *Δsir2* and *Δhst1* mutant cells had no immediate effect on the substrate acetylation (**Figure 5C**, lanes 1 & 3). Loss of *HST2* however led to a clear visualization of acetyllysine signal over the first two (**Figure 5C**, lane 2). This can potentially be explained by the fact that Hst2 is located in the cytoplasm,

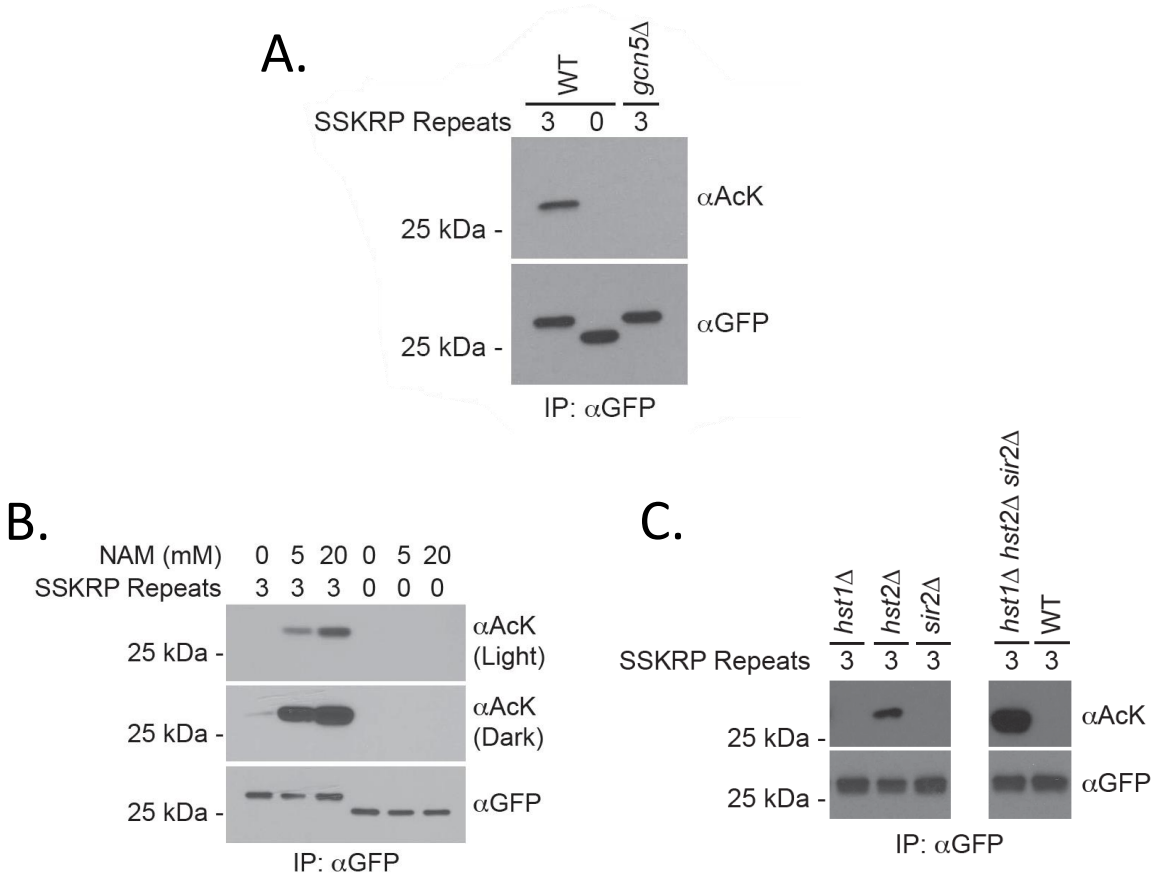

Figure 5. The synthetic substrate is regulated by both Gcn5 and sirtuins. A) The synthetic substrate and control GFP plasmids were transformed into wild type and *gcn5Δ* cell backgrounds. Cells were grown in SC-URA media and harvested during log phase growth. Cells were lysed and proteins were immunoprecipitated with anti-GFP. Samples were separated by SDS-PAGE, transferred to PVDF, blocked and then probed using a site-specific anti-acetyllysine and anti-GFP antibodies. For αGFP, IP'ed samples were diluted 1/25 with SDS sample buffer supplemented with DTT prior to loading. **B)** Wild type cells containing either the synthetic substrate or control GFP plasmids were grown in SC-URA media containing increasing concentrations of nicotinamide. Cells were assayed as in **A**. A light and dark exposure are shown to better represent differences in acetylation. **C)** Indicated mutants were transformed with the synthetic substrate and grown prior to harvesting. Cell extracts were assayed as in **A**. Both panels are cropped from the same blots.

whereas Sir2 and Hst1 are both nuclear (Mead et al. 2007; Froyd & Rusche 2011). As expected, when all three sirtuins were deleted, an even greater signal was observed (**Figure 5C**, lane 4), suggesting sirtuins act redundantly to deacetylate the substrate. These experiments showed that Gcn5 and sirtuins target their substrates based on the presence of a consensus motif, and addition of this consensus sequence can confer acetylation to known non-substrates.

3.2.2 Rapamycin treatment increases substrate acetylation

Gcn5 and the SAGA complex have commonly been shown to activate transcription events in response to certain stresses such as nutrient starvation or heat shock (Huisinga & Pugh 2004). This in turn takes the complex away from other substrates to ensure that genes in response to these stresses are properly expressed. To determine whether Gcn5 can still target its substrates in periods of stress, cells were either treated or not with 200ng/mL rapamycin for 30 min during log phase growth before harvesting. The assay was then analyzed via IP/Western blot. When probing the membrane with a site specific anti-acetyllysine antibody, a band appears in the synthetic construct lanes but not the unmodified GFP lanes (**Figure 6**). Furthermore, a distinct reduction in the acetyllysine signal is observed in the rapamycin treated cells lane (**Figure 6**, lane 2). No difference in loading is observed when probing with anti-GFP. These results are consistent with a model where Gcn5 and SAGA are recruited to response genes in response to certain cellular stresses and are therefore sequestered from other non-histone substrates.

3.2.3 Proximal lysine residue of consensus tail is preferentially acetylated

The IP/Western blot analysis technique is a good indicator of whether we can confer acetylation onto the consensus sequence. However, it remains uncertain that the antibody is reliably detecting the specific site of interest. To confirm whether the acetylation signal

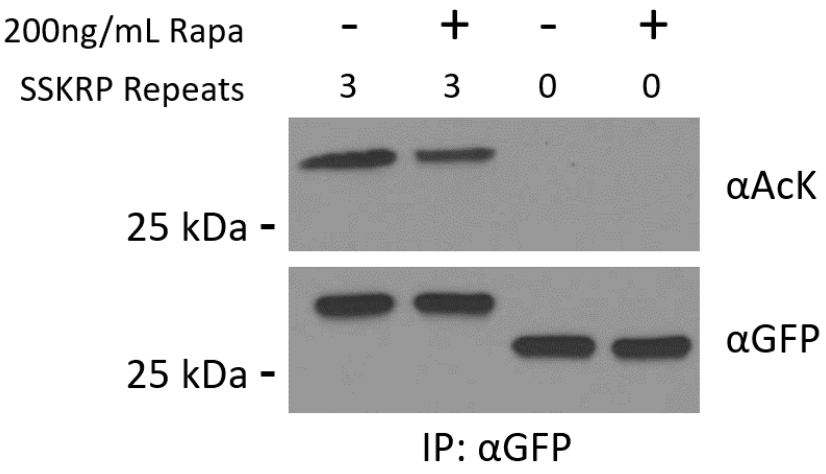

Figure 6. Rapamycin treatment decreases acetylation of the synthetic substrate. Wild type cells expressing either the synthetic substrate or GFP control plasmid were grown in SC-URA media, and either treated or not with Rapamycin (Rapa) for 30 minutes prior to harvesting during log phase growth. Cells were lysed and proteins were immunoprecipitated using anti-GFP. Samples were separated by SDS-PAGE and transferred to PVDF, where membranes were incubated with indicated antibodies. For αGFP, IP'ed samples were diluted 1/25 with SDS sample buffer supplemented with DTT prior to loading.

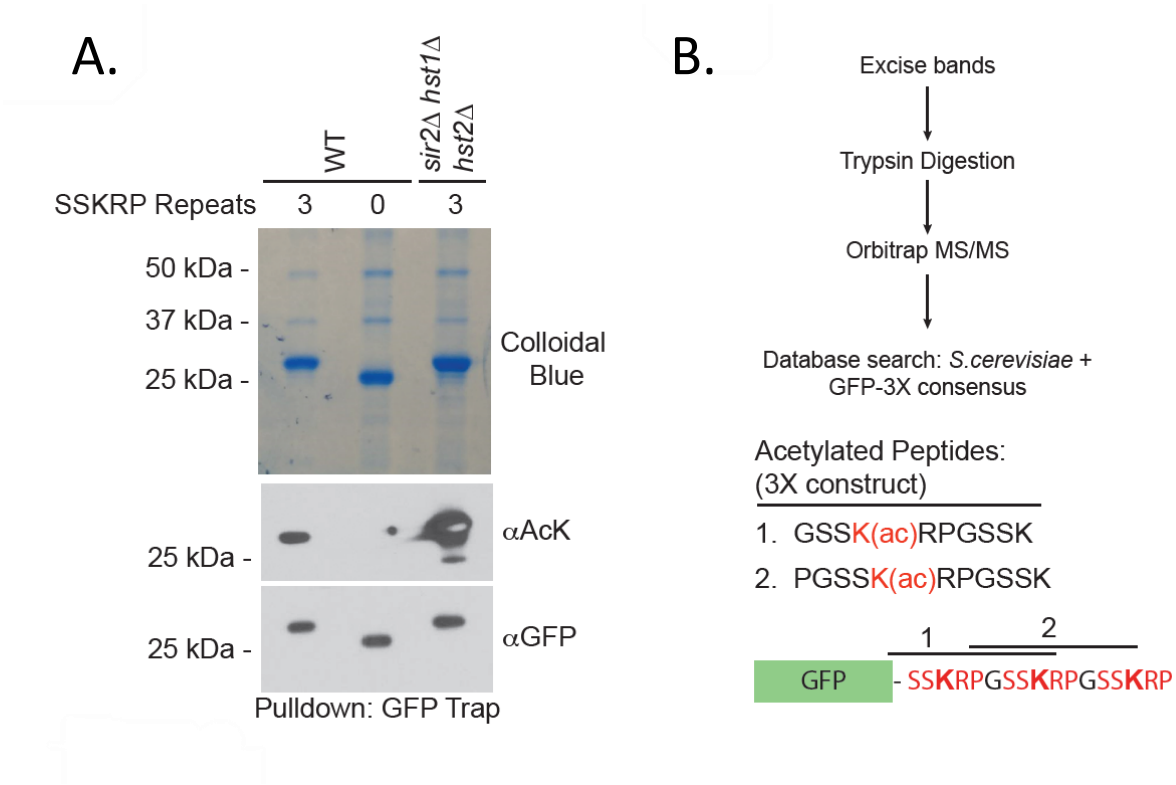

Figure 7. The synthetic substrate is acetylated on the consensus motifs. A) Constructs were immunoprecipitated from the indicated strains using the GFP Trap system and separated via NuPAGE gel. Gel was stained to facilitate excision of bands. A second NuPAGE was run with remaining sample to observe relative acetylation signal. For αGFP, IP'ed samples were diluted 1/25 with SDS sample buffer supplemented with DTT prior to loading. **B)** Brief overview of methods, followed by identified peptides by Orbitrap ms/ms. Observed peptides match first and second repeat of consensus peptide tail.

observed via western blot is resulting of the acetylated consensus peptide repeats being fully acetylated and not the site-specific antibody reacting elsewhere on GFP, the synthetic substrate from wild type and *Δsir2Δhst1Δhst2* cells (**Figure 5C**) were IP'ed and separated using NuPAGE (**Figure 7A**), excised from the gel after staining, and sent for analysis via Orbitrap ELITE mass spectrometry (ThermoFisher Scientific) following trypsin digestion (**Figure 7B**). Only peptides containing the first and second repeats of the 3X consensus peptide tail were detected as acetylated (**Figure 7B**), confirming the target sequence is acetylated *in vivo*. The analysis did not include a peptide containing the third and final site, whether acetylated or not. It is thus unknown if all 3 sites in the consensus peptide tail are acetylated. One possibility of why the third site was not detected could be the size of the peptide, seeing as trypsin digestion leads to peptide generation through cut sites following lysine residues (Zee & Garcia 2012), and when lysines are acetylated, trypsin has difficulty cleaving the peptide thus leading to longer peptides which are not always easily resolved during the analysis.

Up to this point, our synthetic substrate consisted of GFP fused with 3 repeats of the consensus motif. Knowing that at minimum 2 of the sites can be acetylated, different forms of the construct were created to determine whether the 3X was the ideal construct. In a first set of experiments, two additional constructs were designed containing either 1 or 2 repeats of the consensus motif (**Figure 8A**). When assayed via IP/Western, the detectable acetyllysine signal increased as the number of consensus repeats increased, with our initial 3X construct showing the strongest signal (**Figure 8B**), and thus was chosen as the best tool to evaluate acetylation.

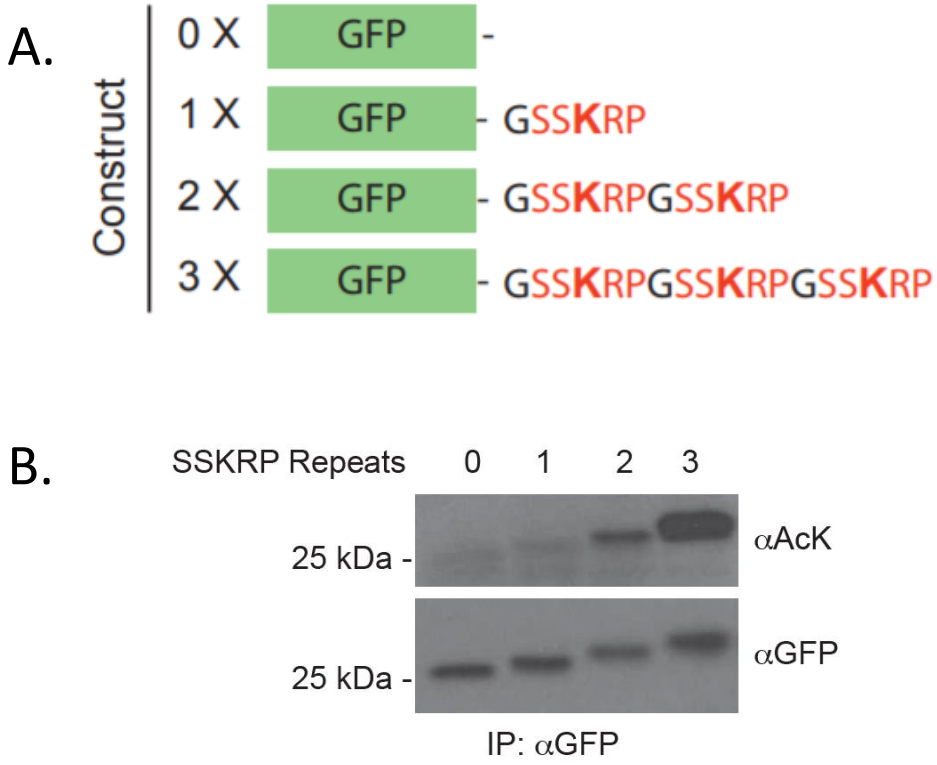

Figure 8. Construct regulation is dependent on number of consensus repeats present. A) multiple constructs were generated with increasing number of consensus motif repeats. **B)** Constructs were transformed into wild type cells and harvested during log phase growth, immunoprecipitated and then run on SDS-PAGE. Following transfer to PVDF, membranes were probed with indicated antibodies. For αGFP, IP'ed samples were diluted 1/25 with SDS sample buffer supplemented with DTT prior to loading.

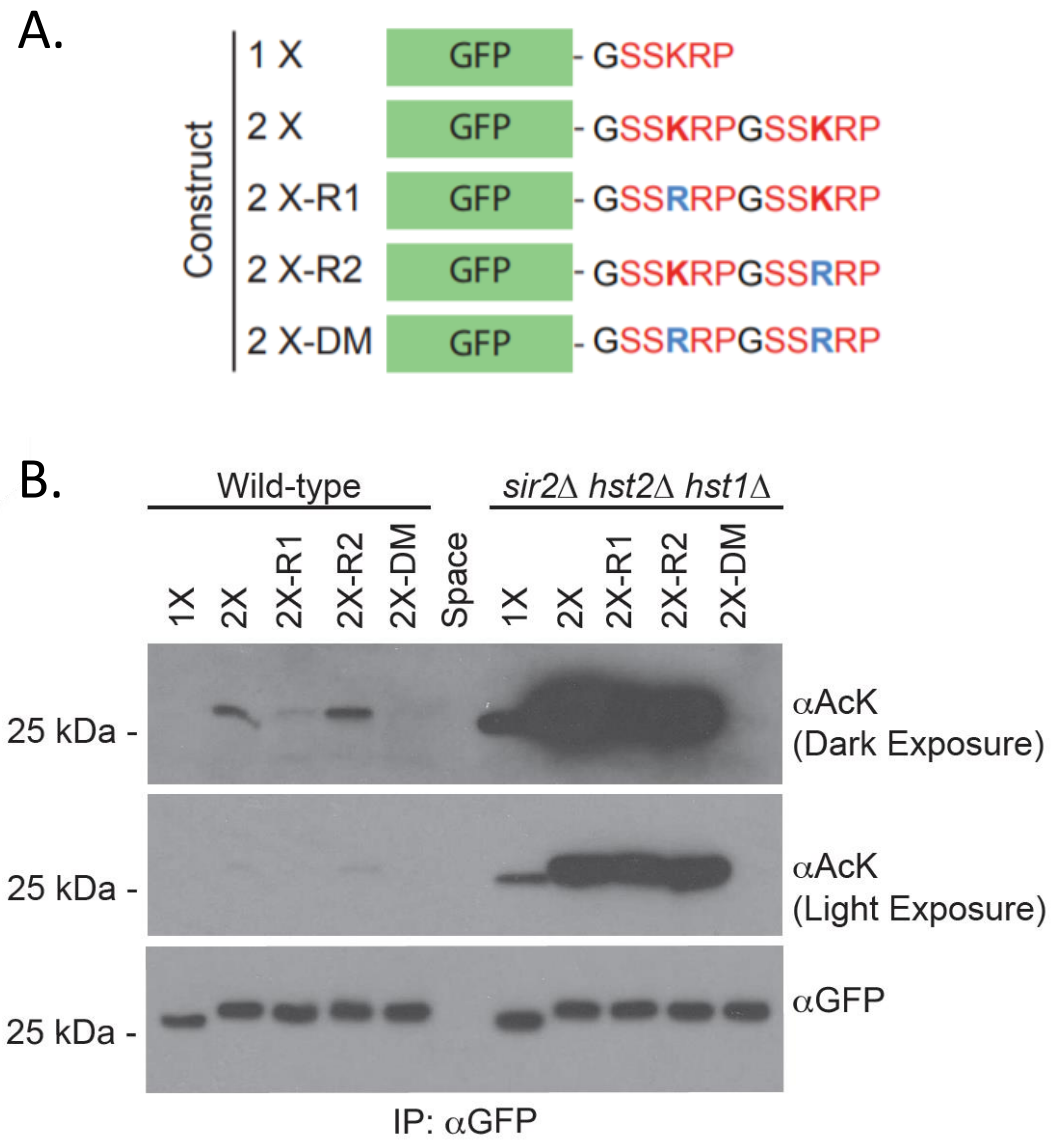

Figure 9. Proximal lysine residue of consensus tail is preferentially acetylated. A) Lysine to arginine mutant constructs were generated in the 2X construct length. **B)** constructs in **A** were transformed into either wild type or sirtuin mutant strain backgrounds, grown and harvested. Immunoprecipitated constructs were run on SDS-PAGE and transferred to PVDF prior to incubation with indicated antibodies. For αGFP, IP'ed samples were diluted 1/25 with SDS sample buffer supplemented with DTT prior to loading.

A second set of constructs were created to determine whether the individual lysine residues of the consensus peptide tail are equally acetylated. These new constructs were based on the 2X construct from the previous set. These variants introduced a lysine to arginine mutation in either the first/proximal (R1), second/distal (R2) or both (DM) consensus repeats (**Figure 9A**). In wild type cells, as observed previously, the 2X repeat construct showed increased acetyllysine signal compared to the 1X construct (**Figure 9B**). When the lysine of the first (N-terminal) peptide repeat is changed to arginine (R1), the signal decreases dramatically compared to the 2X construct (**Figure 9B**, lane 3), yet still more abundant than the 1X. Surprisingly, when the second (C-terminal) peptide's lysine is mutated (R2), there isn't a noticeable change in signal compared to the 2X construct (**Figure 9B**, lane 4), suggesting a model whereby the first/proximal consensus motif is preferentially acetylated. When both lysines of the 2X construct are mutated (DM), there is no acetylation signal (**Figure 9B**). However, when these same constructs are expressed in the sirtuin triple mutant cell background, all constructs are acetylated to the same extent (**Figure 9B**), indicating perhaps that the difference in signal between the R1 and R2 mutants in wild type cells is due to HDAC dynamics, preferring to deacetylate C-terminal lysine residues.

3.2.4 Gcn5 bromodomain is not required for consensus sequence acetylation

In yeast, multiple proteins make use of their bromodomain to bind acetylated lysines, typically on histone tails, to recruit other transcription factors or remodeling complexes to chromatin (Hassan et al. 2002; Shahbazian & Grunstein 2007). As mentioned previously, Gcn5 contains a bromodomain. This could potentially be used to recruit Gcn5 to target proteins and acetylate neighboring sites or proteins (Cieniewicz et al. 2014). This function might explain how despite seeing a preference to acetylate one residue over another in our X2 constructs, there were still multiple acetylations identified via mass spectrometry detection. I therefore decided to

examine how the Gcn5 bromodomain might be impacting how Gcn5 acetylates our 3X consensus repeat peptide on GFP. To do so, I generated a strain in which I introduced a C-terminus truncation to Gcn5 (Δ329-439) in addition to a 3Flag tag. A control Gcn5-3Flag strain was also generated. These strains were then transformed with our synthetic substrate and assayed via IP/Western. Surprisingly, loss of the Gcn5 bromodomain led to a slight increase in acetylation compared to its matched controls (**Figure 10**). This might be explained however by an observed increase in Gcn5 levels when the bromodomain was deleted, as seen when probing with αGcn5 antibody (**Figure 10**, Inputs).

3.2.5 Gcn5 cannot efficiently acetylate the synthetic substrate *in vitro*

Despite having demonstrated that acetylation of the synthetic construct is regulated by Gcn5 and sirtuins, I have yet to show that this activity is direct. To test that Gcn5 directly acetylates the proposed consensus motif, I performed an *in vitro* HAT assay using IP'ed construct from yeast and purified recombinant wild type or catalytic deficient His-tagged Gcn5 from bacteria (see methods). As a control for the activity of the purified enzymes, human recombinant histone H3.3 was also used paired with a pan-acetyllysine antibody for appropriate detection. Western blot analysis shows no acetyllysine signal with either the wild type or mutant Gcn5 on the synthetic substrate (**Figure 11A**). The GFP control lanes also exhibited no acetyllysine signal regardless of enzyme used (**Figure 11A**). When incubated with H3.3, wild type but not the mutant Gcn5 successfully acetylated its target (**Figure 11B**). This indicates that Gcn5 has difficulty acetylating certain substrates on its own, leading us to believe that it depends on other members of the SAGA complex for targeting.

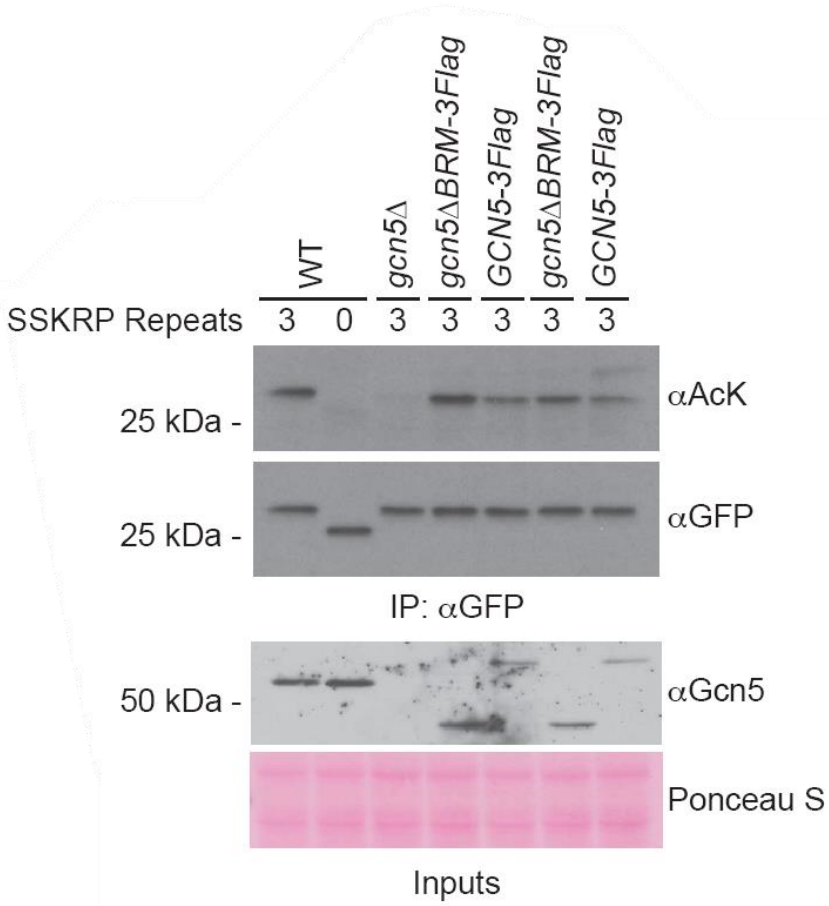

Figure 10. Gcn5 bromodomain is not required for consensus targeting. Indicated mutants expressing the synthetic substrate were grown in SC-URA media and harvested during log phase growth, and subsequently lysed. Proteins were immunoprecipitated using anti-GFP. Samples and inputs were run on SDS-PAGE, followed by transfer to PVDF. Membranes were probed with indicated antibodies. For αGFP, IP'ed samples were diluted 1/25 with SDS sample buffer supplemented with DTT prior to loading.

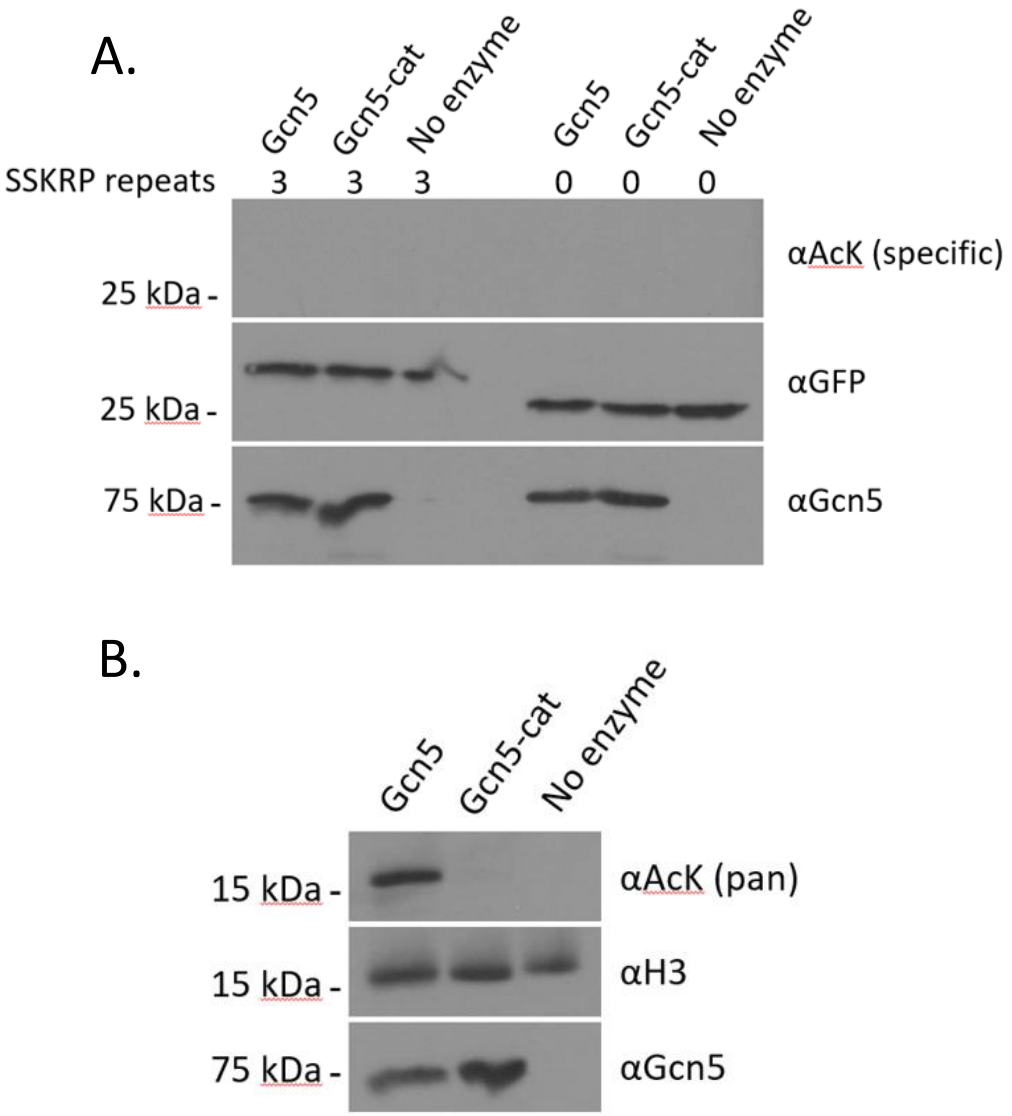

Figure 11. Gcn5 cannot acetylate the synthetic substrate *in-vitro*. A) *In-vitro* acetylation assay was performed with Synthetic substrate and GFP control immunoprecipitated from *gcn5Δ* cells and incubated with recombinant Gcn5, either wild type or a catalytic deficient mutant form (Gcn5-cat), and acetyl-CoA. Samples were run on SDS-PAGE and transferred to PVDF before incubating with indicated antibodies (SSKRP indicates site-specific antibody). **B)** Same as **A**, but with recombinant H3.3 instead of IP'ed substrate. Note: **A** and **B** were cropped from the same blots when same antibodies are used.

3.3 Using the consensus sequence as a tool to evaluate SAGA contribution to substrate targeting

Having demonstrated that Gcn5 and sirtuins can target substrates based on the inclusion of a consensus sequence, the synthetic substrate was next used as a tool to identify regulators of Gcn5 substrate selection. The previous result has shown that Gcn5 on its own cannot efficiently acetylate the synthetic substrate *in vitro*, despite obvious regulation *in vivo*. This then begs the question of what was missing.

3.3.1 SAGA HAT module is important to substrate selection

As mentioned previously, Gcn5 exists in the context of larger protein complexes. In yeast, the most prevalent and best studied is SAGA. As described, the members of these complexes each have specific roles in the complex, and disruption of this complex can lead to misregulation of certain transcriptional events (Wang & Dent 2014). Thus, it is expected that disruption of the SAGA complex can lead to misregulation of non-histone acetylation. One well described example is that of ribosomal protein transcription factor Ifh1, whose acetylation is dependent on Spt7 being intact, and properly assembled into the SAGA complex (Downey et al. 2013). Knowing that Gcn5 has difficulty acetylating our synthetic substrate on its own, I next sought out to determine whether other members of the SAGA complex are required for targeting the consensus motif. From here on out, the synthetic substrate will be used exclusively as a tool to assay the contribution of the non-catalytic members of the SAGA complex.

In this case, I developed mutant strains for individual members of the SAGA complex, as well as *AHC1* and *AHC2*, the unique members of the ADA complex. I also included *CHD1*, a chromatin remodeler that is often associated to the SAGA complex (Pray-Grant et al. 2005; Flanagan et al.

2005; Tran et al. 2000), which binds methylated H3K4, but its inclusion to the complex is uncertain due to its chromatin binding function being debated (Sims et al. 2005; Okuda et al. 2007; Biswas et al. 2007). I then transformed the synthetic substrate into these strains and assayed them via IP/Western blot. The mutants were grouped according to their function in the complex (**Figure 12**). From the DUB module, only *sgf73*Δ showed a slight decrease in substrate acetylation relative to wild type, though this can be explained by a similar decrease in loading (**Figure 12A**). There was also a strong decrease in acetylation in the *spt7*Δ, but there too there is a significant decrease in protein recovered (**Figure 12A**). It is possible that certain mutations lead to disruptions in expression of the construct, as discussed further below. Finally, in the HAT submodule, there is a complete loss in acetylation signal in the *gcn5*Δ as before (**Figure 12B**). There is also a complete loss in *ada2*Δ and *ada3*Δ mutants (**Figure 12B**). Altogether, this data suggests that members of the HAT submodule are the strongest regulators of Gcn5 substrate acetylation, identifying Ada3 as a potential candidate for follow up.

3.3.2 Loss of *ADA3* inhibits recruitment of Gcn5 to the SAGA complex

As Ada2 has already been shown to impact Gcn5 substrate selection (Downey et al. 2015), I decided to turn my focus exclusively on Ada3. Firstly, it was important to determine whether Ada3 also functions similarly to Ada2, wherein it is required for the proper assembly of the SAGA complex. Two separate co-IPs were performed to demonstrate this. The first aimed to determine whether Gcn5 was incorporated into the SAGA complex, which will be observed via co-precipitation with Spt7, a key scaffolding element of the complex; and Sgf73, member of the DUB submodule. What we see here is that as predicted, when immunoprecipitating Sgf73-GFP, Gcn5 and Spt7 are both detected in the sample suggesting the complex is intact. However, when performing this same test in *ada3*Δ mutant cells, the interaction between Gcn5 and Sgf73 disappears (**Figure 13A**) and only Spt7 co-precipitates. Similarly, a second co-IP was

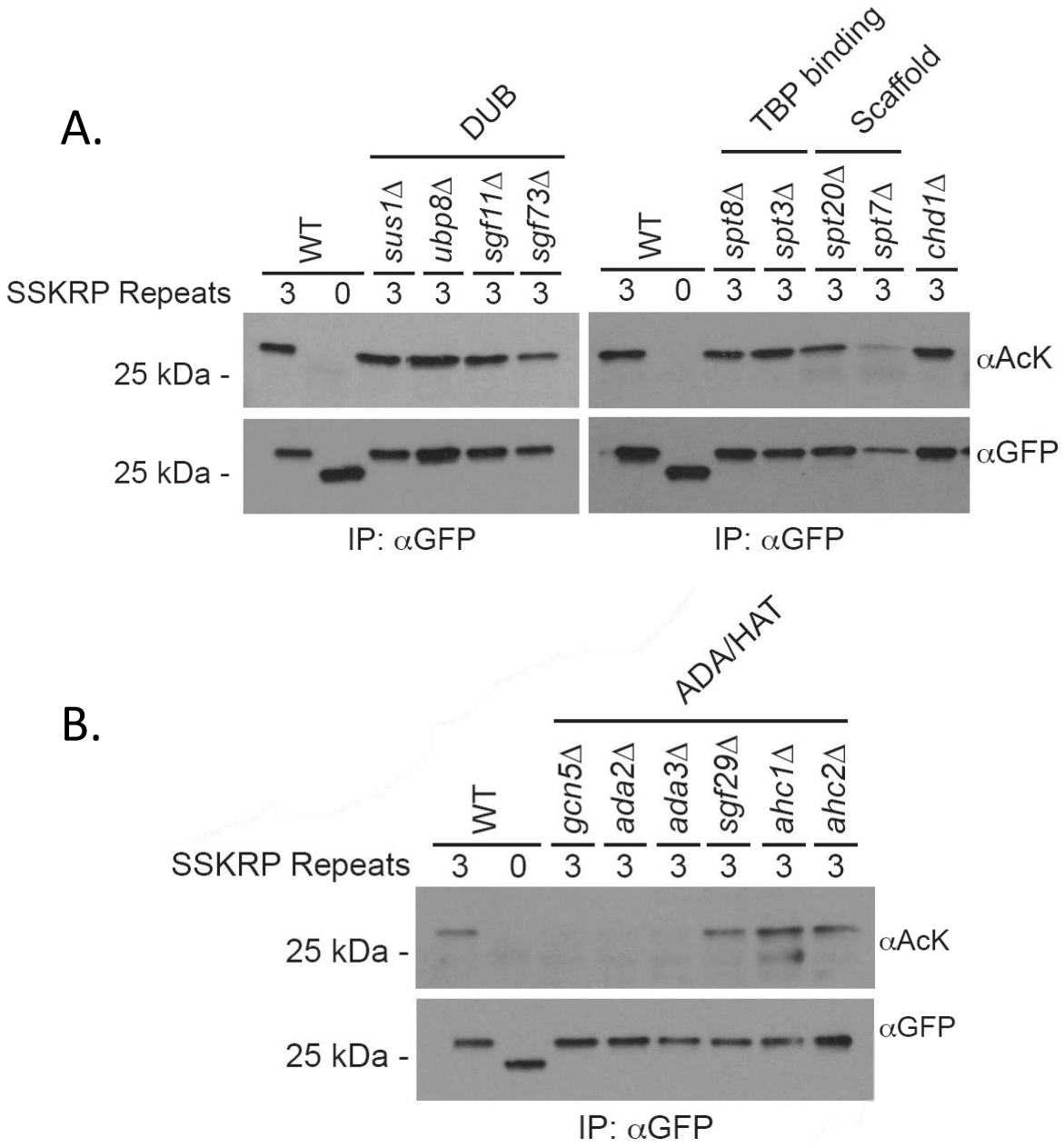

Figure 12. Ada3 is required for Gcn5 consensus targeting. A) & B) The indicated mutants were generated and transformed with the synthetic substrate and assayed via IP/Western blot. Cells were grown and harvested prior to lysis and immunoprecipitation. Samples were separated by SDS-PAGE and transferred to PVDF. Membranes were incubated with indicated antibodies. For αGFP, IP'ed samples were diluted 1/25 with SDS sample buffer supplemented with DTT prior to loading.

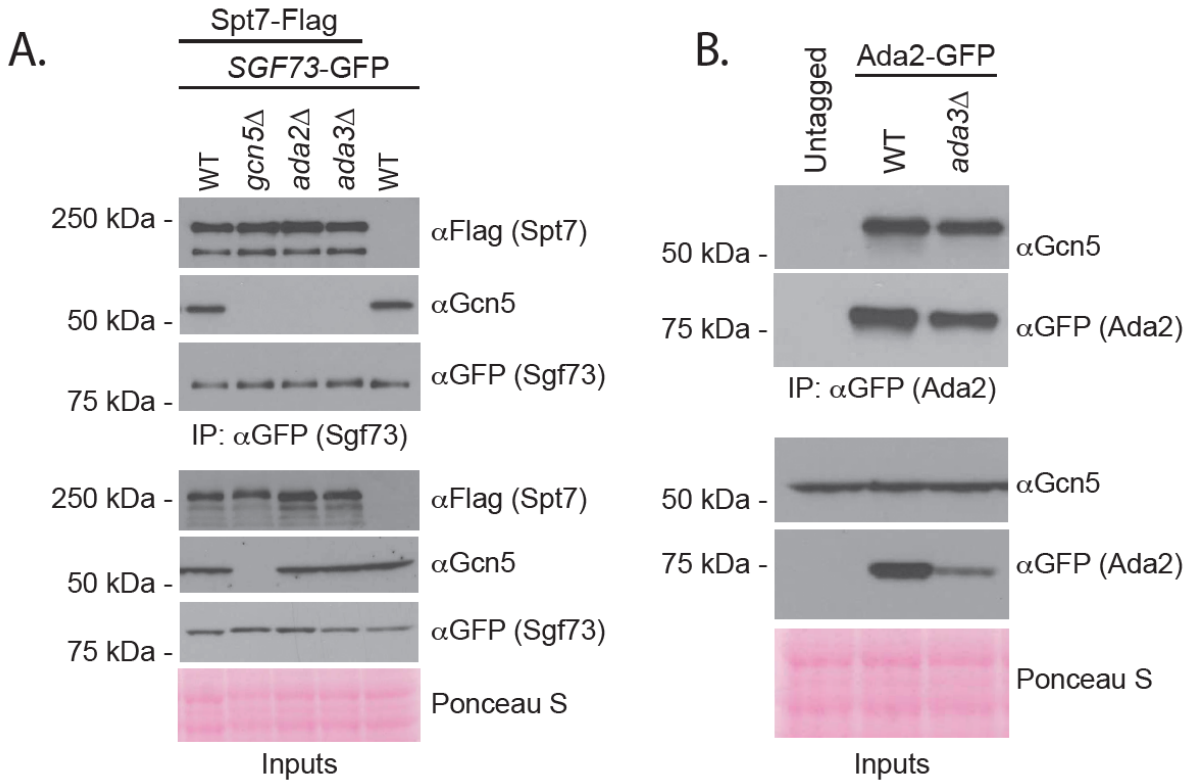

Figure 13. Ada3 regulates Gcn5 interaction with the SAGA complex. A) Sgf73-GFP was immunoprecipitated from the indicated strains and samples were separated by SDS-PAGE. Interactions were determined via detection by indicated antibodies. Inputs confirm presence of protein in cell lysates. Top panels are IP'ed samples and bottom are inputs. **B)** Ada2-GFP was immunoprecipitated as in **A**. Top panels are IP'ed samples and bottom are inputs.

performed to determine whether the interaction between Gcn5 and Ada2 are at all impacted by Ada3. In this case, *ada3*Δ did not affect Gcn5 recruitment to Ada2 (**Figure 13B**). This tells us that the SAGA complex assembly is disrupted and could thus explain an impact on substrate targeting as well as affect other important functions such as transcription. However, these results also indicate that Gcn5 could still be functional in the context of a dimeric group with Ada2, as is discussed further below.

3.3.3 Acetylome profiling identifies novel Ada3 dependent substrates

As Ada3 has now been shown to be a regulator of Gcn5 substrate targeting using our synthetic substrate, and that it is required for proper incorporation to the SAGA complex, the next step was to determine what its relevance is to *in-vivo* substrate targeting. To do so, a set of large scale acetylome profiling screens were performed using wild type cells compared to *ada3Δ* mutants (Rossl et al. 2018). In this case, I decided to use a SILAC mass spectrometry-based approach in order to identify Ada3 dependent acetylation sites. This consisted of harvesting wild type cells grown in media containing heavy isotope labelled lysine (13C6, 15N2) and combining it with equal amount of *ada3Δ* cells grown in regular media, or vice versa (see methods). The generated cell lysates containing a mix of labelled and non-labelled proteins from the respective cell backgrounds are then digested with trypsin to generate peptides, and this peptide pool is subsequently immunoprecipitated using an anti-acetyllysine antibody. In this case, what remains is a collection of acetylated peptides which can be differentiated via mass spectrometry. Using this technique, the mass spectrometer can determine from which cell background (wild type or *ada3Δ*) each acetylated peptide originates, and create ratios based on relative quantity to see how the mutant affects the acetylation of each detected peptide.

Over 2 independent experiments, with each a reverse labelled control and technical duplicate runs, the acetylome profiling was able to detect ratios for 548 acetylated peptides (Rossl et al. 2018), with 38 of these sites showing at least 2-fold downregulation. Unsurprisingly, certain sites were already identified as Gcn5 targets, strengthening the idea of Ada3 as a regulator of substrate selection, but 11 of these sites were in fact novel Ada3 dependent acetylations. The proteins identified as downregulated sites were then analyzed for any overlap in cellular function. GO-term analysis revealed that these proteins so happen to be predominantly involved in translation and chromatin related processes (**Tables 2 & 3**), similar to what was previously identified for Gcn5 targets. What next stands out is the larger number of at least 2-fold upregulated peptides identified, in this case 88 independent sites. Again, there was overlap in previously identified sites, with 21 being newly identified Ada3 dependent sites. GO-term analysis however showed enrichment for proteins involved in glycolysis and gluconeogenesis (**Tables 2 & 3**), which was again unexpected. Altogether, these data support an important role for Ada3 in substrate selection and proper cellular homeostasis.

Table 3. "Biological process" GO-term analysis of Ada3 regulated sites.

Acetylation status	GO-term "Biological process"	Corrected p-value
UP in *ada3*Δ ≥2-fold	Glycolytic process	$1.5 * 10^{-12}$
	Gluconeogenesis	$5.9 * 10^{-8}$
DOWN in *ada3*Δ ≥2-fold	Cytoplasmic translation	$1.5 * 10^{-7}$
	Nucleosome assembly	$6.5 * 10^{-6}$
	Translation	$1.1 * 10^{-5}$
	Chromatin assembly (or disassembly)	$2.7 * 10^{-2}$

Table 4. "Cellular component" GO-term analysis of Ada3 regulated sites.

Acetylation status	GO-term "Cellular component"	Corrected p-value
UP in *ada3*Δ ≥2-fold	Cytoplasm	$2.4 * 10^{-5}$
	Cytosol	$1.8 * 10^{-4}$
	Plasma membrane	$8.5 * 10^{-4}$
DOWN in *ada3*Δ ≥2-fold	Cytosolic large ribosomal subunit complex	$1.0 * 10^{-6}$
	Intracellular ribonucleoprotein	$1.9 * 10^{-6}$
	Ribosome	$2.0 * 10^{-6}$
	Intracellular	$2.1 * 10^{-6}$
	Nuclear nucleosome	$1.5 * 10^{-5}$
	Replication for protection complex	$4.2 * 10^{-4}$
	Chromosome	$3.4 * 10^{-3}$
	Large Ribosomal subunit	$5.5 * 10^{-3}$

4. Discussion

Very little is known about how HAT enzymes selectively choose their non-histone targets for acetylation. Most work done up until recently has aimed to determine specific function of non-histone acetylation events on a site per site basis (Downey & Baetz 2015). Previous work from our group proposed a target consensus sequence to be regulated by the yeast Gcn5 and sirtuin HDACs (Downey et al. 2015). In this study, I sought out to determine whether this proposed consensus site was specifically targeted by Gcn5 and sirtuins and used it to identify Ada3 as a regulator of Gcn5 substrate selection.

4.1 Evaluating the relevance of the consensus sequence *in vivo*

Gcn5 is an acetyltransferase enzyme commonly residing in larger multi-subunit complexes, such as SAGA. It has been previously shown that the other members of the SAGA complex play important roles in gene transcription through various means, such as site recognition and machinery recruitment (Bhaumik & Green 2002; Downey et al. 2013; Balasubramanian et al. 2002). More recently, it has been shown that certain members of the SAGA complex can affect Gcn5 substrate targeting and regulate both histone and non-histone substrate acetylations. To learn more about which of these subunits are important to Gcn5 substrate selection, I made use of a proposed Gcn5-specific consensus sequence S-X-K(ac)-K/R-P (Downey et al. 2015). This consensus sequence was developed using what were deemed high confidence targets of Gcn5 identified via mass spectrometry (≥10-fold regulation) and differed significantly from what had been proposed previously (Rojas et al. 1999). Interestingly the proposed consensus sequence showed significant overlap with one generated for sirtuin HDACs (**Figure 1**) (Downey et al. 2015), and thus an opposite regulation between the HAT and HDACs should be present on proteins containing this same site. This consensus

sequence was then coupled to a GFP fusion protein to create a synthetic substrate that should be directly regulated by Gcn5. The chosen S-S-K(ac)-R-P motif was selected as these amino acids represented the residues most frequent to inhabit their respective positions.

My first efforts went into determining whether the consensus motif could be used to assay Gcn5 activity and predict novel substrates on its own. As such, using Blastp, Spt2 was identified in having the consensus motif, and I successfully showed that it is acetylated at its consensus motif site in a Gcn5 and sirtuin dependent manner (**Figure 3**). Interestingly, Spt2 is an already known target of Gcn5, having been observed when the function of Gcn5 was still being characterized (Pollard & Peterson 1997), and again more recently in a larger screen (Downey et al. 2015), however not at the K166 site identified in this work. I generated a mutant in which the consensus motif was mutated and cannot be acetylated. Now that this construct is made, it can be used to determine the relevance of this site, and whether it is important for Spt2 function, as discussed in future directions below.

As shown in the results section, 4 proteins in *S. cerevisiae* were found to contain the S-S-K-R-P sequence motif, however I was unable to detect acetylation on the remaining 3; Afr1, Far10 and Ydr249c. To combat this, after successfully generating tagged *FAR10*-GFP and *YDR249C*-GFP strains, I made use of the GAL1 inducible promoter system (Longtine et al. 1998) to force the cells to overexpress the *FAR10* or *YDR249C* genes and produce a much larger quantity of protein. The goal of this was to produce enough protein to be able to at minimum match the amount of Spt2 that could be IP'ed, and hopefully increase our chances of observing acetylation via IP/Western. Despite the significant increase in protein levels, there still wasn't any detection by our antibody (**Figure 4**).

As mentioned previously, Far10 is a member of the Far complex, which is found in the endoplasmic reticulum (Pracheil & Liu 2013). Ydr249c, though still uncharacterized, has been shown to physically interact with other cytoplasmic proteins such as Whi3 and Dhh1 (Colomina et al. 2008; Holmes et al. 2013; Miller et al. 2017). This in turn shows that both proteins are largely cytoplasmic whereas Spt2, and Gcn5 alike, are primarily found in the nucleus. In this scenario, it is not expected for Gcn5 and these other two proteins to interact *in vivo* and acetylate them to the point where it is detectable via western blotting, even when the proposed targets are overexpressed.

A new search using Blastp, this time using variations of the core motif, identified a new list of 14 additional proteins to assay similarly to Spt2 and the others. Prioritization was given to proteins that haven't previously been identified in literature as being regulated by either Gcn5 or sirtuins. For example, Nab3 is one of the high confidence targets of Gcn5 (Downey et al. 2015) albeit at a different site than identified here. Of the selected proteins, only Smc6 is also localized to the nucleus like Gcn5, and despite this, no acetylation was observed on any of the candidates, regardless of nicotinamide treatment. In favor of the non-nuclear proteins not being acetylated, localization can once again be attributed to the lack of regulation seen. However as for Smc6, it is possible that the GFP fusion protein was both insoluble during the IP and not functional (Roy & D'Amours 2011) and thus its localization to chromatin and association to the SMC5-SMC6 complex was inhibited. In addition to this, during the development of the antibody, different forms of the consensus motif were used to inoculate the hosts and as such the antibody should recognize these same variations (Rossl et al. 2018), but in our hands have only been able to demonstrate its recognition of the S-S-K(ac)-R-P motif due to this being the core of the synthetic substrate. As such, it is difficult to determine whether the lack of acetylation is due to lack of Gcn5 and sirtuin regulation or lack of detection via antibody.

Additionally, an overall limitation of this screen is the location of the consensus motif itself within the protein. If the consensus sequence resides within a folded or otherwise non-exposed section of the protein, neither Gcn5 nor sirtuins will be able to access the site for regulation. This screen depends on ready access to the site for the enzymes.

Once I had confirmed that acetylation of the consensus sequence is regulated by Gcn5 and sirtuins alike, the goal was to develop a generic substrate that would be targeted by Gcn5 and no other acetyltransferase. I did so by generating a GFP fusion protein coupled to a peptide tail containing 3 repeats of the consensus motif. This construct was then shown to be regulated both by sirtuins and Gcn5 *in vivo* and was easily detectable via IP/Western blot analysis. To best detect the acetylation on the synthetic substrate, we collaborated with BioLegend to develop a site-specific antibody that will detect the acetylated form of our consensus motif (Rossl et al. 2018). In developing the antibody, varying forms of the consensus motif were used in the animal inoculations to ensure that it can target not only the perfect motif as described by me, but versions where certain residues are replaced by their frequent alternatives. Mass spectrometry analysis of the synthetic substrate confirmed that two of the three consensus motif repeats were being acetylated. Although this same analysis showed other sites on GFP to be acetylated, our GFP control construct showed that the signal observed via Western blotting was specific to the consensus motif sites.

I next developed different forms of the synthetic substrate that were used to better understand the dynamics of its targeting by Gcn5. As seen in **Figure 8**, the strongest acetylation is observed in the 3X consensus construct. However, when using the lysine to arginine mutants, which retain the same charge but cannot be acetylated, we see that Gcn5 has difficulty acetylating the more distal site. This may suggest a preference for Gcn5 to acetylate sites that aren't at the C-terminus of proteins. This model is also supported by the previous mass

spectrometry analysis, which did not return the identification of the final site as being acetylated. Surely there should have been the identification of at least that final site acetylated on its own and the first two not. Understandably, trypsin digestion leads to peptides cut only after unmodified lysines and arginines, however if a form existed where only the final (c-terminal) lysine is modified, it should have been identified by the machine.

This could potentially explain why there is very little regulation in the 1X construct, as only one site is present and could be a weaker candidate for Gcn5 regulation. In conjunction to this, as described above, though the deletion of the Gcn5 bromodomain did not show any impact on substrate acetylation, it might still impact the dynamics of how Gcn5 acetylates each individual site in the consensus repeats. A limitation of our system is that we are unable to accurately determine which proportion of the IP'ed protein have all three consensus repeats acetylated, versus having just one or two. It is possible that when the bromodomain is present, Gcn5 is able to acetylate all 3 of the consensus repeats on each protein one after the other, by having the bromodomain bind each consecutive site before acetylating the next (Cieniewicz et al. 2014). However, when the bromodomain is absent, it is possible that there is a larger proportion of the synthetic substrate with only one of three sites acetylated, explained by a decrease in other bromodomain dependent acetylation events (Li & Shogren-Knaak 2009; Syntlchakl et al. 2000). Despite this, my results point toward a model wherein the Gcn5 bromodomain is not required for the acetylation of the synthetic substrate and the proposed consensus sequence. It otherwise leans toward a negative regulation model where its loss leads to an increase in our substrate's acetylation (barring the increase in Gcn5 recovered). However, this may also be explained by a role of the bromodomain to act *in trans*, where it allows for Gcn5 to bind to one protein but acetylate a neighboring one (Li & Shogren-Knaak 2009). In this case, it could be the bromodomain allows for Gcn5 to bind GFP and acetylate nearby proteins,

and when lost, we might see a decrease in other acetylation events in exchange for a minor increase in our substrate's acetylation.

The SAGA complex is most commonly found to regulate transcription of stress related genes (Huisinga & Pugh 2004). As such, in response to certain stresses, Gcn5 is sequestered from certain substrates to ensure the transcription of stress related genes are in order. Previously, it was determined that Gcn5 dependent acetylation of transcription factor Ifh1 will decrease in response to rapamycin treatment (Downey et al. 2013), which triggers nutrient starvation pathways to activate through inhibition of Tor1, a key regulator of intracellular signaling pathways (Hardwick et al. 1999; Beck & Hall 1999; Martin & Hall 2005). I wanted to see if this same effect of decreased non-histone substrate acetylation was true for other targets, in this case, the synthetic substrate. As expected there appeared to be a decrease in acetylation of our synthetic substrate in response to rapamycin treatment, in line with previous findings and confirming our synthetic substrate is treated like other Gcn5 targets.

When trying to determine if the acetylation of the synthetic substrate by Gcn5 is direct, the *in vitro* assay showed no acetylation was present. Considering my hypothesis is that Gcn5 substrate selection is driven by other members of the SAGA complex, this result is consistent with my hypothesis by showing decreased acetylation of its consensus sequence when on its own. This is consistent with previous work on both histones and non-histone substrates (Galdieri et al. 2014; Downey et al. 2013).

What could be viewed as a major limitation to the study is how representative the synthetic substrate is to all Gcn5 substrates. Though I was able to identify a novel site on Spt2 as being regulated by both Gcn5 and sirtuins, it stands to show that all the remaining targets were not shown to be regulated in the same manner. Whilst these can be explained by cellular

localization as discussed, it remains a question whether the proposed consensus sequence itself is truly important for substrate selection. The data suggests that it can be used to direct acetylation by Gcn5 assuming the likelihood of interaction is present suggesting that it is at least targeted and has some relevance *in vivo*. That said, as for using the consensus sequence to develop a synthetic substrate is of another question. At this step, its relevance isn't as important, but more so whether it can be targeted or not. This study has shown that Gcn5 can target the synthetic substrate based on sequence alone. This construct is being used as a tool to better understand how the remaining SAGA subunits affect Gcn5 substrate selection, and not to predict novel substrates. The strength of this tool lies in how it can be used to very quickly and qualitatively see any changes in relative acetylation levels, reflective of Gcn5's ability to target its substrates. Through its use, we can characterize new regulators of Gcn5 substrate selection and use that to expand our knowledge of Gcn5 substrate targeting.

With this in mind, another question is raised regarding the relevance of the consensus sequence to other organisms, namely humans. Considering the degree of homology between the two's orthologs of Gcn5, one could assume that certain characteristics of the consensus sequence could carry over to humans. However, as mentioned, humans express two separate HATs that can be incorporated into the SAGA homologs, hGCN5 and PCAF (Nagy & Tora 2007; Jin et al. 2011; Yang et al. 1996; Yamauchi et al. 2000). Despite their similarities, the two HATs result in the complexes to be recruited to distinct targets. As such, already we have 2 separate proteins in humans with their own distinct sets of targets in addition to multiple complexes each. It is possible some targeting information found in yeast can be carried over for at least hGCN5 due to the higher degree of homology with the yeast Gcn5, however it is fully expected that each of the human enzymes will have their own unique targeted consensus sequences,

influenced by their surrounding proteins. In this regard, the best knowledge that can be shared between the organisms is the well-defined roles of other subunits to substrate targeting.

4.2 Identifying Ada3 as a regulator of Gcn5 substrate selection

Mutant strains for each of the viable SAGA subunit deletions were generated. Unfortunately, these did not include any of the *TAF* genes. Additionally, Ada1 and Tra1 were not mutated as they form important structural elements to the complex which otherwise stops its formation all together when deleted (Sterner et al. 1999). In any case, when the remaining subunits were deleted, the most striking impact was seen in the *ada3*Δ which mirrored the complete loss seen in both *gcn5*Δ and *ada2*Δ (**Figure 12**). This result wasn't exactly surprising. Ada3 is typically associated with both Gcn5 and Ada2, however most work has placed Ada2 as a more important regulator of Gcn5 due to the fact that they interact physically. That said, Ada3 was shown previously to be important for expanding Gcn5 nucleosome targets (Balasubramanian et al. 2002), and more recently also important for regulation of cancer related genes in humans (Chand et al. 2016; Germaniuk-Kurowska et al. 2007). Again, supporting the usefulness of this tool, I was able to identify Ada3 through its complete loss in signal on our substrate, but even more so by the fact that we can observe very minor changes relative to wild type acetylation levels of our substrate, highlighting other subunits which may impact substrate selection, albeit to a lesser degree. This data could then be used to prioritize future experiments where we investigate the contribution of the remaining members of the SAGA complex. One limitation to my synthetic substrate is the use the *ADH1* promoter on the plasmid upstream of the construct. Previous work has demonstrated that transcription of the *ADH1* gene is regulated in part by SAGA, specifically by the SPT module, which is required for the recruitment of TBP to its promoter (Bhaumik & Green 2002). Despite this, no significant

decrease in protein recovered was seen in any of the mutants except *spt7*Δ and thus we can conclude that the system is still valid.

Following this result, I wanted to test whether Ada3 was as important as Ada2 in Gcn5 recruitment to the SAGA complex. To test this, a set of Co-IPs were performed which confirmed that when Ada3 is deleted, Gcn5 is no longer incorporated into the SAGA complex, same as in *ada2*Δ. However, it did not disrupt the interaction between Gcn5 and Ada2. This can be explained by the fact that Gcn5 interacts directly with Ada2 when incorporated into its complexes. A potential follow-up experiment would be to test Gcn5's ability to interact with known partners when not in a complex on its own, or with only Ada2 bound.

With this confirmation, a set of SILAC based experiments were done to identify which acetylated sites are specifically regulated by Ada3, and compare their relative amounts to wild type. The expectations here were that by removing Ada3, any acetylation sites regulated by it should decrease, as it is no longer present to direct Gcn5 to its substrates. In contrast to one's expectations, out of a total 548 acetylated peptides, only 38 acetylations were at least 2-fold downregulated, whereas 88 were upregulated by at least 2-fold. This however was not as surprising as it appears. These results are somewhat in agreement with what was found previously with Ada2, where a portion (albeit smaller) of the identified sites were found to be upregulated in *ada2*Δ (Downey et al. 2015). Of these identified 38 and 88 down- and up-regulated sites, only 11 and 21 respectively were found to be novel acetylation sites. This shows a significant overlap with previously identified Gcn5 and Ada2 dependent sites (Downey et al. 2015), which strongly supports the idea of Ada3 as a regulator of Gcn5 dependent acetylation.

As for why the majority of the identified acetylated peptides were not impacted (≤2-fold), this is due to the fact that there are other HAT enzymes whose activities will go unaffected

by loss of Ada3, but for the sites that had previously been identified as Gcn5 targets, Ada3 is not the only regulator of Gcn5 acetylation. As our group has already characterized Ada2 as being a regulator of Gcn5 substrate selection, it is entirely possible that other members of the SAGA complex whose roles were not as dramatic or detectable via Western/IP from my previous screens are also important. As mentioned previously, Spt7 is required for Gcn5 dependent acetylation of Ifh1 (Downey et al. 2013), and observing my screen data, though the result wasn't as robust as it was for Ada3, there is a decrease in our synthetic substrate acetylation in the *spt7*Δ mutant (barring differences in loading).

One large limitation to our *ada3*Δ SILAC experiments is the low yield in peptides compared to what was previously obtained in the *gcn5*Δ and *ada2*Δ (Downey et al. 2015). This can be due to several reasons. First, in the early steps of sample preparation, during the IP stage where only the acetylated peptides are selected, I made use of a commercially available antibody coupled beads (see methods). This mixture of beads is coupled to a pan-acetyllysine antibody, which is comprised of a mixture of antibodies recognizing individual sites. This antibody can vary between lot numbers and can therefore affect which sites are enriched in the final analysis (Downey & Baetz 2015). Second, and equally important, the analysis was done by a different MS core facility. In such, a different mass spectrometer and model was used. The specificity of these machines varies depending on conditions and parameters used, and maintenance schedule. Though a more specific machine was supposedly used for the *ada3*Δ, it appears that a more accurate comparison between experiments could have been made otherwise.

A separate caveat in the SILAC experiments is due to a difference in strain backgrounds. In the original paper characterizing the SILAC-based acetylome profiling, the *gcn5*Δ and *ada2*Δ strains were developed in a strain background already mutated for sirtuins (*sir2*Δ*hst1*Δ*hst2*Δ)

(Downey et al. 2015). In this case, the relative acetylations would be exaggerated from wild type, leading to much greater ratios and more extreme effects from the mutations. This in turn could highlight proteins which are very minimally acetylated and overestimate their ratios relative to wild type. On the other hand, in my work the *ada3*Δ was not developed in the sirtuin mutant background and thus its possible some of our identified peptides that were deemed not affected (≤2-fold regulation) could have otherwise been considered sites regulated by Ada3. This in turn could have strengthened the overlap in already identified Gcn5 targets and novel Ada3 dependent sites.

Having highlighted both Ada2 and Ada3 as regulators of Gcn5 substrate selection, it begs the question of how it is they do so. Previous work has shown that the interactions of Ada2/3 with Gcn5 can increase it list of histone targets (Balasubramanian et al. 2002). When Gcn5 and Ada2 interact, the Gcn5 histone tail binding pocket is modified and increases its interactions (Balasubramanian et al. 2002). Similarly, when Ada3 is incorporated to the trimeric complex, it increases the binding abilities to nucleosomes, including DNA bound histone tails (Balasubramanian et al. 2002). This data could be applied to the new knowledge of non-histone substrate acetylation, wherein Ada2 and Ada3 increase Gcn5's ability to interact with its substrates through modifications of its binding sites and providing new binding sites across a complex of proteins. A caveat of this work was the exclusion of non-histone substrates as a platform for Gcn5 acetylation. Observing how Ada2 or Ada3 as a dimeric complex with Gcn5 impact non-histone substrates would potentially provide more insight to Gcn5 function. A benefit of the synthetic substrate is that it could be used as a tool to effectively test these same complexes towards non-histone substrates. Interestingly, the same work presented data in which when the whole SAGA complex is present versus just the trimeric Gcn5/Ada2/Ada3, there is a distinct and significant decrease in histone acetylation (Balasubramanian et al. 2002),

indicating an important role of the rest of the SAGA complex to histone selection, and now presumably to non-histone substrate acetylation.

As mentioned previously, Gcn5 along with Ada2/3 can exist in up to 3 complexes in yeast (and more in higher eukaryotes). It is possible that the majority of non-histone substrate acetylation is done by other complexes, such as the ADA complex, which is a much smaller version of the SAGA complex without the transcription coactivator functions. This supports a theory in which SAGA and SLIK are mostly reserved for transcription related events which a much greater degree of specificity and control, however with a small specific list of non-histone substrates. In turn, the smaller ADA complex truer to the trimeric Gcn5/Ada2/Ada3 may have a larger role in freely acetylating a much larger set of substrates.

4.3 Future directions

Identified earlier, a limit of this study was that of the 4 proteins found to contain the consensus sequence, only Spt2 was found to be regulated in a Gcn5 and sirtuin dependent manner. Using the K166R mutants generated, we can determine the function of Spt2 acetylation by Gcn5. To do so, a set of assays can be done to test whether Spt2 can still interact with the SWI/SNF complex and DNA (Pérez-Martín & Johnson 1998). We can also use the constructs to test whether the acetyl mark affects the protein's localization to different cellular compartments, as its been previously shown to be shuttled to the cytosol in response to certain stresses (Dastidar et al. 2012). I also proposed a scenario where Far10 and Ydr249c were not regulated by the HAT or HDACs due to their localization. A potential follow-up experiment would be to generate fusion proteins where each are tagged with GFP as before, as well as a nuclear localization signal. In this case, we would not be concerned about loss of function, but rather to determine solely if Gcn5 could then recognize the consensus motif and lead to their

acetylation. This experiment would fortify the conclusion that inclusion of a consensus sequence is enough to confer acetylation of an otherwise non-substrate. If this system works, and confirms the importance of the consensus motif *in vivo*, we could potentially use this same consensus motif as a tool to drive specific cellular functions.

As seen in this study, incubation of purified Gcn5 and the synthetic substrate was unable to induce its acetylation, despite Gcn5 being functional (see acetylation of control H3.3). This was in line with previous work done showing that Gcn5 requires the other members of the SAGA complex to efficiently acetylate its substrates (Galdieri et al. 2014; Downey et al. 2013). an interesting experiment would set out to determine which subunits of the SAGA complex at a bare minimum are required to observe acetylation of our synthetic substrate. In this case, a first set of experiments where Gcn5 was co-purified alongside Ada2 or Ada3 would be performed. This can be done as a large fusion protein expressing all three subunits, or individually and combined in vitro. Though Gcn5 exists in complex with the HAT submodule of SAGA at a minimum *in vivo*, I expect that only either Ada2 or Ada3 will be necessary to see acetylation of its substrates, though a different subset thereof. This same experiment could be repeated using purified histone H3 instead of the synthetic substrate to confirm the importance of just Ada2/Ada3, and to substantiate or clarify the original findings that Gcn5 requires the SAGA complex to hit all of its sites on H3 (Grant et al. 1997; Grant et al. 1999).

Another interesting assay to demonstrate direct Gcn5 regulation of its substrates, would be to conduct *in vitro* reactions with a number of the identified proteins detected in the SILAC experiments. These would be those that remained unchanged when Ada3 was deleted (≤2-fold regulated) and who overlapped with sites previously found to be Gcn5 targets. These can then be incubated with Gcn5 to see if the identified acetylated sites are direct targets for Gcn5 on its

own, or if another, not yet identified regulator is required for these sites. This would help expand the knowledge of Gcn5 regulators and incite the search for others.

Similarly, though I was unable to properly show direct regulation by Gcn5 on its own, the expectation is that the synthetic substrate would be acetylated once other members of the SAGA complex were present. To do so, an *in vitro* assay using purified Gcn5 alongside both Ada2 and Ada3 could be done. Similarly, these could be repeated with other members to see their contribution. A separate set of *in vitro* assays could aim to determine whether the regulation by sirtuins are direct. In this case, purified either Sir2, Hst1 or Hst2 would be incubated with our substrate in the presence or absence of NAD+ and nicotinamide. Preliminary work by our group (data not shown) suggests that Hst2 can directly deacetylate the synthetic substrate *in vitro*. Though we saw the strongest regulation with Hst2 in our previous experiments, it would be interesting to see to what degree the other two are deacetylating the synthetic substrate, as we know they must be to some extent by the dramatic increase in the triple mutant strain background. As such, this experiment would confirm the direct regulation by sirtuins at our consensus sequence.

Having identified Ada3 through the use of our synthetic substrate, and by very qualitative means, it would be interesting to see the importance of other non-catalytic subunits already proven to affect distinct substrates on general acetylation patterns. In this case, repeating the SILAC based acetylome profiling strains mutated for each of the remaining SAGA subunits, as well as Rtg2 from SLIK and Ahc1/2 from ADA. This could potentially highlight brand new non-histone substrates specific to each subunit, as well as fortify the idea that each complex that Gcn5 resides in act as substrate selection platforms as a whole.

Finally, one interesting follow-up experiment that could be done is to repeat the acetylome profiling, in the *ada2*Δ, *ada3*Δ, and *gcn5*Δ individually (all in the *sir2*Δ*hst1*Δ*hst2*Δ mutant background) using a new antibody during the IP process. In this case, the site-specific antibody. This would potentially highlight novel peptides that would have otherwise not been found by using the commercially available antibodies. Not only would this identify new targets but would confirm the findings in this study by potentially observing acetylations on any of the proteins expressing the consensus sequence, or loose variations thereof, that we could not detect via Western blotting.

4.4 Conclusion

I set out in this study to determine whether an identified consensus motif S-S-K(ac)-R-P is targeted by Gcn5 and sirtuins *in vivo*. Tethering this motif to a known non-substrate was enough to confer acetylation by Gcn5. This study is the first of its kind to propose a functioning consensus motif detected by Gcn5. I next used this synthetic substrate as a tool to determine whether certain members of the SAGA complex act to regulate Gcn5 substrate selection. Using the synthetic substrate as a tool, I identified Ada3 as a key regulator of Gcn5 substrates, and in turn identified novel acetylation sites regulated specifically by Ada3. In addition to this, I also uncovered a novel regulated site based on sequence alone. Overall, this suggests that other HAT enzymes may benefit from a similar system to identify substrate selection regulators.

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Appendix

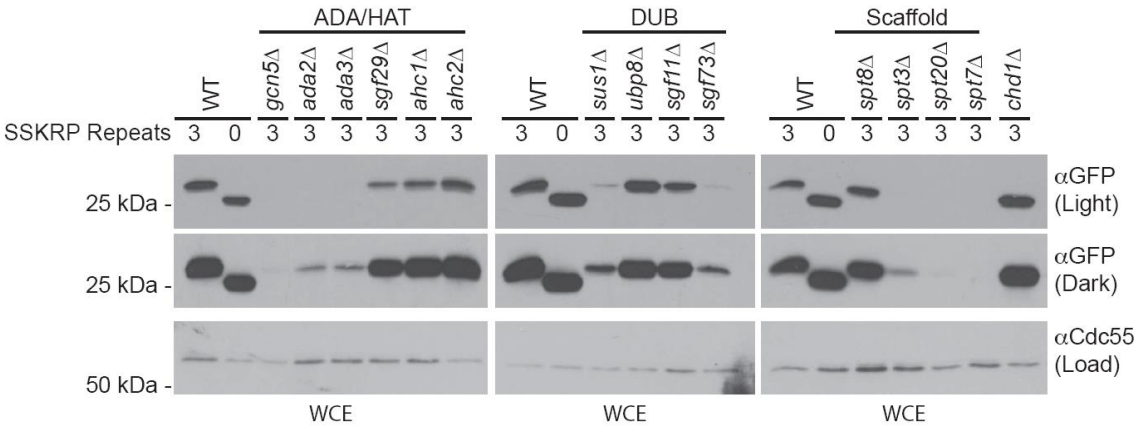

Figure S1. IP/Western blot sample input analysis. Whole cell extracts of SAGA subunit mutants saved prior to immunoprecipitation were separated via SDS-PAGE and transferred to PVDF. Membranes were probed with indicated antibodies. Light and dark exposures of αGFP were used.

Table 5. List of strains used in this study.

Strain Number	Background	Genotype	Source
YMD267	S288C BY47	MatB leu2 ura3 his3-1 met15 SGF73-GFP::HIS3MX	PMID: 25381059
YMD652	S288C BY47	MatB leu2 ura3 his3-1 LYS2+ met15 lys1::NATMX	PMID: 25381059
YMD837	S288C BY47	MATA leu2 ura3 his3 met15 SPT2-GFP::HIS3MX	This Study
YMD848	S288C BY47	MATA leu2 ura3 his3 met15 SPT2-GFP::HIS3MX gcn5::KANMX	This Study
YMD913	S288C BY47	MatB leu2 ura3 his3-1 met15 SGF73-GFP::HIS3MX SPT7-3Flag::HYGMXMX	PMID: 25381059
YMD914	S288C BY47	Mat a leu2 ura3 d his3-1 met15 SGF73-GFP::HIS3MX gcn5::KANMX SPT7-3Flag::HYGMXMX	PMID: 25381059
YMD915	S288C BY47	MatA leu2 ura3 his3-1 met15 SGF73-GFP::HIS3MX ada2::URA3 SPT7-3Flag::HYGMXMX	PMID: 25381059
YMD963	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+	Boone Lab via Rudner Lab
YMD1372	S288C BY47	MatB met LYS2+ his3-1 leu2 ura3 [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1374	S288C BY47	MatB met LYS2+ his3-1 leu2 ura3 [pRS316-ADH1-GFP-CYC1]	This Study
YMD1380	S288C BY47	MatB leu2 ura3 his3-1 LYS2+ met15 lys1::NATMX sir2::HYGMX hst1::G418 hst2::HIS3MX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1389	S288C BY47	MatB gcn5::KANMX his met15 LYS2+ leu2 [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1391	S288C BY47	MatB gcn5::KANMX his met15 LYS2+ leu2 [pRS316-ADH1-GFP-CYC1]	This Study
YMD1457	S288C BY47	Matx leu2 ura3 his3-1 met15 LYS2+ sir2::HYGMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1461	S288C BY47	MatB leu2 ura3 his3-1 hst2::HIS3MX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1465	S288C BY47	MatA leu2 ura3 his3-1 hst1::KANMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1468	S288C BY47	MatB leu2 ura3 his3-1 LYS2+ met15 sir2::HYG hst1::G418 hst2::HIS3MX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1476	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ ada2::KANMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1632	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ ngg1::KANMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1634	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ chd1::KANMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study

YMD1636	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ sus1::KANMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1638	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ spt8::KANMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1640	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ spt3::KANMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1642	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ ubp8::KANMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1720	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ sgf29::KANMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1722	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ sgf73::KANMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1724	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ spt7::KANMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1726	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ spt20::KANMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1744	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ ahc1::KANMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1748	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ ahc2::KANMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1842	S288C BY47	MatB leu2 ura3 his3-1 LYS2+ met15 lys1::NATMX ada3::KANMX	This Study
YMD1891	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ GCN5(Δ329-439)-3Flag::HYGMXMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1895	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ GCN5(Δ329-439)-3Flag::HYGMXMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1899	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ GCN5-3Flag::HYGMXMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1903	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ GCN5-3Flag::HYGMXMX [pRS316-ADH1-GFP-KKK-CYC1]	This Study
YMD1957	S288C BY47	MatB met15 LYS2+ his3-1 leu2 ura3 [pRS316-ADH1-GFP-K-CYC1]	This Study
YMD1959	S288C BY47	MatB met15 LYS2+ his3-1 leu2 ura3 [pRS316-ADH1-GFP-KK-CYC1]	This Study
YMD1964	S288C BY47	MatB leu2 ura3 his3-1 LYS2+ met15 lys1::NATMX sir2::HYGMX hst1::G418 hst2::HIS3MX [pRS316-ADH1-GFP-K-CYC1]	This Study
YMD1966	S288C BY47	MatB leu2 ura3 his3-1 LYS2+ met15 lys1::NATMX sir2::HYGMX hst1::G418 hst2::HIS3MX [pRS316-ADH1-GFP-KK-CYC1]	This Study
YMD2123	S288C BY47	MatB met15 LYS2+ his3-1 leu2 ura3 [pRS316-ADH1-GFP-RK-CYC1]	This Study
YMD2125	S288C BY47	MatB met15 LYS2+ his3-1 leu2 ura3 [pRS316-ADH1-GFP-KR-CYC1]	This Study

YMD2131	S288C BY47	MatB leu2 ura3 his3-1 LYS2+ met15 lys1::NATMX sir2::HYGMX hst1::G418 hst2::HIS3MX [pRS316-ADH1-GFP-RK-CYC1]	This Study
YMD2133	S288C BY47	MatB leu2 ura3 his3-1 LYS2+ met15 lys1::NATMX sir2::HYGMX hst1::G418 hst2::HIS3MX [pRS316-ADH1-GFP-KR-CYC1]	This Study
YMD2216	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ GAL1pr-YDR249C-GFP::HIS3MX	This Study
YMD2217	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ GAL1pr-FAR10-GFP::HIS3MX	This Study
YMD2232	S288C BY47	MatB met LYS2+ his3-1 leu2 ura3 [pRS316-ADH1-GFP-RR-CYC1]	This Study
YMD2240	S288C BY47	MatB leu2 ura3 his3-1 LYS2+ met15 lys1::NATMX sir2::HYGMX hst1::G418 hst2::HIS3MX [pRS316-ADH1-GFP-RR-CYC1]	This Study
YMD2252	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ spt2::KANMX [pRS316-SPT2-GFP]	This Study
YMD2254	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ spt2::KANMX [pRS316-spt2-K166R-GFP]	This Study
YMD2348	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ GYP1-GFP::HIS3MX	This Study
YMD2349	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ SMC6-GFP::HIS3MX	This Study
YMD2350	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ HMG1-GFP::HIS3MX	This Study
YMD2351	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ HSL7-GFP::HIS3MX	This Study
YMD2352	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ CAM1-GFP::HIS3MX	This Study
YMD2353	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ BUD3-GFP::HIS3MX	This Study
YMD2354	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ PPQ1-GFP::HIS3MX	This Study
YMD2355	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ ALG14-GFP::HIS3MX	This Study
YMD2402	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ ADA2-GFP::HIS3MX	This Study
YMD2403	S288C BY47	MatA leu2 ura3 his3-1 met15 LYS2+ ADA2-GFP::HIS3MX ada3::KANMX	This Study
YMD2405	S288C BY47	MatA leu2 ura3 his3-1 met15 SGF73-GFP::HIS3MX SPT7-3Flag::HYGMXMX ada3::KANMX	This Study

Table 6. List of antibodies used in this study.

Antibody	Company/Lab	Catalogue Number/PMID	Use	Dilution Range	Host	Buffer	Can re-use dilution?	Notes
anti-GFP Ab290	AbCam	Ab290	IP	N/A	Rabbit	5 % Milk TBST	Yes	
anti-AcK Agarose	Immunechem	ICP0388	IP	N/A	Rabbit	N/A	N/A	
anti-GFP JL8	Clontech	632381	WB	1:2000	Mouse	5 % Milk TBST	Yes	
anti-AcK	Cell Signaling	9441	WB	1:1000	Rabbit	5 % BSA TBST	Yes	
anti-Gcn5	B-Bridge	62-003	WB	1:2000	Rabbit	5 % BSA or Milk TBST	Yes	
anti-Flag	Sigma	F3165	WB	1:2000	Mouse	5 % Milk TBST	Yes	
anti-Mouse IgG (HRP)	BioRad	172-1011	WB	1:10,000	Goat	5 % Milk TBST	not tested	
anti-Rabbit IgG (HRP)	BioRad	170-6515	WB	1:10,000	Goat	5 % Milk TBST	not tested	
anti-AcK consensus	BioLegend	N/A	WB	1:2000	Mouse	5 % BSA TBST	not tested	custom Ab Clone #A1504708
anti-AcK consensus	BioLegend	N/A	WB	1:2000	Mouse	5 % BSA TBST	not tested	custom Ab Clone #A1504705