Proteotoxicity From Aberrant Ribosome Biogenesis Compromises Cell Fitness

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Proteotoxicity from Aberrant Ribosome Biogenesis Compromises Cell Fitness

A dissertation presented by

Blake Wells Tye

to The Committee on Higher Degrees in Chemical Biology
in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

in the subject of

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Proteotoxicity from Aberrant Ribosome Biogenesis Compromises Cell Fitness

Abstract

Proteins are the workhorses of the cell, carrying out much of the structural and enzymatic work of life. Many proteins function as part of greater assemblies, or complexes, with other proteins and/or biomolecules. As the cell grows and divides, it must double its proteome, which requires synthesis and folding of individual proteins, assembly into complexes, and proper subcellular localization. This is repeated for millions of proteins molecules compromising thousands of potential assemblies, making up a rather tricky set of tasks for cells to carry out simultaneously.

The very machine that carries out protein synthesis—the ribosome—is among the most challenging complexes to assemble and one of the most abundant. Each ribosome requires ~80 unique proteins and 4 unique RNAs to be stitched together in an assembly line that spans the nucleus to the cytosol. Intriguingly, there exist many extracellular insults and genetic mutations that compromise the integrity of ribosome biogenesis, but that impact different cell types and cell states with a range of severities. This represents an intriguing paradox, as the null expectation would be that ribosomes would be critically important to all cells. In this work, I sought to identify the mechanism by which disrupting the integrity of ribosome assembly compromises cell fitness using budding yeast as a model organism with exceptional chemical and genetic tools.

By using these tools in yeast to perturb diverse stages of ribosome assembly one at a time on very short timescales, I was able to study the most proximal consequences. I found that
cells experience a collapse of protein folding homeostasis in conditions that give rise to excess newly-synthesized ribosomal proteins relative to what can be assembled. In detailing this response, I found that cells specifically activate the conserved proteostasis restoration response directed by the transcription factor Heat Shock Factor 1 (Hsf1). In doing so, this work identifies the first such endogenous proteins capable of eliciting Hsf1 activation, and I further explored the possibility that the fate of newly-synthesized proteins may more generally be compromised by other proteotoxic conditions that drive Hsf1 activation. This work implicates protein synthesis in general as a risk for the proteome that may be compromised by various insults, and suggests that the ribosome in particular may be troublesome for rapidly-proliferating cells with high ribosome production.
Acknowledgments

The completion of my PhD and the work of this thesis would not have been possible without the friends, family, colleagues, teachers, and mentors I have had in my decade of experimental science. There is no more fitting expression than “it takes a village…”

I started college at the University of Arizona wanting to be a physician, but as luck would have it, I ended up doing most of my undergrad research in Dr. Indraneel Ghosh’s lab. Neel inspired in me an insatiable love for developing and applying chemical tools to address interesting questions in biology that remains fundamental to my taste in science. Dr. Jim Hazzard, instructor of the core biochemistry lab course, gave me the experience that you could both have fun and take science very seriously.

I interviewed at Harvard in 2013 during Superstorm Nemo as one of only two students who flew in before the visit weekend was canceled. The city was shutdown from more than two feet of overnight snow and I ended up being trapped in Boston while it was probably in the 60s and sunny back in AZ. Nevertheless, the people I met in the Harvard chemical biology program were so fantastic, I couldn’t imagine being scared off by a little frozen water. I’m grateful for the rockstar trainees of chembio; from learning lots of science spanning the purest of chemistry to the purest of biology, to awesome annual karaoke-filled retreats and year-end events, I couldn’t imagine better peers to do a PhD with. The program would not be what it is without the lead faculty—namely Drs. Dan Kahne, Suzanne Walker, and Tim Mitchison—who have kept alive the vision of marrying Cambridge-side chemistry with Longwood-side biology. A very special thanks to the one and only Jason Millberg, who has kept this program in tip-top shape and is no doubt key in bringing in such great students.

The members of my dissertation advisory committee—Drs. Mike Springer, Bob Kingston, and Fred Winston—each brought a unique perspective and personality to my committee that made our meetings highly constructive and enjoyable. You all have been fantastic. Thanks also
to Fred, who always been available to provide wisdom on topics spanning yeast genetics to how to handle professional situations. I spent quite a lot of time downstairs in the Winston lab and always felt welcome. A number of my experiments would not have been a success without Fred and his great lab members, many of whom have become friends.

I have had great collaborators over the years. Nikki Commins, in Dr. Mike Springer’s lab, did some terrific work testing the fitness of mutant yeast strains in the ribosome assembly work. Lillia Ryazanova, in Dr. Martin Wuhr’s lab, did very nice mass spectrometry-proteomics that addressed a tricky reviewer point. Martin also welcomed me into his lab for some interesting proteomics projects that didn’t make it into this thesis, but I learned a lot and had fun thinking about how to carefully define the mammalian nuclear and chromatin proteome. Thank you Martin, for your support there and elsewhere. A special thank you to Dr. David Pincus. In my third year of grad school, I found that I had waded into the very unfamiliar depths known as “proteostasis”. David provided instrumental support in the ensuing years that helped take this work to much higher levels. Dr. Luke Berchowitz provided much-needed edification on how to do biochemistry in the real world. I have enjoyed the opportunity to collaborate in some small way on friends’ fascinating projects: Cathy Gutierrez and Evi Van Itallie.

I came to Boston not knowing a single person, and feel lucky for the amazing friendships I’ve developed here. I met Matt Sonnett at the Harvard orientation and I’m pretty sure we became best friends in a matter of minutes. We’ve enjoyed years of getting wired on iced coffee, talking about all things science and life, and seen dozens of concerts together. I’m incredibly grateful to have Matt in my life and look forward to a long life of friendship. Also in Harvard chembio was Dr. Eileen Moison, who has become a close friend and obliges me on all my naive questions about professionalism. I aspire to have her patience and determination. Dr. Josh Pan has been a great friend and confidant since we met by happenstance at a retreat that neither of us probably belonged at in our first year. We have gotten together quarterly for the last six years, and I have had some of my biggest turnarounds from low-lows through talking
with Josh about life and all the coolest “papes”. I’ve been fortunate to make great friends outside of science here in Boston that have made my time special, including the great folks at CrossFit Fenway who became close friends after an initial shared interest in pummeling ourselves in the gym—Dave, Kris, Matt, Maralene, Chris, among others. The Alumni of Allston, my first group of roommates, for showing me the ropes of Boston/New England life; special shoutout to Dr. Seth and Mrs. Justine Bensussen, who have become very close friends of Danielle’s and mine.

Thank you to Dr. Stirling Churchman, my PhD advisor, from whom I have learned so much. Stirling has always known where to meet my needs, giving me freedom to explore my (really broad) interests but being accessible when I needed her guidance. Certainly not least, I have become an infinitely better science communicator through many, many revisions of writing and the ever-helpful practice talks that result in Churchman lab members giving beautiful talks. I leave the Churchman lab a much better scientist, and by Stirling’s design, I had a lot of fun along the way. It’s no mistake that the lab website mugshots contain big smiles.

Thank you to my labmates in the Churchman lab, past and present, who have been terrific friends, mentors, and colleagues. Drs. Kevin Harlen, Mary Couvillon, Julia di Iulio, Umut Eser, and Stephen Doris provided me with assistance and inspiration as I learned how to do biology, especially genetics and genomics. Subgroup Mito/Ribo—Aaron Aker, Jake Bridgers, Brendan Smalec, Kate Lachance, Drs. Stefan Isaac, Erik McShane, and Iliana Soto—have been a constant source of interesting science and many enjoyable meals. Though our work together didn’t end up in this thesis, thanks to Dr. Jieun Park for taking a detour into phase separation biology with me. A special shoutout to Dr. Heather Drexler, who sat five feet from me for all of grad school. I’m grateful to have gotten to bounce things off of you for so many years and to learn from you as your excellent work unfolded; you have made this whole thing more enjoyable and more fruitful. My friendship with Stephen really took root when I proclaimed at lunch that “Game of Thrones is the only show I’ll watch”. I now count him among my closest friends. Thank you for your years of guidance and wisdom, and a lot of really fun times offline. Thank you to all
others in the lab for making an incredible environment of fun and brilliant people—it’s hard to envision a better group of people work with daily and I will miss you all.

Despite the long distance, some of my oldest friendships have only gotten stronger. Thanks to my oldest friend Daniel Hurtado; no matter how long we go without seeing each other, it’s like we pick up right back where we left off. Thanks to college friends that make up the illustrious and elite Club Andy—Andrew, Colin, Austen, Jack, Chris, Kelly, Mark—for lots of good fun, including some of my most memorable trips in grad school.

To my dad, Scott: thank you for always challenging my mental ability and pushing the boundaries of my knowledge; you inspired my thirst for learning. To this day, people get a kick out of us asking each other spontaneous trivia questions (maybe 1/3rd of which are movie quotes), and seeing who gets stumped first. More importantly, you really taught me how to have a sense of humor from a young age. My stepmom, Nicole, lives and breathes “work hard, play hard”, and I aspire to live the “play” side of my life as fully as she does. Despite having zero science background, she has always asked me to tell her what I’m working on, and to draw it out for her—that meant so much to me. My mom, Lisa, inspired resilience in me and selflessly encouraged my burgeoning curiosity as a kid. My sister, Jocelyn, has been my closest friend for a very long time and has this otherworldly power to inspire me (and others) no matter where I’ve been at in life. Thank you for always being there for me, Joc, and for wanting to hang with your little bro enough to spend many of your own birthdays in Boston. My awesome brother-in-law, Aaron, quickly became one of my role models and has likewise helped me grow in totally unexpected ways. Lastly, my soon-to-be wife, Danielle. We joke about this being *our* PhD, but there is no question that you have been my foundation and my biggest champion on this long journey. I couldn’t imagine doing this PhD, yet alone life, without you by my side, being simultaneously wise beyond your years and side-splittingly funny.

_I would not be who I am, and where I am, without you all. My deepest gratitude._

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Chapter 1  Introduction
Far beyond a passive protein synthesis machine, the ribosome has a rich link to the wellness of a cell. In order for cells to grow and divide, they must continuously replenish their hundreds of thousands or millions of ribosomes with each cell doubling to maintain protein synthetic capacity at sufficient levels. In addition to catalyzing peptide bond formation, the ribosome serves as a platform for interaction with myriad factors that dictate the fate of nascent proteins. As such, cells spend a massive amount of their energetic and biosynthetic budget on ribosome biogenesis. This is no simple task, as each ribosome requires ~80 unique ribosomal protein (r-protein) and four RNA (rRNA) components be stitched together in the right place at the right time along a trajectory that traverses from the nucleolus into the cytosol. Decades of research from many groups has mechanistically detailed much of this assembly line and the hundreds of factors that keep it running smoothly.

Intriguingly, in recent years a number of conditions have been observed to disrupt the integrity of this fundamental process. Though interfering with this process would be expected to have purely deleterious effects to all cells as ribosomes are key components of eukaryotic life, certain cells appear especially sensitive while others are refractory. Human ribosomopathies give rise to quite tissue selective developmental defects, such as in blood cell formation (e.g. Diamond-Blackfan anemia, Shwachman-Diamond syndrome, 5q- syndrome) and craniofacial malformation (Treacher Collins syndrome) while the majority of their other tissues are largely unaffected. Patients with ribosomopathies have a higher cancer incidence, possibly due to selective pressure that these mutations place for loss of p53. (Note: for a review of mutations and clinical manifestations in ribosomopathies, see Aspesi and Ellis2).

Key features of ribosome biogenesis are highly conserved across eukaryotic organisms3. Therefore, the work presented in this thesis was performed using budding yeast to leverage the chemical and genetic tools available to dissect ribosome biogenesis and the cellular response to perturbations that have been found to disrupt the integrity of this process. In particular, genes in haploid yeast can readily have a C-terminal tag appended in a matter of days, which can
provide a handle for conditional removal of a tagged protein of interest from the nucleus by anchor away\(^4\) or degradation by the ubiquitin-proteasome system by auxin-inducible degradation\(^5\) upon addition of a small molecule. The genetic tractability also allows for generating a collection of mutants and sensitive detection of fitness differences in various conditions to tease apart phenotypic importance. Lastly, as it relates to ribosome biogenesis, one of the few small molecule eukaryotic ribosome biogenesis inhibitors functions in budding yeast, and rescuing mutations in the target have been mapped\(^6\). In sum, budding yeast represents an ideal organism to model the observed diversity of perturbations to ribosome biogenesis that would otherwise be intractable.

1.1 Overview of ribosome biogenesis

A rapidly proliferating yeast cell dedicates more than 80% of all RNA synthesis, invoking all three RNA polymerases, and more than 40% of translation, to produce sufficient rRNA and r-proteins to generate >2,000 complete ribosomes a minute\(^7\). In addition, the assembly process enlists more than 200 putative biogenesis factors\(^8\).

R-protein production begins with transcription of r-protein genes by RNA polymerase II, encoding 79 unique proteins. rRNA production begins with transcription of the rDNA loci, which are present in hundreds of copies per cell and form the foundation of the nucleolus. rDNA transcription by RNA polymerase I produces the chimeric 35S precursor (pre-) rRNA, while RNA polymerase III produces the 5S. Dozens of snoRNA-containing ribonucleoproteins (snoRNPs) guide methylation of more than 100 nucleotide 2’-hydroxyl groups while many uridines are substituted for pseudouridines\(^9\); these RNA edits are critical for rRNA folding and assembly. Helicases carry out removal of snoRNPs and allow for commencement of cutting and end trimming of pre-rRNA. Subsequently, the full-length precursor 35S is cut endonucleolitically by factors including Rnt1 and Las1, freeing up 5′ and 3′ ends for processing by exonucleases Rat1,
Rrp17, and exosome members Rrp6 and Dis3/Rrp44. The processed 35S gives rise to the 25S and 5.8S, destined for the large (60S) subunit along with the 5S, as well the the 18S, destined for the small (40S) subunit. As rRNA is transcribed and processed, early assembling r-proteins form on the rRNA along with dozens of assembly factors that shape the pre-40S and pre-60S complexes. Importantly, much of this is conserved, both in terms of factors used and pathway to maturation. Interestingly, human cells and slower growing yeast perform the majority of pre-rRNA processing by nucleases following transcription, in contrast to rapidly growing yeast that perform the majority co-transcriptionally. Nevertheless, rRNA synthesis and processing occurs in the nucleus and is coordinated with assembly of the bulk of the r-proteins at high rates.

Meanwhile, r-protein mRNAs are in the cytosol, where mature ribosomes translate them through canonical cap-dependent translation. Owing in part to the high positive charge important for assembly with negatively-charged rRNA, r-proteins are inherently unstable, and so they employ the help of ribosome-associated chaperones such as Hsp70/Ssb and the nascent polypeptide-associated complex (NAC) to maintain solubility. It is not known, however, whether r-proteins require these factors to fold, or simply fail to remain soluble in their absence after leaving the ribosome. Additionally, dedicated factors, termed escortins or r-protein chaperones, have been recently described in yeast and human cells. To date, r-protein escortins have been described for Rps3 (escortin Yar1), Rps26 (Tsr2, the human homolog of which is associated with Diamond-Blackfan anemia), Rpl3 (Rrb1), Rpl4 (Acl4), Rpl5 and Rpl11 (Syo1), and Rpl10 (Sqt1). These factors bind the nascent chain of one or a few specific r-proteins and chaperone them to their site of assembly in the nucleus or cytosol. Similar to loss of general chaperones, loss of escortins results in insolubility of the target r-protein.

The vast majority of r-proteins are assembled in the nucleolus/nucleoplasm, producing pre-40S and pre-60S particles that are in full exported to the cytosol. Once in the cytosol, the rRNA of the pre-40S is processed to its final (18S) form, and remaining r-proteins such as
Rpl10\textsuperscript{28} and Rpl24\textsuperscript{29} are assembled. The peptide exit tunnel and active site within the new 60S are probed for integrity before entering the mature ribosome pool\textsuperscript{30,31}.

### 1.2 Control and coordination of ribosome biogenesis

The massive investment cells make in r-protein, rRNA, and biogenesis factor production necessitates the ability to coordinate production of these components and change their levels depending on resource availability and cell growth rate. I will focus on the broader concepts, as different organisms utilize relatively distinct methods to regulate ribosome production.

At the highest level, this network is controlled by the transcriptional activity of RNA polymerase I (35S pre-rRNA), RNA polymerase II (r-protein- and biogenesis factor-encoding transcripts, snoRNAs), and RNA polymerase III (5S rRNA)\textsuperscript{7,32}. How precisely these different polymerase activities are coordinated is not clear, but a number of interesting insights have been raised. A key regulator seems undoubtedly to be the kinase Target of Rapamycin (TOR), which is a conserved master regulatory kinase that senses and responds to nutrient availability as a proxy for growth potential\textsuperscript{33,34}. Mechanistically, TOR regulates, directly or indirectly, a network of transcription factors that together influence the activity of all three polymerases. TOR regulates Sfp1 in yeast, which functions homologously to human Myc in regulating r-protein gene transcription by Pol II\textsuperscript{35–37}. Myc itself further influences Pol I and Pol III activity\textsuperscript{38–41}, situating it as a possible cornerstone of coordinating the entire network in humans. Additionally, TOR regulates yeast Hmo1 and the human homolog UBF1, which regulate transcription of rDNA by Pol I. Hmo1 additionally functions at some Pol II promoters of a subset of r-protein genes\textsuperscript{42}. Illustrating the high level coordination by TOR, inhibition by rapamycin results in rapid downregulation of the synthesis of r-proteins\textsuperscript{43}, which is sufficient to cause a shutdown in rRNA processing and subsequently reduced overall rRNA production levels\textsuperscript{44}. Additional mechanisms may link the activity of the polymerases. Recent reports demonstrate that the helicase DDX21
links transcription activity by Pol I and Pol II in human cells\textsuperscript{45}, and in yeast cells the transcription factor Ifh1 that regulates r-protein genes can be titrated along with the rRNA processing factor Utp22\textsuperscript{46}.

Additional evidence exists in yeast cells for possible mechanisms of integrating signals across the RNA polymerases. The transcription factor Rrn3, homologous to human TIF-1A, recruits Pol I to rDNA loci and is required for transcription\textsuperscript{47}, downstream of TOR activity\textsuperscript{48}. Conditions that decrease ribosome production, such as oxidative stress and nutrient deprivation, decrease the formation of Pol I-Rrn3 complexes\textsuperscript{49,50}. Laferte et al.\textsuperscript{51} showed that fusing Rrn3 to Pol I, rendering the complex inseparable, prevents repression of rDNA transcription by Pol I following rapamycin treatment. Remarkably, this was sufficient to attenuate the repression of Pol II, sustaining higher levels of r-protein gene transcripts and suggesting the Pol I may impose upstream control of r-protein production via Pol II. Lastly, some Pol II targets that fall into the ribosome biogenesis regulon and are thus coordinately regulate with r-protein genes encode subunits of Pol I and Pol III, though this would serve as a relatively slower means of coordination than is observed on short timescales.

In addition to transcriptional coordination of ribosome biogenesis, cells utilize post-transcriptional strategies to balance components. The potent downregulation of r-protein gene transcripts by stress\textsuperscript{52} is partly the result of transcriptional downregulation after loss of activators such as Ifh1, Fhl1, and Sfp1 and possible induced binding of the repressor Crf1\textsuperscript{43}. However, it was recently found that stress also substantially decreases the half-lives of r-protein transcripts, suggesting they are targeted for degradation\textsuperscript{53}. Nevertheless, these layers do not result in perfect stoichiometry of r-protein transcripts, and so r-proteins are synthesized at variable levels. The excess free subunits are likely targeted for rapid clearance by the ubiquitin-proteasome system\textsuperscript{54}; a recently described quality control system recognizes excess r-proteins that were induced by overexpression using the E3 ligase Tom1 (human HUWE1). Intriguingly,
even aneuploid cells do not accumulate r-proteins in excess of their stoichiometry, suggesting the importance of clearing excess subunits\textsuperscript{55}.

In sum, eukaryotic cells display myriad approaches to facilitate timely and balanced production of ribosomal components. In response to nutrients and growth cues, TOR functions at a high level to integrate production of all ribosomal components. Post-transcriptionally, the abundance of rRNA is managed by shutting down processing, while r-protein production is regulated by a rapid decline in r-protein mRNA half lives as well as by targeting excess free r-proteins for degradation by the ubiquitin-proteasome system.

1.3 Aberrations in ribosome biogenesis in cellular stress and disease

Genetic, xenobiotic, and extracellular conditions have the potential to interfere with the balanced production and assembly of the ribosome. Many chemotherapeutic drugs have long been thought to function principally on DNA, but more recent evidence has demonstrated major consequences of such agents on RNA metabolism as well. A critical study by Burger et al.\textsuperscript{56} examined the effect of a broad panel of cytotoxic drugs on rRNA processing and found that many of them interfere with rRNA production, either fully halting rRNA production or causing defects in processing. DNA damaging agents such as cisplatin and doxorubicin led to a complete loss of rRNA synthesis, whereas the antimetabolite 5-fluorouracil, topoisomerase inhibitors camptothecin and etoposide, and proteasome inhibitors like MG132 and bortezomib, among others, interfered with variable stages of rRNA processing. Though the authors did not demonstrate these effects were direct, it opens the possibility that cells exposed to such agents may undergo an acute shutdown of rRNA supply, leaving r-proteins in excess.

Many drugs and environmental stress conditions also impact the integrity of the nucleolus and lead to accumulation of precursor rRNA. Heat shock has been best explored on
this front. Early work by Pelham\textsuperscript{57} found that the major cytosolic chaperone, Hsp70, relocates to the nucleolus upon heat shock and facilitates recovery of nucleolar morphology. A broad range of stress conditions has been found to lead disruptions in rRNA processing\textsuperscript{16}, but interestingly not complete shutdown in rRNA production.

Naturally occurring mutations in humans that impact ribosome biogenesis have become described in a number of contexts. These mutations can existing in factors that functions at various stages in ribosome biogenesis. Mutations in genes that function in rRNA production/processing, including \textit{TCOF1}, and \textit{POLR1C} and \textit{POLR1D} (Pol I subunits), give rise to Treacher Collins syndrome, characterized by craniofacial malformations\textsuperscript{58,59}. Mutations in dozens of r-proteins genes as well as the Rps26 escortin-encoding \textit{TSR2} gene (also \textit{TSR2} in yeast) give rise to the red blood cell formation defects in Diamond-Blackfan anemia\textsuperscript{60}, which is mirrored in mutation in the acquired 5q- syndrome (\textit{RPS14})\textsuperscript{61}. R-protein gene mutations in this group include loss-of-function and missense mutations expected to negatively impact the mutant protein’s ability to properly assembly\textsuperscript{60,62}. These mutations therefore seem to influence the abundance/stoichiometry of individual r-proteins that can be assembled into the ribosome, yielding a rather specific anemic phenotype. Mutations in factors that are important for late-stage 60S assembly, such \textit{SBDS} (yeast \textit{SDO1}), \textit{DNAJC21} (yeast \textit{JJJ1}), and \textit{EFL1} (also \textit{EFL1} in yeast), give rise to another form of bone marrow failure (neutropenia, white blood cell insufficiency) and pancreatic insufficiency\textsuperscript{63,64}. Overall, mutations in genes related to ribosome biogenesis recurrently impact skeletal development and lineages (red and white blood cells) that originate in the bone marrow.

A number of models exist that seek to explain the phenotypes observed in ribosomopathies, in particular, the cell-type specific growth defects seen in patients with mutations in genes encoding ribosome biogenesis factors or r-proteins.

The first proposes that certain cell types have high translational demands, and so they may be more sensitive to the reduced translational capacity that would be a byproduct of
successfully making fewer ribosomes. However, as reviewed by Mills and Green\textsuperscript{65} protein synthesis rates of different tissues do not entirely correlate with the tissues that classically show defects in ribosomopathies. It remains possible that existing protein synthesis measurements in other animals do not represent that base case of a developing or grown human\textsuperscript{66,67}.

The second model invokes the role of p53 in cell cycle arrest and apoptosis. When select r-proteins such as L5 or L11 are in excess, they engage the ubiquitin ligase MDM2/HDM2, allowing for the stabilization of p53 and cell cycle arrest/cell death\textsuperscript{68–70}. While loss of p53 in genetic backgrounds of ribosomopathy mutations can rescue some growth defects, it fails to rescue others, suggesting that are are additional, p53-independent means of loss of cell viability\textsuperscript{71–73}.

A more recent notion proposes that such mutations decrease the pool of ribosomes and that select transcripts are especially sensitive to this. Therefore, certain cell types that rely on that “sensitive” transcripts would be especially vulnerable\textsuperscript{65}. The most well-tested form of this hypothesis exists for models of Diamond-Blackfan anemia, where the transcript encoding GATA1, the transcription factor that controls the differentiation trajectory, shows greater than expected translational sensitivity to knockdown of r-protein genes that are mutated in Diamond-Blackfan anemia\textsuperscript{74}. Interestingly, overexpressing GATA1 only partially rescues erythrocyte formation, indicating that either this model is incomplete, or that GATA1 is not the only critical, “sensitive” transcript impacted by these mutations.

An interesting possible hint for another model arises in a disorder related to Diamond-Blackfan anemia: beta-thalassemia. Beta-thalassemia results from mutations that decrease the prevalence of the beta-globin chains that assemble alpha-globin to form hemoglobin, likewise causing a failure to form proper, mature red blood cells. The diminished abundance of beta-globin results in excess free alpha-globin, and this sequesters the chaperone Hsp70 to presumably deal with unstable, aggregated alpha-globin\textsuperscript{75}. Fascinatingly, the phenotype can be rescued by Hsp70 overexpression. The model proposed invokes the need of GATA1 for Hsp70
to protect it from caspases\textsuperscript{76}, thus allowing it to drive the differentiation program. It may then be that Diamond-Blackfan anemia in part exerts its effects by analogously causing an imbalance in ribosomal protein components; Hsp70 likewise seems to be able to suppress the phenotype in ex vivo models\textsuperscript{77}.

As with most areas of biology, it is likely that more than one model contributes to the true underlying mechanism. Indeed, each one has reasonable merits, wherein phenotypes can at least in part be rescued, but also has drawbacks, wherein it fails to explain all genetic contexts and phenotypes. One area of particular interest is in the early phase of disruptions to ribosome biogenesis. Many of the studies that describe mechanisms of the phenotypes above utilize powerful but relatively slow perturbations; these are liable to miss early effects in cell physiology. Alternatively using small molecule drugs like those described above has the major drawback of each such compound likely having myriad other effects, making it challenging to tease apart direct from indirect effects. Perhaps there is room to uncover new biology in this space by using highly selective perturbations and looking very early on.

1.4 Overview of this thesis

In this thesis, I sought to understand the underlying mechanisms by which perturbations to ribosome biogenesis negatively impact cell fitness. Further, I sought an explanation for the anomalous findings of the extent to which such perturbations impact different cell types and states. In Chapter 2, I used chemical-genetic tools in budding yeast to model perturbations to different stages of ribosome biogenesis and found that situations that give rise to excess free r-proteins result in acute loss of protein folding homeostasis due to aggregation of the free r-proteins. In detailing the basis of this response, I found that cells growing faster—and thus, making more ribosomes—experience a greater proteotoxic strain than cells growing slower. This provides a plausible mechanism by which different cell types and states in other
organisms, such as humans, are more or less severely compromised by loss of integrity in ribosome biogenesis, such as rapidly proliferating erythrocyte precursors.

In response to the proteotoxic stress from accumulation of free r-proteins, cells activated a transcriptional response driven by Heat Shock Factor 1 (Hsf1) to upregulate expression of chaperones and the proteasome. Activation of Hsf1 in response to proteotoxic stress, including the namesake heat shock, is an essential and evolutionarily conserved response. Yet, despite a sophisticated understanding of the mechanism of Hsf1 activation and how its target factors resolve toxic misfolded/aggregated protein species, there were previously no known specific endogenous proteins that underlie activation of Hsf1. The identification of newly-synthesized r-proteins serving as a highly specific signal for Hsf1 begged the question of the signal in other conditions, including heat shock, oxidative stress, and proteasome inhibition. In Chapter 3, I provide evidence that ongoing protein synthesis is tightly linked to Hsf1 activation in yeast across a broad spectrum of conditions. I argue that, as with perturbations to r-protein assembly, this may be a more general phenomenon wherein conditions such as heat shock most specifically interfere with the fate of newly-synthesized proteins, rather than unfold mature ones.

I conclude this thesis in Chapter 4 with an outlook on key open questions and future directions. Relating to ribosome biogenesis, I suggest routes forward to expand these findings to human cells and test the hypothesis that proteotoxicity may underlie the tissue-specific pathologies found in patients with mutations in ribosome biogenesis factors. Relating to the link between translation and Hsf1 activity, I suggest experiments to continue testing the notion of newly-synthesized proteins being those most vulnerable to misfolding and aggregation and thus serving as the principle signal for Hsf1’s proteostatic response.
Chapter 2  Proteotoxicity from aberrant ribosome biogenesis compromises cell fitness

Adapted from Tye et al., eLife 8:e43002 (2019).
2.1 Abstract

To achieve maximal growth, cells must manage a massive economy of ribosomal proteins (r-proteins) and RNAs (rRNAs) to produce thousands of ribosomes every minute. Although ribosomes are essential in all cells, natural disruptions to ribosome biogenesis lead to heterogeneous phenotypes. Here, we model these perturbations in *Saccharomyces cerevisiae* and show that challenges to ribosome biogenesis result in acute loss of proteostasis. Imbalances in the synthesis of r-proteins and rRNAs lead to the rapid aggregation of newly synthesized orphan r-proteins and compromise essential cellular processes, which cells alleviate by activating proteostasis genes. Exogenously bolstering the proteostasis network increases cellular fitness in the face of challenges to ribosome assembly, demonstrating the direct contribution of orphan r-proteins to cellular phenotypes. We propose that ribosome assembly is a key vulnerability of proteostasis maintenance in proliferating cells that may be compromised by diverse genetic, environmental, and xenobiotic perturbations that generate orphan r-proteins.

2.2 Introduction

Ribosomes are large macromolecular machines that carry out cellular protein synthesis. Cells dedicate up to half of all protein and RNA synthesis to the production of ribosomal protein (r-protein) and RNA (rRNA) components required to assemble thousands of new ribosomes every minute. rRNAs and r-proteins are coordinately synthesized and matured in the nucleolus and cytosol, respectively, in response to growth cues. R-proteins are co- and post-translationally folded, requiring general chaperones as well as dedicated chaperones called escortins. Thus, ribosome assembly requires the coordinated synthesis and assembly of macromolecules across cellular compartments, and must be performed at extremely high rates.
The balanced synthesis of rRNA and r-protein components in proliferating cells is frequently disrupted by genetic and extracellular insults, leading to a wide range of phenotypes. Environmental stressors, such as heat shock and viral infection, and xenobiotics, such as DNA-damaging agents used as chemotherapeutics, interfere with rRNA processing and nucleolar morphology\textsuperscript{16,56,57,79}. In zebrafish, and possibly in humans, hemizygous loss of r-protein genes can drive cancer formation\textsuperscript{80,81}. Diverse loss-of-function mutations in genes encoding r-proteins, r-protein assembly factors, and rRNA synthesis machinery result in tissue-specific pathologies in humans (ribosomopathies), such as red blood cell differentiation defects in patients with Diamond–Blackfan anemia (DBA)\textsuperscript{26,82,83}. Not all of the phenotypes caused by defects in ribosome biogenesis are wholly deleterious: in budding yeast, loss of r-protein genes increases stress resistance and replicative lifespan and reduces cell size and growth\textsuperscript{35,84,85}, and mutations in r-protein genes in \textit{C. elegans} also extend lifespan. Collectively, then, despite the fact that ribosomes are required in all cells, disruptions in ribosome biogenesis lead to an array of phenotypic consequences that depend strongly on the cellular context.

Phenotypes resulting from perturbations to ribosome assembly have both translation-dependent and -independent origins. As expected, when ribosomes are less abundant, biomass accumulation slows and growth rates decreases. Furthermore, reduced ribosome concentrations alter global translation efficiencies, impacting the proteome in cell state–specific ways\textsuperscript{26,65}. In many cases, however, cellular growth is affected before ribosome pools have appreciably diminished, indicating that perturbations of ribosome assembly have translation-independent or extraribosomal effects. The origins of these effects are not well understood, but may involve unassembled r-proteins. In many ribosomopathies, excess r-proteins directly interact with and activate p53, presumably as a consequence of imbalanced r-protein stoichiometry. However, p53 activation is not sufficient to explain the extraribosomal phenotypes observed in ribosomopathies or in model organisms experiencing disrupted ribosome biogenesis\textsuperscript{73}. Interestingly, r-proteins produced in excess of one-another are normally
surveyed by a ubiquitin-proteasome-dependent degradation\textsuperscript{54}, which appears to prevent their aberrant aggregation\textsuperscript{86,87}.

To determine how cells respond and adapt to perturbations in ribosome assembly, we took advantage of fast-acting chemical-genetic tools in \textit{Saccharomyces cerevisiae} to rapidly and specifically disrupt various stages of ribosome assembly. These approaches capture the kinetics of cellular responses, avoid secondary effects, and are far more specific than available fast-acting chemicals that disrupt ribosome assembly, such as transcription inhibitors, topoisomerase inhibitors, and nucleotide analogs. Furthermore, by performing this analysis in yeast, which lacks p53, we obtained insight into the fundamental, p53-independent consequences of perturbations of ribosome biogenesis.

We found that in the wake of perturbed ribosome assembly, cells experience a rapid collapse of protein folding homeostasis that independently impacts cell growth. This proteotoxicity is due to accumulation of excess newly-synthesized r-proteins, which are found in insoluble aggregates. Under these conditions, cells launch an adaptive proteostasis response, consisting of Heat Shock Factor 1 (Hsf1)-dependent upregulation of chaperone and degradation machinery, which is required for adapting to r-protein assembly stress. Bolstering the proteostasis network by exogenously activating the Hsf1 regulon increases cellular fitness when ribosome assembly is perturbed. The high degree of conservation of Hsf1, proteostasis networks, and ribosome assembly indicates that the many conditions that disrupt ribosome assembly and orphan r-proteins in other systems may also drive proteostasis collapse, representing a key extraribosomal vulnerability in cells with high rates of ribosome production.
2.3 Results

2.3.1 Imbalanced rRNA:r-protein synthesis elicits upregulation of proteostasis machinery via Heat Shock Factor 1 (Hsf1)

Ribosome biogenesis commences in the nucleolus, where rRNA is synthesized and processed, and many r-proteins are assembled concomitantly (Figure 2.1A). As a first class of disruption to ribosome biogenesis, we examined the consequences of imbalances in rRNA and r-protein production. Specifically, we focused on nuclease factors involved in several different stages of processing rRNAs for the large (60S) ribosomal subunit: endonuclease Las1, 5’-exonucleases Rat1 and Rrp17, and 3’-exonuclease Rrp44/Dis3 (exosome)\textsuperscript{14,32,88}. We tagged the target molecules with an auxin-inducible degron (AID), which allows rapid depletion of a tagged protein upon addition of the small molecule auxin\textsuperscript{5}, thereby acutely shutting down production of mature rRNA (Figure 2.1B). The rRNA processing factors were depleted by 75–90% within 10–20 min of auxin addition, and precursor rRNA (pre-rRNA) accumulated by 20 min, confirming that depletion of these factors rapidly interfered with rRNA processing (Figure 2.1C,D). Depletion also led to a detectable reduction in the level of free 60S subunits, indicating that the cell was failing to assemble new 60S, but had no effect on the mature ribosome pool (Figure A1.1A).

To determine whether cells respond directly to disrupted rRNA production, we explored the immediate transcriptional response following depletion of these factors. For this purpose, we auxin-treated (or mock-treated) each strain for 20 min, and then performed gene expression profiling by RNA-seq. WT cells exhibited no alteration of the transcriptome in the presence of auxin, whereas each AID-tagged strain exhibited the same compact response. Remarkably, the induced genes are known targets of Heat Shock Factor 1 (Hsf1), a conserved master transcription factor that controls protein folding and degradation capacity in stress, aging, and disease\textsuperscript{89} (Figure 2.1E). Hsf1 directly controls \textasciitilde50 genes encoding proteostasis factors,
Figure 2.1  Imbalanced rRNA:r-protein synthesis elicits upregulation of proteostasis machinery via Heat Shock Factor 1 (Hsf1)

(A) Brief schematic overview of ribosome biogenesis. (B) Auxin-inducible degradation (AID) of rRNA processing factors. The C-terminus of the protein is genetically tagged with the AID tag (IAA7-V5) in cells co-expressing the E3 ligase adapter OsTIR1. Addition of auxin allows recognition and degradation of AID-tagged proteins by the proteasome. (C) Depletion of AID-tagged rRNA processing factors following addition of auxin (100 µM) detected by anti-V5 immunoblot. (D) Pre-rRNA accumulation following rRNA processing factor depletions. RNA from mock and auxin (20 min) treated cells was analyzed by Northern blot with a probe (800, see Supplementary Table 3) that recognizes full-length pre-rRNA (35S) and processing intermediates (27S-A2 and 23S)\textsuperscript{16}. (E) Upregulation of Hsf1 targets in rRNA processing factor-depleted cells. RNA-seq density plots of log\textsubscript{2} fold
change after 20 min auxin treatment (versus mock-treated control), determined from n=2 biological replicates. Hsf1 targets, n=42; Msn2/4 targets, n=207; all others, n=4,912. The oxidative agent diamide (15 min, 1.5 mM) was used as a comparative control. The WT strain treated with auxin also expressed OsTIR1 but lacked any AID-tagged factor. (F) Schematic illustrating that rapamycin and CHX treatment acutely shutdown r-protein synthesis ahead of rRNA synthesis leading to an imbalance in ribosome components. (G) Northern blots of pre-rRNA and Hsf1-dependent BTN2 from WT cells treated with rapamycin (200 ng/ml) or cycloheximide (CHX, 200 µg/ml) for the indicated times. Heat shock (HS, 37°C, 15 min) and azetidine-2-carboxylic acid (AZC, 10 mM, 30 min) were used as positive controls for Hsf1 activation.

including protein folding chaperones (SSA1/4 (Hsp70), HSP82 (Hsp90), co-chaperones), aggregate clearance factors (BTN2, HSP42, HSP104), the transcription factor that regulates proteasome abundance (RPN4), and ubiquitin (UBI4)90,91. Upregulation of Hsf1-dependent genes coincided with an increase in Hsf1 occupancy at their promoters (Figure A1.1B) and was independent of the translational stalling pathway (Rqc2, Figure A1.1C). Hsf1-target transcripts, measured by Northern blot, were maintained at high levels over an 80-min time-course of auxin treatment (Figure A1.1D). AID-tagged Rrp17 acted as a partial loss-of-function allele, as indicated by the accumulation of pre-rRNA even in the absence of auxin and reduced cell growth (Figure 2.1D and data not shown), potentially explaining the mild and more transient upregulation of Hsf1 target transcripts following auxin addition in the strain expressing this protein. Nevertheless, depletion of all four rRNA processing factors each led to strong and specific activation of the Hsf1 regulon.

Importantly, we ruled out the possibility that the depletion strategy itself resulted in Hsf1 activation. Depletion of several factors not involved in rRNA processing via AID did not activate Hsf1, including the RNA surveillance exonuclease Xrn1, mRNA decapping enzyme Dxo1, and transcription termination factor Rtt103 (Figure A1.2A,B). Additionally, nuclear depletion of an rRNA processing factor using an orthogonal method that does not require proteasome-mediated
degradation (“anchor-away”)\textsuperscript{4} likewise led to Hsf1 activation, whereas anchor-away depletion of another nuclear protein did not (Figure A1.2C-F).

Stress conditions and xenobiotics in yeast characteristically activate a “general” environmental stress response (ESR), driven by the transcription factors Msn2/4, which rewire metabolism and fortifies cells against further stress\textsuperscript{52}. Strikingly, Msn2/4-dependent ESR genes were not activated after depletion of rRNA processing factors (Figure 2.1E). By contrast, treatment of WT cells with the oxidative agent diamide for 15 min potently activated both Hsf1- and Msn2/4-dependent genes, as expected (Figure 2.1E). Highly specific activation of Hsf1 in the absence of ESR has only been observed in circumstances in which cellular proteostasis is acutely strained: treatment with azetidine-2-carboxylic acid (AZC), a proline analog that interferes with nascent protein folding, resulting in aggregation\textsuperscript{92}, or overexpression of an aggregation-prone mutant protein\textsuperscript{93}. Comparison of the kinetics of pre-rRNA and Hsf1-dependent transcript accumulation revealed that cells activate Hsf1 within minutes after rRNA processing is disrupted, indicating a rapid strain on proteostasis, as observed in instantaneous heat shock (Figure A1.1E).

The results of acute disruption of rRNA processing suggest that Hsf1 is activated by an excess of newly-synthesized r-proteins relative to rRNAs. To determine whether the reverse phenomenon (i.e., a surplus of rRNAs relative to new r-proteins) could also activate Hsf1, we treated cells with rapamycin to inhibit r-protein expression by inactivating TORC1 (Figure 2.1F). During the first 15–30 min of low-dose rapamycin treatment, cells strongly repress synthesis of r-proteins while maintaining normal levels of rRNA transcription\textsuperscript{44}. Precursor rRNA accumulated due to r-protein limitation, as expected, but the Hsf1-dependent gene BTN2 was not upregulated during rapamycin treatment (Figure 2.1G). Similarly, halting translation, and thus r-protein synthesis, with cycloheximide (CHX) resulted in pre-rRNA accumulation but no upregulation of BTN2. On the basis of these findings, we conclude that when r-proteins are in
excess relative to what can be assembled into ribosomes, yielding orphan r-proteins, cells activate a proteostatic stress response driven by Hsf1.

2.3.2 Orphan r-proteins are sufficient to activate the Hsf1 regulon

As an orthogonal means of testing the model that orphan r-proteins activate the Hsf1 regulon, we directly inhibited assembly of r-proteins. To this end, we treated cells with a small molecule, diazaborine (DZA), that blocks cytoplasmic assembly of several r-proteins into the 60S subunit by specifically inhibiting the ATPase Drg1 (Figure 2.2A). Screens for DZA resistance have yielded only mutations in factors involved in drug efflux and the gene encoding the drug’s mechanistic target, DRG1, indicating that the compound is highly specific. Over a time-course of moderate, sublethal DZA treatment, the Hsf1-dependent transcripts BTN2 and HSP82 strongly accumulated by 15 min, whereas the Msn2/4-dependent transcript HSP12 exhibited no response (Figure 2.2B). Moreover, Hsf1-dependent transcripts returned to basal levels at 90 min, indicating that Hsf1 activation was an adaptive response. Importantly, a DZA-resistant point mutant of Drg1 (V725E) restored cell growth and reduced accumulation of Hsf1-dependent transcripts, confirming that DZA contributes to Hsf1 activation via the expected mechanism (Figure A1.3). Consistent with a functional role of Hsf1 activation, we found that DZA treatment protected cells from subsequent lethal heat stress (thermotolerance) (Figure A1.4). In cells treated with DZA for 15 or 45 min, RNA-seq revealed activation of the same response that was induced by depletion of rRNA processing factors: upregulation of Hsf1-dependent proteostasis genes in the absence of Msn2/4-dependent general stress genes (Figure 2.2C). Furthermore, by 45 min, cells upregulated proteasome subunits ~2-fold, consistent with the early Hsf1-dependent upregulation of the proteasome-regulatory transcription factor RPN4 (Figure 2.2D). Consistent with the exceptional specificity of this perturbation in eliciting an Hsf1-dependent response, we found that the canonical unfolded
protein response (UPR), which responds to misfolded proteins in the endoplasmic reticulum, was not activated by either DZA or depletion of rRNA processing factors (Figure A1.5).

![Figure 2.2](image)

**Figure 2.2** Orphan r-proteins are sufficient to activate the Hsf1 regulon

(A) Schematic describing that diazaborine (DZA) inhibits Drg1, preventing r-protein assembly into pre-60S subunits. (B) Kinetics of Hsf1 activation following DZA treatment. Northern blot of Hsf1-dependent BTN2 and HSP82 and Msn2/4-dependent HSP12 transcripts from cells treated with DZA (15 µg/ml) for the indicated time. Diamide (1.5 mM) was used as a positive control for Hsf1 and Msn2/4 activation. (C) Upregulation of Hsf1 targets in DZA-treated cells. RNA-seq density plots of log2 fold change after 15 or 45 min DZA treatment (versus DMSO-treated control), determined from n=2 biological replicates. (D) Upregulation of proteasome subunits during RPAS. Swarm plot of log2 fold change after 15 or 45 min DZA or 15 min diamide treatment for transcripts encoding proteasome subunits (n=27). (E) Schematic describing how escortins Tsr2, Yar1, and Sqt1 chaperone newly-synthesized Rps26, Rps3, and Rpl10, respectively, to assembling ribosomes. (F) Western blots showing depletion of AID-tagged Tsr2, Yar1, and Sqt1 and
Northern blots for Hsf1-dependent BTN2 and HSP82 and Msn2/4-dependent HSP12 transcripts at the indicated time after auxin addition. Unt, untreated; HS, heat shock.

As another means to inhibit r-protein assembly, we depleted dedicated r-protein chaperones, called escortins\(^{21,22}\). Each escortin binds a specific newly-synthesized r-protein and brings it to the assembling ribosome, preventing aberrant aggregation (Figure 2.2E). We generated AID-tagged strains for the Rps26 escortin Tsr2, whose mutation in human cells leads to DBA\(^{26}\). We also analyzed two other escortins, Sqt1 (Rpl10) and Yar1 (Rps3), and performed a time-course of auxin treatment for all three. Each escortin was depleted ~70% by 20 min. Northern blots revealed accumulation of BTN2 and HSP82 mRNAs by 10–20 min, with no change in the level of Msn2/4-regulated HSP12 mRNA (Figure 2.2F). Both Rps26 and Rps3 are assembled into the pre-40S in the nucleus, whereas Rpl10 is the last r-protein assembled into the ribosome in the cytoplasm. Thus, either by inhibition of Drg1 or depletion of escortins, orphan r-proteins are sufficient to activate the Hsf1 regulon. Accordingly, we refer to the stress imparted by orphan r-proteins as ribosomal protein assembly stress (RPAS).

### 2.3.3 Compromised r-protein gene expression and translational output during RPAS

In addition to the upregulation of the Hsf1 regulon in RPAS, we also observed downregulation of some genes. Intriguingly, the set of downregulated genes comprised mostly r-protein genes (Figure 2.3A,B). Under many stress conditions, both r-protein genes and assembly factor genes, collectively termed the ribosome biogenesis (RiBi) regulon, are repressed through Tor-dependent signaling\(^{35,36,96}\) (e.g., oxidative stress by diamide, Figure 2.3A,B). Therefore, we suspected that the specific downregulation of r-protein genes, but not assembly factors, in RPAS would not be executed through Tor. Indeed, cells treated with DZA for 15 or 45 min exhibited no change in the level of the TORC1 activity reporter, phosphorylated (phos-) Rps6\(^{97}\) (Figure 2.3F).
Figure 2.3 Compromised r-protein gene expression and translational output during RPAS

(A) Swarm plot of log$_2$ fold change of r-protein encoding transcripts in the condition indicated on the x-axis (n=136). (B) Swarm plot of log$_2$ fold change of transcripts encoding ribosome biogenesis (RiBi) factors, excluding r-protein genes, in the condition indicated on the x-axis (n=169). (C) Schematic of transgene system for estradiol-inducible expression of V5-tagged ORFs. (D) Western blot showing time-course of induction of Rps3-V5 after the indicated time of beta-estradiol (100 nM) addition. (E) Strains containing the indicated V5-tagged transgene were induced for 10 min with
estradiol and then treated with vehicle (-) or 15 µg/ml DZA (+) for 20 min and analyzed by western blot (upper) and quantified relative to vehicle control (lower). Bar height indicates the average and error bars the standard deviation of n=3 biological replicates. The dashed line corresponds to the hypothetical maximal reduction amount (to 80% of control) in protein produced as a result of ribosome dilution alone in 20 min (one fourth of a cell cycle). (F) WT cells were treated with vehicle (DMSO) or DZA for 15 or 45 min and analyzed by western blot. Rapamycin (rap, 200 ng/ml, 45 min) was used as a positive control for altering Gcn2 and TORC1 activity.

Many stress conditions lead to global translational repression, mediated in part by the kinase Gcn2, and enable specialized or cap-independent translation programs that aid in coping with the stress. Previous experiments with DZA showed that translation is downregulated shortly after treatment. To determine whether translation is repressed in RPAS, we monitored the synthesis of various V5-tagged ORFs. Transcription of V5-tagged transgenes was activated by the synthetic transcription factor Gal4–estradiol receptor (ER)–Msn2 activation domain (AD) (GEM) upon the addition of estradiol. Under normal conditions, we found that the V5-tagged proteins began to accumulate after 10 minutes. To determine the effect of RPAS on translational output, we briefly treated ORF-V5 strains with estradiol followed by DZA for 20 minutes and assessed the level of protein accumulation. All ORFs, including GFP-V5, accumulated to lower levels when cells were treated with DZA, consistent with a rapid reduction in translational output under RPAS. Because DZA could achieve a maximal reduction of 20% in the ribosome pool in a 20-minute experiment, this >50% reduction in synthesis cannot be explained by a diminishing ribosome pool. Interestingly, the reduction in translational capacity is not mediated through the kinase Gcn2 as in other stresses such as carbon or nitrogen starvation and oxidative stress, as phosphorylated (phos-) eIF2α did not accumulate during DZA treatment. In sum, we observed compromised r-protein gene transcription and global translational output during RPAS independent of canonical signaling pathways.
2.3.4 Aggregation of orphan r-proteins during RPAS

Hsf1 responds to an increased prevalence of misfolded or aggregated proteins, and activates a transcriptional program to resolve these issues. Several r-proteins are found to aggregate in the absence of general cotranslational folding machinery, post-translational escortins, or nuclear import machinery\(^{17,18,21}\). Further, excess r-proteins are targeted for degradation by Excess Ribosomal Protein Quality Control (ERISQ), a ubiquitin-proteasome mediated pathway, in the absence of which r-proteins likewise prevalently aggregate\(^{86,87}\). We therefore hypothesized that following disruptions to ribosome assembly, newly synthesized orphan r-proteins would aggregate. Supporting this idea, we found that Hsf1 activation by DZA required ongoing translation: pre-treatment with CHX prevented upregulation of Hsf1 targets, supporting the model of proteotoxic orphan r-proteins (Figure 2.4A). Similarly, Hsf1 activation by depletion of the rRNA processing factor Rat1 was fully inhibited by CHX pre-treatment (Figure A1.6A).

To test for the presence of protein aggregation in DZA-treated cells, we used a sedimentation assay that separates soluble proteins from large, insoluble assemblies (Figure 2.4B)\(^{104}\). As a positive control, we induced global protein misfolding by AZC and observed gross protein aggregates associated with disaggregases Hsp70 and Hsp104 (Figure 2.4C). By contrast, RPAS induced by DZA treatment resulted in no such gross protein aggregation, even at 40 minutes.

We next asked whether newly-synthesized r-proteins aggregated during RPAS. Using the estradiol induction system for V5-tagged ORFs, we followed the fate of newly-synthesized r-proteins in mock- or DZA-treated cells. We found that newly-synthesized Rps26, Rpl10, and Rpl3 shifted 3–5-fold to the insoluble fraction upon DZA treatment (Figure 2.4D,F). Interestingly, the levels of Rpl4 and Rps3 in the pellet increased modestly if at all, possibly due to their distinct
Figure 2.4  Aggregation of orphan r-proteins during RPAS

(A) Cells were mock or CHX (200 µg/ml) treated for 3 min prior to addition of DZA for 20 min and Hsf1 target were detected by Northern blot. HSE-Venus, Venus transgene downstream of four Hsf1 binding sites (Heat Shock Element, HSE). (B) Schematic of the protein aggregation assay. Proteins extracted from cryogenically lysed cells were fractionated by centrifugation at 20,000 g for 20 min to pellet insoluble proteins. (C) Cells were treated with DZA for 0, 20, or 40 min. Input and insoluble proteins (pellet) were resolved by SDS-PAGE. AZC (10 mM, 40 min) was used as a control to compare DZA
results to a general increase in aggregates in the pellet, by Ponceau staining, and Hsp70 and Hsp104 sedimentation. 10X more of the pellet sample than input sample was loaded to increase sensitivity. (D) Strains expressing the indicated V5-tagged r-protein (or GFP as a control) were induced for 10 min with estradiol followed by vehicle (DMSO) or DZA treatment for 20 min. Input and pellet samples for all were analyzed by Western blot. 10X more of the pellet sample than input sample was loaded to increase sensitivity. (E) Same as (D), except cells were continuously induced for 5 h with estradiol to label the mature protein pool prior to DMSO or DZA treatment. (F) Quantification of the indicated V5-tagged proteins in the pellet fraction versus the input (from panels D and E), normalized to the pellet to input ratio of Rpb1. The ratio was set to 1 for DMSO treated cells. Bar height indicates the average and error bars the range of n=2 biological replicates. (G) Box plot depicting results of quantitative mass spectrometry on proteins that pellet following 20 min mock (DMSO) or DZA treatment. Fold change (DZA/mock) of each protein was calculated for input and pellet fractions and r-proteins (pink) were compared to all other proteins (grey). ***, p-value < .0001 (Wilcoxon rank-sum test). (H) List of r-proteins that assemble in the nucleus and cytosol and ribosome biogenesis factors with greatest increase in abundance in the pellet fraction (>1.5-fold in two biological replicates) detected in DZA-treated cells by mass spectrometry (data as in G). See Supplementary Table A1.6 for full dataset.

biochemical characteristics, protection from aggregation by chaperones, or rapid assembly into precursor ribosome subunits. Treating extracts with the nuclease benzonase did not solubilize aggregated r-proteins, indicating that they were not in RNA- or DNA-dependent assemblies (Figure A1.6B). To compare these results with the behavior of mature, assembled r-proteins, we grew V5-tagged Rpl10 and Rpl3 strains continuously for 5 hours in estradiol prior to DZA treatment. Under these conditions, most of the tagged r-proteins should reside in mature ribosomes, with a small fraction existing unassembled. After DZA treatment, only a modest amount of tagged r-proteins were present in the pellet, likely due to the small unassembled fraction (Figure 2.4E,F). We performed quantitative mass spectrometry to test the generality of r-protein aggregation during RPAS, and found that a broad complement of r-proteins accumulate in aggregates following DZA treatment (Figure 2.4G). Despite observing 3-5-fold increases of newly-synthesized Rps26, Rpl3, and Rpl10 in the aggregate fraction following DZA
treatment (Figure 2.4F), none of these proteins were in the highest ranking aggregating proteins in the mass spectrometry data (Figure 2.4H). As the mass spectrometry data are not specifically assaying newly-synthesized proteins, the fold increase in aggregation is likely an underestimate, which would explain the discrepancy. Nevertheless, we observed a clear and general shift of r-proteins to the aggregate fraction following DZA treatment, beyond those that are directly downstream of Drg1 (the target of DZA) function in the cytosol (Figure 2.4G,H). Together, we conclude that RPAS results in specific aggregation of orphan r-proteins.

2.3.5 RPAS disrupts nuclear and cytosolic proteostasis

In addition to finding r-proteins, particularly those that are in the large 60S subunit, amongst the strongest aggregators in DZA, we found a prominent group of nucleolar ribosome biogenesis factors (Figure 2.4H, Figure A1.7). This group contained 17 proteins, including 66S (pre-60S) associated factors such as Nop53, Nsa2, Mak16, and Cic1. Intriguingly, a number of factors involved in rRNA processing were found to be strong aggregators in DZA, including four of the components of the nuclear exosome: Lrp1, Rrp41, Rrp43, and the catalytic Rrp6. These data suggest that, in addition to causing aggregation of r-proteins downstream of Drg1 function in the cytosol, DZA treatment leads to aggregation of r-proteins assembled in the nucleus and collateral aggregation of nucleolar ribosome biogenesis factors (Figure 2.4F–H).

Misfolded and aggregated proteins in the cell are often toxic and have the potential to sequester proteins with essential cellular activities\textsuperscript{105–107}. Accordingly, in addition to upregulating proteostasis factors, cells utilize spatial quality control mechanisms to minimize the deleterious effects of aggregates. For example, cells triage proteins into cytosolic aggregate depots, referred to as Q-bodies or CytoQ, where the Hsp40/70 chaperones and Hsp104 disaggregate collaborate to resolve and refold misfolded proteins\textsuperscript{108,109}. Aggregates also form in the nucleus,
in the intranuclear quality control compartment (INQ), which is thought to be involved in their degradation\textsuperscript{109–111}.

We used confocal fluorescence microscopy to follow the localization of the Hsp70 co-chaperone Sis1, which recognizes substrates and participates in nuclear aggregation and degradation\textsuperscript{112–114}. In normal growing populations, Sis1-YFP was distributed evenly throughout the nucleus except in the nucleoli; the nucleolar protein Cfi1-mKate, which localized at the periphery of the nucleus, exhibited little or no colocalization with Sis1. Upon treatment with DZA, Sis1 drastically relocalized within the nucleus, moving to the nuclear periphery, where it formed
Figure 2.5  RPAS disrupts nuclear and cytosolic proteostasis

(A) Fluorescence micrographs of cells expressing Sis1-YFP and the nucleolar marker Cfi1-mKate after treatment with DZA (5 µg/ml, 30 min) with or without pre-treatment with CHX (200 µg/ml, 5 min). (B) Quantification of Sis1 relocalization to the nuclear periphery was done via fluorescence line scans and computed as the ratio of Sis1 signal at the periphery (p) versus the center (c) of the nucleus (n>30 cells per condition). (C) Image segments (50 pixels) centered on the middle of the nucleus were extracted in both the Sis1-YFP and Cfi1-mKate channels for individual cells (n = 25 cells for both conditions). Images were stacked and average intensity was projected. The Cfi1 ring under control conditions results from the composite of images: in most cells it appears localized to one side, but always at the periphery of the nucleus. Fluorescent line scans quantify the localization patterns. (D) Micrographs of cells expressing Hsp104-mKate were imaged live in untreated conditions or after DZA treatment (5 µg/ml, 30 min). Below micrographs, quantification of number of Hsp104 foci and Sis1 peripheral localization (n>30 cells/condition).

A ring-like structure (Figure 2.5A–C). At the same time, Cfi1 relocalized from the periphery towards the middle of the nucleus, adjacent to the Sis1 ring structure. The effect of DZA on Sis1 and Cfi1 was completely blocked by inhibiting translation with CHX, consistent with the idea that newly synthesized orphan r-proteins drove the response. The subnuclear relocalization of Sis1 in response to RPAS is consistent with a role in the INQ, though the ring-like structure is distinct from the single subnuclear puncta observed following heat shock. In addition, we analyzed the localization of the disaggregase Hsp104, which colocalizes with aggregates and resolves them, including in a variety of proteotoxic stresses. Untreated cells contained one or two Hsp104 foci. Treatment with DZA increased the number of cytosolic Hsp104 foci, to seven or eight per cell, likely reflecting CytoQ body formation in response to orphan r-proteins (Figure 2.5D). Based on these data, we conclude that the orphan r-proteins produced as a result of DZA treatment disrupt proteostasis in the cytosol and the nucleus.
2.3.6 Hsf1 and Rpn4 support cell fitness under RPAS

To determine the physiological relevance of Hsf1 activation in response to RPAS, we tested the fitness of *hsf1* mutants and deletions of single Hsf1-dependent genes in DZA. Because *HSF1* is an essential gene, we studied a hyperphosphorylated mutant of Hsf1, *hsf1* po4*, in which all serines are replaced with phospho-mimetic aspartates; this strain grows normally in basal conditions but is a hypoinducer of Hsf1 target genes under heat shock and has a tight temperature-sensitive growth defect\(^{118}\). We found that *hsf1* po4* cells grew at wild-type rates at 30°C but were very sick under proteotoxic conditions (AZC or 37°C), demonstrating that the *hsf1* po4* allele lacks the ability to cope with proteotoxic stress (Figure 2.6A). *hsf1* po4* were nearly incapable of growth in DZA (Figure 2.6B), highlighting the critical role of wild-type Hsf1 in the adaptation to RPAS.

To identify which Hsf1 targets are critical for RPAS adaptation, we investigated the fitness consequence of loss of single Hsf1-dependent genes. In this analysis, we focused on genes whose loss in basal conditions is minimally perturbing but are likely to have important functions in coping with proteotoxic stress. In particular, we deleted factors involved in aggregate formation and dissolution (*HSP104, BTN2, HSP42, HSP26*) and proteasome-mediated degradation (*RPN4, TMC1, PRE9*); in addition, we deleted the Hsf1-independent gene *HSP12* as a negative control. Because many of these single-gene deletions do not have gross phenotypes, we used a competitive fitness assay to sensitively detect small differences in cell fitness\(^{119,120}\). Individual deletion strains expressing mCherry (mCh) were co-cultured with a wild-type reference strain expressing YFP without treatment (YPD), at 37°C, in 5 mM AZC, DMSO (vehicle), or in 15 or 30 µg/ml DZA. Competitions were maintained over the course of 5 days, and the relative proportion of wild-type and mutant cells was monitored by flow cytometry (Figure 2.6C). Deletion of most factors had no effect on fitness under any condition tested, likely due to redundancy in the mechanisms responsible for restoring proteostasis (Figure
A1.8). However, loss of the transcription factor *RPN4*, which controls the basal and stress-induced levels of the proteasome \(^{96,121}\), conferred a substantial growth defect in the presence of DZA (~25-fold more severe than in the absence of drug on day 3), at 37°C, and in the presence of AZC (Figure 2.6D), suggesting that the proteasome plays a critical role in the response to RPAS. We also found that loss of the only non-essential proteasome subunit, *PRE9*, made cells DZA-resistant (Figure A1.8). Resistance to some proteotoxic stressors has been observed in weak proteasome mutants, such as *pre9*, and may

![Figure 2.6](image)

**Figure 2.6**  Hsf1 and Rpn4 support cell fitness under RPAS
(A) Growth defects of *hsf1 po4* cells. Left panels, wild-type (*HSF1*) and mutant (*hsf1 po4*, all serine to aspartate) cells were serially diluted 1:10 onto YPD plates and incubated at 30 or 37°C for 2 days. Right panel, cells were grown for 24 h in the presence of the indicated concentration of AZC and relative growth (compared to untreated) was determined by OD$_{600}$. Line represents the average and error bars the range of n=2 biological replicates. (B) Cells were grown for 24 h in the presence of the indicated concentration of DZA and relative growth (compared to untreated) was determined by OD$_{600}$. Line represents the average and error bars the range of n=2 biological replicates. (C) Schematic of competitive fitness assay. Wild-type (WT) cells expressing YFP and query cells expressing mCherry (mCh) were co-cultured in each condition over 5 days. Abundance of YFP+ and mCh+ cells was determined daily by flow cytometry. (D) The log$_{10}$ ratio of mCh+ (query) to YFP+ (WT reference) of wild-type (*RPN4*) and *rpn4Δ* cells after 3 days of co-culture in YPD, YPD at 37°C, 5 mM AZC, vehicle (DMSO) and DZA (15 µg/ml). Box plot of n=8 biological replicates with outliers shown as diamonds. (E) Growth of cells expressing a synthetic Hsf1 construct severed from negative regulation by chaperones (*Hsf1$^{DBD}$-VP16) was expressed under an estradiol-responsive promoter. Pre-conditioning was performed with estradiol (2 nM) for 3 h prior to addition of DMSO, DZA (8 µg/ml), or AZC (2.5 mM) for an additional 21 h. Growth was determined as OD$_{600}$ normalized to DMSO control. Bar height depicts the average and error bars the standard deviation of n=3 biological replicates. Values below indicate the average % increase in growth by estradiol pre-conditioning versus mock. *, all p<0.01 (Student’s t-test). (F) Results of experiments performed identically as described in A, but with an isogenic strain containing *HSF1* under its WT promoter instead of the *Hsf1$^{DBD}$-VP16* under an estradiol-responsive promoter. n.s., not significant, all p>0.1 (Student’s t-test).

be the result of compensation by alternate proteasome subunits or elevated basal levels of other proteostasis factors in this mutant$^{122-125}$. As with DZA, *rpn4* and *pre9* cells are sensitive and resistant, respectively, to endoplasmic reticulum (ER) folding stress, which involves clearance of misfolded ER proteins by the proteasome$^{126,127}$. In sum, these data demonstrate that Hsf1 and its target Rpn4, which controls proteasome abundance, support cellular fitness under RPAS.
2.3.7 Proteostatic strain contributes to the growth defect of cells under RPAS

We hypothesized that the proteotoxic stress created by orphan r-proteins contributes to the growth defect of cells under RPAS beyond what would be expected from the effects of a reduced ribosome pool. Because Hsf1 responds to and is required for growth under RPAS, we uncoupled Hsf1 from the proteostasis network and placed it under exogenous control to test whether enhanced proteostasis would modulate the DZA-induced growth defect. For this purpose, we placed a chimeric fusion of the Hsf1 DNA-binding domain with the transactivation domain VP16 (Hsf1\textsuperscript{DBD}-VP16) under the control of an estradiol-responsive promoter in a strain lacking wild-type \textit{HSF1}, allowing exogenous upregulation of the Hsf1 regulon by addition of estradiol. The Hsf1\textsuperscript{DBD}-VP16 strain was more sensitive to DZA than the wild-type strain, further supporting the importance of wild-type \textit{HSF1} in the RPAS response (Figure 2.6E,F). To determine whether upregulation of the Hsf1 regulon alleviates the DZA growth defect, we preconditioned cells with a 3-hour estradiol treatment, and then measured cell growth after 21 hours of exposure to DZA, AZC, or DMSO (vehicle). Pretreatment with estradiol yielded a >40% growth enhancement in DZA that was independent of changes to cell size (Figure A1.9). Similar effects were observed after growth in AZC, which induces global proteotoxicity, whereas only a 9% growth rate increase was observed for vehicle-treated cells (Figure 2.6E,F). These data suggest that the proteotoxic stress of RPAS slows growth, which can be rescued by exogenous amplification of the proteostasis network.

2.3.8 Cells producing fewer ribosomes show reduced proteostatic strain in RPAS

Our data demonstrate that rapidly proliferating yeast cells experience an acute loss of proteostasis when ribosome assembly is disrupted. We asked whether cells producing fewer ribosomes would experience an attenuated proteotoxic stress during RPAS. To this end, we
analyzed wild-type yeast grown in rich medium containing the optimal carbon source glucose or the suboptimal (respiratory) carbon source glycerol\textsuperscript{128}. Under these conditions, cells doubled every 1.6 and 3.7 hours, respectively. When challenged with DZA, cells grown in glycerol demonstrated a lower level of Hsf1 target gene activation (Figure 2.7A).

### Figure 2.7  Cells producing fewer ribosomes show reduced proteostatic strain in RPAS

(A) Wild-type cells were grown to mid-log in rich medium with either 2% glucose or glycerol and treated with DMSO (vehicle, -) or DZA (+) for 15 min. Shown are Northern blots for Hsf1 target genes \textit{HSP82} and \textit{BTN2}. (B) Wild-type and \textit{sch9Δ} cells were both grown to mid-log in rich medium with 2% glucose and treated with DMSO (vehicle, -) or DZA (+) for 15 min. Shown are Northern blots for Hsf1 target genes \textit{HSP82} and \textit{BTN2}. 
Model of how disruptions to ribosome biogenesis leads to RPAS and the impacts on cellular physiology. During proliferation, cells rapidly produce ribosomes through coordinated synthesis of r-proteins (purple circles) in the cytoplasm and rRNAs in the nucleolus. Perturbations that result in orphan r-proteins result in proteotoxic stress following r-protein aggregation (left panel). In the cytoplasm, aggregates are visible via Hsp104 foci and translation is downregulated. In the nucleus, Hsp40 Sis1 (orange), and possibly Hsp70, are targeted to aggregates and the nucleolus moves from the nuclear periphery, to adjacent to Sis1-marked “rings”. Concomitantly, pre-rRNA accumulates, r-protein genes are transcriptionally repressed, and Hsf1 is liberated from Hsp70 sequestration to activate target genes encoding protein folding and degradation machinery. Proteostasis collapse stalls growth independently from reduced pools of ribosomes (right panel).

To analyze the impact of reduced ribosome biogenesis without changing the carbon source, we analyzed cells lacking the gene SCH9, whose product controls ribosome production at the transcriptional level, in glucose-containing medium. As with wild-type cells in glycerol, sch9Δ cells showed lower levels of Hsf1 target gene activation by DZA \(\text{(Figure 2.7B)}\). Importantly, we observed that DZA treatment altered the processing of rRNA under all conditions \(\text{(Figure A1.10)}\), validating that ribosome assembly was being disrupted. Thus, the proteotoxic strain was stronger in cells with higher rates of ribosome production, indicating that proliferating cells are at a stronger risk of experiencing RPAS.

2.4 Discussion

Here, we report an extraribosomal consequence of aberrant ribosome assembly: collapse of proteostasis resolved by an Hsf1-dependent response. We propose a model wherein excess orphan r-proteins that arise from aberrations in ribosome biogenesis drive proteotoxicity and impact cellular fitness under r-protein assembly stress \(\text{(Figure 2.7C)}\). In turn, the master proteostasis transcription factor Hsf1 is activated to increase the abundance of folding and degradation machineries, likely following sequestration of chaperones such as
Hsp40 and Hsp70 by r-protein aggregates (Zheng et al., 2016). The proteostatic response supports cell fitness and is capable of protecting cells from r-protein assembly stress. Thus, proliferating cells accept a tradeoff between the risk of proteotoxicity and the growth benefits of high ribosome production. The resulting balancing act is vulnerable to disruption by a variety of genetic and chemical insults, necessitating protective mechanisms capable of restoring the balance. Interestingly, several r-proteins are produced in excess, for instance in human tissue culture cells, and are rapidly targeted for degradation by the ubiquitin-proteasome system. We therefore propose that in the perturbations modeled in this work, cells are challenged with a larger proportion of orphan r-proteins that overwhelms the canonical clearance mechanisms, necessitating an increase in proteostasis capacity, consistent with the importance of both Hsf1 and Rpn4 in RPAS (Figure 2.6B,D).

It is possible that, rather than aggregated r-proteins, pre-40S/60S precursors accumulated in the nucleolus elicit RPAS. Though we cannot definitively test this alternative model, we find it unlikely for several reasons. First, many lines of evidence point towards Hsf1 activation requiring accumulation of misfolded/aggregated proteins that titrate chaperones away from binding and inactivating Hsf1, making it difficult to envision a model wherein precursors per se drive Hsf1 activation independent of r-protein aggregation. Second, the RPAS response is also activated by depletion of rRNA processing factors, which remove the platform (rRNA) for precursor assembly altogether. Third, in the case of DZA treatment, we found many additional r-proteins that aggregate beyond those that are downstream of Drg1 function, including many that assemble at the earliest stages of precursor formation in the nucleolus. Thus, we favor a model wherein aberrations in ribosome biogenesis that affect both rRNA production and r-protein assembly lead to RPAS due to aggregation of orphan r-proteins in the nucleus and cytosol.

Given the conservation of proteostasis mechanisms and ribosome biogenesis, we suspect that disrupted ribosome assembly might also cause proteotoxic stress in other
eukaryotes. Certainly, many conditions have the potential to orphan r-proteins, thereby straining proteostasis. For example, DNA-damaging chemotherapeutic agents like etoposide, camptothecin, and 5-fluorouracil and transcription inhibitors like actinomycin D disrupt the nucleolus and rRNA processing. Indeed, several Hsf1 targets are seen upregulated by and may be important in responding to DNA damaging agents. Environmental stressors such as heat shock also deform the nucleolus, and many other stressors in yeast cause accumulation of pre-rRNA. Imbalanced production of r-proteins arises in mutations found in ribosomopathies, as well as in aging and cancer. Because ribosome biogenesis is not a constitutive process, but instead fluctuates in response to nutrient availability, stress, cell growth, and differentiation cues, these conditions are likely to acutely challenge ribosome biogenesis and lead to periodic disruptions to proteostasis. The severity of the resulting phenotype may relate to cell growth rate and the required level of ribosome production in a cell type/state (Figure 2.7A,B), which suggests a possible mechanism for why certain cell types are especially vulnerable to disrupted ribosome biogenesis, such as in ribosomopathies.

Proteotoxic stress has been extensively linked to overall disruption of cellular homeostasis. While the molecular basis for how protein aggregates compromise cell health is not fully understood, one demonstrated possibility is that aggregates sequester other proteins with essential functions. Thus, the proteotoxic stress elicited by RPAS has the potential to severely disrupt cellular homeostasis, consistent with our findings that alleviating proteotoxic stress enhances cell growth under RPAS (Figure 2.6E). Differences among cell types in the ability to withstand proteotoxic conditions might contribute to the phenotypic variability in response to ribosome assembly defects.

The gene expression response mounted by cells experiencing RPAS provides clues regarding how the cell deals with toxic orphan r-proteins. The requirement for an Hsf1-mediated response suggests that upregulation of the folding and/or degradation machinery contributes to this resolution. The extreme sensitivity of rpn4 cells to RPAS suggests an
important role for proteasome-mediated degradation of orphan r-proteins. Consistent with this, yeast and human cells degrade r-proteins produced in excess, and cells lacking this quality control mechanism contain aggregated r-proteins\textsuperscript{54,86,87}. Indeed, the proteotoxicity of excess r-proteins may explain why cells evolved mechanisms to prevent their accumulation above stoichiometric levels, even in aneuploid cells\textsuperscript{55}.

Activation of the Hsf1 regulon in RPAS is the consequence of newly-synthesized r-proteins that cannot reach their normal destination and therefore fail to assemble into a cognate complex, leading to their aggregation. Similarly, the mitochondrial unfolded protein response is activated when assembly of mitochondrial complexes is disrupted\textsuperscript{138}. Blocking import of organellar proteins into the ER or mitochondria results in cytosolic proteotoxic stress\textsuperscript{123,139–141}. Thus, aberrant accumulation of orphan proteins – that is, those that do not arrive at their appropriate complex or subcellular location – is a hallmark of proteostasis loss, which is resolved by pathways tailored for each cellular compartment. Given that the nucleolus is morphologically disrupted and recruits chaperones such as Hsp70 under stress, including heat shock and proteasome inhibition\textsuperscript{57,79,129}, it is tempting to speculate that RPAS is responsible, at least in part, for Hsf1 activation in response to various stress stimuli. Consistently, new r-proteins undergo ubiquitination, localize in protein aggregates, and associate with chaperones under heat shock\textsuperscript{142–144}. R-proteins, due to their exceptionally high abundance, complex assembly pathway, and aggregation-prone nature, represent a particularly vulnerable group of proteins.

Particular cell types and cell states, such as tumor cells or differentiating erythropoietic precursors, have exceptional demand for high ribosome production\textsuperscript{65,145}. Intriguingly, both of these cell states are unusually sensitive to disruption of proteostasis. Erythroid differentiation is highly reliant on Hsp70 availability, as evidenced by the fact that Hsp70 sequestration can result in the anemic phenotype of beta-thalassemia\textsuperscript{75}. Similarly, cancer cells are sensitized to small molecules that dampen the proteostasis network\textsuperscript{146,147}. In this work, we showed that
exogenous activation of the Hsf1 regulon protects yeast from RPAS. Future studies should seek to determine whether an analogous strategy can therapeutically mitigate phenotypes of disrupted ribosome biogenesis in disease processes.

2.5 Methods

Yeast strain construction and growth

Strains were constructed by standard transformation techniques. Gene tagging and deletion was carried out using PCR products or integrating plasmids, and transformants were verified by colony PCR and western blotting where relevant. The Hsf1 activity reporters contain four Hsf1 binding sites (heat shock element, HSE) upstream of a reporter gene. The HSE-GFP and HSE-mVenus reporters were integrated at URA3 and LEU2, respectively, and were used interchangeably depending on experimental requirements. OsTIR1 driven by the GPD1 promoter was integrated at LEU2. The AID tag was added to a TIR1-containing strain by transformation with the V5-IAA7::KANMX6 cassette. Further transformation of AID strains often resulted in loss of OsTIR1 activity, reflected by failure to deplete the tagged protein in auxin; accordingly, such transformations were not performed. The DRG1 and DRG1V725E strains were constructed in a diploid by deletion of one DRG1 allele followed by transformation with the WT or mutant allele on a URA3-marked CEN/ARS plasmid (see “Cloning”). Clones containing only the plasmid-borne copy were isolated by sporulation and tetrad dissection. Estradiol-inducible expression strains were generated with a plasmid containing the V5-tagged ORF downstream of the GAL1 promoter that integrates at HIS3 in a background expressing the Gal4-ER-Msn2AD transcription factor (Stewart-Ornstein et al., 2012). All strains and plasmids are listed in Supplementary Tables A1.1 and A1.2, respectively.

All experiments were performed at 30°C with cultures were grown in standard YPD (1% yeast extract, 2% peptone, 2% dextrose, pH 5.5) medium unless indicated otherwise. Where
indicated, SCD (0.2% synthetic complete amino acids [Sunrise], 0.5% ammonium sulfate, 0.17% yeast nitrogen base, 2% dextrose, pH 5.5) medium was used. Heat shock was performed by adding an equal volume of 44°C media to the culture and immediately shifting to a 37°C incubator.

**Drug treatments**

Treatments were generally carried out in log-phase cultures at OD ~0.4–0.6, depending on the length of treatment, such that cultures remained in log growth during the course of the experiment. For drugs dissolved in DMSO, vehicle-only controls contained the same final volume of DMSO. Auxin (indole-3-acetic acid, Sigma-Aldrich) was prepared fresh daily at 100 mM in ethanol and added at a final concentration of 100 µM. Diazaborine (DZA, Calbiochem) was prepared at 15 mg/ml in DMSO (stored at -20°C, protected from light) and used at the indicated concentration. Cycloheximide (Sigma-Aldrich) was purchased as a 100 mg/ml DMSO stock and added at a final concentration of 100 µg/ml (for sucrose gradients) or 200 µg/ml (for stress experiments). AZC (L-azetidine-2-carboxylic acid, Sigma-Aldrich) was prepared at 1 M in water and used at the indicated concentration. Diamide (Sigma-Aldrich) was prepared at 1 M in water and added at a final concentration of 1.5 mM. Rapamycin (LC Laboratories) was prepared fresh daily in ethanol and used at a final concentration of 200 ng/ml (to inhibit r-protein synthesis) or 1 µg/ml (for anchor-away, in a rapamycin-resistant *tor1-1* background). Beta-estradiol (Sigma-Aldrich) was prepared as a 1000X stock for each experiment in ethanol and added to the indicated final concentration.

**Cloning**

*DRG1*, including promoter and terminator regions, was PCR amplified from genomic DNA with tails containing *Bam*HI and *Not*I sites and cloned into pBluescript KS. The *DRG1*<sup>V725E</sup> mutant was constructed by Q5 site-directed mutagenesis. WT and mutant were subcloned
using the same restriction sites into pRS316 (URA3 CEN/ARS) and verified by sequencing of
the full insert. V5-tagged ORFs were ordered as gBlocks (IDT) with a C-terminal 6xGly-V5 tag
and XhoI and NotI sites and cloned into pNH603 under the GAL1 promoter. RP ORFs had the
sequence of the genomic locus and GFP encoded enhanced monomeric GFP (F64L, S65T,
A206K).

**Total protein extraction and western blotting**

Each western blot was performed with a minimum of two biological replicates unless
otherwise stated and a representative blot is shown. Protein extraction was adapted from the
alkaline lysis method\textsuperscript{149}. One milliliter of a mid-log culture was harvested in a microfuge,
aspirated to remove supernatant, and snap-frozen on liquid nitrogen. Pellets were
resuspended at RT in 50 µl 100 mM NaOH. After 3 min, 50 µl 2X SDS buffer (4% SDS, 200
mM DTT, 100 mM Tris pH 7.0, 20% glycerol) was added, and the cells were lysed on a heat
block for 3 min at 95°C. Cell debris was cleared by centrifugation at 20,000 g for 5 min.

Extracts were resolved on NuPAGE Bis-Tris gels (Invitrogen), transferred to
nitrocellulose on a Trans-Blot Turbo (Bio-Rad), and blocked in 5% milk/TBST (0.1% Tween-
20). AID-tagged and V5-tagged proteins were detected with mouse anti-V5 (Invitrogen, R960-
25, 1:2,000). Pgk1 was detected using mouse anti-Pgk1 (Abcam, ab113687, 1:10,000). Rpb1
was detected with rabbit anti-Rpb1 (y-80, Santa Cruz Biotechnology, sc-25758, 1:1,000).
Hsp104 was detected with rabbit anti-Hsp104 (Enzo Life Sciences, ADI-SPA-1040, 1:1,000).
Hsp70 was detected with mouse anti-Hsp70 (3A3, Abcam, ab5439, 1:1,000). Rps6
phosphorylated at Ser235/236 was detected with rabbit anti-phos-Rps6 (D57.2.2E, Cell
Signaling Technology, 1:2,000). eIF2α phosphorylated at Ser51 was detected with rabbit anti-
phos-eIF2α (Invitrogen, 44-728G, 1:1,000). Pgk1 and Rpb1 were used as loading controls.
Cy3-labeled secondary antibodies were used, and immunoreactive bands were imaged on a
Typhoon.
Proteomics

Samples were prepared essentially as previously described\textsuperscript{150,151}. Soluble (input) and pelleting proteins were extracted exactly as in section “Protein aggregation assay.” About 200 µg of protein were cleaned with a chloroform/methanol precipitation\textsuperscript{152}. Proteins were resuspended in 6 M GuHCl, diluted to 2 M GuHCl with 10 mM EPPS at pH = 8.5, and digested with 10 ng/µL LysC (Wako) at 37 °C overnight. Samples were further diluted to 0.5 M GuHCl and digested with an additional 10 ng/µL LysC and 20 ng/µL sequencing grade Trypsin (Promega) at 37 °C for 16 h. TMT tagging, and peptide desalting by stage-tipping was performed as previously described\textsuperscript{150,151}. LC-MS. LC-MS experiments were performed on a Thermo Fusion Lumos equipped with an EASY-nLC 1200 System HPLC and autosampler (Thermo). During each individual run, peptides were separated on a 100–360 µm inner-outer diameter microcapillary column, which was manually packed in house first with ∼0.5 cm of magic C4 resin (5 µm, 200 Å, Michrom Bioresources) followed by ∼30 cm of 1.7 µm diameter, 130 Å pore size, Bridged Ethylene Hybrid C18 particles (Waters). The column was kept at 60 °C with an in house fabricated column heater\textsuperscript{153}. Separation was achieved by applying a 6–30% gradient of acetonitrile in 0.125% formic acid and 2% DMSO at a flow rate of ∼350 nL/min over 90 min for reverse phase fractionated samples. A voltage of 2.6 kV was applied through a PEEK microtee at the inlet of the column to achieve electrospray ionization. The data were acquired with a MultiNotch MS3 method essentially as previously described\textsuperscript{154}. Five SPS precursors from the MS2 were used for the MS3 using MS1 isolation window sizes of 0.5 for the MS2 spectrum and isolation windows of 1.2, 1.0, and 0.8 m/z for 2+, 3+ and 4-6+ peptides respectively. An orbitrap resolution of 50k was used in the MS3 with an AGC target 1.5e5 and a maximum injection time of 100 ms. Proteomics data were analyzed essentially as previously
described\textsuperscript{155}. Protein-level data are presented in Supplementary Table A1.6. Raw signal-to-noise measurements for each TMT channel (corresponding to one sample) were normalized but dividing each protein by the sum of all signal in that channel and multiplying by 10e6, resulting in parts per million (ppm). Gene ontology (GO) term enrichment was performed using the Saccharomyces Genome Database GO term finder tool on the 51 proteins whose input-normalized fold change in the pellet of DZA-treated cells was >1.5X in both replicates (see Supplementary Table A1.6). The list of all proteins quantified in the dataset was used as the background set.

**Total RNA extraction and Northern blotting**

Each Northern blot was performed with a minimum of two biological replicates unless otherwise stated and a representative blot is shown. Two milliliters of a mid-log culture were harvested in a microfuge, aspirated to remove supernatant, and snap-frozen on liquid nitrogen. RNA was extracted by the hot acid-phenol method and ethanol precipitated. RNA purity and concentration were determined on a NanoDrop.

Typically 5 µl (5 µg) of RNA was mixed with 16 µl sample buffer (10 µl formamide, 3.25 µl formaldehyde, 1 µl 2X MOPS, 1 µl 6X DNA loading dye, 0.75 µl 200 µg/ml ethidium bromide) and denatured for 10 min at 65°C. After chilling briefly on ice, samples were loaded onto a 100 ml 1.2% agarose/1X MOPS gel and electrophoresed for 90 min at 100V in 1X MOPS in a Thermo EasyCast box. Some gels contained 6% formaldehyde and ran for 5 h, but a 90 min run without formaldehyde gave sharper, more even bands. We also found that low EEO agarose gave the best results. RNA integrity and equal loading were examined by imaging ethidium bromide to visualize rRNA bands. RNA was fragmented in the gel for 20 min in 3 M NaCl/10 mM NaOH before downward capillary transfer on a TurboBlotter apparatus using the manufacturer’s blotting kit. Transfer ran for 90 min in 3 M NaCl/10 mM NaOH, and then the membrane was UV crosslinked. Pre-5.8S rRNA was resolved by running 1 µg RNA (in
1X TBE-urea loading buffer) on a 6% TBE-urea gel in 0.5X TBE. RNA was electroblotted to a membrane and UV-crosslinked.

RNA was detected with either small DNA oligonucleotides or large (100–500 bp) double-stranded DNA (see Supplementary Table A1.3). For oligo probes, the membrane was pre-hybridized at 42°C in ULTRAhyb-Oligo buffer (Thermo Fisher Scientific). The oligo was 5’ end–labeled in a reaction containing 25 pmol oligo, 10 U T4 PNK, 2 µl gamma-32P-ATP (PerkinElmer), and 1X PNK buffer. Probe was hybridized overnight and washed twice in 2X SSC/0.5% SDS at 42°C for 30 min before exposure on a phosphor screen and imaging on a Typhoon. For dsDNA probes, the membrane was pre-hybridized at 42°C in 7.5 ml deionized formamide, 3 ml 5 M NaCl, 3 ml 50% dextran sulfate, 1.5 ml 50X Denhardt’s, 750 µl 10 mg/ml salmon sperm DNA, 750 µl 1 M Tris 7.5, 75 µl 20% SDS. Probes were made in a reaction containing 50 ng of a PCR product as template, random hexamer primers, Klenow (exo-), and 5 µl alpha-32P-ATP (PerkinElmer). Denatured probes were hybridized overnight and washed twice in 2X SSC/0.5% SDS at 65°C for 30 min before exposure on a phosphor screen and imaging on a Typhoon scanner.

**Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR)**

ChIP was performed based off of standard approaches. Fifty milliliters of a mid-log culture were crosslinked in 1% formaldehyde for 30 min at RT and quenched in 125 mM glycine for 10 min. Cells were pelleted and washed twice in ice-cold PBS before snap-freezing on liquid nitrogen. Chromatin was extracted in LB140 (50 mM HEPES pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS and 1X protease inhibitor cocktail [cOmplete EDTA-free, Roche]) by glass bead beating. Chromatin was sonicated to 100–300 bp on a Bioruptor (Diagenode) and diluted 1:10 in WB140 (LB140 without SDS). Diluted chromatin (1.5 ml, corresponding to ~6 ml of the original cell culture volume) was incubated overnight at 4°C with 1 µl rabbit anti-Hsf1 serum (kind gift from Dr. David Gross,
Louisiana State University), or normal rabbit serum as a negative control. Twenty-five microliters of washed Protein A Dynabeads (Invitrogen) were added, and the sample was incubated for 4 h. One wash each was performed for 5 min in WB140 (140 mM NaCl), WB500 (500 mM NaCl), WBLiCl (250 mM LiCl), and TE. Samples were eluted from beads in TE/1% SDS and de-crosslinked overnight at 65°C, followed by RNase A and proteinase K treatment and cleanup on columns. Input and IP DNA were quantified using Brilliant III SYBR Green Master Mix (Agilent Technologies) in technical triplicate for each biological replicate sample. A dilution curve was generated for each input. Data are recorded for each IP as percent of input using Ct values. Primers are available in Supplementary Table A1.3.

**Protein aggregation assay**

Insoluble proteins were isolated using the protocol described in¹⁰⁴. Twenty-five milliliter cultures were grown to mid-log and treated as indicated, pelleted for 1 min at 3,000 g, and rinsed once in 1 ml ice-cold WB (20 mM HEPES pH 7.5, 120 mM KCl, 2 mM EDTA). The pellet was resuspended with 100 µl SPB and dripped into 2 ml safe-lock tubes filled with liquid nitrogen along with a 7 mm stainless steel ball (Retsch). Cells were cryogenically lysed on a Retsch Mixer Mill 400 by four cycles of 90 sec at 30 Hz and re-chilled on liquid nitrogen between each cycle. The grindate was thawed with 400 µl SPB (WB + 0.2 mM DTT + 1X protease inhibitors [cOmplete EDTA-free, Roche] + 1X phosphatase inhibitors [PhosSTOP, Sigma-Aldrich]) for 5 min on ice with repeated flicking and gentle inversion. Where indicated, 2 µl benzonase (Sigma-Aldrich) was included in SPB to degrade RNA and DNA for 10 min on ice. The lysate was clarified for 30 sec at 3,000 g to remove cell debris. Twenty microliters of extract was reserved as input. The remaining extract was centrifuged for 20 min at 20,000 g to pellet insoluble proteins. The supernatant was decanted and the pellet rinsed with 400 µl ice-cold SPB with brief vortexing and centrifuged again for 20 min. The pellet was resuspended in 200 µl IPB (8 M urea, 2% SDS, 20 mM HEPES pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM
DTT, 1X protease inhibitors) at RT. The input was diluted with 160 µl water and 20 µl 100% TCA and precipitated for 10 min on ice, centrifuged for 5 min at 20,000 g and washed with 500 µl ice-cold acetone. Inputs were resuspended in 100 µl IPB. Input and pellet fractions were centrifuged for 5 min at 20,000 g, RT. Ten microliters of input (0.5%) and pellet (5%, 10X) were used for western blotting as above.

**Sucrose gradient sedimentation**

Fifty-milliliter cultures were grown to mid-log and treated as indicated, followed by addition of CHX to 100 µg/ml and incubation for 2 min. All following steps were performed on ice or at 4°C. Cells were pelleted for 2 min at 3,000 g, washed once in 10 ml buffer (20 mM Tris pH 7.0, 10 mM MgCl₂, 50 mM KCl, 100 µg/ml CHX), and once in 1 ml buffer. Cells were pelleted in a microfuge and snap-frozen on liquid nitrogen. Cells were lysed by addition of 400 µl glass beads and 400 µl lysis buffer (20 mM Tris pH 7.0, 10 mM MgCl₂, 50 mM KCl, 100 µg/ml CHX, 1 mM DTT, 50 U/ml SUPERaseIn [Thermo Fisher], 1X protease inhibitors) followed by bead beating for six cycles (1 min on, 2 min off) on ice. Lysate was clarified 10 min at 20,000 g. A continuous 12 ml 10–50% sucrose gradient was prepared in 20 mM Tris pH 7.0, 10 mM MgCl₂, 50 mM KCl, 100 µg/ml CHX on a BioComp Gradient Station, and 200 µl (~20 A₂₆₀ units) lysate was layered onto the top and spun for 3 h at 40,000 rpm in a SW41 rotor. Absorbance profiles and fractions were collected on a BioComp Gradient Station.

**Competitive fitness and growth assays**

Fitness experiments were performed as described¹²⁰. Query strains (WT and deletions) expressing *TDH3p-mCherry* were co-cultured with a reference strain expressing *TDH3p-YFP*. All strains were inoculated from single colonies into liquid YPD and grown to saturation. Query and reference strains were mixed 1:1 (v:v) at a total dilution of 1/100 and grown for 6 hours to an OD₆₀₀ of 0.2–0.5. Co-cultured cells were diluted 1/10 to a final OD₆₀₀ of 0.02–0.05 in YPD
alone or YPD with: 0.1% (v/v) DMSO (vehicle), 15 µg/mL DZA, 30 µg/mL DZA, or 5 mM AZC and grown at 30°C. Samples were also diluted in YPD and grown at 37°C. Samples were cocultured for 5 days and diluted 1/100 into fresh media every 24 h. At each time point, an aliquot of each sample was transferred to TE and quantified by flow cytometry on a Stratedigm S1000EX cytometer. Manual segmentation was used to identify the query and reference strain populations. Data are available in Supplementary Table A1.5.

To determine relative growth of HSF1 and hsf1 po4* (Figure 2.6A,B) and DRG1 and DRG1 V725E (Figure A1.3), overnight cultures were diluted to OD_{600} ~0.05 in the indicated condition, grown for 24 h, and OD_{600} measured. “Relative growth” is the OD_{600} for each condition relative to the vehicle control of that strain.

For estradiol pre-conditioning (Figure 2.6E,F and Figure A1.9), overnight cultures grown in SCD were back diluted 1:100 in fresh SCD to ensure mock and estradiol cultures were at the same starting dilution. The culture was immediately split into two flasks (20 ml each), and one was treated with 20 µl 2 µM estradiol (final concentration 2 nM). Mock and estradiol-treated cultures were grown for 3 h and then treated with DMSO (vehicle), 8 µg/ml DZA, or 2.5 mM AZC, grown for an additional 21 h, and OD_{600} measured. “Relative growth” is the OD_{600} for each condition relative to the mock (no estradiol), DMSO only control. Cultures were also assessed for relative cell size distribution by measuring side scatter on a Stratedigm S1000EX cytometer.

Serial dilution plating assay (Figure 2.6A) was performed by diluting overnight cultures to OD_{600} ~1.0 in fresh media and serially diluting 1:10 on a 96-well plate. The cultures were stamped onto plates using a “frogger” device and grown as indicated.

Thermotolerance (Figure A1.4) was performed by diluting overnight cultures to OD ~0.05 and growing for 5.5 h. The culture was split and treated with the indicated concentrations of DZA for 45 min. One milliliter was removed and immediately placed on ice as a pre-heat shock control. One milliliter was placed at 50°C for 15 min on a heat block with thorough mixing
every 5 min and then placed on ice. Cells were serially diluted 1:10,000 (for pre-heat shock cultures) or 1:100 (for post-heat shock cultures) and 200 µl were spread onto YPD plates. Plates were incubated at 30°C for 2 days and colonies were counted. Reported are the number of colonies formed on each post-heat shock plate, which corresponds to approximately 100,000 cells that were exposed to heat shock as determined from the pre-heat shock plates.

**Fluorescence microscopy**

Preparing anchor-away strains expressing FRB-GFP–tagged proteins for microscopy was performed as described⁴. Briefly, 1 ml of cells was harvested, fixed in 1 ml -20°C methanol for 6 min, and resuspended in TBS/0.1% Tween with DAPI. Fixed, DAPI-stained cells were spotted onto a 2% agarose pad on a glass slide and topped with a cover slip. Samples were imaged for both GFP and DAPI on a Nikon Ti2 microscope with a 100x objective and an ORCA-R2 cooled CCD camera (Hamamatsu).

Confocal microscopy of Sis1-YFP, Cfi1-mKate, and Hsp104-mKate was performed live by allowing low density cultures grown in SCD at room temperature to settle in 96-well glass bottom plates coated with concanavalin A. For treatments, medium was removed and fresh SCD containing the indicated drug was added to the well. Imaging was performed on a Nikon Ti microscope with a 100x 1.49 NA objective, a spinning disk confocal setup (Andor Revolution) and an EMCCD camera (Andor).

**RNA-seq**

RNA was depleted of ribosomal RNA using Yeast Ribo-Zero Gold (Illumina). For all auxin-related experiments, libraries were prepared from biological duplicates (individual strain isolates grown and treated on separate days) using the TruSeq Stranded Kit (Illumina). The diamide RNA-seq data are of libraries were prepared using another RNA-seq library construction protocol, as previously described¹⁵⁶ and were not done in replicate as the RNA-
seq data recapitulated the well-characterized transcriptional response to diamide\(^5\). All libraries were sequenced on an Illumina NextSeq platform.

**RNA-seq data analysis**

Raw fastq files were processed as follows. The adapter sequence (AGATCGGAAGAG) was removed using Cutadapt (v1.8.3) with option “-m 18” to retain reads >18 nt. Reads were then quality-filtered using PRINSEQ and alignment was performed with TopHat (v2.1.0). The resulting BAM files from each lane on the flow cell were merged, sorted, and indexed with SAMtools. The number of reads for each genomic feature (e.g. transcript), was quantified using HTSeq count. The GTF file was ENSEMBL release 91 for *Saccharomyces cerevisiae*

Quantification and differential expression for auxin experiments were carried out using DESeq2\(^1\) with drug treatment as the variable: two biological replicates each of mock-treated and auxin-treated. RNA abundance changes were reported using the log\(_2\) fold change calculated by DESeq2 for auxin/untreated for each transcript. For +/- diamide datasets, RNA abundance was determined using RPKM and reported as log\(_2\) fold change (diamide vs. untreated) for each transcript. Quantified RNA-seq data can be found in Supplementary Table A1.4.

Transcript classes were defined as follows. “Hsf1 targets”: identified using an approach that defines transcripts that fail to be activated when Hsf1 is depleted prior to acute heat shock\(^9\). “Msn2/4 targets”: classification from\(^9\). “All others”: all other genes characterized as “Verified ORFs” by SGD, excluding those in “Hsf1 targets” and “Msn2/4 targets” classes. “Proteasome subunits”: the 27 genes encoding the 27 subunits of the 26S proteasome. “R-protein genes”: the 136 genes encoding the 79 subunits of the ribosome (ribosomal proteins). “Other ribosome biogenesis (RiBi) genes”: 169 unique genes from the SGD GO term “ribosome biogenesis” with r-protein genes removed. “Hac1-dependent UPR genes”: core set of UPR
genes induced by Hac1 overexpression, tunicamycin treatment, and DTT treatment\textsuperscript{158}. Gene lists can be found in Supplementary Table A1.4.

**Data availability**

All RNA-seq data generated in this work are available on Gene Expression Omnibus under the accession number GSE114077.

### 2.6 Contributions and acknowledgements

L. Stirling Churchman and I conceived of and designed the study. I drafted figures and LSC and I wrote and edited the manuscript, with input from other authors. I performed all experiments and analyses with the following exceptions. Lillia V. Ryazanova performed the mass spectrometry-based proteomics experiment with input and supervision from Martin Wühr. Nicoletta Commins performed the competitive fitness experiment and gating of the resulting raw flow cytometry data with input and supervision from Michael Springer. David Pincus provided key feedback on experimental design, as well as yeast strains, especially *HSF1* mutant strains as attributed in the original publication, and performed microscopy on Sis1, Hsp104, and Cfi1 reporter strains.

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GM117333 to LSC, R01-GM120122 to MS, R35-GM128813 to MW), the DOE (DE-SC0018420 to MW), and the NSF (Graduate Research Fellowship to BWT).
Chapter 3    Ongoing protein synthesis underlies Hsf1 activation by proteotoxic stress
3.1 Abstract

The integrity of the proteome relies on the cell’s ability to resolve exogenous perturbations that disrupt protein folding homeostasis (proteostasis). As such, eukaryotic cells have a dedicated transcriptional regulatory factor, Heat Shock Factor 1 (Hsf1), which is activated in response to conditions that strain proteostasis, such as heat shock and oxidative agents. Activation of Hsf1 leads to transcriptional induction of protein folding chaperones and degradation machinery that can refold or degrade misfolded and aggregated proteins that arise in these conditions, thus restoring proteostasis. Despite the sophistication of this homeostatic mechanism, the precise cellular proteins that misfold or aggregate as a consequence of heat shock and other stress and thus necessitate Hsf1 activation has not been elucidated. Previous work in mammalian cells has shown that Hsf1 activation by stress was blocked by inhibiting translation with cycloheximide. Thus, there is a possible link between ongoing protein synthesis and proteostatic stress. Here, we systematically explore this link in budding yeast. We find that Hsf1 activation is blocked by inhibiting translation with cycloheximide or rapamycin across diverse stresses, and that this appears to be independent of a labile intermediate protein that must be translated. Cells that are naturally growing slower and translating at reduced levels show attenuated Hsf1 activation in proteotoxic stress relative to rapidly growing cells, recapitulating this link in a physiological setting. We propose that ongoing protein synthesis renders cells vulnerable to proteotoxic stress, thus underlying Hsf1 activation.

3.2 Introduction

Misfolding and aggregation deprive cells of active proteins, but also can be toxic, perhaps as the result of causing co-aggregation of other essential cellular proteins\textsuperscript{105–107}. As such, cells invest heavily in protein folding and degradation machinery. However, upon a change in condition, such as heat shock or oxidative stress, that results in an excess of aberrant
proteins beyond what the cell’s basal factors can resolve, the master eukaryotic transcription factor Heat Shock Factor 1 (Hsf1) is activated. Subsequently, Hsf1 drives transcriptional activation of target genes consisting of more chaperone and degradation machinery. The increase in chaperones such as Hsp70 (yeast Ssa1-4) and Hsp90 (Hsc/p82), co-chaperones such as Hsp40s (Sis1, Ydj1), the disaggregate Hsp104, and the downstream accumulation of proteasomes, allows cells to deal with the increase in aberrant proteins and restore proteostasis. As Hsf1 is itself normally held inactive by Hsp70, once chaperones again reach excess relative to other protein clients, Hsf1 is turned back off.

Despite the many details we understand about this sophisticated homeostatic mechanism, it remains unclear what necessitates Hsf1 activation in these stress conditions. Importantly, multiple data points indicate that Hsf1 is a bona fide misfolded/aggregated protein sensor. First, overexpression of a mutant, aggregation-prone protein or treatment of cells with the strained proline analog azetidine-2-carboxylic acid (AZC), which causes nascent chain aggregation when incorporated, are sufficient to activate a highly specific Hsf1-dependent response. Second, its activity is controlled by titration of chaperones Hsp70/90, which interact with exposed hydrophobic regions of proteins as would be expected for misfolded/aggregated proteins.

Early work on Hsf1 in mammalian tissue culture cells demonstrated that treatment with the translation elongation inhibitor cycloheximide (CHX) prior to heat shock, oxidative stress/protein disulfide altering compounds, or proteasome inhibition blocked induced DNA binding by Hsf1 that coincides with target gene activation. Additionally, it was found that heat shock and the protein disulfide alkylating agent, iodoacetamide, could be titrated to high enough levels to overcome the CHX-induced block on Hsf1 DNA binding. These results led to a number of hypotheses that could explain the putative link between translation and Hsf1 activation in stress. Chief among these were suggestions that newly-synthesized proteins are more labile than mature folded proteins and thus underlie Hsf1 activation, and/or that inhibiting
translation frees of chaperones that normally would be engaged in folding newly-synthesized proteins to deal with increased protein unfolding without requiring induction of new chaperones.

However, these ideas were ultimately not expanded upon. We therefore wanted to expand upon these findings to further explore the link between ongoing protein synthesis and Hsf1 activity. In particular, we sought to test the conservation of this link in a distinct species, budding yeast, across many conditions in parallel; utilize orthogonal means of translation repression to rule out the possibility that translation stalling by CHX might have anomalous effects on Hsf1 activity; to ensure that gene induction by other stress-responsive transcription factors was unaffected; and to find a physiological condition where the link between lower translation and lower Hsf1 activity was recreated.

### 3.3 Results

It was previously shown that treating mammalian tissue culture cells with the translation elongation inhibitor cycloheximide (CHX) prior to heat shock, oxidative stress, or proteasome inhibition blocked activation of Hsf1, primarily at the level of induced DNA binding\(^ {160,164-167} \). To assess whether this phenomenon extended to other organisms and stress conditions, we used the budding yeast *Saccharomyces cerevisiae*. Pretreatment with CHX for 3 min completely blocked activation of Hsf1 as seen by lack of accumulation of three target transcripts (Figure 3.1). This includes heat shock, oxidative stress by diamide, and proteasome inhibition by MG132, consistent with prior results in mammalian cells. In addition, CHX blocked Hsf1 activation by the denaturant ethanol\(^ {168} \) and the ribosome assembly inhibitor diazaborine\(^ {169} \). We considered it critical to rule out that CHX may non-specifically interfere with stress signaling; the transcript HSP12, activated by the general environmental stress responsive transcription factors Msn2/4, accumulated as normal even in the presence of CHX (Figure 3.1). These data suggest that translation inhibition by CHX blocks Hsf1 in yeast across a broad range of proteotoxic
conditions, and that this is not due to causing a general defect in transcriptional stress responses.

![Figure 3.1](image)

**Figure 3.1** Translation inhibition by cycloheximide (CHX) prevents Hsf1 activation across diverse proteotoxic stressors

Cells were grown to mid-log and treated with either 0.2% v/v vehicle (DMSO, -) or 200 µg/ml cycloheximide (CHX, +) for 3 min prior to treatment with the indicated condition. Shown are Northern blots of RNA from cells treated as indicated, probed for Hsf1-dependent (purple) and Msn2/4-dependent (green) transcripts. *HSE-mVenus*, Hsf1 reporter transgene of *mVenus* driven by four repeats of the Heat Shock Element (Hsf1 binding site). Treatments: AZC (10 mM, 30 min), diamide (1.5 mM, 30 min), diazaborine (15 µg/ml, 30 min), ethanol (5%, 20 min), MG132 (50 µM, 30 min), glucose starvation (shift to 0%, 30 min), heat shock (37°C, 20 min). Note that a *pdr5Δ* strain was used for MG132 to facilitate uptake.

As CHX inhibits translocation of actively engaged ribosomes and thus may have anomalous consequences, we sought to test an orthogonal means of downregulating translation. To this end, we used the TOR inhibitor rapamycin, which represses translation initiation.\textsuperscript{34,170} Wild-type cells were pretreated for 30 min with rapamycin prior to heat shock, which completely blocked accumulation of an Hsf1 activity reporter (Figure 3.2A).
Figure 3.2  Rapamycin pre-treatment prevents Hsf1 activation by heat shock independent of phosphorylation

(A) Wild-type cells grown to mid-log were either treated with mock or 200 ng/ml rapamycin for 30 min prior to instantaneous upshift to 37°C for 20 min. The abundance of the Hsf1 reporter transcript HSE-mVenus was assessed by Northern blot and normalized to SCR1. Values plotted are shown as fold change relative to no rapamycin, no heat shock control cells. (B) Same as in A, except using a strain that contained a non-phosphorylatable Hsf1 mutant, where all serine and threonine residues are mutated to alanine with the exception of S225, which is required for DNA-binding\textsuperscript{118}.

Previous work argued that TOR directly regulates Hsf1 activity via phosphorylation\textsuperscript{171,172}. Therefore, to rule out that the block by rapamycin pretreatment of Hsf1 activation was the consequence of altered Hsf1 phosphorylation, we repeated the experiment with a mutant Hsf1 that has all serine and threonine residues mutated to the non-phosphorylatable residue alanine, except for the DNA-binding domain S225. In response to heat shock, this mutant protein shows no signs of phosphorylation\textsuperscript{118}. We found that rapamycin likewise blocked Hsf1 activation in this mutant strain, arguing against this phenomenon being a consequence of altered phosphorylation when TOR is inhibited (Figure 3.2B). Together, these data demonstrate by an orthogonal means that Hsf1 activation is predicated on ongoing protein synthesis.

The link between ongoing protein synthesis and Hsf1 activation in stress may suggest that a labile molecule is required to be synthesized as part of the direct Hsf1 activation pathway. We considered it prudent to find a condition in which Hsf1 could still be activated in the absence of translation. A study in mammalian cells showed that heat and the sulfhydryl alkylating agent
iodoacetamide could be titrated to a level that would rescue Hsf1 activity in the presence of CHX\textsuperscript{160,167}. However, these studies used DNA binding of Hsf1 as the readout for rescuing activation, but given DNA binding and transcriptional activation can be uncoupled\textsuperscript{173,174}, we looked to ensure that such a condition would rescue transcriptional activation by Hsf1. We focused on titrating the protein denaturant ethanol, and found that, though Hsf1 activation by 5% ethanol was completely blocked by CHX, titrating to 7.5% ethanol led to full restoration of Hsf1 target transcript accumulation (Figure 3.3). This demonstrates that Hsf1 is competent for activation in the absence of translation, and argues against the requirement for a labile signaling molecule.

![Figure 3.3 Rescue of Hsf1 activation in the absence of translation by titrating ethanol](image)

Mid-log phase cells were treated with 0.2% v/v DMSO (-) or 200 ug/ml CHX (+) for 3 min. The cultures were then split and treated with the indicated final concentration of ethanol for 20 min. Shown are Northern blots for Hsf1-dependent transcripts (purple).

The experiments above use the activity of Hsf1 as a proxy for the extent of proteotoxic stress that cells experience. As an alternative measure, we tested for the presence of protein aggregates induced by heat shock with and without translation inhibition by CHX. In particular, we used the disaggregate protein Hsp104 as a marker for protein aggregate formation, as it has been used extensively for this purpose in the past due to its ability to non-specifically bind to aggregated proteins\textsuperscript{108,115–117}. Such an experiment has been previously reported, but in one case CHX was found to block Hsp104 foci formation by heat shock, while the other found no such effect\textsuperscript{117,175}. In using a concentration of CHX known to maximally inhibit translation\textsuperscript{176} for 3
min prior to heat shock, we found that the absence of translation led to a block in Hsp104 foci formation by heat shock (Figure 3.4). This suggests, consistent with the former study\textsuperscript{117}, an absence of protein aggregation when translation is not active, further supporting the model that proteotoxic stress is linked to ongoing protein synthesis.

![Figure 3.4](image)

**Figure 3.4** Diminished heat shock-induced protein aggregation in the absence of translation

Cells expressing a C-terminal mCherry fusion of Hsp104 were treated with 0.2% DMSO (mock) or 200 ug/ml CHX for 3 min and then either maintained at 30°C or instantaneously shifted to 37°C for 20 min.

We considered that slow-growing cells with lower protein synthesis levels would experience diminished proteotoxic stress than rapidly proliferating cells. To this end, we grew cells in glucose or glycerol containing medium, resulting in doubling times of 1.6 and 3.7 hours, respectively. Cells were subsequently exposed to a variety of proteotoxic conditions, and those that were grown in glycerol showed reduced accumulation of Hsf1 target transcripts, including in heat shock, proteasome inhibition, and oxidative stress (Figure 3.5A). Importantly, cells grown in glycerol did not have lower amounts of Hsf1 protein present, and the abundance of Hsf1 targets is not basally higher in glycerol (Figure 3.5B,C). In sum, these data provide a physiological context that recreates the link between ongoing protein synthesis and proteotoxic load in stress conditions.
Figure 3.5  Attenuated Hsf1 activation recreated in slow versus fast growing cells

(A) Cells were grown to mid-log in either 2% glucose (G, 1.6 h doubling time) or 2% glycerol (Y, 3.7 h doubling time) and exposed to the indicated stressors (same parameters as Figure 1.1) for 30 min. A pdr5Δ strain was used for MG132 treatment to facilitate uptake. Shown are Northern blots for Hsf1-dependent transcripts (purple). (B) Cells were grown to mid-log in 2% glucose or glycerol and the abundance of Hsf1-FLAG-V5 was assessed by western blot. Shown are three biological replicates for each condition. (C) Mass spectrometry-proteomics data for cells grown in glucose and glycerol, normalized to parts per million and shown in log_{10}. Hsf1 targets are shown in orange. Data are from Paulo et al.177

We envision two potential non-exclusionary models that could explain the link between ongoing protein synthesis and Hsf1 activation/protein aggregation in proteotoxic stress. First, since nascent and newly-synthesized proteins are key substrates of chaperones, the absence of translation may free up chaperones, obviating the need for Hsf1 to activate the synthesis of more chaperones. Second, newly-synthesized proteins are those most prone to misfold/aggregate and thus elicit Hsf1 activation upon stress. More specifically, the latter model posits that the principle signal that activates Hsf1 in a condition such as heat shock would be aggregating of newly-synthesized proteins, rather than unfolding and subsequent aggregation of mature proteins.
Figure 6. Interfering with organellar import or assembly of newly-synthesized proteins activates Hsf1

Density plot of gene expression data for the indicated perturbation for Hsf1 targets (n=42) and all other transcripts. "Protein import into endoplasmic reticulum": ribosome profiling data after 30 min of depleting the signal recognition particle protein Srp72 by auxin-inducible degradation\textsuperscript{178}. "Protein import into mitochondria": RNA-seq data after 4 h of overexpressing Psd1\textsuperscript{141}. "Ribosomal protein assembly": RNA-seq data after 15 min of Drg1 inhibition (diazaborine) treatment\textsuperscript{169}.

The hypothesis that newly-synthesized proteins elicit proteotoxic stress proposes that such stresses would interfere with the normal fate of new proteins, including folding, protein complex assembly, or subcellular localization. Thus, an expectation of this hypothesis would be that interfering with these processing steps of new proteins should result in Hsf1 activation. We previously showed that interfering with assembly of newly-synthesized ribosomal proteins into ribosome complexes results in aggregation of these orphan proteins and subsequent Hsf1 activation\textsuperscript{169}. Furthermore, analyzing existing data in which the import of newly-synthesized proteins into the endoplasmic reticulum\textsuperscript{178} or mitochondria\textsuperscript{141} was disrupted by depleting the signal recognition particle or overexpressing an import substrate revealed a similar signature of Hsf1 activation (Figure 3.6). These data provide three independent means of supporting the
notion that orphan newly-synthesized proteins are themselves competent to drive proteotoxic stress.

3.4 Discussion

The work presented here expands on previous observations linking ongoing protein synthesis with both the formation of protein aggregates and activation of Hsf1 by stressors. In particular, these results suggest a general phenomenon whereby Hsf1 activation by stress is a consequence of ongoing protein synthesis. This phenomenon has now been expanded to budding yeast and tested against a broad range of proteotoxic conditions. Importantly, a number of key alternative hypotheses were ruled out. First, the ability of Msn2/4 to be activated in CHX-treated cells by stress demonstrates that CHX is not merely acting non-specifically to repress stress-induced transcriptional programs. Second, rapamycin inhibition of Hsf1 activation by heat shock, independent of Hsf1 phosphorylation itself, argues against an anomalous consequence of inhibiting ribosome elongation. Third, rescue of Hsf1 activation by high concentration ethanol argues against the requirement for synthesis of a labile signaling molecule. Though it is possible that Hsf1 activation may proceed by a distinct mechanism at low and high concentrations of ethanol, the most parsimonious explanation is that higher ethanol concentration overcomes the threshold by unfolding a greater pool of proteins. Fourth, CHX-dependent inhibition of aggregate formation, marked by Hsp104, is consistent with previous results and suggests that translation inhibition overall prevents proteostasis collapse in heat shock. Finally, the diminished activation of Hsf1 in cells that are growing slower and translating less (glycerol) provides the first such link in a physiological condition.

A number of lines of evidence point to the prospect that newly-synthesized proteins, rather than mature proteins, underlie Hsf1 activation. Using heat shock as a prototype, the energetic input required to transiently unfolded mature proteins and expose hydrophobic
segments that can either self associate and form aggregates, or directly engage the activity of chaperones such as Hsp70, would be expected to be a greater barrier than for nascent or incompletely folded proteins. A telling experiment was performed by Beckmann et al.\textsuperscript{163}, who found that upon stress, human Hsp70 remains engaged with newly-synthesized proteins for an extended period of time. They note that, as the temperature of heat shock increased, the proportion of mature proteins that engaged Hsp70 increased, and contend that at lower temperatures, newly-synthesized proteins are likely dominant. Further, in yeast, newly-synthesized proteins seed protein aggregate formation\textsuperscript{117} and are the predominant target of stress-induced protein degradation by the ubiquitin-proteasome system, whereas mature proteins are spared\textsuperscript{179}. In sum, these data point to newly-synthesized proteins being labile in stress, and the potential for such proteins to underlie Hsf1 activation is seen in three distinct scenarios (Figure 3.6).

It is instructive to consider how each type of perturbation that activates Hsf1 in a translation-dependent fashion (Figure 3.1) may be related to newly-synthesized proteins. AZC, of course, results directly in misfolding and aggregation of newly-synthesized proteins when it is incorporated in lieu of proline into nascent chains. Indeed, a large increase in ubiquitination can be seen on nascent chains in AZC-treated cells\textsuperscript{180}. Diazaborine interferes with the assembly of newly-synthesized ribosomal proteins\textsuperscript{6}. Heat shock and ethanol, on the other hand, may biochemically interfere with the folding of nascent chains by decreasing the enthalpic advantage of burying hydrophobic segments, allowing for interactions between exposed hydrophobic segments between incompletely folded polypeptides\textsuperscript{168}. A key job of the proteasome is to clear out newly-synthesized proteins, such as those protein complex subunits that are produced in stoichiometric excess\textsuperscript{54,180}; inhibition by MG132 may result in accumulation of these orphan proteins that were destined for degradation. Diamide may have myriad effects, including reducing disulfide bonds, alkylate free cysteines, creating inter-protein disulfide crosslinks, and deplete cellular glutathione\textsuperscript{181,182}. It may be that disulfide bond formation happens rapidly during
protein synthesis or that proper disulfide bond formation is critical for folding of the protein\textsuperscript{183}. Though the precise mechanism of diamide may be hard to pinpoint, the heavy metal arsenite, which forms bulky adducts around cysteine thiols\textsuperscript{184}, has been found in yeast to cause aggregation of newly-synthesized proteins\textsuperscript{175}. In sum, each perturbation has a plausible means by which it could interfere with the fate of newly-synthesized proteins.

Regardless of whether newly-synthesized proteins serve as a principle cause of proteotoxic stress or not, it may also be true that decreased translation frees up chaperones that help to offset the need for Hsf1 activation in stress. Preliminary experiments looking at ribosome-associated Hsp70s (Ssa and Ssb) did not show a decrease in association in the presence of CHX (data not shown). Chaperones associated with newly-synthesized proteins that have departed the ribosome may, however, have greater availability in these conditions. Future experiments should seek to explore this possibility further.

In any regard, the link between the level of protein synthesis and the cell’s experience of proteotoxic insults has interesting implications for cell physiology. In one regard, previous work has shown that inhibiting translation in cancer cells leads to inactivation of constitutively activated Hsf1\textsuperscript{185}. This may then be the result of cancer cells having higher than normal protein synthesis levels, which renders cells reliant on increased Hsf1 activity. Indeed, imbalance in newly-synthesized protein components has been proposed to underlie the proteotoxic stress and sensitivity of aneuploid cells to drugs targeting proteostasis factors\textsuperscript{55,186,187}. Protein synthesis thus represents a liability to the integrity of the proteome.

3.5 Methods

Yeast cell growth

Saturated overnight cultures were grown in yeast extract-peptone-2\% glucose (YPD) overnight, back diluted into fresh medium containing either 2\% glucose or 2\% glycerol as
indicated, and grown to mid-log. For cycloheximide (CHX) treatment, cultures were split, and one half was treated with 0.2% DMSO (vehicle) and the other half was treated with a final concentration of 200 ug/ml CHX (from 100 mg/ml stock) for 3 min. Cells were then exposed to stressors as indicated in the figure legends. Rapamycin was used by treating cells for 30 min with a final concentration of 200 ng/ml rapamycin freshly prepared in ethanol. Heat shock was performed by addition of an equal volume of 44°C medium and shifting to a 37°C incubator.

**Yeast strains**

Figures 3.1, 3.3, 3.5 used a BY4741 strain with the Hsf1 reporter 4xHSE::mVenus::LEU2 integrated at the LEU2 locus (strain YBT256169), with the exception of the MG132 experiments performed by transforming the same reporter construct into pdr5Δ from the haploid deletion collection (strain YBT257). Figure 3.2 used previously-reported W303a derivatives118: wild-type HSF1 with the 4xHSE::mVenus reporter (strain DPY304, David Pincus, University of Chicago); non-phosphorylatable HSF1 with reporter (all serine/threonine mutated to alanine, except the DNA-binding S225, strain DPY416, David Pincus). Figure 3.4 used a strain that had the endogenous HSP104 locus tagged with a C-terminal mCherry::HIS3 cassette (strain YBT230). The Hsf1 western blot used a strain containing a C-terminal FLAG-V5 tag118 (David Pincus).

**Total RNA extraction and Northern blotting**

RNA was extracted and analyzed by Northern blot as described in the Methods section of Chapter 2. Quantification was performed using ImageJ.

**Total protein extraction and western blotting**

Proteins were extracted and analyzed by western blot as described in the Methods section of Chapter 2. Hsf1-FLAG-V5 was detected using mouse anti-FLAG (Millipore Sigma, F1804, 1:1,000). Pgk1 was detected using mouse anti-Pgk1 (Abcam, ab113687, 1:10,000).
Fluorescence microscopy

Cells expressing Hsp104-mCherry were treated with 1/10th volume of 37% formaldehyde for 10 min and washed twice in PBS. Samples were mounted onto 2% agarose pads on a glass slide and cover with a glass coverslip. Images were acquired on a Nikon Ti2 microscope with a 100x objective and an ORCA-R2 cooled CCD camera (Hamamatsu).

Genomics data analysis

For Srp72 depletion, ribosome profiling data in Table S3 from Costa et al.\textsuperscript{178} were used, and the fold change values were determined by dividing the 30 min auxin time point (“sec63BirA_srp72-AID_30mAuxin_2mCHX_2mBiotin_input_rpkm”) by the 0 min auxin time point (“sec63BirA_srp72-AID_0mAuxin_2mCHX_2mBiotin_input_rpkm”). For Psd1 overexpression, RNA-seq data in Table S1 from Weidberg et al.\textsuperscript{141}, column “GalPsd1.Empty.logFC”, were used. “Hsf1 targets” and “All others” gene groups were defined as in Chapter 2.

3.6 Contributions and acknowledgements

This work was performed independently with input from L. Stirling Churchman. I thank members of the Churchman lab and David Pincus for feedback. Microscopy was performed at the Nikon Imaging Center at Harvard Medical School.
Chapter 4  Outlook
The work presented in this thesis has two key findings. First, Chapter 2 demonstrates that perturbations to ribosome assembly lead to collapse of proteostasis when orphan r-proteins aggregate and seemingly compromise essential cellular processes. This provides a plausible mechanism that may play into why, for instance, ribosomopathies result in a rather specific ablation of cell types like erythrocytes, which appear to be sensitive to insults that sequester Hsp70\textsuperscript{75}. Second, and more generally, these results identify newly-synthesized r-proteins as specific endogenous proteins competent to cause proteostasis collapse and require the transcriptional response by Hsf1. Chapter 3 sought to expand on this and develops a more general link between ongoing protein synthesis and the cell’s experience of proteotoxic stress that may be related to newly-synthesized proteins being compromised in stress.

Given that many canonical proteotoxic stress conditions, such as heat shock and oxidative stress, can disrupt ribosome assembly, I propose that these perturbations may in part cause proteotoxic stress and elicit Hsf1 activation due to ribosome assembly defects. Future work should investigate whether newly synthesized ribosomal proteins aggregate in these conditions and whether cells experience proteotoxicity in the absence of ribosome production.

Outstanding questions in r-protein assembly stress:

1. **What is the basis and function for the highly selective repression of r-protein gene transcription during RPAS?** Albert *et al.*\textsuperscript{188} provided evidence for aggregation of a key transcription factor that regulates r-protein gene expression, Ifh1, during RPAS. Interestingly, this effect appeared to be selective for Ifh1, as the other r-protein gene transcription factors, Rap1 and Fhl1, remained associated with promoters. A possible way to test the consequence of repressing r-protein genes would be to use CARA cells, which expresses a fusion of the RNA polymerase I subunit Rpa43 with the transcription factor Rrn3\textsuperscript{51}. In response to stress, this strain fails to repress synthesis of both rRNA
and r-proteins. It would be of interest to test the fitness of this strain relative to wild-type cells in RPAS.

(2) **How is global translation repressed in RPAS?** The independence of Gcn2-mediated translational repression suggests that it may follow a novel mechanism. One intriguing possibility is that, as with Ifh1 aggregation driving inactivation of r-protein genes in RPAS, translation factors may likewise aggregate as has been seen in extreme heat shock with eIF3 and eIF4G\textsuperscript{104,189}. Translational repression by stress granule assembly is also a possibility\textsuperscript{190}, though I did not observe a loss of polysomes (Figure A1.1A).

(3) **Do r-proteins aggregate in other stress conditions, such as heat shock?** Hsp70 in mammalian cells has been found to move to the nucleolus upon heat shock\textsuperscript{57}; it may be that the nucleolus, a phase separated body, is disrupted by heat, disrupting r-protein assembly.

(4) **What is the fate of aggregated orphan r-proteins?** The reduced fitness of \textit{rpn4}\textsubscript{Δ} cells, which fail to induce an increase in proteasome abundance in stress\textsuperscript{95}, suggests that ubiquitin proteasome-dependent degradation of r-proteins is critical in RPAS. This would be consistent with the recently described ERISQ pathway in yeast and human cells, which clears excess free r-proteins made in steady state conditions using the E3 Tom1/HUWE1\textsuperscript{86,87}. However, chaperones may be required to extract proteins from aggregates ahead of degradation; it remains unclear why mutants such as \textit{hsp104}\textsubscript{Δ} do not show reduced fitness in RPAS.

(5) **Does RPAS occur in human cells?** Key to dissecting this question may be the observation that slower growing yeast cells experience a diminished proteotoxicity by various stressors. Therefore, this should be tested in cells with both fast and slow doubling rates, and can be probed with ribosome biogenesis inhibitors\textsuperscript{191,192}, other chemotherapeutic drugs that impact ribosome biogenesis\textsuperscript{56}, and by rapid conditional depletion of assembly factors using auxin or the recently described dTAG approach\textsuperscript{193}. 

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(6) If RPAS does occur in human cells, can Hsf1 or target gene activity be modulated to suppress the cell-type specific phenotypes, such as failure of hematopoietic progenitors to make red blood cells in Diamond-Blackfan anemia (DBA)? I found in yeast that upregulating Hsf1 activity could enhance cell growth in the face of RPAS (Figure 2.2E). It would be prudent to test whether this also works in human cells, such as by overexpressing Hsf1 in ex vivo hematopoietic stem cells from patients with DBA or by using small molecules that sustain Hsf1 activity, like riluzole\textsuperscript{194}. If this works, such an approach could be carried forward to preclinical testing as a possible therapeutic approach. The key risk here is that continuous sustained Hsf1 activity may have negative consequences to other cells\textsuperscript{195}, though riluzole itself is an approved drug for treatment of amyotrophic lateral sclerosis.

Outstanding questions related to protein synthesis and proteotoxic stress:

(1) Can disruption of assembly of complexes other than the ribosome lead to loss of proteostasis and subsequent Hsf1 activation? R-proteins are relatively positively charged and highly abundant, making them prone to aggregation. Highly abundant proteins destined for assembly into protein complexes, and thus containing charged or hydrophobic surfaces, may likewise serve as Hsf1 activating signals should their assembly be disrupted. Indeed, certain proteins seem to require an excess of the components with which they complex and to assemble co-translationally, and in their absence, they aggregate\textsuperscript{196}. Candidates of particular interest would be histones, as they are likewise highly abundant and contain flexible and highly positively charged tails that allow them to interact with negatively charged DNA, as r-proteins do with RNA. This and other complexes could be tested by using auxin-inducible degradation or anchor away to sequester single components of the complex and test for Hsf1 activation. Membrane proteins may also be of interest\textsuperscript{197}.  

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(2) **Do newly-synthesized proteins aggregate in stress?** Short-term metabolic labeling followed by stress treatment could allow this to be tested by differential centrifugation, similar to described\(^8\). Challenges for this would be to find conditions where cells do not need to go through nutrient depletion to facilitate uptake of metabolic label, and finding differential centrifugation conditions that provide low levels of background.
Appendix 1  Supplementary materials related to Chapter 2
Figure A1.1  Kinetics of Hsf1 activation
(A) Absorbance profiles of sucrose gradients (10-50%) of extracts from \textit{RAT1}^{AID} cells mock or auxin treated for 20 min. Shown are two biological replicates. (B) ChIP-qPCR data of Hsf1 at the indicated promoter region of cells untreated, auxin treated, or heat shocked (37°C) for 20 min. Bar height indicates the average and error bars the standard deviation of n=3 biological replicates. (C) Rat1 anchor-away cells (see also Figure A1.2) were depleted of Rat1 by rapamycin treatment (1 µg/ml, 40 min) or heat shocked for 20 min. Deletion of \textit{RQC2} did not alter the activation of the Hsf1 targets \textit{HSP82} and \textit{BTN2}. Shown are two biological replicates. (D) WT or the indicated AID-tagged strains were treated with auxin for the indicated times and accumulation of Hsf1 targets \textit{HSP82} and \textit{BTN2} and Msn2/4 target \textit{HSP12} was followed by Northern blot. As a control for Hsf1 and Msn2/4 activation, RNA from WT cells untreated (unt) or treated with diamide (dia, 1.5 mM, 20 min) was included on each blot. RNA was from the same cells used in Figure 2.1C to allow direct comparison. (E) RNA from (D) was probed for pre-5.8S rRNA species (probe 017^{108}). Note that the \textit{BTN2} blot is the same as in (D) and is included for comparison of kinetics.
Figure A1.2  Specificity of Hsf1 activation by depletion of rRNA processing factors

(A) RTT103AID and DXO1AID cells were treated with auxin for the indicated times and assayed for accumulation of Hsf1 targets by Northern. RNA from RAT1AID was included as a positive control. Western blots (below) show depletion of AID-tagged proteins. This experiment was not repeated. (B) XRN1AID cells were treated with auxin for the indicated time and the indicated RNAs detected by Northern. Consistent with the role of Xrn1 in RNA turnover, known target transcripts modestly accumulated during the time course of Xrn1 depletion. GAL10 and GAL10-IncRNA are established Xrn1 substrates that accumulate with kinetics similar to those of Hsf1- (BTN2 and HSP82) and Msn2/4-dependent transcripts (TPS2 and HSP12). Thus, these RNAs accumulated in the absence of normal Xrn1-mediated decay. (C) Fluorescence micrographs of Rat1-FRB-GFP and Nrd1-FRB-GFP at indicated time points after rapamycin (1 μg/ml) addition. Nuclei were stained with DAPI. Cells co-express Rpl13a- 2xFKBP12 as an anchor and harbor the tor1-1 mutation, rendering Tor1 insensitive to rapamycin. Addition of rapamycin induces dimerization of FRB-tagged protein to the anchor and rapid nuclear export during export of Rpl13a. (D) Schematic of Hsf1 activity reporter transgene HSE-
GFP consisting of GFP driven by four repeats of the Hsf1 binding site (Heat Shock Element, HSE). (E) Northern blot for HSE-GFP after rapamycin treatment for the indicated time or heat shock (HS, 37°C, 20 min) as a control. (F) Northern blot for Hsf1-dependent gene HSP82 from wild-type or anchor-away strains untreated, treated for 45 min with rapamycin (1 µg/ml), or 45 min rapamycin followed by 20 min diamide (1.5 mM). Nrd1 is a nuclear non-coding RNA transcription termination factor.
Figure A1.3 On-target inhibition of Drg1 by DZA

(A) Schematic of the yeast Drg1 protein, with the two ATPase domains shown in red. The V725E mutation in the second ATPase domain confers DZA resistance\(^6\). (B) Growth of WT and \textit{DRG1 V725E} strains after 24 h in the indicated concentration of DZA relative to vehicle-only controls. Line indicates the average and error bars the range of \(n=2\) biological replicates. (C) Northern blot for Hsf1 target genes in WT and \textit{DRG1 V725E} cells treated with DZA (15 \(\mu\)g/ml) for the indicated times. (D) Quantification of Northern blots for the indicated Hsf1 target transcripts, normalized against \textit{SCR1}. Line indicates the average and error bars the range of \(n=2\) biological replicates.
Figure A1.4  DZA treatment enhances thermotolerance

WT cells treated with the indicated concentration of DZA for 45 min were exposed to 50°C HS for 15 min. Colony forming units were determined by plating approximately 100,000 cells. Bar height indicates the average and error bars the standard deviation of n=3 biological replicates.
Figure A1.5  The endoplasmic reticulum unfolded protein response (UPR) is not activated during RPAS

Swarm plot of log₂ fold change of Hac1-dependent UPR transcripts in the condition indicated on the x-axis (n=23). RNA-seq data for cells treated with tunicamycin (5 µg/ml, 4 h) and dithiothreitol (DTT, 5 mM, 4 h), established inducers of the UPR, are from Pincus et al. (2014).
Figure A1.6  Aggregation of orphan r-proteins during RPAS

(A) $RAT^{AID}_1$ cells were mock or CHX (200 µg/ml, 3 min) treated before addition of auxin or heat shock (37°C) for 20 min. Northern was performed for the Hsf1 reporter transgene $HSE$-$GFP$ consisting of GFP downstream of four Hsf1 binding sites (Heat Shock Element, HSE), and $HSP82$. (B) Treating extracts with benzonase does not prevent the aggregating behavior of newly synthesized Rpl10 when treated with DZA. Experiment was performed as in Figure 4 except extracts contained benzonase to degrade RNA and DNA.
Figure A1.7 Gene ontology analysis of top aggregating proteins in DZA-treated cells detected by mass spectrometry

Gene ontology (GO) term enrichment for aggregated proteins (>1.5-fold in two biological replicates, n=51) detected in DZA-treated cells by mass spectrometry (data as in Figure 2.4). Shown are the top 5 terms for the "Process" (blue) and "Component" (red) categories with p-value and the fold enrichment relative to all proteins detected (n=2491).
Figure A1.8  Competitive fitness of strains lacking single Hsf1-dependent genes

Log$_{10}$ ratios of query (mCh) to WT reference (YFP) cells after the indicated number of days of co-culture, normalized to the ratio at t=0. Each dot represents one replicate for a total of 8 replicates per competition. Conditions: YPD, 37°C, AZC (5 mM), DMSO (vehicle, 0.2%), DZA15 (DZA 15 µg/ml), DZA30 (DZA 30 µg/ml). The query parent (WT), $btn2\Delta$, $hsp104\Delta$, $hsp42\Delta$, and $hsp12\Delta$ all grew identically under all conditions, suggesting these mutants had no growth defect in any condition. $tmc1\Delta$ exhibited a mild
but reproducible defect in DZA (~4% slower per doubling, $p=2.2 \times 10^{-8}$ by two-sided Student’s t-test in “DZA30”, no defect in DMSO).
Figure A1.9  Growth improvement is not due to changes in cell size

The size of distribution of cells from Figure 2.6E was determined by flow cytometry by side scatter, plotted in log-space for each condition without or with estradiol pre-conditioning.
**Figure A1.10  Disrupted rRNA processing in DZA-treated cells**

(A) Samples from Figure 7A were probed for 35S and 27S-A2 (probe 800,\textsuperscript{16}) and 7S pre-rRNA (probe 017,\textsuperscript{198}). SCR1 control is as in Figure 7A for comparison, and mature 5.8S rRNA is detected from a short exposure of probe 017. (B) Samples from Figure 7B were probed for 35S and 27S-A2 (probe 800,\textsuperscript{16}) and 7S pre-rRNA (probe 017,\textsuperscript{198}). SCR1 control is as in Figure 7B for comparison, and mature 5.8S rRNA is detected from a short exposure of probe 017.
### Supplementary Table A1.1  Yeast strains

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### Supplementary Table A1.2  Plasmids

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### Supplementary Table A1.3  Primers

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Supplementary Table A1.4 Gene annotation lists and RNA-seq data (electronic attachment)

Tab “Gene_Lists” contains members of groups used for analysis. Subsequent tabs contain RNA abundance measurements determined by DESeq2 or RPKM calculations.

Supplementary Table A1.5 Flow cytometry data from competitive fitness experiments (electronic attachment)

Query (mCh) and reference (YFP) counts for each competition at t=0, 1, 2, 3, 4, 5 days. Each mutant query had four isolates (“Iso1-4”) that were tested in two technical replicates (“Rep1-2”), for a total of eight replicates per experiment. The normalized, log_{10} transformed values were used to generate plots.

Supplementary Table A1.6 Summary of proteomics data of input and pellet proteins (electronic attachment)

The value of each protein is normalized to the total signal in each sample (TMT channel) to determine relative abundance within each sample (parts per million, ppm).
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