Winter 12-15-2021

The Effects of Molecular Chaperone Modulation on Protein Folding, Prion Formation, and Prion Propagation in Saccharomyces cerevisiae

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by
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A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

December 2021
St. Louis, Missouri

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<u>Acknowledgments</u>

First, I would like to thank the vast community of people who have supported me over the past seven years and throughout the rest of my life. As everyone knows, I am an open book, over-sharer, and an affirmation champion, which means I inadvertently pull a lot of people into my orbit. I appreciate the kind words and support I have received from every one of you. Every time we met for coffee or lunch or caught up in a hallway or talked on the phone I was buoyed by the support and encouraging words we shared. Thank you all so much for supporting me and for letting me support you.

Next, I'd like to thank you, Heather, for your mentorship and friendship. When I was looking for a lab I didn't expect that my PI would care about my whole being. I expected that a PI would just care about my productivity and scientific development. I have been really lucky to have a PI that cares about my mental and physical health and development as well as who I am becoming in a professional sense. You have been extremely supportive in my changing career goals, allowing and supporting me in exploring many different career avenues throughout my time in the lab. Within lab, you have taught me so much about the importance of thinking critically and not just asking the interesting questions, but finding the right ways to answer those questions. I will certainly take the lessons I have learned from you about research, mentoring, and how to treat others with me throughout the rest of my life.

I also would like to thank my thesis committee. All of you have been so encouraging of my work and supportive of me. I appreciate the thoughtful questions you have asked in order to help me think deeper about my projects. Hani, thank you for your guidance as my chair, helping me to become a better scientist, and supporting me through the rough patches. Chris, thank you

for asking difficult questions and being such a helpful collaborator. Chuck and Gretchen, thank you for joining my committee later in my grad career, getting caught up so quickly, and giving such thoughtful feedback. Jim, thank you for always caring about my well-being, giving me opportunities to explore different careers, allowing me to give back to the St. Louis community through your outreach projects, and letting me intern in your lab back in 2012. You've taught me that science isn't just about research; it's also about caring about the people who do the research and giving everyone opportunities to explore science.

I could not have gotten to this point in my graduate career without Sally Vogt and the other coordinators in DBBS. Thank you so much, Sally, for reminding me to turn in documents over and over again, for not getting angry at me when I kept forgetting, and for always treating me with such genuine warmth and understanding. I have so appreciated you shepherding me, a very unruly and annoying student, through this program with so much grace and care.

To my lab, thank you for being a place where I can be my whole, entire, loud, clumsy self every single day. Through every bit of broken glassware and loudly belted broadway tune you have…tolerated me, and that is truly a gift! You have all made me a better friend and scientist, and, as George Gershwin would say, "who could ask for anything more?" To Ankan, thank you for supporting me through this last season of my grad work. Going to lab would have been so much harder without our daily conversations and catch-ups. I've appreciated all the help and understanding you have given me throughout my graduate career, but especially over the last year and a half. Your insights in science and life have helped make me a better person, thank you. To Melanie, thank you for being the very best work-spouse a person could ask for. Getting to have lunch with you and hear about your life and share about mine was truly the highlight of my days. After you graduated it was definitely harder to go in every day and your presence has

been sorely missed. Thank you for being a true friend to me and for really understanding my experience. I've been so lucky to have such a kind, caring, and talented friend and scientist to go through grad school with, thank you.

I would not be here without my parents. Dad, you have taught me the value of hard work and dedication, as well as valued my goals throughout my life. I know it makes you so proud to tell people that I am a scientist at WashU, but I know you would be just as proud of me no matter what I was doing. Your love and support of me have allowed me to love and support myself and for that I am forever grateful. Mom, you have held so many titles throughout my life. Mom, friend, confidante, cheerleader, counselor, driver, I could go on forever. Thank you for being a constant in my life. You have shown me how to go through life with humor and grace, even when circumstances were making it really hard to have much of either. Thank you both for loving me for exactly who I am. Thank you for showing me the world so that I could find my place in it. Along the way you both have taught me the most important lesson of all, how to hold myself to high standards while still allowing myself to have a lot of fun. Thank you for only letting me bring one textbook on our spring break trip when I was in fifth grade. Thank you for racing me through the Louvre so that I could see the Mona Lisa, but also making sure we had enough time to ditch our tour group and go to the Hard Rock Café. Thank you for telling me bedtime stories completely in spoonerisms. Thank you for helping me write silly songs about science. Thank you for riding go-carts with me, taking me to virtually every arcade in Gulf Shores, and continuing to go mini-golfing with me when I visit. Thank you for always asking me how the movie ended when I tell you I watched Titanic. These may seem like small things, but they are big parts of what made me who I am and I wouldn't be who I am without you two. Thank you.

I would be remiss if I did not take some time (and a few pages) to thank all my friends. First, thank you Joanna for being my best friend. You have been around since the very beginning. I remember when you found out I was a biology major in college and would come by my room to complain about your biology lab TA. You would talk about how you had "made a scientific discovery" when really you probably just messed up the experiment (sorry!) and that your TA had not thought you were funny at all. Every one of those conversations were so funny and will be etched in my mind forever. Every time you visited me at WashU you would beg me to find a lab that could find out your blood type and no matter how many times I told you that isn't how it worked you never let up. Thank you for always making me laugh and keeping me on my toes. Past all that, you have been so supportive and loving to me throughout my time at WashU. You have never failed to check in on me or call me to cheer me up when I was spending long hours in lab. You have supported me through every twist and turn of my grad career, from leaving one lab to rotate again, to saying I was going to quit over and over, you have helped me think through every situation with calmness and clarity. You are one of those friends who doesn't just watch a person experience things, but jumps in after them to experience it with them. Thank you for doing that for me over and over again.

I'd also like to thank Lindsey, Lindsey, and Rebecca. I don't know what I can say that can adequately describe what you have done for me. Thank you for making me go out to events with you after a bad break-up. Thank you for commiserating with me over failed experiments. Thank you for talking with me about everything that's been wrong about the world in thought-provoking and challenging ways. By creating a comfortable space and challenging me you have helped and allowed me to develop my thoughts further in a safe environment. I can truthfully say that your friendship was one of the reasons I decided to stay at WashU when I really thought

about leaving during my second year. Thank you for helping me remember and discover what was and is important to me.

I'd also like to thank Colin and Patrick for their steadfast friendship. I never would have thought that y'all watching Rainer and I through my constantly open windows (because Rainer tore down the blinds) would have led to such an amazing friendship. You have been the people that just keep showing up for me, both literally and figuratively. From showing up at my apartment to see if I really was in my PJs by 6pm (I was) to supporting me through my transition, you have always been there. You are the people I call when things are going well, but you are even more the people I call when things aren't. Thank you for talking through so many tough times with me and being so understanding. Thank you for making me have fun when I was working too much and for always making the best yeast jokes. Thank you for being part of my family.

To Kate Lipovsky-Durkee, I don't even know where to begin. You are someone who gives everything you do 110% and that has been true throughout our friendship. You have been SO supportive of me over the years. From riding in the party car where you told me you were SURE I'd get into WashU, to my first year when I got a C in a class and you told me it would all be fine, to celebrating successful experiments and me passing the class when I took it again with a disco ball, you have believed in and celebrated me when I found it very difficult to do those things myself. Thank you so much for caring so much and having so much faith. I know it can't be easy and I want you to know that it is SO appreciated.

To Ashley and Emily, thank you for welcoming me into your lives and giving me an outlet to relieve stress. I have so appreciated going to your house for parties, talking about anything other than science, singing, playing games, and now playing with baby Evan. Thank

you for integrating me into your community of friends. Emily, I have so appreciated your ability to seamlessly welcome new people into your orbit and I am glad to count myself as one of the people you did this to.

Next I would like to thank the communities I have been lucky enough to be a part of during my time at WashU. First, thank you to the WashU Med School Musical community. You have made my time at WashU so much fun. I have loved spending every weekend from September to April singing, dancing, and acting with you all. My mind has been broadened by every interaction with you all and I am forever grateful for the time we had together. To Lindsey B., thank you for pushing me to be a better vocal coach, forcing me to hone my conducting skills when you dipped out on rehearsals (she actually gave me a lot of notice when she had to leave me hanging), and making being duo music directors so laidback and fun. I'd also like to thank the WashU Connections community. From being a member to being part of the leadership, I have loved every moment I spent with all of you. Learning and growing would not have been the same without all the conversations and fellowship we enjoyed together. I will take the things I learned at all our meetings with me throughout the rest of my life.

I also want to take a little space to thank my wonderful companions, Rainer and Canyon. Rainer, I could not have imagined the adventures we would have together when I got you when I was 17. You went with me to Charlottesville, Bowling Green, and St. Louis and kept me on my toes at every turn. You were always a source of happiness and stress relief for me. Coming home was always the best part of my day because I got to see your wagging tail and tippy-tapping feet on the other side of the door. I loved taking you on meandering walks and hearing you howl at firetrucks and ambulances. I was so lucky to have such a wonderful reason for maintaining work-life balance. Thank you for loving me so steadfastly every single day. Thank you for forgiving

me for long experiments. Thank you for reminding me about the simple things in life, like the healing powers of feeling the breeze on my face and eating an ice cream cone. By stealing sweet potatoes and giving them to your smaller doggy companions you taught me that helping others is as rewarding as helping myself. By getting Halloween candy down and spraying nerds all over the apartment you taught me that sometimes you just need to throw yourself a party. By stealing a peanut butter and jelly sandwich from Rachel's backpack you taught me that sometimes it is easier to ask forgiveness than permission. You were truly a one in a million dog. I can't imagine ever encountering a better good boy than you. Thank you for being my first and best dog. I will love you forever. Canyon, oh man, dude. You are truly the worst and the best all rolled into one. You have stayed by my side throughout so much of this writing process and demanded many petting breaks. Through your steadfast focus on the squirrels and opossums you have taught me so much about dedication and hard work. Through your constant crying at me for pets and attention you have taught me to advocate for myself. By alerting us when Rainer was sick so that we could rush him to the hospital you taught me about the importance of advocating for others. Thank you for finally calming down a little. I love you so much.

Last and certainly, certainly, certainly not least I have to thank my amazing wife, Rachel Ryan. You have been so patient throughout this process. Every time I came home late, every time we had to get fast food for dinner because I was coming home at 1 am, every time my experiments made us late for events or parties or dinner with friends, you have been so kind and understanding. You have always taken the time to listen to how I am feeling and to support me when experiments didn't work or gave me results I didn't appreciate. You have gone to lab with me both when I was finding it difficult to go on my own and when we were going somewhere and I just needed to "run in real quick" (which always took at least 45 minutes) and you only

sometimes complained. You have reminded me of my worth when it was almost impossible for me to see it on my own. You have made life so fun and meaningful for me. The good has been great because of you and the bad has stung less because of you. Thank you for supporting me throughout this sometimes ugly process of becoming and for seeing the very best in me when I couldn't. I can't believe that after watching this happen you still want to get a Ph.D., but I will steadfastly support you every step of the way.

Clearly, graduate school has been less about experiments for me and more about growing into a person who thinks critically, not just about science, but about the world, too. Thank you to everyone who has had a hand in my growth, both in lab and outside of it.

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December 2021

Dedicated to Rainer.

I couldn't have done this without you; I will love and miss you forever.

ABSTRACT OF THE DISSERTATION

The Effects of Molecular Chaperone Modulation on Protein Folding, Prion Formation, and Prion

Propagation in *Saccharomyces cerevisiae*

by

Leeran Blythe Dublin Ryan

Doctor of Philosophy in Biology and Biomedical Sciences

Developmental, Regenerative, and Stem Cell Biology

Washington University in St. Louis, 2021

Professor Heather True, Chair

Proper and efficient protein folding is vital for cell survival. Many factors affect protein folding fidelity and prion formation, including molecular chaperone availability and activity. Research has shown that modulating chaperone availability and function can affect protein misfolding and aggregation, as well as *de novo* prion formation and propagation. However, the factors involved and underlying mechanisms influencing prion formation and protein folding are largely unknown. The following work aims to elucidate these areas.

The Nascent Polypeptide-Associated Complex (NAC) is the first point of chaperone contact for nascent polypeptides. Previous work has shown that disruption of the NAC leads to improved viability in cells experiencing protein misfolding stress. This counterintuitive result led us to investigate the ability of NAC deletion to improve survivability of cells expressing misfolding human proteins. This work resulted in the identification of multiple NAC deletion strains that improve viability in cells expressing disease-causing alpha-synuclein and expanded polyglutamine proteins. Also, this work identified changes in *de novo* induction of a yeast prion and morphological changes in expanded polyglutamine aggregates as a result of NAC disruption.

Overall, this work reveals the potential of NAC disruption as a therapeutic target for neurodegenerative diseases and sets the stage for investigating the mechanism by which NAC disruption improves viability in cells expressing disease-causing, aggregating proteins.

Mutations in another chaperone, DNAJB6, have been shown to cause Limb-Girdle Muscular Dystrophy Type 1D (LGMDD1). While we know that these mutations are associated with LGMDD1, the mechanism by which they induce disease remains unknown. Because substrates of DNAJB6 have not been identified, we have turned to a homologous protein in yeast, Sis1, with known client proteins to better understand the effect of these mutations. We have also developed a Sis1-DNAJB6 chimeric protein (SDSS) to evaluate these mutations. This chimeric protein includes the J, G/M, and C-terminal domains of Sis1, and the G/F domain, in which many LGMDD1-associated mutations are found, of DNAJB6. Previous work has shown that when LGMDD1-associated mutations are introduced in Sis1 or SDSS there is disruption of client processing by Sis1. This body of work identifies multiple second-site suppressors that, when introduced in combination with LGMDD1-associated mutations, are capable of recovering client processing by Sis1 and SDSS. Overall, this work shows that second-site suppressors may be capable of recovering DNAJB6 activity when introduced in combination with LGMDD1-associated mutations. Moreover, it provides an experimental model for the continued investigation of these second-site suppressors and identification of similar therapeutic avenues for potentially treating patients with other LGMDD1-associated mutations in the future.

Chapter 1: Background and Significance

1.1 Overview

Proteins must achieve particular folds in order to participate in normal cellular functions. This process is integral to maintaining life. Molecular chaperones facilitate proper protein folding both co- and post-translationally. Modulation of expression and activity of these molecular chaperones can alter the protein folding environment. This can lead to protein misfolding and aggregation in a subset of diseases called chaperonopathies. Protein misfolding and aggregation often induces diseases such as Huntington's Disease, certain forms of muscular dystrophy, Alzheimer's Disease, and many others. Significant questions remain on how proper protein folding is achieved, what causes *de novo* protein misfolding and aggregation, what changes in molecular chaperones can be tolerated or beneficial to cells, and how diseases caused by molecular chaperone perturbations can be successfully treated. This dissertation seeks to better understand how molecular chaperone modulation can change the protein folding landscape and identify potential strategies for restoring molecular chaperone activity in disease-causing mutants.

1.2 Protein Folding

1.2.1 Protein Translation

Proper protein folding is vital for virtually all cellular processes and, thus, for cell survival. *Saccharomyces cerevisiae* has provided a robust and tractable model system for studying protein translation and folding. Therefore, the processing of proteins will herein be described within this model organism unless otherwise stated. Every protein is created through translation of messenger RNA (mRNA) by the ribosome. The ribosome reads each triplet of nucleic acid code, fits a tRNA to that unique code, and a corresponding amino acid is added to a chain of previously decoded amino acids, known as a polypeptide. This process creates long

chains of amino acids that must then be folded to create a functional protein. As shown in Figure 1.1A, proteins sample many different fold conformations and adopt folds that reduce free energy. Molecular chaperones facilitate this sampling and promote protein folding to the native and functional state.

1.2.2 Co-translational and post-translational protein folding

As the ribosome decodes mRNA and amino acids are added, the polypeptide elongates through the ribosome exit tunnel and has been shown to adopt various folds within the ribosome itself (Woolhead et al., 2004; Lu & Deutsch, 2005; Kosolapov & Deutsch, 2009; Wruck et al., 2021). As the protein emerges from the ribosome there are multiple factors poised to interact with the nascent polypeptide (Figure 1.1B). First, the nascent polypeptide associate complex (NAC) binds to the amino acid chain to prevent errant binding or folding of the polypeptide in the crowded cytosolic landscape. In fact, research has shown that the NAC extends into the ribosome exit tunnel and binds to polypeptides as short as two amino acids to begin this process even before the polypeptide exits the ribosome (Gamerdinger et al., 2019). Furthermore, the NAC is involved in targeting proteins to the mitochondria, as the NAC has been shown to interact with mitochondrial translocation complexes TOM and SAM (Ponce-Rojas et al., 2017). Second, other factors bound to the ribosome come into contact with the nascent polypeptide. The signal recognizing particle (SRP) is one such factor. Polypeptides containing a signal sequence are bound and recognized by SRP, which then initiates the transport of the ribosome-nascent chain complex (RNC) to the endoplasmic reticulum (ER). There, SRP binds to the signal recognition particle receptor (SR) and recruits Sec61p, which will coordinate translocation of the protein into the ER. Other protein folding chaperones, such as SSB, interact with the emerging polypeptide as well. SSB is an Hsp70 protein that associates with the ribosome and interacts with

nascent polypeptides to assist with co-translational folding (Willmund et al., 2013). SSB association with the ribosome and binding to nascent polypeptides is greatly enhanced by the presence of the ribosome-associated complex (RAC) which is composed of an Hsp40, Zuo1, and an Hsp70, Ssz (Gumiero et al., 2016). The RAC interacts with SSB and stimulates ATP hydrolysis by SSB. The RAC binds directly to the ribosome near the ribosome exit tunnel, suggesting the RAC and SSB are important for co-translational protein folding. Recent research has shown that some protein complexes assemble during translation, revealing a role for protein complex binding partners in co-translational protein folding (Shiber et al., 2018). Simultaneous deletion of multiple co-translational components (namely the NAC and SSB) results in drastic growth defects, showing that the roles of these factors overlap and further cementing the importance of co-translational protein folding in proteostasis (Koplin et al., 2010).

As proteins are continuously sampling various folding conformations there is not a clear demarcation between co- and post-translational folding. Instead there is a spectrum of molecular chaperone interactions that are at one end almost exclusively involved in early co-translational folding, at the other almost exclusively involved in post-translational folding, and in the middle participating in both stages of protein folding. Hsp70s and Hsp40s are molecular chaperone proteins that work together to bind and fold a variety of polypeptides, both associated with the ribosome and downstream of translational machinery. Hsp70s directly bind folding intermediates, while Hsp40s are cochaperones that regulate the protein binding activity of Hsp70s. Hsp40s are also known as J-proteins, as they all have a J-domain that is responsible for interacting directly with the Hsp70 nucleotide binding domain (NBD) (Craig & Marszalek et al., 2014). This interaction stimulates hydrolysis of an Hsp70 bound ATP to ADP, which triggers the Hsp70 to stably bind the folding intermediate substrate in its closed state. After this, a nucleotide

exchange factor (NEF), another cochaperone, binds to the Hsp70 and catalyzes exchange of the bound ADP for an ATP, which triggers the release of the substrate. This sudden release of the protein allows spontaneous folding to occur. Proteins that require additional folding after interactions with the Hsp70-Hsp40 system are recruited to cylindrical chaperonin complexes by an ATP-independent chaperone known as prefoldin (Kim et al., 2013). Chaperonin complexes are ATP-dependent, large, hollow, double-ring structures that encapsulate nascent proteins, allowing them to fold in a controlled environment away from the crowded and somewhat chaotic protein folding environment within the cytosol (Kim et al., 2013). Chaperonins are composed of several subunits per ring (seven to nine) and there are two groups chaperonins are classified as, separated by their requirement for a lid-shaped cochaperone and differences in the composition of their ring structures (Frydman, 2001, Kim at al., 2013). Group I chaperonins require lid-shaped co-chaperones and include the bacterial chaperonin GroEL, the mitochondrial chaperonin Hsp60, and the chloroplast chaperonin Cpn60 (Tilly et al., 1981; Kim et al., 2013). The lid-shaped co-chaperones that interact with these Group I chaperonins are GroES, Hsp10, and Cpn10/Cpn20, respectively (Kim et al., 2013). Group II includes the archaea chaperonin thermosome and the eukaryotic TRiC/CCT (TCP-1 ring complex/chaperonin-containing TCP-1) chaperonin, both of which have lid domains. The TRiC/CCT chaperonin is known to participate in co- and post-translational folding of many cytosolic proteins, including actin and tubulin, which require the TRiC/CCT complex for folding (Llorca et al., 2000; Frydman, 2001). Prefoldin, which has a heterohexameric structure in eukaryotes, binds to unfolded actin and alpha and beta tubulin during translation and releases them when they have been delivered to TRiC/CCT (Hansen et al., 1999). Interestingly, TRiC/CCT is known to interact with the N-terminus of mutant huntingtin, which includes the expanded polyglutamine sequence, in yeast

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and human cell culture and induces non-toxic aggregation of the protein (Behrends et al., 2006; Tam et al., 2009). This interaction has been shown to reduce cytotoxicity of the protein (Kitamura, et al., 2006; Tam et al., 2006).

Hsp90 (homologous to Hsp82 in *Saccharomyces cerevisiae*) is a posttranslational molecular chaperone that interacts with nascent proteins as a homodimer. In eukaryotes, Hsp90 has three domains: a N-terminal domain, which has an essential ATP-binding site, a middle domain that is required for substrate interaction and regulation of ATP hydrolysis, and a C-terminal domain that houses the interaction site that allows dimerization of the chaperone (Kim et al., 2013). There are multiple co-chaperones that regulate Hsp90 activity. Hsp90 organizing protein (HOP, homologous to Sti1 in yeast) has one domain each for interacting with Hsp70 and Hsp90, which allows it to coordinate client protein transfer to the Hsp90 homodimer from the Hsp70-Hsp40 system (Kim et al., 2013). HOP, as well as another co-chaperone, Cdc37, inhibits the ATPase activity of the Hsp90 homodimer, which serves to stabilize the open conformation of the complex (Prodromou et al., 1999; Siligardi et al., 2002, Roe et al., 2004). To balance this system there are two co-chaperones, Hsp90 ATPase (Aha1), which binds first to one middle domain, then to one N-terminal domain within the dimer, stimulating ATP hydrolysis and transition to the closed state, and p23 (Sba1 in yeast), which binds to the N domains of the dimer and stabilizes the closed ATP-bound state of the system (Retzlaff et al., 2010; Schopf et al., 2017).

Finally, yeast have a AAA+ (ATPase Associated with diverse cellular Activities) protein known as Hsp104 that acts as a disaggregase (Parsell et al., 1994). Hsp104 is a protein with two nucleotide binding domains (NBD1 and NBD2) separated by a middle domain (MD) and bookended by an amino-terminal domain (NTD) and a short carboxy-terminal domain

(CTD) (Schirmer et al., 1996). Each of the NBD domains contain Walker motifs (Walker A and Walker B) and an arginine finger residue, all of which are important for ATP binding and hydrolysis (Sweeny and Shorter, 2016). The NBD domains also contain pore loops rich in tyrosine residues that engage folding substrates (Sweeny and Shorter, 2016). The proximity of these regions to one another couples ATP hydrolysis and the structural changes that occur as a result with substrate refolding (Sweeny and Shorter, 2016). While some aspects of Hsp104 assembly and function are understood, much remains unknown. ADP or ATP binding by NBD2 is crucial for the hexameric complex to form, but NBD1 nucleotide binding is not required (Parsell et al., 1994). Furthermore, it is known that Hsp104 disassembles both amorphous and organized aggregate structures, though by different mechanisms. Amorphous aggregates can be disassembled through non-cooperative activity of the hexamer subunits, however more structured aggregates require cooperative activity of the hexamer subunits and substrates are pulled partially or completely through the pore made by the hexamer structure (Lum et al. 2004, 2008; Haslberger et al. 2008; Tessarz et al. 2008; DeSantis et al, 2012; Sweeny et al. 2015; Gates et al. 2017). Hsp104 is required for yeast [*PSI*⁺] prion propagation (along with propagation of other yeast prions), as the hexameric complex formed by Hsp104 is thought to break large [*PSI*⁺] aggregate structures into smaller oligomers or prion seeds that can be passed through the budding neck to daughter cells (Romanova and Chernoff, 2010).

1.2.3 Chaperonopathies

Chaperonopathies are conditions caused by pathology of molecular chaperones, either through inherited or acquired genetic mutation or post translational modifications (Macario and Conway de Macario, 2019). These chaperone mutants can lead to overexpression, underexpression, destabilization, or other changes that perturb the normal function of the

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chaperone, causing disease. Many different types of diseases can have pathological molecular chaperone hallmarks, including congenital conditions (like EVEN-PLUS syndrome), many neurodegenerative diseases (such as Parkinson's disease with Lewy bodies), myopathies (like Limb-Girdle Muscular Dystrophy type 1D), autoimmune disorders caused by chaperones stimulating the immune system, and certain cancers (Macario and Conway de Macario, 2019).

EVEN-PLUS syndrome is a rare congenital condition characterized by multiple facial and skeletal malformations (Royer-Bertrand et al., 2015). Currently five cases of this condition have been studied and all five probands have mutations in *HSPA9*, a gene that encodes the mitochondrial Hsp70 mHSP70/mortalin (Royer, Bertrand et al., 2015; Younger & Vetrini, et al., 2020). In vitro studies of two of the mHSP70 mutations, R126W and Y128C, have revealed that the mutations lead to decreased thermal stability, decreased ATP hydrolysis activity, and increased aggregation propensity of mHSP70 (Moseng et al., 2020). This work shows that dysfunction of a molecular chaperone can contribute to disease through multiple mechanisms.

It has been shown that many Hsps, including Hsp27, Hsp40, Hsp60, Hsp70, and Hsp90, localize to Lewy bodies, indicating that molecular chaperones may be involved in Parkinson's disease progression (McLean et al., 2002; Uryu et al., 2006; Leverenz et al., 2007). Further research in human cell line models, yeast, mice, and *Drosophila melanogaster* have shown that overexpression of Hsp70 leads to improved outcomes, including reduction of oligomeric alpha-synuclein and rescue of alpha-synuclein-induced cytotoxicity (Auluck et al., 2002; Klucken et al., 2004; McLean et al., 2004; Flower et al., 2005; Luk et al., 2008; Outeiro et al., 2008). Furthermore, induction of a dominant negative Hsp70 in *drosophila melanogaster* accelerated alpha-synuclein toxicity (Auluck et al., 2002). These data show that, when overexpressed, molecular chaperones can have a protective role in neurodegenerative conditions and that

downregulation of some molecular chaperones may be involved in acceleration of Parkinson's disease.

Limb-Girdle Muscular Dystrophy type 1D (LGMDD1) is a dominantly inherited degenerative muscular disease characterized by progressive weakness and wasting of the proximal muscles surrounding the hips and shoulders (Straub et al., 2018; Zima et al., 2019). A wide variety of mutations across multiple domains of an Hsp40 protein, DNAJB6, have been notably associated with LGMDD1 (Harms et al., 2012; Sarparanta et al., 2012; Sato et al., 2013; Couthouis et al., 2014; Suarez-Cedeno et al., 2014; Yabe et al., 2014; Nam et al., 2015; Palmio et al., 2015; Ruggieri et al., 2015; Bohlega et al., 2018; Kim et al., 2018; Zima et al., 2019; Palmio et al., 2020). Initially, mutations within the glycine/phenylalanine-rich (G/F) domain were reported in multiple cases of LGMDD1, spanning many families and locations, including Asia, Europe, North America, and Saudi Arabia. Several of these mutations have been introduced into a homologous yeast protein, Sis1, and a Sis1-DNAJB6 construct, SDSS, and subsequently shown to disrupt client processing in yeast (Figure 1.4B, C, and D) (Stein et al., 2014). These experiments will be discussed more extensively later in this chapter and in chapter 3. More recently, mutations associated with LGMDD1 were found in the J-domain of DNAJB6, specifically in a section of the J-domain that shows close proximity to the G/F domain (Palmio et al., 2020). In vitro cell culture experiments show that when two of these mutants, DNAJB6-A50V and DNAJB6-E54A, are individually expressed the cells show impaired anti-aggregation activity in the presence of either expanded polyglutamine Huntingtin protein or TDP-43 (Palmio, 2020). Studies conducted using the yeast homolog Sis1 show that expression of the A50V mutation (Sis1-A49V in yeast) leads to impaired substrate refolding and alters client processing by Sis1 (Pullen et al., 2020). Together, these results, show that G/F and J domain mutations can

alter and disrupt client processing and indicate that alterations of protein domain interactions can be detrimental to chaperone function and contribute to disease.

One example of a chaperone stimulating the immune response and causing an autoimmune disorder is anti-Hsp60 activity in patients with atherosclerosis. Hsp60 is a mitochondrial chaperonin that has a bacterial homolog, GroEL. Atherosclerosis is thought to be initiated by T cells that recognize Hsp60 on endothelial cells, identifying Hsp60 as the autoantigen for atherosclerosis (Jakic et al., 2019). Because Hsp60 has high homology with GroEL it is thought that this autoimmunity can arise from high infection of bacteria. Indeed, high infection rates have been correlated with incidences of atherosclerosis (Campbell and Rosenfeld, 2015). After infection with *Chlamydia trachomatis*, for example, Hsp60 antibodies were found at high levels in patient sera, likely because the immune systems of these patients developed antibodies against GroEL (Sanchez-Campillo et al., 1999). Because Hsp60 is present on the plasma membrane of vascular endothelial cells to signal stress, T-cells immunized against Hsp60 recognize and attack these cells, leading to endothelial dysfunction and contributing to atherosclerosis (Campbell and Rosenfeld, 2015). This example shows that chaperonopathies can be acquired as a result of environmental stressors and complex extracellular processes.

Finally, evidence has been shown that members of the Hsp70 family are upregulated in many types of cancer cells including prostate, breast, leukemia, lung, colon, and gastric cancers (Kumar et al., 2016). It is known that Hsp70 expression is induced by cellular stress and that overexpression of Hsp70 reduces apoptosis, so it is not surprising that overexpression of Hsp70s could be beneficial to cancers. In fact, knockdown of Hsp70 has been shown to sensitize cancer cells and reduce tumor size in animal models (Schmitt et al., 2006). The mechanisms by which Hsp70s assist cancer growth and drug resistance are currently unclear; however current research

suggests that certain cancers can be considered acquired chaperonapathies, as the overexpression of Hsp70s heavily contribute to the growth and treatment-resistance of some cancer cells (Macario and Conway de Macario, 2019).

1.3 Prions and Prion Propagation

1.3.1 Mammalian prions

Prions are proteins that misfold, self-template, and aggregate (Atkinson et al., 2016). While functional prions exist, there are many prions that cause cellular dysfunction in neurons, and neuronal cell death (Atkinson et al., 2016). Prion proteins have a natively-folded, soluble state, but when misfolded, typically in a beta-sheet rich conformation, they template other soluble prion protein molecules to misfold and form highly organized aggregate structures, called amyloids. The mammalian prion protein (PrP) misfolds to cause the human diseases Creutzfeldt-Jakob Disease (CJD), Gerstmann-Straussler-Scheinker disease (GSS), Familial Fatal Insomnia (FFI), kuru, and variant CJD (vCJD) (Venneti, 2010). PrP aggregates also cause a wide range of diseases in non-human mammals, including Scrapie in sheep and goats, Transmissible Mink Encephalopathy, Chronic Wasting Disease in deer, and Bovine Spongiform Encephalopathy in cattle (Marin-Moreno et al., 2017). The pathogenic form of PrP is known as PrP^{Sc}, so named for Scrapie in sheep and goats (Prusiner, 1998). The soluble form of PrP is known as PrP^{C} and acts as a cell-surface glycoprotein (Sarnataro et al., 2017).

1.3.2 Yeast prions

A variety of prions also exist in yeast and fungi, in which they confer epigenetic inheritance of certain traits to progeny (Shorter and Lindquist, 2005). Presently, nine amyloid-based prions have been discovered in *Saccharomyces cerevisiae* (Chernoff et al., 2020). This variety represents a number of proteins with diverse cellular processes, different levels of

expression in wild yeast and laboratory strains, and positive and negative biological effects as a result of amyloid formation (Chernoff et al., 2020).

1.3.3 [*PSI*+] and [*RNQ*+]

[*PSI*+] is a yeast prion that has been shown to impart transient beneficial characteristics, such as enhanced growth in adverse environments, that can be adopted as permanent traits independent of [*PSI*+] status (True et al., 2004). Furthermore, it is known that environmental stressors can induce [*PSI*+] formation (Tyedmers et al., 2008; Westergard and True, 2014). Together these studies indicate that yeast prions can be induced by adverse events and utilized to increase current and future survivability. [*PSI*+] is the prion form of the protein Sup35. Sup35 is the yeast homolog of human eRF3 and, along with Sup45 (human eRF1), is involved in stop codon recognition during translation termination. Because of this unique role in protein translation, yeast have been genetically manipulated to develop a unique [*PSI*+]-dependent colorimetric assay that will be discussed later in this chapter.

[*RNQ*+], another yeast prion, is known to be important for [*PSI*+] propagation (Derkatch et al., 2004). Rnq1 protein, the role of which is unknown, misfolds and aggregates to form [*RNQ*+]. [*RNQ*+] has been shown to template [*PSI*+] and greatly enhances [*PSI*+] formation (Keefer et al., 2016). Furthermore, aggregation of ectopic huntingtin exon1 with an expanded polyglutamine region is dependent on the presence [*RNQ*+] (Meriin et al., 2002).

1.3.4 Molecular chaperones required for prion propagation

Interestingly, the Hsp40 Sis1 is important for maintenance of both the [*PSI*+] and [*RNQ*+] prions, though studies have shown that [*RNQ*+] is more drastically affected by reduction of Sis1 (Higurashi et al., 2008). It has been shown that Sis1 binds to Rnq1 in [*RNQ*+] cells and is required for propagation of [*RNQ*+], making [*RNQ*+] propagation a potential readout for Sis1

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function (Higurashi et al., 2008). In *sis1Δ* cells, DNAJB1, the human homolog of Sis1 and potent molecular chaperone, can compensate for Sis1 loss, further establishing the potential for [*RNQ$^+$*] propagation as an experimental model for studying Hsp40 activity (Lopez et al., 2003). While the [*PSI$^+$*] prion is not "cured" as quickly by Sis1 deletion or repression as [*RNQ$^+$*], [*PSI$^+$*] propagation is greatly hindered by repression of Sis1 (Higurashi et al., 2008). In fact, upon Sis1 repression, [*RNQ$^+$*] propagation ceases after 20 generations and [*PSI$^+$*] propagation ceases only after 80 generations (Higurashi et al., 2008).

Both [*RNQ$^+$*] and [*PSI$^+$*] require the activity of molecular chaperone Hsp104. Deletion of *HSP104* eliminates both [*PSI$^+$*] and [*RNQ$^+$*], however overexpression of HSP104 also eliminates [*PSI$^+$*], but does not affect [*RNQ$^+$*] (Liebman and Chernoff, 2012). As described previously, Hsp104 interacts with Hsp40 and Hsp70 to promote disaggregation of aggregate proteins. It is thought that this disaggregase activity is required for virtually all yeast prion propagation because it breaks large aggregates into smaller aggregates that can be passed on to daughter cells without diluting prion units (Liebman and Chernoff, 2012). Less is known about the mechanism by which Hsp104 overexpression eliminates [*PSI$^+$*]. Microscopic assays with fluorescently-labeled Sup35 show that upon Hsp104 overexpression reveal [*PSI$^+$*] aggregates that are visible in some cells and become unresolvable in other cells (Greene et al., 2020). Upon starvation stress however, these aggregates reappear. It is unknown if this phenotype is due to Hsp104 completely dismantling Sup35 aggregates into monomeric protein, or to Hsp104-mediated oligomerization of aggregates such that they cannot be visualized through fluorescent microscopy. An aggregate "trimming" role of Hsp104 has been proposed to explain this observation, in addition to its already established severing activity (Shorter and Lindquist, 2004; Shorter and Lindquist, 2006), however more evidence and investigation is needed to support this (Greene et al., 2020).

1.3.5 Prion variants

Both [*RNQ*⁺] and [*PSI*⁺] prions can form different variants that have many distinct phenotypes and are thought to result from discrete protein conformations (Huang et al., 2013; Dergalev et al., 2019; Huang and King, 2020). These variants can be distinguished using methods such as thermostability assays (Huang et al., 2013), semi-denaturing agarose gel electrophoresis (SDD-AGE) (Huang et al., 2013; Huang and King, 2020), colorimetric assays (Huang et al. 2013; Huang and King, 2020), structural analysis (Dergalev et al., 2019), fluorescent microscopy (Huang et al., 2013; Huang and King, 2020) and, in the case of [*RNQ*⁺], the ability to induce other prion formation (Huang et al. 2013). Known [*RNQ*⁺] variants are single or multi-dot low, medium, high, and very high, so named for their appearance as one or several puncta when evaluated by fluorescent microscopy (single or multi-dot) and their ability to induce [*PSI*⁺] formation (low, medium, high, or very high) (Bradley et al., 2002; Sharma & Liebman, 2013).

Known [*PSI*⁺] variants can be observed by a colorimetric assay that takes advantage of the stop codon recognition property of Sup35 and is shown in Figure 1.2. In this assay, red yeast colonies are populated by monomeric, or soluble, Sup35 and are [*psi*⁻] and white, light pink, and dark pink colonies are populated by both monomeric and aggregated Sup35 and are [*PSI*⁺] (Liebman and Chernoff, 2012). This change in color occurs because a premature stop codon has been introduced in a gene (*ADE1*) in the adenine biosynthesis pathway. When Sup35 exists as a monomer it participates in the recognition of this premature stop codon in the *Ade1* gene (known specifically as *ade1-14*), Ade1 is not produced, and the adenine biosynthesis pathway is disrupted, which causes a red byproduct to accumulate in the cell (Liebman and Chernoff, 2012). This is why [*psi*⁻] colonies appear red in color. Furthermore, because the adenine biosynthesis

pathway is disrupted these cells cannot grow on media lacking adenine (Liebman and Chernoff, 2012). When Sup35 is aggregated in [*PSI*⁺] cells, the protein is titrated away from its role in recognizing stop codons and there is readthrough of the premature stop codon in the *ade1-14* allele, Ade1 is made, and the adenine biosynthesis pathway is restored (Liebman and Chernoff, 2012). These cells do not accumulate a red byproduct, so they appear white (or pink) and can grow on media lacking adenine (Liebman and Chernoff, 2012). [*PSI*⁺] strains are separated primarily by color from very weak to strong [*PSI*⁺]. Very weak [*PSI*⁺] corresponds to dark pink colonies, weak [*PSI*⁺] are medium pink, medium [*PSI*⁺] are lighter pink, and strong [*PSI*⁺] are pale pink or white in color.

[*PSI*⁺] variants can also be distinguished by mitotic stability, measured by the frequency of [*PSI*⁺] loss, levels of monomeric Sup35, and the level of nonsense codon suppression (Derkatch et al., 1996; Dergalev et al., 2019; Huang and King, 2020). It is known that monomeric Sup35 and faithful translation termination rates are higher in weak [*PSI*⁺] and that strong [*PSI*⁺] is more mitotically stable than weak [*PSI*⁺] (Derkatch et al., 1996; Uptain et al., 2001). Furthermore, in vitro studies have shown that when prion formation is seeded by strong [*PSI*⁺], prion fibers form more efficiently than when seeded by weak [*PSI*⁺] (Uptain et al., 2001).

1.3.6 *Saccharomyces cerevisiae* **as a model for Huntington's disease**

Not only is [*RNQ*⁺] required for other yeast prion formation, it is required for the formation of mutant Huntingin protein aggregation in yeast (Meriin et al, 2002). Yeast present a compelling model for studying amyloid diseases. As yeast prions are not cytotoxic when expressed at homeostatic levels, they can be utilized to better understand the effects of chaperone alteration on aggregate formation and propagation. Because yeast present a tractable genetic

model, chaperone expression is easily altered and studied. Furthermore, the ability to preferentially select yeast that have taken up a plasmid allows for the integration and study of human aggregating proteins within wild type (WT) yeast cells, as well as strains that have been genetically altered to variably express molecular chaperones.

A variety of neurodegenerative and amyloidogenic proteins have been introduced and studied in yeast. These studies have identified new potential therapeutic targets and revealed mechanisms by which human proteins cause toxicity (Di Gregorio and Duennwald, 2018). Huntington's Disease has been modeled in *Saccharomyces cerevisiae* with great success. Huntington's Disease is an autosomal dominant neurodegenerative disease that is caused by expanded CAG repeats (>39) in the *HTT* gene (McColgan and Tabrizi, 2018). These repeats result in an abnormally long stretch of glutamines in the huntingtin protein, which makes the protein less stable, and, over time, more prone to aggregation. A common Huntington's Disease model in yeast is generated by the introduction of constructs expressing part of WT Huntingtin (htt) exonI with 25 CAG repeats, resulting in 25 glutamines (25Q) or mutant Huntingtin (mhtt) 103 CAG repeats, resulting in 103 glutamines (103Q). Each construct is denoted as htt25Q (or 25Q htt exon I) and htt103Q (or 103Q htt exon I), respectively. Flanking regions of this core protein have a profound effect on aggregation and toxicity and it has been shown that N-terminal-FLAG-tagged and C-terminal CFP-tagged htt103Q under the control of a Gal1 promoter is cytotoxic and forms aggregates when induced (Duennwald et al., 2006). This construct and its non-cytotoxic 25Q counterpart are utilized in Chapter 2 of this dissertation. Studies using these and similar constructs have revealed mechanistic insights into htt103Q aggregation and toxicity. Cells expressing htt103Q were found to be defective in respiration, which is caused by impairment in mitochondrial respiratory chain complex II and III activities,

which are integral to ATP production (Solans et al., 2006). The same group of researchers found that overexpression of Hap4, which participates in mitochondrial gene expression regulation, suppresses PolyQ toxicity, likely through enhancing couple mitochondrial respiration (Ruetenik et al., 2016). Moreover, it was shown that mutant huntingtin toxicity is achieved through different mechanisms in various yeast strains, leading to the hypothesis that htt103Q could cause toxicity through distinct mechanisms in neurons of varying brain compartments or cells in other tissues (Serpionov et al., 2017). Duennwald and Lindquist used a yeast model to show that htt103Q fragments entrap the proteins Np14, Ufd1, and p97, which causes a profound defect in endoplasmic reticulum-associated degradation (ERAD) (2008). They also show that after only eight hours of htt103Q induction both the profound defect in ERAD and upregulation of ER stress proteins BiP, PDI, and CHOP can be detected (Duennwald and Lindquist, 2008). Lastly, they show that the heat-shock response (HSR), which is normally activated by unfolded proteins in the cytosol is unaffected by htt103Q aggregation (Duennwald and Lindquist, 2008). Interestingly, molecular chaperones, such as Sis1 and TriC, have been implicated in prevention of and the cellular response to expanded polyglutamine aggregation (Tam, et al., 2009; Klaips, et al. 2020). Altogether, these studies show the rich potential of *Saccharomyces cerevisiae* as a model for examining the mechanisms by with expanded polyglutamine aggregates and discovering potential avenues for ameliorating the toxic effects of this aggregation.

1.4   The Nascent Polypeptide-Associated Complex

1.4.1 NAC subunits and co-translational protein folding

The Nascent Polypeptide-Associated Complex (NAC) is the first point of contact for newly synthesized proteins as they exit the ribosome. The NAC is a heterodimeric complex and its deletion causes embryonic lethality in multicellular eukaryotes (Deng and Behringer, 1995;

Bloss et al., 2003). The heterodimeric structure is formed by the binding of an alpha subunit and a beta subunit, known as NACA and BTF3 respectively in humans and Icd-2 and Icd-1 in *C. elegans* (Arsenovic et al., 2012). These subunits form a beta-barrel structure through a conserved NAC binding domain in each subunit consisting of 6 antiparallel beta-strands (Kogan et al, 2014). In *Saccharomyces cerevisiae* there is one alpha subunit, Egd2, and two beta subunits, Egd1 and Btt1. Btt1 and Egd1 have 64.3% homology with stark differences in their C-terminal regions and Btt1 arose from a yeast-specific genome duplication event (Ott et al, 2015). Egd1 is present at 100x the concentration of Btt1 in the cell (Reimann et al., 1999). Both the beta and alpha subunits reversibly bind the NAC to the ribosome in a one to one ratio, however it is currently known that the beta subunit binds with multiple ribosomal proteins (Rpl31, Rpl25/35), while the alpha subunit interacts with the ribosomal protein Rpl17 only (Pech et al., 2010). Both the alpha and beta subunits bind nascent polypeptides as they exit the ribosome and it is currently theorized that the NAC does this to prevent off pathway folding or interactions with other proteins in the cytoplasm. All three NAC subunits in yeast have been shown to form homodimers that associate with translating ribosomes, thus complicating and diversifying the system (Wang et al., 2010; Del Alamo et al., 2011).

Analysis of NAC subunit specificity has shown that the Egd1-Egd2 NAC, Btt1-Egd2 NAC, and homodimers of each subunit have differing preferences for nascent polypeptides, the diversity of which stretches to encompass almost every nascent polypeptide in the cell (del Alamo et al, 2011). Microarray data of mRNA recovered from RNCs isolated by their association with specific TAP-tagged chaperones shows that Btt1 preferentially binds to RNCs translating shorter, more intrinsically disordered, less hydrophobic proteins with higher translation rates (del Alamo et al, 2011). Conversely, Egd1 binds to RNCs translating longer, less

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intrinsically disordered, more hydrophobic proteins with slower translation rates (del Alamo et al., 2011). Egd2 alone was associated with RNCs enriched with secretory pathway proteins, notably proteins targeted to the ER (del Alamo et al, 2011). This further suggests that Egd2 can function as a homodimer.

1.4.2 NAC interactions with SRP and the ER

The NAC is also involved in ribosome shuttling to the endoplasmic reticulum (ER) and mitochondria (Gamerdinger et al., 2015; Zhang et al., 2012; Ponce-Rojas et al., 2017). In *Saccharomyces cerevisiae*, evidence suggests that the NAC modulates activity of the signal recognition particle (SRP) and ribosomal ER-targeting in a variety of ways. The SRP is a ribonucleoprotein that recognizes and binds to signal sequences of nascent proteins and targets those proteins and the ribosome translating them to the ER. The NAC is required for SRP recruitment to ribosomes translating proteins with signal sequences while the signal sequence is still in the ribosome exit tunnel (Zhang et al., 2012). The NAC is able to bind to these signal sequences as well, and both the NAC and SRP are able to bind to the ribosome and nascent polypeptides simultaneously (Zhang et al., 2012). Lastly, this work showed that even when the ribosome has docked to the ER membrane the NAC stays bound to the ribosome (Zhang et al, 2012). It is hypothesized that this allows the NAC to bind to nascent polypeptides and protect the exposed sequence from inappropriate or off-pathway interactions during recruitment to the ER membrane and during translocation of the protein into the ER. In *C. elegans*, beta-*nac* depletion has been shown to increase ribosome localization to the ER membrane, suggesting NAC is a negative regulator of ribosomal binding to the ER (Gamerdinger et al, 2015). Further work has shown that NAC regulates SRP-independent binding of the ribosome to the ER and induces ribosome release from the ER after translation termination (Gamerdinger et al, 2015).

Furthermore, NAC overexpression reduces SRP-dependent binding to the ER (Gamerdinger et al, 2015). Altogether, this research shows that NAC is a potent regulator of ribosome targeting and binding to the ER, and that the activity of the complex heavily modulates SRP-dependent ribosome targeting to the ER (Gamerdinger et al, 2015). More recently, detailed in vitro examination of the NAC and SRP interaction demonstrated that even at small concentrations the NAC is capable of reducing SRP interaction with SRP receptor in the presence of a non-functional signal sequence, while the NAC has no effect on SRP interaction with SRP receptor in the presence of a functional signal sequence (Hsieh et al, 2020). This work confirms that NAC and SRP co-bind to ribosomes and, through single molecule FRET and TIRF microscopy, that the NAC induces conformational changes in SRP, which regulates SRP and SRP receptor interaction (Hsieh et al, 2020). Moreover, the NAC was shown to greatly reduce SRP association with the ribosome until the nascent polypeptide chain has grown long enough to exit the ribosome, thus delaying targeting of the ribosome to the ER until the signal sequence can be recognized by SRP (Hsieh et al, 2020).

1.4.3 NAC interactions with the mitochondria

Egd2 (NAC α subunit) has been implicated in yeast ribosome targeting to the mitochondria for over twenty years (George et al, 1998). Continued research implicated NAC in ribosomal association with the mitochondrial surface (as deletion of the NAC resulted in fewer ribosomes present on mitochondria) and as a stimulator of protein import into the mitochondria (George et al, 2002; Funfschilling and Rospert, 1999). More recent work in *Saccharomyces cerevisiae* has shown that the NAC, specifically the Egd2 and Btt1 heterodimer, physically interacts with Sam37 and they work together to navigate the early stages of mitochondrial protein import. (Ponce-Rojas et al, 2017). Sam37 is a subunit of the sorting and assembly

machinery (SAM) complex in the mitochondria. Sam37 contributes to the stability of the complex as well as the physical interaction between the translocase of the outer membrane (TOM) and SAM complexes. Furthermore, it has been shown that the NAC interacts with the mitochondrial receptor OM14 (Lesnik et al, 2014). These findings indicate that the NAC is heavily involved in many steps required for co-translational mitochondrial protein targeting and import. Based on this conclusion it is unsurprising that, upon analysis of mRNAs present in the ER lumen of *C. elegans* depleted of beta-NAC, it was found that mitochondrial proteins were strongly mistargeted to the ER, along with cytosolic and nuclear proteins (Gamerdinger et al, 2015).

1.4.4 Effects of NAC overexpression

Work in *C. elegans* has shown that overexpression of the NAC over a particular threshold is lethal and that moderate (2- to 3-fold) overexpression of the complex results in developmental delays (Gamerdinger et al, 2015). Somewhat surprisingly, the same body of work reported that alpha and beta *nac* depletion separately and in combination reduces lifespan and that combinatorial *nac* depletion induces endoplasmic reticulum and mitochondrial stress in wild type animals (Gamerdinger et al, 2015). Beta-*nac* suppression specifically leads to high levels of ER stress reporter induction (Gamerdinger et al, 2015). Potentially conflicting data collected in *Saccharomyces cerevisiae* indicates that *nac* deletion did not result in transcriptional activation of the unfolded protein response (UPR) (del Alamo et al, 2011). These data suggest the NAC is vital for development and global homeostasis, however its importance in specific cell-type or organ system homeostasis and the potential benefits from overexpressing or suppressing the NAC remain unknown.

Overexpression of the N-terminus of the beta-NAC subunit has been shown to reduce poly-glutamine aggregation in *C. elegans* (Shen et al., 2019). The work by Shen at al. shows that the positively charged N-terminus of beta-NAC delays poly-glutamine aggregation independently of its previously defined ribosome-associated roles.

1.4.5 Effects of NAC deletion

While NAC deletion in higher order eukaryotes has been shown to cause embryonic lethality, deletion of the NAC and NAC subunits has little to no effect on growth in *Saccharomyces cerevisiae*. In fact, NAC deletion and disruption is beneficial to in *S. cerevisiae* in the presence of aggregating prion protein. Previously, a screen in the True lab was conducted to find cellular modifications that rescued [*PSI*⁺]-induced cytotoxicity. [*PSI*⁺] formation does not normally induce cytotoxicity, however when the aggregating protein (Sup35) is grossly overexpressed in [*PSI*⁺] cells, cell death occurs. This screen sought to identify gene disruptions that abolished cytotoxicity without curing the prion status of the cells, thereby discovering potential therapeutic targets to treat a wider variety of protein misfolding conditions. One gene disruption discovered in this screen was that of *egd1* (Figure 1.3A). This result was highly surprising, as Egd1 is an essential protein-folding chaperone involved in multiple cellular processes. Further work showed that two NAC deletion strains in particular, *egd1Δegd2Δ* and *egd1Δbtt1Δ*, robustly rescued cell viability in [*PSI*⁺] cells overexpressing SUP35 (Figure 1.3A) (Keefer & True, 2016). While deletion of the NAC subunits did not cure the [*PSI*⁺] prion, aggregate formation and distribution in the *egd1Δegd2Δ* strain was altered as compared to WT and other NAC deletion strains (Figure 1.3B) (Keefer & True, 2016). It was hypothesized that deletion of the NAC subunits in the *egd1Δegd2Δ* and *egd1Δbtt1Δ* strains delayed the joining of newly synthesized Sup35 to preexisting prion aggregates, based on data showing delayed

detection of *de novo* expressed Sup35 in the pelleted fraction of [*PSI⁺*] cell lysates of the *egd1Δegd2Δ* and *egd1Δbtt1Δ* strains (Figure 1.3C) (Keefer & True, 2016). It was determined that this change in the *egd1Δegd2Δ* strain could be due to changes in chaperone balance in the strain. Analysis of Ssb localization showed that Ssb was heavily localized to polysomes and nearly absent from Sup35 aggregates in [*PSI⁺*] cells, as compared to a more moderate distribution of Ssb to both polysomes and Sup35 aggregates in WT cells (Figure 1.3D) (Keefer &True, 2016). Importantly, while the *egd1Δbtt1Δ* strain did not induce changes in Ssb localization to polysomes compared to WT, the strain did have a marked decrease in Ssb localization to Sup35 aggregates in [*PSI⁺*] cells, similar to the *egd1Δegd2Δ* strain (Keefer & True, 2016). Lastly, this work showed robust resistance to general protein misfolding cytoxicity induced by canavanine treatment in a variety of NAC deletion strains (Figure 1.3E) (Keefer & True, 2016). Overall, this work revealed the NAC as a potential target for disruption in the treatment of protein misfolding diseases.

1.5 Limb-Girdle Muscular Dystrophy Type 1D (LGMDD1)

1.5.1 Limb-Girdle Muscular Dystrophies

As stated previously in this chapter, Limb-Girdle Muscular Dystrophy (LGMD) is a group of muscular weakness and wasting disorders that are characterized by shoulder and pelvic girdle onset (Liewluck and Milone, 2018). LGMDs are separated into two categories, LGMD Type 1 (LGMD1) and LGMD Type 2 (LGMD2). Within these types are subtypes 1A-1H and 2A-2Z, respectively (Liewluck and Milone, 2018). LGMD1 and LGMD2 differ in a number of features, including that LGMD1 is an autosomal dominant condition while LGMD2 is autosomal recessive and the LGMD subtypes are separated by known causative genes.

1.5.2 J-Proteins

LGMD type 1D (LGMDD1) is caused by mutations in the DNAJB6 gene. DNAJB6 is an Hsp40, or J-protein, involved in protein folding in conjunction with Hsp70s. Mutations in DNAJB6 associated with LGMDD1 have been found in the G/F and J domains of the protein (Harms et al., 2012; Sarparanta et al., 2012; Sato et al., 2013; Couthouis et al., 2014; Suarez-Cedeno et al., 2014; Yabe et al., 2014; Nam et al., 2015; Palmio et al., 2015; Ruggieri et al., 2015; Bohlega et al., 2018; Kim et al., 2018; Zima et al., 2019; Palmio et al., 2020). Hsp40s/DNAJs/J-proteins are an expansive group of proteins that share a characteristic J-domain. This domain is located at the N-terminus, 70 amino acids in length, and, most importantly, stimulates the Hsp70 ATPase activity. There is a conserved HPD-motif (histidine, proline, aspartic acid) within the J-domain that is crucial for stimulating the ATPase activity of Hsp70 (Tsai and Douglas, 1996). This motif is at position 31-33 in the amino acid sequence of DNAJB6. There are three DNAJ protein subtypes, I or A, II or B, and III or C. All three subtypes contain the conserved J-domain, though the J-domain is not necessarily located at the N-terminus in subtype III (or C) (Kampinga and Craig, 2010). Both subtypes I (or A) and II (or B) contain a conserved glycine/phenylalanine (G/F)-rich domain, the function of which is unclear, and a C-terminal region that identify and bind to client proteins (Liewluck and Milone, 2018). Lastly, subtype I (or A) contains a cysteine-rich reason bordered by the G/F-rich domain and the C-terminal substrate binding region.

1.5.3 DNAJB6 structure and expression

DNAJB6 is a Type II (or B) protein and therefore contains a J-domain with the conserved HPD motif, G/F domain, and a C terminal domain. DNAJB6 exists within the cell in two isoforms, DNAJB6a and DNAJB6b, created by alternative splicing. The main difference between these two isoforms is the length of the C-terminal domain. DNAJB6a is the longer

isoform and has a nuclear localization signal, while DNAJB6b is shorter and has been shown to have cytosolic and nuclear localization (Bengoechea et al., 2015). DNAJB6 is expressed in most human tissues; interestingly, however, DNAJB6 expression is relatively low in mouse and human skeletal muscle (Ding et al., 2016).

1.5.4 DNAJB6 mutations in LGMDD1

Mutations in the G/F and J domains of DNAJB6 have been found in patients with LGMDD1. Discovery of new mutations is ongoing, as are studies to uncover the mechanisms by which these mutations modulate DNAJB6 activity. Many of these mutations have been shown to reduce the anti-aggregation activity of DNAJB6 (Sarparanta et al., 2012; Palmio et al., 2018; Bengoechea et al., 2015; Sarparanta et al., 2020; Stein et al., 2014, Pullen et al., 2020). Unfortunately, there is no known client of DNAJB6 in humans, making it difficult to directly study the functional changes caused by these mutations. Sis1 is a Type II HSP40 in yeast that interacts with the HSP70 Ssa1 and has known client proteins, which include Rnq1 and Sup35. As stated previously, Sis1 is required for propagation of both [RNQ^+] and [PSI^+] prions. Because Sis1 has homology with DNAJB6 and another human J-protein, DNAJB1, this system has been exploited repeatedly to better understand the effect of LGMDD1 mutations. These studies have shown that multiple G/F and J domain mutations alter prion and non-prion client processing (Stein et al., 2014; Pullen et al., 2020; Bengoechea et al., 2020). Because DNAJB1 is more closely homologous to DNAJB6 and can compensate for the cytotoxic loss of Sis1, Stein et al. introduced the disease related DNAJB6 mutations F89I, F93L, and P96R into DNAJB1 (2014). They then introduced these constructs into a yeast *sis1Δ* [RNQ^+] model. They found that WT Sis1 and WT DNAJB1 were able to maintain the propagation of the [RNQ^+] prion, but that cells expressing the DNAJB1 proteins containing the F93L and P96R mutations displayed a

significant reduction in [RNQ^+] propagation (Stein et al., 2014). Expression of the DNAJB1 protein with the F89I mutation (DNAJB1-F90I) proved cytotoxic, so they were unable to access [RNQ^+] in these cells (Figure 1.4A) (Stein et al., 2014). While unfortunate for continued experimentation, this result allowed for a unique opportunity to probe for intragenic second-site suppressors of this strong phenotype.

Because the DNAJB6-F89I mutation showed the strongest phenotype, Stein et al. wanted to further investigate its effect on [RNQ^+] and [PSI^+] propagation. To do this they created a chimeric protein that contained the G/F domain of DNAJB6 and the J, G/M, and CTD of Sis1 (Figure 1.4B) (Stein et al., 2014). This protein was named SDSS, which denotes the origin of each domain. It was reasoned that SDSS would combine the disease relevant domain of DNAJB6 and the prion propagation and viability roles of Sis1. Indeed, *sis1Δ* cells expressing SDSS-F89I were viable, and while WT SDSS, SDSS-F93L, and SDSS-P96R were able to maintain prion propagation of single dot medium [RNQ^+], weak [PSI^+], and a lab-created [PSI^+] strain known as Sc37, SDSS-F89I expressing cells were cured of prions (Figure 1.4C and D) (Stein et al. 2014). Interestingly, this loss of prion propagation was not seen in cells expressing the SDSS mutants and containing multi-dot high [RNQ^+], strong [PSI^+], or a lab-created [PSI^+] strain known as Sc4 (Figure 1.4C and D) (Stein et al. 2014). This result shows that these mutant chaperones may be able to interact with and influence some protein conformers but not others. The clear viability phenotype in DNAJB1 with the F89I mutation combined with the clear propagation of particular prion strains defect phenotype in SDSS-F89I present an intriguing system for study, which will be discussed in Chapter 3.

1.5.5 Intragenic second-site suppressors

Intragenic second-site suppressors are secondary mutations that partially or completely revert a mutant protein back to WT function. Discovery of second-site suppressors often leads to new insights in protein function, domain interactions, and potential therapeutic avenues. As the role of the G/F domain remains unclear, second-site suppressors of G/F domain mutants could advance our understanding of G/F domain function and its potential interaction with other DNAJB6 domains. Investigation into the effects of second site suppressors of the DNAJB6 F89I mutation will be further discussed in Chapter 3 of this work.

1.6   Summary and Significance

Protein folding is critical for cell survival and as such the cell has developed many ways to improve and enhance the protein folding environment. Molecular chaperones that engage in co- and post-translational protein folding are factors that often improve protein folding and promote homeostasis and proteostasis. Even with this network of molecular chaperones there are proteins that misfold and aggregate, often harming the cellular environment and causing irreversible damage to the organism. Outstanding questions in this field include identifying how modulation of molecular chaperones can affect the protein folding environment and how perturbations in molecular chaperone activity can be addressed. This dissertation attempts to address these questions.

Chapter two of this document investigates the NAC and how deletion of particular NAC subunits delays and alters protein aggregation and cytotoxicity of human disease-causing proteins. It also reveals changes in yeast prion aggregation and induction as a result of NAC subunit deletion. This work illuminates chaperone complex modulation as a potential suppressor of neurodegenerative disease or other protein conformational disorders. Chapter three identifies

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and evaluates second-site suppressors of DNAJB6 mutations. These studies were conducted in the DNAJB6 and Sis1 chimeric protein SDSS and the yeast Hsp40 Sis1. Two second site suppressors are shown to rescue the client-associated activity of SDSS and Sis1 that are disrupted by disease associated mutations. These data show the potential of second-site suppressors as potent therapies for rescuing protein activity and open new avenues of research into the mechanism by which they rescue SDSS and Sis1 activity. Overall, this work addresses important areas of molecular chaperone modulation and activity and may serve to inform further research into reduction and prevention of protein misfolding-induced cellular toxicity.

1.7 Figures
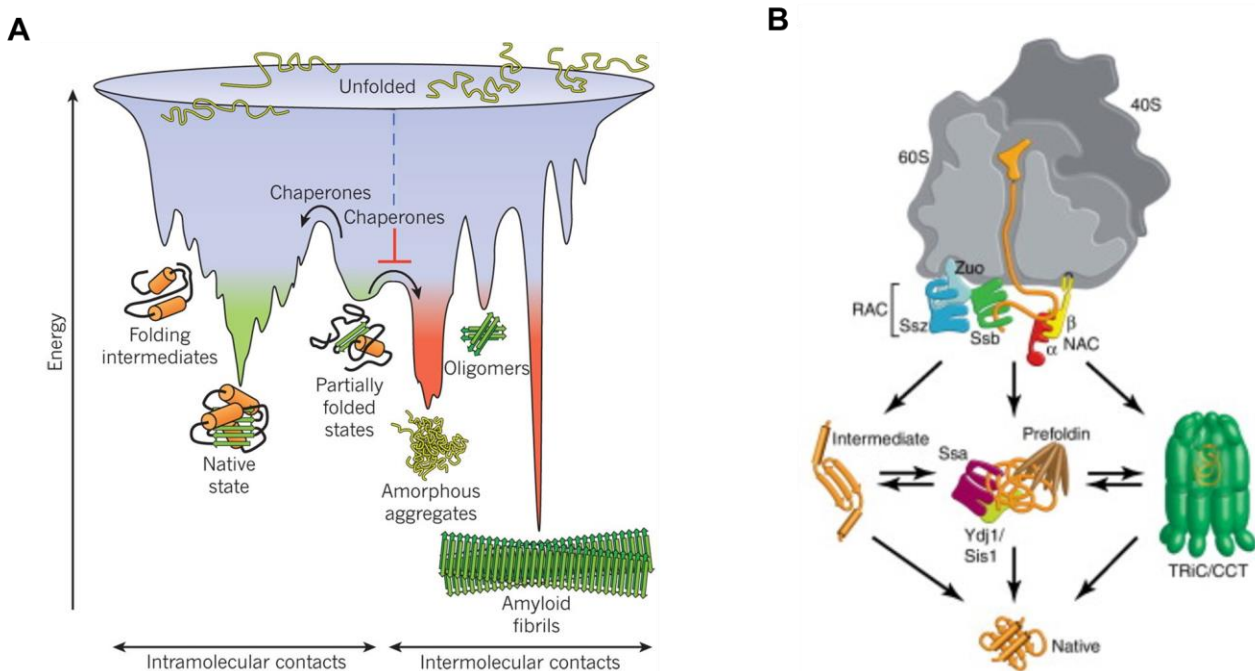
Figure 1.1 Molecular chaperones effects on protein folding. Illustrations adapted (Hartl et al., 2011; Preissler and Deuerling, 2012); (A) Proteins can adopt innumerable folding conformations characterized by various levels of free energy and intra- or inter-molecular contacts. Proteins sample various folds as they attempt to fold in the lower free energy, native state. Conversely, proteins may, through intermolecular interactions, form amorphous aggregates, oligomers, or amyloid fibrils. Molecular chaperones are involved in promoting native state folding conformations and disrupting and reducing amorphous aggregate, oligomer, and amyloid fibril conformations. (B) Molecular chaperones exist as a network of co- and post-translational protein interactors. Bound to the ribosome and the first point of nascent protein contact is the NAC (nascent polypeptide-associated complex). The RAC (ribosome associated complex) consists of Zuo, which binds to the ribosome, Ssz, and Ssb, which interacts with nascent polypeptides. Prefoldin, Ssa, and Ydj or Sis1 interact with and assist in folding proteins either after they exit the ribosome, as they exist in the cytosol as folding intermediates, or before or after interactions with TRiC/CCT. Each of these interactions promotes folding of the protein to its native state.
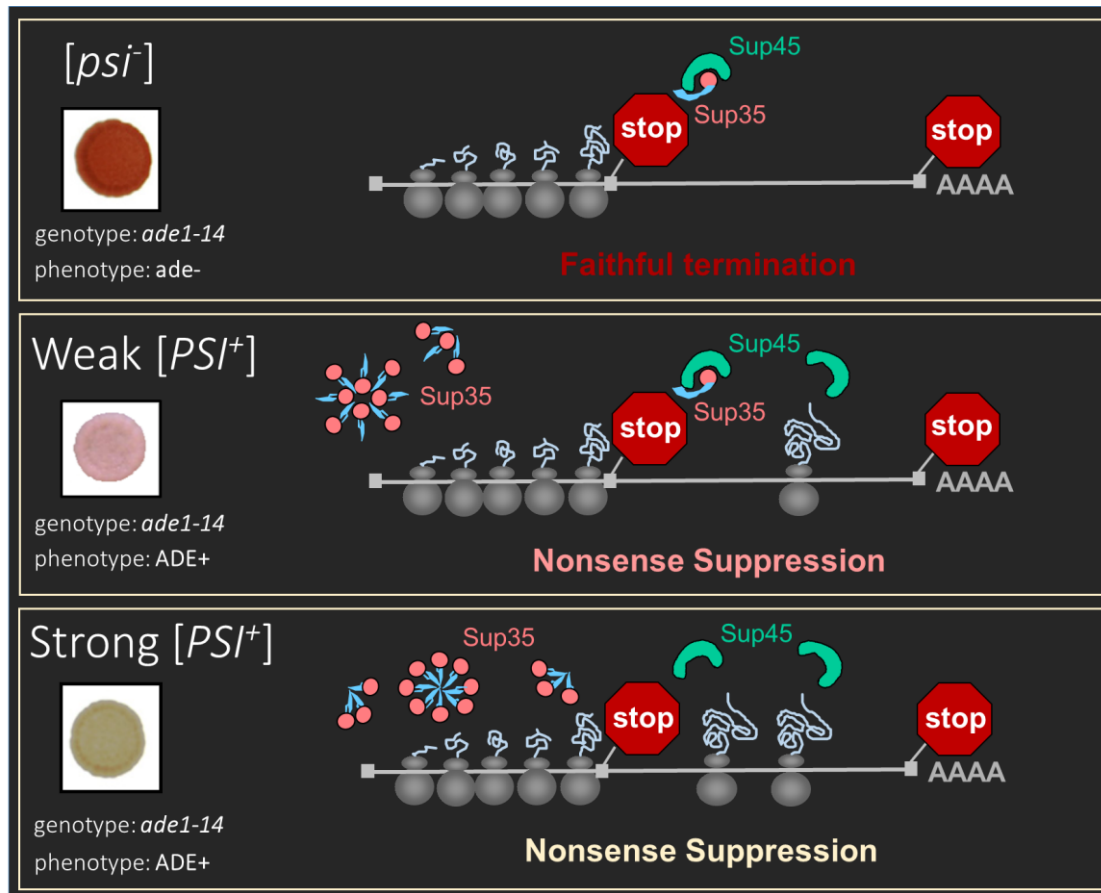
Figure 1.2 [*PSI*+] colorimetric phenotypes. Illustration adapted (True, 2003; Keefer, 2017). In the absence of prion Sup35 participates in stop codon recognition of the premature stop codon in *ade1-14*. There is faithful termination of translation, Ade1 is not made, a red byproduct builds up in the cell, and the cells cannot grow on media lacking adenine. When prions are present Sup35 is titrated away from stop codon recognition, there is nonsense suppression of the premature stop codon, Ade1 is made, there is no build-up of red byproduct, and the cells can grow on media lacking adenine. Different prion variants are associated with various levels of nonsense suppression, leading to variations on color. The weak [*PSI*+] prion variant leads to lower levels of nonsense suppression, some buildup of red byproduct, making colonies appear pink in color. The strong [*PSI*+] variant causes higher levels of nonsense suppression and very little red byproduct, resulting in a white colony phenotype.

A

pCUP1-SUP35 — WT [*psi-*]

Empty vector — WT

pCUP1-SUP35 — WT

pCUP1-SUP35 — egd1Δegd2Δ

pCUP1-SUP35 — egd1Δbtt1Δ

NACΔ

egd1Δ^t

SD-Trp   SD-Trp + 350μM CuSO₄

B

5μm

[*psi-*]   WT   egd1Δegd2Δ   egd1Δbtt1Δ   NACΔ

GFP

DIC

C

2hr   3hr   4hr

T S P   T S P   T S P

WT

egd1Δegd2Δ

egd1Δbtt1Δ

NACΔ

WB: Sup35-GFP

D

Ssb in polysome relative to monosome

WT   egd1Δegd2Δ   NAC

E

WT

egd1Δ

egd2Δ

egd1Δegd2Δ

btt1Δ

egd1Δbtt1Δ

egd2Δbtt1Δ

NACΔ

ssb1Δ

SD-Arg   SD-Arg + 550μM canavanine

Figure 1.3 NAC deletion effects on [*PSI*⁺] formation, chaperone localization, and protein folding environment. Figure adapted from Keefer and True, 2016. (A) NAC subunits were deleted in [*PSI*⁺] cells and *SUP35* overexpression was induced by spotting on selective media containing $CuSO_4$. The original screen Egd1 disruption is represented as *egd1Δᵗ*. (B) WT and NAC deletion strains were transformed with a copper-inducible plasmid expressing GFP-tagged *SUP35*, grown overnight in selective media, induced by $CuSO_4$, and imaged. (C) [*PSI*⁺] WT and NAC deletion strains were grown overnight in selective media and expression of GFP-tagged *SUP35* was induced by $CuSO_4$. GFP-tagged Sup35 was detected by western blot. (D) Ssb levels in WT and NAC deletion strains were detected in monosome and polysome fractions by western blot and are represented as ratios of Ssb expression in polysome relative to monosome. (E) WT and NAC deletion strains were spotted on canavanine containing media to measure viability during global protein misfolding stress.
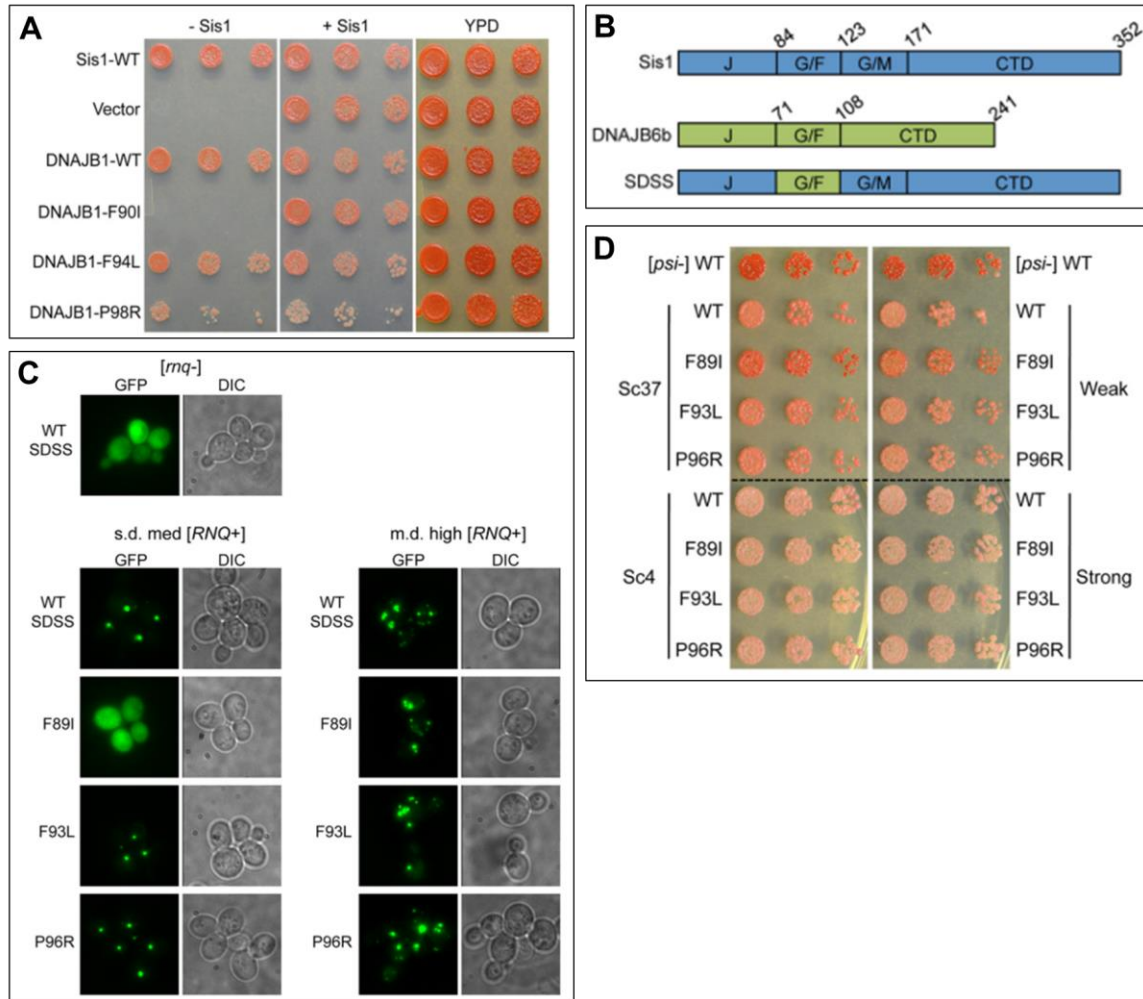
Figure 1.4 Disease-associated mutations in *DNAJB6* cause client processing disruptions in yeast. Figure adapted from Stein et al., 2014. (A) Homologous mutations to DNAJB6-F89I, DNAJB6-F93L, and DNAJB6-P96R were introduced in DNAJB1. Yeast expressing these mutant proteins and DNAJB1-WT were spotted to measure viability in the presence and absence of Sis1. (B) Diagram showing the construction of Sis1-DNAJB6 chimeric protein SDSS. (C) WT SDSS and SDSS mutants SDSS-F89I, SDSS-F93L, and SDSS-P96R were expressed in *sis1Δ* [*rnq⁻*], single dot medium [*RNQ⁺*], and multidot high [*RNQ⁺*] strains. Each strain expressed a copper-inducible Rnq1-GFP as well and prion aggregation was evaluated via fluorescent microscopy. (D) WT SDSS and SDSS mutants SDSS-F89I, SDSS-F93L, and SDSS-P96R were expressed in *sis1Δ* [*psi⁻*], Sc37, Sc4, weak [*PSI⁺*], and strong [*PSI⁺*] strains. Strains were spotted on rich media and evaluated for prion propagation by color.

Chapter 2: Disruption of the Nascent Polypeptide-Associated Complex Leads to Reduced Polyglutamine Aggregation and Toxicity

Leeran B. Dublin and Heather L. True

2.1 Abstract

The nascent polypeptide-associate complex (NAC) is a heterodimeric chaperone complex that binds near the ribosome exit tunnel and is the first point of chaperone contact for newly synthesized proteins. Deletion of the NAC induces embryonic lethality in many multi-cellular organisms. Previous work has shown deletion of the NAC rescues cells from prion-induced cytotoxicity. This counterintuitive result led us to hypothesize that NAC disruption would improve viability in cells expressing human misfolding proteins. Here, we show that NAC disruption not only improves viability in cells expressing alpha-synuclein and expanded polyglutamine, but also leads to delayed and reduced aggregation of expanded polyglutamine and changes in polyglutamine aggregate morphology. Moreover, we show that NAC disruption leads to changes in *de novo* yeast prion induction. These results indicate that the NAC plays a critical role in aggregate organization and has potential as a therapeutic target in neurodegenerative disorders.

2.2 Introduction

Protein folding is vital for cell survival and maintaining cellular homeostasis. Some proteins have amyloidogenic amino acid regions, which make them prone to misfolding and aggregation, causing disease (Dobson, 2017). For example, Huntington's Disease is caused by elongated repeats of glutamine (polyQ) in the Huntingtin protein that lead to misfolding and aggregation of the protein (Huang et al., 1998). The accumulation of these Huntingtin aggregates in neurons entangles cellular resources and alters cellular processes, causing stress and eventual cell death (Ravikumar et al., 2004, Koga et al., 2011, Ashkenazi et al., 2017, Vidal et al., 2012, Lajoie and Snapp, 2012, Duennwald and Lindquist, 2008). Currently there are no therapies

available for Huntington's Disease and the process by which Huntingtin protein aggregates and causes disease is not fully understood. Many studies on the effects of the expanded polyQ region of Huntingtin protein (htt103Q) have been conducted in yeast models, showing that polyglutamine toxicity disrupts ER stress responses, such as ER-associated degradation (ERAD) (Duennwald and Lindquist, 2008).

Molecular chaperones operate as a vast and complex network, assisting in co- and post-translational protein folding. Furthermore, molecular chaperones are known to assist in ribosome shuttling to the endoplasmic reticulum and mitochondria by recognizing signal sequences on nascent polypeptides (Zhang et al., 2012; Gamerdinger et al., 2015; Ponce-Rojas et al., 2017). Many molecular chaperones are required for cellular growth and deleting these chaperones results in cytotoxicity (Deng and Behringer, 1995; Barrott and Haystead, 2013; Helary et al., 2019).

Surprisingly, past research has shown that deleting portions of a co-translational molecular chaperone complex, the nascent polypeptide-associated complex (NAC), rescues yeast prion induced cytotoxicity by altering the balance and localization of other molecular chaperones (Keefer and True, 2016). The NAC is highly conserved and deletion of the complex is embryonically lethal in many multicellular organisms (Deng and Behringer, 1995; Bloss et al., 2003). The NAC is comprised of two classes of subunits known as alpha (α) and beta (β). Egd2 is the α subunit, while in yeast there are two β subunits, Egd1 and Btt1. Btt1 arose in yeast after a genome duplication event, is known as the β' subunit, and is 100 fold less concentrated in the cell than Egd1. The NAC is most prominently present in the cell as a heterodimer of one α subunit and one β subunit (Reimann et al., 1999; Del Alamo, et al., 2011). The β subunit reversibly binds the complex to the ribosome in a one to one ratio near the ribosomal exit tunnel

(Pech et al., 2010). Both the α and β subunits are the first point of contact for nascent polypeptides within the exit tunnel and as they emerge from the ribosome (Gamerdinger et al., 2019). The NAC also assists in the recognition of peptide signals and shuttling the ribosome to the ER and mitochondria (Zhang et al., 2012; Gamerdinger et al., 2015; Ponce-Rojas et al., 2017). Our group previously showed that partial deletion of the NAC rescued cytotoxicity in a yeast prion model (Keefer and True, 2016). Work from other groups has shown that overexpression of the NAC prevents aggregation by other misfolding proteins in yeast and other organisms (Gamerdinger et al., 2015; Shen et al., 2019). Because of this work we wanted to evaluate NAC modulation effects on human misfolding protein-induced cytotoxicity in yeast cells.

Herein, we report that partial deletion of the NAC improves cellular growth in yeast expressing a toxic expanded polyQ protein and reduces aggregation of polyglutamine. We also show that partial deletion of the NAC reduces induction of certain yeast prion strains. These results identify the NAC as a potential therapeutic target to reduce amyloidogenic protein aggregation and further elucidate the mechanism by which NAC disruption improves the cellular environment in the face of protein misfolding stress.

2.3 Results

2.3.1 NAC modulation effects on yeast expressing human misfolding proteins

We set out to investigate the ability of NAC modulation to improve viability in yeast cells expressing human misfolding proteins. Previously, we have shown that NAC deletion rescued prion-induced cytotoxicity, while others have shown that NAC overexpression delayed protein aggregation (Keefer and True, 2016, Shen et al., 2019). Based on this work, we hypothesized that modulating NAC subunit expression would rescue toxicity induced by human

misfolding proteins. To test this hypothesis, we expressed multiple disease-causing human misfolding proteins in the NAC deletion strains as well as WT yeast strains overexpressing NAC subunits. We expressed two galactose-inducible partial huntingtin proteins, htt exon1 103Q and htt exon1 25Q, as well as multiple galactose-inducible alpha-synuclein proteins, including wild type and two disease-causing mutants (A30P and A53T) in a complete set of NAC deletion strains and a WT strain. We spotted the *egd1Δbtt1Δ, egd1Δegd2Δ, nacΔ,* and WT transformants on selective media containing glucose or galactose to evaluate toxicity induced by these alpha-synuclein proteins (Figure 2.1A) and expanded polyglutamine (Figure 2.1B). We also transformed a WT 74-D694 yeast strain with plasmids expressing NAC β-subunit Egd1 or NAC α-subunit Egd2. We then transformed these strains with a plasmid expressing htt exon1 103Q or an empty vector and spotted them to evaluate toxicity (Figure 2.1C). We were surprised to see partial rescue of htt exon1 103Q toxicity in the *egd1Δbtt1Δ* and *egd1Δegd2Δ* strains but not in strains overexpressing Egd1 or Egd2. Other work has shown that overexpression of the NAC α and β subunits suppressed expanded polyglutamine aggregation in *C. elegans* (Shen et al., 2019). Interestingly, we also saw improved viability in the *egd1Δegd2Δ* and *nacΔ* strains expressing alpha-synuclein WT and disease-causing mutant constructs when compared to WT expressing the same constructs (Figure 2.1A).

2.3.2 NAC disruption delays and reduces polyglutamine aggregation

Intrigued by these results, we investigated aggregation of htt exon1 103Q. While the exact mechanism by which polyQ-expanded htt exonI causes toxicity is unknown, we know the aggregation of polyQ is a contributing factor and marker of toxicity (McColgan and Tabrizi, 2018). To visualize htt exon1 103Q aggregation in *egd1Δbtt1Δ* and *egd1Δegd2Δ* we induced expression of the protein for 6 and 20 hours using a galactose inducible promoter. We then

conducted microscopy and visualized the CFP-tagged htt exon1 103Q protein. We saw significantly reduced aggregation in the *egd1Δbtt1Δ* strain as compared to WT and *egd1Δegd2Δ* at both 6 and 20 hours of induction (Figure 2.2A). WT cells expressing htt exon1 103Q for 6 hours showed that an average of 37% cells contained aggregates while only 4.5% of the *egd1Δbtt1Δ* cells contained aggregates (Figure 2.2B). Even after inducing expression of htt exon1 103Q for 20 hours, 41% WT cells contained aggregates while 13% of the *egd1Δbtt1Δ* cells contained aggregates (Figure 2.2C).

2.3.3 NAC disruption changes polyglutamine aggregate distribution

Interestingly, we saw morphological differences between the htt exon1 103Q aggregates present in the mutant strains as compared to the aggregates in wild type cells. To quantify aggregate phenotype, we evaluated each cell expressing htt exon1 103Q and ranked them as 0-3 depending on the number of aggregates present in the cell (Figure 2.2D). These analyses were of keen interest, as many groups have hypothesized and shown that soluble polyglutamine oligomers are cytotoxic, while large insoluble, polyglutamine aggregate structures may pose less risk to cellular homeostasis (Takahashi et al., 2008, Lajoie and Snapp, 2010, Lajoie and Snapp, 2013, Leitman et al., 2013). At 6 hours htt exon1 103Q induction, the aggregate populations in WT and *egd1Δbtt1Δ* aggregate populations were similar, as cells containing one aggregate comprise about half of the aggregate population in both strains (Figure 2.2E). However, cells containing three or more aggregates appear in 49% of cells with aggregates in the *egd1Δegd2Δ* strain at the 6 hour induction timepoint (Figure 2.2E). At 20 hours htt exon1 103Q induction, aggregate populations observed in WT and *egd1Δegd2Δ* are similar (cells with one aggregate: 42% and 40%, two aggregates: 18% and 23%, and three or more aggregates: 39% and 37%) (Figure 2.2E). Conversely, the most abundant aggregate population in the *egd1Δbtt1Δ* strain after

20 hours induction is one aggregate, which make up 60% of the aggregate population in *egd1Δbtt1Δ* cells (Figure 2.2E).

2.3.4 NAC disruption-induced reduction of polyglutamine aggregation is not caused by reduced polyglutamine expression

We considered the possibility that the reduction of htt exon1 103Q aggregation in *egd1Δbtt1Δ* cells could be caused by reduced expression of htt exon1 103Q, as reduced polyglutamine expression has been shown to reduce and reverse polyglutamine aggregate formation (Yamamoto et al., 2000, Becanovic et al., 2015, Yang et al., 2016). To determine htt exon1 103Q expression we performed western blots for the FLAG-tag attached to the N-terminus of the htt exon1 25Q and htt exon1 103Q proteins induced in the NAC deletion strains for 6 hours. We saw no significant difference between htt exon1 103Q expression in WT and the *egd1Δbtt1Δ* strain at 6 hours induction (Figure 2.3A). We also detected htt exon1 103Q aggregates after 6 and 20 hour induction in WT, *egd1Δbtt1Δ*, *egd1Δegd2Δ*, and *nacΔ* strains by filter trap assay. Filter trap assays allow for the visualization and semi-quantification of large protein aggregates, as these aggregates, unlike monomeric proteins, are unable to pass through cellulose acetate membranes (Duennwald, 2013). We expected to see a reduction of aggregated htt exon1 103Q by filter trap assay in the *egd1Δbtt1Δ* strain compared to WT at both 6 and 20 hours of induction. We saw this result at the 6-hour time point (Figure 2.3B), but did not at the 20-hour time point (data not shown). Quantification of aggregated htt exon1 103Q signal intensity showed a 53% percent reduction in the *egd1Δbtt1Δ* strain compared to WT after 6 hours induction (Figure 2.3C).

2.3.5 NAC disruption alters [*PSI*$^+$] variant induction

We wanted to delve deeper into the change in aggregate morphology we saw with microscopy. We utilized the well-characterized [*PSI*$^+$] prion model in yeast, as *de novo* [*PSI*$^+$] strain induction can be used to better evaluate aggregate morphological differences as a result of chaperone deletion. Furthermore, we wanted to investigate whether NAC disruption would change aggregation morphology of another protein (Sup35) compared to WT. *Saccharomyces cerevisiae* harbor a wide array of prions, which are normally non-cytotoxic. Non-cytotoxic prions have potential as reporters for cellular protein folding environmental conditions. One prion, [*PSI*$^+$], has been used to develop a colorimetric assay that allows for the evaluation of prion induction and prion strain determination.

Like polyglutamine, proteins that misfold to form prions can fold and aggregate in different conformations, creating different prion strains. These strains can be differentiated by a myriad of assays and, in the yeast [*PSI*$^+$] prion system, by colony color. Sup35 is a protein involved in stop codon recognition with Sup45. Sup35 misfolds and aggregates to form the yeast prion [*PSI*$^+$]. The 74-D694 yeast strain contains the *ade1-14* allele. *ADE1* encodes a protein that is involved in the adenine biogenesis pathway, and *ade1-14* contains a premature stop codon. When Sup35 is natively folded the premature stop codon in *ade1-14* is recognized and adenine biosynthesis is interrupted. This results in the accumulation of a red byproduct in cells and results in their inability to grow on media lacking adenine. When Sup35 misfolds and aggregates there is readthrough of the premature stop codon in *ade1-14*, resulting in completion of the adenine biogenesis pathway, no accumulation of red pigment in yeast cells, and colony growth on media lacking adenine. [*PSI*$^+$] prion strains include very weak, weak, medium, and strong

[*PSI*$^+$] and each strain can be recognized by the accumulation of pigment in yeast colonies, ranging from [*psi*$^-$] cells appearing red and strong [*PSI*$^+$] cells appearing very light or pale pink.

Given the observed changes in htt exon1 103Q aggregate morphology, we hypothesized that loss of the NAC would cause changes in the distribution of *de novo* [*PSI*$^+$] prion strains when compared to *de novo* [*PSI*$^+$] prion strains induced in WT yeast. In order to induce prion formation we expressed a Sup35 plasmid in WT and the NAC deletion strains (*nacΔ*, *egd1Δegd2Δ*, and *egd1Δbtt1Δ*) and grew the resulting transformants overnight in 1M KCl selective media. 1M KCl was selected because it has been shown to cause osmotic stress (Slaninova, et al., 2000) and induce [*PSI*$^+$] (Tyedmers, et al., 2008). We then plated the transformants and determined the [*PSI*$^+$] strain induced for a minimum of 100 colonies per yeast strain. The WT and *nacΔ* strains showed similar [*PSI*$^+$] strain distributions (Table 2.1), however both the *egd1Δegd2Δ* and *egd1Δbtt1Δ* strains had notable increases in very weak and weak [*PSI*$^+$], and decreases in medium and strong [*PSI*$^+$] when compared to WT (Table 2.1). While not perfectly correlative, this shift reflects the polyglutamine morphological differences we detected in *egd1Δbtt1Δ,* indicating partial NAC deletion leads to potentially widespread changes in protein folding.

2.4 Discussion

Our work shows that disruption of the NAC can be beneficial to cell survival in the face of protein misfolding and aggregation stress. We have provided evidence that deletion of NAC subunits leads to improved viability in cells overexpressing alpha-synuclein and htt exon1 103Q. Furthermore, we have shown that htt exon1 103Q aggregation is delayed in *egd1Δbtt1Δ* when compared to htt exon1 103Q aggregation in WT, though expression of htt exon1 103Q is

unchanged. Filter trap assays show that less htt exon1 103Q protein is aggregated after 6 hours induction in *egd1Δbtt1Δ* cells when compared to htt exon1 103Q aggregated protein in WT cells. Interestingly, NAC disruption leads to changes in htt exon1 103Q aggregate morphology as well. Lastly, we show that NAC disruption changes [*PSI*⁺] strain distributions when compared to WT. This suggests that the NAC is an important factor in aggregate organization and that NAC disruption may be capable of inducing widespread alterations in aggregate morphology.

Clearly, these counterintuitive results warrant further investigation. Beyond the main results showing deletion of *EGD1* and *BTT1* leads to delayed and decreased aggregation of htt exon1 103Q, we noticed that complete NAC deletion and deletion of *EGD1* and *EGD2* do not result in robust rescue of cellular viability when htt exon1 103Q is expressed (Figure 2.1B). This indicates that individual NAC subunits may have cellular roles that are important in protein aggregation and maintaining cellular viability. Previous work showing variable rescue of canavanine toxicity suggested this as well (Figure 1.3E) (Keefer and True, 2016).

Overall, this work shows that disruption of a chaperone complex has potential as a therapeutic target in protein misfolding and aggregation conditions. Further investigation is needed to understand how deletion of *EGD1* and *BTT1* leads to delayed and reduced htt exon1 103Q aggregation, htt exon1 103Q aggregate morphology changes, and *de novo* [*PSI*⁺] strain induction. Potential avenues to address this will be proposed in Chapter 4.

2.5 Materials and methods

2.5.1 Yeast strains, plasmids, cultures, and transformations

All yeast strains are derivatives of 74-D694 (*ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52*). Single and combinatorial genetic deletions of *EGD1, EGD2, and BTT1* were made as previously described (Keefer & True, 2016). Plasmids expressing huntingtin exon I fusions

controlled by the inducible *GAL1* promoter (p416Gal1-FLAG-htt25QΔPro-CFP and p416Gal1-FLAG-htt103QΔPro-CFP) were kindly gifted by S. Lindquist and were made as previously described (Duennwald et al, 2006). Plasmids expressing alpha-synuclein constructs controlled by the inducible *GAL1* promoter (pRS426-Gal1-SNCA^{WT}-GFP, pRS426-Gal1-SNCA^{A30P}-GFP, pRS426-Gal1-SNCA^{A53T}-GFP) were kindly gifted by M. Jackrel. Egd1 and Egd2 overexpression plasmids are under control of their endogenous promoters (p413-Egd1, p413-Egd2) and were made using standard molecular biology techniques and confirmed by sequencing.

2.5.2 Antibodies

A monoclonal anti-FLAG antibody was obtained from Sigma-Aldrich. A monoclonal anti-PGK1 antibody was obtained from ThermoFisher Scientific. A rabbit anti-mouse secondary antibody was obtained from ThermoFisher Scientific.

2.5.3 Prion manipulation

WT and NAC deletion yeast strains transformed with p426-Sup35 plasmid were grown at 30°C for 16hrs with rotation in 1M KCl SD-ura media to induce *de novo* prion formation. OD600 of each culture was determined and they were then plated on 150mm ¼ YPD solid media plates at dilutions that would render 200-500 colonies. These plates were then incubated at 30°C for 6 days and subsequently incubated at 4°C overnight for further color development. Each colony was evaluated for color and scored [*psi*$^-$] if red and [*PSI*$^+$] if pink, white, or sectored. At least 100 [*PSI*$^+$] colonies were picked and pinned on ¼ YPD, SD-ade, and 5mM GdnHCl and grown for 5 days at 30°C in order to evaluate [*PSI*$^+$] strength and color change due to [*PSI*$^+$] induction. Each experiment was repeated at least 3 times.

2.5.4 Fluorescent microscopy

Cells expressing p416Gal1-FLAG-htt25QΔPro-CFP, p416Gal1-FLAG-htt103QΔPro-CFP, or p414Gal1-EV were grown overnight in SD-ura. Cells were washed in S-ura, then grown for indicated incubation time in S-ura, 2% galactose to induce expression of htt exon1 25Q or htt exon1 103Q. Cells were imaged after 6 and 20 hours of induction using a x63 objective and cyan filter on Zeiss LSM 880 airyscan two-photon confocal microscope. Images were blinded to researcher and aggregation of htt exon1 103Q was assessed phenotypically. Collected data were graphed and analyzed using GraphPad Prism version 8.1.1 for Windows, GraphPad Software, La Jolla, California USA. Results represent a compilation of data collected during 3 separate experiments.

2.5.5 Yeast phenotypic and growth assays

WT and all combinatorial deletions of NAC subunits harboring p416Gal1-FLAG-htt25QΔPro-CFP, p416Gal1-FLAG-htt103QΔPro-CFP, pRS426-Gal1-SNCA^{WT}-GFP, pRS426-Gal1-SNCA^{A30P}-GFP, pRS426-Gal1-SNCA^{A53T}-GFP, or p416Gal1-EV were grown overnight in SD-ura. Cultures were normalized to 0.1 OD600 and spotted in 5 5-fold dilutions onto YPD, SD-ura, S-ura, 2% galactose, 1% raffinose, S-ura, 2% galactose, 0.1% raffinose, and S-ura, 2% galactose. A WT yeast strain harboring p413-Gal1-FLAG-103Q-GFP and pRS316-Egd2 or pRS316-Egd1 were grown overnight in SD-ura-his. Cultures were normalized to 0.1 OD600 and spotted in 5 5-fold dilutions onto YPD, SD-ura-his, S-ura-his, 2% galactose, 1% raffinose, S-ura-his, 2% galactose, 0.1% raffinose, and S-ura-his, 2% galactose. Induction plates were grown for 5 days at 30°C and plates containing glucose were grown for 3 days at 30°C. Experiments were repeated at least 3 times.

2.5.6 Protein analysis

Cells harboring p416Gal1-FLAG-htt25QΔPro-CFP, p416Gal1-FLAG-htt103QΔPro-CFP, or p416Gal1-EV were grown overnight in SD-ura, washed 2x with S-ura, and induced for 6 or 20 hours in S-ura, 2% galactose. Cell lysates were prepared as previously described. Total protein concentrations were determined by Bradford assays, normalized to ensure equal loading, and mixed with SDS-PAGE sample buffer. Samples were boiled for 10 minutes at 100°C before loading onto 10% polyacrylamide gel and run at constant current of 100V. Blots were transferred overnight, incubated with primary and secondary antibodies, and visualized with enhanced chemiluminescence and film (GeneMate). Bands were analyzed and quantified with ImageJ and normalized to PGK1. Experiments were repeated at least 3 times.

2.5.7 Filter trap assay

Followed modified protocol from Sin et al., 2018. Cells harboring p416Gal1-FLAG-htt25QΔPro-CFP, p416Gal1-FLAG-htt103QΔPro-CFP, or p414Gal1-EV were grown overnight in SD-ura, washed 2x with S-ura, and induced for 6 hours in S-ura, 2% galactose. Cell lysates were prepared as previously described. Lysates were normalized to 500ug total protein in Filter Trap Assay Buffer and serially diluted three times at 1:2 dilutions. Resulting 2µ filter was incubated with primary and secondary antibodies, and visualized with enhanced chemiluminescence and film (GeneMate). Bands were analyzed and quantified with ImageJ. Ponceau staining was used as a loading control. Experiments were repeated at least 3 times.

2.6 Acknowledgements

2.7 Figures
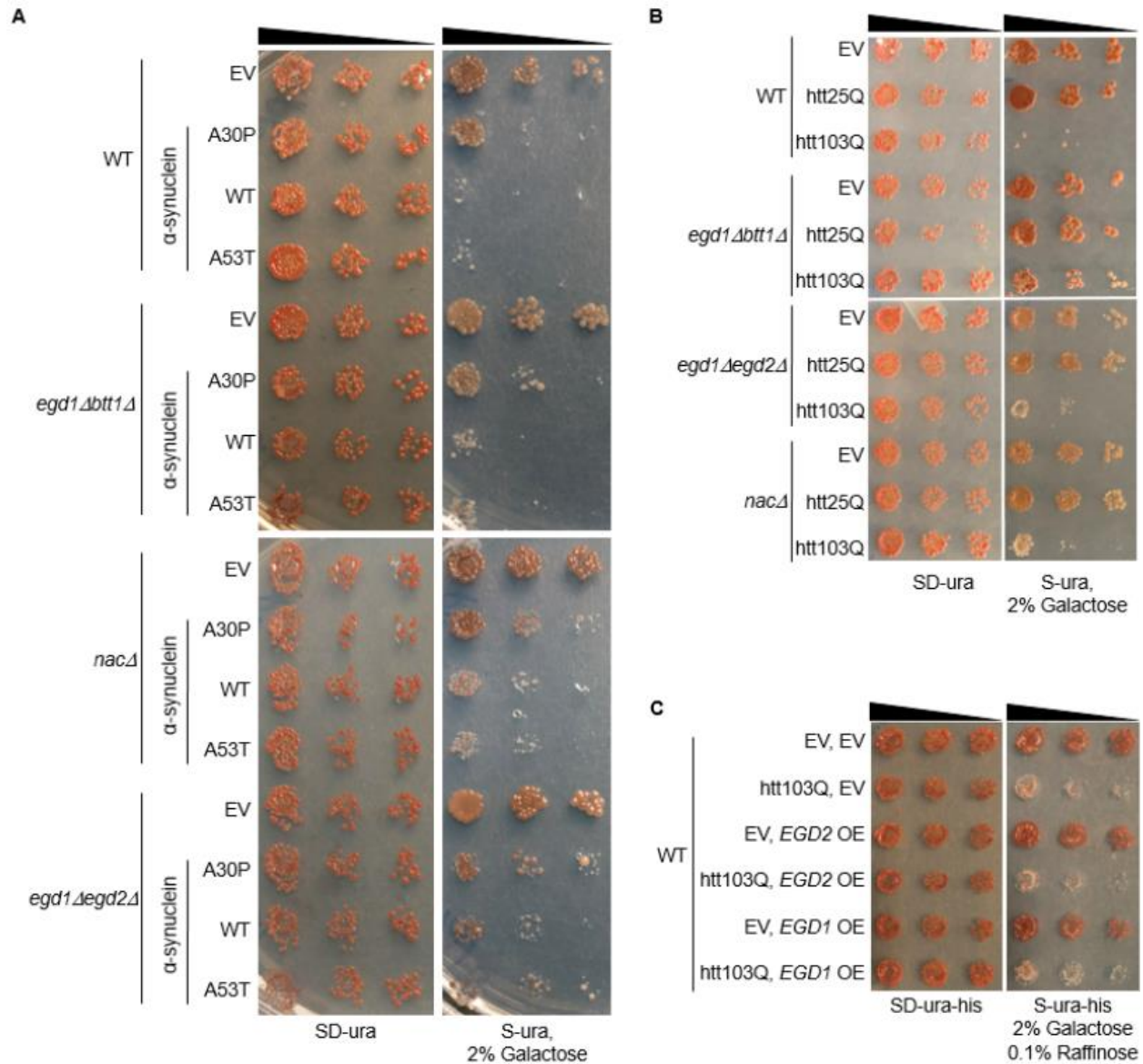
Figure 2.1 NAC modulation effects on amyloid-induced cytotoxicity (A) Yeast 74-D694 WT and nac deletion strains expressing Gal1-inducible EV or A30P, WT, or A53T α-synuclein constructs were serially diluted 5-fold and spotted onto ¼ YPD (not shown), SD-ura, and S-ura, 2% galactose (first 3 spots shown) to monitor α-synuclein toxicity and the ability of nac deletion to rescue α-synuclein cytotoxicity (n=3). (B) Yeast 74-D694 WT and nac deletion strains expressing Gal1-inducible EV, htt25Q, or htt103Q constructs were serially diluted 5-fold and spotted onto ¼ YPD (not shown), SD-ura, and S-ura, 2% galactose (first 3 spots shown) to monitor expanded polyglutamine toxicity and the ability of nac deletion to rescue expanded polyglutamine cytotoxicity (n=3). (C) A yeast 74-D694 WT strain expressing EV, EGD2, or EGD1 constructs in combination with Gal-inducible EV or htt103Q constructs were serially diluted 5-fold on ¼ YPD (not shown), SD-ura-his, and S-ura-his, 2% galactose, 0.1% raffinose

(first three spots shown) to evaluate expanded polyglutamine toxicity and the ability of EGD1 and EGD2 overexpression to rescue expanded polyglutamine cytotoxicity (n=3).
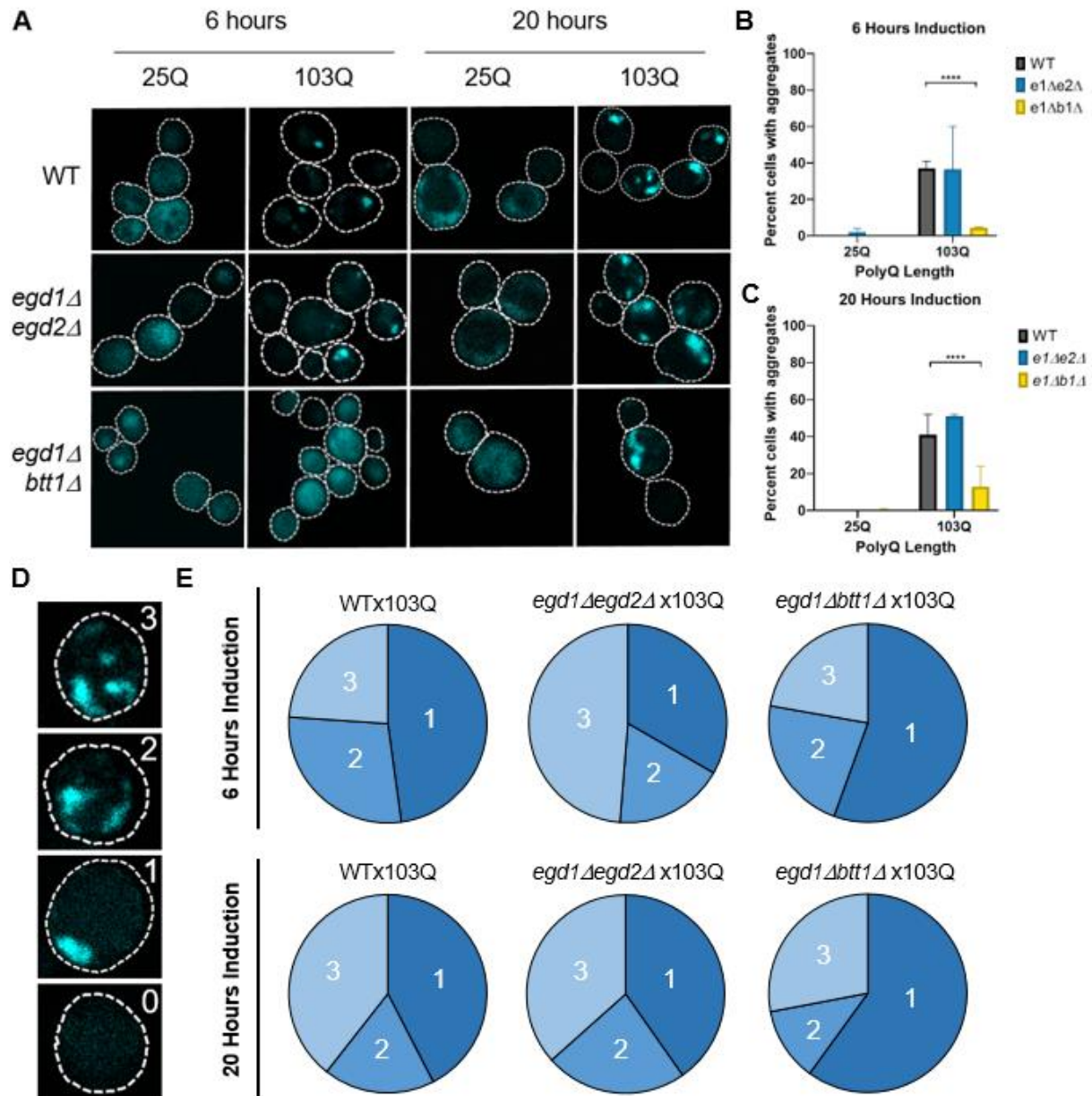
Figure 2.2 NAC deletion effects on polyQ aggregation and aggregate distribution. (A) WT and NAC deletion strains expressing Gal1-htt25Q-CFP or Gal1-htt103Q-CFP constructs were grown in selective media in the presence of 2% galactose for 6 and 20 hours. Two-dimensional z-stack images were taken with a Zeiss LSM 880 Airyscan Two-Photon Confocal Microscope with a 63x oil immersion objective. The *egd1Δbtt1Δ* strain showed a smaller population of cells with aggregates than the WT or *egd1Δegd2Δ* strains at both time points. (B) Quantification of percent cells expressing Gal1-htt25Q-CFP or Gal1-htt103Q-CFP constructs containing aggregates after 6

hours induction by 2% galactose in selective media. Significance was determined by Fisher's exact test (****=<0.0001). (C) Quantification of percent cells expressing Gal1-htt25Q-CFP or Gal1-htt103Q-CFP constructs containing aggregates after 20 hours induction by 2% galactose in selective media. Significance was determined by Fisher's exact test (****=p value<0.0001). (D) Representative images of cells with 0, 1, 2, or 3 aggregates. Microscopy of WT and NAC deletion strains expressing Gal1-htt25Q-CFP or Gal1-htt103Q-CFP constructs for 6 and 20 hours were evaluated based on this scale. (E) WT and NAC deletion strain cells expressing Gal1-htt103Q-CFP constructs for 6 or 20 hours and containing aggregates were evaluated as having 1, 2, or 3 or more aggregates. The resulting population distributions are represented as percentages in pie graphs.
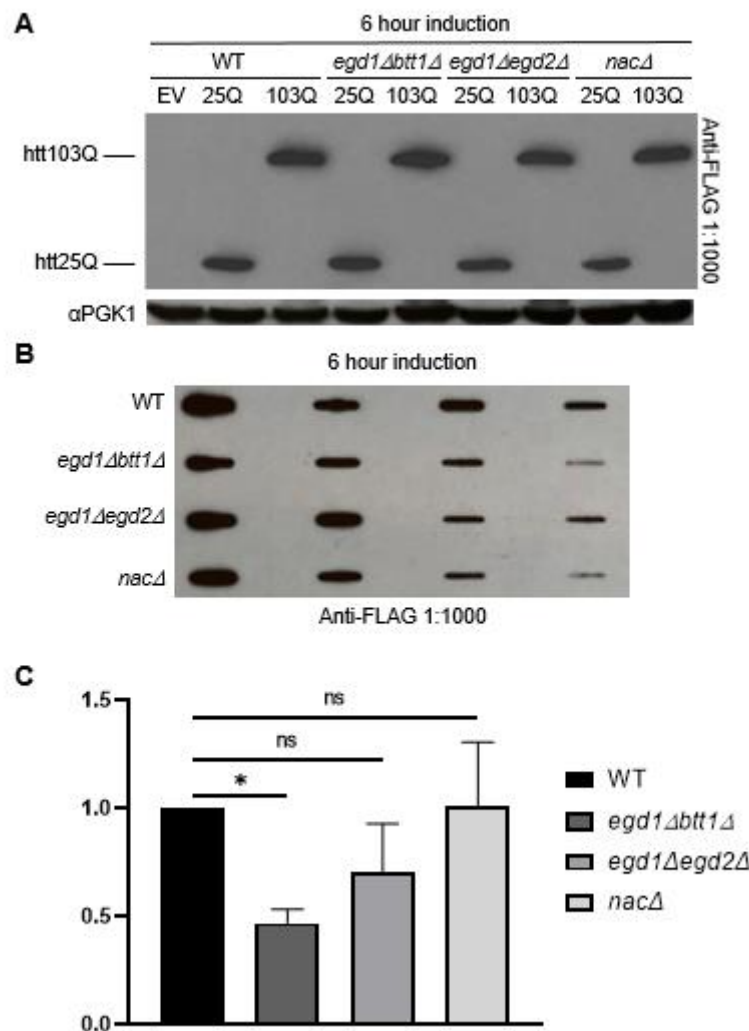
Figure 2.3 WT and NAC deletion strains express similar levels of polyQ protein and polyQ aggregation is reduced in *egd1Δbtt1Δ*. (A) WT and NAC deletion strains expressing Gal1-FLAG-htt25Q-CFP or Gal1-FLAG-htt103Q-CFP constructs were grown in selective media in the presence of 2% galactose for 6 hours prior to SDS-PAGE and Western blotting for FLAG and PGK1. Western blots are representative images of three independent experiments. (B) WT and

NAC deletion strains expressing Gal1-FLAG-htt25Q-CFP (not shown) or Gal1-FLAG-htt103Q-CFP constructs were grown in selective media in the presence of 2% galactose for 6 hours prior to Filter Trap Assay and Western blotting for FLAG. All strains in figure are expressing Gal1-FLAG-htt103Q-CFP. Western blot is a representative image of three independent experiments. (C) Quantification and normalization to WT of 3 independent Filter Trap Assay Western blots represented in 3(B), data are represented as mean ± SEM, significance was determined by paired t-test, *=p value<0.05, and ns=p value>0.05.

[*PSI*⁺] strain	Wild Type	*nacΔ*	*egd1Δegd2Δ*	*egd1Δbtt1Δ*
Very Weak	8.0±5.0%	10.7±4.3%	19.1±6.1%	17.1±3.8%
Weak	26.9±6.2%	28.8±5.2%	38.0±3.9%	35.3±3.0%
Medium	54.1±8.4%	52.6±7.9%	40.7±3.2%	41.6±3.7%
Strong	11.0±2.6%	8.0±1.1%	2.3±2.3%	6.1±2.4%

Table 2.1 [*PSI*⁺] distribution is altered in NAC deletion strains. WT and NAC deletion strains expressing a pEMBL-SUP35 construct were grown overnight in selective media containing 1M KCl, plated, and evaluated for [PSI+] prion strength by a previously described colorimetric assay. Over 100 colonies were evaluated from three independent experiments and ±values=SEM.

Log #	Plasmid	Auxotrophic Marker	Control
SL7711	p416-Gal1-FLAG-25Q-CFP	URA3	X
SL7712	p416-Gal1-FLAG-103Q-CFP	URA3	
SL7874	p413-Gal1-FLAG-103Q-GFP	HIS3	
SL9250	P413-Gal1	HIS3	X
SL9256	p416-Gal1	URA3	X
5320	pRS316-Egd2	URA3	
5328	pRS316-Egd1	URA3	
6244	p426-Sup35	URA3	
6890	pRS426-Gal1-SNCA^{WT}-GFP	URA3	
6891	pRS426-Gal1-SNCA^{A30P}-GFP	URA3	
6892	pRS426-Gal1-SNCA^{A53T}-GFP	URA3	

Table 2.2 Plasmids used in this study. The left-most column indicates the True Lab log number of each plasmid, the second from the left column contains the plasmid names, the third from the left column indicates the auxotrophic marker of each plasmid, and the right most column identifies the control plasmids.

Log #	Strain Information
3233	74D-694, [*RNQ*⁺], [*psi*⁻], Mat A
3234	74D-694, [*RNQ*⁺], [*psi*⁻], *egd1Δ*, Mat A
3235	74D-694, [*RNQ*⁺], [*psi*⁻], *egd2Δbtt1Δ*, Mat A
3236	74D-694, [*RNQ*⁺], [*psi*⁻], *nacΔ*, Mat A
3237	74D-694, [*RNQ*⁺], [*psi*⁻], *egd2Δ*, Mat A
3238	74D-694, [*RNQ*⁺], [*psi*⁻], *egd1Δegd2Δ*, Mat A
3239	74D-694, [*RNQ*⁺], [*psi*⁻], *btt1Δ*, Mat A, HIS+
3240	74D-694, [*RNQ*⁺], [*psi*⁻], *egd1Δbtt1Δ*, Mat A HIS+

Table 2.3 Strains used in this study. The left most column indicates the True Lab log number of each strain and the right column contains relevant strain information.

Supporting Figure S1: Ponceau staining shows similar protein concentrations loaded in filter trap assay. WT and NAC deletion strains expressing Gal1-FLAG-htt25Q-CFP (not shown) or Gal1-FLAG-htt103Q-CFP constructs were grown in selective media in the presence of 2% galactose for 6 hours prior to Filter Trap Assay and Western blotting for FLAG. After developing 2µ filter was soaked in Ponceau staining to show total protein load. All strains are expressing Gal1-FLAG-htt103Q-CFP.

Chapter 3: Evaluating Second-Site Suppressors of LGMDD1 Mutations

This work is based on a second-site suppressor screen described in section 3.3.1. All experiments in subsequent sections are original to this work and were solely conducted by Leeran Blythe Dublin-Ryan.

3.1 Abstract

Chaperonopathies are conditions caused by mutations in molecular chaperones. Limb-Girdle Muscular Dystrophy Type 1D (LGMDD1) is a chaperonopathy associated with many mutations in the Hsp40 DNAJB6, spanning the J and G/F domains of the protein. Functional studies of DNAJB6 in multicellular organisms are challenging, as a known substrate of DNAJB6 has not yet been discovered. Sis1 is a yeast homolog of DNAJB6 with known client substrates. The existence of a homolog in *Saccharomyces cerevisiae* provides a compelling system for studying the effects of LGMDD1-associated mutations. Previous work has shown that mutations in the G/F-domain of DNAJB6 cause disruption of chaperone activity and client processing in Sis1 and a DNAJB6/Sis1 chimeric protein, SDSS. We show here that multiple second-site suppressors in the J-domain of Sis1 are capable of rescuing the activity defects caused by two different G/F-domain mutations. This work shows the viability of second-site suppressors in rescuing Hsp40 activity and shows great potential as a therapeutic strategy in the treatment of LGMDD1.

3.2 Introduction

Because chaperones are vitally important for innumerous protein folding events, mutations in chaperones can lead to modulation of activity and disease. Diseases induced by chaperone mutations are known as chaperonopathies (Macario and Conway de Macario, 2007). For example, LGMDD1 is associated with a myriad of mutations in the G/F and J domains of DNAJB6 (Harms et al., 2012; Sarparanta et al., 2012; Sato et al., 2013; Couthouis et al., 2014; Suarez-Cedeno et al., 2014; Yabe et al., 2014; Nam et al., 2015; Palmio et al., 2015; Ruggieri et al., 2015; Bohlega et al., 2018; Kim et al., 2018; Zima et al., 2019; Palmio et al., 2020). DNAJB6

is an Hsp40 that participates with Hsp70s to induce proper protein folding (Cyr et al., 1992; Freeman et al., 1995).

While it is known that many mutations in DNAJB6 are causative of LGMDD1, little is known about the cellular mechanism by which mutations in the protein induce disease phenotypes. Likewise, a known substrate of DNAJB6 remains elusive, making studying the mechanisms of the protein more difficult. Luckily, there is a yeast homolog of DNAJB6, the essential Hsp40 Sis1, with multiple known substrates, including Rnq1 and Sup35, which form the prions [RNQ^+] and [PSI^+], respectively. The introduction of LGMDD1-associated mutations in a Sis1 and DNAJB6 chimeric protein, SDSS, resulted in a reduced ability to propagate [RNQ^+] and [PSI^+] prions (Stein, et al., 2014). While DNAJB6 could not be used in these experiments because it cannot compensate for other Sis1 activities involved in cellular viability, the presence of another human J-protein, DNAJB1, is able to confer viability in *sis1Δ* cells and was used for further experimentation (Stein et al., 2014). One experiment revealed that DNAJB1-F90I (homologous to the LGMDD1-associated mutation DNAJB6-F89I) showed robust loss of viability (Stein et al., 2014). This result was of particular interest because it is useful for a second-site suppressor screen.

Suppression genetics has been used as a tool for understanding interactions between different proteins and different domains of the same proteins for decades (Hodgkin, 2005). A second-site suppressor is a secondary mutation that reverts a phenotype caused by a primary mutation back to the WT phenotype. There are two types of second-site suppressor mechanisms of action. Second-site suppressors can act in an intragenic manner, meaning the second-site suppressor is located in the same gene as the primary mutation, or in an intergenic manner,

meaning the second-site suppressor is located in a different gene than the primary mutation. Most Type I and Type II Hsp40s form functional homodimers, including Sis1, DNAJB1, and DNAJB6, so an intragenic second-site suppressor could act within one Hsp40 molecule or cause reversion to the WT phenotype through extra-molecular interactions between two Hsp40 molecules (Kampinga and Craig, 2010; Sha et al., 2000; Hu et al., 2008; Söderberg et al., 2018).

A requirement for second-site suppressor screening is a fairly robust primary phenotype, which we found in the stark DNAJB1-F90I-induced loss of viability. The yeast model organism in which this phenotype was found provided a tractable genetic system for a second-site suppressor screen. Moreover, the previous work that established clear [*RNQ*[+]] and [*PSI*[+]] propagation defects resulting from LGMDD1 mutations provided an experimental scaffolding to evaluate the effects of resulting second-site suppressors. Here we report the discovery of multiple second-site suppressors of DNAJB1-F90I. We also show that two of these second-site suppressors are particularly effective in rescuing activity defects caused by two LGMDD1-associated mutants. This work shows the potential of second-site suppressors as viable therapies and sets the stage for further studies to understand the mechanism by which these second-site suppressors act.

3.3 Results

3.3.1 Identification of second-site suppressors of DNAJB1-F90I

As previously described in Chapter 1, there are many mutations in J and G/F domains of DNAJB6 that are associated with LGMDD1 (Harms et al., 2012; Sarparanta et al., 2012; Sato et al., 2013; Couthouis et al., 2014; Suarez-Cedeno et al., 2014; Yabe et al., 2014; Nam et al., 2015; Palmio et al., 2015; Ruggieri et al., 2015; Bohlega et al, 2018; Kim et al., 2018; Zima et al., 2019; Palmio et al., 2020). These LGMDD1-associated mutations in the DNAJB6 J and G/F

domains have been extensively studied in yeast and multicellular organisms, however our understanding of their effect at the cellular level in the context of disease is limited. Kevin Stein (a former graduate student in the True lab) found that some G/F domain mutants caused disruption in propagation of particular [RNQ^+] and [PSI^+] variants (Stein et al., 2014). He also found that introducing an F90I mutation in DNAJB1 (homologous to DNAJB6-F89I) caused a viability defect in *sis1Δ* cells (Figure 3.1A). This strong phenotype was well-suited for a second-site suppressor screen, which is beneficial for uncovering new insights into protein domain interactions and effects of primary mutations.

Sis1 is required for viability and DNAJB1 can compensate for Sis1 loss. Therefore, Kevin performed error-prone PCR on a DNAJB1-F90I construct, creating a library of DNAJB1-F90I constructs that had additional mutations introduced. He grew a 74-D694 [RNQ^+], [psi^-], *sis1Δ* strain that was transformed with a URA3-marked, WT *SIS1* plasmid (pRS316-SIS1), transformed the strain with the library of potential DNAJB1-F90I mutants, and selected for loss of the WT *SIS1* plasmid by growing the transformants on 5-fluoroorotic acid (5-FOA). The viable colonies resulting from this process should only contain the DNAJB1-F90I library constructs. Resulting colonies were then tested to determine if their viability was due to rescue by a second-site suppressor. From this screen Kevin identified six second-site suppressors (Figure 3.1A), two of which (DNAJB1-R22W and R26G) were used in the subsequent experiments described herein.

3.3.2 Two second-site suppressors are located near a conserved Hsp40 motif

The DNAJB1-R22W and DNAJB1-R26G (homologous to DNAJB6-K21W and DNAJB6-K25G and Sis1-K24W and Sis1-K28G, Figure 3.1B) second-site suppressors are of particular interest because they are located near the HPD motif, which is a conserved motif

important for J-protein interaction with HSP70s (Tsai and Douglas, 1996), and multiple potential (unconfirmed) LGMDD1-associated mutations. We set out to evaluate the ability of these second-site suppressors to rescue chaperone activity by assessing the prion propagation defects caused by LGMDD1-associated mutations in the DNAJB6 G/F domain. Thus, we decided to use the SDSS chimeric protein that compensates for *sis1* loss and harbors the DNAJB6 G/F domain. This protein is constructed of the Sis1 J-domain, the DNAJB6 G/F domain, the Sis1 G/M domain, and the Sis1 CTD (Figure 3.1B) and has been successfully used to study DNAJB6 G/F domain mutations in the past (Stein et al, 2014). The second-site suppressors were introduced in SDSS-F89I, which is homologous to DNAJB1-F90I (Figure 3.1B), and in SDSS-WT. This resulted in four plasmid constructs, each expressing one of the following proteins: SDSS-K24W, SDSS-K28G, SDSS-K24W-F89I, and SDSS-K28G-F89I.

3.3.3 Second-site mutations K24W and K28G have varied effects on [*PSI*$^+$] propagation defect resulting from F89I mutation

As described in Chapters one and two, yeast prions are non-cytotoxic and many are clients of Sis1. Specifically, [*RNQ*$^+$] and [*PSI*$^+$] require Sis1 for propagation (Sondheimer et al., 2001; Higurashi et al., 2008). SDSS-WT is able to compensate for *sis1* loss and SDSS-F89I was previously shown to disrupt weak [*PSI*$^+$] and Sc37 propagation (Figure 1.4D) (Stein et al., 2014). Therefore, we transformed weak [*PSI*$^+$] and Sc37 *sis1Δ* strains harboring pRS316-*SIS1* with plasmid constructs expressing SDSS-WT, SDSS-F89I, SDSS-K24W, SDSS-K28W, SDSS-K24W-F89I, and SDSS-K28W-F89I, grew the resulting transformants on 5-FOA to select for the loss of pRS316-*SIS1*, and performed a viability assay with the resulting colonies (Figure 3.2). After growth on 5-FOA these colonies only express the *SDSS* constructs, which are on TRP1-marked plasmids. [*PSI*$^+$] propagation was disrupted as a result of SDSS-F89I expression (Figure 3.2), consistent with previous results (Stein et al., 2014). Interestingly, we saw that Sc37

propagation was partially maintained in colonies containing SDSS-K24W-F89I (Figure 3.2). Furthermore, we observed uniform maintenance of [*PSI*⁺] propagation in colonies containing SDSS-K28G-F89I (Figure 3.2).

Intrigued by the heterogeneity of results in the SDSS-K24W-F89I Sc37 colonies, we decided to take a closer look at [*PSI*⁺] propagation in these strains. To do this we grew the Sc37 strain containing TRP1-marked plasmids expressing SDSS-WT, SDSS-F89I, SDSS-K24W, SDSS-K28W, SDSS-K24W-F89I, and SDSS-K28W-F89I and the weak [*PSI*⁺] strain containing TRP1-marked plasmids expressing SDSS-WT, SDSS-F89I, SDSS-K28W, and SDSS-K28W-F89I in SD-trp media overnight and plated them at concentrations that would result in 200-500 colonies per plate. We then evaluated the resulting colonies by color, recording the number of [*PSI*⁺] and [*psi*⁻] colonies. As expected, the SDSS-WT containing colonies maintained [*PSI*⁺] at nearly 100% in both the Sc37 and weak [*PSI*⁺] strains (Figure 3.3). Expression of SDSS-F89I led to almost total loss of [*PSI*⁺] propagation in the Sc37 strain (Figure 3.3A) and loss of weak [*PSI*⁺] propagation in approximately 75% of colonies (Figure 3.3B). The introduction of K28G in combination with F89I led to robust rescue of the SDSS-F89I [*PSI*⁺] propagation defect in both the weak [*PSI*⁺] and Sc37 strains (Figure 3.3). While the addition of K24W to SDSS-F89I led to a modest increase in weak [*PSI*⁺] propagation, this change was not significant when compared to the strain expressing SDSS-F89I alone (Figure 3.3A).

3.3.4 K24W and K28G rescue [*PSI*⁺] propagation defect caused by another LGMDD1-associated mutation

Encouraged by these results, we wanted to investigate whether the K24W and K28G second-site suppressors were effective in combination with other LGMDD1 mutations. Because these second-site suppressors are not currently predicted to interact with the G/F domain and are

within close proximity to the HPD-motif, we hypothesize they may alter aspects of J-protein interaction with Hsp70s. Because of this we expected that they may be effective in rescuing defects resulting from other LGMDD1-associated mutations. Thus, we introduced the second-site suppressors in Sis1 in combination with F106L and F115I, which are homologous to LGMDD1-associated mutations DNAJB6-F93L and DNAJB6-F100I (Figure 3.1B).

We transformed the Sc37 and Weak [*PSI*+] *sis1Δ* strains expressing Sis1-WT on a URA3-marked plasmid with TRP1-marked plasmids expressing Sis1-WT, Sis1-F106L, Sis1-F115I, Sis1-K24W, Sis1-K28G, Sis1-K24W-F106L, Sis1-K24W-F115I, Sis1-K28G-F106L, and Sis1-K28G-F115I. We then grew these transformants on 5-FOA to select for loss of the URA3-marked *SIS1-WT* plasmid. Cells that grew on 5-FOA containing medium were grown in SD-trp liquid medium overnight, diluted to an OD of 0.1, serially diluted 5-fold, and spotted on SD-trp, ¼ YPD and SD-ura (to confirm loss of the URA3-marked SIS1 plasmid). Expression of the Sis1-F106L mutation did not cause loss of the [*PSI*+] prion (data not shown). This result meant we could not evaluate the addition of the second site suppressors, as there was no apparent phenotypic difference between Sis1-WT and Sis1-F106L expressing colonies. However, expression of Sis1-F115I resulted in partial loss of [*PSI*+] propagation in both the Sc37 and weak [*PSI*+] strains. In addition, the introduction of K28G in Sis1-F115I led to rescue of the partial [*PSI*+] propagation defect in both the Sc37 and weak [*PSI*+] strains (Figure 3.4). Introduction of K24W in Sis1-F115I resulted in a mixed population of red and pink colonies in both the Sc37 and [*PSI*+] strains, indicating some [*PSI*+] propagation loss (Figure 3.4).

Once again, we wanted to further evaluate [*PSI*+] propagation in these cells, so we grew Sc37 and Weak [*PSI*+] *sis1Δ* strains transformed with TRP1-marked plasmids expressing Sis1-

WT, Sis1-F115I, Sis1-K24W, Sis1-K28G, Sis1-K24W-F115I, and Sis1-K28G-F115I in SD-trp medium overnight. We plated the cultures on ¼ YPD 150mm plates at concentrations that would render 200-500 colonies per plate, and evaluated the resulting colonies by color. We photographed representative plates to show the resulting phenotypes (Figure 3.5A), and recorded the number of [*PSI*$^+$] and [*psi*$^-$] colonies in the weak [*PSI*$^+$] strains transformed with TRP1-marked plasmids expressing Sis1-WT, Sis1-F115I, Sis1-K24W, Sis1-K28G, Sis1-K24W-F115I, and Sis1-K28G-F115I (Figure 3.5B). In both the Sc37 and weak [*PSI*$^+$] strains we noted the marked increase of red ([*psi*$^-$]) colonies upon the introduction of the Sis1-F115I mutation, which was reduced when introduced in combination with K28G (Figure 3.5A). Furthermore, while the expression of Sis1-F115I led to a 20% reduction in colonies propagating [*PSI*$^+$] as compared to Sis1-WT colonies, introduction of either K24W or K28G in combination with F115I led to the rescue of this mutation-induced [*PSI*$^+$] propagation defect (Figure 3.5B). These results revealed that the two second-site suppressors are effective in rescuing defects induced by multiple LGMDD1-associated mutations and warrant further investigation into the mechanism of action of this rescuing effect.

3.4   Discussion

While these data are encouraging, much work is left to be done to understand the effects of these second-site suppressors on LGMDD1-associated mutations. Through these experiments we have learned that the K28G second-site suppressor may be more effective in rescuing defects caused by LGMDD1-associated mutations. We have also seen that these second-site suppressors may be capable of rescuing defects resulting from a wide range of LGMDD1-associated mutations. This could be because these second-site suppressors affect the tertiary or quaternary structures of the SDSS or Sis1 mutants and, possibly, DNAJB6 mutants. Conversely, the second-

site suppressors could modulate interactions with Hsp70, perhaps creating conditions that are favorable for some recovery of chaperone activity by the protein.

The next steps of this project are to use an arsenal of in vitro assays to evaluate the second-site suppressors effect on various aspects of the Hsp40/70 cycle, including ATPase activity, substrate-binding activity, HSP40/70 binding, and Hsp40 dimerization. We will also examine the ability of the second-site suppressors to influence [RNQ^+] prion propagation in order to determine if the results we have seen are substrate specific. We hypothesize that the addition of second-site suppressors will result in key differences in at least one stage of the HSp40/70 cycle.

3.5 Materials and methods

3.5.1 Yeast cultures, transformations, and plasmid shuffling

Spottings and [PSI^+] induction assays were performing using derivatives of 74-D694 (ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52), are [psi^-], [PSI^+], [rnq^-], or [RNQ^+], and were kindly gifted by Sue Liebman and Jonathan Weissman. Yeast strains were cultured using standard techniques. YPD (1% yeast extract, 2% peptone, 2% dextrose) or synthetic defined (SD) media (0.67% yeast nitrogen base without amino acids, 2% dextrose) lacking specific amino acids. In order to study the function of second-site suppressors in SDSS and Sis1 we used *sis1Δ* cells that were previously transformed with a pRS316-*SIS1* plasmid. We then transformed these cells with a pRS314 plasmid carrying *sdss* or *sis1* mutants as described in the results section. We plated these transformants first on SD-trp media to select for the mutant plasmids, then grew the colonies on plates containing 5-fluoroorotic acid (5-FOA) to select for colonies that had lost the pRS316-*SIS1* plasmid. All plasmid transformations were performed using the standard PEG/LiOAc method. The SDSS chimera plasmid was made and used in the True lab by

Kevin Stein (Stein et al., 2014). The pRS316-*SIS1* plasmid was kindly gifted by E. Craig. Any other plasmids used are described in the results section and were constructed using standard molecular techniques.

3.5.2 Plasmid construction

SDSS mutants were created using site directed mutagenesis as described previously (Stein et al., 2014; Pullen, 2020). Using pRS314-*SIS1*, F106L and F115I mutants were generated using the Quick Change Mutagenesis Kit (Agilent Technologies #200517). Second-site suppressors were created using the Takara in-fusion cloning site-directed mutagenesis system. Mutagenesis was confirmed by sequencing the entire coding region of Sis1.

3.5.3 Yeast spotting assay

Cultures were grown overnight in selective media. OD600 of each culture was determined. Each culture was normalized to an optical density of 0.1 and spotted in 5 5-fold serial dilutions on ¼ YPD, SD-ura, 5-FOA, and SD-trp using an ethanol-sterilized 48-pin replicator. Spottings were grown at 30°C for 5 days, incubated overnight at 4°C for further color development before being photographed. Each experiment was repeated 3 times.

3.5.4 [*PSI*$^+$] propagation frequency

74-D694 yeast containing the indicated strains of [*PSI*$^+$] and expressing the indicated *sis1* or *sdss* mutants were grown at 30°C for 16hrs with rotation. OD600 of each culture was determined and they were then plated on 150mm ¼ YPD solid media plates at dilutions that would render 200-500 colonies. These plates were then incubated at 30°C for 6 days and subsequently incubated at 4°C overnight for further color development. Each colony was evaluated for color and scored [*psi*$^-$] if red and [*PSI*$^+$] if pink, white, or sectored. Each experiment

was repeated at least 3 times. Resulting data were analyzed and graphed using GraphPad Prism version 8.1.1 for Windows, GraphPad Software, La Jolla, California, USA.

3.6   Acknowledgements

3.7 Figures

A
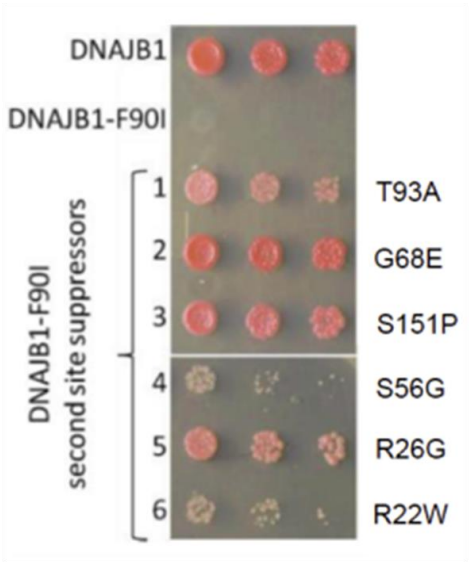
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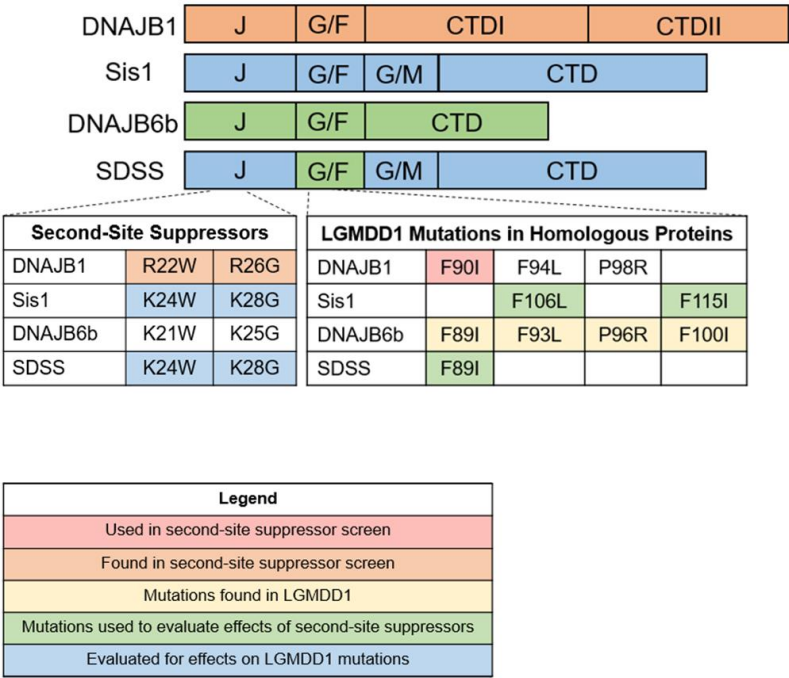
Figure 3.1 Identification of intragenic second-site suppressors and Hsp40s homology. (A) Screen performed by Kevin Stein. Spottings are from the same plate and are labeled by second-site suppressor. (B) Top illustration denotes the different domains of DNAJB1 (orange), Sis1 (blue), DNAJB6b (green), and SDSS (blue J, G/M, and CTD; green G/F). Middle insets indicate two second-site suppressors in each Hsp40 and relevant LGMDD1 mutations in each Hsp40. Bottom is the legend for the colors used to highlight specific second-site suppressors and LGMDD1 mutations in the middle insets.
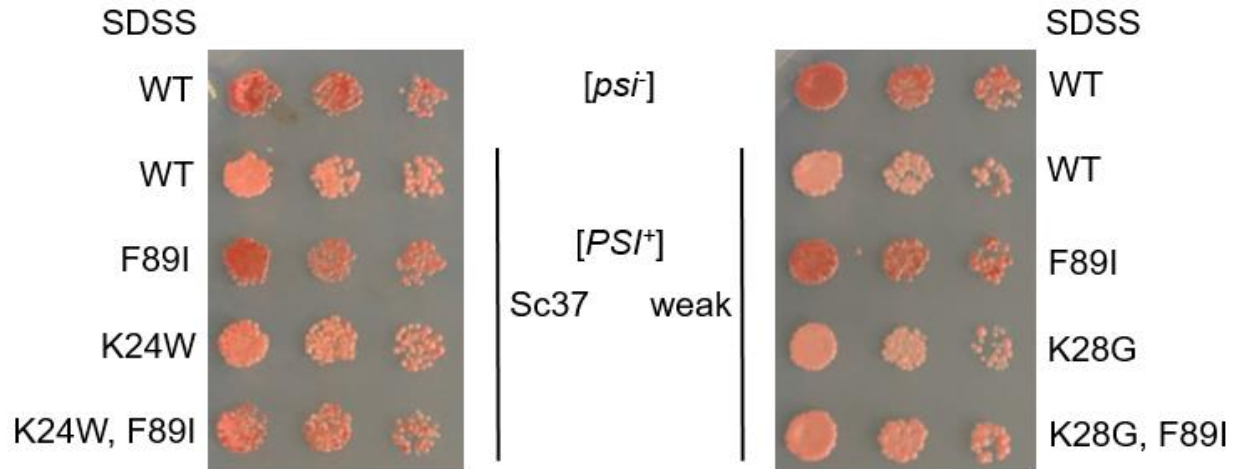
Figure 3.2 Second-site suppressors K24W and K28G do not affect viability and rescue prion propagation disrupted by F89I mutation. Yeast *sis1Δ* cells harboring the indicated SDSS expressing constructs in Sc37 and weak [*PSI*⁺] were serially diluted 5-fold and spotted on media selecting for the constructs (n=3).
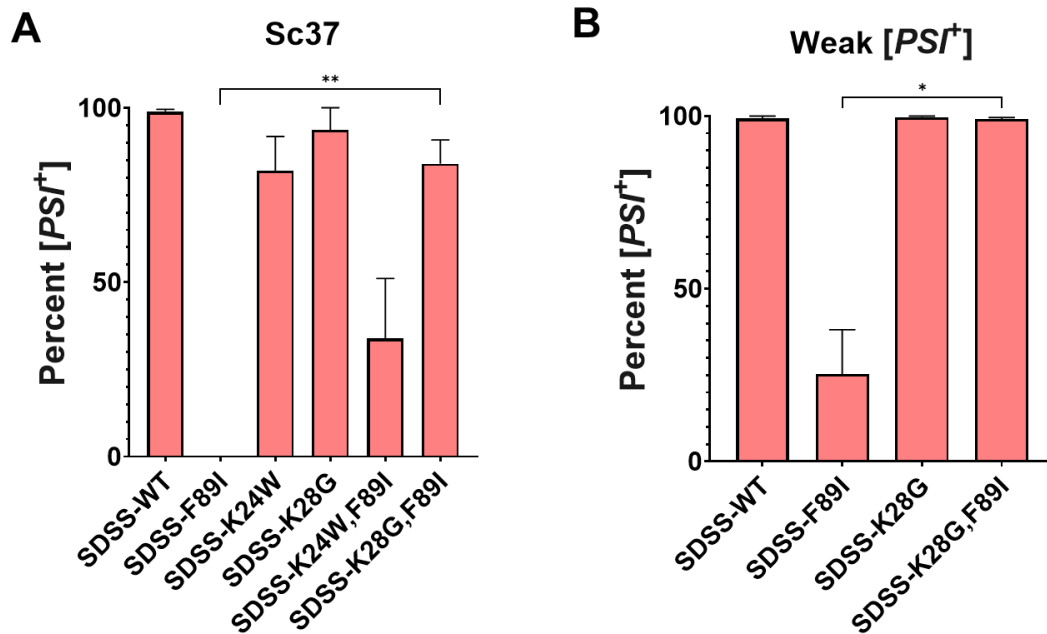
Figure 3.3 Second-site suppressor mutation K28G rescues [*PSI*⁺] propagation defect resulting from F89I mutation. Yeast *sis1Δ* cells harboring the indicated SDSS expressing constructs in (A) Sc37 and (B) weak [*PSI*⁺] were grown overnight in selective media and plated at concentrations to render 200-500 colonies per plate. Plates were incubated and subsequent colonies were evaluated by color to determine if they were [*PSI*⁺] or [*psi*⁻] (n=3). * indicates p value < 0.05, ** indicates p value < 0.01.

Weak [*PSI*⁺]

Sc37

Sis1-WT

Sis1-F115I

Sis1-K24W

Sis1-K28G

Sis1-K24W, F115I

Sis1-K28G, F115I
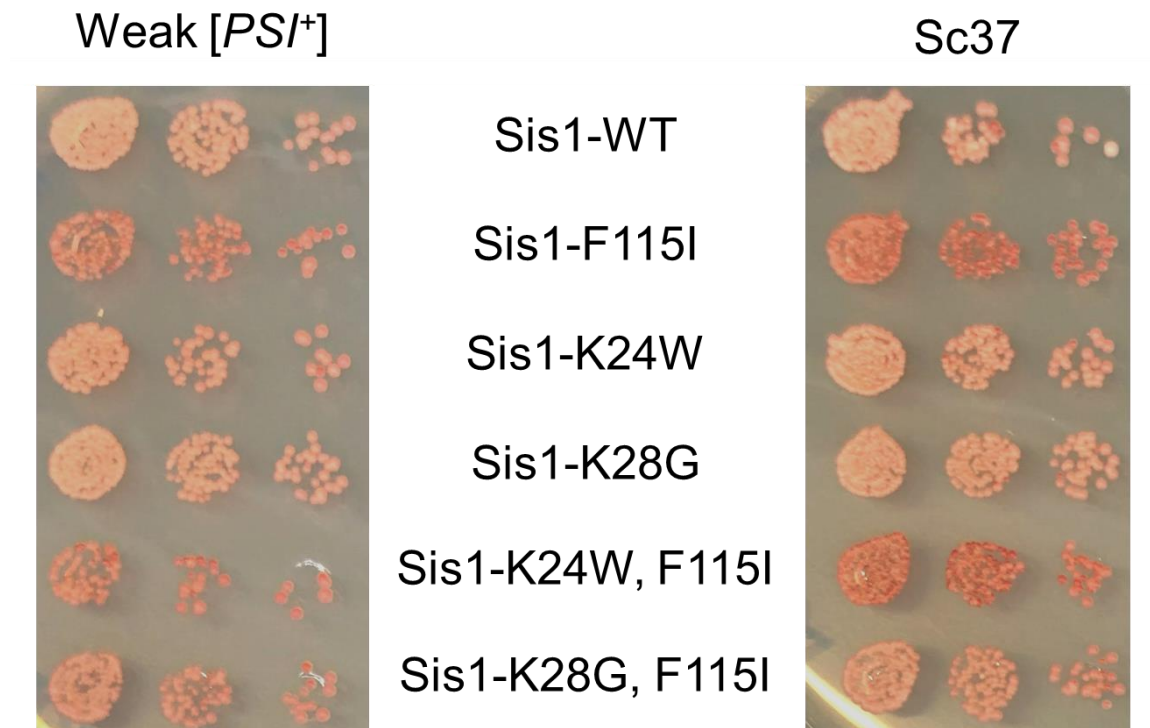
Figure 3.4 Sis1-F115I does not affect viability and induces prion loss, which second-site suppressors K24W and K28G partially rescue. Yeast *sis1Δ* cells harboring the indicated Sis1 expressing constructs in Sc37 and weak [*PSI*⁺] were serially diluted 5-fold and spotted on media selecting for the constructs (n=3).

A

Sis1

| | WT | F115I | K28G | K28G-F115I |

weak [*PSI*+]

Sc37

B

Weak [*PSI*+]

percent [*PSI*+]

Sis1-WT
Sis1-F115I
Sis1-*K24W*
Sis1-*K28G*
Sis1-*K24W-F115I*
Sis1-*K28G-F115I*
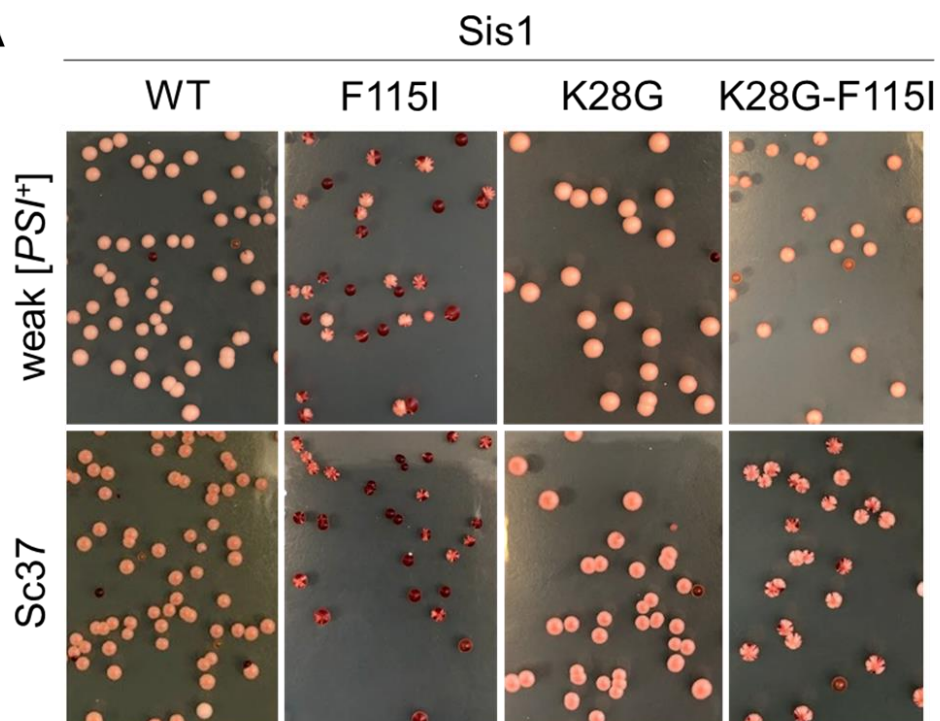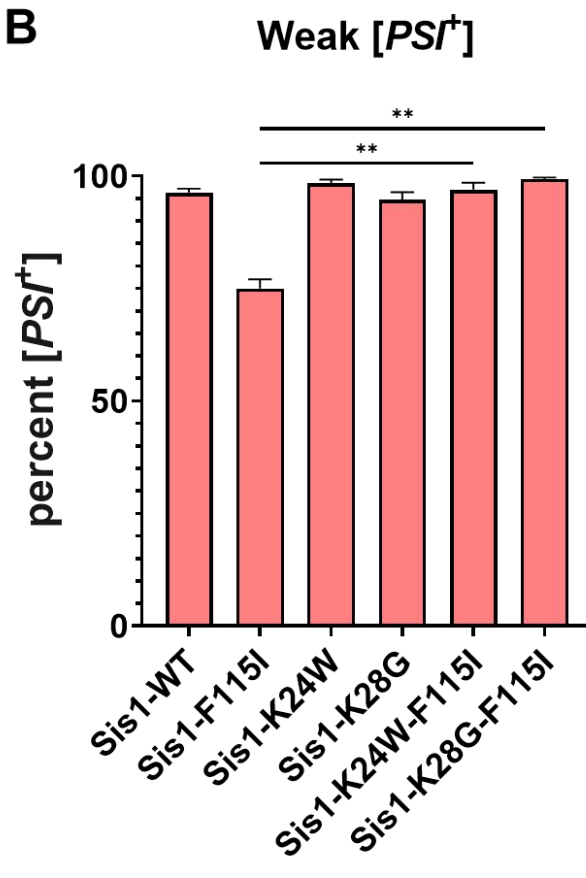
Figure 3.5 Sis1-F115I induces prion loss, which is significantly rescued by introduction of second-site suppressors K24W and K28G. Yeast *sis1Δ* cells harboring the indicated Sis1 expressing constructs in Sc37 and weak [*PSI*⁺] were grown overnight in selective media and plated at concentrations to render 200-500 colonies per plate. Plates were incubated and subsequent colonies were evaluated by color to determine if they were [*PSI*⁺] or [*psi*⁻] (n=3). Phenotypes can be seen in (A) and the resulting percent [*PSI*⁺] in the weak [*PSI*⁺] transformants can be seen in (B). (n=3), ** indicates p value < 0.01.

Log #	Plasmid	Auxotrophic Marker	Control
5691	pRS316-*SIS1*	URA3	
6404	pRS314-*SIS1*	TRP1	X
6405	pRS314-*sis1-F106L*	TRP1	X
6552	pRS314-*sis1-F115I*	TRP1	X
6581	pRS314-*SDSS*	TRP1	X
6600	pRS314-*sdss-F89I*	TRP1	X
6863	pRS314-*sdss-K24W*	TRP1	X
6864	pRS314-*sdss-K28G*	TRP1	X
6865	pRS314-*sdss-G68E*	TRP1	X
6868	pRS314-*sdss-K24W-F89I*	TRP1	
6869	pRS314-*sdss-K28G-F89I*	TRP1	
6870	pRS314-*sdss-G68E-F89I*	TRP1	
6893	pRS314-*sis1-K24W*	TRP1	X
6894	pRS314-*sis1-K28G*	TRP1	X
6895	pRS314-*sis1-K24W-F106L*	TRP1	
6896	pRS314-*sis1-K28G-F106L*	TRP1	
6897	pRS314-*sis1-K24W-F115I*	TRP1	
6898	pRS314-*sis1-K28G-F115I*	TRP1	

Table 3.1 Plasmids used in this study. The left-most column indicates the True Lab log number of each plasmid, the second from the left column contains the plasmid names, the third from the left column indicates the auxotrophic marker of each plasmid, and the right most column identifies the control plasmids.

Log #	Strain Information	Plasmid
2384	74D-694, Weak [*PSI*+], *sis1Δ*, Mat alpha, ADE+, URA+	5691
2470	74D-694, Sc37 [*PSI*+], *sis1Δ*, Mat A, ADE+, URA+	5691
2879	74D-694, [*rnq*-], [*psi*-], *sis1Δ*, Mat alpha, URA+	5691
3562	74D-694, Weak [*PSI*+], *sis1Δ*, Mat alpha, ADE+, URA+	6581
3563	74D-694, Weak [*PSI*+], *sis1Δ*, Mat alpha, ADE+, URA+	6600
3564	74D-694, Weak [*PSI*+], *sis1Δ*, Mat alpha, ADE+, URA+	6869
3565	74D-694, Weak [*PSI*+], *sis1Δ*, Mat alpha, ADE+, URA+	6864
3566	74D-694, Sc37 [*PSI*+], *sis1Δ*, Mat A, ADE+, URA+	6581
3567	74D-694, Sc37 [*PSI*+], *sis1Δ*, Mat A, ADE+, URA+	6600
3568	74D-694, Sc37 [*PSI*+], *sis1Δ*, Mat A, ADE+, URA+	6863
3569	74D-694, Sc37 [*PSI*+], *sis1Δ*, Mat A, ADE+, URA+	6864
3570	74D-694, Sc37 [*PSI*+], *sis1Δ*, Mat A, ADE+, URA+	6868
3572	74D-694, Sc37 [*PSI*+], *sis1Δ*, Mat A, ADE+, URA+	6869
3573	74D-694, [*rnq*-], [*psi*-], *sis1Δ*, Mat alpha, URA+	6581

Table 3.2 Strains used in this study. The left most column indicates the True Lab log number of each strain, the middle column contains relevant strain information, and the right most column identifies the plasmid harbored in each strain.

Chapter 4: Conclusions and Future Directions

4.1 Summary

Chapter 1 outlines the current understanding of molecular chaperones and their roles in protein folding and misfolding, chaperonopathies, and maintaining cellular homeostasis. Furthermore, the chapter reviews prion proteins, prions in yeast, human aggregate-prone proteins, and using yeast as a model system for investigating protein misfolding and aggregation. Chapter 2 presents data suggesting that disruption of a particular molecular chaperone complex, the NAC, is a possible therapeutic target for polyglutamine misfolding. This work shows that deletion of two NAC components, Egd1 and Btt1, leads to delayed and reduced polyglutamine aggregation and improved viability in the face of aggregation-induced cytotoxicity. Moreover, this combinatorial deletion changes the distribution of polyglutamine aggregates within the cell. Lastly, this chapter shows that NAC disruption changes the distribution of [*PSI*$^+$] strains as well. This work builds on a growing project showing chaperone disruption can be effective in combating toxic aggregation. Chapter 3 uses an innovative genetic system to investigate possible mechanisms for rescuing LGMDD1-associated mutation effects. This chapter shows that two second-site suppressors near the conserved HPD-motif are able to rescue [*PSI*$^+$] propagation defects caused by multiple LGMDD1-associated mutations. This work shows great promise for both in vitro studies to better understand the mechanism by which they rescue and translation into more complex disease models, such as tissue culture or animal models.

While the goals and findings of these chapters are different and seemingly disconnected, they both identify potential therapeutic avenues for treating protein misfolding diseases. We hope that future work will identify the mechanisms by which these genetic modulations affect

protein aggregation and chaperone activity and that they will lead to novel and effective therapeutic strategies.

4.2 Future Directions

Studying human diseases at the organism or tissue level can be extremely challenging; yeast present a system in which genetic manipulation is more accessible and mechanistic studies are less complex and laborious to implement. Thus, it is not surprising that many mechanistic insights have been made using yeast models. The work described in chapters 2 and 3 sets the stage for examining the mechanisms of action that lead to reduced polyglutamine aggregation and cytotoxicity and rescue of defects resulting from LGMDD1-associated mutation.

4.2.1 Elucidating the mechanism by which NAC disruption affects polyglutamine aggregation and toxicity

Determine the importance of EGD2 *expression in the* egd1Δbtt1Δ *strain effects on polyglutamine aggregation and toxicity*

Chapter 2 highlights the novel discovery that NAC disruption affects polyglutamine aggregation and toxicity, however the mechanism by which this occurs remains unknown. In yeast there are three NAC subunits, Egd1, Egd2, and Btt1. Egd1 and Btt1 are known as the β and β' subunits and are responsible for reversibly binding the complex to the ribosome (Reimann et al., 1999). Because the *egd1Δbtt1Δ* strain lacks these subunits we have hypothesized that Egd2 is not interacting with nascent polypeptides at the ribosome, but this has not been confirmed. Furthermore, even if our hypothesis is correct and Egd2 is not localizing to the ribosome and interacting with nascent polypeptides, Egd2 has other roles in the cell (Shi et al., 1995; Franke et al., 2001; Meury et al., 2010) and it is important to understand if these roles are affecting polyglutamine aggregation and toxicity. It is known that Egd2 has a UBA domain, which stabilizes the protein (Panasenko et al., 2006). Deletion of the Egd2 UBA domain has been

shown to alter Egd2 chaperone activity and stability (Panasenko et al., 2006; Panasenko et al., 2009; Ott et al., 2015). In order to assess whether Egd2 activity and stability are required for the effects of the *egd1Δbtt1Δ* strain on polyglutamine aggregation, an *egd2-UBAΔ* construct would be expressed in the *nacΔ* strain, along with expanded-polyQ. If expression of this construct in the *nacΔ* strain resulted in recapitulation of the *egd1Δbtt1Δ* phenotypes then it would be determined that the presence of Egd2, but not its chaperone activity or stability, is required for the phenotypes observed in the *egd1Δbtt1Δ* strain expressing expanded polyglutamine. Conversely, if the *nacΔ* strain expressing the *egd2-UBAΔ* and expanded polyglutamine constructs does not phenocopy the *egd1Δbtt1Δ* strain it would be determined that stable Egd2 is required for the phenotypes we have observed in the *egd1Δbtt1Δ* strain expressing expanded polyglutamine. Because the reduction in expanded polyglutamine-induced cytotoxicity seen in the *egd1Δbtt1Δ* strain was not observed in the *egd1Δegd2Δ* and *nacΔ* strains we hypothesize that the presence of stable Egd2 is required for the *egd1Δbtt1Δ* phenotypes.

Investigate egd1Δbtt1Δ *effects on protein misfolding responses*

It is known that modulation of molecular chaperones can change the effectiveness (Chernoff et al., 1999) and cellular distribution of other molecular chaperones (Keefer and True, 2016). While previous work has shown that NAC deletion does not globally change the expression levels of many other molecular chaperones (Ssb, Ssa, Sis1, Hsp104) (Keefer and True, 2016), it remains unknown if disruption of the NAC leads to modulation of other components of the molecular chaperone and protein folding network. It is known that heat stress in HeLa cells leads to downregulation of Egd2 and that depletion of Egd2 in HeLa cells activates multiple ER stress proteins, including BiP, CHOP, and phosphorylated PERK (Hotokezaka et al.,

2009). Because expanded polyglutamine has been shown to activate the Unfolded Protein Response pathway (Duennwald et al., 2006) we hypothesize that the mechanism by which *egd1Δbtt1Δ* reduces expanded polyglutamine cytotoxicity and aggregation could involve alterations to the activation of the UPR. To test this hypothesis, western blots of the WT and nac deletion strains before expression of expanded polyglutamine, 6 hours after induction, and 20 hours after induction would be performed and the resulting membranes would be probed for Kar2 (yeast homolog of Bip) and Ire1. There are many possible outcomes from this experiment. The deletion of *egd1* and *btt1* could create a cellular environment where Egd2 is no longer localized to ribosomes and instead participates in suppressing the UPR. This would lead to low signal of Kar2 and Ire1 at every time point. The deletion of *egd1* and *btt1* could destabilize Egd2, leading to induction of the UPR, which would result in increased Kar2 and Ire1 at every time point. There are also possibilities of one of the above scenarios occurring, but the expression of expanded polyglutamine modulates Egd2 stability in a way that leads to downstream changes in UPR activation. In any case these experiments would likely provide new insights into NAC disruption's effect on the cellular response to expanded polyglutamine expression.

Determine ability of Egd1 and Btt1 expression to rescue expanded polyglutamine aggregation and toxicity

It has been determined that loss of *egd1* and *btt1* leads to delayed aggregation and reduced cytotoxicity of expanded polyglutamine, however it remains unknown if loss of certain cellular roles of these subunits could be sufficient for these phenotypes. This could be important, as total loss of the NAC is lethal in multicellular organisms (Deng and Behringer, 1995; Bloss et al., 2003) and interruption of certain cellular roles could allow for effective treatment of protein

misfolding diseases without harming the cell. Both Egd1 and Btt1 have ribosome binding domains that can be disrupted while maintaining the domains required for dimerization and interacting with nascent polypeptides (Ott et al., 2015). Constructs of either ribosome-binding impaired Egd1 or Btt1 could be introduced in the *egd1Δbtt1Δ* strain, along with expression of expanded polyglutamine. The strains could then be examined via fluorescent microscopy, western blotting, and spotting assays to determine the ability of expanded polyglutamine to aggregate and cause toxicity. If the cells expressing expanded polyglutamine and the Egd1 or Btt1 mutants phenocopy wild type expressing expanded polyglutamine then it could be concluded that the presence of Egd1 or Btt1 in the cell is sufficient to rescue expanded polyglutamine aggregation and toxicity. If the cells expressing expanded polyglutamine and the Egd1 or Btt1 ribosome-binding impaired mutants continue to reduce cytotoxicity and expanded polyglutamine aggregation, then it can be determined that Egd1 or Btt1 ribosome binding is required for polyglutamine aggregation and cytoxicity. Either result would allow for better understanding the importance of ribosome binding in the activity of the NAC and the relevance of this activity in protein aggregation. It is possible that abolishing NAC association with the ribosome could allow localization of the complex to expanded polyglutamine, which could invoke stronger reduction of aggregation and toxicity reduction.

Elucidate Egd2 localization in egd1Δbtt1Δ *cells expressing expanded polyglutamine*

Finally, it is important to understand the localization of Egd2 in *egd1Δbtt1Δ* cells expressing expanded polyglutamine. A fluorescent tag should be added to Egd2 and confocal microscopy should be performed on *egd1Δbtt1Δ* cells expressing tagged Egd2 and expanded polyglutamine for 6 and 20 hours. Egd2 could be localized to polyglutamine aggregates, in

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which case Egd2 would appear in and overlapping with punctate structures. Egd2 could be localized to ribosomes, in which case expression would likely appear diffuse. Egd2 could be acting in its role as a transcription factor (Shi et al., 1995), in which case it would be localized to the nucleus. Any of these outcomes would present new insights into the mechanism by which the *egd1Δbtt1Δ* strain reduces aggregation and cytotoxicity.

4.2.2 Determining the mechanism by which second-site suppressors K24W and K28G rescue prion propagation defect induced by LGMDD1-associated mutations

Evaluate the ability of Sis1-K24W-F115I and Sis1-K28G-F115I to stimulate Hsp70 ATPase activity

An important step in the Hsp40/70 cycle is the stimulation of Hsp70 ATPase activity by Hsp40 (Craig & Marszalek et al., 2014). Hsp40 stimulation of Hsp70 ATPase activity regulates Hsp70 binding to substrate. Furthermore, recent research has shown that inhibition of the HPD-motif, which is critical for ATPase stimulation by Hsp40, is beneficial in a LGMDD1 mouse disease model (Bengoechea et al., 2020). We would hypothesize that because the K24W and K28G mutations are located spatially proximal to the HPD-motif they will affect ATPase stimulation of Hsp70 by Sis1-K24W-F115I and Sis1-K28G-F115I. In order to assess ATP hydrolysis, Sis1-WT, Sis1-F115I, Sis1-K24W, Sis1-K28G, Sis1-K24W-F115I, and Sis1-K28G-F115I would be purified and the various Sis1 substrates would be used to perform ATPase assays with Ssa1 and [RNQ^+] prion seeds.

Determine the ability of Sis1-K24W-F115I and Sis1-K28G-F115I to dimerize

Sis1 has been reported to dimerize through a dimerization domain located on the C-terminus of the protein (Sha et al., 2000). Dimerization of the protein is vitally important for substrate

binding of Sis1, and thus, of the Hsp40/70 cycle. Furthermore, it is known that dimerization is reduced in many LGMDD1-associated mutants, including Sis1-F115I (Bhadra et al., in prep). We hypothesize that the addition of either second-site suppressor will modulate dimerization of Sis1. While the second-site suppressors are not located near the C-terminus they could still affect the structure and stability of the dimer through intragenic and extragenic interactions. To do this experiment Sis1-WT, Sis1-F115I, Sis1-K24W, Sis1-K28G, Sis1-K24W-F115I, and Sis1-K28G-F115I would be purified and the resulting proteins would be used to conduct binding assays. These binding assays would show dimerization efficiency of these proteins to make homodimers. Furthermore, we could test the ability of the second-site suppressors to dimerize with Sis1-WT, or in trans with the LGMDD1-associated mutation, such as testing the ability of Sis1-F115I to dimerize with Sis1-K28G.

Determine the ability of Sis1-K24W-F115I and Sis1-K28G-F115I to act on substrate

Because Rnq1 is a known client protein of Sis1, Sis1 efficiency can be determined by measuring the speed with which Rnq1 aggregates form in vitro. To do this the fluorescent emission of Thioflavin T, which associates with beta-sheet rich structures, is measured. As more [RNQ^+] aggregates form the fluorescence emission of Thioflavin T increases. Binding of Sis1 to Rnq1 slows formation of [RNQ^+] aggregates, therefore Sis1 activity and effectiveness can be determined based on the fluorescent emission of Thioflavin T. We would hypothesize that Sis1-K24W-F115I and Sis1-K28G-F115I would show delayed [RNQ^+] aggregation compared to Sis1-F115I. This experiment would require isolation of Sis1-WT, Sis1-K24W, Sis1-K28G, Sis1-F115I, Sis1-K24W-F115I, Sis1-K28G-F115I and unseeded RNQ monomer.

4.3 Conclusion

The previously outlined experiments are a basis for uncovering the mechanisms by which NAC disruption and second-site suppressors improve the cellular protein folding environment in the face of cellular stress. These experiments should aid in the discovery of relevant information to further each of these projects and perhaps identify new therapeutic strategies for treating neurodegenerative disorders and chaperonopathies. These experiments are crucial for furthering our understanding of how NAC disruption and the second-site suppressors of LGMDD1-associated mutants affect protein homeostasis.

In summation, this body of work identifies novel effects of chaperone modulation on protein folding. NAC modulation is shown in this work to be a potential therapeutic strategy for reducing expanded polyglutamine aggregation and toxicity. Likewise, two second-site suppressors have been shown to rescue defects induced by LGMDD1 mutations and thus can be evaluated for their potential as therapeutics to treat LGMDD1.

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