UNDERSTANDING THE ROLE OF YDJ1 ACETYLATION ON CHAPERONE BINDING AND TRANSLATION IN YEAST

by

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ABSTRACT

COURTNEY M. SHRADER. Understanding the Role of Ydj1 Acetylation on Chaperone Binding and Translation in Yeast. (Under the direction of DR. ANDREW TRUMAN)

Proteostasis is critical for cell viability and as such is well-conserved throughout all organisms. In mammalian cells, newly synthesized proteins ("clients") are processed sequentially by Hsp40, Hsp70 and Hsp90 chaperones to become folded and active. Proteomic studies have uncovered a large number of post-translational modifications (PTMs) on chaperones, referred to as the "Chaperone Code". While several groups have uncovered the role and regulation of PTMs on Hsp70 and Hsp90, very little is known about the PTMs on Hsp40. In this study, we set out to clarify the role of lysine acetylation on the major yeast Hsp40, Ydj1. We mutated a series of acetylation sites on the yeast Hsp40 Ydj1 J-domain to either arginine to block any acetylation or glutamine to mimic constitutive acetylation. Cells in which acetylation was prevented appeared to have no visible phenotype, whereas acetyl-mimic mutants displayed a variety of phenotypic defects consistent with loss of Ydj1 function. Proteomic analysis of Ydj1 interactions identified 306 proteins, about 30% of which were altered in response to mutation of acetylation sites. Interestingly, proteomic data clearly showed that Ydj1 acetylation promoted dissociation with Ssa1 while only mildly altering interaction with the yeast Hsp90, Hsc82. *In vitro* studies confirmed that acetyl-mimic mutations on Ydj1 disrupt interaction with Ssa1 and consequently refolding of a model luciferase substrate. Excitingly, an analysis of novel Ydj1 interactors identified by MS confirmed that Ydj1 acetylation impacts the stability of key ribosomal subunits. Taken together, our results suggest that Ydj1 acetylation may be a novel regulatory mechanism to regulate translation and client maturation.

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DEDICATION

I would like to dedicate this to my father, Richard Shrader, for his constant support and encouragement when I had bad days among the good days. I could not have done this without his steadfast support. And never forgotten, special thanks to Rob, for always believing in me and telling me how proud I made you.

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LIST OF ABBREVIATIONS

Ac- Acetylation

ER- Endoplasmic reticulum

GO- Gene ontology

HPD- Histidine, Proline, Aspartic Acid region

HSF- Heat shock factor

HSP- Heat shock protein

HSP40- Heat shock protein 40

HSP70- Heat shock protein 70

HSP90- Heat shock protein 90

HSR- Heat shock response

JDP- J-domain protein

LB- Luria broth

MS- Mass spectrometry

ncAAs- non-canonical amino acids

PTM- Post-translational modification

SBD- Substrate binding domain

SC- Synthetic complete

sHSP- Small heat shock protein

YPD- Yeast peptone dextrose (yeast media)

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

1.1.1. Heat Shock Protein 70 (Hsp70)

The 70 kDa heat shock (Hsp70) proteins are a highly expressed, highly conserved family of molecular chaperones. They are essential for cell viability and are present in all cells. They bind to a range of client proteins, performing housekeeping tasks related to the entire life cycle of a protein from initial folding, transportation across cellular compartments, and, ultimately, protein degradation [1]. Structurally, they are comprised of a 44 kDa N-terminal domain that has intrinsic ATPase activity, an 18 kDa substrate-binding domain, and a C-terminal lid domain that contains an EEVD motif that is essential for function and is required for binding of many of the co-chaperone proteins [2-5]. Hsp70 works as a molecular clamp, changing conformation upon the binding/hydrolysis of ATP, which traps bound clients in the peptide-binding domain. This allosteric regulation of Hsp70 is mediated by both ATP hydrolysis and by specific co-chaperones and is essential for Hsp70 function [2-5].

1.1.2. Hsp70s in yeast

In the budding yeast *Saccharomyces cerevisiae,* there are seven cytosolic Hsp70 family proteins, Ssa1-4 (which provide essential cell viability), Ssb1-2, and Ssz1 (ribosomally-bound) [6-8]. The Ssa1-4 proteins arose from genome duplication and are highly conserved, with Ssa1 sharing 99%, 84%, and 85% amino acid identity with Ssa2, 3, and 4, respectively [8]. The highest sequence variation occurs within the substrate binding domain (SBD), specifically the outer-facing region of the “lid”. While the lack of conservation of

sequence in this area might suggest low functional importance, a more likely scenario is that it dictates the nature of client proteins that bind to each Ssa isoform [9]. Although highly conserved, the Ssa1-4 proteins differ in expression level in the cell. Ssa1 and 2 are expressed at constitutively at high levels presumably for housekeeping purposes, whereas Ssa3 and 4 are highly stress-inducible [10]. Cells lacking Ssa1 and 2 show dramatic upregulation of Ssa3 and 4 levels as a compensatory mechanism. Interestingly, cells overexpressing Ssa1 but lacking Ssa2-4 are fully viable and have no significant phenotypes [10]. In our recent studies, we have focused our attention on the major constitutively expressed isoforms of Hsp70, Ssa1 (in yeast), and Hsc70 (in mammalian cells).

1.1.3. Hsp40s in yeast

Hsp40s are a large family of proteins that function as a partner to Hsp70s [11]. These proteins (co-chaperones) have been assigned several different roles within the cellular environment, however, Hsp40s aid in the direct assistance to Hsp70. Hsp40s work to regulate the ATPase activity of Hsp70 by binding to Hsp70 and stimulating ATPase activity [11]. These Hsp40s are well conserved with their yeast counterparts; the human homologue of Ydj1 is DNAJA1 and has been observed in pancreatic cancer cell growth [12]. Ydj1 has the primary role of presenting unfolded clients to Hsp70. Once Ydj1 delivers the client protein and binds to Hsp70, the ATPase activity begins and the chaperone is able to initiate the folding cycle. Structurally, Hsp40s are also referred to as J-proteins (Ydj1 belongs to this class of proteins), named for their J-domain. The J-domain is an area that is comprised of an ~ 70 amino acid sequence and is incredibly conserved across many organisms. The J-domain is named after the *Escherichia coli* protein, DnaJ

[5]. In addition to their conserved J-domain, another region of well-conservation is the histidine, proline, and aspartic acid tripeptide (HPD region), that loops between the main helices [5]. This is a critical motif for the function of J-domain proteins (JDP). The J-domain is the responsible region for the stimulation of Hsp70's ATPase activity. These exposed residues form an interaction surface with Hsp70 [5].

1.1.4. J-proteins: Ydj1 and DNAJA1

The heat shock proteins (Hsps)/molecular chaperones are vital to studying processes in eukaryotic and prokaryotic systems. Processes such as protein folding, assembly of larger multi-unit proteins, transport and others that vary, thus is a variety of Hsps suited for each of these [13]. Many of the Hsps work together at different steps in order to fulfil their cellular process. One of Hsp70s major role is to bind unfolded polypeptides until they reach their destination, or until the polypeptide is fully assembled. In the budding yeast *S. cerevisiae*, there are over 10 Hsp70 proteins (cytosolic, mitochondrial, and ER) and the encoding of different homologues suggest specialization, and this is extended to other Hsps within the cell.

While there are the major chaperone families such as Hsp70s, Hsp60s and others, these chaperones do not function alone. These Hsps require help in the form of co-chaperones. The first co-chaperone was identified in *E. coli*, and is such named Dnaj [14, 15] and are often referred to as Hsp40s. After its discovery, it has been shown that DnaJ works with the bacterial Hsp70 (DnaK) and one other protein, GrpE to modulate protein folding in addition to several other processes [13]. Ydj1 is the yeast homologue of DnaJ, which is required for normal cellular growth and works with Hsp70 to regulate its activity [13, 14].

Ydj1 is mainly cytosolic, and it is partially membrane-bound via a farnesylation modification [13, 16]. Ydj1 stimulates the ATPase activity of Hsp70, which leads to the client dissociating from Hsp70.

As a class I JDP, DNAJA1 can transiently interact with other JDPs to form complexes that bind to a range of unfolded or aggregated proteins which are then targeted to Hsp70 [17]. Overall, the main roles of Hsp70 and its partnered JDPs are as follows: protection of substrates that are sent for degradation due to aggregation, recruitment of the ubiquitin machinery to substrates, and the shuttling of substrates to the proteasome, which keeps them from aggregation and premature degradation [17].

DNAJA1 is found in the cytosol like its yeast homolog Ydj1. Unlike many other Hsps that exhibit specificity towards particular client proteins, DNAJA1 is more promiscuous in its binding to clients [18]. DNAJA1 has been implicated in various diseases, particularly neurodegenerative disorders characterized by tau aggregation and amyloidogenesis [19]. Abisambra et al. demonstrated that DnaJA1, which acts as a regulator of Hsc and Hsp70, influences tau stability. The interaction between Hsp70 and the tau-DnaJA1 complex prevents tau degradation, suggesting that blocking the binding of DnaJA1 to Hsp70 could redirect tau towards a pathway of clearance. Other studies have also implicated DnaJA1 in the pathogenesis of other proteins involved in neurodegenerative disorders, such as amyloid beta (Abeta) [20]. Overall, DNAJA1 plays a critical role in cellular homeostasis and the pathogenesis of neurodegenerative diseases, highlighting its importance as a potential therapeutic target.

1.1.5. Hsp90 and the co-ordination of protein folding

Hsp90 is a 90kDa molecular chaperone that works to fold and activate client proteins via a nucleotide-dependent cycle [21]. Human Hsp90s are classified according to their cellular localization: Hsp90α and Hsp90β are located in the cytoplasm; others such as HSPC4 are ER residents [22]. In the cell, Hsp90 is one of the most abundant proteins [23] and has two isoforms. Human Hsp90 has a constitutively expressed form, Hsp90β, and an inducible form Hsp90α [23]. In *Saccharomyces cerevisiae,* gene duplication has given rise to a constitutively expressed isoform, the 83kDa heat shock cognate protein (Hsc82), and a heat-induced isoform, Hsp82 [23-25]. Around 15% of all client proteins in yeast and humans are impacted by the function of Hsp90, both direct and indirect [21]. Kinases, telomerases, transcription factors, and certain viral proteins are some of the client proteins of Hsp90 [26]. Upon client binding, Hsp90 undergoes conformational changes driven by the hydrolysis of ATP [21]. This ATPase cycle is regulated by co-chaperones such as Sti1, Aha1, and Sba1 [27]. In all cells, Hsp40s are the first proteins to bind misfolded proteins and transport them to Hsp70 for the first step of protein folding. The next stage relies on the handover of the client from Hsp70 to Hsp90. Historically, this step has been mechanistically. This prompts the question of what alternative mechanism yeast must employ to transfer clients from Hsp70 to Hsp90.

1.1.6. Proteostasis

Protein folding and proteostasis are critical for cell viability, and are well-conserved throughout all organisms. Heat shock proteins (Hsps) are directly involved in protein biogenesis– from their synthesis as a nascent polypeptide to chain to the assembly of

multimeric complexes [28]. Their role in this process has earned them the of name molecular chaperones. Molecular chaperones and co-chaperones orchestrate this complex process and work together to fold nascent polypeptides. The first Hsp40 co-chaperone was identified in *Escherichia coli*, and was subsequently named DnaJ due to its role in DNA replication [14, 15]. Given the essential nature of molecular chaperones, DnaJ-like proteins are found in all organisms, from bacteria to humans [28]. One of the most well studied J-proteins is budding yeast DnaJ gene (*YDJ1*), which was identified and isolated from a yeast expression library [13]. Its human equivalent, DNAJA1, was isolated from a human umbilical vein endothelial cDNA library [29]. The human homolog shows 41% identity to *E. coli* DnaJ, with the highest degree of homology at the N-terminus, which is a characteristic shared by other known DnaJ homologs [29].

Ydj1 and DNAJA1 facilitate protein folding and prevent aggregation. They are the initial step in protein folding, binding to newly synthesized and unfolded proteins. They transport the bound client to Hsp70, where the client then forms a transient complex with the open peptide binding sites of Hsp70. This process stimulates the Hsp70 ATPase causing the lid of Hsp70 to close, stabilizing the client interaction and promoting protein folding [5].

1.1.7. Use of mass spectrometry to study proteins

Classic research methods have been used for decades; one example is the study of a protein at its individual level. Decades later, the techniques have vastly improved and with the discovery of particular methods such as electrospray and matrix-assisted laser desorption ionization (MALDI) [30], mass spectrometry (MS) is now at the forefront of

proteomic studies. MS is used in three major areas: characterization of recombinant proteins and other macromolecules, protein identification (small-scale or large-scale proteomic studies) and is the choice for detection of posttranslational modifications [30]. MS is a versatile tool, and can be used in several applications such as the identification of a single protein up to several thousands of complex proteins [31]. Mass spectrometry measures the mass-to-charge (m/z) values and signal intensities [31], and this allows for the major characterization of proteins and their features.

Traditionally, the most commonly used technology in MS is referred to as “bottom-up”, and is a quantitative technique that relies on data-dependent acquisition (DDA) [31]. This can be used on complex samples like biological tissue or fungal material and the proteins are extracted from the sample and digested into peptides using enzymes such as trypsin. These peptides are around 7-30 amino acids, and work best for analyzing. Once the peptides are detected, their sequences are then assigned downstream to be analyzed further. The “bottom-up” phrase is a reference to the information that can be inferred from the peptide analysis, and thus is not a complete picture. While it is best to analyze a complete protein, peptides do have some advantages such as less variety in sizes and are able to be separated by other techniques such as reversed-phase high-performance liquid chromatography (HPLC) [31].

While these peptide fragments can be analyzed, it is important to note that there are many factors affecting a protein and its function. The dynamic landscape of proteins can include mutations, RNA processing, and posttranslational modifications which can leads to just one single gene that can produce various proteoforms – but are functionally distinct [32]. These proteoforms can have an impact on processes such as gene regulation and a

protein's activity, and as such maintains an important role in understanding the activity and mechanisms of proteins [32]. "Top-down" mass spectrometry is the measurement of an intact protein mass or its proteoform. This means there are no enzymatic digestion steps, and there are no fragments of peptides being measured and yields additional information. Detection of posttranslational modifications, relationships or cross-talk between PTMs, and the characterization of drug target interactions are just some of the additional pieces of information that can be gained by utilizing "top-down" mass spectrometry[32].

1.1.8. The heat shock response (HSR)

Cells continually undergo proteotoxic stresses such as heat shock, and as such, they must respond to this to maintain their viability. When cells encounter stresses such as heat shock, the heat shock response is activated. The heat shock response (HSR) is a highly conserved transcriptional program [33] and one of the most defining features of HSR is the rapid increase in expression of the heat shock proteins (molecular chaperones) [33]. Molecular analysis of the Hsp genes has uncovered other players important to the HSR. The heat shock element (HSE) has been identified and is a stress-responsive promoter that is a major factor allowing for inducibility of heat shock [34]. These are located upstream of the transcription initiation site and bind to heat shock transcription factors (HSFs) [34].

A conserved regulator of the HSR is the transcription factor heat shock factor 1 (HSF1), which is a multi-domain protein with a highly conserved DNA-binding domain [33]. Most vertebrates express several members of the HSF gene family, but in *S. cerevisiae*, Hsf1 is encoded by one essential gene: *HSF1* [33, 34]. As such, Hsf1 and the DNA binding site have shown some of the most conservation [35]. It is been shown that

mammalian and yeast Hsf1 facilitates the expression of specialized genes involved in proteostasis and forms a tightly-knit group that is centered around Hsp70, 40, and Hsp90. Preliminary data has shown that upon deletion of Ydj1, cells become incredibly sensitive to heat stress, implying that Ydj1 does indeed play a primary role in the HSR. Out of all HSF-induced genes, Hsp26 (a small heat shock protein) is the most highly induced. A lack of Hsp26 induction (detected via Western Blotting or beta-galactosidase assay) can indicate a malfunction in Hsf1 [36].

1.1.9. The Chaperone Code

Improvements in proteomic technologies have led to the discovery of a huge number of post-translational modifications on chaperones [37, 38]. To date, the number of identified PTMs found on the Hsp70 family is a rather large number; in Hsc70 it has been shown that 60% of the Ser, Thr, and Tyr residues are identified as phosphorylated sites [38]. Other chaperones that show an extensive number of modified residues are Hsp90, with 56 % modification [37, 38]. These seem relatively high, but there are other proteins found in the cell with an astonishingly high number of modified residues: the DNA damage response protein 53BP1, with 93% of its residues modified [38]. The discovery of these numerous PTMs have led to the Chaperone Code, where multiple cellular signals converge on chaperones and allow the fine-tuning of their interactions and alter the flow of information needed through the cellular pathways [38]. Indeed, many of these modified residues are well-conserved, suggesting their importance in regulation, still, little is known about the regulations of these sites. It is likely that there is a combination of factors that make these PTMs important in the regulation of chaperones, such as individual sites

regulating specific functions and some sites may be connected to other neighboring modifications, creating a cascade [38].

Although substantial research on the PTMs of chaperones is ongoing, there is less known about the role and regulation of PTMs on other chaperones/co-chaperones such as Ydj1 and DNAJA1. Molecular chaperones are regulated by various PTMs that include phosphorylation, methylation, and acetylation. Other regulatory PTMs such as farnesylation also work to modulate interaction with other chaperones and client proteins. While Ydj1 is primarily cytosolic, farnesylation facilitates the attachment of Ydj1 to the Endoplasmic Reticulum and the perinuclear membrane [39]. There is a CAAX farnesylation motif on the C-terminus of Ydj1, which allows farnesylation to occur [40]. The regulation of this is still being investigated, however previous studies have shown that Ydj1 farnesylation is required for regulation and activation of the select Hsp90 clients [39].

Recent studies have shown that Hsc70 and its co-chaperone DNAJA1 are deacetylated by the deacetylase HDAC6, a process that increases interaction between Hsc70 and DNAJA1 [41]. Understanding these modifications and how they are regulated may provide insight into their processes and potential novel ways to inhibit diseases such as cancer. While chaperone code research is still in its infancy, research will help elucidate the roles and regulations of these PTMs, and will lend toward novel findings in the global regulation of all PTMs.

1.1.10. Acetylation

Cells respond to their environmental cues in many ways, but PTMs are a rapid way to respond to the ever changing environment, particularly stress [42]. Lysine acetylation is a

way cells can respond, and it is a powerful PTM that is well-characterized in the regulation of histones as well as playing a role in metabolism and stress response [43, 44]. A main role for lysine acetylation is influencing gene expression, and the proper orchestration between lysine acetyltransferases (KATs) and lysine deacetylases (KDACs) works to maintain the proper levels and regulation of histone acetylation, which in turn promotes processes such as cell proliferation, growth, and differentiation [43]. Abnormal function of these enzymes can result in diseases such as cancer [43]. While histone acetylation appears to be the norm, there are a number of nonhistone proteins that have been shown to undergo acetylation [43]. In Saccharomyces cerevisiae, around 4000 lysine acetylation sites have been uncovered, and many of these occur on mitochondrial proteins [43].

Reversible lysine acetylation/deacetylation is remarkably well-conserved from bacteria to mammals and suggests that its functions have been maintained throughout evolutionary history [42, 45]. Acetylation and deacetylation have been shown to play a role in processes such as DNA repair, chromatin remodeling, cellular metabolism, protein folding and transcription (via histone acetylation/deacetylation) [42, 45]. More recently, studies have shown that deacetylation of yeast Hsp70 is a requirement to mount an appropriate heat shock response [42]. In mammalian cells, lysine deacetylase activity is required for the interaction between Hsp70 and its DNAJA1 co-chaperone [41].

1.2. Rationale

This work has the potential to solidify Ydj1/DNAJA1 as an integrator of signaling whose hierarchy of modifications (chaperone code) connects multiple pathways that include heat, DNA damage, and cell cycle progression. While this study seeks to uncover the basic mechanisms in the regulation of Ydj1 acetylation and its physiological relevance,

the results of this study will guide future studies focused on exploiting co-chaperone acetylation as a novel anti-cancer therapeutic strategy.

CHAPTER 2: OVERALL HYPOTHESIS AND SPECIFIC AIMS

2.1. Hypothesis

That acetylation in the J-domain of the Hsp70 co-chaperone Ydj1 disrupts interaction with chaperones and client proteins, fine-tuning Ydj1 function.

2.2. Research Focus

To observe the impact of mutating Ydj1 acetylation sites and observing their overall effect on in vitro and in vivo chaperone function.

2.3. Specific Aims

2.3.1. Specific aim 1: To determine the impact of Ydj1 J-domain acetylation on the yeast stress resistance.

2.3.2. Specific aim 2: To determine the effect of Ydj1 J-domain acetylation on the protein-protein interactions of Ydj1.

2.3.3. Specific aim 3: To understand the role of Ydj1 J-domain acetylation on *in vitro* chaperone function.

CHAPTER 3: CHARACTERIZING THE IMPACT OF YDJ1 J-DOMAIN ACETYLATION ON THE YEAST STRESS RESISTANCE

3.1. MATERIALS AND METHODS

3.1.1. Plasmids and yeast strains

Plasmid pYCP-GPD-YDJ1-FLAG was constructed by VectorBuilder (https://en.vectorbuilder.com/). This plasmid expresses C-terminally tagged Ydj1 from the constitutive GPD promoter and contains the *HIS3* selectable marker. Site-directed mutagenesis of lysines K23, K24, K32, K37, K46, and K48 to an R (non-acetylatable) or Q (acetyl-mimic) was completed by Genscript (https://www.genscript.com/). For yeast experiments, plasmids expressing Ydj1-FLAG were then transformed into BY4742 *ydj1*Δ strain.

3.1.2. *E. coli* transformation

Competent E. coli cells were thawed on ice and 50 µl pipetted into a chilled 1.5ml Eppendorf tube. An appropriate amount of plasmid DNA was added and the cells were incubated on ice for 30 minutes. The cells were heat-shocked at 42˚C for 90 seconds and placed on ice for 3 minutes. The cells were then resuspended in 500 µl of SOC medium (2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 2.5mM KCl, 10 mM MgCl2, 20 mM MgSO4, 20 mM D-glucose) and incubated at 37˚C for 1 hour. The cells were centrifuged at 6,000g for 30 seconds. The pellet was resuspended in 100 µl of the supernatant and then plated on LB plus ampicillin to a final concentration of 100µg/ml and incubated at 37˚C overnight.

3.1.3. Preparation of plasmid DNA from bacteria

Approximately 20-100 µg of high-copy plasmid DNA was obtained from 5ml or 3ml overnight cultures of E.coli using QIAprep® Miniprep or Midiprep kits respectively as described in the appropriate QIAprep® handbooks (QIAGEN Ltd.).

3.1.4. Growth media and culture conditions

Yeast cultures were either grown in rich media (YPD) or synthetic-defined minimal media (SD). The recipes for all yeast media are given below (all % values are w/v):

YPD: 2% D-glucose, 2% Bacto-peptone, 1% Bacto-yeast extract.

SD: 2% D-glucose, 0.67% yeast nitrogen base (without amino acids) plus one or more of the following auxotrophic amino acids where required; adenine (20mg/l), L-histidine (20mg/l), L-leucine (30mg/l), L-lysine (30mg/l), L-tryptophan (20mg/l), uracil (20mg/l).

Dropout media: 6.7g of SD base (Clontech) and appropriate amount of complete synthetic media supplement lacking the required amino acids were added per litre of required media. Plates were left on the bench for 1-2 days after pouring to dry. Liquid media cultures were grown at the appropriate temperature with rapid agitation in a media/volume ratio of 1/5 of the flask volume. Yeast strains were maintained in frozen stocks in 2X YPD + 15% glycerol at –70°C. All solutions and glassware were sterilized by autoclaving at 15psi for 20min.

3.1.5. Yeast transformation

Transformation of *S. cerevisiae* strains with DNA was performed chemically as described by [46].

3.1.6. Yeast serial dilutions

Yeast strains were cultured at 30°C overnight in 10ml YPD (1% yeast extract, 2% glucose, 2% peptone) or SC (0.67% yeast nitrogen base without amino acids and carbohydrates, 2% glucose) with the appropriate selection media. The next morning, yeast cultures were diluted into 50ml of the appropriate selection media and were grown to reach an OD_{600} of 0.5. For growth assays, a 1/10 serial dilution was performed in a 96-well plate and then replicated onto YPD or the appropriate SC media. Cells were grown to an OD_{600} of 0.5-1.0 where high temperature, and stressing reagents such as cell wall damaging, DNA damaging, and oxidative stress were spotted onto SC (5mM caffeine, 50ug/ml calcofluor white, 100mM HU, 10mM H_2O_2, 0.02% SDS, and 100J/m^2) and subjected to 37℃. Plates were incubated at 30℃ and 37℃ respectively for 3 days, then imaged.

3.1.7. Heat treatment of yeast cells

Yeast strains were cultured overnight in 10ml of YPD or selective media at 30℃. The following morning, the cells were diluted into 100ml of fresh media, and incubated for 3 hours. After 3 hours, the cells were split into two 250ml flasks, and cells to undergo heat shock were incubated at 25℃ for the final hour to reach an OD_{600} of 0.5-1.0. Cells that did not undergo heat shock were left to incubate at the optimal temperature of 30℃. Heat shock treatment yeast were then incubated in an environment at 39℃ for 90 minutes to elicit a heat shock response. The protein was then extracted, and 20ug was separated using a 4%-

12% NuPAGE SDS-PAGE gel (Thermo). Western Blot analysis was performed using an Hsp26 primary antibody (1:2000, a gift from Dr. Johannes Buchner).

3.1.8. Protein extraction from yeast

Yeast strains were cultured overnight in 10ml of YPD or selective media at 30℃. The following morning, the cells were diluted into 50ml of fresh media, and incubated until an OD_{600} of 0.5-08 was reached. The cells were harvested by centrifugation (5 minutes at 3000 rpm) and pellets were washed with sterile, distilled water. Pellets were then resuspended in yeast protein extraction buffer (1M Tris HCl, pH 7.5, 5M NaCl, 1M DTT, supplemented with NP-40 and glycerol) with a protease inhibitor cocktail (Pierce). Glass beads with a 0.5mm diameter were added to lyse cells using a bead beater (Biospec products). The supernatant was then centrifuged for 10 minutes at 14,800 rpm to remove any cellular debris and transferred to a 1.5ml Eppendorf. Protein extraction was stored at -20℃.

3.1.9. SDS-PAGE/Western Blot analysis

Protein extracts were made as previously described. 20ug of protein was separated by 4-12% Bis-Tris NuPAGE gel (Thermo). Proteins were detected using the following antibodies; anti-Flag tag (1:2000, Sigma), anti-GAPDH (1:2000, Invitrogen), anti-Hsp26 (1:4000, Pineda), and anti-PGK1 (1:4000, Invitrogen). Blots were imaged on a Bio-Rad Imager after being treated with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo). The blots were then subsequently stripped and re-probed with relevant antibodies using a mild or harsh stripping buffer (glycine, SDS, Tween20 pH of 2.2 or 10% SDS, 0.5M Tris HCl, pH 6.8 with β-mercaptoethanol respectively).

3.1.10. Sequence alignment of Ydj1 and DNAJA1

Yeast and human Hsp40 sequences were obtained from Saccharomyces Genome Database (SGD, https://www.yeastgenome.org/) and Universal Protein Resource (UniProt, https://www.uniprot.org/) and BLAST (https://www.uniprot.org/blast) was used to align the two sequences.

3.2. RESULTS

3.2.1. Identifying functionally important sites of post-translational modification on Ydj1

To identify important sites of post-translational modification on Ydj1, we considered two criteria: 1) if sites were conserved between yeast and mammalian cells and 2) the location of the sites in important Ydj1 regulatory domains. Figure 3.1 shows the domain diagram of Ydj1 and DNAJA1 annotated with known PTMs, obtained from the Global Proteome Machine Organization database (GPMdb, https://gpmdb.thegpm.org/). Both Ydj1 and DNAJA1 have a range of PTMs including phosphorylation, methylation, acetylation and farnesylation (Figure 3.1). Interestingly, DNAJA1 has far more identified

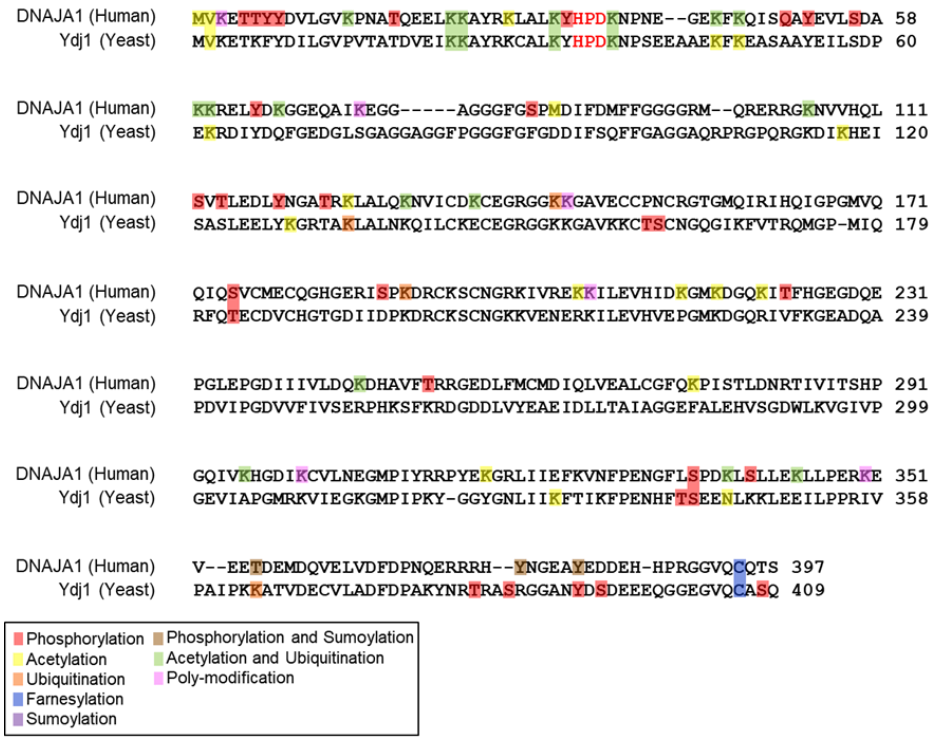

Figure 3.1: Conservation of DNAJA1 and Ydj1 PTMs. Alignment was built using DNAJA1 and Ydj1. Sequences were aligned using BLAST (Accession numbers BAA02656.1 and NP_014335.1) and each PTM was identified using GPMdb and colored accordingly to the figure legend.

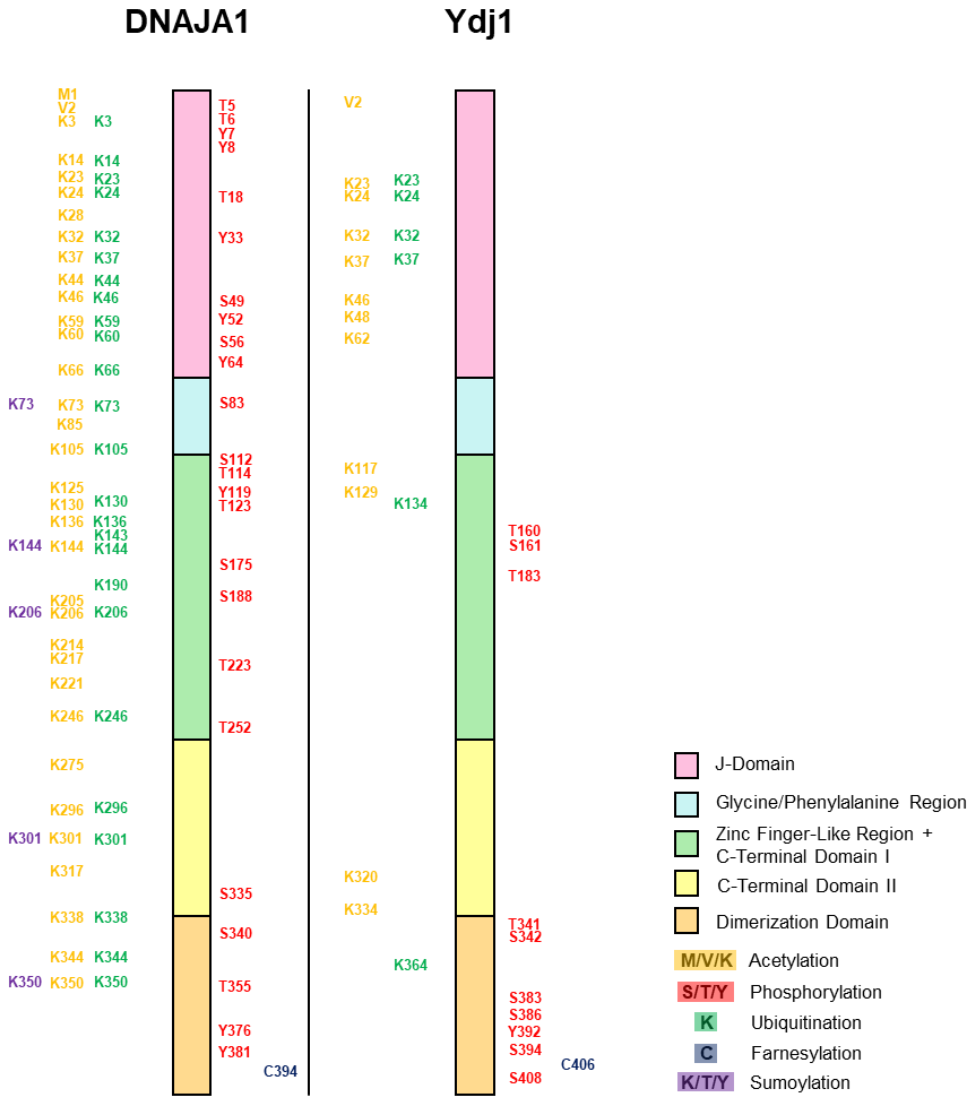

Figure 3.2: The post-translational modifications of DNAJA1 and Ydj1. Shown above is a domain representation of the Hsp40s DNAJA1 and Ydj1, and the known PTMs labeled with the residue number. Domains are colored as before, in Figure 1A. PTMs are labeled as follows: acetylation in yellow, phosphorylation in red, ubiquitination in green, farnesylation in dark blue, and sumoylation in purple.

PTMs than Ydj1 (89 vs 30), which may be attributed to either modification of these sites not being conserved or simply a lack of experimental evidence (Figure 3.2). Ydj1 and DNAJA1 show a high level of conservation, particularly over the J-domain (Figure 3.1, amino acid positions 1-70). Importantly, acetylation was well-conserved between Ydj1 and DNAJA1, particularly over this J-domain region. Interestingly, previous studies had

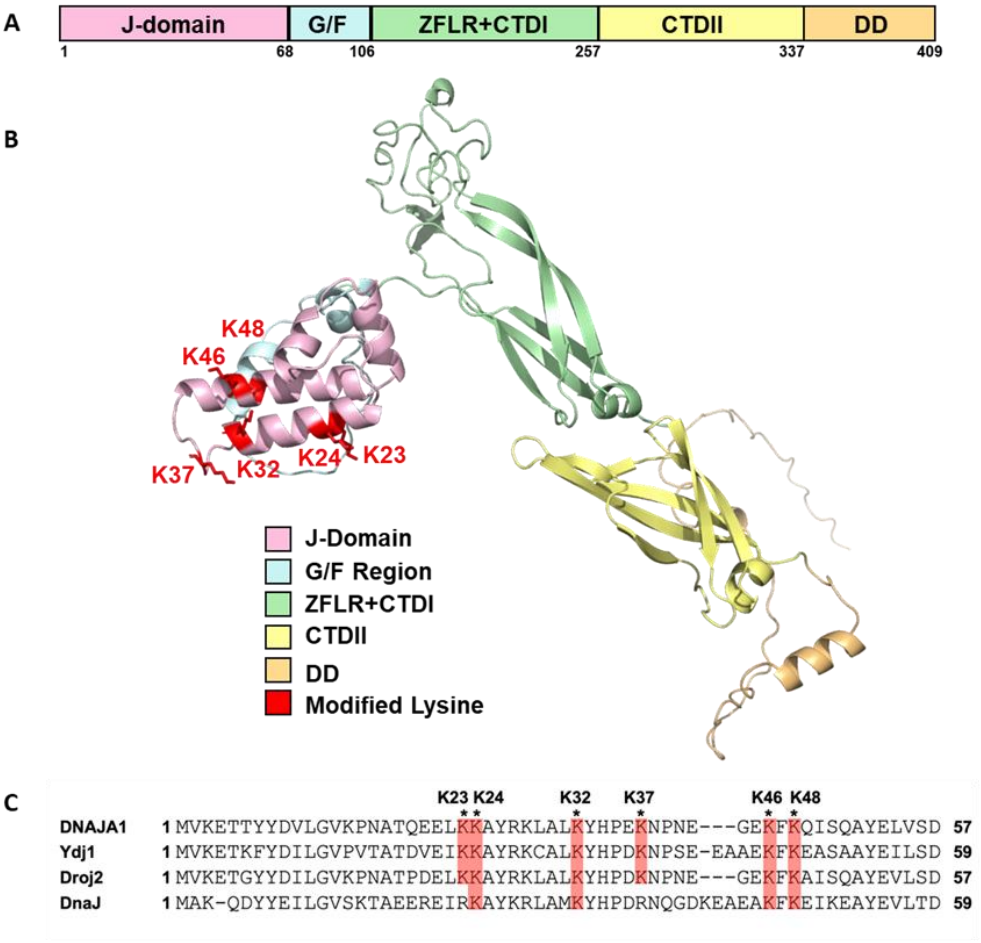

Figure 3.3: Ydj1 structure and mutation of conserved Lysine in the HPD region affects co-chaperone function. (A) Schematic of the Ydj1 structure spilt into its domains; J-domain in pink, the G/F region: glycine and phenylalanine rich region in cyan; the ZFLR: the zinc finger-like region in green; CTDI: carboxyl-terminal domain I in green; CTDII: carboxyl-terminal domain II in yellow; and DD: the dimerization domain in orange. (B) Crystalized Ydj1 (PDB: 1NLT) structure shown as according to A's coloring. The six lysine sites are highlighted in red. (C) Amino acid alignment of the J-domain showing conservation of the lysine sites.

revealed a role for acetylation in mediating the DNAJA1-Hsp70 interaction, but specific sites of acetylation were not identified [41].

Taking conservation and domain localization together, we decided to focus our studies on six acetylation sites (K23, K24, K32, K37, K46, and K48) (Figure 3.3). Ydj1 mediates

its cellular functions via interaction with yeast Hsp70 (Ydj1). To understand if the selected acetylation sites may potentially impact interaction with Hsp70, we mapped these acetylation sites on a complex structure of Ssa1 with Ydj1 (Figure 3.4A). Interestingly, all six acetylation sites lie along the interaction surface of Ssa1-Ydj1. Two of these sites, K23 and K37 participate in hydrogen boding to amino acids located on Ssa1 (Figure 3.4B).

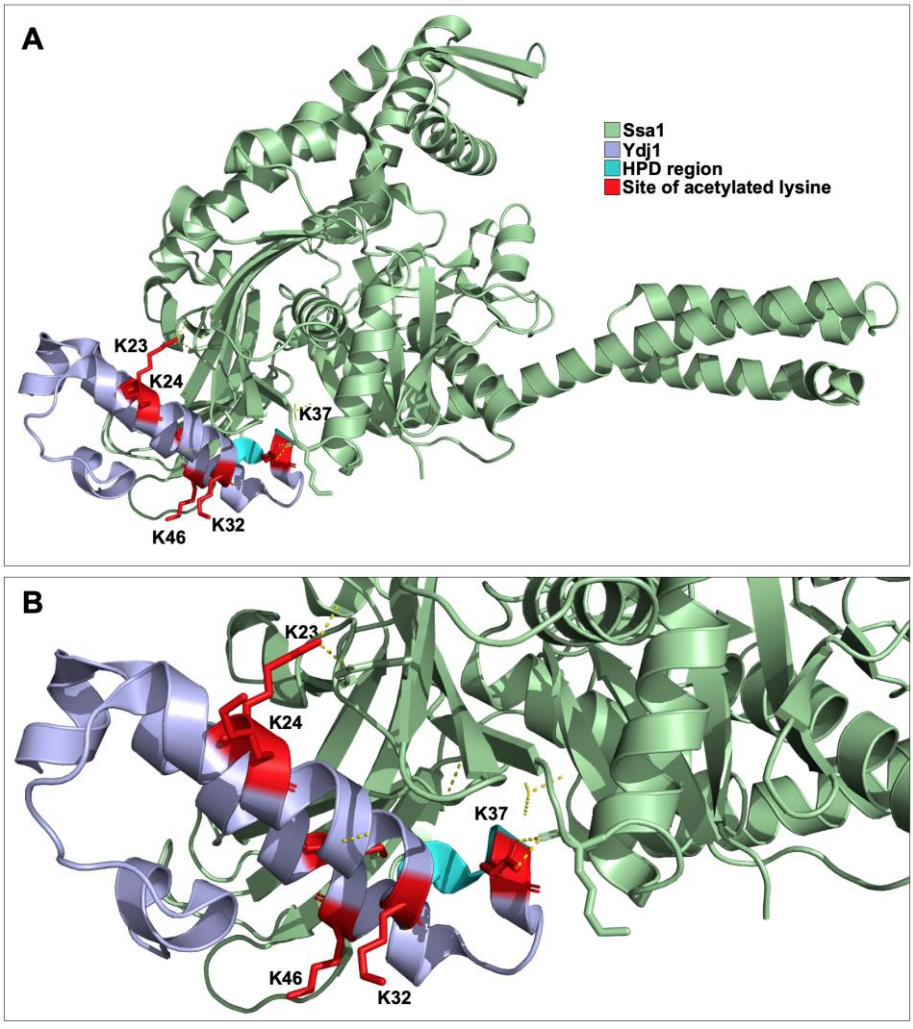

Figure 3.4: The acetylated lysine sites on Ydj1 are found on the interaction surface between Ydj1 and Ssa1. A: Modeled structures of DnaK in complex with the J-domain of DnaJ (PDB:5NRO). Crystalized Ydj1 structure (purple) shown with Ssa1 (green), and the location of the HPD region. The HPD region is shaded in blue and the six acetylated sites are highlighted in red. The residues K23 and K37 exhibit hydrogen bonding. B: The same structure as seen in A, just zoomed in for clarity.

Based on the interaction seen in this structure, it is suggested that acetylation of any of these six sites would most likely disrupt the binding of Ydj1 to Ssa1.

3.2.2. J-domain lysine acetylation is required for Ydj1 function

To determine the effect of J-domain acetylation on Ydj1 function, we constructed a series of Ydj1 point mutations. The six selected lysines were individually mutated to either arginine (non-acetylatable) or glutamine (acetyl-mimic). We also mutated all six lysines in combination to either arginine (6KR) or glutamine (6KQ). A functional Ydj1 is required for growth in several stress conditions such as high temperature, DNA damaging agents (such as hydroxyurea) and agents that disrupt the cell wall (calcofluor white, SDS) [40]. In order to probe the impact of acetylation on Ydj1 function, cells lacking Ydj1 were transformed with plasmids expressing either an empty control plasmid, WT Ydj1 or acetyl

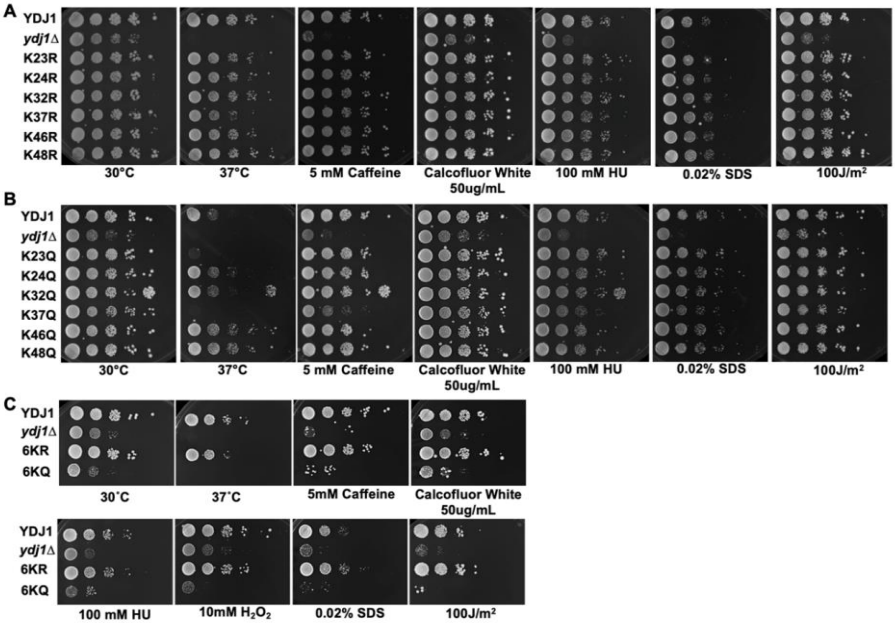

Figure 3.5: Mutation of Ydj1 acetylation sites impacts the yeast stress resistance. *ydj1*Δ expressing FLAG-Ydj1 containing single mutations in acetylation sites were grown to exponential phase and then 10-fold serially diluted onto media containing the indicated stressor. Plates were imaged after 3 days. A) shows acetylation deficient (KR) mutants, B) shows acetyl-mimicking mutants (KQ) and C) shows impact of mutating all six sites to either Q or R

site mutants (K23, K24, K32, K37, K46, K48 R/Q versions). Although cells lacking Ydj1 were sensitive to all stresses tested in comparison to WT, prevention of Ydj1 acetylation had little impact on yeast stress resistance (Figure 3.5A and C). In contrast, mutation of the selected lysines to glutamine (KQ) had a more pronounced impact on the function of Ydj1. K23Q, K37Q (and K24Q to a lesser degree) cells were unable to grow at elevated temperatures (Figure 3.5B). Interestingly this effect was not observed on many of the other stresses tested. Although K37Q displayed a partially caffeine-sensitive phenotype, it phenocopied WT Ydj1 function for all other stresses tested (Figure 3.5B). K23Q and K24Q cells in contrast grew at WT rates in response to caffeine, calcofluor white (CFW), SDS and UV treatment (Figure 3.5B). Mutation of all six sites to arginine (6KR) had no impact on the cellular response to the stresses examined (Figure 3.5C). In contrast, the 6KQ produced the most pronounced loss of function phenotype, displaying sensitivity to all stresses tested (Figure 3.5C).

PTMs can impact a range of protein properties including protein stability. Since these mutants are defective for growth under several conditions, we considered the possibility that mutation of Ydj1 acetylation sites might alter Ydj1 stability. To assess this, we extracted total protein from WT Ydj1, *ydj1* Δ, and cells expressing single and combined KR and KQ mutations. We analyzed Ydj1-FLAG abundance via western blotting, probing with FLAG (Sigma) and using GAPDH (Invitrogen) antibodies as a loading control. As seen in Figure 3.6, the levels of protein differ with specific mutations. Interestingly, the K37Q shows an increased protein abundance, and there is a shift in molecular weight with the mutants K46Q, K48Q, and 6KQ. In contrast, all R mutants had WT levels of Ydj1 except K46R, which displayed lowered levels of Ydj1 (Figure 3.6).

3.2.3. Ydj1 acetylation impacts the heat shock response

Several of the acetyl-site mutants examined displayed a temperature-sensitive defect suggesting an inability to mount a correct heat shock response. Yeast responds to thermal stress by expressing chaperones that refold heat-denatured proteins. Expression of these proteins such as Hsp26 are thus a reliable way to assess the molecular heat shock response in yeast. To assess if Ydj1 acetylation impaired the core heat shock response we measured Hsp26 induction in the full suite of Ydj1 mutants. Yeast cells were grown as described in

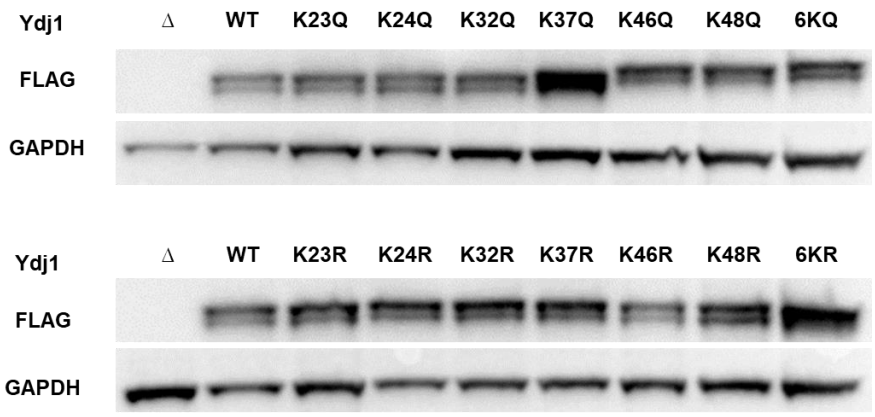

Figure 3.6: Protein expression of Q and R mutants. Total protein was extracted from WT Ydj1, *ydj1*Δ, and cells expressing R, Q, 6KR, and 6KQ mutations. Lysates were analyzed via western blotting.

section 3.1.4, and we extracted total protein from FLAG-WT and mutant yeast. Lysates were processed via SDS-PAGE/Western Blotting and were probed with anti-sera to Hsp26 (a gift from Johannes Buchner) and anti-PGK (Invitrogen) antibodies. Interestingly, in contrast to WT and *ydj1*Δ cells, K23Q and K32Q along with all KR mutants showed lowered Hsp26 induction (Figure 3.7). K37Q which displayed the strongest phenotypic growth defects in Ydj1 in our previous experiments (Figure 3.5B) demonstrated an increased Hsp26 induction (Figure 3.7B).

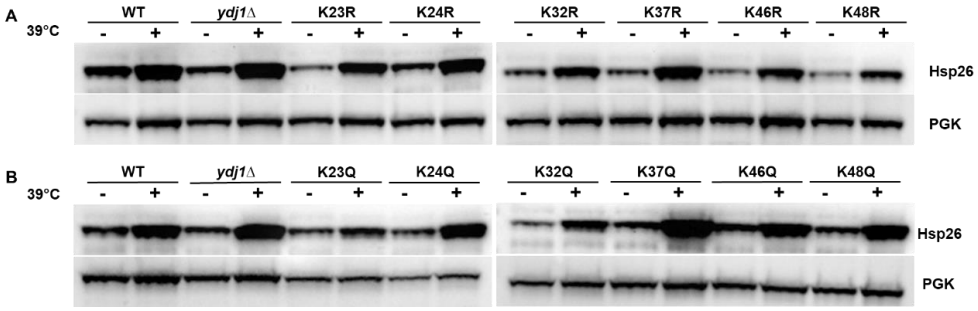

Figure 3.7: Ydj1 acetylation impacts the heat shock response. Yeast cells expressing WT Ydj1, *ydj1*Δ, R, or Q mutations were grown to mid-log phase at 25C, then subjected to heat shock at 39C for 90 minutes. Protein was extracted and total cell lysates were analyzed by Western Blotting with an Hsp26 antibody, and PGK used as a loading control.

3.3. DISCUSSION

Research over the past decade has revealed the presence of a "Chaperone Code", PTMs that regulate molecular chaperone function [38, 47, 48]. Ydj1 has 30 PTMs, 12 of these are acetylation sites, 6 of these are ubiquitination sites, 10 of these are phosphorylation sites, with one succinylation, and a farnesylation site. Out of these 30 PTMs, almost nothing is known about their role and regulation. This sub-aim set out to determine the relevance of Ydj1 acetylation. We identified six acetylation sites that were highly conserved and exist on the J-domain of Ydj1, a region critical for the interaction between Ssa1 and Ydj1. The J-domain of Ydj1 forms an interaction surface that allows the HPD region to bind to and stimulate the ATPase activity of Ssa1, thereby closing Ssa1's lid and trapping the client protein [5]. This activity is highly regulated, though there is still some debate as to the current mechanisms behind this. After structural analysis, we observed these sites to lie extremely close (and even some cases on) the interaction surface between the two proteins. Mutation of these sites to either prevent or mimic acetylation at these sites produced an

interesting spectrum of phenotypes. Surprisingly, prevention of acetylation produced no phenotype whatsoever. In contrast, K23Q, K37Q and 6KQ showed a complete loss of growth at high temperatures. On the crystal structure of the Ydj1-Ssa1 complex, K23 and K37 lie directly on the interaction surface and participate in hydrogen bonding with Ssa1. It is highly likely that the phenotypic defects seen in these mutants are a result of loss of this interaction.

PTMs such as lysine ubiquitination play an important role in the regulation of protein stability for example the rise and fall of cyclins throughout the cell cycle. It is interesting to note that the K37Q mutant displays higher abundance than WT Ydj1. While it is possible that K37 is also the site of ubiquitination and mutation leads to stabilized Ydj1, the levels of Ydj1 in the K37R mutant do not corroborate this theory. It is of course possible that K37Q prevents ubiquitination at a lysine not studied in this project-this will be investigated in future studies. Farnesylation of Ydj1, which can be detected as a doublet on Western Blots, can be clearly seen in all mutant samples. This suggests that while acetylation may impact association with Ssa1, farnesylation is unaffected.

Other chaperones found in the cell can be regulators for stresses such as temperature. Small heat shock proteins exhibit chaperone activity and work to protect proteins from irreversible aggregation [49]. Hsp26 is a small heat shock protein that is a temperature-regulated molecular chaperone and forms large oligomeric complexes, and these complexes are activated by heat shock temperatures. It is suggested the role of these sHsps are to create a protective form around proteins that can be re-folded to their native state with Hsp70 [49]. Binding to non-native proteins occurs *in-vivo*, and structural changes occur under heat shock conditions, making it an efficient chaperone only at high

temperatures. This makes Hsp26 an intrinsic sensor for detecting heat shock conditions [49]. Under heat shock conditions, the heat shock transcription factor (HSF) binds to the heat shock element (HSE) and works to regulate transcription that aids in the expression of heat shock proteins [33, 50]. In yeast, the master regulator of the HSR is the transcription factor HSF1, and recognizes HSEs, a stress-responsive promoter increasing the number of protective heat shock proteins. Under normal cellular conditions, Hsp70 binds to Hsf1, which suppresses its activity [51], and during heat shock, unfolded clients can bind to Hsp70, knocking off Hsf1, which allows it to bind to HSEs allowing activation of transcription [51]. In the K23Q and K24Q mutants, we saw a lowered Hsp26 induction, and compared to the WT and cells lacking Ydj1, the induction of Hsp26 was stronger. Because of the nature of Hsp26 as an indicator of heat shock, it may be that the cells are forming reversible aggregates, and can overcome the stress of heat shock. In the other cases of the K23Q and K24Q, which show a lowered Hsp26 induction, it may suggest there are other factors at play and there is a combination of things happening within the cell. Specifically, the loss of interaction between Ydj1-Ssa1 is perhaps causing a reduced number of interactions, and even under the stress of heat, there are less aggregates forming due to the loss of contact between the two proteins.

CHAPTER 4: ANALYSIS OF THE IMPACT OF ACETYLATION ON YDJ1'S INTERACTOME USING MASS SPECTROMETRY

4.1. MATERIALS AND METHODS

4.1.1. Yeast strains, growth conditions and plasmids

Yeast cultures were grown in SC (0.67% yeast nitrogen base without amino acids and carbohydrates, 2% glucose) with the appropriate selection for the 6KR and 6KQ strains. Cpr6 and Sti1 plasmids (pRS416GPDHis-Cpr6 and pRS415GPDHis-Sti1, a gift from Dr. Jill Johnson) were transformed into FLAG-Ydj1 strains using the PEG/lithium acetate method. Cells were then restreaked onto plates lacking histidine and uracil. Transformants were then grown to mid-log phase. SC-his/ura media and protein was extracted as described in Chapter 3.1.5. RPL6A, RPL6B, RPS0A, RPS3, and GDC11 strains were obtained from Yeast ORF collection and grown in the appropriate media. RPS0A, RPS3, and GCD11 plasmids were then transformed into FLAG-Ydj1 strains using the PEG/lithium acetate method. Cells were then restreaked onto plates lacking histidine and uracil. Transformants were grown to mid-log phase, then induced with 2g of galactose for 4 hours to allow for protein expression.

4.1.2 Western Blotting

Proteins were quantitated and analyzed via western blotting as described in Chapter 3.1.6. Proteins were detected with the following antibodies: anti-FLAG tag (1:2000, Sigma), anti-HIS (1:2000, Qiagen), anti-GAPDH (1:2000, Invitrogen), and anti-HA (1:2000, ThermoFisher). The blots were imaged using a ChemiDoc MP imaging system (Bio-Rad) after treatment with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo). The blots were then subsequently stripped and re-probed with relevant

antibodies using a mild or harsh stripping buffer (glycine, SDS, Tween20 pH of 2.2 or 10% SDS, 0.5M Tris HCl, pH 6.8 with β-mercaptoethanol respectively).

4.1.3. Purification and preparation of 6KR/KQ Ydj1 complexes for mass spectrometry analysis

Cells expressing either FLAG-Ydj1 6KQ or 6KR were grown overnight in SC-HIS (0.67% yeast nitrogen base without amino acids and carbohydrates, 2% glucose) media then reinoculated into 50 or 100mL of SC-HIS and grown to an OD600 of 0.5-0.8. Cells were then harvested and FLAG-tagged proteins were isolated as follows: protein was extracted via bead beating in 600ul of lysis buffer (1M Tris HCl, pH 7.5, 5M NaCl, 1M DTT, supplemented with NP-40) and a protease inhibitor cocktail (Pierce). 1000ug of protein extract was incubated with 20ul of anti-FLAG M2 magnetic beads (Sigma) and 200ul of lysis buffer rotating O/N at 4 ℃. The next day, supernatant was removed and Anti-Flag M2 beads were collected via magnet.

Immunoprecipitates were digested with 0.4 µg of trypsin-LysC mix (Promega) overnight at 37˚C. Tryptic peptides were desalted with Pierce C18 Desalting Spin Columns (Thermo Fisher Scientific) according to manufacturer's protocol, dried down on SpeedVac, and resuspended in mobile phase A (0.2% formic acid in water) immediately prior to mass spectrometric analysis.

4.1.4. Mass Spectrometry: Liquid chromatography-tandem mass spectrometry peptide analysis

Mass spectrometry was performed at the University of Oklahoma by Luca Fornelli and team. Resuspended cross-linked peptides were separated by nanoflow reversed-phase liquid chromatography (LC). An Ultimate 3000 UHPLC (Thermo Scientific) was used to

load ~1 ug of peptides on the column and separate them at a flow rate of 300 nL/min. The column was a 15 cm long EASY-Spray C18 (packed with 2 µm PepMap C18 particles, 75 µm i.d., Thermo Scientific). The analytical gradient was performed by increasing the relative concentration of mobile phase B (0.2% formic acid, 4.8% water in acetonitrile) the following steps: from 2% to 30% in 32 min, from 30% to 50% in 5 min, and from 50 to 85% in 5 min (for washing the column). The 4 min wash at high organic concentration was followed by moving to 15% in 2 minutes, increasing to 70% in 1 min for a secondary wash before re-equilibration of the column at 2% B for 7.5 min, for a total run time of 68 min. A 2.2 kV potential was applied to the column outlet using an in-house nanoESI source based on the University of Washington design for generating nano-electrospray.

All mass spectrometry (MS) measurements were performed on a tribrid Orbitrap Eclipse (Thermo Scientific). Broadband mass spectra (MS1) were recorded in the Orbitrap over a 375-1500 m/z window, using a resolving power of 120,000 (at 200 m/z) and an automatic gain control (AGC) target of 4e5 charges (maximum injection time: 50 ms). Precursor ions were quadrupole selected (isolation window: 1.6 m/z) based on a data-dependent logic, using a maximum duty cycle time of 3 s. Monoisotopic precursor selection and dynamic exclusion (60 s) were applied. Peptides were filtered by intensity and charge state, allowing the fragmentation only of precursors from 2+ to 7+. Tandem mass spectrometry (MS2) was performed by fragmenting each precursor passing the selection criteria using both higher energy collisional dissociation (HCD) with normalized collision energy (NCE) set at 30% and electron transfer dissociation – higher energy collisional dissociation (EThcD), with ETD reagent target set at 5e5, reaction time calculated on the basis of a calibration curve and supplemental collisional activation set at NCE=10%. The

AGC target for both HCD and EThcD MS2 was set at 8e4 (maximum injection time: 55 ms), and spectra were recorded at 15,000 resolving power.

4.1.5. MS data analysis, statistical analysis and visualization

For general identification of all-proteins included in the samples, HCD fragmentation data were processed with Protein Discoverer 2.4 utilizing Sequest HT and MS Amanda search engines. For both Precursor Mass Tolerance was 10 ppm and Fragment Mass Tolerance was 0.2 Da. Carbamidomethylation (C) was allowed as a static modification and dynamic modifications were as follows: Oxidation(M), Acetyl (protein N-term). Identified peptides were validated using Percolator and target FDR value was set to 0.01 (strict) and 0.05 (relaxed). Finally, results were filtered for high confidence peptides using consensus steps. Control peptide error rate strategy was used and 0.01 (strict) and 0.05 (relaxed) values were used for Target FDR for both PSM and Peptide levels. Changes in protein abundance between the two strains were statistically tested by ANOVA using the built in function within Proteome Discoverer.

Gene Ontology was performed using the GO Slim Mapper on the Saccharomyces Genome Database (SGD) (https://www.yeastgenome.org/goSlimMapper). Classification of interactors were also organized according to the GO Slim Mapper on the SGD.

Visualization of the interactome was performed using GraphPad Prism 9 software.

Data points indicating an interaction of $\log_2 > 1$ or $\log_2 < -1$ were colored according to various parameters to indicate an increase in interaction or a decrease in interaction.

4.1.6. Purification of FLAG-Ydj1 complexes from yeast for IP analysis

Cells transformed with the pAG4313 plasmid carrying FLAG-tagged Ydj1 or Ydj1 Δ (strain YAT 427) were grown overnight in SC-HIS (0.67% yeast nitrogen base without amino acids and carbohydrates, 2% glucose) media then reinoculated into 50 or 100mL of SC-HIS or appropriate media and grown to an OD_{600} of 0.5-0.8. Cells were then harvested and FLAG-tagged proteins were isolated as follows: protein was extracted via bead beating in 600ul of lysis buffer (1M Tris HCl, pH 7.5, 5M NaCl, 1M DTT, supplemented with NP-40 and glycerol) with a protease inhibitor cocktail (Pierce). 200ug – 1000ug of protein extract was incubated with 20ul of anti-FLAG M2 magnetic beads (Sigma) and 200ul of lysis buffer rotating O/N at 4 ℃. The next day, supernatant was removed and Anti-Flag M2 beads were collected via magnet. The beads were washed 5 times with 1X TBS, After the last wash, 65ul of elution buffer (1X TBS and Flag Peptide) was added and was vortexed for 30 minutes. Supernatant containing FLAG-tagged Ydj1 was transferred to a fresh Eppendorf tube and 15ul of 4X NuPAGE LDS sample buffer (Invitrogen) was added and denatured by boiling for 5 minutes. 10-20ul of sample was then analyzed via SDS-PAGE, with the respective antibodies.

4.2. RESULTS

4.2.1. Acetylation impacts the Ydj1 interactome

The interactions of chaperones and co-chaperones are modulated by cellular stress and the chaperone code [38]. Our previous experiments had shown the Ydj1 acetylation impacted Ydj1 stability and the heat shock response. To clarify whether this effect may be through altered Ydj1 interactions we compared the interactomes of Ydj1-FLAG 6KQ and Ydj1-FLAG 6KR. Ydj1-FLAG complexes from cells expressing the 6KQ or 6KR mutants were isolated via FLAG-Dynabeads. These complexes were digested via trypsin and peptides were comparatively analyzed by LC-MS. Protein interactions of Ydj1 were normalized against the bait (Ydj1) and then the $\log_2$ ratio of interaction (6KQ/6KR) was calculated. A change in interaction between 6KQ and 6KR samples was considered significant if the normalized $\log_2$ (6KQ/6KR) value was >1 or <-1. The interactors were then sorted according to their Gene Ontology terms and were plotted with the y-value corresponding to a change in chaperone interaction (Figure 4.1).

Over 300 Ydj1 interactors were identified and over 30% of these were altered by mutation of acetylation sites. 44 of these interactors increased upon Ydj1 acetylation, and is shown in green. 55 of these interactors decreased upon Ydj1 acetylation, leaving the remaining 216 interactors unchanged. Each category showed a variety of changes, with the cellular respiration/metabolism and translational category showing the most interactors. In the cellular respiration/metabolism category, there were a total of 107 interactors; 15% showed an increase in interaction upon acetylation, while 21% showed a decrease in interaction upon acetylation. The remaining 62% of interactors showed no change. The translational category showed the most interactors, a total of 130; 13% of these showed an

increase in interaction upon acetylation, while 10% of these showed a decrease in interaction upon acetylation. The remaining 76% of interactors showed no change.

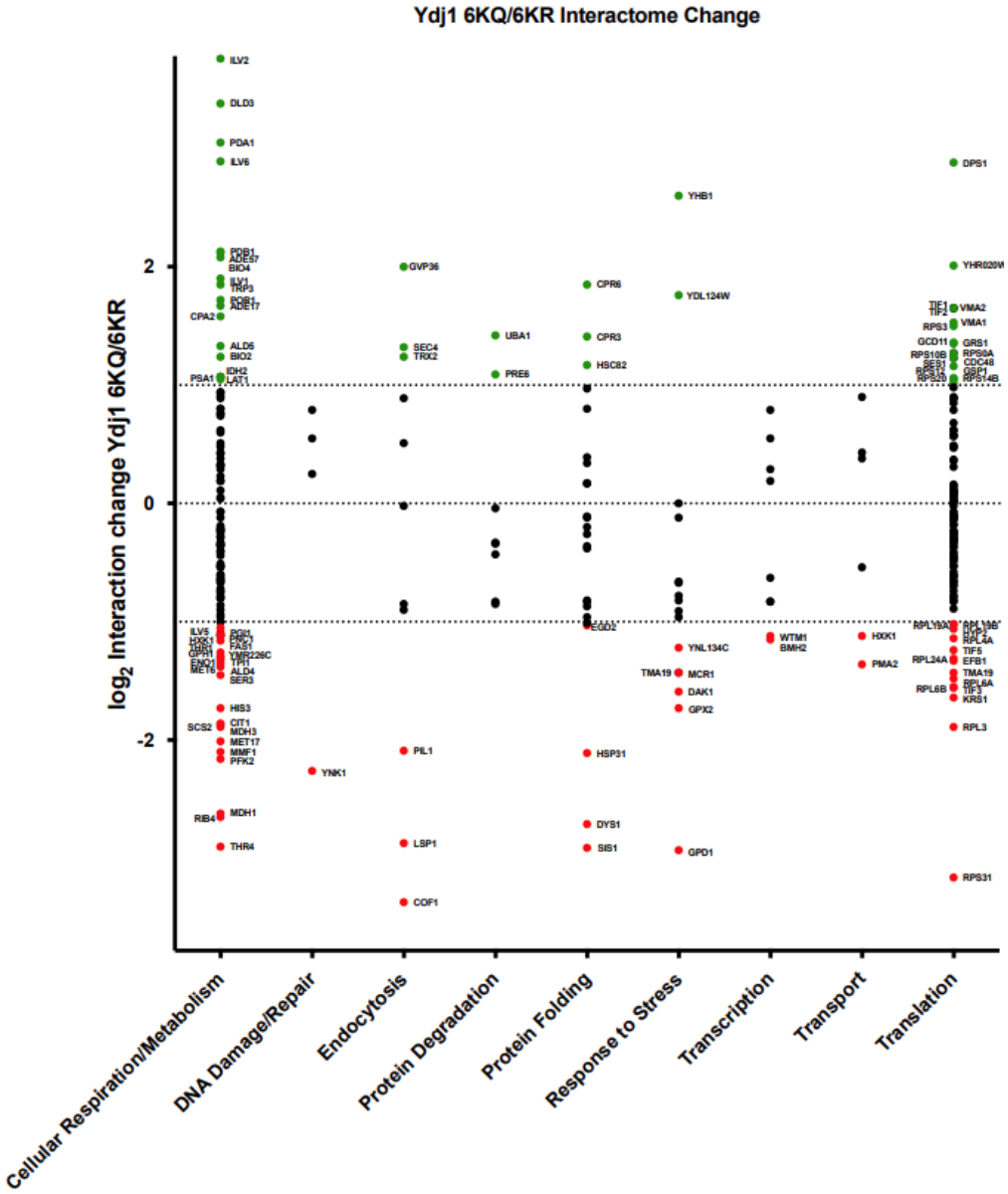

Figure 4.1: Global changes in the Ydj1 interactome under acetylation status. Ydj1 complexes were purified from yeast (6KR and 6KQ mutants) and were analyzed via quantitative mass spectrometry. Interactors were organized into functional categories and were plotted with the y-value corresponding to change in chaperone interaction and the x-axis value corresponding to the total protein abundance change. The dotted lines represent a two-fold interaction change up or down. Interactors were colored according to a significant increase in abundance or decrease in abundance as follows: red (decreased interaction), green (increased interaction), or black (no significant change in interaction).

The rest of the categories showed varying changes. Endocytosis interactors showed 45% of its interactors unchanged with a 27% change in increased interactors, and a 27% change in decreased interactors. Protein degradation interactors showed 75% unchanged with a 25% change in increased interactors. Protein folding showed 15% increase in interaction, 17% decrease in interaction with the remaining interactors unchanged, at 69%; Response to stress showed 50% of interactors unchanged, with a 10% increase in interaction, and a 37% decrease in interaction. The DNA damage, transcription and transport categories were mostly unchanged, with a 25%, 22% and 33% decrease in interaction, respectively.

4.2.2. Interactions between Ydj1 and chaperones/co-chaperones are altered by acetylation

Ydj1 mediates the majority of its functions through interactions with Hsp70 and Hsp90 chaperones as well as assorted co-chaperone proteins [5]. We focused our analysis of interesting hits from the MS screen to chaperone/co-chaperone interactors (Figure 4.2.) In our interactome study, Ydj1 co-purified with Hsp70 paralogs Ssa1 and 2 and Hsp90 paralogs Hsc82 and Hsp82 (Figure 4.2.). We also identified important members of the proteostasis network Cpr6, Sis1, Ssb1/2, Sse1/2, Ssc1, Ssz1, Tsa1, Frp1, Pdi1, Hsp26, Hsp60, Hsp104 and Cpr3 (Figure 4.2). While the majority of Ydj1 chaperone and co-chaperone interactions were unchanged between the two interactomes, increased interaction was seen between Ydj1 and Hsc82, Cpr6 and Cpr3 (Figure 4.2) in the 6KQ sample. In contrast, the interaction between Ydj1 and the partially-redundant co-chaperone Sis1 was decreased in the 6KQ sample.

Proteomic analysis of chaperones is complicated by the presence of highly similar, partially redundant isoforms that vary in expression level [9]. To clarify whether Ydj1-Ssa1 interaction was perturbed by acetylation we performed a direct immunoprecipitation experiment. Yeast expressing WT, 6KQ or 6KR Ydj1-FLAG were grown to mid-log, protein was extracted and Ydj1 complexes isolated by FLAG-Dynabeads. Immunoprecipitates were probed for the presence of Ssa1 due antisera specific to this isoform. An increased Ydj1-Ssa1 interaction was observed in the 6KR mutant, while almost no equivalent interaction was seen in 6KQ cells (Figure 4.3).

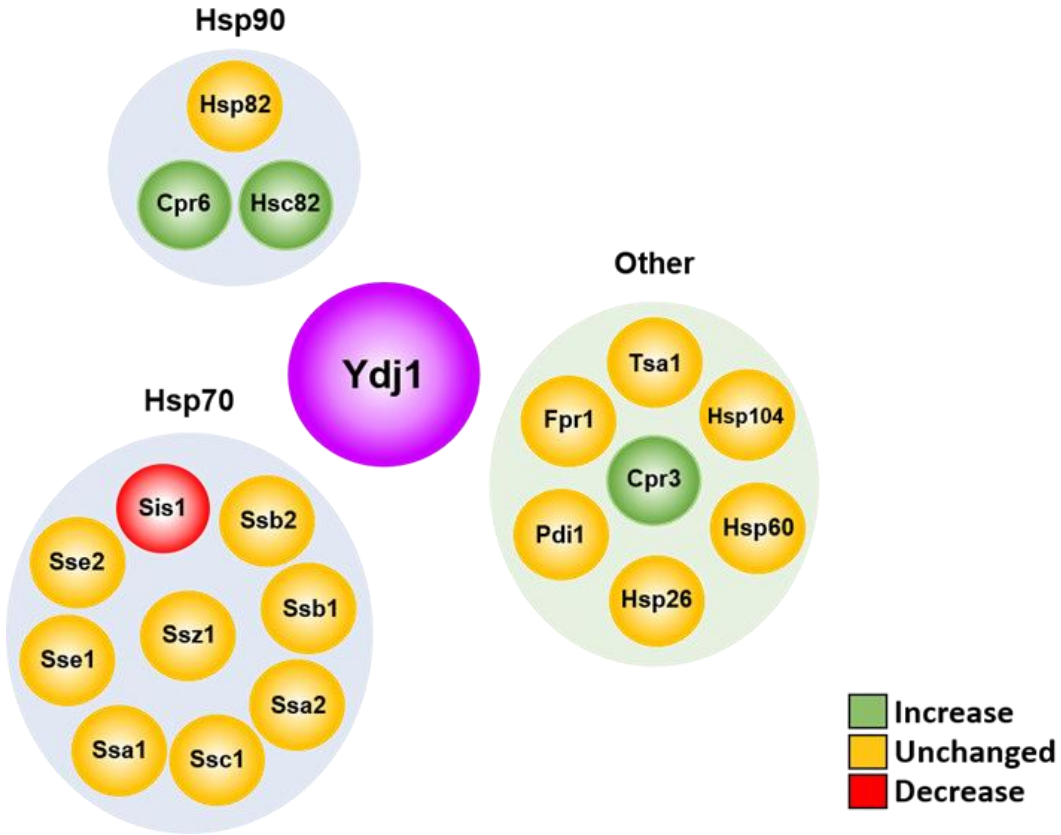

Figure 4.2: Chaperone and co-chaperone interactors. A Classification of direct interactors of Ydj1. Color indicates interaction change under acetylated or non-acetylated status. Green indicates an increase in interaction, red indicates a decrease in interaction, and yellow indicates interaction is unchanged

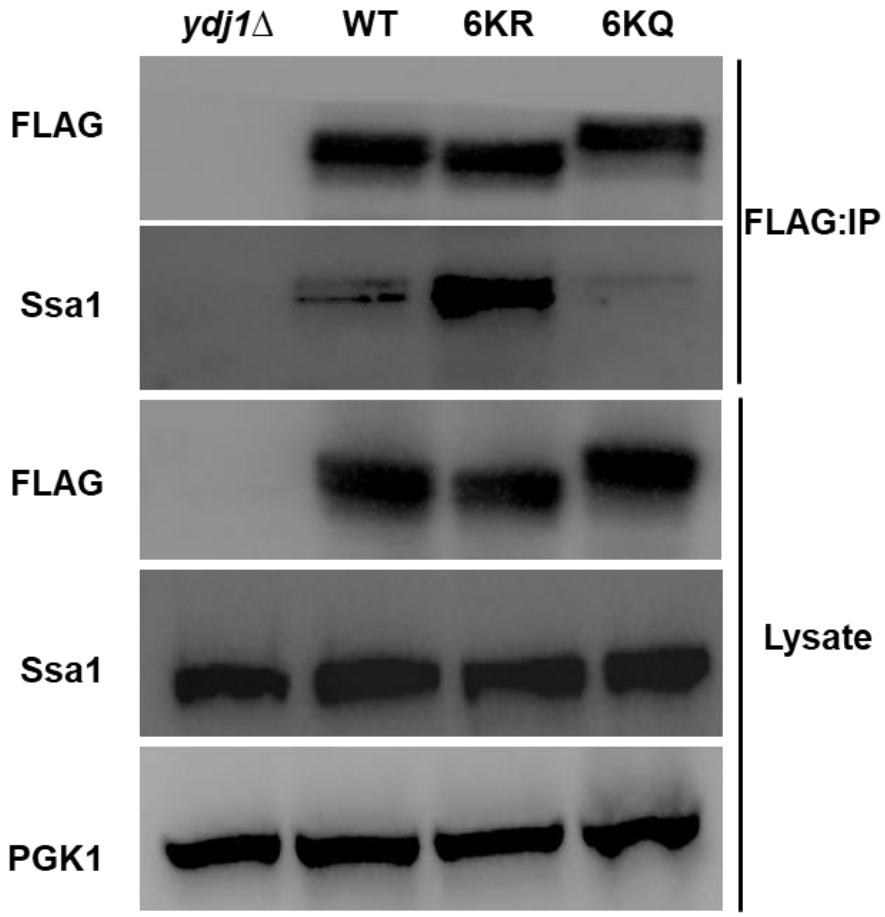

Figure 4.3: Direct interaction of Ydj1 and Ssa1. Analysis of Ydj1-Ssa1 interactions in yeast via co-immunoprecipitation. Yeast cells expressing WT, 6KQ or 6KR Ydj1-FLAG were grown to mid-log, protein was extracted and Ydj1 complexes isolated via FLAG-magnetic beads. Immunoprecipitates were probed with anti-FLAG and anti-Ssa1 to analyze interaction.

4.2.3. Acetylation of Ydj1 alters apparent interaction with ribosomal subunits

Chaperones bind newly-translated proteins and help them achieve their native, active state [52]. It is thus unsurprising that we identified a number of proteins involved in protein translation in our proteomics screen. In particular, Ydj1 co-purified with both ribosomal subunits with 7 of these proteins found in the small subunit, and 7 from the large subunit, representing 17% of the ribosome. There were complex differences in how these

interactions changed between the 6KQ and 6KR samples (Figure 4.1). For example, while a large number (76%) of ribosomal interactions were independent of Ydj1 acetylation, 10% of interactions decreased and 13% increased upon Ydj1 acetylation (6KQ/6KR). To understand if there was a spatial specificity to these differential changes, we mapped the identified Ydj1-binding ribosomal components to the structure of the 80S ribosome (PDB: 4V7R) ribosomal complex (Figure 4.4). Proteins clustered together in the small (40S) subunit of the ribosome tended to increase with interaction with the 6KQ mutant, whereas

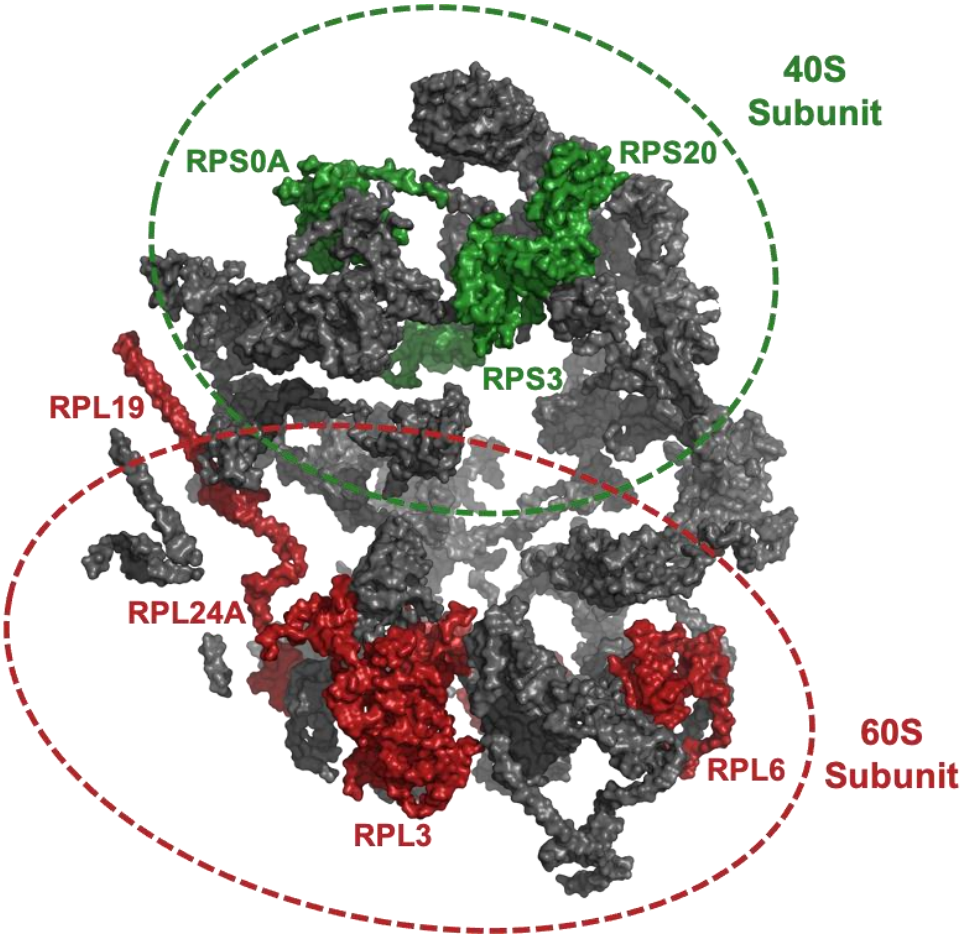

Figure 4.4: Ydj1 acetylation alters interaction with the ribosome. The yeast 80S ribosome (PDB: 4V7R) was mapped with protein interactors that showed an increase or decrease in interaction upon Ydj1 acetylation. Interactors in green are those that increased in interaction, while those in red are interactors that showed a decrease in interaction.

those in the large (60S) subunit decreased in interaction.

Changes in protein interactions in proteomic studies can be a result of either changes in the strength of association or simply the altered abundance of the interactor. To determine whether ribosomal protein hits in our screen were caused by changes in protein abundance, we utilized the Dharmacon Yeast ZZ-tag ORF collection (https://horizondiscovery.com/en/non-mammalian-research-tools/products/yeast-orf-collection). These plasmids contain express ZZ-HA-HIS-tagged yeast ORFs from the inducible galactose promoter.

We selected five ribosomal proteins (RPS0A, RPS3A, GCD11, RPL6A and RPL6B) from our proteomics screen to validate based on properties such as expression in the cell and molecular weight. We transformed ZZ-RPS0A, RPS3A, GCD11, RPL6A and RPL6B plasmids into WT BY4742 yeast and performed an initial expression test (Figure 4.5).

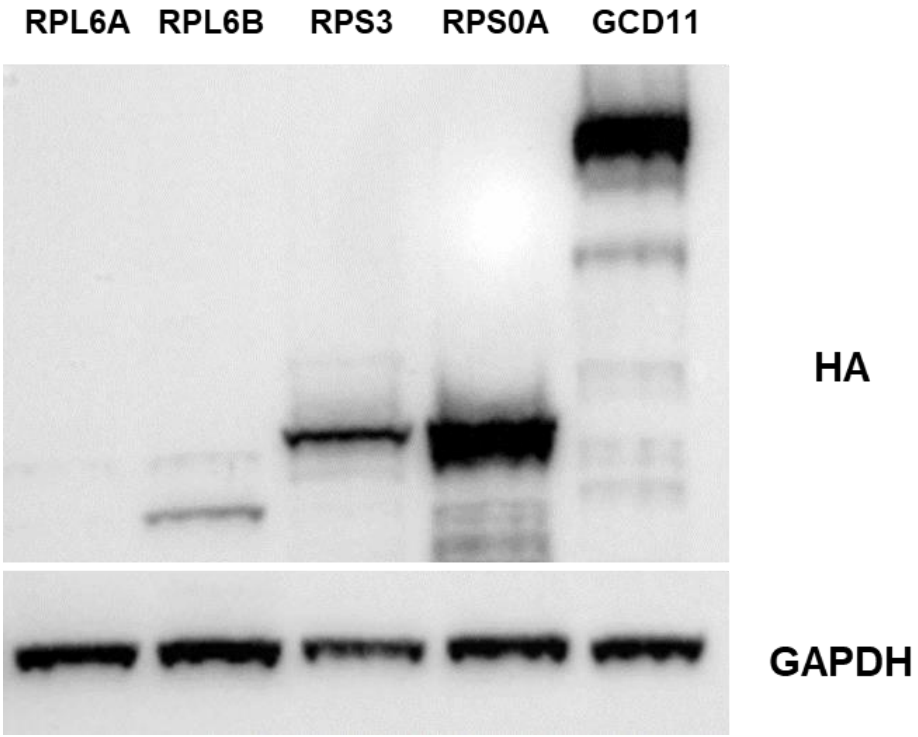

Figure 4.5: Protein expression of ZZ-tagged ribosomal proteins. ZZ-tagged ribosomal proteins (from the ZZ-tag collection) were expressed in FLAG-Ydj1 constructs and Ydj1 Δ cells. Total protein was extracted and analyzed via Western Blotting, probing with anti-HA and anti-GAPDH as a loading control.

Yeast cells were grown to mid-log and then induced in galactose media for 4 hours. Cells were disrupted, proteins were separated by SDS-PAGE and analyzed via Western Blotting

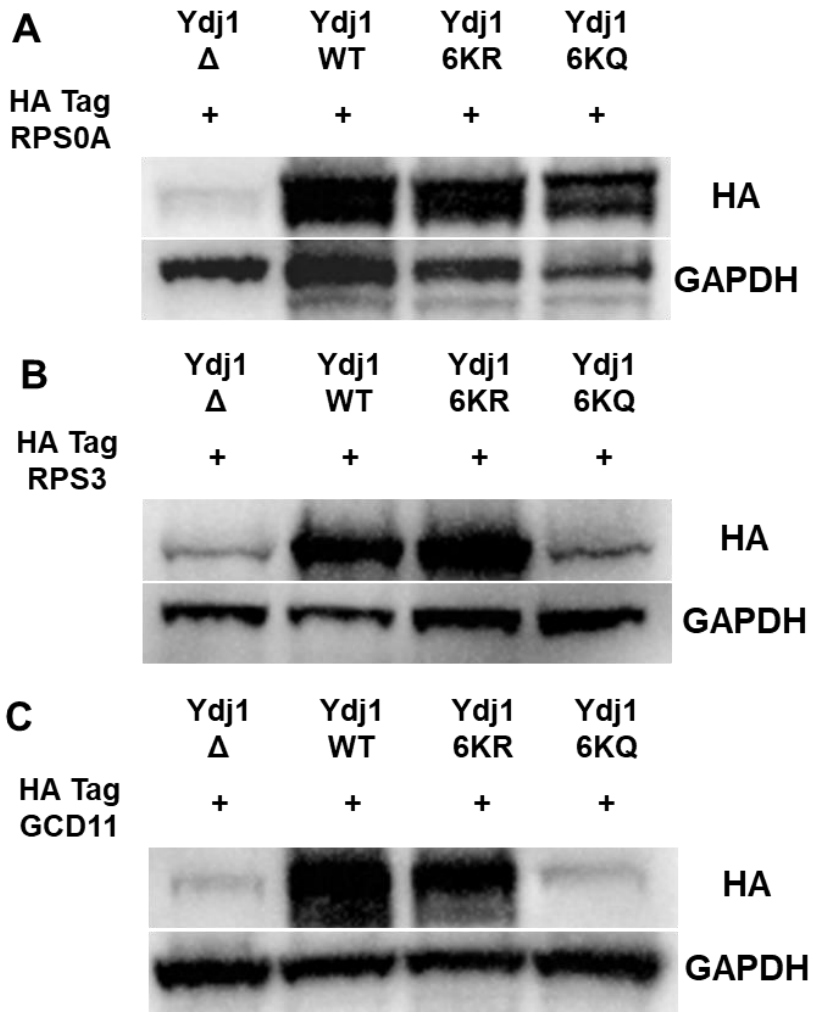

Figure 4.6: Ydj1 acetylation alters the interaction with ribosomal proteins. The levels of protein with ZZ-tagged proteins RPS0A, RPS3, and GCD11, A, B and C respectively. Total protein was extracted and analyzed via Western Blotting. Lysates were probed with anti-HA and anti-GAPDH, and levels of protein varied based on translational protein. Ydj1 Δ transformed with each ZZ tag showed a reduced protein level, while those of the Ydj1 6KQ also showed a reduced protein level

with an anti-HA antibody and anti-GAPDH for loading control. Rpl6a and Rpl6b showed a faint band at their expected molecular weights. Rps3, Rps0a and Gcd11 expressed at

higher levels all at the correct molecular weight. Based on these results, we decided to analyze Rps3, Rps0a and Gcd11 levels in WT, *ydj1*Δ, 6KR and 6KQ yeast.

The stability of Rps0A was dramatically reduced in cells lacking Ydj1 and moderately reduced in 6KQ cells (Figure 4.6A). Excitingly, the stability of Rps3 and Gcd11 was also clearly dependent on Ydj1 function, with lowered expression of both proteins in cells either lacking Ydj1 or expressing the KQ mutant (Figure 4.6B-C).

4.3. DISCUSSION

Post-translational modification of chaperones impacts their protein interactions with clients and co-chaperones. We anticipated that acetylation of Ydj1 would have a similar impact. In our proteomic study, we identified 314 interactors of Ydj1, with 215 of these being unchanged between 6KQ and 6KR samples. Overall, there were a total of 99 interactors that showed a change in interaction under acetylated status Out of these 99 changed interactors, 44 of these showed an increase in interaction, and 55 of these showed a decrease in interaction. Based on previous knowledge of acetylation, it is not surprising that there was a large pool of interactors. Ydj1 is partnered with Ssa1 and their collaboration leads to protein reactivation and folding, however, Ydj1 is promiscuous in its binding leading to a large pool of clients. Since Ydj1 associates with Ssa1, and most recently has been shown to create transient ternary complexes with Hsp82 [53], we would expect to see a change in these chaperone interactions.

Interestingly, despite our prediction from structural information, interaction between Ssa1 and Ydj1 appeared to be unchanged in the 6KQ sample. Based on our previous experience, we postulated that this may be due to complexities in identifying and

quantifying the highly similar Ssa isoforms. To bypass this issue, we performed co-immunoprecipitation/Western Blot analysis of the Ydj1-Ssa1 interaction using antisera specific to the Ssa1 isoform. Consistent with our original hypothesis, acetylation of Ydj1 indeed disrupted interaction with Ssa1. It is interesting to note that the 6KR displayed a higher-than-normal Ssa1-Ydj1 interaction, which may suggest that WT Ydj1 is basally acetylated to some extent.

We saw an expected increase in Ydj1-Hsc82 interaction in the 6KQ sample. While Hsp40s are responsible for bringing partially folded clients to Hsp70, these partially folded clients either need to be fully folded by Hsp70, or handed off to Hsp90, to fully activate certain proteins such as glucocorticoid receptors. It has been shown that Hsp70 and Hsp90 directly interact via the middle domain of Hsp90 and there is evidence that Hsp40s directly interact with Hsp90, also via its middle domain [53]. It is suggested that the client is first bound by Hsp40, and then delivered to Hsp70. This in turn activates the ATPase activity of Hsp70 and both interact with the middle domain of Hsp90. While forming this transient complex, Hsp70 and Hsp40 eventually dissociate, and Hsp90 will finalize the folding process. This process is conserved, and has been shown in yeast, with Ydj1 directly interacting with Hsp82 [53]. It is possible that acetylation of Ydj1 is the catalyst to allow client transfer between Hsp70 and Hsp90 in yeast.

Chaperones bind newly synthesized protein, helping them fold and gain their native state and thus intimately tied to the ribosomal site. Mutations in Ydj1 (*ydj1-151*, a temperature-sensitive mutant) has been shown to prevent efficient translation by delaying translation of two heterologously expressed proteins, firefly luciferase, and GFP [54].

Additionally, Ydj1 has shown sensitivity to translation inhibitors such as hygromycin B and cycloheximide [55].

Protein synthesis is carried out by the ribosome; an RNA-protein complex consisting of a small and large subunit [56]. Ribosome assembly contains more than 200 assembly factors, 79 ribosomal proteins, rRNAs and other ribosomal-related proteins that participate in the complex pathway to achieve protein synthesis [56, 57]. Assembly of the ribosome begins with transcription of rRNA in the nucleolus, then undergoes complex folding pathways, nucleotide modification and binding to ribosomal proteins [57]. In *S. cerevisiae*, the small subunit (40S) has 33 ribosomal proteins, and an 18S ribosomal RNA. The large subunit (60S) has 46 ribosomal proteins and 3 ribosomal RNAs. The small subunit is the location of the decoding site, where the anticodon of an amino-acyl tRNA base pairs with its respective codon found in the mRNA [56]. The large subunit contains the peptidyl transferase center (PTC), which is the active site of the unit. This is where rRNA catalyzes the formation of peptide bonds and hydrolyzes the peptidyl-tRNA bonds [56]. Since Ydj1 is not known to directly associate with the ribosome, it is interesting that interactions within the translational category showed various changed hits.

Upon further examination, many of the interactors of Ydj1 identified in our screen were structural components of the ribosome, both from the small and large subunit. Kolhe et al. [58] has shown using polysome fractionation that Hsp90 is primarily associated with the small subunit, while the nascent chain binding chaperone Hsp70 was found to be associated with all the ribosome fractions. If Hsp90 is found associating with the 40s subunit, and Hsp70 is found all over, it would make sense that Ydj1 would interact with these ribosomal proteins. It is likely that the nascent polypeptides may be “generic” and Ydj1- Hsp70 are

working to fold the nascent polypeptide found in the exit of the ribosome. We saw that the stability of Rps0A, Rps3 and Gcd11 were drastically reduced in cells lacking Ydj1. The 6KQ cells showed a reduced stability of these proteins as well, which is the opposite of what we see in our mass spec data, where these proteins are shown to be increased in interaction. Overall, this suggests that these proteins are dependent upon Ydj1's function, and the loss of interaction seen in Ydj1 delete cells suggest that these ribosomal proteins (Rps0A and Rps3) are genuine, novel clients of Ydj1.

Sis1 is an essential Hsp40 that is associated with the ribosome and inhibition of Sis1 results in translational defects [59-61]. Interestingly, while Sis1 was thought to interact with Ssb, it was found that Sis1 stimulates the ATPase activity of Ssa1 instead [59]. The translation factor PAB1, along with other ribosome associated proteins Sis1 and Ssa1 have been shown to interact on translating polysomes. Pab1 and Sis1 both interact with Ssa1, but Sis1 and Pab1 do not directly interact. It is likely they interact via mediation of an Hsp70. Overall, Horton et al.[59] showed that Ssa has a specific interaction on the translating polysomes with Pab1 and Sis1.

Interestingly, Sis1 and Ydj1 have both been implicated in the construction of stress granules, suggesting a functional overlap [61]. Both proteins were found to accumulate in stress granules, and defects in these proteins showed a decrease in the disassembly and the clearance of stress granules. Defects in Ydj1 showed accumulation of stress granules and a reduced recovery of translation under stressed conditions. Defects in Sis1 did not affect the recovery of translation, but instead showed a reduction in targeting the stress granules to a specific location [61]. It is interesting that each of these proteins can play a role in the same process, but show a very different outcome.

In our study, we observed for the first-time interaction between these two J-proteins, an interaction which was mediated by acetylation. The biological importance of this interaction has yet to be determined and will be the focus of future studies. It should be noted that while there are substantial differences between the 6Q and 6R interactomes, 65% of Ydj1 interactions remained unchanged. This data along with the relatively subtle phenotypes observed suggests that acetylation may be selectively fine-tuning Ydj1 function.

CHAPTER 5: CHARACTERIZING THE ROLE OF YDJ1 J-DOMAIN ACETYLATION ON *IN VITRO* CHAPERONE FUNCTION

5.1. MATERIALS AND METHODS

5.1.1. Bacterial Plasmids

For bacterial expression, the *YDJ1* gene was PCR amplified from yeast genomic DNA to produce overhangs containing XbaI and XhoI restriction sites on the 5' and 3' ends of the PCR product. After digestion with XbaI and XhoI, the YDJ1-containing fragment was inserted into bacterial expression plasmid pRSETA (Thermo). K23R/Q, K37R/Q and 6KQ/R versions of these plasmids was engineered by Genscript.

5.1.2. Expression of recombinant Ydj1 from bacteria

HIS-Ydj1 plasmids were transformed into BL21-competent cells. His-tagged Ydj1, K23R, K23Q, K37R, K37Q, 6KR, and 6KQ were grown to an OD600=0.6 in 2YT media supplemented with 100ug/ml ampicillin at 37 °C. Once the appropriate OD was reached, protein expression was induced by adding IPTG at a 1mM final concentration. Cultures were incubated at 37 °C with shaking for an additional 4 hours. Induced cells were then harvested via centrifugation (5000 rpm, 5 minutes) and sonicated in intervals of 30 seconds on ice until clear. Protein extracts were stored at -20°C until prepared for Western Blot analysis.

5.1.3. Biolayer interferometry (BLI) analysis

Biolayer interferometry (BLI) was carried out by Dr. Sue Wickner and Joel Hoskins at the NIH National Cancer Institute in Bethesda, MD. Briefly, the BLI was carried out

according to [62]. BLI was used to monitor the interaction between Ssa1, His-Ydj1 and mutant proteins using a Sartorius Octet R4 instrument and Ni-NTA biosensors at 23˚C. Ydj1 (5ug/ml) was loaded on the biosensors and the association of Ssa1 (1uM) with Ydj1 was monitored over time followed by dissociation in the absence of Ssa1. All Ydj1 and Ssa1 BLI steps were performed in 20 mM Tris pH 7.5, 50 mM KCl, 10 mM MgCl2, 0.01% Triton X-100 (vol/vol), 0.02% Tween-20 (vol/vol) and 1 mg/mL BSA. For each experiment, nonspecific binding was monitored using a reference biosensor subjected to each of the above steps in the absence of the biotinylated protein, and the nonspecific binding signal was subtracted from the corresponding experiment. For steady-state analysis of kinetic association data, the association phase from three replicates at each mutant concentration was fit using a single exponential equation in Prism 9 for Mac (GraphPad Software version 9.4.1), and the plateau value determined from the fit was plotted versus the concentration of mutants. The resulting binding curve was analyzed using a one-site specific binding model in Prism to determine the Kd (equilibrium binding constant) and Bmax (maximum specific binding) values.

5.1.4. Luciferase refolding assay

Luciferase reactivation assay was carried out by Dr. Sue Wickner and Joel Hoskins at the NIH National Cancer Institute in Bethesda, MD. Briefly, the luciferase reactivation was carried out according to [62]. Luciferase (8 μM) was chemically denatured in 5M guanidine hydrochloride, 25 mM Hepes pH 7.5, 50 mM KCl, 0.1mM EDTA, 4 mM DTT for 10 min at 23 °C. For reactivation, denatured luciferase was diluted 100-fold into 25 mM Hepes pH 7.5, 0.1 M KOAc, 5 mM DTT, 10 mM Mg(OAc)2, 100 μg/ml BSA, 2 mM

ATP, an ATP regenerating 17 system (10 mM creatine phosphate, 3 μg creatine kinase), 3 μM Ssa1, 0.3 μM His-Ydj1 WT, His-Ydj1 6KR, His-Ydj1 6KQ, and individual sites K23R, K23Q, K37R, and K37Q. Aliquots were removed at the indicated times and light output was measured using a Tecan Infinite M200Pro in luminescence mode with an integration time of 1000 ms following the injection of luciferin (50 μg/ml). Reactivation was determined compared to a non-denatured luciferase control.

5.1.5. Measurement of Ssa1 ATPase activity

Ssa1 ATP hydrolysis measurements were carried out by Dr. Sue Wickner and Joel Hoskins at the NIH National Cancer Institute in Bethesda, MD. Briefly, the ATPase assay was carried as in [62]. Steady-state ATP hydrolysis was measured at 37 °C in 20 mM Tris pH 7.5, 50 mM KCl, 2 mM DTT, 0.005 % (vol/vol) Triton X-100, 5 mM MgCl2, and 2 mM ATP using a pyruvate kinase/lactate dehydrogenase enzyme-coupled assay and 1 μM Ssa1, His-Ydj1WT or mutants.

5.1.6. Incorporation of acetylated lysines at specific sites using genetic code expansion

C321.ΔA.exp cells were transformed with plasmids pEVOL-AcKRS (Addgene) and FLAG-Ydj1-K37Ac carrying the amber codons (TAG) at K37. Cells were grown in LB supplemented with 100ug/mL ampicillin and 34ug/mL chloramphenicol at 30℃ until they reached an OD_{600}= 0.6. To induce protein expression, cells were supplemented with 0.2% arabinose, 1mM IPTG, and 5mM acetyl-lysine (AcK). Incubation was continued at 30℃ for 6-8 hours. Cells were pelleted (5000 rpm, 5 minutes) and pellets were resuspended with 2mL lysis buffer (B-PER, protease inhibitor cocktail and supplemented with universal

nuclease and lysozyme). Suspension was sonicated in 30 second intervals and placed on ice until clear. The samples were then centrifuged at 14,800 rpm for 5 minutes to remove excess debris. Proteins were stored in -20 ℃ until ready for Western Blot analysis.

5.1.7. Analysis of recombinant protein expression

Following sonication, protein lysates were quantitated via Coomassie Blue then NuPage LDS sample buffer was added and were heated to 100 ℃ for 5 minutes. To analyze protein expression, 20ug of protein was separated via SDS-PAGE. Bands were visualized by Coomassie staining. For Western Blot analysis, 20ug of protein was separated as above and blotting was performed as described using an anti-HIS antibody (Quigen) to determine protein expression.

5.2. RESULTS

5.2.1. Expression of recombinant Ydj1 acetylation mutants in bacteria

Our results in Chapter 3 suggested that Ydj1 acetylation disrupts interaction with Ssa1, altering the stability of client proteins and the heat shock response. We decided to corroborate these findings with studies on in vitro chaperone function. FLAG-Ydj1 (WT, K23R, K23Q, K37R, K37Q, 6KR and 6KQ) were all induced in BL21 cells and analyzed by SDS-PAGE/Western Blotting (Figure 5.1). Robust expression of WT and all mutant versions of Ydj1 was observed in bacteria, setting the stage for our in vitro assays (Figure 5.1). We previously observed that K37Q, K46Q, K48Q and 6KQ mutants show reduced mobility on SDS-PAGE and that the abundance of K37Q was higher than WT when expressed in yeast (Figure 3.6). When expressed in bacteria, this reduced mobility was also observed in the 6KQ mutant (Figure 5.1).

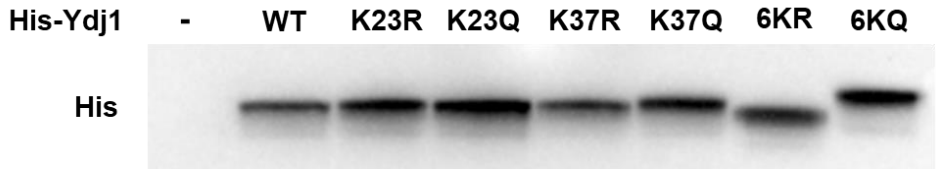

Figure 5.1: Bacterial expression of His-Ydj1. His-tagged Ydj1 was expressed in BL21 cells, and total protein was extracted from cells expressing the R or Q mutations at sites of interest.

5.2.2. Mutant Ydj1 proteins show defective chaperone activity in vitro

In line with what we have seen in Chapter 3, acetylation impacts the basic function of Ydj1, which suggests a conformational change is occurring. His-tagged Ydj1 (Figure 5.1) was sent to our collaborators, Dr. Sue Wickner and Joel Hoskins at the NIH National Cancer Institute in Bethesda, MD. Since we have seen acetylated Ydj1 leads to growth defects, and changes within the interactome, we wanted to investigate the function of Ydj1

and its ability to stimulate the ATPase activity of Hsp70. The steady-state ATP hydrolysis was measured using mutant Ydj1 and Ssa1 (Figure 5.2). Similarly, Ydj1 WT stimulates ATP hydrolysis by Ssa1, as well as 6KR (Figure 5.2A). However, the 6KQ mutant does not, and shows a drastic reduction in the stimulation of ATP hydrolysis. It is likely that due to the location of the multiple mutations, especially near the HPD region these mutants would be defective due to their proximity to Hsp70s binding site. We then wanted to see how the single sites would behave in stimulating ATP hydrolysis.

Ydj1 WT is able to stimulate ATP hydrolysis by Ssa1, as well as the mutants K23R, K23Q, and K37R (Figure 5.2B). K37Q has a reduced ability to stimulate ATP hydrolysis, consistent with our results from Chapter 3, where the K37Q mutants is impacted for growth.

To determine if mutant Ydj1 was defective for chaperone activity, we tested them via an in-vitro chaperone assay. We monitored guanidine hydrochloride (GuHCl) denatured luciferase and the Ydj1 mutants combined with Ssa1. Firefly luciferase has been used as a model substrate to asses chaperone activity and protein folding [63, 64]. The His-tagged Ydj1 WT is active in luciferase reactivation in-vitro, showing its effectiveness with binding to Ssa1 (Figure 5.3A). The 6KQ mutant shows no activity and is dead while the 6KR mutant shows more activity than the WT.

These results are in agreeance with what we saw with the ATPase activity, and we further wanted to test each of the single sites. From Figure 5.3B, we can see that the Ydj1 WT is active in luciferase reactivation, while the K37Q mutant is dead. This aligns with the residue's location, next to the HPD region with mutations in this region likely to affect its binding to Hsp70 and its folding activity. The K23Q and K23R show a similar activity

to the WT. Interestingly, the K37R is more active than the WT. The R's showing a higher activity can also be seen in Figure 5.2B, where the K37R had a higher activity in ATP hydrolysis. Overall, these results suggest that these residues (perhaps the K23 and K37) are important for Ydj1-Ssa1 interaction.

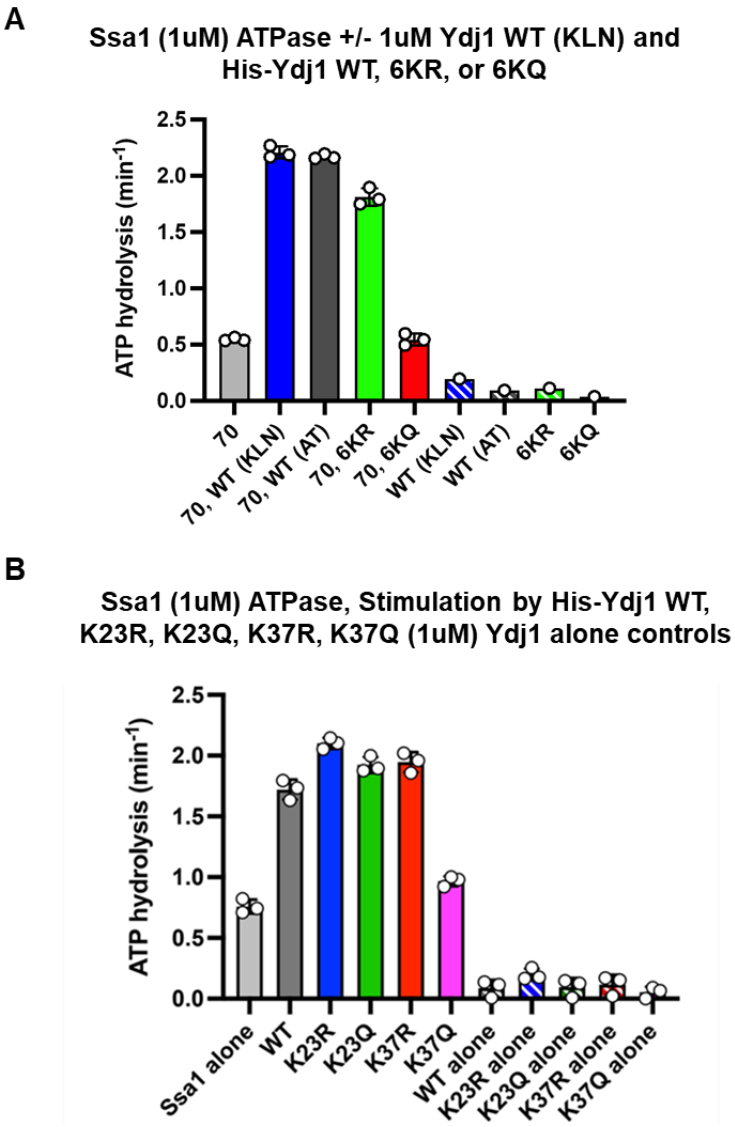

Figure 5.2: ATPase Activity of His-Ydj1. The steady-state ATP hydrolysis was measured using 1uM Ssa1, WT, or mutant Ydj1. Results are shown as the means +/- SD for three or more replicates.

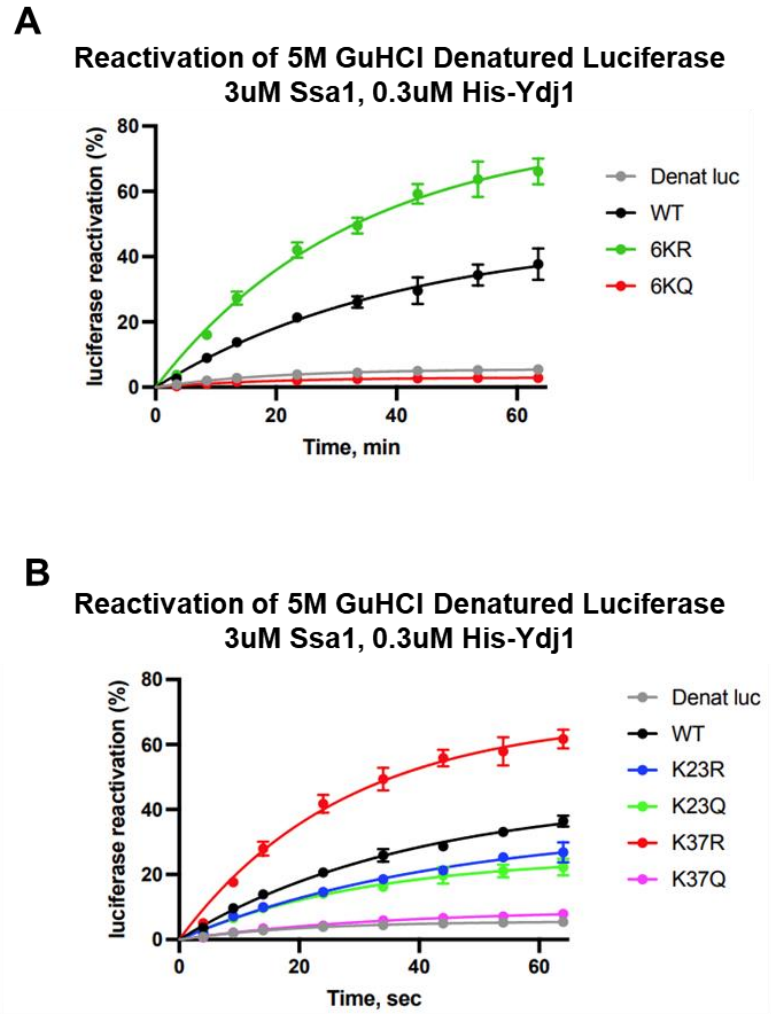

Figure 5.3: Time course of luciferase refolding activity of His-Ydj1. Time course of luciferase refolding of His-tagged Ydj1 WT, K23R, K23Q, K37R, and K37Q (0.3uM) in the presence of constant levels of Ssa1 (3uM). Results are shown as the means +/- SD for three or more replicates.

To confirm our hypothesis of residues K23 and K37 playing an important role in Ydj1-Ssa1 interaction, we took the His-tagged mutants and carried out a binding assay with Ssa1. We wanted to monitor the interaction between Ydj1 and Ssa1, and monitor the activity of the mutated Ydj1. As expected, we saw that Ssa1 effectively binds to His-Ydj1 WT (Figure 5.4) and the Ydj1 37Q mutant is defective and unable to bind to Ssa1. This is consistent with our hypothesis that the Q mutations cause defects in binding and growth. This is also consistent with our hypothesis that mutations in the region near the HPD cause

a disruption in interaction between Ydj1-Ssa1. Overall, these results suggest that these residues (K23 and K37) are important in the binding to Ssa1.

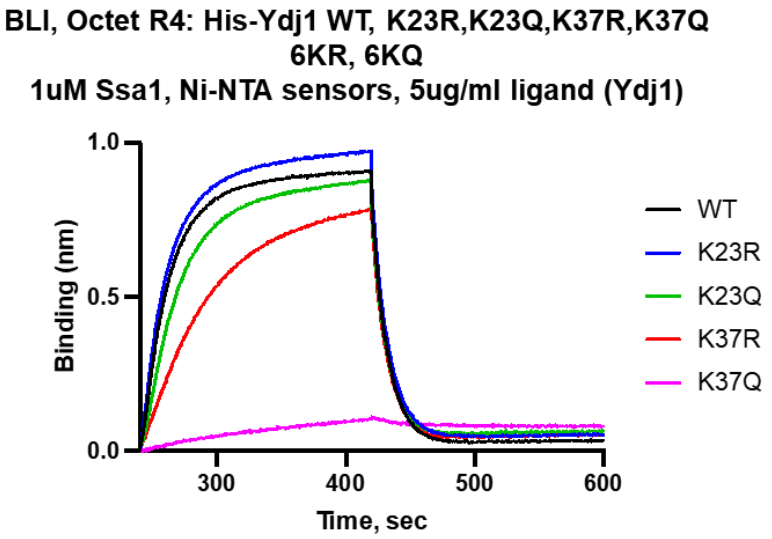

Figure 5.4: Ydj1-Ssa1 binding is impacted by acetylation. The ability of His-tagged Ydj1 WT, and mutated Ydj1 to bind with Ssa1 was analyzed using BLI

5.2.3. Creating acetylated Ydj1 at sites of interest using genetic code expansion

Site specific lysine acetylation has been used in previous studies to synthesize acetylated histones [65]. The genetic code expansion is useful in studies where there are limitations to mimics such as a KQ mutation. We can create recombinant acetylated Ydj1 mutants in bacteria using the genetic code expansion. The acetylated Ydj1 will be achieved using the genetic code expansion and aminoacyl-tRNA synthetase/tRNA-CUA pairs, using [65-68] as references for methodology and reagents. These acetylated Ydj1 mutants will be produced by replacing the selected lysine codons (K23 and K37) with the amber codon (CUA) on standard expression plasmids. From there, we will express the proteins from these acetylated mutants (K23 and K37) in C321.ΔA.exp cells. This will give us a true acetylated version of Ydj1 K23 and K37 to observe in the in-vitro assays previously carried

out with the Q mimics. The purified Ydj1 will then be assessed for its ability to bind to specific clients, its structure change and interactions with other chaperones and co-chaperones.

Protein expression was induced in the co-transformants carrying the pEVOL-AcKRS and Ydj1 carrying amber codons as described in 5.1.6. Induction for 8 hours at 30 °C was carried out first, according to [65]. We were not able to see induction of the proteins, so we carried out induction overnight, at 30 °C, to see if we could get protein expression. Finally,

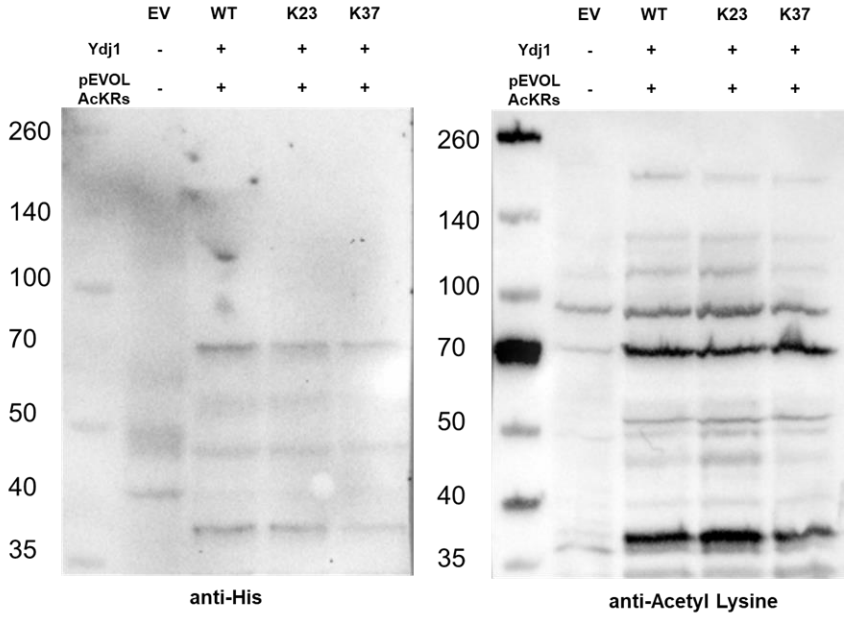

Figure 5.5: Protein expression of Ydj1 constructs carrying amber codons. C321.ΔA.exp cells carrying the indicated plasmids were induced for protein expression, and protein was extracted as described in 5.1.6. Lysates were analyzed via Western Blot, and probed with the respective antibodies to show protein expression.

we carried out induction overnight, at 16 °C, and we were unable to see protein expression. Figure 5.5 shows the Western Blot analysis of the Ydj1 constructs carrying the amber codons, His-Ydj1 WT and an empty vector. The His-Ydj1 constructs show a band around 52kDa, but the amber constructs did not show a clear band at this molecular weight.

Further, when we probed with acetyl lysine, we saw no clear induction of the acetylated proteins, suggesting the proteins carrying the amber codons did not take in the acetyl lysine.

5.3 DISCUSSION

In accordance with our cellular data, Ydj1 K23Q and K37Q have a reduced ability to bind Ssa1 and stimulate its ATPase activity. This is also apparent in the inability of these mutants to promote the correct folding of the model luciferase substrate. It is also interesting to note that the R mutations consistently showed a higher level of activity than the WT, matching the data from the Ydj1-Ssa1 IP in Chapter 3. Although K to Q mutations have been used successfully for decades to mimic acetylation it is always desirable to create a protein with the natural modification of the sites for studies [42]. We sought to use genetic code expansion with the aid of previous literature to produce site-specific acetylated Ydj1 [65]. Despite several attempts, we were unsuccessful in producing acetylated Ydj1. It is challenging to speculate the cause of this issue, but may be linked to the bacteria strain used or the amount of acetyl-lysine added to the media. Going forward we hope to optimize this experiment to validate our current in vitro experiments.

5.4. CONCLUSION

Overall, our results in vitro and in vivo suggest that acetylation of the residues located in the J-domain particularly K23 and K37 disrupt Ssa1-Ydj1 interaction thus decreasing its cellular activity. Our data thus produce a conundrum-why would the cell want to inhibit Ydj1 activity? There are several possible answers to this question. Over a decade ago, researchers discovered that the G1 cyclin Cln3 competed with Ydj1 for binding to Ssa1

and that this process was critical for cell cycle progression [69]. Follow up studies determined that phosphorylation of Ssa1 on T36 was required for this process, allowing displacement of Ydj1 from Ssa1 and allowing Cln3 to bind and be targeted for destruction [70]. It is thus possible that Ydj1 acetylation is part of this mechanism to regulate Ssa1-Ydj1 interaction during cell cycle progression. Previous studies have underpinned the importance of acetylation on the heat shock response. In mammalian cells, HDAC6 deacetylase activity is required for HSP70-DNAJA1 interaction [41]. Similarly, in yeast our lab has shown that deacetylation of Ssa1 is needed for a robust heat shock response. This evidence, combined with our data suggest that deacetylation of both Ssa1 and Ydj1 may be coordinated to increase Ssa1-Ydj1 interaction and trigger refolding of important client complexes.

While proteomic technologies continue to advance, there are still obstacles to overcome. Limitation of technology, particularly the challenges of custom site-specific render it difficult to determine the stoichiometry of Ydj1 acetylation in growing yeast cells. Although KQ mutants are impaired for Ydj1 function while the KR mutants are not, the Ssa1-Ydj1(KR) interaction in yeast is substantially stabilized compared to WT. These two pieces of data suggest that only a small (but non-zero) amount of Ydj1 is acetylated at any time.

Despite our cellular and in vitro evidence pointing to a loss of activity in KQ mutants, it is clear that this is not the complete story. A large number of Ydj1 interactions are not altered by acetylation, and several interactions are even stabilized by Ydj1 acetylation. Our mass spectrometry evidence thus suggests that Ydj1 acetylation may work to fine tune the specificity of Ydj1-client interactions.

Ydj1 mediates the majority of its functions through interaction with Hsp70 and Hsp90, yet the mechanism by which clients are sequentially shuttled from Hsp70 to Hsp90 remains undetermined. In this study we have shown that acetylation of Ydj1 inhibits interaction with Ssa1 while supporting interaction with Hsc82. While further work is needed, it is exciting to speculate that Ydj1 acetylation is this molecular trigger for client transfer. We intend to examine this possibility by examining the impact of similar site mutations in human DNAJA1.

Our proteomics screen identified a large number of novel Ydj1 interactors, many of which were altered by Ydj1 acetylation. Of particular interest was interaction with proteins involved in translation. The differential behavior of large and small ribosomal subunits upon ydj1 acetylation suggests that modification of Ydj1 may be important for ribosomal assembly/disassembly. Going forward, we hope to measure translational fidelity and chaperone-polysome association in yeast expressing acetylation site mutants to provide mechanistic insight.

Acetylation plays an important role in human pathologies. An HDAC6 inhibitor was shown to alter the tau interactome leading to the degradation of pathological Tau in an AD animal model. When tau undergoes acetylation, it recruits chaperone proteins Hsp70, Hsp40 and Hsp110 and this complex binds to acetylated tau, leading it to the clearance, via the proteasomal pathway. This is an interesting link between acetylation and the regulation of the Hsp70-Hsp40 chaperone network. This suggests that chaperones/co-chaperones within this network need to be acetylated in order to regulate their recruitment in the modulation of protein degradation.

Ydj1 (and DNAJA1) have been shown to interact with pathological proteins such as Tau, ABeta, and HTT, however, there are no mutations found that link them to neurodegenerative diseases [20, 71]. However, changes in their expression in the cell have been found to affect the progression and toxicity of certain neurodegenerative diseases [71]. DNAJA1 has been shown to bind to the Parkinson Disease associated α-syn fibrils which in turn increases Hsc70 binding to the fibrils. When these are added directly to cells incubated with α-syn fibrils it led to an improved cell viability [71]. It has been shown that regulation of DNAJA1 expression can affect the aggregation of these types of proteins, specifically ABeta and tau [20, 71]. When DNAJA1 homologs in *C. elegans* is knocked down, the toxicity of ABeta increases, while knockdown of DNAJA1 in HeLa cells expressing tau led to an increase in tau levels which leads to the suggestion that these J-proteins play a key role in the modulation of ND-associated proteins [71].

The regulation of the chaperone network via acetylation may prove to be a path to novel therapies for ND's. If we can regulate the function of these chaperones, specifically the function of co-chaperones, we may be able to fine-tune how these cells process stress and the damaging events such as aggregates and fibril buildups. Traditional therapies have not been successful in the treatment of these types of diseases, perhaps there is hope for a novel therapeutic in the alteration of the chaperone network.

Table 1. Yeast Strains Used in This Study

Strain	Strain Name	Genotype	Reference/Source
yAT28	*ydj1Δ*	MATα S288c (BY4742) ydj1Δ::KanMX4	This study

Table 2. Plasmids Used in This Study

Plasmid	Description	Reference/Source
pAT743	YDJ1-Flag	Vector Builder
pAT744	YDJ1-Flag K23Q	Vector Builder
pAT745	YDJ1-Flag K24Q	Vector Builder
pAT746	YDJ1-Flag K32Q	Vector Builder
pAT747	YDJ1-Flag K37Q	Vector Builder
pAT748	YDJ1-Flag K46Q	Vector Builder
pAT749	YDJ1-Flag K48Q	Vector Builder
pAT750	YDJ1-Flag 6KQ	Vector Builder
pAT751	YDJ1-Flag K23R	Vector Builder
pAT752	YDJ1-Flag K24R	Vector Builder
pAT753	YDJ1-Flag K32R	Vector Builder
pAT754	YDJ1-Flag K37R	Vector Builder
pAT755	YDJ1-Flag K46R	Vector Builder
pAT756	YDJ1-Flag K48R	Vector Builder
pAT757	YDJ1-Flag 6KR	Vector Builder
pAT870	pRSETA	This Study
pAT983	HIS-Ydj1 (WT) pRSETA	GenScript
pAT984	HIS-Ydj1 (K23Q) pRSETA	GenScript
pAT985	HIS-Ydj1 (K23R) pRSETA	GenScript
pAT986	HIS-Ydj1 (K37Q) pRSETA	GenScript
pAT987	HIS-Ydj1 (K37R) pRSETA	GenScript
pAT988	HIS-Ydj1 (6KQ) pRSETA	GenScript
pAT989	HIS-Ydj1 (6KR) pRSETA	GenScript
pAT992	pRSETA, Empty Vector (transformed in BL21)	This Study
pAT993	HIS-Ydj1 (WT) pRSETA (transformed in BL21)	This Study
pAT994	HIS-Ydj1 (K23Q) pRSETA (transformed in BL21)	This Study
pAT995	HIS-Ydj1 (K23R) pRSETA (transformed in BL21)	This Study
pAT996	HIS-Ydj1 (K37Q) pRSETA (transformed in BL21)	This Study
pAT997	HIS-Ydj1 (K37R) pRSETA (transformed in BL21)	This Study
pAT998	HIS-Ydj1 (6KQ) pRSETA (transformed in BL21)	This Study
pAT999	HIS-Ydj1 (6KR) pRSETA (transformed in BL21)	This Study
pAT1001	pEVOL-AcK R's	Addgene
pAT1097	pJD RAUG/FAUG	Dr. John Lorsch
pAT1098	pJD RAUG/FUUG	Dr. John Lorsch
pAT1157	pBG1805-GAL1 promoter-ZZ-HA-RPL6A	Dharmacon, Yeast ORF Library
pAT1158	pBG1805-GAL1 promoter-ZZ-HA-RPL6B	Dharmacon, Yeast ORF Library
pAT1159	pBG1805-GAL1 promoter-ZZ-HA-RPS0A	Dharmacon, Yeast ORF Library
pAT1160	pBG1805-GAL1 promoter-ZZ-HA-RPS3	Dharmacon, Yeast ORF Library
pAT1161	pBG1805-GAL1 promoter-ZZ-HA-GCD11	Dharmacon, Yeast ORF Library

Table 3. Antibodies Used in This Study

Antibody	Company	Dilution	Source
Anti-FLAG	Sigma (F3165-1MG)	1:2000	This study
Anti-HIS	Qiagen (Cat#34670)	1:2000	This study
Anti-GAPDH	Invitrogen (MA5-15738)	1:3000	This study
Anti-PGK	Invitrogen (459250)	1:4000	This study
Anti-HSP26	N/A	1:4000	Johannes Buchner[36]
Anti-HA	ThermoFisher (Cat#26183)	1:2000	This study
Secondary Antibody- Mouse	GE (NA931V)	1:5000	This study
Secondary Antibody-Rabbit	GE (NA934V)	1:5000	This study

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