The *Saccharomyces cerevisae* acetyltransferase NuA4 Regulates Stress Granule Formation in Response to Glucose Deprivation

By

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ABSTRACT

When cells are exposed to stress conditions such as heat stress (HS) and glucose deprivation (GD), stress granules (SG) form in the cytoplasm that act to sequester and protect translationally stalled mRNAs from degradation. Presently it is poorly understood how the cell regulates the formation of SGs, however numerous post translational modifications (PTMs) have been detected on SG proteins in both yeast and man, including lysine acetylation. Disruption of lysine deacetylases (KDACs) has been reported to impact SG function in mammalian cells, by an unknown mechanism. Likewise, existing data indicates that the *S. cerevisiae* lysine acetyltransferase (KAT) NuA4 interacts with and acetylates several SG proteins *in-vitro*. In this work I demonstrate that NuA4 is required for GD SG assembly, and is involved in regulating SG formation in response to HS and NaN_3 treatment. Further, I show that NuA4 is not contributing to GD SG assembly through inhibition of translation initiation, regulation of the glucose sensing pathway Snf1/AMPK, or modulation of Pab1 protein levels. The identification of glucose dependent acetylation sites on Pab1 and the discovery that Pab1 *in-vivo* acetylation state is dependent on NuA4 implies that acetylation of SG proteins, and Pab1 in particular, maybe a key regulator of assembly. Interestingly, my studies reveal that KDACs inhibit SG formation in unstressed conditions. Collectively this work establishes a role for NuA4 in regulating SG assembly and suggests that lysine acetylation is playing a conserved and critical role in mRNA metabolism.

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List of Abbreviations:

+Glucose	Media Containing Glucose
-Glucose	Media Lacking Glucose
2µ	High copy plamid
Acetyl-CoA	Acetyl Coenzyme-A
Ac-K	Acetyl-Lysine
AD	Alzheimers Disease
43ALS	Amyotrophic Lateral Schlerosis
AMPK	AMP Kinase
BF	Bright Field
cAMP	Cyclic AMP
cDNA	Complementary deoxyribonucleic acid
CEN	Centromere(low copy plasmid)
CHX	Cyclehexamide
Co-IP	Co-immunoprecipitation
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
DTT	Dichlorodiphenyltrichloroethane
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EtOH	Ethanol
FITC	Fluorescein isothiocyanate
G6PDH	Glucose-6-phosphate dehydrogenase
GD	Glucose Deprivation
GFP	Green fluorescent protein
GNAT	Gcn5-related N-acetyltransferases
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
H4	Histone 4
HEPEs	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPR	Horseradish peroxidase
HS	Heat Shock
IgG	Immunoglobulin G
IP	Immunoprecipitation
IRES	Internal ribosome entry sites
KAT	Lysine acetyltransferase
KDAC	Lysine deacetylase
LC-MS/MS	Liquid chromatography–mass spectrometry
mCHIP	Modified chromatin Immunoprecipitation

mRNA	Messenger ribonucleic acid
MS	Mass Spectrometry
MYC	Myelocytomatosis oncogne
MYST	MOZ, Ybf2/Sas3, Sas2, and Tip60
NAD+	Nicotinamide adenine dinucleotide
NaN3	Sodium Azide
NLS	Nuclear localization sequence
OD_{260}	Optical Density at 260nm
OD_{600}	Optical Density at 600nm
PAGE	Polyacrylamide gel electrophoresis
PB	Processing Body
PCR	Polymerase chain reaction
PKA	Protein Kinase A
PTM	Post translational modification
qPCR	quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNP	Ribonucleoprotein Particle
RPM	Rotations per minute
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SC	Synthetic Complete
SD	Standard Deviation
SDS	Sodium monododecyl sulfate
SG	Stress Granule
SPG	Stationary-phase granule
TAP	Tandem affinity purification
TDH	Glyceraldehyde-3-phosphate dehydrogenase
TOR	Target of rapamycin
UV	Ultra-Violet
WCE	Whole cell extract
WT	Wild-type
YP	Yeast peptone
YPD	Yeast peptone dextrose

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1.0 Introduction

When eukaryotic cells are exposed to environmental stressors such as nutrient limitation, heat stress, or cytotoxic drugs they induce a series of regulatory mechanisms which promote energy conservation and survival. One mechanism by which this is accomplished is the rapid accumulation of proteins and mRNA into ribonucleoprotein (RNP) particles called stress granules (SG). SGs are associated with several cellular processes including microRNA (Leung 2006), cell signalling (Arimoto 2008, Takahara and Maeda 2012), and cellular transport (Dewey 2012). Interestingly, SGs are a hallmark of many neurodegenerative diseases (Ginsberg, Galvin et al. 1998, Nonhoff, Ralser et al. 2007, Ito and Suzuki 2011) and are linked with chemotherapy resistance in cancer (Arimoto 2008, Fournier, Gareau et al. 2010, Gareau, Fournier et al. 2011, Fournier, Coudert et al. 2013, Thedieck, Holzwarth et al. 2013). Despite the connection between SGs and diseases, a regulatory mechanism of SG formation remains unknown. SGs are highly dynamic structures whose rapid assembly could be regulated by an equally dynamic mechanism consisting of Post Translational Modifications (PTMs) including phosphorylation and acetylation. In fact, it has been established that phosphorylation of the translation initiation factor eIF2α is required for SG assembly (Anderson and Kedersha 2006). Likewise, existing literature indicates that the Lysine Deacetylases HDAC6 in mammals (Kwon, Zhang et al. 2007), and SIRT6 in yeast and mammals (Jedrusik-Bode, Studencka et al. 2013) localize to and affect SG assembly, by a yet to be discerned mechanism. A recent screen published by the Baetz lab identified that the *Saccharomyces cerevisiae* lysine acetyltransferase NuA4 co-purifies several SG proteins (Mitchell, Huard et al. 2013). In the following thesis I will be discussing the novel role of NuA4 in regulating SG formation upon glucose deprivation (GD).

1.2 Yeast

Saccharomyces cerevisiae, also known as baker's yeast, is a model organism that has been used to investigate a variety of cellular processes (reviewed in Yand and Seto 2007; Henry, Kohlwein et al 2012). In fact, studies in yeast have allowed scientists to understand and order the eukaryotic cell cycle (Costanzo, Nishikawa et al. 2004). *S. cerevisiae*, a eukaryote, is easily used as a model organism to study human disorders due to the small genome size, short generation time, ease of recombination, haploid or diploid chromosome number, and extensive homology with human cells. Compared with the approximately 40 000 genes found in humans, the yeast genome consists of a more manageable 6000 genes (Walberg 2000). Indeed the amount of genetic redundancy in yeast is very low, allowing genetic manipulations such as knock-outs to be performed relatively easily as compared to mammalian systems (Costanzo, Baryshnikova et al. 2011). For example, while one Poly A binding protein (Pab1) exists in yeast there are 3 cytoplasmic and an additional testes specific isoform in humans (Katzenellenbogen, Vliet-Gregg et al. 2010). In human cells, genetic redundancy buffers the effect of knock-outs since more than one gene often codes for a particular pathway. Furthermore, approximately twenty percent of the genes implicated in human diseases have homologous genes in yeast (Walberg 2000). Interestingly much of the early characterisation of lysine acetylation and the mRNA lifecycle was first performed using *Saccharomyces cerevisiae*. Here I harness the power of budding yeast to elucidate the role of NuA4 on stress granule formation.

1.3 KATs/KDACs, NuA4 and acetylation

Lysine Acetylation -A dynamic Posttranslational Modification (PTM)

Lysine acetylation is catalyzed by enzymatic complexes called lysine acetyltransferases (KATs), which transfer of an acetyl moiety from Acetyl coenzyme A (Acetyl-coA) onto the epsilon amino group of lysines (Polevoda and Sherman 2002). Conversely, Lysine Deacetylases (KDACs) are responsible for catalyzing the removal of acetyl-coA from the epsilon amino group of Lysine residues (Bernstein, Tong et al. 2000). Post-translational acetylation and deacetylation were first described on histone proteins whereby the addition or removal of the acetyl moiety regulates the conversion between heterochromatin and euchromatin (reviewed in(Oliver and Denu 2011, Peserico and Simone 2011, Van Opdenbosch, Favoreel et al. 2012). Due to their role in histone modification, KATs and KDACs were initially referred to as Histone Acetyltransferases (HATs) and Histone Deacetylases (HDACs) respectively.

The addition or removal of an acetyl moiety respectively abrogates or regenerates the positive charge of lysine residues effectively altering coulombic interactions. This impacts protein and cellular function through numerous molecular mechanisms including regulating protein-protein interactions, protein activity, protein stability and protein localization. Within the nucleus acetylation functions to neutralize the interaction between the positively charged lysine residues of histone tails and the negatively charged phosphate backbone of DNA allowing for the unwinding of DNA and increased polymerase accessibility (Peserico and Simone 2011). Additionally, acetylation is capable of promoting protein-protein interactions through bromodomains by creating binding sites. For example, in order for p53 mediated activation of apoptosis and quiescence, p53 must first be acetylated which allows for it to interact with the bromodomain of CBP (Mujtaba, He et al. 2004).

In addition to regulating cellular interactions acetylation is capable of regulating protein activity, stability, and localization. For example, the acetylation of the cyclin depdendent kinase Cdk1 is essential for yeast cell viability (Choudhary, Kumar et al. 2009). Likewise, acetylation of several enzymes involved in cellular metabolism including phosphoenolpyruvate carboxykinase (PEPCK) in yeast (Zhao, Xu et al. 2010, Jiang, Wang et al. 2011) and the M2 homolog of Pyruvate Kinase in humans (Lv, Li et al. 2011) results in their subsequent degradation. Additionally, the tumor suppressor protein p53 is activated through acetylation by KATs during DNA damage events (Gu and Roeder 1997) leading to the initiation of apoptosis or quiescence (Prives and Hall 1999). Finally, acetylation regulates the nucleolus to nucleoplasm translocation of the WRN DNA helicase in response to DNA damage events (Blander, Zalle et al. 2002). Taken together these studies suggest that acetylation is an important regulator of a variety of cellular activities including a growing number of non nuclear processes.

KATs/KDACs:

In *S.cerevisiae* there are nine KAT proteins that have confirmed *in-vivo* activity (Esa1 (Smith, Eisen et al. 1998), Sas3 (Takechi and Nakayama 1999), Eco1 (Toth, Ciosk et al. 1999) Elp3 (Wittschieben, Otero et al. 1999), Hat1 (Kleff, Andrulis et al. 1995), Hpa2 (Angus-Hill, Dutnall et al. 1999), Rtt109 (Scholes, Banerjee et al. 2001), Sas2 (Ehrenhofer-Murray, Rivier et al. 1997), and Gcn5 (Grant, Duggan et al. 1997)), one putative KAT (Spt10 (Eriksson, Mendiratta et al. 2005)) and one additional protein that displays *in-vitro* KAT activity (Taf1 (Durant and Pugh 2006)). The yeast KATs function in enzymatic complexes within cells and can be classified into three families initially identified in humans. The largest KAT superfamily is GNAT (Gcn5-related N-acetyltransferases) and in yeast consists of Gcn5, Hat1, Elp3, Eco1, and

Hpa2 (Roth, Denu et al. 2001). GNAT family members are involved in many cellular processes including Eco1 which has an established role in sister chromatid cohesion in both yeast and humans (Kim and Yang 2011). The MYST (MOZ, Ybf2/Sas3, Sas2, and Tip60) family members in yeast includes: Sas3, Sas2, and Esa1. Members of the GNAT and MYST superfamilies contain members in all eukaryotes (reviewed in (Aka, Kim et al. 2011) and share several sequence motifs including an essential acetyl co-A binding domain (Lafon, Chang et al. 2007). Finally, the p300/CBP family consists of Rtt109, Taf1, and Spt10 in yeast (Steger, Eberharter et al. 1998, Roth, Denu et al. 2001, Lee and Workman 2007) and contains no sequence similarities with either the GNAT or MYST superfamilies. Rtt109 is fungi specific and a structural homolog of p300 and CBP (Tang, Holbert et al. 2008), both of which are conserved from worms to humans (reviewed in (Goodman and Smolik 2000).

S. cerevisiae also has ten KDACs which are categorized into three groups based on the homology of their sequences (Gregoretti, Lee et al. 2004). The first two classes are zinc-dependent KDACs. Rpd3, Hos1, and Hos2 are Class I HDACs, while Hda1 and Hos3 are classified as Class II KDACs. Interestingly the core subunits of both complexes contain Rpd3, Sin3, and Ume1 (Carrozza, Florens et al. 2005, Keogh, Kurdistani et al. 2005, Shevchenko, Roguev et al. 2008). Hda1 interacts with two regulatory subunits (Hda2 and Hda3) (Wu, Carmen et al. 2001) to form a heterotrimeric complex that has been shown to regulate the expression of several genes including a subset which are also regulated by Rpd3 (Bernstein, Tong et al. 2000). The remaining Class III enzymes consist of the Sirtuins (Sir2 and Hst1-4) which use NAD^+ as a co-activator during deacetylation (Shore 2000, Smith, Avalos et al. 2002).

The NuA4 lysine Acetyltransferase

The yeast nucleosome acetyltransferase histone H4 complex (NuA4) is a highly conserved 13 subunit MYST family KAT complex (Clarke, Lowell et al. 1999) in *S. cerevisiae* (**Figure 1**). The human homolog of NuA4, Tip60 contains subunits that are homologous to 12 subunits of NuA4 and all of the SWR1 chromatin remodelling complex subunits (Doyon and Cote 2004). NuA4 activity was first observed *in-vitro* whereby the addition of purified NuA4 was shown to acetylate histone H4 in a histone and acetyl-coA dependent manner (Steger, Eberharter et al. 1998). NuA4 activity is linked to a variety of cellular processes including, but not limited to: stress response (Chittuluru, Chaban et al. 2011, Lu, Lin et al. 2011), autophagy (Yi, Ma et al. 2012), gluconeogenesis (Lin, Lu et al. 2009) , and transcription (Doyon, Selleck et al. 2004). Despite its role as a major transcriptional regulator (via H4 acetylation) microarray analysis indicates that NuA4s role in global gene expression is small (Choy et al., 2001; Krogan et al., 2004; Lindstrom et al., 2006; Zhang et al., 2004). This suggests that NuA4 may regulate most cellular activities by acetylating non-histone targets rather than acting to control transcription.

The NuA4 complex consists of 7 non-essential subunits (Eaf7, Eaf3, Eaf5, Eaf1, Eaf6, Yaf9, and Yng2) and 6 essential subunits (Arp4, Epl1, Act1, Swc4, Tra1, and Esa1) (Doyon and Cote, 2004, (Chittuluru, Chaban et al. 2011)) (**Figure 1**) which are organized into two subcomplexes. The piccolo-NuA4 subcomplex consisting of Esa1, Epl1, Yng2, and Eaf6 is the catalytic subcomplex capable of acetylating global histone H4 (Boudreault, Cronier et al. 2003). The remaining subunits of NuA4 make up the recruitment module of NuA4 which is thought to function to target NuA4 to specific genomic loci and non-histone protein targets. For instance, Arp4, an essential subunit and member of the recruitment module, recruits NuA4 to double

stranded DNA breaks upon DNA damage leading to histone H4 acetylation and DNA double-strand break repair (Bird, Yu et al. 2002).

Esa1, the essential catalytic subunit of NuA4 (**Figure 1**) functions to acetylate protein targets by forming a ternary Esa1-acetyl-coA-histone complex (Allard, Utley et al. 1999, Clarke, Lowell et al. 1999, Yan, Barlev et al. 2000, Berndsen, Albaugh et al. 2007). Yeast containing a temperature-sensitive allele of *ESA1*, *esa1-L254P* mutant, lose viability and *in-vivo* catalytic activity (as measured through H4 acetylation) when grown at the restrictive temperature (Clarke, Lowell et al. 1999). This strain will allow me to determine whether NuA4 catalytic activity is involved in regulating a particular cellular process.

Eaf1 is the scaffolding subunit of the NuA4 complex (**Figure 1**). When *EAF1* is deleted the NuA4 complex loses its integrity and the piccolo NuA4 subcomplex dissociates from the recruitment module (Auger, Galarneau et al. 2008, Mitchell, Lambert et al. 2008). *eaf1Δ* mutants exhibit several cellular defects including slow growth, sensitivity to heat, caffeine, and DNA damage-inducing drugs (hydroxyurea and MMS), and mictrotubule destabilization (Chang, Bellaoui et al. 2002, Dudley, Janse et al. 2005, Auger, Galarneau et al. 2008, Mitchell, Lambert et al. 2008, Sinha, David et al. 2008, White, Riles et al. 2009).

Eaf7 is a nonessential subunit of NuA4 found within the recruitment module (**Figure 1**) (Auger, Galarneau et al. 2008, Mitchell, Lambert et al. 2008). Deletion of *EAF7* results in only mild fitness defects (Deutschbauer, Jaramillo et al. 2005). The relative robustness of *eaf7*Δ strains validates their use in corroborating phenotypic data obtained using *eaf1Δ* strains.

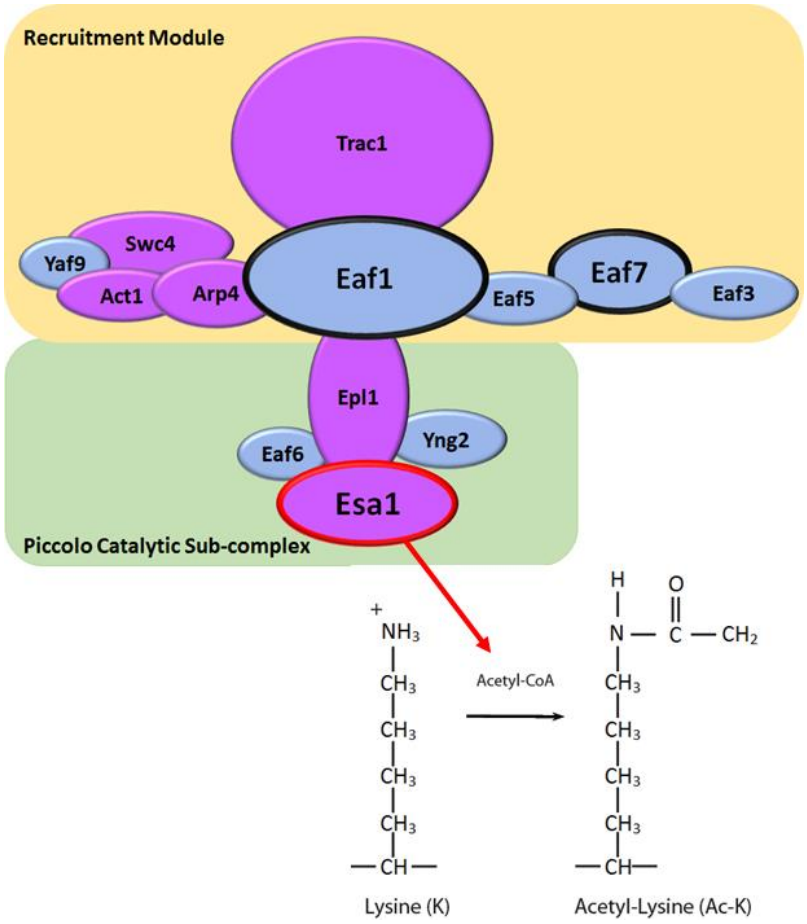

Recruitment Module
Trac1
Swc4
Yaf9
Act1
Arp4
Eaf1
Eaf5
Eaf7
Eaf3
Epl1
Yng2
Eaf6
Esa1
Piccolo Catalytic Sub-complex
Acetyl-CoA
Lysine (K)
Acetyl-Lysine (Ac-K)

Figure 1: Schematic representation of NuA4. Essential NuA4 subunits are purple. Non-essential subunits are coloured in blue. The recruitment module and piccolo catalytic sub-complexes are highlighted in yellow and green respectively. Important subunit deletion mutants used in this study are outlined in black. The Catalytic subunit Esa1 is outlined in red and its catalytic mechanism is likewise shown.

Non-Histone Acetylation Targets

Recent high-throughput systematic screens intended to identify acetylated lysines on peptides in bacteria and eukaryotes have revealed that acetylation is a conserved and ubiquitous post-translational modification comparable to phosphorylation (Kim, Sprung et al. 2006, Choudhary, Kumar et al. 2009, Zhang, Sprung et al. 2009, Wang, Zhang et al. 2010, Zhao, Xu et al. 2010, Weinert, Wagner et al. 2011, Henriksen, Wagner et al. 2012) . Although a large number of chromatin associated proteins are common to all of these screens, the majority of acetylated proteins identified are associated with a variety of non-chromatin related functions including, but not limited to: stress responses, cell cycle, kinase regulation, and metabolism.

NuA4 and Glucose Deprivation

NuA4-mediated acetylation has been linked with the environmental stress response in yeast. It has been demonstrated that in addition to its role as a transcriptional activator, NuA4's catalytic activity regulates several proteins involved in glucose metabolism and glucose sensing pathways in yeast. For instance, Pck1, a gluconeogenesis regulator, is an *in-vivo* acetylation target of Esa1 (Lin, Lu et al. 2009). Likewise, NuA4 has been shown to inhibit the activity of the AMP Kinase Snf1 in glucose replete conditions through the acetylation of the regulatory subunit Sip2 (Lu, Lin et al. 2011). This inhibition is lost when cells are exposed to a glucose starvation, resulting in increased Snf1 activity within a cell. Interestingly, groups have shown that a 10 minute glucose deprivation results in the formation of stress granules (described below) (Buchan, Yoon et al. 2011). Taken together, this data suggests that NuA4 is playing a central role in glucose signaling and may have additional yet to be characterized targets.

Glucose Sensing and Regulatory Pathways in *S. cerevisiae*

As the favoured carbon source in yeast the presence or absence of glucose effects the expression of a variety of genes. Specifically, in the presence of glucose the expression of genes involved in gluconeogenesis, the respiratory pathway (cytochrome proteins), and alternate carbon source utilization are repressed while genes involved in glycolysis and fermentation are induced (Johnston 1999). This glucose-dependent induction or repression is accomplished by one of three regulatory signalling cascades: PKA, TOR, or SNF1/AMPK. Interestingly, these three pathways converge in regulating the localization of the transcription factor Msn2, a transcriptional activator of stress responsive genes (Medvedik, Lamming et al. 2007). When glucose is depleted the signalling cascades are altered to induce the expression of genes involved in gluconeogenesis and alternate carbon utilization, and repress genes involved in glucose metabolism. In addition to their roles in regulating the expression of stress-response genes, these pathways involve the phosphorylation of a number of different substrates linked with a wide variety of cellular processes (reviewed in (Ferretti, Larocca et al. 2012)).

In the presence of glucose (absence of an environmental stress) constitutive adenylate cyclase (Cyr1) activity results in high levels of cyclic AMP (cAMP). Molecules of cAMP bind to and cause Bcy1 (the inhibitory subunit of PKA) to dissociate from PKA leading to its activation (Thevelein and de Winde 1999). Active PKA phosphorylates and inhibits Msn2 and Msn4 resulting in their nuclear export. Simultaneously, when glucose is abundant Sch9 kinase activates TOR resulting in the cytoplasmic sequestration and inhibition of Msn2 and Msn4 (Beck and Hall 1999). The SNF1/AMPK signalling pathway is an important mechanism involved in glucose sensing. In the presence of glucose Hxk2 activates the Glc7-Reg1 protein phosphatase which then inhibits Snf1 resulting in low activity. Additionally, low Snf1 activity is maintained by the acetylation of the regulatory subunit Sip2 by NuA4 (Lu, Lin et al. 2011).

Upon glucose deprivation the membrane bound Gpr1 (G-protein coupled receptor) no longer interacts with glucose resulting in the inhibition of the downstream G-protein Gpa2 and deceased Cyr1 activity (Xue, Batlle et al. 1998, Kraakman, Lemaire et al. 1999, Wang, Pierce et al. 2004). Low levels of cAMP result in low PKA activity and nuclear retention of unphosphorylated Msn2 and Msn4 (Broek, Samiy et al. 1985, Broek, Toda et al. 1987). Likewise, in the absence of glucose TOR is no longer activated resulting in the nuclear retention of Msn2 and Msn4 (Santhanam, Hartley et al. 2004). With regards to the Snf1 pathway, when glucose levels are low NuA4 and Glc7-Reg1 are unable to repress Snf1 activity. This results in high AMPK activity. (**Figure 2**).

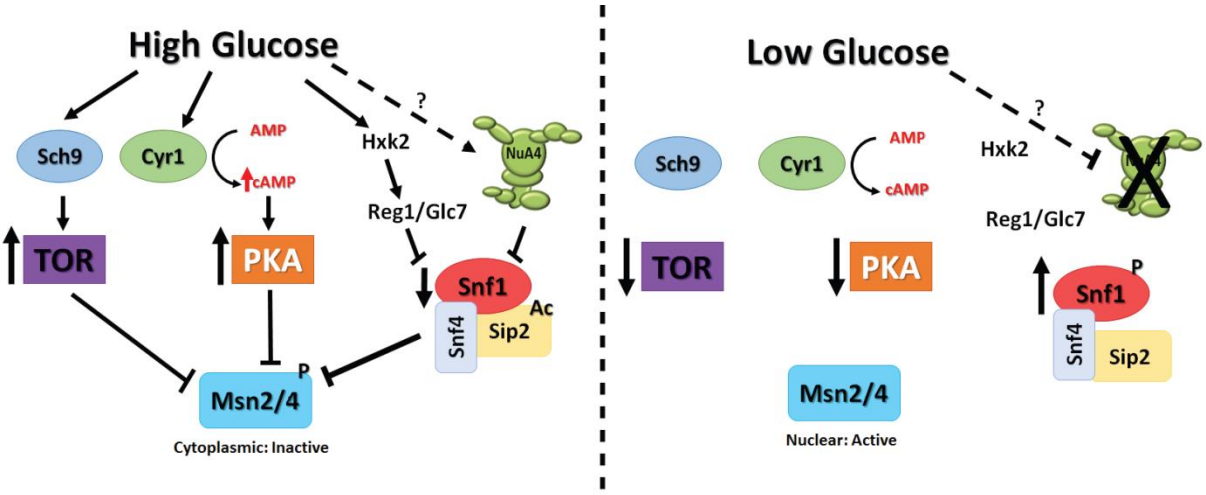

High Glucose
Sch9
Cyr1
AMP
cAMP
Hxk2
?
NuA4
Reg1/Glc7
TOR
PKA
Snf1
Ac
Snf4
Sip2
P
Msn2/4
Cytoplasmic: Inactive
Low Glucose
Sch9
Cyr1
AMP
cAMP
Hxk2
?
Reg1/Glc7
TOR
PKA
P
Snf1
Snf4
Sip2
Msn2/4
Nuclear: Active

Figure 2: Glucose dependent regulation of PKA, TOR, and Snf1. In the presence of glucose TOR and PKA activity is high while Snf1 activity is repressed leading to Msn2/4 phosphorylation and nuclear exclusion. When glucose levels are low Snf1 activity is high and TOR and PKA activity is low resulting in the nuclear retention of unphosphorylated Msn2/4.

1.4 Stress Granules

Messenger RNA (mRNA) Lifecycle

Before a newly transcribed messenger RNA (mRNA) can emerge from the nucleus it must undergo a series of post-transcriptional modification including 3' poly adenylation and the addition of a 5' cap. As polyadenylation takes place the Poly A Binding Protein (Pab1 in yeast) binds to the poly A tail and acts to stabilize the mRNA by preventing deadenylation and subsequent degradation (Sachs, Davis et al. 1987). Once in the cytoplasm Pab1 forms stable interactions with the 5' cap and translation initiation factors to form a closed loop structure which acts to further stabilize the mRNA and promote the formation of polysomes ((Tarun, Wells et al. 1997, Wells, Hillner et al. 1998, Amrani, Ghosh et al. 2008, Safaee, Kozlov et al. 2012) reviewed in (Sachs, Sarnow et al. 1997, Amrani, Sachs et al. 2006)). Under normal growth conditions post-transcriptional mRNA regulation and translation readily occur. However, exposure to environmental stressors initiates a cascade of intracellular responses culminating in the stalling of translation initiation (Li, King et al. 2013). Once translation initiation is stalled the mRNA is sorted into one of two highly dynamic cytoplasmic ribonucleoprotein (RNP) particles: Stress Granules (SG) and Processing Bodies (PB) (Anderson and Kedersha 2006). SG and PB have opposite effects on the mRNAs that accumulate within them. PBs are characterised by the presence of exonucleases, deadenylases, and other mRNA degradation machinery that act to destroy mRNA (Ingelfinger, Arndt-Jovin et al. 2002, Sheth and Parker 2003, Kshirsagar and Parker 2004, Shah, Zhang et al. 2013). Conversely, SGs sequester mRNA stalled in translation initiation and prevent its potential degradation in PBs ((Teixeira, Sheth et al. 2005, Swisher and Parker 2010) reviewed in (Buchan and Parker 2009)). To date the functional dynamics of PBs and SGs remain poorly understood. Identification of a regulatory mechanism of RNP formation is important to decipher the exact function of SGs and PBs in a cell. (**Figure 3**)

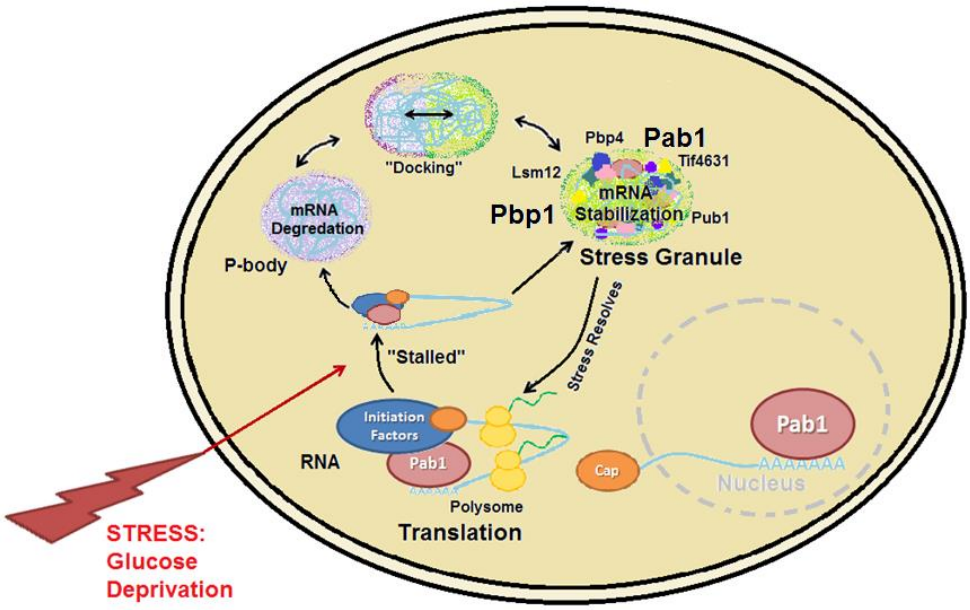

"Docking"
Pbp4
Pab1
Tif4631
Lsm12
mRNA
Stabilization
Pbp1
Pub1
Stress Granule
mRNA
Degredation
P-body
"Stalled"
Stress Resolves
Initiation
Factors
RNA
Pab1
Cap
Pab1
AAAAAAA
Nucleus
Polysome
Translation
STRESS:
Glucose
Deprivation

Figure 3: mRNA Lifecycle. Newly transcribed mRNA emerges from the nucleus with a 5' cap and a 3' poly-A tail to which Pab1 is bound. In the cytosol, Pab1 interacts with translation initiation factors forming a 'closed loop' structure on which polysomes form. Upon environmental stress translation stalls and mRNA is sorted into Stress Granules (SGs) or Processing Bodies (PBs). Upon stress resolution mRNAs sequestered in SG are translated once more.

Processing Bodies vs Stress Granules

Processing Bodies (PBs) are homogenous spheroid particles that increase in both size and number in response to cellular stress (Kedersha, Stoecklin et al. 2005, Teixeira, Sheth et al. 2005, Wilczynska, Aigueperse et al. 2005). PBs were first characterised by the presence of the 5'-3' mRNA decay machinery, the RNA-induced silencing complex, and the nonsense-mediated decay pathways. The cytoplasmic distribution of the mammalian exonuclease Xrn1 provided the first clues to the presence of a distinct cytoplasmic site of mRNA decay (Bashkirov, Scherthan et al. 1997). Subsequently other decay factors including the Lsm1-7 proteins and the decapping enzymes Dcp1 and Dcp2 were also found to have a similar cytoplasmic distribution (Bashkirov, Scherthan et al. 1997, Eystathioy, Chan et al. 2002, Ingelfinger, Arndt-Jovin et al. 2002, van Dijk, Cougot et al. 2002, van Dijk, Le Hir et al. 2003). These cytoplasmic foci were termed Processing Bodies (PBs) when it was discovered that mRNAs containing 5'-3' exonuclease resistant oligo-G tracts accumulated at these foci in yeast (Sheth and Parker 2003). Later studies determined that along with the exonuclease Xrn1, PBs contain additional enzymes responsible

for both the general mRNA decay pathway and the nonsense-mediated decay pathway (Unterholzner and Izaurralde 2004). In mammalian cells PBs also contain proteins involved in the RNA-induced silencing complex (microRNA (miRNA) and argonaute ((Liu, Valencia-Sanchez et al. 2005, Sen and Blau 2005), GW182 (required for RNA silencing), and 4-ET (eIF4E binding protein) (Liu, Rivas et al. 2005, Rehwinkel, Behm-Ansmant et al. 2005).

Stress Granules (SGs) are heterogenous particles that are characterized by an arrest in translation as well as the accumulation of mRNAs, translation initiation factors, and RNA binding proteins (RBPs) in distinct cytoplasmic foci (reviewed in (Stoecklin and Kedersha 2013). SGs were first observed in the cytoplasm of tomato plants in response to heat shock (Nover, Scharf et al. 1989). Further characterization revealed that SGs form in response to a variety of environmental stressors including, but not limited to: oxidative stress, UV irradiation, hypoxia, and glucose deprivation (reviewed in (Anderson and Kedersha 2006).

It is hypothesized that SGs function as triage centres for mRNAs during stress in which mRNAs are either returned to translation, retained in a non-translating state, or sorted to PBs for degradation during PB-SG docking ((Hoyle, Castelli et al. 2007, Buchan, Muhlrad et al. 2008, Buchan, Nissan et al. 2010) (**Figure 3**). In addition to the small ribosomal subunit and early translation initiation factors several yeast proteins localise to glucose deprivation SG (**Table 1**). Interestingly, several of these proteins found in yeast have mammalian homologs which localize to SGs in humans (Buchan, Nissan et al. 2010) emphasizing the conservation between yeast and humans. It is important to note that the type of stress dictates both the protein and the mRNA composition that accumulates in a SG. For instance, Pub1 and Pbp1 are required for the formation of glucose starvation stress granules; however, they are not required for the formation of SG derived from a heat stress (Buchan, Muhlrad et al. 2008).

Although SGs form in response to a variety of cellular stresses their exact function remains elusive. It has been suggested that SGs reserve cellular energy by storing important mRNAs and translation initiation factors during stress (Decker and Parker 2012). In fact, mRNPs that are localized in SGs are primed for re-entry into translation once a stressor is removed (Buchan and Parker 2009). Interestingly, it has also been shown that SGs are correlated with increased cell survival during stress ((Baguet, Degot et al. 2007, Kwon, Zhang et al. 2007, Eisinger-Mathason, Andrade et al. 2008, Buchan and Parker 2009). SG assembly is not required for the repression of global translation (Buchan, Muhlrad et al. 2008), instead SGs may act of sites of non-canonical stress-responsive mRNA translation (reviewed in (Buchan and Parker 2009)). Another proposed function of SGs is their involvement in the sequestration and storage of a variety of signalling molecules. In fact the incorporation of pro-apoptosis factors and signalling proteins into SG may prevent cell death during stress ((Kwon, Zhang et al. 2007, Arimoto 2008) reviewed in (Anderson and Kedersha 2009)).

Perhaps most importantly, SGs are key phenotypical markers of several cancers in which their assembly correlates with increased resistance to chemotherapeutic drugs (Fournier, Gareau et al. 2010) (Fournier, Coudert et al. 2013, Thedieck, Holzwarth et al. 2013). Likewise, SGs are associated with several neurodegenerative diseases including Alzheimers Disease (AD) and Amyotrophic Lateral Sclerosis (ALS) (Castellani, Gupta et al. 2011) (Li, King et al. 2013). Specifically, groups have been able to show that TDP-43 and FUS, proteins implicated in ALS mutagenesis, and tau, a protein associated with neurodegeneration and AD, are able to co-localize with SGs ((Liu-Yesucevitz, Bilgutay et al. 2010, Vanderweyde, Yu et al. 2012) reviewed in (Wolozin 2012) and (Li, King et al. 2013)). The extensive homology between yeast and

humans suggests that the model organism *Saccharomyces cerevisiae* can be used to decipher SG dynamics.

Protein		Reference #
Yeast	**Mammalian homolog**	
40S Ribosomal subunit		(Kedersha, Chen et al. 2002, Kimball, Horetsky et al. 2003, Grousl, Ivanov et al. 2009)
eIF4A (Tif1, Tif2)		(Kedersha, Chen et al. 2002, Kimball, Horetsky et al. 2003, Buchan, Yoon et al. 2011)
eIF4B (Tif3)		(Kedersha, Chen et al. 2002, Kimball, Horetsky et al. 2003, Buchan, Yoon et al. 2011)
eIF4E (Cdc33)		(Kedersha, Chen et al. 2002, Kimball, Horetsky et al. 2003, Hoyle, Castelli et al. 2007)
eIF4G1 (Tif4631)		(Kedersha, Chen et al. 2002, Kimball, Horetsky et al. 2003, Hoyle, Castelli et al. 2007)
eIF4G2 (Tif4632)		(Kedersha, Chen et al. 2002, Kimball, Horetsky et al. 2003, Hoyle, Castelli et al. 2007)
Pab1	PABP	(Hoyle, Castelli et al. 2007)
Pbp1	ATAXIN-2	(Buchan, Muhlrad et al. 2008)
Pub1	TIA-1	(Buchan, Muhlrad et al. 2008)
Lsm12	LSM12	(Swisher and Parker 2009)
Pbp4		(Swisher and Parker 2009)
Ngr1	TIAR	(Buchan, Muhlrad et al. 2008)

Ded1	DDX3	(Hilliker, Gao et al. 2011)
Ecm33		(Mitchell, Jain et al. 2013)
Slf1		(Mitchell, Jain et al. 2013)
Ksp1		(Mitchell, Jain et al. 2013)
Sro9		(Mitchell, Jain et al. 2013)
Tae2		(Mitchell, Jain et al. 2013)

Table 1: Proteins that localize to glucose deprivation stress granules. # Lists primary reference showing the protein localizes to SG upon glucose deprivation.

Stress Granule Assembly, Disassembly and Dynamic Nature

To date little is understood about SG assembly, disassembly, and regulation. Existing data states that both yeast and mammalian SGs and PBs are distinct structures that form independently ((Buchan, Kolaitis et al. 2013, Shah, Zhang et al. 2013) reviewed in (Stoecklin and Kedersha 2013). Noteably, studies have shown that the PKA and TOR pathways are not involved in stress granule formation in response to glucose deprivation (Tudisca, Simpson et al. 2012, Shah, Zhang et al. 2013). While confusion exists as to whether an eukaryotically-conserved mechanism of SG assembly exists, recently published data suggests that SG disassembly is mediated by autophagy in both yeast and mammals (Buchan, Kolaitis et al. 2013).

When cells are exposed to an environmental stress such as heat stress or nutrient deprivation SGs form rapidly (within 10-30min). To date the major contributing factor in SG assembly remains the phosphorylation of the translation initiation factor eIF2α by a family of stress-activated kinases including Protein Kinase R (PKR), GCN2, the heme-regulated inhibitor, and PKR-like ER kinase (Anderson and Kedersha 2006). Interestingly, the type of stress not only

dictates the species of mRNA and proteins that aggregate into SGs, but the kinase responsible for eIF2α phosphorylation (reviewed in (Kedersha and Anderson 2007)). Mechanistically, phyosphorylation of eIF2α reduces the availability of the eIF2–GTP–tRNAi^{Met} ternary complex, and subsequently blocks translation initiation and promotes polysome disassembly (Kedersha, Gupta et al. 1999, McEwen, Kedersha et al. 2005). Following translation inhibition the glutamine-rich prion-like domains of Pub1 and Pbp1 promote self-aggregation as well as the aggregation of additional proteins and mRNAs into SGs (Gilks, Kedersha et al. 2004). Interestingly, the self-aggregation of the RNA binding protein G3BP in mammals is regulated by the phosphorylation at serine 149 (Tourriere, Gallouzi et al. 2001) suggesting that PTMs are an important factor in SG assembly.

Lysine Acetylation and Stress Granules

The Baetz lab and others have identified lysine acetylation on Pab1, Pbp1, Pbp4, and Lsm12 (Henriksen, Wagner et al. 2012, Mitchell, Huard et al. 2013) and mammalian acetylome studies indicate that acetylation of SG proteins is a conserved process (Choudhary, Kumar et al. 2009, Brook, McCracken et al. 2012, Henriksen, Wagner et al. 2012) (**Table 2**). These acetylome studies, along with the dynamic nature of SGs, suggests a possible role for KATs and/or KDACs in SG assembly, dynamics or function. To date, there are a handful of studies linking these signalling enzymes to SG assembly and dynamics. One of these studies found that HDAC6 is an important regulator of mammalian stress granule formation in 293T cells (Kwon, Zhang et al. 2007). A second study identifies that Hos2, a KDAC in yeast, is able to localize to cytoplasmic RNP granules in quiescent cells (Liu, Chiu et al. 2012), however the assembly of these stationary-phase granules (SPGs) is not linked with SG formation. Recently one group was able to show that the KDAC SIRT6 regulates stress granule formation in *C. elegans* and

mammals (Jedrusik-Bode, Studencka et al. 2013). Finally, the Baetz lab established that NuA4 co-immunopurifies stress granule (SG) proteins and that Pab1 is an *in-vitro* target of NuA4 (Mitchell, Huard et al. 2013), suggesting a potential role for NuA4 in SG dynamics. Despite the identification of additional associations between KATs/KDACs and SGs a precise mechanism of SG formation remains unknown.

Yeast Name	Mammalian Homolog	Published Lysine Acetylation Sites	Reference
eIF4A (Tif1, Tif2)		K23, K226, K279, K304	(Henriksen, Wagner et al. 2012)
eIF4B (Tif3)		K155	(Henriksen, Wagner et al. 2012)
eIF4E (Cdc33)		K9, K36, K114, K162, K172	(Henriksen, Wagner et al. 2012)
eIF4G1 (Tif4631)		K153, K689, K839, K844	(Henriksen, Wagner et al. 2012)
Pab1	PABP	K7,K94, K105, K131, K268, K288, K382, K390	(Brook, McCracken et al. 2012, Henriksen, Wagner et al. 2012, Mitchell, Huard et al. 2013)
Pbp1	Ataxin-2	K260, K191, K433	(Henriksen, Wagner et al. 2012)
Pbp4		K39, K51	(Henriksen, Wagner et al. 2012)
Ded1	DDX3	K92, K164, K264	(Henriksen, Wagner et al. 2012)
Ksp1		K438	(Henriksen, Wagner et al. 2012)
Sro9		K79, K143, K229, K290	(Henriksen, Wagner et al. 2012)
Lsm12	LSM12	K52, K171	(Choudhary, Kumar et al. 2009, Henriksen, Wagner et al. 2012)

Table 2. Published lysine acetylation sites on known glucose deprivation stress granule proteins. Black sites are from yeast acetylome study (Choudhary, Kumar et al. 2009, Henriksen, Wagner et al. 2012). Red sites are NuA4 *in-vitro* targets identified the NuA4 mChIP-KAT-MS study (Mitchell, Huard et al. 2013).

Hypothesis: I hypothesize that the NuA4 lysine acetyltransferase complex impacts stress granule formation in response to glucose deprivation through the acetylation of Pab1.

Specific Aims:

Aim 1: To decipher the impact of NuA4 activity on stress granule formation under various stresses.

Aim 2: To identify a mechanism by which NuA4 mediates glucose deprivation stress granule assembly in yeast.

2.0 Methods

2.1 Yeast strains and Media

Yeast strains and plasmids used in this study are listed in **Table** B1 and **Table** B2 respectively. Epitope tag integrations made for this study were generated using the standard PCR-mediated gene insertion technique (Longtine, McKenzie et al. 1998) and confirmed by PCR and Western blot analysis. Note that endogenously tagged proteins are expressed from their own genomic loci at WT levels while strains transformed with plasmids to over-express Pab1 protein levels supplement the endogenous Pab1 expression with additional Pab1 expression. The strains were grown in either Synthetic Complete (SC) media or Yeast Peptone Dextrose (YPD) media as specified.

2.2 Fluorescence Microscopy of Stress Granule Formation

Single deletion mutant strains used to assess SG formation were grown in SC media lacking uracil at 30°C allowing for the expression of the Pab1-GFP::URA::CEN plasmid. Integrated SG and PB strains were grown in YPD media. For Pab1 overexpression experiments cells were grown in SC media lacking both URA and LEU permitting the expression of Pab1-

GFP::URA::CEN(PKB192) , or control plasmids [empty vector::URA (PKB23) and empty vector::LEU (PKB21)]. Cells exposed to glucose deprivation were grown in 50mL cultures to an OD_{600} of 0.3-0.5, pelleted, washed in fresh media lacking glucose, and resuspended in 50mL of glucose deplete media for 10 minutes. Cells exposed to heat stress (46°C), ethanol (15%), and NaN_3 (0.5%) stresses were likewise grown in 50mL cultures until an OD_{600} of 0.3-0.5 and exposed to each stressor for 30 minutes. All cells were immediately imaged live in absence of fixation agents. Briefly, 5mL of culture (control and stress conditions) was gently spun down (3000rpm, 2 minutes at 30°C) and re-suspended in 200µl of synthetic media. 10µl were spotted onto a glass plate and imaged immediately using brightfield and FITC filters. Microscopy was performed using a Leica DMI 6000 florescent microscope (Leica Microsystems GmbH, Wetzler Germany), equipped with a Sutter DG4 light source (Sutter Instruments, California, USA), Ludl emission filter wheel with Chroma band pass emission filters (Ludl Electronic Products Ltd., NY, USA) and Hamamatsu Orca AG camera (Hamamatsu Photonics, Herrsching am Ammersee, Germany). Z-stacked images (100x objective, no binning) were collected using Velocity 4.3.2 Build 23 (Perkin Elmer).

2.3 Image Quantification and Analysis

The quantified data sets presented in this study represent the analysis of three replicates consisting of ~100 cells each to a total of ~300 cells per experiment. All images were subjected to deconvolution using Velocity 4.3.2 Build 23 (Perkin Elmer) (PSF: 63 or 100x_oil_535m (fitc,yfp)). Images were analysed using ImageJ to determine the percentage of cells that form SGs. Briefly, deconvolved images were opened in ImageJ and converted to 8-bit types followed by smoothing and thresholding as previously described (Kedersha, Tisdale et al. 2008) to score SG formation. Statistics were completed using excel and online software including: <

http://www.graphpad.com/quickcalcs/ttest1/?Format=SD > and < http://www.physics.csbsju.edu/stats/anova.html >.

2.4 Ribosomal Profiling

The polysome profile experiments presented in this thesis reflect the work completed by Dr. Sylvain Huard. 400mL of cells were grown at 30°C to an O.D of 0.4, split equally and pelleted (3min 3000rpm). Each pellet was washed with 25ml pre-warmed (30°C) YPD or pre-warmed (30°C) YP followed by brief centrifugation (3min 3000rpm). Pellets were then resuspended in 25mL of YPD or YP and re-inocculated into 175mL cultures of YPD or YP. After 10 minutes cells (unstressed and GD) were collected in cold centrifuge bottles containing 2ml of 10mg/mL cycohexamide (CHX) (3min, 3000rpm, 4°C). Cells were then washed with 100mg/mL CHX and subsequently pelleted and frozen at -80°C. Whole cell extracts (WCEs) were obtained by lysing cells (using glass beads) in buffer (20 mM HEPES (pH 7.4), 2 mM MgOAc, 100 mM KOAc, 100 mg/ml cycloheximide, 0.5 mM DTT) six times for 20s at 1min intervals. WCEs were isolated by poking a hole through the bottom of each eppendorf tube using a red hot needle (21G11/2) and centrifuging at 1000rpm for 1min, collecting the WCE through the needle hole in a fresh 1.5mL eppendorf tube. The lysate was cleared by brief centrifugation (5min, 10000rpm, 4°C) followed by longer centrifugation (20min, 10000rpm 4°C). The supernatant was transferred to a new 1.5mL Eppendorf tube and the A_{260} was measured. 10 A_{260} units were loaded onto 15-45% linear sucrose gradients.

Sucrose gradients were made by adding first low and then higher percentage sucrose solutions (Basic Buffer (in RNAse free water): 15mM Tris-HCl, pH 7.4, 300mM NaCl, 15mM $MgCl_2$) to tubes that were subsequently capped to insure that there was no air between the cap and the solution.

Gradients were fractionated by adding 200uL of lysate to the inside of each tube followed by centrifugation (90min, 39000rpm, 4°C) using the SW41-Ti rotor (no brake). Samples were then removed from the rotor and carefully placed on ice. 1mL fractions were collected for each sample using a fractions collector (Brandel-Amersham) and the quality was monitored at 254nm using an ISCO UA-6 UV detector.

2.5 Tandem Affinity Purification (TAP)-tagged and Green Fluorescent Protein (GFP)-Trap Immunoprecipitation

Cells were grown in YPD to an O.D of 0.6 after overnight pre-growth followed by dilution to an O.D of 0.2. During TAP co-immunoprecipitations (co-IPs) approximately 20mg of WCE was incubated with 40µl of magnetic Dynabeads (catalog no. 143-01; Dynal, Invitrogen) crossed-linked to rabbit immunoglobulin G (IgG) (catalog no. PP64; Chemicon) at 4°C with end-over-end rotation for 2 or 24 hours (each completed in triplicate). Following incubation, the Dynabeads were collected using a magnet, washed 3 times with 1mL of ice cold wash buffer (100mM HEPES pH 8.0, 20mM magnesium acetate, 10% glycerol (V/V), 10mM EGTA, 0.1mM EDTA, 300mM sodium acetate, 0.5% Nonidet P-40) making sure to transfer the incubated WCE to new Eppendorf tubes in between each wash. The washed beads were then re-suspended in 40µl of 1×loading buffer (50mM Tris pH 6.8, 2% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, 10% glycerol) and eluted from the beads by heating at 65°C for 10 min. The eluted samples were transferred into new Eppendorf tubes, and 2-β-mercaptoethanol was added to each sample at a final concentration of 200mM.

GFP IPs were completed by adding 20µl of GFP-trap magnetic beads (Chromotek, Germany; Cat. #: GTM-20) to 10mg of WCEs and incubated for 2 hours with end over end rotation

at 4°C. Samples were collected using a magnet and washed 3 times with 1mL of ice cold wash buffer (100mM HEPES pH 8.0, 20mM magnesium acetate, 10% glycerol (V/V), 10mM EGTA, 0.1mM EDTA, 300mM sodium acetate, 0.5% Nonidet P-40). The incubated WCE was only transferred to new Eppendorf tubes during the final wash. The washed beads were re-suspended in 20µl of 2×loading buffer (50mM Tris pH 6.8, 2% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, 10% glycerol) and heated at 65°C for 10 minutes.

2.6 Immunoblotting

50µg to 150µg of protein from WCE or all protein eluted from beads from IP samples were separated by SDS-PAGE. Proteins and acetylation were detected using the following antibodies as indicated; anti-acetyl lysine (Cell Signaling; Cat. #: 9681; dilution 1/500), GFP fusion protein was detected using α-GFP (Roche; Cat. #: 11814460001; dilution 1/3000), TAP fusion protein was detected using α-TAP (Thermo Scientific, CAB1001; dilution 1/5000), MYC fusion protein was detected using α-MYC (Roche Applied Science 11667149001; dilution 1/800), and Pab1 protein was detected using α-Pab1 (1:10,000, Antibodies online). G6PDH was used as a loading control and was detected with anti-G6PDH (Sigma, A9521 1/10,000). HPR coupled secondary antibodies used in this study were: goat anti-mouse IgG (BioRad, catalog no. 170-6516, 1:5000) and goat anti-rabbit IgG (Chemicon, catalog no. AP307P, 1:5000). Membranes were developed using Western Chemiluminescent HRP Substrate Detection System (Millipore, catalog no. WBKLS0500) and imaged using either chemiluminescence imaging on a Molecular Imager ChemiDoc XRS System (BioRad) or LI-COR Odyssey Fc system (LI-COR Biosciences, USA). The secondary antibodies used for chemiluminescence were: goat anti-rabbit IgG (Chemicon, catalog no. AP307P, 1:5000) and goat anti-mouse IgG (BioRad, catalog no. 170-6516, 1:5000). Secondary antibodies used with the LI-COR system were: Alexa Flour 680 goat anti rabbit, and

Alexa Flour 790 goat anti mouse. Band intensity of blots exposed using the LI-COR system were quantified using Image Studio V2.0 and standardized to G6PDH levels. Briefly, equal-sized boxes were drawn around each Pab1 protein band and standardized to loading control bands (G6PDH): Pab1 band intensity/Corresponding G6PDH band intensity. Total relative band intensity corrected to the loading control was used to measure Pab1 protein levels.

2.7 Quantitative real-time PCR

qPCR experiments presented in this thesis reflect the work completed by Dr. Sylvain Huard. 13ml of cells from 50ml of mid-log-phase culture (OD_{600} between 0.6 to 0.8) grown in YPD at 30°C were collected by centrifugation and washed twice with 5ml of cold water. Cells were re-suspended in 2ml of Tri-Reagent (Sigma-Aldrich, USA; Cat. #: T9424) and followed by the addition of 1ml of acid-washed glass beads (Fisher Scientific, USA; Cat. #: 35535). The cell pellet was then frozen in liquid nitrogen and store at -80C until the samples were ready for processing. All the centrifugations were performed at 4°C. The samples were allowed to be thawed at room temperature (RT), vortexed at maximum speed for 5min, followed by the addition of 500µl of chloroform and vortexed again for 15 sec. The samples were incubated for 10min at room temperature and then centrifuged at 3200g for 20min. The aqueous layer was transferred equally between two RNAse-free eppendorf tubes. 1ml of isopropanol was added and incubated on ice for 10 min. The RNA was centrifuged at 12000g for 25 min. The pellet was washed with 500µl of ethanol 75% made with DEPC water. The pellet was again centrifuged at 7500g for 5min, dried and resuspended in a final volume of 40µl of nuclease-free water (Ambion, USA; Cat. #: AM9937). 10ug of RNA (ND-1000 spectrophotometer, NanoDrop Technologies, USA) was treated with DNAse (RQ1 RNAse-free DNAse, Promega, USA; Cat. #: M6101) for 30 min at 37°C as per the manufacturer's instructions. The RNA was precipitated by adding 400µl of Nuclease-

free water and 500µl of phenol/chloroform. The RNA was vortexed at maximum speed for 1min and centrifuged at 12000g for 5 min. The aqueous layer was transferred to a new RNAse-free eppendord, and 5µl of NaAc 3M and 1ml of Ethanol 100% was subsequently added. The precipitated RNA was incubated at -80°C overnight and centrifuged at 12000g for 30 min. The pellet was washed with 500µl of 70% ethanol made with Nuclease-free water, centrifuged again at 7500g for 5 min, dried and resuspended in 20µl Nuclease-free water. The RNA was normalized at 200 ng/µl with Nuclease-free water. RT-PCR was performed as per the manufacturer's instructions with 2.5µg of RNA in a final volume of 20µl (High Capacity cDNA RT Kit, Applied Biosystems, USA; Cat. #: 4368814). qPCR was performed with the cDNA as per the manufacturer's instructions in a final volume 10µl (SsoFast EvaGreen *Supermix; Bio-Rad, USA; Cat. #: 172-5201). PAB1* mRNA expression was normalized to WT expression of the reference gene *TDH3* (Glyceraldehyde-3-phosphate dehydrogenase). qPCR experiments were completed using CFX manager software (*Bio-Rad*). *Primer used for the qPCR:*

PAB1 forward: 5'-TGCCACCGACGAAAACGGAA- 3'; PAB1 reverse: 5'-AGCTTCCTTGGCAGCACCTT-3'

TDH3 forward 5'-CTGTCAAGTTGAA CAAGGAAACCAC-3'; TDH3 reverse 5'-CAACGTGTTCAACCAAGTCGACAA-3'

2.8 Variable Glucose Pab1 Interactome

The interactome and acetylome of Pab1 in glucose replete or deprived conditions was generated by using the modified Chromatin Immunoprecipitation (mCHIP) (Lambert, Mitchell et al. 2009) a method to purify endogenously TAP-tagged *PAB1*. Cells were grown at 30°C in 500mL YPD cultures to mid-log phase (OD_{600} ~ 0.6-0.9) and were collected by centrifugation (3000 rpm,

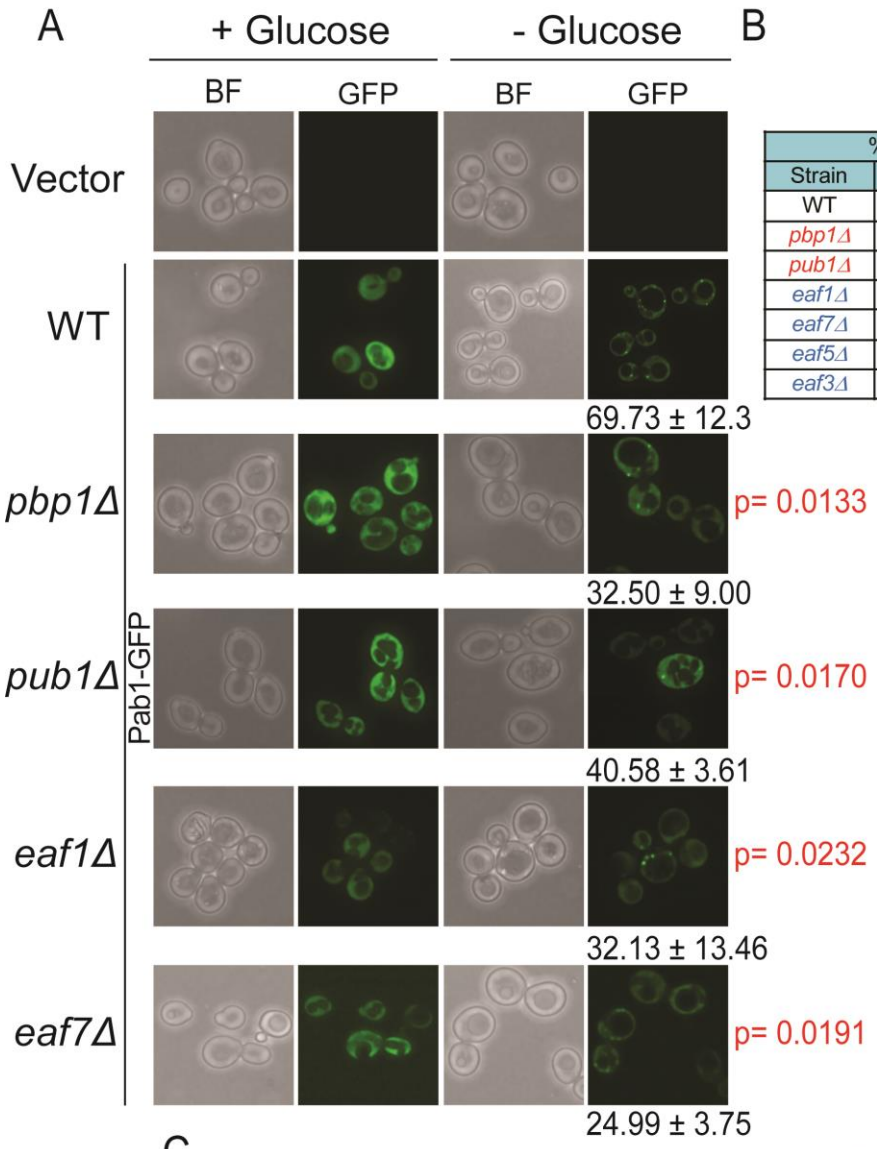
3 minutes, 4°C) followed by a wash in 25mL of ice-cold distilled water and subsequent freezing in 1.5mL Eppendorf tubes using dry ice. Cells subjected to glucose deprivation were grown to an OD_{600} of 0.5, pelleted, washed YP media (lacking glucose), and resuspended in 500mL cultures of YP media for 10, 30, or 60 minutes and collected as despcribed above. Pelleted cells were stored at -80°C. Cell lysates were obtained by re-suspending frozen cells in 300µL of lysis buffer (100mM HEPES pH 8.0, 20mM magnesium acetate, 10% glycerol (V/V), 10mM EGTA, 0.1mM ETDA, 300mM sodium acetate, and fresh protease inhibitor cocktail (Sigma, P8215)) plus an equal volume of acid washed glass beads (Fisher Scientific, 35-535). Cells were lysed by vortexing (6×1 minute vortex with 1 minute incubation on ice in between each pulse). The crude whole cell extract (WCE) was separated from the glass beads into new Eppendorf tubes by poking a hole through the bottom of each existing Eppendorf tube using a 21G1½ (Becton Dickinson, catalog no. 305167) needle heated with a flame and centrifuging at 1000 rpm for 1 minute at 4°C. WCEs were subjected to sonication (3x20sec; 1 minute incubation on ice between each pulse) using a Misonix Sonicator 3000 at setting four. Prior to centrifugation (10min, 3000rpm, 4°C), Nonidet P-40 was added to a final concentration of 1% and incubated with end-over-end at 4°C for 10 minutes. The WCE was then clarified by centrifugation (15 minutes, 13200 rpm, 4°C) and the supernatant was transferred into new1.5mL Eppendorf tubes. Protein concentration for each sample was determined by Bradford Assay (Bio-Rad, 500-0006). NuPAGE Novex gradient gel SDS/PAGE (4–12%Bis•Tris Gel; Invitrogen, NP0321) was used to separate proteins and visualized by silver stain. Lanes were excised for identification by mass spectroscopy, and processed by LC-MS/MS on an LTQ-Orbitrap XL mass spectrometer (Thermo-Electron) as previously described (Mitchell, Huard et al. 2013).

3. Results

3.1 NuA4 is required for Pab1-GFP assembly into Glucose Deprivation Stress Granules

The co-precipitation of core stress granule proteins by Esa1-TAP (Mitchell, Huard et al. 2013) and the known roles of NuA4 in stress response (Chittuluru, Chaban et al. 2011) (Lu, Lin et al. 2011) suggested that NuA4 may have a role in stress granule dynamics. Further, NuA4 has established roles in glucose metabolism (Lin, Lu et al. 2009). Therefore, I first sought to determine whether NuA4 has a role in SG formation upon GD. Wild type, NuA4 non-essential mutant cells (*eaf1Δ, eaf7Δ, eaf3Δ,* and *eaf5Δ*) and *pub1Δ* and *pbp1Δ* deletion mutants of SG components that display decreased GD SG (Buchan et al 2008), were transformed with a Pab1-GFP expressing plasmid (Swisher and Parker 2010). Pab1-GFP cytoplasmic foci (SGs) were monitored in both media containing glucose and after 10 minute glucose deprivation as previously described (Buchan et al 2010). As expected, upon GD while 69.73 % of wild type cells displayed Pab1-GFP cytoplasmic foci or SGs, this is significantly reduced, but not eliminated in *pub1Δ* and *pbp1Δ* cells (Buchan et al 2008). Upon glucose deprivation *eaf1Δ, eaf7Δ, eaf5Δ, and eaf3Δ* cells show a decrease in Pab1-GFP localization to SG to a similar extent as control cells (**Figure 4)**. Due to the known phenotypic growth defects associated with *eaf1Δ* cells (Auger, Galarneau et al. 2008, Mitchell, Lambert et al. 2008) I monitored SG formation after 10, 30, and 60 minutes of GD (**Figure A1)**. This time-course analysis revealed that the defect I observed in *eaf1Δ* mutant cells is not simply explained by the slow growth phenotype.

Rather, as all non-essential NuA4 mutant strains, including those with no growth defects, displayed significant reduction in SG formation it suggests that NuA4 is required for SG formation in response to GD.



% Cells with Stress Granules		
Strain	+ Glucose	– Glucose 10 min
WT	10.21 ± 1.38	69.73 ± 12.3
pbp1Δ	12.98 ± 5.69	32.5 ± 9.00
pub1Δ	17.71 ± 10.43	40.58 ± 3.61
eaf1Δ	13.67 ± 9.47	32.13 ± 13.46
eaf7Δ	10.81 ± 4.06	24.99 ± 3.75
eaf5Δ	11.62 ± 2.43	27.99 ± 3.38
eaf3Δ	10.06 ± 1.40	26.25 ± 8.58

Figure 4. NuA4 is required Pab1-GFP assembly into stress granules (SG) upon glucose deprivation (GD). Cells were transformed with *PAB1-GFP::URA::CEN* plasmid (PKB192) and subjected to glucose deprivation for 10 minutes (-glucose) or control conditions (+glucose). SGs were quantified in a blind manner using Image J software exactly as described (Kedersha, Tisdale et al. 2008). 3 replicates of n ≥ 300 for a minimum of 900 cells total . **A)** Images of the Bright Field (BF) and GFP images of the indicated strains: WT (YKB1079), *pbp1Δ* (YKB3217), *pub1Δ* (YKB3218), *eaf1Δ* (YKB3453), *eaf7Δ* (YKB3292). Numbers indicate the % of cells that form SG ± SD. p-value computed using unpaired t-tests against WT. **B)** Table lists % of cells with SG foci ± SD for mutants listed with glucose (+Glucose) and without glucose (-Glucose 10 min). Indicated strains are the same as shown in A with the addition of *eaf5Δ* (YKB3290) and *eaf3Δ* (YKB3291). **C)** One-way ANOVA confirms that GD SG defects displayed by NuA4 mutants similar to ***pbp1Δ*** cells (YKB3217) and ***pub1Δ*** cells (YKB3218). ANOVA computed using online software: <http://www.physics.csbsju.edu/stats/anova.html >. WT=Wild-type. SG=Stress Granule. SD=Standard Deviation.

To confirm that GD SG assembly defects are due to the acetyltransferase activity of NuA4, I also asked if temperature sensitive *esa1-L254P* mutant (*esa1^{ts}*)(Clarke, Lowell et al. 1999) cells expressing endogenously tagged Pab1-GFP display defects in SG formation. If NuA4 catalytic activity is involved in GD SG assembly one would expect that upon 10 minutes of GD, *esa1^{ts}* cells grown at increasingly restrictive conditions (increasing temperatures) should show a temperature-dependent decreased ability to form SGs. Indeed this is what I find. *esa1^{ts}* cells grown at permissive (25^{O}C), semi-permissive (30^{O}C), and restrictive temperatures (37^{O}C) do not show any defects in SG assembly under non-stressed conditions (**5A**). However, upon GD (**5B**) mutant cells grown at the restrictive temperature (37^{O}C) have a SG assembly defect similar to that of other NuA4 mutants (**Figure 4**). Interestingly, when *esa1^{ts}* mutants are grown at 30^{O}, a semi-permissive temperature, there is a smaller, yet significant decrease in the percentage of cells that form SGs ($p<0.05$). Finally, when *esa1^{ts}* cells are subjected to GD at 25^{O}C (catalytically active NuA4) they are able to form SGs to similar extent as wild type cells. Taken together with the data presented above, these results indicate that the NuA4 complex is required for the assembly of Pab1-GFP into GD SGs.

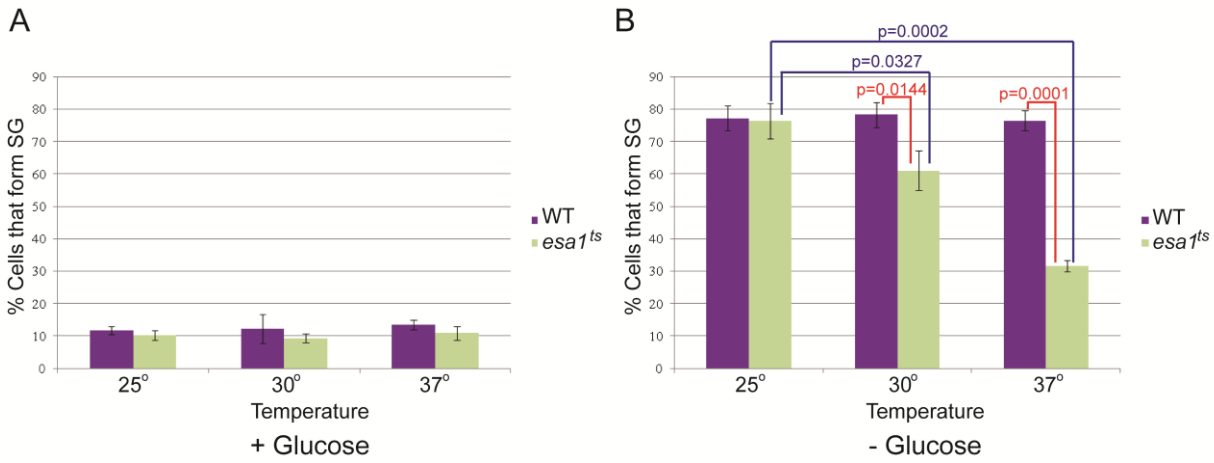

A
% Cells that form SG
90
80
70
60
50
40
30
20
10
0
25°
30°
37°
Temperature
+ Glucose
WT
esa1ts
B
p=0.0002
p=0.0327
p=0.0144
p=0.0001
% Cells that form SG
25°
30°
37°
Temperature
- Glucose
WT
esa1ts

Figure 5. NuA4 catalytic activity is required for Pab1-GFP assembly into glucose deprivation stress granules (GD SG). WT (YKB 1079) *and esa1^{ts}* (YKB3855) strains expressing Pab1-GFP from its endogenous genomic location, were grown at permissive (25^{O}C), semi-permissive (30^{O}C), and restrictive (37^{O}C) temperatures and subjected to glucose deprivation for 10 minutes (-glucose) and stress granules were quantified in a blind manner using Image J software. **A)** Graph indicating the % of cells with SG foci in unstressed conditions (+ Glucose). **B)** Graph indicating the % of cells with SG foci in stressed conditions (- Glucose). p-values computed using unpaired t-tests against WT. Error bars indicate SD. 3 replicates of n ≥ 300 for a minimum of 900 cells total**.** WT=Wild-type. SG=Stress Granule . SD= Standard Deviation.

3.2 NuA4 is required for GD Stress Granule Assembly

To determine if in addition to regulating the localization of Pab1-GFP to SGs upon GD, NuA4 impacts overall GD SG formation I examined the localization of endogenously tagged core stress granule proteins Pbp1-GFP, Pub1-GFP along with endogenously tagged Pab1-GFP ((Hoyle, Castelli et al. 2007, Buchan, Muhlrad et al. 2008) in wild-type, *eaf1Δ* and *eaf7Δ* strains. I determined that NuA4 mutant cells display a decreased ability to form Pub1-GFP, Pbp1-GFP and Pab1-GFP foci upon GD ($p<0.05$) (**Figure 6**). Collectively, with the work presented in section 3.1, my data demonstrates that NuA4 is not just impacting Pab1 localization to cytoplasmic foci, but is likely required for SG assembly upon GD.

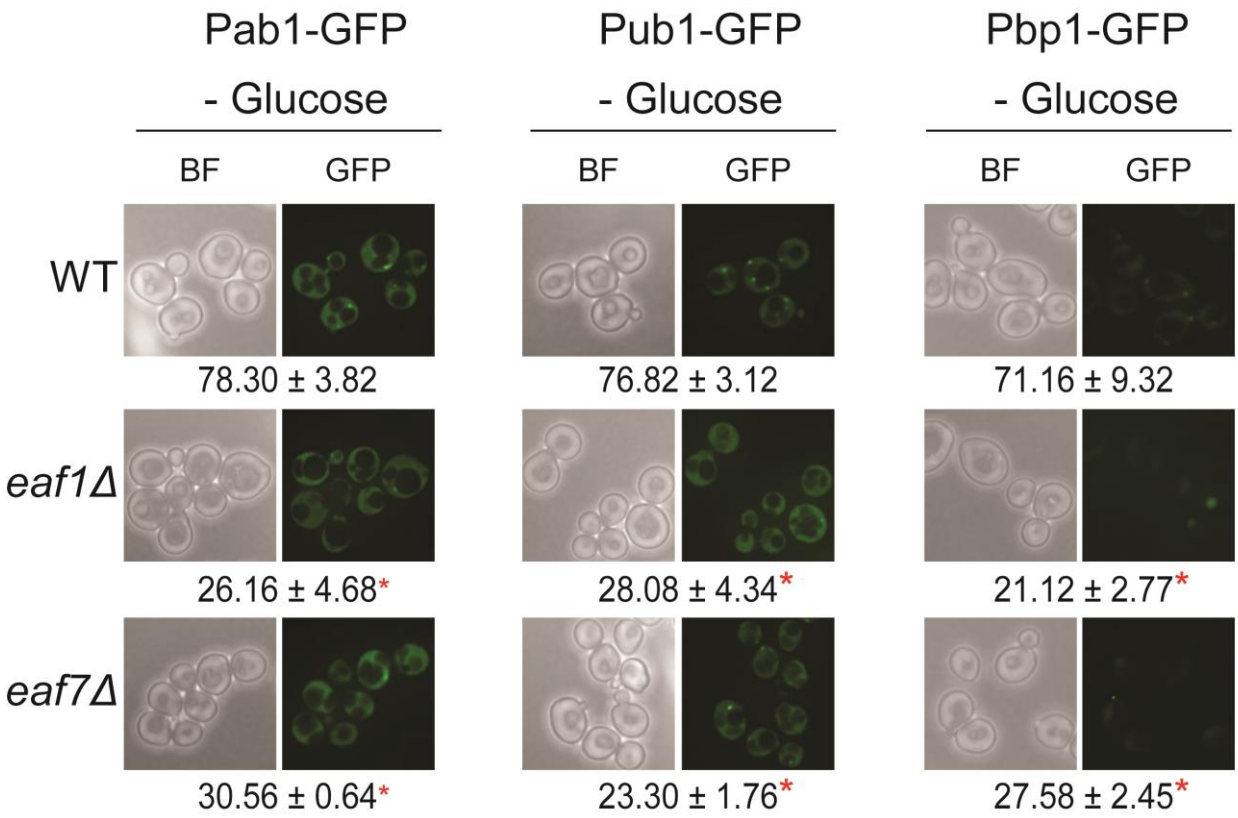

Pab1-GFP
- Glucose
Pub1-GFP
- Glucose
Pbp1-GFP
- Glucose
BF
GFP
BF
GFP
BF
GFP
WT
78.30 ± 3.82
76.82 ± 3.12
71.16 ± 9.32
eaf1Δ
26.16 ± 4.68*
28.08 ± 4.34*
21.12 ± 2.77*
eaf7Δ
30.56 ± 0.64*
23.30 ± 1.76*
27.58 ± 2.45*

Figure 6. NuA4 is required for glucose deprivation stress granule (GD SG) assembly. WT, *eaf1Δ,* and *eaf7Δ* strains expressing Pab1-GFP[WT(YKB3114), *eaf7Δ*(YKB3336), *eaf1Δ* (YKB3382)], Pub1-GFP [WT(YKB3115), *eaf7Δ* (YKB3337), *eaf1Δ* (YKB3339)] and Pbp1-GFP [WT(YKB3258), *eaf7Δ* (YKB3335), *eaf1Δ* (YKB3338)] from their endogenous genomic location, were subjected to glucose deprivation for 10 minutes (-glucose) and stress granules were quantified in a blind manner using Image J software. Representative Brightfield (BF) and GFP images are shown and the % of cells with SG foci ± SD is indicated beneath. 3 replicates of n ≥ 300 for a minimum of 900 cells total . p-values computed using unpaired t-tests against WT. *p<0.05. WT=Wild-type. SG=Stress Granule .SD=Standard Deviation.

3.3 NuA4 mildly affects SG formation in response to NaN_3 exposure and Heat Stress but not Ethanol Stress

Though I have discovered a novel regulatory link between NuA4 and GD SG assembly, I next sought to determine if NuA4 impacts SG formation upon additional environmental stressors. I subjected wild type (WT), *eaf1Δ* and *eaf7Δ* cells transformed with a plasmid containing Pab1-GFP (Swisher and Parker 2010) to heat stress (HS), ethanol (EtOH), and sodium azide (NaN_3) treatment and monitored the cellular localization of Pab1-GFP as previously described (Buchan et al 2010). Analysis reveals that NuA4 mildly affects the formation of SGs in response to heat stress and sodium azide treatment, but has no effect on SG formation in response to ethanol (EtOH) stress (**Figure 7**). Specifically, NuA4 mutants exposed to a heat stress of 46^{O}C and 0.5% NaN_3 treatment show a modestly decreased and increased ability to form SG respectively. Together this shows that NuA4 may have multiple roles in SG dynamics including, but not limited to: the inhibition of SG formation upon NaN_3 stress, and the regulation of SG assembly upon HS and GD.

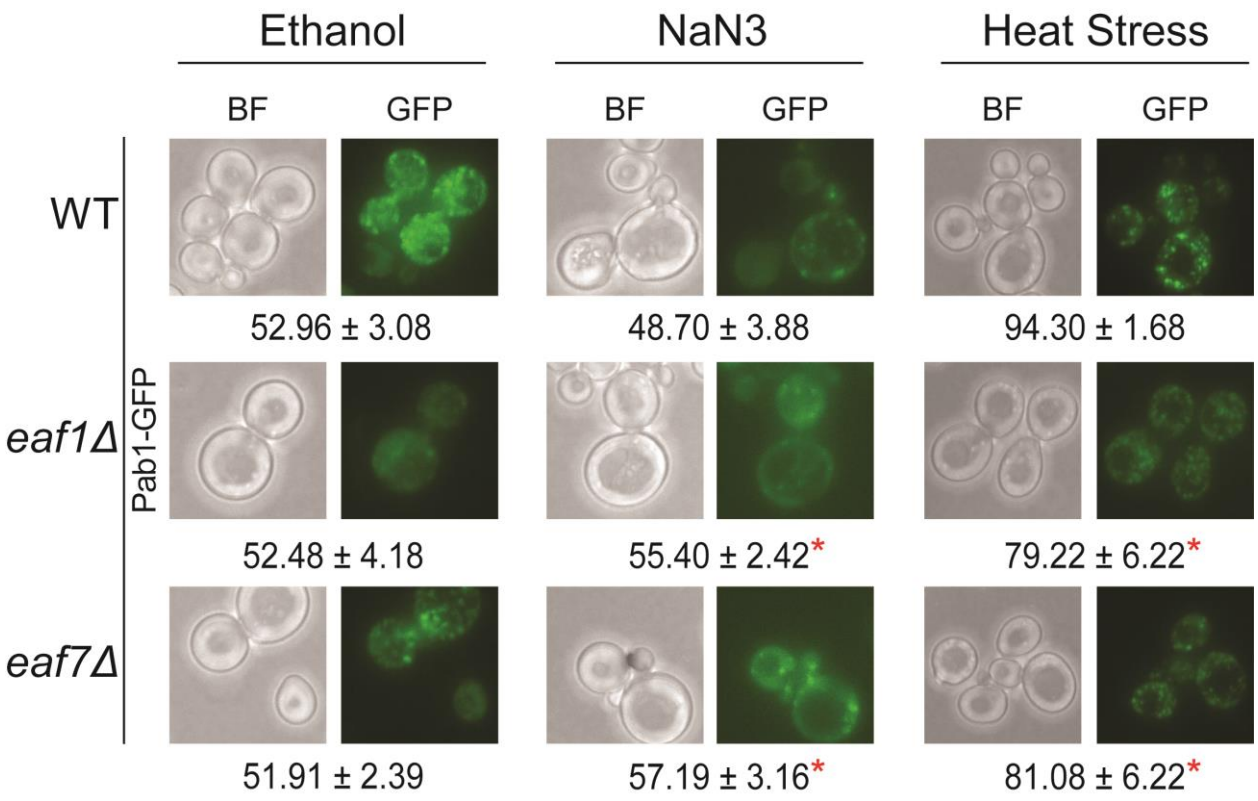

Ethanol
NaN3
Heat Stress
BF
GFP
BF
GFP
BF
GFP
WT
Pab1-GFP
52.96 ± 3.08
48.70 ± 3.88
94.30 ± 1.68
eaf1Δ
52.48 ± 4.18
55.40 ± 2.42*
79.22 ± 6.22*
eaf7Δ
51.91 ± 2.39
57.19 ± 3.16*
81.08 ± 6.22*

Figure 7. SG formation in response to Ethanol, NaN_3, and Heat Stress. WT (YKB1079), *eaf1Δ* (YKB3453), and *eaf7Δ* (YKB3292) cells were transformed with a *PAB1-GFP::URA::CEN* plasmid (PKB192) and exposed to Ethanol (15% Ethanol), NaN_3 (0.5% NaN_3), or Heat Stress (46°) for 30 minutes. Representative Brightfield (BF) and GFP images are shown and the % of cells with SG foci ± SD is indicated beneath. SGs were quantified in a blind manner using Image J software. 3 replicates of n ≥ 300 for a minimum of 900 cells total. p-values computed using unpaired t-tests against WT. * $p < 0.06$. WT=Wlid-type. SG=Stress Granule. SD=Standard Deviation. NaN_3=sodium azide.

3.4 Multiple KATs and KDACs inhibit SG formation, but only NuA4 and Gcn5 are required for GD SG assembly

To determine if other KAT/KDACs in *S. cerevisiae* play a role in GD SG formation I screened a library of single non-essential KAT and KDAC mutants (**Table 3**). The KAT/KDAC library was transformed with a Pab1-GFP expressing plasmid and screened for SG under both glucose replete and depleted conditions. My screen revealed that NuA4 is the main KAT involved in GD SG formation. Interestingly, an additional KAT Gcn5 shows a modest, yet statistically significant decrease in SG formation ($p<0.05$). Remarkably, this screen reveals that KDACs may have a role in repressing SG formation under non-stress conditions. When grown in media containing glucose *hos3Δ* and *hst4Δ* KDAC single deletion strains show a decreased ability to repress SG formation compared to WT cells ($p<0.06$) (**Table 3**). However, it is important to note that these cells do not show any statistically differences in GD SG assembly from WT cells. Interestingly, similar results were identified in a recently published screen showing that some yeast KAT and KDAC deletion mutants display constitutive SGs under non-stressed conditions (Buchan, Kolaitis et al. 2013). Together, my and the Buchan ((Buchan, Kolaitis et al. 2013)) systematic analyses reveal that while numerous KDACs are required to repress SG assembly under non-stress conditions, my screen determines that NuA4 is the primary KAT required for GD SG formation.

KDAC mutant *rpd3Δ* , which was specifically identified by the Buchan screen (Buchan, Kolaitis et al. 2013), displays the greatest increase in SG formation under non-stress conditions. Further, many other phenotypes of NuA4 mutants can be rescued by deletion of *RPD3* which suggests that Rpd3 is the KDAC opposing NuA4 KAT activity for many acetylation sites (Biswas, Takahata et al. 2008). Hence, I sought to determine if deletion of *RPD3* (increased

acetylation) rescues the GD SG defect observed in *eaf7Δ* cells (Section 3.2). I generated single (*rpd3Δ, eaf7Δ*) and double mutants (*eaf7Δ rpd3Δ)* in which the endogenous Pab1 is GFP-tagged and assessed SG assembly under both glucose and glucose deprived conditions. I determined that deletion of *RPD3* suppresses the GD SG formation defect observed in the NuA4 mutant *eaf7Δ* (**Figure 8**) suggesting that Rpd3 promotes SG disassembly by deacetylating NuA4-dependent acetylation sites that mediate SG assembly. Alternatively, Rpd3 may be the main KDAC involved in repressing GD SG formation.

Strain	+ Glucose		– Glucose 10 min	
	% SG	Unpaired t-test	% SG	Unpaired t-test
WT	10.21 ± 1.38	*p-value	69.73 ± 12.3	*p-value
*pbp1Δ**	12.98 ± 5.69	0.4586	32.5 ± 9.00	0.0134
*pub1Δ**	17.71 ± 10.43	0.2845	40.58 ± 3.61	0.0170
eaf1Δ	13.67 ± 9.47	0.5651	32.13 ± 13.46	0.0233
eaf7Δ	10.81 ± 4.06	0.8204	24.99 ± 3.75	0.0038
eaf5Δ	11.62 ± 2.43	0.4315	27.99 ± 3.38	0.0048
eaf3Δ	10.06 ± 1.40	0.9012	26.25 ± 8.58	0.0074
spt10Δ	20.66 ± 13.54	0.2543	66.20 ± 18.34	0.7956
gcn5Δ	21.49 ± 11.61	0.1700	45.15 ± 7.65	0.0424
rtt109Δ	16.25 ± 6.52	0.1916	60.94 ± 11.05	0.4093
elp3Δ	16.05 ± 4.88	0.1168	57.12 ± 4.49	0.1706
hat1Δ	17.64 ± 6.21	0.1131	58.77 ± 12.35	0.3373
sas2Δ	17.31 ± 8.72	0.2361	60.27 ± 4.81	0.2825
sas3Δ	14.74 ± 5.38	0.2306	56.63 ± 10.95	0.2403
hpa2Δ	16.95 ± 7.13	0.1832	60.35 ± 15.07	0.4506
hpa3Δ	15.53 ± 4.62	0.1286	72.13 ± 9.88	0.8052
atf2Δ	14.52 ± 10.53	0.5209	73.53 ± 11.67	0.7177
rpd3Δ	26.49 ± 13.10	0.0990	63.93 ± 11.92	0.5890
hos1Δ	16.33 ± 4.23	0.0758	64.11 ± 8.59	0.5518
hos2Δ	12.67 ± 1.30	0.0879	67.66 ± 5.30	0.8022
hos3Δ	24.67 ± 5.58	0.0121	60.11 ± 13.06	0.4056
sir2Δ	17.59 ± 7.23	0.1575	65.14 ± 6.38	0.5969
hst1Δ	16.94 ± 4.70	0.0760	58.75 ± 6.16	0.2390
hst2Δ	18.01 ± 8.83	0.2052	66.12 ± 4.45	0.6576
hst3Δ	17.31 ± 8.65	0.2330	61.66 ± 6.17	0.3672
hst4Δ	18.37 ± 5.25	0.0598	65.20 ± 11.97	0.6713
hda1Δ	13.79 ± 6.85	0.4250	58.57 ± 1.14	0.1927

Table 3. Multiple KATs and KDACs inhibit stress granule formation, but only NuA4 and Gcn5 are required for glucose deprivation stress granule (GD SG) assembly. WT (YKB1079), *pbp1⊿* (YKB3217), *pub1⊿* (YKB3218) and indicated KAT (blue) and KDAC (green) mutants (see **Table B1**) were transformed with a *PAB1-GFP::URA::CEN* plasmid (pKB192) and subjected to glucose deprivation for 10 minutes (-glucose) or control conditions (+glucose). 3 replicates of n ≥ 300 for a minimum of 900 cells total. Stress granules were quantified in a blind manner using Image J software. %SG indicates average % of cells with SG ± SD. Unpaired t-tests were performed against WT for each condition, and p-value for the 95% confidence is listed. Any mutant displaying a 2-fold change and/or a p-value <0.06 is highlighted in yellow. * indicates control cells. WT=Wild-type. SG=Stress Granule. SD=Standard Deviation. KAT=Lysine Acetyltransferase. KDAC=Lysine Deacetylase.

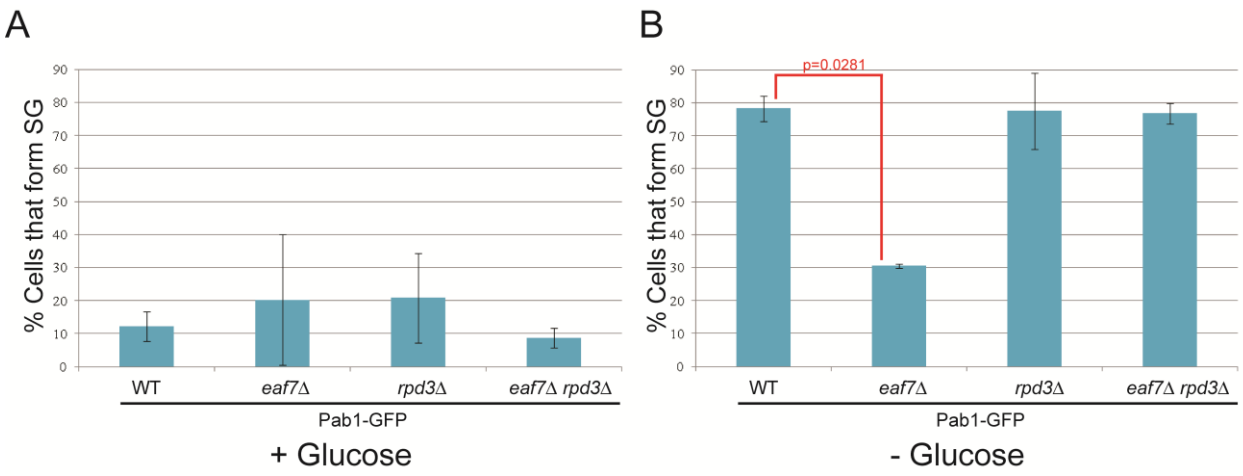

A
% Cells that form SG
90
80
70
60
50
40
30
20
10
0
WT
eaf7Δ
rpd3Δ
eaf7Δ rpd3Δ
Pab1-GFP
+ Glucose
B
p=0.0281
% Cells that form SG
WT
eaf7Δ
rpd3Δ
eaf7Δ rpd3Δ
Pab1-GFP
- Glucose

Figure 8. Deleting the KDAC Rpd3 rescues the glucose deprivation stress granule (GD SG) assembly defect of *eaf7Δ* cells. WT (YKB3114), single deletion (*eaf7Δ* (YKB3336), *rpd3Δ* (YKB3341)), and double deletion (*eaf7Δ rpd3Δ* (YKB3853)) strains expressing Pab1-GFP from their endogenous genomic location, were subjected to glucose deprivation for 10 minutes (-Glucose) or control conditions (+Glucose) and stress granules were quantified in a blind manner using Image J software. **A)** Graph indicating the % of cells with SG foci in unstressed conditions (+ Glucose). **B)** Graph indicating the % of cells with SG foci in stressed conditions (- Glucose). p-values computed using unpaired t-tests against WT. Error bars indicate SD. 3 replicates of n ≥ 300 for a minimum of 900 cells total. WT=Wild-type. SG=Stress Granule . SD= Standard Deviation.

3.5 NuA4 does not regulate processing body assembly in response to glucose deprivation

To determine if in addition to regulating GD SG assembly NuA4 mediates PB formation I endogenously tagged two known PB proteins (Edc3 and Lsm1 (Ingelfinger, Arndt-Jovin et al. 2002, Fenger-Gron, Fillman et al. 2005)) with a green fluorescent tag (GFP) in both wild-type and *eaf7Δ* backgrounds. Subjection of these cells to a 10 minute glucose deprivation followed by immediate imaging (as described for SG) reveals that although GD results in an increase in PB foci (Buchan, Kolaitis et al. 2013), deletion of *EAF7* has no impact on either Lsm1-GFP or Edc3-GFP cytoplasmic foci (PB) formation (**Figure 9**). Indeed this finding supports recent literature in yeast showing that SGs and PBs are distinct structures that form independently (reviewed in (Stoecklin and Kedersha 2013)). Hence similar to PKA which is required for PB formation, but not SG formation ((Tudisca, Simpson et al. 2012)), NuA4 only impacts GD SG assembly further solidifying the idea that though these foci do at points colocalize, that their assembly is through distinct pathways.

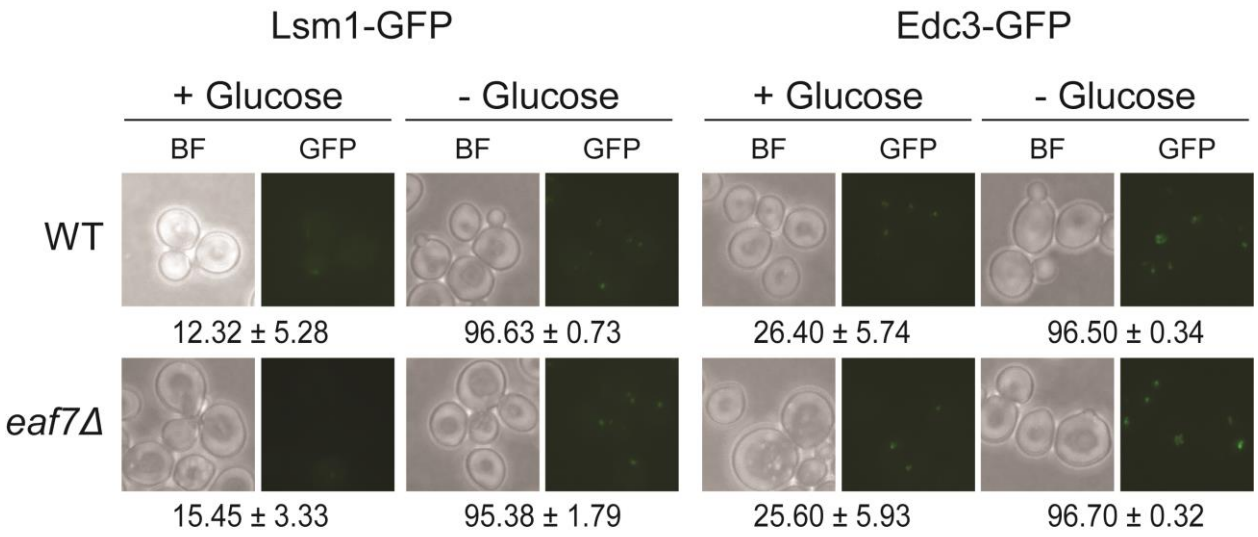
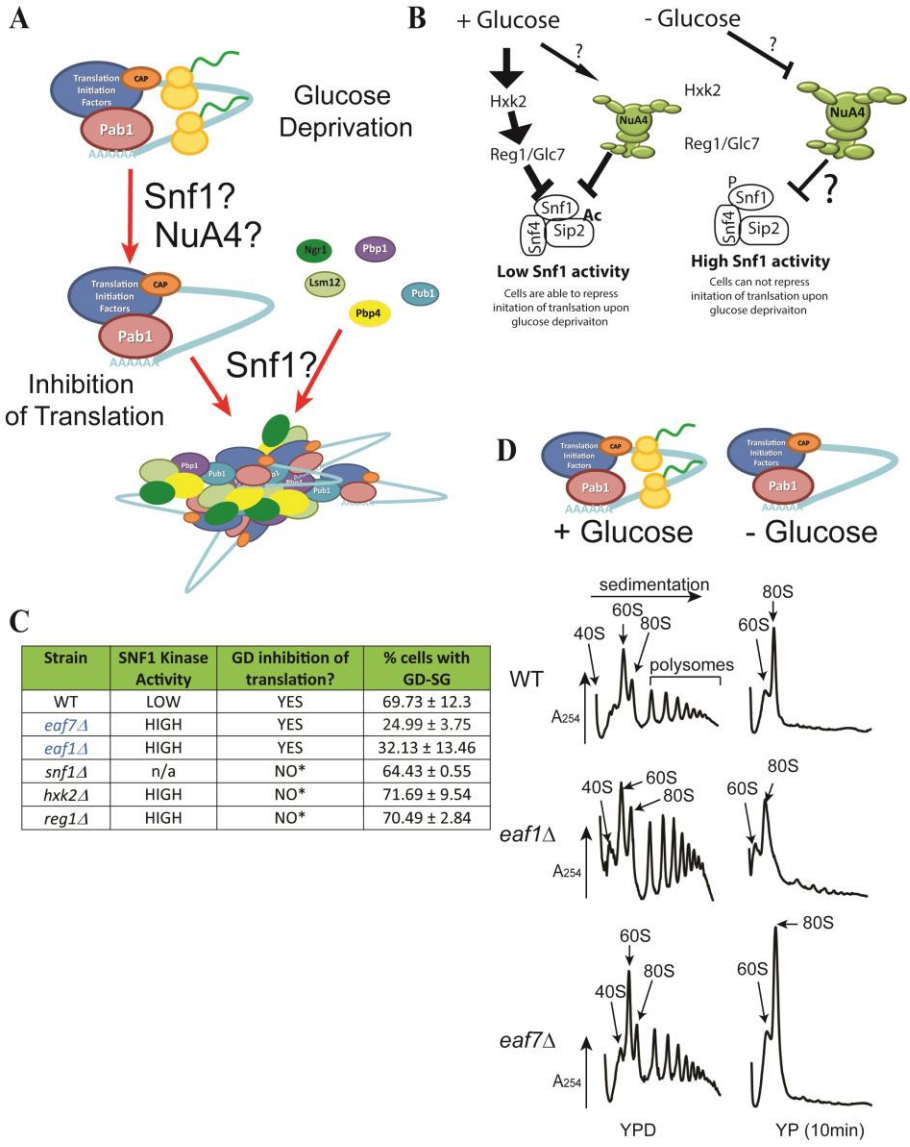

Lsm1-GFP
Edc3-GFP
+ Glucose
- Glucose
+ Glucose
- Glucose
BF
GFP
BF
GFP
BF
GFP
BF
GFP
WT
12.32 ± 5.28
96.63 ± 0.73
26.40 ± 5.74
96.50 ± 0.34
eaf7Δ
15.45 ± 3.33
95.38 ± 1.79
25.60 ± 5.93
96.70 ± 0.32

Figure 9. NuA4 does not regulate processing body assembly in response to glucose deprivation (GD). WT and *eaf7Δ* strains expressing Lsm1-GFP [WT(YKB3710), *eaf7Δ* (YKB3718)] and Edc3-GFP [WT(YKB3711), *eaf7Δ* (YKB3743)] from their endogenous genomic location, were subjected to glucose deprivation for 10 minutes (-glucose) and processing bodies were quantified in a blind manner using Image J software as described (Kedersha, Tisdale et al. 2008). Representative Brightfield (BF) and GFP images are shown and the % of cells with PB foci ± SD is indicated beneath. 3 replicates of n ≥ 300 for a minimum of 900 cells total. p-values computed using unpaired t-tests against WT (data not shown). WT=Wild-type. SD=Standard Deviation. PB= Processing Body.

3.6 NuA4 does not regulate stress granule formation through the inhibition of translation initiation or the SNF1 pathway

Despite growing evidence in yeast that SGs are sites of non-canonical translation (reviewed in (Buchan and Parker 2009)), traditional models indicate that in order for mRNA to assemble into SGs translation initiation must be stalled. To elucidate whether the decrease in GD SG formation observed in NuA4 mutants is due to an inhibitory role in translation initiation I examined if deletion of *EAF1* or *EAF7* affects bulk translation by ribosomal profiling. Polysomal analysis completed by Dr Sylvain Huard reveals that like WT cells, NuA4 mutants are capable of stalling translation initiation in response to a 10 minute glucose deprivation (**Figure 10D**). Therefore, NuA4 does not mediate GD SG formation through the inhibition of translation initiation.

Of the three primary glucose sensing and signalling pathways in yeast (PKA, TOR, and SNF1 (**Figure 2**)) TOR and PKA do not to regulate GD SG formation (Tudisca, Simpson et al. 2012, Shah, Zhang et al. 2013). Intriguingly, the final glucose sensing pathway (SNF1/AMPK) is regulated by NuA4 depending on glucose abundance. Specifically, NuA4 acetylates Sip2, the regulatory subunit of Snf1, in glucose conditions leading to Snf1 inhibition (**Figure 2**) (Lu et al 2011). To test if the SNF1 pathway is required for GD SG formation, I asked if *hxk2Δ*, *reg1Δ* and *snf1Δ* mutants impact Pab1-GFP foci formation upon GD. In *hxk2Δ* and *reg1Δ* mutant cells, Snf1 activity is elevated (Ashe, De Long et al. 2000); but *snf1Δ* cells demonstrate no activity. Deletion of these key regulatory proteins in the SNF1 pathway has no effect on SG formation (**Figure 10C**). These experiments imply the SNF1 pathway does not regulate GD SG assembly, indicating that NuA4 regulates GD SG formation independently of its role in the SNF1 pathway.



C

Strain	SNF1 Kinase Activity	GD inhibition of translation?	% cells with GD-SG
WT	LOW	YES	69.73 ± 12.3
eaf7Δ	HIGH	YES	24.99 ± 3.75
eaf1Δ	HIGH	YES	32.13 ± 13.46
snf1Δ	n/a	NO*	64.43 ± 0.55
hxk2Δ	HIGH	NO*	71.69 ± 9.54
reg1Δ	HIGH	NO*	70.49 ± 2.84

Figure 10. The Snf1 pathway is not required for glucose deprivation stress granule (GD SG) assembly and mutants of NuA4 are able to inhibit translation initiation upon glucose deprivation (GD).
A) Schematic model illustrating the hypothesis that Snf1 Kinase or NuA4 could be regulating SG formation through inhibition of translation or that Snf1 has additional targets. **B)** Schematic illustrating the regulation of Snf1 Kinase activity. Mutants with high Snf1 activity do not repress translation initiation upon glucose deprivation (Ashe et al 2000). **C)** Snf1 and its regulators Reg1 and Hxk2 are not required for GD SG assembly. WT (YKB1079), *eaf1Δ* (YKB3453), *eaf7Δ* (YKB3292), *snf1Δ* (YKB3389), *hxk2Δ* (YKB3390), and *reg1Δ* (YKB3388) strains were transformed with *PAB1-GFP::URA::CEN* plasmid (PKB192), subjected to glucose deprivation for 10 minutes (-glucose) and stress granules were quantified in a blind manner using Image J software. %SG indicates average % of cells with SG ± SD. 3 replicates of n ≥ 300 for a minimum of 900 cells total. Inhibition of translation was determined experimentally using ribosomal profiling (Ashe, De Long et al. 2000) **D)** Eaf1 and Eaff7 are not required for inhibition of translation upon GD. Polyribosome traces of WT (YKB1079), ***eaf1Δ*** (YKB3453) and ***eaf7Δ*** (YKB3292) cells that were grown in YPD and resuspended in YP medium lacking (YP) or containing (YPD) glucose for 10 minutes. The peaks that contain the small ribosomal subunit (40S), the large ribosomal subunit (60S), and both subunits (80S) are indicated by arrows. The polysome peaks are bracketed. The yeast polysome profile has been generated after the lysate has been loaded onto a 15-45% sucrose gradient and centrifuged in a SW41 rotor for 90 min at 39,000 RPM at 4°C. The gradient was then collected from the top, and the A254 was measured continuously to generate the traces. Work of Sylvain Huard. WT=Wild-type. SG=Stress Granule. SD=Standard Deviation.

3.7 Eaf7 and Pab1 co-purify in unstressed and GD cells

As NuA4 is not impacting GD SG assembly through either SNF1 or inhibition of translation, another possibility is that NuA4 is impacting SG assembly directly through interaction and acetylation of SG core proteins. Indeed, the Baetz lab has recently shown that Esa1-TAP can co-purify SG proteins Pab1, Lsm12, Pbp1 and Pbp4 (Mitchell L et al 2013). To confirm this interaction and to determine if the full NuA4 or piccoloNuA4 interact with Pab1, I performed a reciprocal co-Immunoprecipitations with Pab1-TAP and Eaf7-Myc, a subunit of NuA4 not found in piccolo NuA4 (**Figure 11**, **lane 4**). I confirmed that Pab1 co-IPs Eaf7 and hence the larger NuA4 complex. Further, both brief (**Figure 11, lane 5)** and sustained (**Figure 11, lane 6-7**) glucose deprivation results in an increased interaction between Eaf7 and Pab1. This suggests that upon GD NuA4 may have an important and long-lasting role in regulating Pab1.

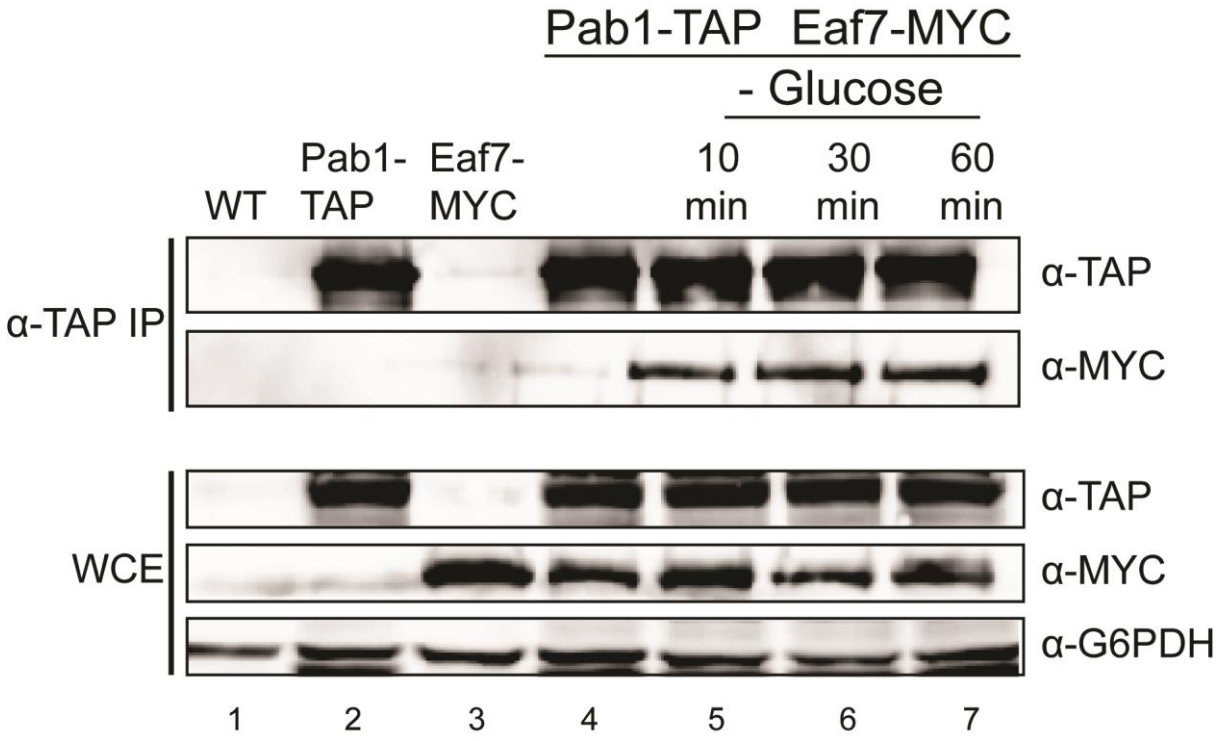

Pab1-TAP Eaf7-MYC
- Glucose
WT
Pab1-TAP
Eaf7-MYC
10 min
30 min
60 min
α-TAP IP
α-TAP
α-MYC
WCE
α-TAP
α-MYC
α-G6PDH
1
2
3
4
5
6
7

Figure 11. Pab1- Eaf7 Interaction increases upon glucose deprivation (GD). C-terminally TAP tagged Pab1 was immunopurified from WT (YKB 1079), control strains (*PAB1-TAP* (YKB1194), *EAF7-MYC* (YKB518)), and a strain background containing C-terminally MYC tagged Eaf7 (*PAB1-TAP EAF7-MYC* (YKB1195)) under unstressed and stressed (- Glucose 10-60 minute) conditions. Immunopreciptations (IPs) and whole cell extracts (WCEs) were analysed by Western blot and probed with anti-TAP (α-TAP), MYC (α-MYC), or glyceraldehyde-6-dehydrogenase (α -G6DPH) antibodies. 90% of the eluted IP sample was loaded for α-MYC probing, while 10% was loaded for probing with α-TAP. 50ug WCE was loaded for probing with α-TAP and α-G6PDH, while 150ug WCE was loaded for probing with α-MYC. Experiment presented is one of 6 replicates which all displayed similar results. WT=Wild-type.

3.8 NuA4 regulates GD SG assembly independently of its role in Pab1 protein level control

Western blot analysis of the whole cell extracts from wild-type and NuA4 mutant cells reveals that NuA4 impacts Pab1-GFP protein levels (**Figure 12A lane 3, 11B; Figure A4**). Interestingly, q-RT-PCR analysis completed by Dr. Sylvain Huard indicates that NuA4 mutants *eaf1Δ* and *eaf7Δ* do not affect *PAB1* mRNA expression (**Figure 12C**). Taken together this data suggests that NuA4 may regulate GD SG assembly by directly impacting Pab1 protein levels. To determine if this is the case I transformed WT, Pab1-GFP, and Pab1-GFP *eaf7Δ* cells with a Pab1-GFP plasmid to over-express Pab1. After completing a western blot to ensure Pab1 protein over-expression under standard glucose conditions (**Figure 13A and B; Figure A3A and B**), I subjected these transformed cells to a 10 minute GD followed by microscopy to determine if increased Pab1 protein levels can rescue the SG assembly defect observed in NuA4 mutant cells. Analysis reveals that although the over-expression of Pab1 protein increases the percentage of cells that form SG in both unstressed (+Glucose **Figure 13C; Figure A3C**) and stressed (-Glucose, **Figure 13D; Figure A3D**) conditions, it does not rescue the defect observed in *eaf7Δ* cells ($p<0.06$) (**Figure 13D; Figure A3D**). Collectively, this data suggests that the regulation of GD SG formation by NuA4 is independent of its role in Pab1 protein level control.

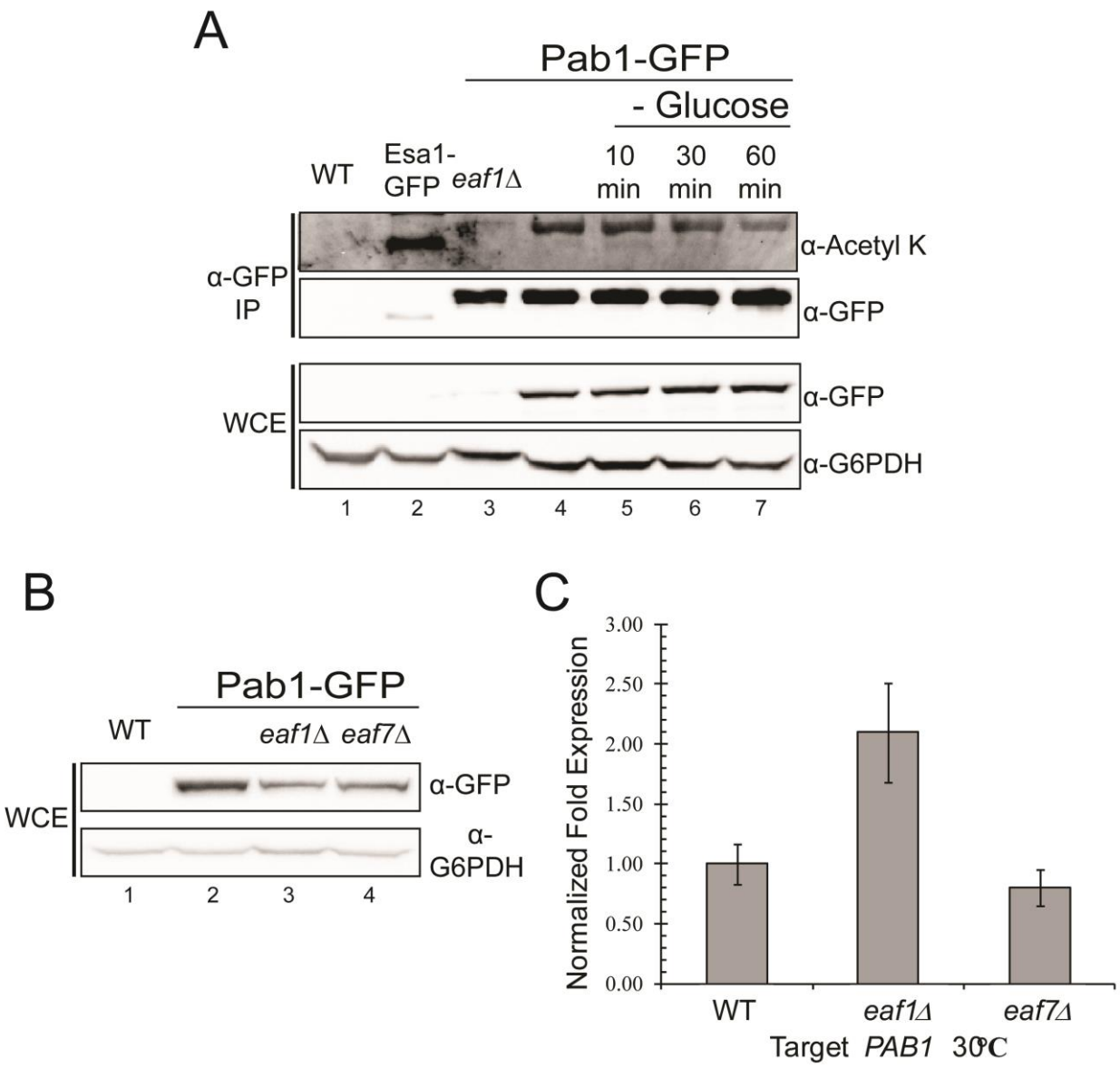

A
Pab1-GFP
- Glucose
WT
Esa1-GFP
eaf1Δ
10 min
30 min
60 min
α-GFP IP
α-Acetyl K
α-GFP
WCE
α-GFP
α-G6PDH
1 2 3 4 5 6 7
B
Pab1-GFP
WT
eaf1Δ eaf7Δ
WCE
α-GFP
α-G6PDH
1 2 3 4
C
Normalized Fold Expression
3.00
2.50
2.00
1.50
1.00
0.50
0.00
WT
eaf1Δ
eaf7Δ
Target PAB1 30℃

Figure 12. The *in-vivo* acetylation and protein level of Pab1 is dependent on NuA4. **A)** Pab1 *in-vivo* acetylation state is dependent on Eaf1 and decreases upon prolonged glucose starvation. GFP Trap precipitation of WT (YKB 1079), *ESA1-GFP* (YKB2002), *PAB1-GFP eaf1Δ* (YKB3382), and *PAB1-GF*P (YKB3114)], followed by western blotting to probe for acetylated lysine residues (α-Acetyl K) (90% IP) and α-GFP (10% IP), and α-G6DPH (50ug WCE) and α-GFP (75ug WCE). **B)** NuA4 regulates Pab1-GFP protein levels. 50ug WCE from the indicated strains [WT (YKB1079), *PAB1-GFP eaf1Δ* (YKB3382), and *PAB1-GFP eaf7Δ* (YKB3336)] were separated by SDS-PAGE and Western blot analysis was performed using the indicated antibodies. Images shown in Panels A-C are representative of three replicates. **C)** *PAB1* mRNA expression is not decreased in *eaf1*Δ or *eaf7*Δ mutants. Yeast cells were harvested at mid-log phase from cultures grown at 30°C. mRNA was isolated and *PAB1* mRNA level was assessed by quantitative PCR and standardized to the WT *PAB1* transcription level. *PAB1* mRNA expression was normalized to the reference gene *TDH3*. Three biological replicates were performed with technical duplicates. Work of Sylvain Huard. WT=Wild-type. IP=Immunoprecipitation. WCE=Whole Cell Extract.

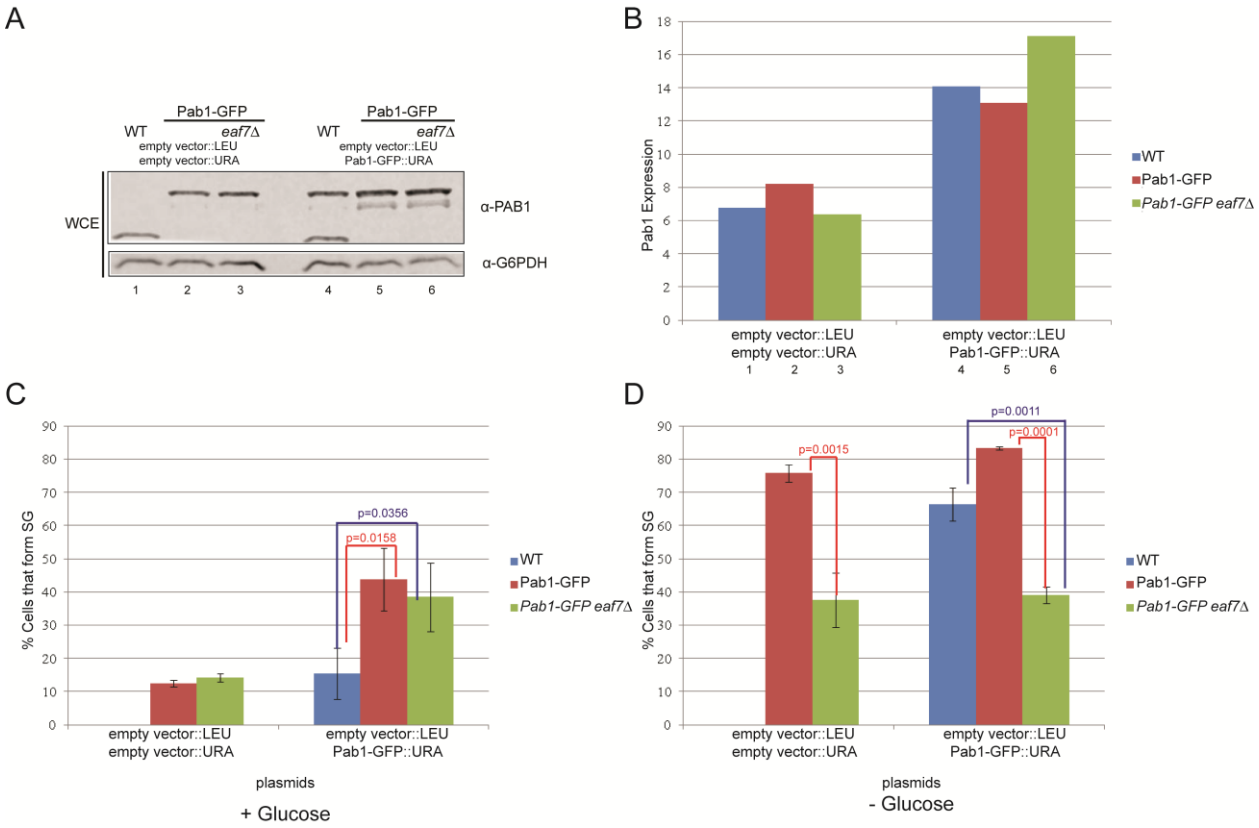

A
Pab1-GFP
WT
eaf7Δ
empty vector::LEU
empty vector::URA
Pab1-GFP
WT
eaf7Δ
empty vector::LEU
Pab1-GFP::URA
WCE
α-PAB1
α-G6PDH
1 2 3 4 5 6
B
Pab1 Expression
empty vector::LEU
empty vector::URA
1 2 3
empty vector::LEU
Pab1-GFP::URA
4 5 6
WT
Pab1-GFP
Pab1-GFP eaf7Δ
C
% Cells that form SG
p=0.0356
p=0.0158
empty vector::LEU
empty vector::URA
empty vector::LEU
Pab1-GFP::URA
plasmids
+ Glucose
WT
Pab1-GFP
Pab1-GFP eaf7Δ
D
% Cells that form SG
p=0.0011
p=0.0001
p=0.0015
empty vector::LEU
empty vector::URA
empty vector::LEU
Pab1-GFP::URA
plasmids
- Glucose
WT
Pab1-GFP
Pab1-GFP eaf7Δ

Figure 13. Pab1 overexpression does not rescue the glucose deprivation stress granule (GD SG) assembly defect of *eaf7Δ* cells. Wild-type (WT (YKB 1079)), *PAB1-GFP* (YKB3114), and *PAB1-GFP eaf7Δ* (YKB3336) strains were transformed with *PAB1-GFP::CEN::URA* (PKB192) plasmids to over-express Pab1 and empty *LEU* (PKB21) and empty *URA* (PKB23) plasmids as controls. **A)** Western blot showing Pab1 protein expression in transformed cells. Blots imaged using LI-COR software. **B)** Graph displaying quantified Pab1 protein expression from the blot shown in A. Signal intensity was calculated using Image Studio software and Pab1 expression was determined by normalizing Pab1 signal to loading controls. 50ug WCE was loaded and run on 7.5% gels and probed with α-Pab1 and α -G6PDH antibodies. **C)** Graph indicating the % of cells with SG foci in unstressed conditions (+ Glucose). p-values computed using unpaired t-tests against WT cells. Error bars indicate SD. 3 replicates of n ≥ 300 for a minimum of 900 cells total. Stress granules were quantified in a blind manner using Image J software. **D)** Graph indicating the % of cells with SG foci in stressed conditions (- Glucose). p-values computed using unpaired t-tests against WT cells. Error bars indicate SD. 3 replicates of n ≥ 300 for a minimum of 900 cells total. Stress granules were quantified in a blind manner using Image J software. WT=Wild-type. SG=Stress Granule. SD=Standard Deviation. WCE=Whole Cell Extract.

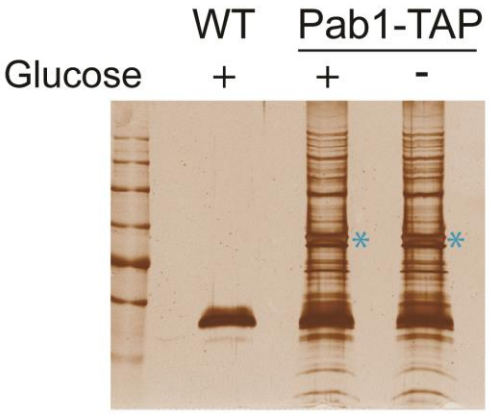
3.9 Pab1 in vivo acetylation state is dependent on NuA4

Existing literature indicates that Pab1 is acetylated and that it is an *in-vitro* substrate of NuA4 (Choudhary, Kumar et al. 2009, Mitchell, Huard et al. 2013). To determine if Pab1 *in-vivo* acetylation state and levels are dependent on NuA4 or GD I performed GFP-immunoprecipitations of WT, Esa1-GFP (acetylation control (Mitchell, Huard et al. 2013)), and Pab1-GFP cells. Resolution of the purified proteins by SDS-PAGE followed by western blotting probing for acetylated lysine residues verified that Pab1-GFP is acetylated. Likewise, analysis reveals that the *in-vivo* acetylation state of Pab1 is dependent on NuA4 (**Figure 12A lane 3**). Interestingly, the acetylation level of Pab1-GFP decreases upon prolonged glucose deprivation stress exposure (**Figure 12A lanes 5-7**). In contrast, although acetylation was detected by Western blot analysis on Pub1, Pbp4 and Pbp1, but not Lsm12, these acetylations were not dependent on NuA4 (Personal communication of Dr. Huard, data not shown). Collectively this data indicates that multiple SG proteins are acetylated, but only the acetylations on Pab1 are dependent on NuA4.

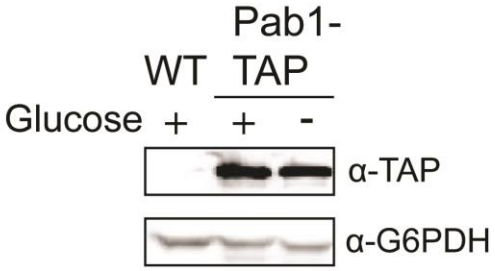
To further verify if SG proteins are acetylated and to determine if GD has an effect, I TAP (Tandem Affinity Purification) tagged and subsequently purified SG proteins for mass spectrometry (**Figure 14A** and **B**). Additional acetylation sites on Pab1 were identified (**Figure 14C**). Interestingly, two of these acetylation sites (K164 and K504) are glucose dependent. In the presence of glucose (+Glucose) Pab1 is acetylated at K164, however upon GD (-Glucose) Pab1 becomes acetylated at K504, and loses the K164 acetylation (**Figure 14C**). Together with existing data, my experiments reveal that SG proteins are acetylated and that Pab1 is an *in-vivo* and *in-vitro* target of NuA4. Although additional experiments are required to elucidate the

biological consequences of Pab1 acetylation, the data presented in this thesis suggest that NuA4 mediated acetylation of Pab1 may be involved in GD SG assembly.

A



B



C

Protein	Acetylation Site		
	+ Glucose	-Glucose 30 min	Both Conditions
Pab1	K164	K504	K7

D

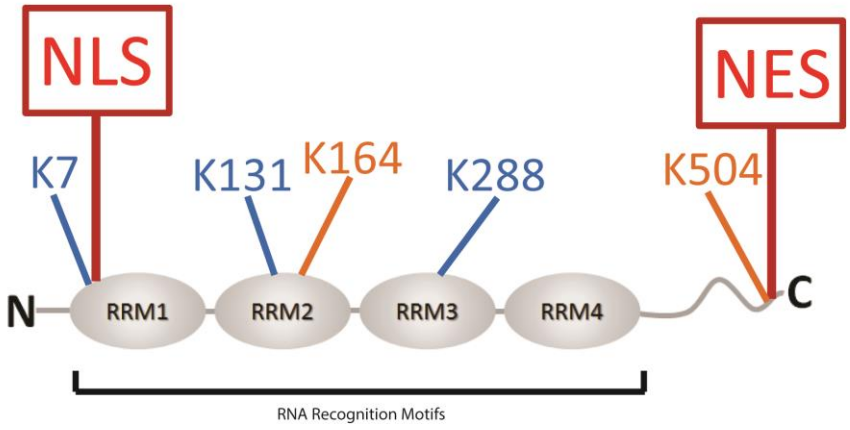


Figure 14. Pab1 acetylation sites identified by mass spectrometry. A and **B)** WT (YKB799) and *PAB1-TAP* (YKB1194) were mChIPed from 250mL of log phase cells grown in YPD and resuspended in YP medium lacking (-Glucose) or containing (+Glucose) for 10 minutes. 95% of the Immunoprecipitations (IP) were resolved on SDS-PAGE and analyzed by silver stain **(A)** and 5% was analyzed by Western (α-TAP probes IP; α-G6PDH the extracts pre-IP to ensure equivalents amount used) **(B)**. Each Pab1-TAP IP lane of the silver stain gel was sliced into 8 fractions and MS was performed to identify proteins and acetylations. A blue asterisk (*) Identifies Pab1-TAP on the silver stain. **C)** Table of additional acetylation sites identified by Mass Spectrometry. **D)** Cartoon schematic of Pab1 depicting lysine acetylation sites identified by mass spectrometry (K7, K164, K504), and two additional acetylation sites which are conserved with lysine residues on human PABP (K131 and K288) (Choudhary, Kumar et al. 2009, Henriksen, Wagner et al. 2012). Sites highlighted in orange are glucose dependent. IP=Immunoprecipitation. WCE=Whole cell extract. WT=Wild-type.

4. Discussion

Exposure of eukaryotic cells to environmental stress triggers the suppression of mRNA translation initiation followed by the rapid and reversible reorganization of mRNPs into distinct cytoplasmic SGs (reviewed in (Anderson and Kedersha 2006)). Recently, it has been shown that the *S. cerevisiae* acetyltransferase NuA4 is capable of co-purifying several SG proteins. Moreover, Pab1, a SG component, was identified as being an *in-vitro* acetylation target of NuA4 (Mitchell, Huard et al. 2013). Likewise, several groups have identified acetylation sites on several SG components, including Pab1, in both yeast and mammals (Choudhary, Kumar et al. 2009, Henriksen, Wagner et al. 2012) suggesting that acetylation of Pab1 may regulate some aspects of Pab1 function, including its role in SG assembly. Focussing on Pab1, in this study I not only verify and *in-vivo* interaction between NuA4 and Pab1 (**Figure 11**), but I demonstrate that NuA4 is a novel regulator of SG assembly in response to GD (**Figures 4-6**). Importantly, my work is the first to identify a potential signaling pathway regulating the assembly of SGs in yeast.

4.1 NuA4 is required for GD SG formation.

Despite growing interest in SG assembly, identification of a regulatory mechanism of formation remains elusive. Existing evidence suggests that KATs and/or KDACs may have essential roles in the mRNA lifecycle. For instance, HDAC6 (a KDAC in mammals) has been shown to regulate SG assembly by mediating the movement of SG components along microtubules in 293T cells in response to a variety of stresses (Kwon, Zhang et al. 2007). Likewise, the KDAC SIRT6 has been shown to regulate SG assembly in *C. elegans* and mammals by a yet to be discerned mechanism (Jedrusik-Bode, Studencka et al. 2013). The identification of a physical interaction between NuA4 and SG proteins, including Pab1 (Mitchell, Huard et al. 2013), suggests a possible role for NuA4 in SG dynamics in yeast. Indeed, my study demonstrates that NuA4

mutants display a significant reduction in the percentage of cells that produce SGs in response to 10 minutes of GD (**Figures 4-6**). Specifically, *eaf1Δ*, *eaf7Δ*, and *esa1*^{ts} (grown at restrictive temperatures) mutant cells show an approximately 40% decrease in the ability to form SG in response to GD as compared to WT cells; a reduction similar to that observed in the *pbp1Δ* and *pub1Δ* control cells (Takahara and Maeda 2012).

4.2 How does NuA4 regulate GD SG formation?

Despite the existence of three glucose regulatory and sensing pathways in yeast, TOR and PKA have been shown to not regulate GD SG assembly (Tudisca, Simpson et al. 2012, Shah, Zhang et al. 2013). Likewise, I have shown that the other major glucose sensing pathway SNF1, whose activity is regulated by NuA4, is not involved in GD SG assembly (**Figure 10C**). Traditionally it has been thought that in order for mRNA and its associated proteins to sort into SGs, global translation initiation must be inhibited (Kedersha, Gupta et al. 1999, McEwen, Kedersha et al. 2005). Ribosomal profiling completed by Dr. Sylvain Huard indicates that like WT cells, *eaf1Δ* and *eaf7Δ* mutants are capable of inhibiting global translation initiation in response to 10 minutes of GD (**Figure 10D**). Interestingly, my data suggests that global translation initiation does not have to be suppressed in order for GD SGs to form (**Figure 10C**). This theory supports existing evidence showing that non-canonical translation of anti-apoptosis and stress-related genes still occurs during stress (reviewed in (Buchan and Parker 2009)). Indeed, it has been shown that 10-15% of mRNAs containing active internal ribosome entry sites (IRESs) in cell lines are preferentially and differentially translated upon stress (Spriggs, Stoneley et al. 2008). Further studies will be required to verify this theory and to potentially identify specific mRNA species that are translated during GD.

As NuA4 is not regulating known glucose sensing pathways how else could it be impacting GD-SG assembly? Recently that the Baetz lab determined that NuA4 interacts with several SG proteins including Pab1, Pub1, Lsm12, and Pbp4 (Mitchell, Huard et al. 2013) and determined that Pab1 is an *in-vitro* acetylation target of NuA4. Pab1, the *S. cerevisiae* Poly-A-binding protein, functions to stabilize mRNA and is sorted to SGs in response to a variety of stresses including GD (Sachs, Davis et al. 1987, Swisher and Parker 2010). Collectively with data presented above this suggests that NuA4 may impact SG assembly by interacting with and modifying Pab1; however, more work needs to be completed to determine if this is true. Here I confirmed that Pab1 and Eaf7 (subunit of NuA4) co-purify under non-stress conditions (**Figure 11 lane 4**) and have shown that the interaction between Eaf7 and Pab1 increases upon GD (**Figure 11 lanes 5-7**). Though other SG proteins are acetylated in vivo, only the *in-vivo* acetylation state of Pab1-GFP appears to be dependent on NuA4 (**Figure 12A lane 3**).

Although I reproducibly show an increased co-IP between Eaf7 and Pab1 upon GD (**Figure 11 lanes 5-7**) there does not appear to be a change in Pab1 acetylation state immediately upon GD as detected by anti-acetyl antibodies (**Figure 12 lane 5**). One possible explanation for this is that the antibody used to probe for global acetylation state is not sensitive enough to detect subtle changes in acetylation of specific sites. In fact, my MS data indicates that at least two acetylation sites on Pab1, K164 and K504, are potentially glucose dependent (**Figure 14C**). Therefore, while some lysine residues may in fact become acetylated upon GD, these differences might not be detectable by western blot.

NuA4 mediated acetylation of Pab1 could function to alter Pab1 stability by preventing ubiquitination and subsequent degradation. Alternatively, NuA4-dependent acetylation of Pab1 may be necessary to "prime" Pab1 for efficient incorporation into SGs upon GD and this

acetylation is eventually reversed in order to promote disassembly. Indeed, global Pab1-GFP acetylation state decreases upon prolonged GD when SGs are beginning to disassemble (**Figure 12 lane 6-7**). Though the NuA4-Pab1 interaction is still maintained upon prolonged GD, the decrease in detectable Pab1-acetylation state by western analysis may be due to the counteractivity of KDACs including Rpd3 which may be involved in SG disassembly upon prolonged GD exposure (discussed below).

Despite showing that NuA4 regulates GD SG assembly (**Figures 4-6**), Pab1 protein levels (**Figure 12A, B; Figure A4**), and Pab1 acetylation state (**Figure 13**), these functions appear not to be linked. My experiments show that at least two of these functions: regulation of GD SG assembly and Pab1 protein level control, are independent. Although NuA4 regulates Pab1 protein levels in both endogenously GFP-tagged (**Figure 12A, B; Figure A4**) and Pab1 over-expressing strains (**Figure A3**), live cell fluorescence microscopy using Pab1 over-expressing cells demonstrates that despite an increase in the number of cells that form SG in both unstressed (+ Glucose) and stressed (- Glucose) conditions, this increase is not enough to rescue the defect observed in NuA4 mutant cells (**Figure 13C** and **D; Figure A3 C** and **D**).

If Pab1 protein levels are not impacting SG assembly, how else could NuA4 acetylation of Pab1 be contributing to SG formation? One possible mechanism through which NuA4 regulates GD SG formation is directly through acetylation of SG proteins, and in particular Pab1. If this is the case, acetylation neutralizes the positive charge of lysine residues thereby increasing the likelihood of forming hydrophobic interactions with other SG proteins like Pub1 and Pbp1. Since SG formation is mediated by the self-aggregation of Pub1 and Pbp1 through their prion-like domains (Gilks, Kedersha et al. 2004), increased hydrophobic interactions between Pab1, Pbp1, and Pub1 could promote further assembly into SGs. Another possibility is that acetylation of Pab1

by NuA4 may regulate Pab1 nuclear localization whereby upon GD acetylation could result in the translocation of Pab1 from the nucleus and aggregation into SG. Interestingly, the K7 acetylation site identified by MS is located near the Nuclear Localization Sequence (NLS) of Pab1 (Dunn, Hammell et al. 2005) (**Figure 14D**), suggesting this site may have an important role in Pab1 nuclear localization. Alternatively, NuA4 acetylation of Pab1 may not impact SG assembly, but other functions of Pab1. For example, acetylation of Pab1 could alter its function in stabilizing mRNAs through controlling poly-A-tail length (Amrani, Minet et al. 1997). Interestingly, the acetylation site K164 identified by MS, along with two additional conserved lysine residues (K131 and K288) (Henriksen, Wagner et al. 2012), are located within RNA binding motifs of Pab1 and may be important for Pab1 function. Further studies must be completed to determine if NuA4 regulation of GD SG assembly and Pab1 acetylation are linked, to determine the specific biological consequences of specific acetylation sites on Pab1, and to identify the specific mechanism by which NuA4 regulates GD SG assembly. These questions could be studied by monitoring the formation of GD SGs in yeast containing acetylation site-specific point mutants. If acetylation of Pab1 is not contributing to GD SG assembly, due to the high incidence of acetylation on SG proteins, another likely scenario is that acetylation of other SG protein(s) may be contributing to SG assembly (see below).

4.3 A Conserved role for KATs and KDACs in SG dynamics.

To determine if additional KATs or KDACs are involved in GD SG assembly I completed a microscopy based screen of single deletion mutants of all non-essential KATs and KDACs in yeast. This screen reveals that in addition to NuA4, Gcn5 may be involved in GD SG assembly (**Table 3**). Although the ~25% reduction in GD SG assembly observed in the *gcn5Δ* mutant is

smaller than that of NuA4 mutants (~40% decrease) my results suggests that Gcn5 may play a minor role in GD SG assembly.

Exposure of *eaf1Δ* and *eaf7Δ* mutant strains to additional environmental stresses suggests that NuA4 mediated regulation of SGs is not limited to GD. In this study I demonstrate that NuA4 mutants exposed to a heat stress (HS) of 46^{O}C for 30 minutes show a ~13% reduction in the ability to form SG ($p<0.05$). Conversely, treating *eaf1Δ* and *eaf7Δ* cells to a 30 minute incubation with 0.5% NaN_3 resulted in a ~8% increase in the ability to form SGs. Finally, following exposure to 15% ethanol for 30 minutes, WT and NuA4 mutants did not display any phenotypic differences in SG assembly (**Figure 7**). Along with my data on GD, these findings point to a model in which the type of stress dictates NuA4s, and/or other KATs and KDACs regulatory effect on SG assembly. This suggests that cells possess several differing regulatory mechanisms that function to mediate the activity of different KATs or KDACs in response to different environmental stresses. Indeed, this supports my finding that NuA4 and Gcn5 are required for GD SG assembly, but the KDAC Hos2 is required for stationary-phase granule (SPG) assembly (Liu, Chiu et al. 2012). Likewise, such an idea can explain how different stresses dictate alternate species of mRNA and proteins that aggregate into SGs, as well as the different kinases responsible for eIF2α phosphorylation (reviewed in (Kedersha and Anderson 2007)). Despite these findings additional studies must be completed to identify each of the stress-specific regulatory mechanisms of SG assembly.

Remarkably, my KAT/KDAC screen revealed that deacetylases may function to inhibit SG assembly in non-stressed cells (**Table 3**), a concept supported by existing literature (Buchan, Kolaitis et al. 2013). My results suggest that Rpd3, Hos3, and Hst4 in particular may be involved in repressing SG formation. While *hos3Δ* and *hst4Δ* strains demonstrated significant increases in the percentage of cells forming SGs under normal conditions ($p<0.06$), all other single KDAC

deletion strains tested did not show a statistically significant difference. In particular, *rpd3Δ* mutant cells demonstrated a ~16% increase (the largest of all the KDAC mutants tested) in SG assembly in non-stressed conditions as compared to WT cells. These findings point to a possible reciprocal mechanism in which KDACs act to suppress SG formation in the absence of stress, while KATs promote the assembly of SGs in the presence of stress. To further test this model I created a KAT and KDAC double deletion mutant (*eaf7Δ rpd3Δ*) which I subjected to 10 minutes of GD followed by fluorescent live cell imaging. Interestingly, *eaf7Δ* and *rpd3Δ* single mutants display a slightly decreased % of cells that form SGs in unstressed conditions as compared to those analyzed in **Table 3**. This difference may be due to the absence or presence of a Pab1-GFP expressing plasmid. Indeed, I have shown that Pab1 over-expression results in an increase in SG formation in unstressed conditions (**Figure 13C**). Analysis reveals that deleting the HDAC Rpd3 suppresses the GD SG assembly defect observed in *eaf7Δ* cells (**Figure 8**). This result suggests that in addition to repressing SG assembly in unstressed cells, Rpd3 may be the main KDAC involved in GD SG disassembly. Collectively, my data suggests that KATs and KDACs may have distinct stress-specific functions. Indeed, such a hypothesis could not only explain the large number of acetylated SG proteins (**Table 2**), but it could justify why I have shown that the KDACs Rpd3, Hos3, and Hst4 function to repress SG assembly under non-stress conditions in yeast, while others have shown that the KDAC SIRT6 mediates SG assembly in *C. elegans* and mammals (Jedrusik-Bode, Studencka et al. 2013).

Due to the complexity of SG dynamics it is likely that NuA4 is not the only enzyme involved in regulating GD SG assembly in *S. cerevisiae*. Indeed, I have shown that Gcn5 is also involved in regulating SG formation upon GD. Likewise, other groups have demonstrated that KDACs localize and regulate SG dynamics (Kwon, Zhang et al. 2007, Jedrusik-Bode, Studencka

et al. 2013). Therefore, it is likely that KATs and KDACs work together to promote SG assembly and disassembly. Additional studies must be completed to discern a precise mechanism.

4.4 Conclusion

The objective of this study was to decipher the role of NuA4 on stress granule assembly and dynamics. I have been able to show that in *S. cerevisiae* NuA4 mutants display significant defects in GD SG formation. Although NuA4 may be involved in regulating SG assembly in response to a variety of stresses, I have determined that NuA4 and to a lesser extent Gcn5, function to promote GD SG assembly. Alternatively, KDACs including Rpd3, Hos3, and Hst4 may act to promote GD SG disassembly in addition to suppressing SG formation in unstressed cells. Though a precise regulatory mechanism remains unknown, my data suggests a model in which NuA4 may regulate GD SG assembly by increasing its interaction with Pab1 and acetylating it at additional sites under glucose deprivation, resulting in increased hydrophobic interactions with proteins like Pbp1 and Pub1, and subsequent targeting into SGs (**Figure 15**).

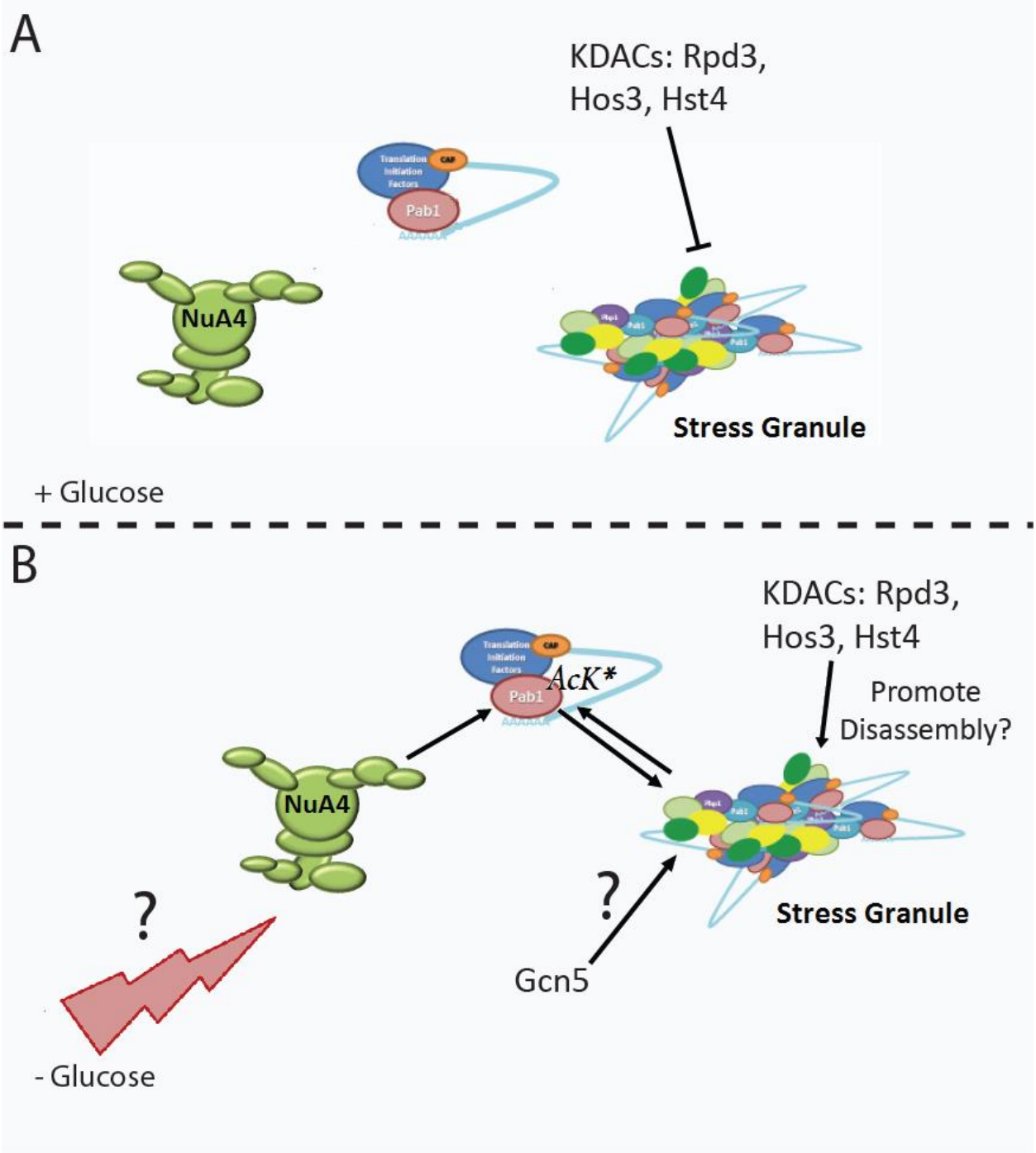

A
KDACs: Rpd3,
Hos3, Hst4
Translation Initiation Factors
CAP
Pab1
NuA4
Stress Granule
+ Glucose
B
KDACs: Rpd3,
Hos3, Hst4
Promote
Disassembly?
AcK*
NuA4
?
?
Gcn5
Stress Granule
- Glucose

Figure 15. One possible model of NuA4 mediated regulation of glucose deprivation stress granule (GD SG) assembly. A) Under non-stressed conditions (+ Glucose) KDACs including, but not limited to Rpd3, Hos3, and Hst4 function to repress SG formation. **B)** Upon glucose deprivation (- Glucose) NuA4 interacts with Pab1 and acetylates it at specific lysine residues. Acetylated Pab1 forms hydrophobic interactions with Pbp1, Pub1, and other SG proteins and aggregates into cytoplasmic stress granules (SG). The KAT Gcn5 also functions to promote GD SG assembly, but to a lesser extent than NuA4, and through a yet unknown mechanism. Over time KDACs including Rpd3 may act to promote GD SG disassembly by deacetylating Pab1 and/or other proteins. KAT=Lysine Acetyltransferase. KDAC=Lysine Deacetylase. GD=Glucose Deprivation.

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Appendix A:

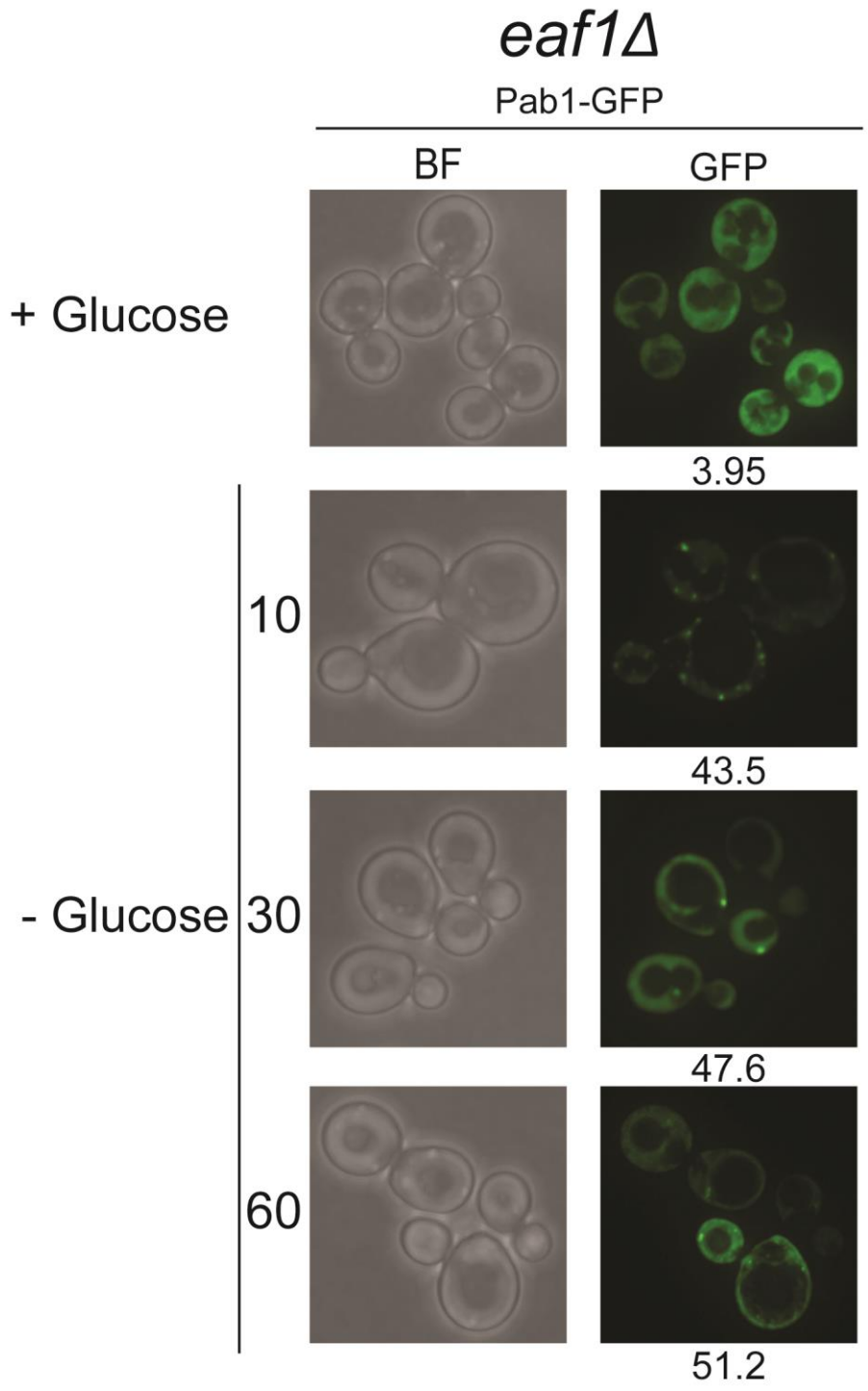

Figure A1. *eaf1Δ* strain glucose deprivation (GD SG) assembly defect is not due to phenotypic growth delay.
Representative Bright Field (BF) and GFP images of *eaf1Δ* (YKB) cells transformed with *PAB1-GFP::URA::CEN* plasmid (PKB192) and subjected to glucose deprivation for 10, 30, or 60 minutes (-glucose) or control conditions (+glucose). SGs were quantified in a blind manner. 1 replicate, n=300 cells for each condition. Numbers indicate the % of cells that form SG. SG=Stress granule.

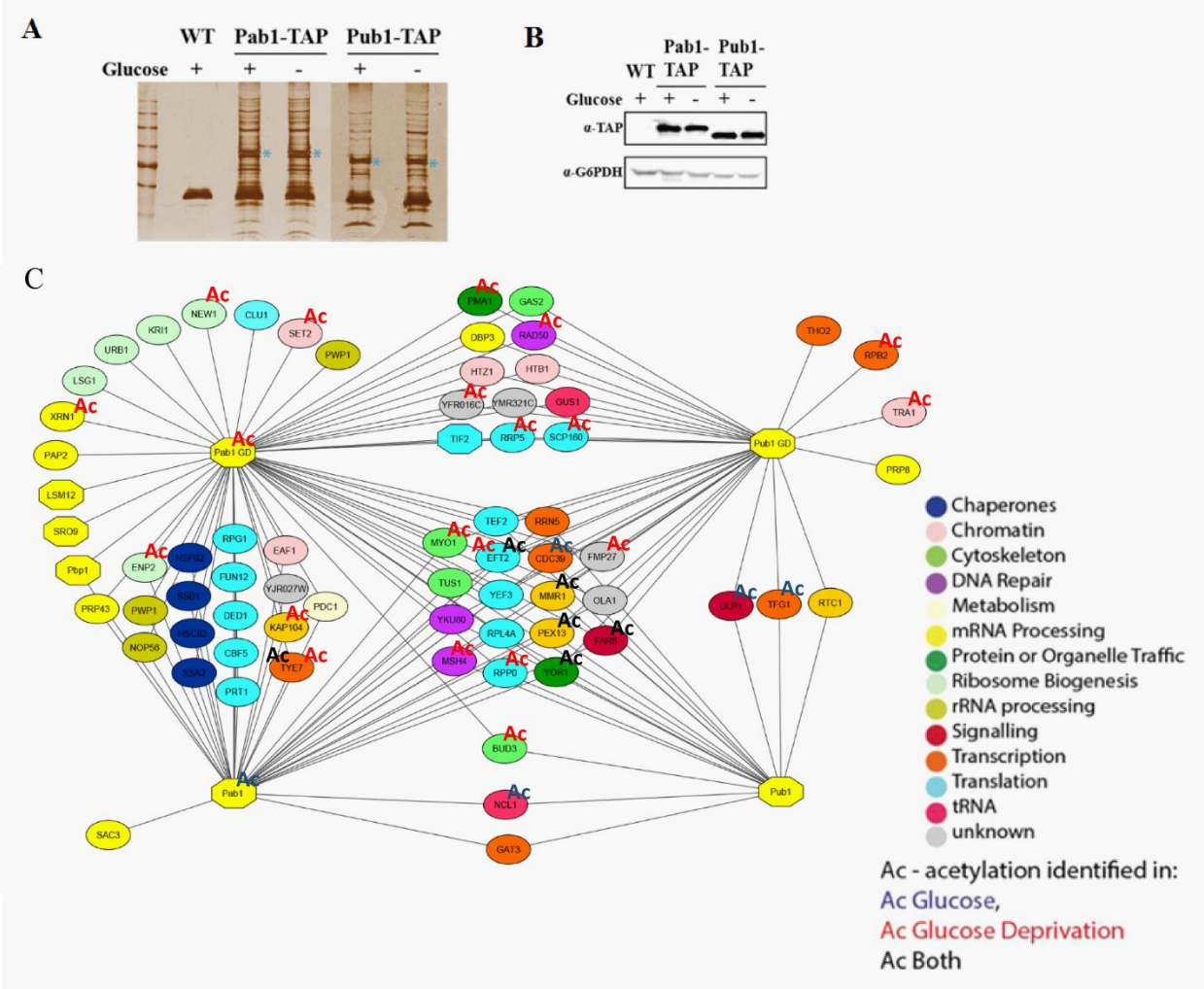
A
WT
Pab1-TAP
Pub1-TAP
Glucose + + - + -
B
WT
Pab1-TAP
Pub1-TAP
Glucose + + - + -
α-TAP
α-G6PDH
C
Pab1 GD
Pub1 GD
Pab1
Pub1
Chaperones
Chromatin
Cytoskeleton
DNA Repair
Metabolism
mRNA Processing
Protein or Organelle Traffic
Ribosome Biogenesis
rRNA processing
Signalling
Transcription
Translation
tRNA
unknown
Ac - acetylation identified in:
Ac Glucose,
Ac Glucose Deprivation
Ac Both

Figure A2. Glucose deprivation (GD) remodels the Pab1 and Pub1 interactomes. A and **B)** WT (YKB779), *PAB1-TAP* (YKB1194), and *PUB1-TAP* (YKB3259) were mChIPed from 250mL of log phase cells grown in YPD and resuspended in YP medium lacking (-Glucose) or containing (+Glucose) for 10 minutes. 95% of the Immunoprecipitations (IP) were resolved on SDS-PAGE and analyzed by silver stain **(A)** and 5% was analyzed by Western (α-TAP probes IP; α-G6PDH the extracts pre-IP to ensure equivalents amount used) **(B**). **C)** Pab1 and Pub1 Interactome Network. This preliminary network only contains proteins that were either identified in two different IPs or had known or putative roles in SG or RNA processing. Nodes are colour coded for process. Proteins that have been localized to stress granules are octagon in shape. If an acetylation was identified in Glucose condition it is colored in BLUE, glucose deprived it is coloured in RED, if in both conditions it is colored in BLACK. “Pab1 GD” and “Pub1 GD” are precipitations that occurred from glucose starved cells.

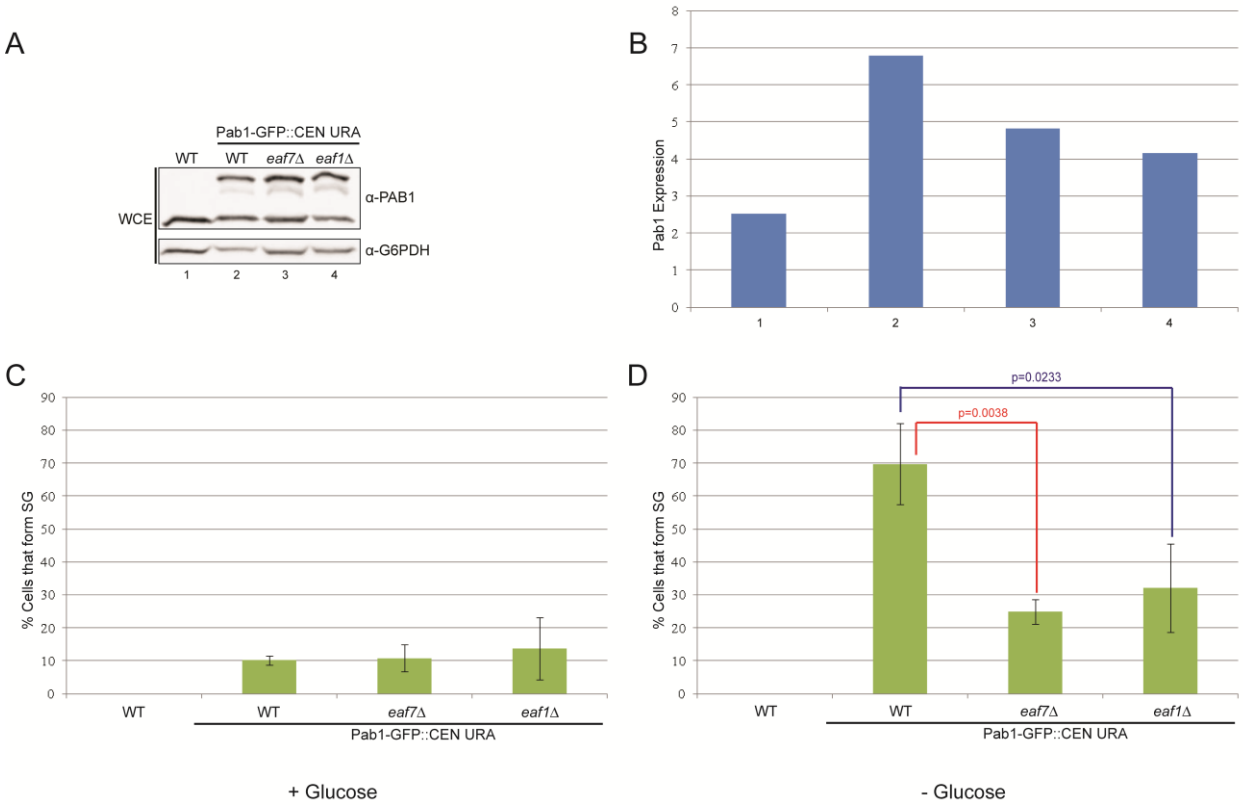
A
Pab1-GFP::CEN URA
WT
WT
eaf7Δ
eaf1Δ
WCE
α-PAB1
α-G6PDH
1
2
3
4
B
Pab1 Expression
C
% Cells that form SG
WT
WT
eaf7Δ
eaf1Δ
Pab1-GFP::CEN URA
+ Glucose
D
p=0.0233
p=0.0038
% Cells that form SG
WT
WT
eaf7Δ
eaf1Δ
Pab1-GFP::CEN URA
- Glucose

Figure A3. Pab1 overexpression does not rescue the glucose deprivation stress granule (GD SG) assembly defect of *eaf7Δ* and *eaf1Δ* cells. Wild-type (WT (YKB 1079)), *eaf7Δ* (YKB3292), and *eaf1Δ* (YKB3453) strains were transformed with *PAB1-GFP::CEN::URA* (PKB192) and empty *LEU* (PKB21) and empty *URA* (PKB23) plasmids. **A)** Western blot showing Pab1 protein expression in transformed cells. Blots imaged using LI-COR software. **B)** Graph displaying quantified Pab1 protein expression from the blot shown in A. Signal intensity was calculated using Image Studio software and Pab1 expression was determined by normalizing Pab1 signal to loading controls. 50ug WCE was loaded and run on 7.5% gels and probed with α-Pab1 and α -G6PDH antibodies. **C)** Graph indicating the % of cells with SG foci in unstressed conditions (+ Glucose). p-values computed using unpaired t-tests against WT cells. Error bars indicate SD. 3 replicates of n ≥ 300 for a minimum of 900 cells total. Stress granules were quantified in a blind manner using Image J software. **D)** Graph indicating the % of cells with SG foci in stressed conditions (- Glucose). p-values computed using unpaired t-tests against WT cells. Error bars indicate SD. 3 replicates of n ≥ 300 for a minimum of 900 cells total. Stress granules were quantified in a blind manner using Image J software. WT=Wild-type. SG=Stress Granule. SD=Standard Deviation. WCE=Whole Cell Extract.

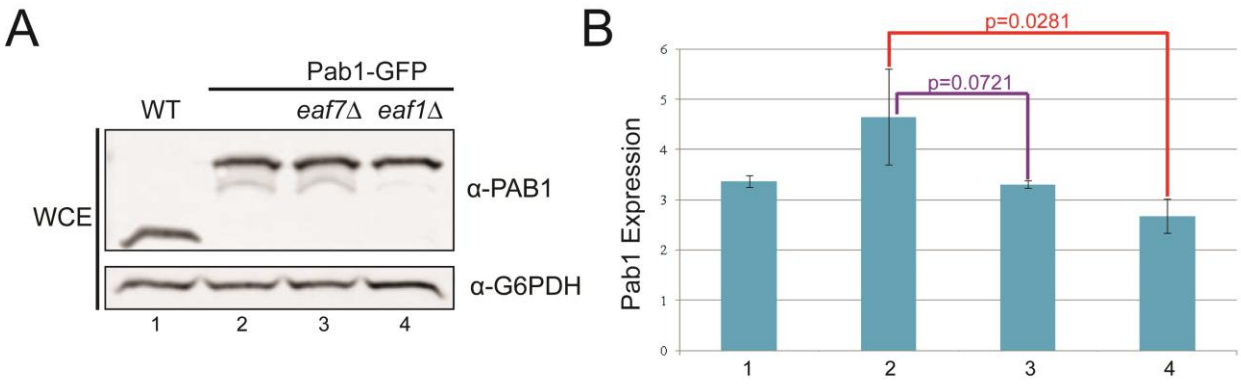
A
Pab1-GFP
WT
eaf7Δ
eaf1Δ
WCE
α-PAB1
α-G6PDH
1
2
3
4
B
p=0.0281
p=0.0721
Pab1 Expression
0
1
2
3
4
5
6
1
2
3
4

Figure A4. NuA4 regulates Pab1 protein levels in endogenously GFP tagged strains. Wild-type (WT (YKB 1079)), *PAB1-GFP* (YKB3114), *PAB1-GFP eaf7Δ* (YKB3336), and *PAB1-GFP eaf1Δ* (YKB3382) strains were lysed and 50ug WCE was loaded and run on 7.5% gels and probed with α-Pab1 and α -G6PDH antibodies. **A)** Western blot showing Pab1 protein expression in transformed cells. Blots imaged using LI-COR software. **B)** Graph displaying quantified Pab1 protein expression from the blot shown in A. Signal intensity was calculated using Image Studio software and Pab1 expression was determined by normalizing Pab1 signal to loading controls p-values computed using unpaired t-tests against WT cells. Error bars indicate SD. Graph displays average of 3 replicates. WT=Wild-type. SD=Standard Deviation. WCE=Whole Cell Extract.

Appendix B

Table B1: Strain List.

YKB	Auxotrophies	Reference
YKB 779	*MATa ade2-101 his3-Δ200 lys2-801 leu2-Δ1 ura3-52 trp1-Δ63*	(Sikorski and Hieter 1989)
YKB 1079	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0*	OpenBiosystems
YKB1194	*MATa ade2-101 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PAB1-TAP::TRP*	TAP collection
YKB3259	*MATa ade2-101 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PUB1-TAP::TRP*	TAP collection
YKB3453	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 eaf1Δ::NAT*	This study
YKB3292	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 eaf7Δ::KAN*	OpenBiosystems
YKB3290	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 eaf5Δ::KAN*	OpenBiosystems
YKB3291	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 eaf3Δ::KAN*	OpenBiosystems
YKB3218	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 pub1Δ::KAN*	OpenBiosystems
YKB3217	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 pbp1Δ::KAN*	OpenBiosystems
YKB3855	*MATa leu2Δ lys2Δ0 Pab1-GFP::HIS esa1ΔHIS3 esa1-L245P::URA3*	This study
YKB3114	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 PAB1-GFP::HIS*	GFP collection
YKB3382	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 PAB1-GFP::HIS eaf1Δ::NAT*	This study
YKB3336	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 PAB1-GFP::HIS eaf7Δ::KAN*	This study
YKB3115	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 PUB1-GFP::HIS*	GFP collection
YKB3339	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 PUB1-GFP::HIS eaf1Δ::NAT*	This study
YKB3337	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 PUB1-GFP::HIS eaf7Δ::KAN*	This study
YKB3258	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 PBP1-GFP::HIS*	GFP collection
YKB3338	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 PBP1-GFP::HIS eaf1Δ::NAT*	This study
YKB3335	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 PBP1-GFP::HIS eaf7Δ::KAN*	This study
YKB3300	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 spt10Δ::KAN*	OpenBiosystems
YKB3489	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 gcn5Δ::KAN*	OpenBiosystems
YKB3482	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 elp3Δ::KAN*	OpenBiosystems
YKB3485	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 hat1Δ::KAN*	OpenBiosystems
YKB3488	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 hpa2Δ::KAN*	OpenBiosystems
YKB3297	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 hpa3Δ::KAN*	OpenBiosystems
YKB3298	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 atf2Δ::KAN*	OpenBiosystems
YKB3484	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 rtt109Δ::KAN*	OpenBiosystems
YKB3486	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 sas2Δ::KAN*	OpenBiosystems
YKB3487	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 sas3Δ::KAN*	OpenBiosystems
YKB 3303	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 rpd3Δ::KAN*	OpenBiosystems
YKB3492	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 sir2Δ::KAN*	OpenBiosystems
YKB3490	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 hos1Δ::KAN*	OpenBiosystems
YKB3301	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 hos2Δ::KAN*	OpenBiosystems
YKB3302	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 hos3Δ::KAN*	OpenBiosystems

YKB3293	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 hst1Δ::KAN*	OpenBiosystems
YKB3294	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 hst2Δ::KAN*	OpenBiosystems
YKB3295	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 hst3Δ::KAN*	OpenBiosystems
YKB3296	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 hst4Δ::KAN*	OpenBiosystems
YKB3491	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 hda1Δ::KAN*	OpenBiosystems
YKB3710	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 LSM1-GFP::HIS*	GFP collection
YKB3718	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 LSM1-GFP::HIS eaf7Δ::KAN*	This study
YKB3711	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 EDC3-GFP::HIS*	GFP collection
YKB3743	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 EDC3-GFP::HIS eaf7Δ::KAN*	This study
YKB3853	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 PAB1-GFP::HIS eaf7Δ::KAN rpd3Δ::KAN*	This study
YKB3341	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 PAB1-GFP::HIS rpd3Δ::KAN*	This study
YKB3389	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 snf1Δ::KAN*	OpenBiosystems
YKB3388	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 reg1Δ::KAN*	OpenBiosystems
YKB3390	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 hxk2Δ::KAN*	OpenBiosystems
YKB518	*MATa ade2-101 his3-Δ200 lys2-801 leu2-Δ1 ura3-52 trp1-Δ63 EAF7-MYC::KAN*	(Mitchell, Lambert et al. 2008)
YKB1195	*MATa his3-Δ200 ade2-101 ura3-52 lys2-801 leu2-Δ1 trp1-Δ63 PAB1-TAP::HIS EAF7-MYC::KAN*	This study
YKB2002	*MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 ESA1-GFP::HIS*	(Mitchell, Lambert et al. 2008)

Table B2: Plasmid List.

PKB	Other Name	Markers	Reference
PKB192	pRP1362	*CEN PAB1-GFP URA3*	(Brengues and Parker 2007)
PKB21	pRS415	*CEN6 LEU2*	Lab stock
PKB23	pRS416	*CEN6 URA3*	Lab stock

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Cold Spring Harbour NY, USA. Oral Presentation.

Presentations:

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Oral presentation to faculty.
Received third prize among biochemistry M.Sc students.

University of Ottawa Faculty of Medicine Poster Day. Spring 2013
Poster presentation.

Citizenship: Canadian.