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INSIGHTS INTO STRESS-INDUCED CONDENSATION OF MRNA AND PROTEIN

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Erst die Arbeit, dann das Vergnügen.

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Most of all, I am indebted to my family–Gottlieb, Annette, and Kristof–for their love and support in all aspects of my life. My parents are my role models, and I can only hope to be like them when I grow up. My brother is my best friend. This work is not possible without them; therefore, it is for them.

ABSTRACT

mRNA and protein clump—or condense—in response to cellular stress across the tree of eukaryotic life. Yet, despite decades of inquiry and its universal evolutionary conservation, the function of stress-induced condensation remains enigmatic. The aim of this thesis is to gain insights into this fundamental phenomenon, using both cell biological and reductionist biophysical perspectives. Outstanding issues in the field of mRNA condensation are disagreements of which transcripts condense in response to stress, mechanistic understanding of how mRNA condenses and accumulates into microscopically visible stress granules, and the functional consequences of mRNA condensation. Outstanding issues in the field of protein condensation are a lack of high resolution understanding of the structures of condensates, how the structures of condensates may differ in different stress contexts, and how Nature encodes condensation into a protein's primary sequence. Furthermore, how organisms modulate condensation by altering the chemical environment of the cell remains understudied.

In Chapter 2, I summarize our understanding of stress-induced condensation of mRNA and protein, detail active areas of inquiry, and raise grand challenges plaguing the field from answering these questions.

In Chapter 3, we interrogate mRNAs condensation during stress using budding yeast as a model organism. I show that most mRNAs condense following exposure to multiple divergent stresses. Rather than length being the defining predicter of mRNA condensation, we find that transcriptionally induced mRNAs escape condensation. Mechanistic work reveals that an increased abundance of ribosome-free mRNA is not sufficient to explain stress-induced mRNA condensation. Rather than simply being a byproduct of stress-triggered translational downregulation, our data supports a model in which mRNA condensation helps focus the cell's translational machinery to produce proteins needed to mount its stress response.

In Chapter 4, I probe the molecular mechanisms of protein condensation using polyadenylate-

binding protein (Pab1 in budding yeast) as a model. I advance our understanding of Pab1 condensation mechanism by identifying putative, specific crosslinks connecting Pab1 protomers in the condensate. Supporting the thermodynamic specificity model of Pab1 condensation, I use HDX-MS to probe the hydrogen bond networks of Pab1 condensates formed at different temperatures and find that different condensation onset temperatures causes different condensate structures. HDX-MS study of Pab1 condensates from orthologs with different condensation onset temperatures informs how Nature encodes condensation in primary sequence.

In Chapter 5, I investigate how Nature may utilize transition metal signaling to modulation condensation. Using Pab1 from budding yeast as a model system, I find that Zn^{2+} specifically promotes Pab1 condensation. Transition metals may be a broadly applicable class of signaling molecules, aiding the cell to transduce stress signals into condensate formation.

CHAPTER 1

INTRODUCTION

1.1 What is biomolecular condensation and what does it do?

Biomolecular condensation refers to the nonstoichiometric clumping of protein and mRNA into massive, membraneless assemblies. [Banani et al., 2017]. Condensates include many critical cellular compartments, such as the nucleoli and p bodies [Banani et al., 2017]. Condensation also appears to be critical for cells to adapt to stress, as their formation is universally conserved across eukarya in response to a a wide array of different stresses [Kedersha et al., 2013]. Stress granules (SGs) are cytoplasmic, stress-induced condensates, which are operationally defined as microscopically visible foci of mRNA and certain marker proteins which form in response to stress [Kedersha et al., 2000].

The concept of biomolecular condensation has been received with great interest by the scientific community, as it provides a novel mechanism by which cells can organize in space and time. By concentrating certain biomolecules in specific locations, these compartments are thought to be able to serve specific function for the cell [Banani et al., 2017]. Traditionally, eukaryotic organisms have been thought to dominantly utilize phospholipid membranes to generate concentration gradients. Thus, the reckoning that we as a field were oblivious to ubiquitous layers of cellular organization is extremely exciting. In fact, condensates are sometimes referred to as "membraneless organelles." However, despite decades of study, questions as basic as the functions of stress-induced condensates remain enigmatic [Glauninger et al., 2022].

1.2 Uncertainty in the function of stress-induced condensates and stress granules

A wide range of perspectives exists as to the potential functions of stress-induced condensates and stress granules, from deleterious or coincidental to functional. One model is that stress conditions cause widespread protein misfolding, exposing hydrophobic patches which aggregate [Cherkasov et al., 2013, Vabulas et al., 2010]. In this model, the misfolding of protein and resulting condensation are part of the toxicity of the stress. A separate view is that the formation of stress granules occurs when increased concentrations of ribosome-free mRNA are present in a cell, such as following the widespread translational attenuation during stress conditions [Hofmann et al., 2021]. Here, the condensation of ribosome-free mRNA occurs as a byproduct of stress conditions, without necessarily positing a function or detriment. Yet another view is that stress-induced condensation is an evolved cellular response enabling cells to adapt to stress conditions [Riback et al., 2017, Keyport Kik et al., 2023, Iserman et al., 2020].

The importance of understanding to what degree stress-induced condensation is deleterious, coincidental, or functional is underscored by the association of perturbed condensation and various disease states [Boija et al., 2021, Eiermann et al., 2020, Wolozin and Ivanov, 2019]. Emerging efforts to pharmaceutically drug condensates will be stymied if we do not know which direction (if any) to modulate them [Mitrea et al., 2022]! Contributing to the uncertainty regarding the functional understanding of condensation is a dearth in knowledge of which biomolecules condense in stress, and how.

1.3 Mechanisms of condensation

Many cellular condensates, including stress granules, are believed to form at least partially through liquid-liquid phase separation (LLPS) [Hofmann et al., 2021]. LLPS describes the

demixing of a solution into 2 distinct liquid states, such as oil and water [Hyman et al., 2014]. LLPS in biology has been embraced as a revolutionary concept because it enables the thermodynamically driven (no energy input needed) compartmentalization of biomolecules without membranes. In the case of stress granules, a flurry of recent work has led to a model of G3BP1/2 phase separation as the central nexus of SG formation [Yang et al., 2020, Guillén-Boixet et al., 2020, Sanders et al., 2020]. Yet, future work is required to understand the degree to which LLPS underlies SG formation across stresses and organisms and the detailed molecular mechanisms by which condensates form and functin *in vivo* [Mateju and Chao, 2022].

For a simple single protein system, phase diagrams are a powerful tool to understand the concepts of liquid-liquid phase separation (Figure 1.1, from Alberti et al. [2019]). Important concepts include understanding that LLPS can only occur above a certain saturation concentration (c_{sat}), which is dependent on the environmental condition the biomolecule is exposed to—for example, temperature or pH. There exists a critical point at which the 2-phase regime no longer occurs. Whether the system behaves via an upper or lower critical solution condition determines which regime it falls in. For example, Figure 1.1 depicts an upper critical solution concentration system, as there is a critical point above which LLPS does not occur. In contrast, Pab1 condenses as a lower critical solution temperature system [Riback et al., 2017]. The fact that LLPS behavior is tuned by environmental conditions is critical for biology, as this implies that condensing proteins must be tuned to appropriately function in the cellular environment. This further implies that condensing proteins have evolved relative to the environmental conditions experienced by the organism, a concept critical to Chapter 4. Moreover, the inverse must also be appreciated: the extreme sensitivity of LLPS to solution condition implies that cells may have evolved to adjust their chemical environments to modulate LLPS in response to stress, a concept critical to Chapter 5.

A key feature of LLPS is that the c_L (concentration of the light phase) and c_D (concen-

Figure 1.1: LLPS phase diagram. c_L and c_D represent the concentration of the light and dense phases respectively. The critical point is the environmental condition above or below (depending on whether the system acts as an upper or lower critical solution conditions system) which phase separation cannot occur. Figure taken from Alberti et al. [2019].

tration of the dense phase) remain constant at different overall protein concentrations at a certain environmental condition (Figure 1.1). The light phase has concentration c_L, while the dense phase has concentration c_D. Instead, as total protein concentration increases (moving along a tide line, as depicted in orange in Figure 1.1), the relative volume fraction of light and dense phase change.

1.4 Specific questions

In this work, we aim to address the following questions to gain further insight into the stress-induced condensation of mRNA and protein:

1. Which mRNAs condense during stress, and how?

2. How do the molecular mechanisms of Pab1 condensation vary across temperatures and orthologs?

3. Could transition metal signaling play a role in modulating condensation during stress?

To answer these questions, we apply cell biological and biophysical approaches to study the cellular stress response to heat shock in budding yeast *S. cerevisiae*. First, Chapter 2 reviews the state of knowledge of stress-induced condensation of mRNA and protein and posits grand challenges impeding the field. Next, Chapter 3 studies stress-induced mRNA condensation. We show that most of the transcriptome condenses in a length-independent manner in response to stress. The transcriptionally-induced stress response messages escape condensation to be robustly translated. Different mRNAs condense in response to different stresses, arguing for stress-induced mRNA condensation being an adaptive response which helps the cell adapt to new environments. Chapter 4 studies the molecular mechanisms of Pab1 condensates. Using hydrogen-deuterium exchange mass spectrometry (HDX-MS), we show that Pab1 condensates formed at different temperatures and from different species have distinct structures, supporting the thermodynamic selectivity mechanism of Pab1 condensation. Additionally, we identify specific contacts which may form the underlying architecture linking Pab1 protomers within the condensate. Finally, Chapter 5 investigates transition metal signaling as a putative mechanism tuning condensation in stress. Using *in vitro* biochemical reconstitution, we show that Zn^{2+} promotes Pab1 condensation, suggesting the presence of a specific binding site and is consistent with a putative role of transition metal signaling in stress response.

CHAPTER 2

STRESSFUL STEPS: PROGRESS AND CHALLENGES IN UNDERSTANDING STRESS-INDUCED MRNA CONDENSATION AND ACCUMULATION IN STRESS GRANULES

This chapter has been adapted from [Glauninger et al., 2022].

2.1   Summary

Stress-induced condensation of mRNA and protein into massive cytosolic clusters is conserved across eukaryotes. Known as stress granules when visible by imaging, these structures remarkably have no broadly accepted biological function, mechanism of formation or dispersal, or even molecular composition. As part of a larger surge of interest in biomolecular condensation, studies of stress granules and related RNA/protein condensates have increasingly probed the biochemical underpinnings of condensation. Here, we review open questions and recent advances, including the stages from initial condensate formation to accumulation in mature stress granules, mechanisms by which stress-induced condensates form and dissolve, and surprising twists in understanding the RNA components of stress granules and their role in condensation. We outline grand challenges in understanding stress-induced RNA condensation, centering on the unique and substantial barriers in the molecular study of cellular structures, such as stress granules, for which no biological function has been firmly established.
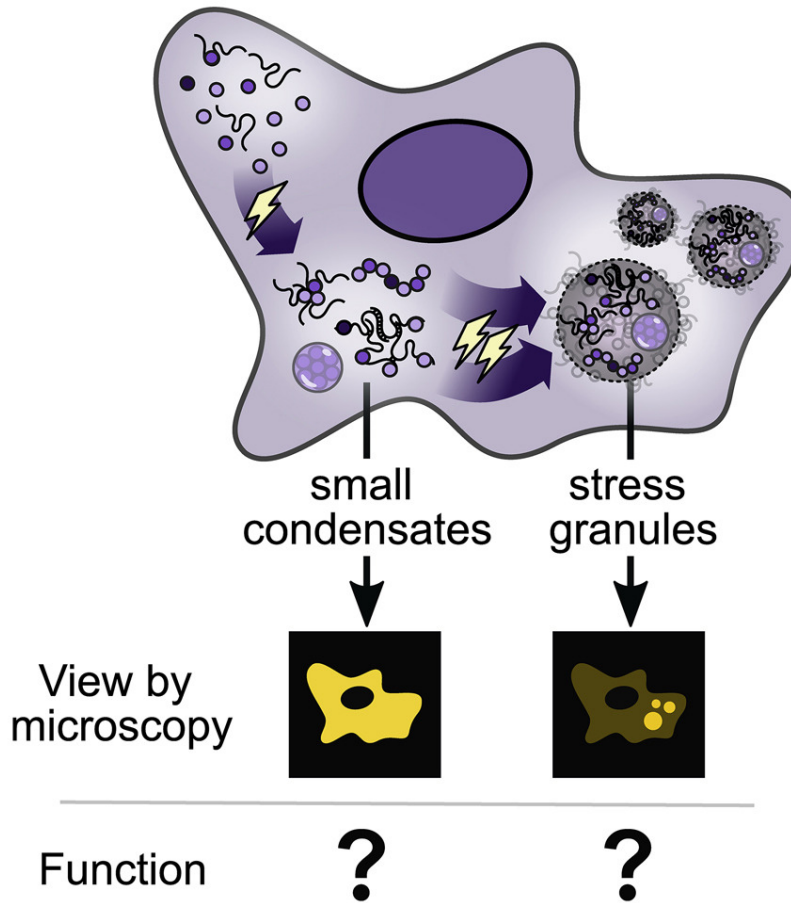
Figure 2.1: Graphical abstract
What is the function of stress-induced condensation?

2.2 Introduction

From humans and other vertebrates to single-celled yeasts, from plants to protozoa, the onset of primordial stresses such as heat shock, oxidizing agents, hypoxia, and starvation is rapidly followed by the intracellular condensation and accumulation of myriad proteins and mRNAs in cytosolic clusters [Cherkasov et al., 2013, Decker and Parker, 2012, Farny et al., 2009, Jain et al., 2016, Kedersha et al., 2000, 1999, Kramer et al., 2008, Nover et al., 1989, Wallace et al., 2015]. These enigmatic structures, called stress granules when they grow large enough to resolve by microscopy, have become standard examples of so-called membraneless organelles alongside nucleoli, processing (P) bodies, paraspeckles, and others [Alberti and Carra, 2018,

Boeynaems et al., 2018, Brangwynne, 2013, Gomes and Shorter, 2019, Guo and Shorter, 2015, Lyon et al., 2021, Mitrea and Kriwacki, 2016]. Stress granules and their condensed molecular precursors have become a nexus of extraordinary recent activity because of the involvement of protein and RNA liquid-liquid phase separation (LLPS) in their formation [Guillén-Boixet et al., 2020, Molliex et al., 2015, Riback et al., 2017, Sanders et al., 2020, Van Treeck et al., 2018, Wheeler et al., 2016, Yang et al., 2020] and hints that dysregulation of condensation and stress granule formation contribute to disease [Bosco et al., 2010, Patel et al., 2015].

However, despite sustained and vigorous inquiry, a remarkable array of foundational questions remain unanswered. What do stress granules do, if anything? What are the functional consequences of condensation, and what functions do specific mechanisms of condensation, such as LLPS, carry out? (Throughout this review, we explicitly intend "condensate" to be a catch-all term for membraneless clusters without any further stipulation as to their structure, process of formation, or adaptive significance (Table 2.1), largely following standard usage [Banani et al., 2017, Lyon et al., 2021]. What biological roles are played by molecular-level condensation events versus subsequent merging of these condensates into larger, microscopically visible structures? How do condensation and accumulation occur and are these processes mediated mainly by intrinsic molecular forces or extrinsic cellular machinery such as cytoskeleton-associated motors? To what extent are stress-triggered condensation and stress granule accumulation processes and participants conserved over evolutionary time?

Among the deepest challenges in studying stress granules is that, in the absence of molecular functions and cellular phenotypes, the phenomenon itself is operationally rather than biologically defined: a stress granule consists of anything which forms microscopically visible foci that colocalize with established stress granule markers (cf. Table 2.1). Although these structures have been hypothesized to play a variety of cellular roles, their function remains unclear [Buchan et al., 2011, Ivanov et al., 2019, Kedersha and Anderson, 2002, 2009, Ked-

What is a condensate?
Biomolecular condensates are membraneless clusters of biomolecules such as proteins and nucleic acids. Classic examples are nucleoli, stress granules, P bodies, and germline P granules, among many others. "Biomolecular condensate" serves as an umbrella term for these structures which is agnostic as to their specific size, function, mechanism of formation, material state, or method of experimental study. The term arose, in part, due to the growing realization that more specific terms referring to mechanism (e.g., liquid-liquid phase separation [LLPS]), material state (e.g., droplet, hydrogel), or function (compartment, membraneless organelle) often implied more than is presently known. Importantly, many biomolecular condensates have been near-exclusively studied by specific methods. Stress granules, for example, are operationally defined by formation of foci resolvable by fluorescence microscopy that contain specific marker proteins and poly(A)+ RNA. Failure to detect microscopic foci is routinely taken to indicate the absence of stress granules, although submicroscopic assemblies may be present. Rather than overturn this well-established operational definition, here we use the umbrella term condensates to refer to assemblies whether or not they are visible by microscopy. We use "accumulation" as a general term for processes in which smaller condensates are brought together to form larger structures.

Table 2.1: What is a condensate?

ersha et al., 2000]. That stress granules are termed "membraneless organelles," where the latter word explicitly means a cellular structure that performs distinct functions, has served to create the unfortunate impression that this fundamental question has been answered.

This question of function applies not only to stress granules but also to the broader study of cytoplasmic ribonucleoprotein (RNP) foci including P-bodies, RNA transport granules, and P granules. In some cases, such as RNA transport granules in neurons, the question of function has been more directly addressed [Kiebler and Bassell, 2006, Pushpalatha and Besse, 2019]. However, in many cases, function is still presented as a model. P-bodies were long presumed to be sites of RNA degradation [Aizer et al., 2014, Franks and Lykke-Andersen, 2007, Sheth and Parker, 2003], but this model has been challenged [Eulalio et al., 2007,

Hubstenberger et al., 2017]. Additionally, work on G3BP1 aggregates in axons shows that condensates composed of canonical stress granule proteins may play a role under nonstress conditions, introducing basal stress granule-like condensates [Sahoo et al., 2018, 2020]. The questions and challenges regarding stress granules raised here apply to other biomolecular condensates, purported membraneless organelles, and contexts beyond cell stress.

As efforts to develop a parts list for stress granules [Buchan et al., 2011, Cherkasov et al., 2015, Jain et al., 2016, Wallace et al., 2015] have proceeded alongside attempts to recapitulate in vitro certain molecular events such as stress-reactive condensation and RNA recruitment [Begovich and Wilhelm, 2020, Iserman et al., 2020, Riback et al., 2017, Van Treeck et al., 2018], evidence has emerged for multiple quasi-independent contributing pathways, multiple molecular stages, and multiple levels of organization in stress granules and their precursors. This will serve as our jumping-off point. Given the multiple levels of molecular organization known to contribute to stress-induced RNA condensation, how do these levels interrelate, and at what level are adaptive features best understood?

Throughout this review, we intend a larger question to lurk in the reader's mind. How can the characterization, interrogation, isolation, and reconstitution of stress-induced protein/RNA condensates and stress granules be effectively guided and evaluated in the absence of established functions, biological activities, or cellular phenotypes?

2.3   Multiple stages of stress-induced RNA condensation and stress granule formation

What is the relationship between protein/mRNA biomolecular condensation and stress granule formation? Although these processes are sometimes considered synonymous and although how initial condensates accumulate in microscopically visible foci remains largely unknown, the existence of multiple stages in stress granule formation has long been understood (Figure 2.2). Existing models commonly reflect hierarchical organization in stress granules, with

some stable components ("core") surrounded by more dynamic components ("shell") [Jain et al., 2016, Wheeler et al., 2016] or nanoscopic "seeds" interacting and merging to form stress granules [Padrón et al., 2019, Panas et al., 2016].
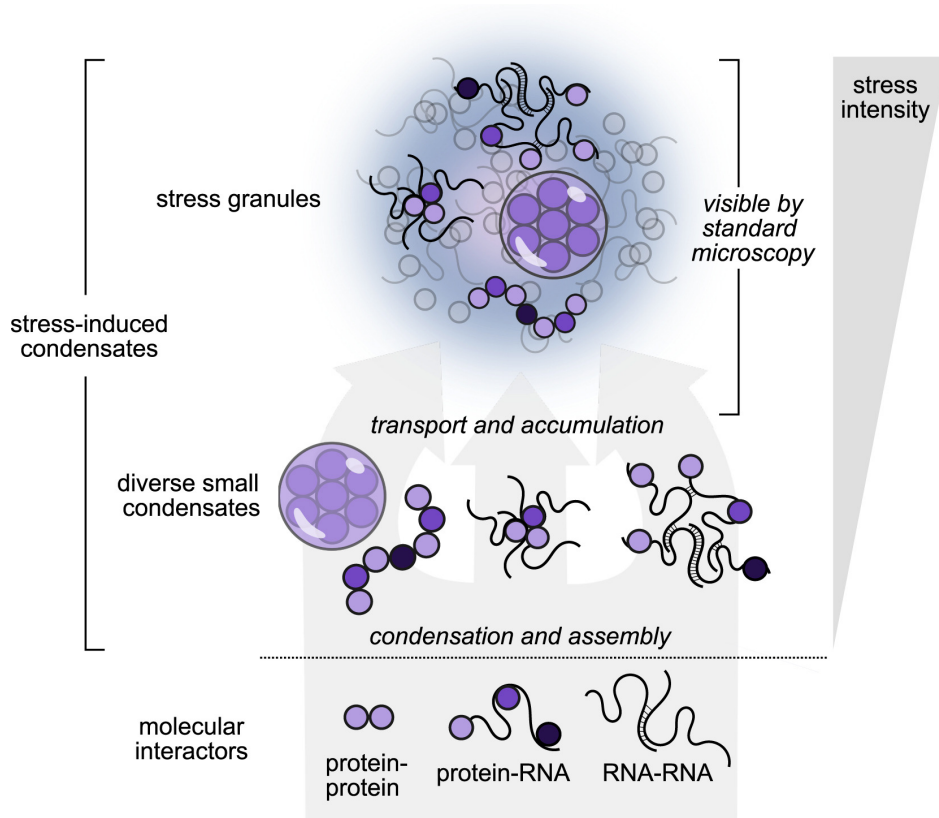
Figure 2.2: Stress-triggered protein/mRNA condensation and stress granule formation occur in stages, depend on stress intensity and identity, and involve multiple types of molecular interactions

Evidence for these multiple stages comes from several independent sources. First, individual core markers for stress granules such as poly(A)-binding protein, G3BP, and Ded1 can be purified recombinantly and will autonomously condense in response to stress-associated physiological cues (e.g., heat shock, presence of long ribosome-free mRNA) *in vitro* [Guillén-Boixet et al., 2020, Iserman et al., 2020, Kroschwald et al., 2018, Riback et al., 2017, Yang et al., 2020]. These *in vitro* results suggest that condensation *in vivo* may not depend on interactions between a large set of stress granule components, at least at initial stages.

Second, although formation of canonical microscopically visible stress granules can be blocked by translation elongation inhibitors [Kedersha et al., 2000, Nadezhdina et al., 2010, Namkoong et al., 2018, Wallace et al., 2015], the stress-triggered condensation, as measured by biochemical fractionation, of stress granule components such as poly(A)-binding protein proceeds virtually unaffected by such inhibition, indicating that accumulation of condensates into stress granules is a separate step [Wallace et al., 2015]. This suggests that formation of canonical stress granules involves cell-biological transport processes that bring multiple components together in the cytosol [Panas et al., 2016]. In support of this model, depolymerization of microtubules disrupts stress granule accumulation [Ivanov et al., 2003,], and stress granules tether to the endoplasmic reticulum and lysosomes using specific factors for intracellular transport [Liao et al., 2019]. Similarly, in contrast to *in vitro* ATP-independent condensation processes, ATP-driven mechanisms are required for stress granule formation in cells [Jain et al., 2016]. Transport and accumulation of small condensates and other components is a separate process from the initial condensation events that also accompany stress.

Finally, the appearance of canonical stress granules generally depends on stress intensity and duration, and in important cases, low levels of stress cause condensation of protein constituents but not their stress granule accumulation. For example, heat shock in budding yeast leads to biochemically detectable condensation of certain proteins after 8 min at 37°C or 42°C and accumulation of certain proteins in cytosolic foci, but formation of classic stress granules marked by poly(A)-binding protein requires pushing temperatures to 44°C–46°C at this timescale [Cherkasov et al., 2013, Wallace et al., 2015]. Limitations of imaging techniques may contribute to this discrepancy to some degree (see our discussion of grand challenges below), and exciting developments of improved microscopy-based methods—such as lattice light-sheet microscopy or fluorescence cross-correlation spectroscopy—may help minimize these concerns in the future [Guillén-Boixet et al., 2020, Peng et al., 2020]. However, the

differential accumulation of protein factors at different levels of stress intensity [Grousl et al., 2013] rules out simplistic notions that, for example, stress granules are merely small at first and grow larger with intensifying stress. More evidence for an ordered assembly of stress granules comes from time-resolved proximity labeling experiments, which identified the interactome of the stress granule component eIF4A1 during heat shock of HEK293 cells [Padrón et al., 2019]. This study found that certain canonical stress granule components interacted with eIF4A1 before others. Thus, assembly proceeds in separable stages, ending with accumulation in large foci under severe stress.

The existence of assembly stages naturally raises the question: at what stages might specific functions be carried out? A deeper question haunting the field is: what do stress granules actually do?

2.4 Elusive functions of stress granules and stress-triggered RNA condensation

No commonly accepted function for stress granules yet exists. Many functions have been proposed, implicating stress granules in a range of roles, including sequestration of mRNAs and proteins, protection of mRNAs and proteins from degradation, promotion of enzymatic activities by increasing local concentration, minimization of cellular energy expenditure, and acting in translational quality control, signaling, and cargo delivery [Aronov et al., 2015, Buchan and Parker, 2009, Escalante and Gasch, 2021, Ivanov et al., 2019, Kedersha and Anderson, 2002, Kedersha et al., 2013, Mahboubi and Stochaj, 2017, Moon et al., 2020]. Stress granules have also been implicated in suppressing cell death by sequestering proapoptotic factors such as receptor of activated C kinase 1 (RACK1) [Arimoto et al., 2008, Tsai and Wei, 2010]. Similarly, a recent study found that stress granule formation suppressed pyroptosis, a form of cell death associated with inflammation, by sequestering the protein DEAD-box helicase 3 X-linked (DDX3X) [Samir et al., 2019]. However, the large variety of

functions proposed for stress granules, combined with some conflicting findings, have made it difficult to form an overarching model of stress granule function [Mateju and Chao, 2022].

For instance, an oft-speculated function for RNA condensation is transiently protecting transcripts from degradation during stress [Hubstenberger et al., 2017, Moon et al., 2019, Sorenson and Bailey-Serres, 2014]; however, other work finds no effect on mRNA half-life following stress granule inhibition [Bley et al., 2015]. Another model holds that RNA condensation contributes to selective translation of non-condensed transcripts. Stress-induced transcripts are often translated in the midst of global translational shutoff. Some transcripts that are highly translated during stress, such as HSP70 and HSP90, do not associate with stress granules, suggesting a connection between translation and escaping condensation [Kedersha and Anderson, 2002, Stöhr et al., 2006, Zid and O'Shea, 2014]. Certain translation initiation factors also condense, raising the possibility that a combination of protein and RNA sequestration can help promote selective translation during stress [Iserman et al., 2020, Wallace et al., 2015]. However, stress granules are not required for global translational shutoff; hence, this selective translation would occur on top of a more dominant effect [Escalante and Gasch, 2021]. Additionally, translation has been observed inside stress granules, complicating this model [Mateju et al., 2020].

A potential resolution to these conflicting results may be that particular functions are carried out at specific stages of organization. For example, stabilization of RNA by sequestration can conceivably occur at the premicroscopic condensate level, whereas other proposed functions may require collection of components into a larger and more molecularly diverse body (Figure 2.3). Hypothetically, a study in which perturbations block stress granule accumulation but not initial condensation, with no effect on RNA stabilization, would reach different conclusions than a study in which perturbations block both processes. An expanded understanding of assembly stages, a deepened grasp of the molecular drivers of these stages and a widened array of perturbations capable of targeting specific stages and

molecular determinants will be needed to sort out these questions.
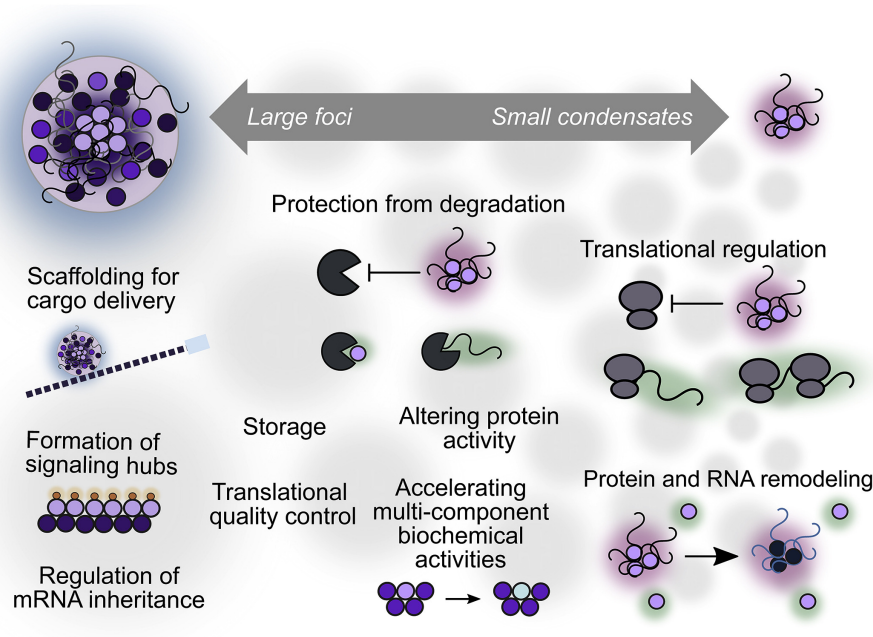
Figure 2.3: Formation of canonical stress granules (visible by standard microscopy, composed of a large number of components) may not be required for many attributed functions

Less discussed in the field are the issues inherent in studying biological phenomena whose functional contributions, if any, are unclear. Purification and reconstitution strategies, deprived of an activity-based standard for measuring success, must instead rely on morphological or compositional metrics whose relationship with biological function remains to be established [Begovich and Wilhelm, 2020, Freibaum et al., 2021]. The lack of functional insight is compounded by the remarkable lack of standard cellular phenotypes in the study of stress granules. Because not all of a given protein or RNA localizes to stress granules, determining a function must come from specifically perturbing condensation behavior without influencing activity, localization, or expression level. Even at the condensate level, phenotypes have been difficult to establish, although an allelic series of mutations that suppress poly(A)-binding protein's heat-triggered condensation in vitro and in vivo also suppress growth during heat stress [Riback et al., 2017]. The rarity of such phenotypes, particularly for stress granules, has led to a lingering question of whether stress granules may often simply be byproducts of

other cellular changes [Mateju and Chao, 2022].

2.5 Informing functions of stress-triggered condensation through the lens of disease

Some promising directions in uncovering stress granule function have come through study of disease contexts. Stress granules are induced by viral infection, where their formation has been proposed to help restrict viral replication [Eiermann et al., 2020]. In fact, many viruses have developed strategies for preventing stress granule formation by, for instance, sequestering or cleaving key stress granule components [Katoh et al., 2013, White et al., 2007]. What function do stress granules serve that viruses are so intent on disrupting? One possibility is that stress granules could sequester viral RNA, similar to their proposed function in storing cellular mRNAs [Burgess and Mohr, 2018, Law et al., 2019]. However, as discussed above, it is difficult to conclude whether recruitment of viral RNA to stress granules is required for proposed functions without mutations that specifically perturb stress granule formation while preserving separate molecular functions of stress granule components. One such perturbation comes from recent work showing that chikungunya virus promotes stress granule disassembly through the ADP-ribosyl hydrolyase activity of nonstructural protein 3 (nsP3) [Abraham et al., 2018, Akhrymuk et al., 2018, Jayabalan et al., 2021]. Removing this activity from nsP3 preserves stress granules during infection, providing a manipulatable system for future studies of stress granule function without deletion of any host machinery.

The stressful environments inhabited by tumors—such as nutrient deprivation, hypoxia, increased reactive oxygen species, and perturbed protein folding resulting from the dysregulation of metabolism and growth in malignancy—makes cancer biology a useful model for studying the functions of stress-induced condensation [Ackerman and Simon, 2014, Anderson et al., 2015, Clarke et al., 2014, Gorrini et al., 2013]. Moreover, certain chemotherapy drugs trigger cancer cells to form stress granules, which are generally thought to be prosurvival,

leading to condensation modulation as a potential target for therapeutics [Fournier et al., 2010, Gao et al., 2019, Kaehler et al., 2014]. In contrast, another chemotherapy agent, sodium selenite, triggers noncanonical stress granules lacking certain components whose stress granule localization has been linked to cell survival. These noncanonical stress granules have thus been suggested to be less functional in the stress response [Fujimura et al., 2012]. Additional work aimed at understanding the precise differences in stress-induced condensation between the considered prosurvival canonical and the noncanonical stress granules, at both the stress granule and premicroscopic condensate level, will help inform the functions of condensation in response to stress and perhaps even inform the importance of its organization at the size/spatial levels.

Further underscoring the potential role of condensation in the pathogenesis of cancer, recent work studying myeloid malignancies has shown that specific driver mutations upregulate stress granule formation, which is linked to increased stress adaptation and cancer development [Biancon et al., 2022]. Additionally, work with disease mutations related to neurodegenerative diseases suggests a relationship between maladaptive protein aggregates and adaptive condensates like stress granules, suggesting that maladaptive aggregates may occur when stress granules are not properly disassembled [Gal et al., 2016, Gwon et al., 2021, Mackenzie et al., 2017]. Even so, our understanding of these maladaptive protein aggregates will be limited without a deeper understanding of the function of adaptive condensates. Without understanding the functions of stress-induced condensation, we can only speculate on the pathophysiology of persistent stress granules.

Although many studies of stress granules focus on proteins which, when fluorescently tagged, are easily visible microscopically, RNA sits at the center of stress granule formation and function. We thus begin with a consideration of how our understanding of RNA's role has changed as new methods have come into use.

2.6 The role of RNA: Old observations and emerging results

The accumulation of poly(A)-RNA is among the defining features of stress granules. Moreover, the role of mRNA in stress granule formation has long been known. Among the most crucial experiments is the demonstration that translational inhibition affects stress granule formation in a mechanistically specific way: elongation inhibitors such as cycloheximide and emetine, which freeze ribosomes on mRNA, block stress granule formation, whereas puromycin, which prematurely terminates translation and frees mRNA of ribosomes, promotes stress granule formation [Bounedjah et al., 2014, Kedersha et al., 2000, Namkoong et al., 2018, Wallace et al., 2015]. Inhibition of transcription also inhibits stress granule formation [Bounedjah et al., 2014, Khong et al., 2017], further underscoring the role of RNA, at least at the accumulation stage.

However, which RNAs? How does RNA contribute to condensation and stress granule formation? To what extent does RNA drive condensation or accumulation and to what extent is it passively dragged along?

Early important results showed that prominent stress-induced mRNAs are selectively excluded from stress granules in both plant and mammalian cells [Kedersha and Anderson, 2002, Nover et al., 1989, Stöhr et al., 2006, Zid and O'Shea, 2014]. Because stress granules are, by most metrics, accumulation sites for translationally repressed mRNAs, and because it is both biologically appealing and empirically established in some systems that stress-induced transcripts are well translated [Preiss et al., 2003, Zid and O'Shea, 2014], these early results placed stress granules at the center of translational regulation during stress.

However, these foundational results have not survived into the recent era dominated by high-throughput studies, where transcriptome-scale effects can be observed. Modern studies do not find substantial depletion of stress-induced mRNAs from stress granules; instead, recent studies employing diverse approaches have converged on transcript length as the key correlate of mRNA recruitment to stress granules. Messenger RNA length is the dominant

correlate of their enrichment in the transcriptome associated with purified stress granule cores and stress-associated RNA granules [Khong et al., 2017, Matheny et al., 2019, 2021, Namkoong et al., 2018]; in *in vitro* systems, increasing RNA length promotes RNA/protein phase separation organized by the stress-granule hub G3BP1 [Guillén-Boixet et al., 2020, Yang et al., 2020], and single-molecule studies show that mRNA length correlates with the dwell time of mRNAs on stress granules and other condensed structures [Moon et al., 2019].

An increased concentration of ribosome-free mRNA following stress-induced translational shutdown is considered the key trigger for stress granule formation [Hofmann et al., 2021], and inhibition of translation initiation triggers condensation, such as in stress, eIF2α phosphorylation, or inhibition of the initiation factor eIF4A [Buchan et al., 2008, Iserman et al., 2020, Kedersha et al., 1999, Mazroui et al., 2006, Riback et al., 2017]) (Figure 2.4). This model is supported by several lines of evidence: (1) global translation initiation downregulation and subsequent polysome collapse is associated with RNA condensation during stress [Cherkasov et al., 2013]), (2) prevention of polysome collapse during stress blocks stress granule formation [Kedersha et al., 2000], (3) transfection of translationally arrested cells with free mRNA triggers stress granule formation [Bounedjah et al., 2014], and (4) inhibiting eIF4A, an essential translation initiation factor, promotes stress granule formation [Dang et al., 2006, Low et al., 2005, Mazroui et al., 2006, Tauber et al., 2020]. Alongside these data, early and still-current alternative models in which RNA length plays a minimal role exist. For example, stalled preinitiation complexes (PICs) that accumulate during stress may in part form the core of stress granules [Kedersha et al., 2002] (Figure 2.4).

Beyond ribosome-free RNA, a role of RNA length makes intuitive biophysical sense because the number of opportunities for either RNA-RNA or protein-RNA interactions—i.e., valence—naturally scales with length, all else being equal [Jain and Vale, 2017]. Evidence for a role from RNA-RNA interactions is circumstantial, resting on partial recapitulation of some stress granule transcriptome features in vitro using only purified RNA [Van Treeck et al.,
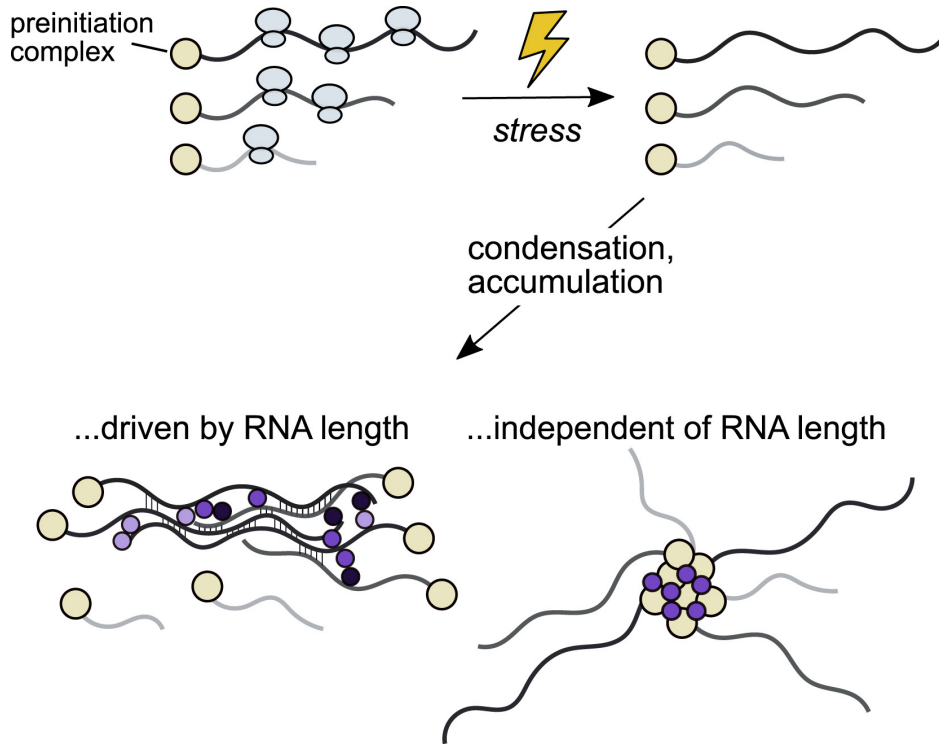
Figure 2.4: The mechanisms of stress-triggered condensation and stress granule formation remain an area of active inquiry

2018], the dependence of in vitro phase separation on long, unfolded RNAs [Guillén-Boixet et al., 2020, Yang et al., 2020] and RNA helicases [Tauber et al., 2020]. Further discussion of the available evidence supporting the roles of RNA-RNA or protein-RNA interactions can be found in several informative reviews [Campos-Melo et al., 2021, Hofmann et al., 2021, Ripin and Parker, 2022, Van Treeck and Parker, 2018].

Although a dominant role for RNA length is sensible biophysically, it is puzzling biologically. The overwhelming consensus holds that stress granules are accumulation sites for mRNA whose translation is suppressed during stress. However, the length-driven model (and existing results supporting it) suggests that induction of long transcripts during stress would be futile for protein production because long transcripts would be immediately recruited into translationally silent stress granules. However, although evidence that long transcripts are translationally silenced during stress after their stress granule recruitment is lacking, it has

been hypothesized that shorter transcripts may be associated with rapid responses, which could help resolve the paradox [Lopes et al., 2021].

However, an important caveat is that mRNA length is also a natural confounding variable in experiments and analyses. Sedimentation by centrifugation is employed in most transcriptome-scale studies aimed at isolating stress granule-associated mRNAs, mirroring the use of sedimentation in proteome-scale studies of stress granule-associated proteins [Cherkasov et al., 2015, Jain et al., 2016, Wallace et al., 2015]. However, unlike proteins, long RNAs, due to their size—an mRNA weighs roughly an order of magnitude more than the protein it encodes—will tend to sediment whether or not they are in a condensate. Consequently, comparing stress and nonstress conditions is crucial to determining the extra sedimentation due to stress. However, as others have pointed out [Namkoong et al., 2018], the original study [Khong et al., 2017] reporting yeast and mammalian stress granule transcriptomes, and reporting the profound effect of length, did not include nonstress controls. Long RNAs may stick nonspecifically to affinity reagents in pulldowns due to their valence or increased structure [Sanchez de Groot et al., 2019]. Although subsequent controlled work in mammalian cells has confirmed the accumulation of longer RNAs in granules following ER or oxidative stress [Matheny et al., 2019, Namkoong et al., 2018], the effects are more modest, and no nonstress control is yet available in yeast. Reduced translational efficiency (TE) has also been reported to be a major contributor to stress granule RNA accumulation. However, the two measures of TE used—codon optimality and ribosome density—have long been known to be inversely correlated with transcript length [Arava et al., 2005, Duret and Mouchiroud, 1999, Weinberg et al., 2016], raising the question of whether TE is a causal contributor to mRNA recruitment or a spurious correlation. Sedimentation-independent methods to examine recruitment of mRNAs, such as mRNA fluorescence in situ hybridization (FISH) in intact cells, have covered only a handful of targets [Khong et al., 2017, Matheny et al., 2019], reported only a modest stress granule recruitment effect from

length, and concluded that "length, per se, is not the major driving force in stress granule enrichment" [Matheny et al., 2021]. Large-scale, well-controlled, and systematic studies of the effect of length will be useful in resolving lingering uncertainty.

Given the sharp change in the apparent biology of RNA recruitment to stress granules from early to present-day studies, the limited set of transcriptome-scale studies available at this writing, and the challenging nature of isolating molecular components of functionally ill-defined structures, the RNA components of stress-induced condensates and stress granules will continue to be an area of intense investigation.

2.7   Mechanisms of dissolution

How do stress-induced RNA condensates dissolve after stress, as cells return to basal operations? Dissolution appears to be a regulated, controlled process that relies on specific proteins [Hofmann et al., 2021, Marmor-Kollet et al., 2020]. Proteins categorized as molecular chaperones and autophagic proteins have been implicated in stress granule dissolution, as have proteins associated with posttranslational modifications (PTMs) such as sumoylation, ubiquitination, and phosphorylation [Buchan et al., 2013, Cherkasov et al., 2013, Gwon et al., 2021, Keiten-Schmitz et al., 2020, Marmor-Kollet et al., 2020, Maxwell et al., 2021, Shattuck et al., 2019, Yoo et al., 2022]. Work in yeast has revealed that heat-induced (42°C) protein aggregates are entirely reversible, which is incompatible with autophagy and suggests that different fates occur in different stresses [Wallace et al., 2015]. Recent work shows that molecular chaperones can dissolve stress-triggered protein condensates orders of magnitude more efficiently than misfolded reporter proteins *in vitro*, suggesting that molecular chaperones may have evolved to interact with stress-induced condensates [Yoo et al., 2022]. Additionally, recent work in mammalian cells has shown that stress granules can be eliminated through either an autophagy-independent disassembly process or autophagy-dependent degradation, depending on the severity and acuteness of the initial stress [Gwon et al., 2021, Maxwell

et al., 2021]. This work suggests that the disassembly of stress granules is related to the initial stress, suggesting that different methods of assembly may require different methods of disassembly.

The kinetics of stress granule dissolution may be tied to a functional role, such as translational control. If stress-induced condensates are sites of storage, the contents must be disassembled in a timely manner. It has been proposed that stress granules dissolve in discrete steps, where an initial shell is pulled away followed by a core, with particular proteins being recruited at distinct stages [Wheeler et al., 2016]. Proteins necessary for cell recovery from stress, such as translation initiation factors, may need to be dispersed earlier than other stress granule core proteins that are dissolved more slowly. In fact, proper disassembly of stress granules was shown to be required for recovering cellular activities, such as translation, after stress [Maxwell et al., 2021]. The dissolution of stress-induced condensates may be related to maladaptive insoluble protein aggregates that are often associated with diseases, motivating a further understanding of the mechanism and function of dissolution [Hofmann et al., 2021].

However, as the function of stress granules remains unclear, the lack of functional assays demands careful experimental perturbations and cautious conclusions. For example, condensates that are no longer visible by microscopy may still occupy a conformation distinct from a monomeric form. New findings about the material state and assembly process of stress-induced condensates will illuminate the dissolution process, addressing questions such as whether the multiple steps of dissolution are equivalent to the stages of assembly or if a change in material state may lead to a different dissolution process. On this front, the role of LLPS in stress granule formation may have crucial consequences for how these structures dissolve.

2.8 Examining the role of liquid-liquid phase separation in stress-induced condensation

LLPS is a thermodynamically driven mechanism by which a solution of a compound demixes into a dilute and a dense phase above a certain critical concentration [Hyman et al., 2014]. A host of stress granule-associated proteins have been shown to undergo phase separation *in vivo* and *in vitro* [Guillén-Boixet et al., 2020, Iserman et al., 2020, Kroschwald et al., 2018, Molliex et al., 2015, Riback et al., 2017, Sanders et al., 2020, Yang et al., 2020], and it is widely held that stress granule assembly is driven by LLPS (reviewed in [Hofmann et al., 2021]). Recent work has converged on G3BP as a central node in LLPS-driven stress granule formation [Guillén-Boixet et al., 2020, Sanders et al., 2020, Yang et al., 2020]; however, G3BP is dispensable for stress granule formation in response to certain stressors, such as heat and osmotic shock [Kedersha et al., 2016, Matheny et al., 2021]. Thus, G3BP-focused models of stress granule formation may overly simplify the complex process of stress-induced condensation.

Using LLPS as an assembly mechanism provides key advantages beneficial for responding to stress. The ultra-cooperativity of LLPS enables proteins to precisely sense and respond to small changes in their environments [Yoo et al., 2019]. For instance, in yeast, Ded1 autonomously condenses in response to temperature stress. Ded1 from a cold-adapted yeast condenses at lower temperatures than that of *S. cerevisiae*, whereas Ded1 from a thermophilic yeast condenses at higher temperatures [Iserman et al., 2020]. This correlates with the fact that each yeast species has evolved to trigger its heat shock response relative to its environmental niche. Other key advantages of LLPS include that it enables passive (energy independent) cellular reorganization and that it is reversible. Following the removal of the stress stimulus, LLPS would no longer be energetically favored, and the system would spontaneously return to basal conditions.

Biomolecular condensation can result in the concentration of protein and RNA molecules

into phases with a variety of material states. How could a condensate's material state—how liquid-like or solid-like it is—affect its function? More solid-like condensates have been linked to disease, as pathogenic mutations of certain condensing proteins such as fused in sarcoma (FUS) increase aging and a loss of liquid-like properties over time [Patel et al., 2015]. This thinking extends to RNA condensates as well, as it has been proposed that RNA helicases prevent RNA-RNA entanglement to maintain a liquid-like condensed state [Tauber et al., 2020,]. Further, the viscoelasticity of the nucleolus has been linked with enabling the vectorial release of properly folded ribosomes [Riback et al., 2022]. However, the material state of stress-induced condensates does not appear to be widely conserved across eukaryotes, which like other evolutionarily variable features would usually be taken as evidence that the material state is not central to function. For instance, yeast stress granules are more solid-like than those of metazoa [Kroschwald et al., 2015], although there are methodological caveats [Wheeler et al., 2016]. Reconstituted heat-induced condensates of the yeast stress granule protein Pab1 are solids [Riback et al., 2017] that are not spontaneously reversible, although these condensates are readily dispersed by endogenous molecular chaperones [Yoo et al., 2022]. Even within an organism, pH-induced condensates of the yeast stress granule protein Pub1 are more liquid-like than those induced by heat shock—and only the heat-induced condensates depend on chaperones [Kroschwald et al., 2018]—yet both conditions are thought to be physiologically relevant.

The apparent lack of conservation of the material state can be rationalized when we consider that a condensate's material state appears irrelevant for many of the functions ascribed to stress granules. For example, if the role of stress-induced condensation is to temporarily store housekeeping mRNA to enable the preferential translation of stress-response messages, how liquid-like the storage compartment is may be of minor importance. Additionally, if the function is to sequester certain proteins to perturb a given signaling pathway in the cytoplasm, the key feature is to deplete the protein from the dilute phase, and the liquidity

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of the dense phase is less relevant. On the other hand, if the material state is particularly relevant for the potential pathogenicity of condensates, then the evolutionary pressures on material state in different organisms may differ substantially even if stress granules have a conserved cellular function.

2.9   Hazards in defining stress granule composition

Defining the composition of stress granules is complicated by a number of factors, even setting aside the existential problem of what constitutes a biologically important structure in the absence of well-established functions and phenotypes. Nevertheless, the obvious consistency and evolutionary conservation of the accumulation of some proteins and RNAs into large foci has led to a sustained effort to identify lists of molecular components involved in the lifecycle of stress granules. Individual mRNAs and proteins can be localized to microscopically visible foci of stress granule markers [Cherkasov et al., 2015, Khong et al., 2017, Mateju et al., 2020, Moon et al., 2019, 2020, Wallace et al., 2015, Wilbertz et al., 2019]. On a larger scale, the stress granule interactome has been defined using a variety of techniques, many of which rely on using individual stress granule components, such as poly(A)-binding protein, G3BP1, TIA1, and eIF4A, as bait proteins and then assessing the mRNAs and proteins that interact with that bait. The interactors have been identified through immunoprecipitations, purification of particles containing a bait fused to a fluorescent protein, and biotin proximity labeling [Hubstenberger et al., 2017, Khong et al., 2017, Namkoong et al., 2018, Padrón et al., 2019, Somasekharan et al., 2020]. Additionally, proximity labeling methods have found similar interactomes between stress granule proteins prior to stress and during stress [Markmiller et al., 2018, Youn et al., 2018]. This may indicate that stress granules are mainly stabilized by enhancements of basal interactions or that the interactions which distinguish stress granules are labile or refractive to these methods.

The different levels of organization in stress-triggered condensation and stress granule

formation, along with diverse methods whose relative accuracy can be difficult to establish, given the ill-defined nature of the target, combine to create a challenging experimental landscape (Figure 2.5). Unlike a membrane-bound mitochondrion or a relatively compositionally stable ribosome, stress-induced condensates and stress granules lack features that might simplify their description.
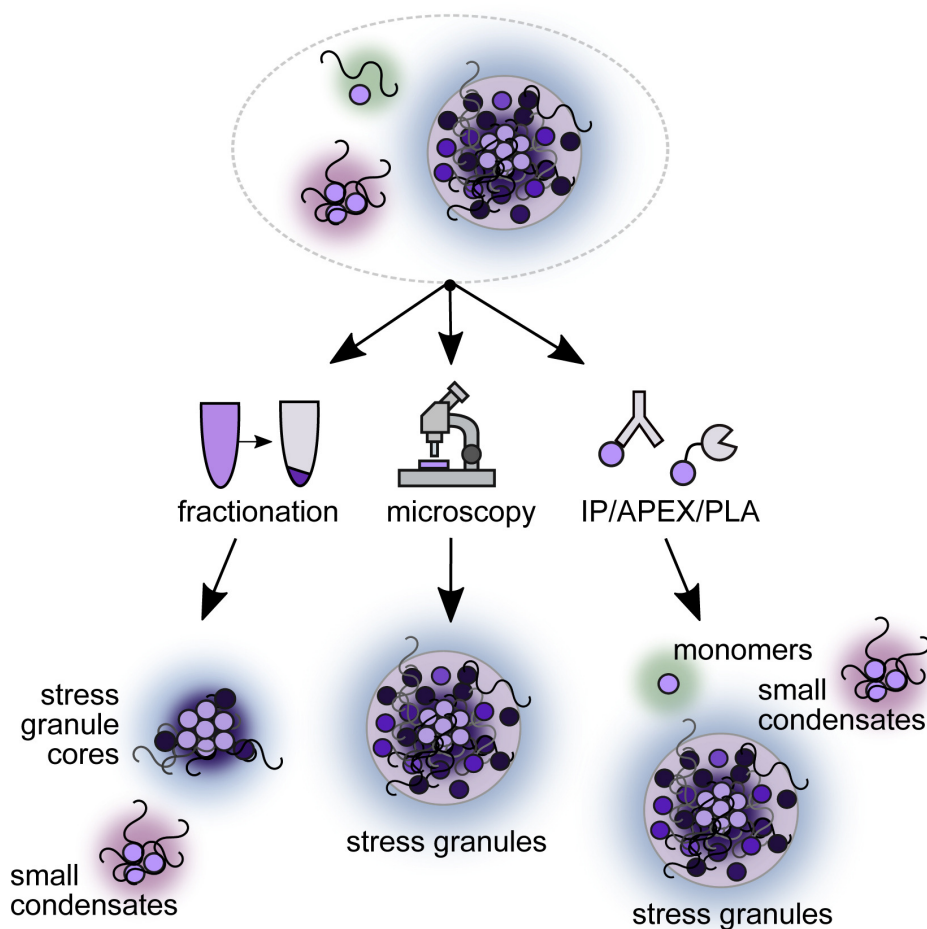
Figure 2.5: Different methods used to probe stress-induced condensation capture and report on different stages of stress-induced condensation and stress granule formation, providing complementary information

A hallmark of biomolecular condensation is that many of the components of the condensate individually associate through weak, dynamic interactions [Alberti and Hyman, 2021]. No biologically clear cutoff for interaction strength exists, making it unclear how to decide if

a given component is part of the structure or not. For instance, many transcripts have been observed to associate only briefly with stress granule proteins [Wilbertz et al., 2019]. How long must an mRNA reside at a stress granule to be considered a component? Additionally, consistent but weak associations may be lost during the isolation steps necessary for sequencing, mass spectrometry, or other biochemical methods. Perhaps, certain molecular components form a scaffold to which client proteins are recruited [Campos-Melo et al., 2021, Shiina, 2019, Zhang et al., 2019]. Differences in interaction strength may reveal biologically important differences; for example, major molecular chaperones associate with stress granules by colocalization [Cherkasov et al., 2013] but do not co-fractionate with stress-triggered condensates [Wallace et al., 2015]. Should such chaperones be considered a component of stress granules, merely associates, or something else? Here, again, functional assays would sharpen these distinctions in crucial ways.

Because stress granules are operationally defined as microscopic foci marked by specific proteins, the definition of the structure is unfortunately entwined with technical limitations and with compositional preconceptions. Failure to observe foci microscopically, for example, at low levels of stress, are consistent with two distinct biological possibilities: the absence of condensates entirely or the formation of structures below the diffraction limit which still retain key properties of larger condensates [Guzikowski et al., 2019]. Likewise, failure to observe colocalization with a specific marker molecule may reflect legitimate biological variation either in the marker itself or in the structure being marked.

Finally, the composition of stress granules is not static but depends on the nature of the stress and also changes over time [Aulas et al., 2017, Buchan et al., 2011, Padrón et al., 2019, Reineke and Neilson, 2019, Zhang et al., 2019]. Cells have evolved a variety of strategies to deal with changing environments. In the face of brief stresses, it may be advantageous to store transcripts until the stress has passed, allowing for a faster restoration of growth, whereas prolonged stress may necessitate more drastic reprogramming of cellular processes

[Arribere et al., 2011]. Consequently, deciding whether a molecular species is or is not a part of the stress granule transcriptome/proteome, reducing the problem to a yes or no, may obscure more biology than it illuminates.

2.10 Grand challenges in studying stress-induced protein/mRNA condensation

As is now apparent, stress granules and their molecular precursors represent an exemplary system in which field-level challenges find crisp expression. Here, we identify grand challenges in the study of these structures (Figure 2.6).
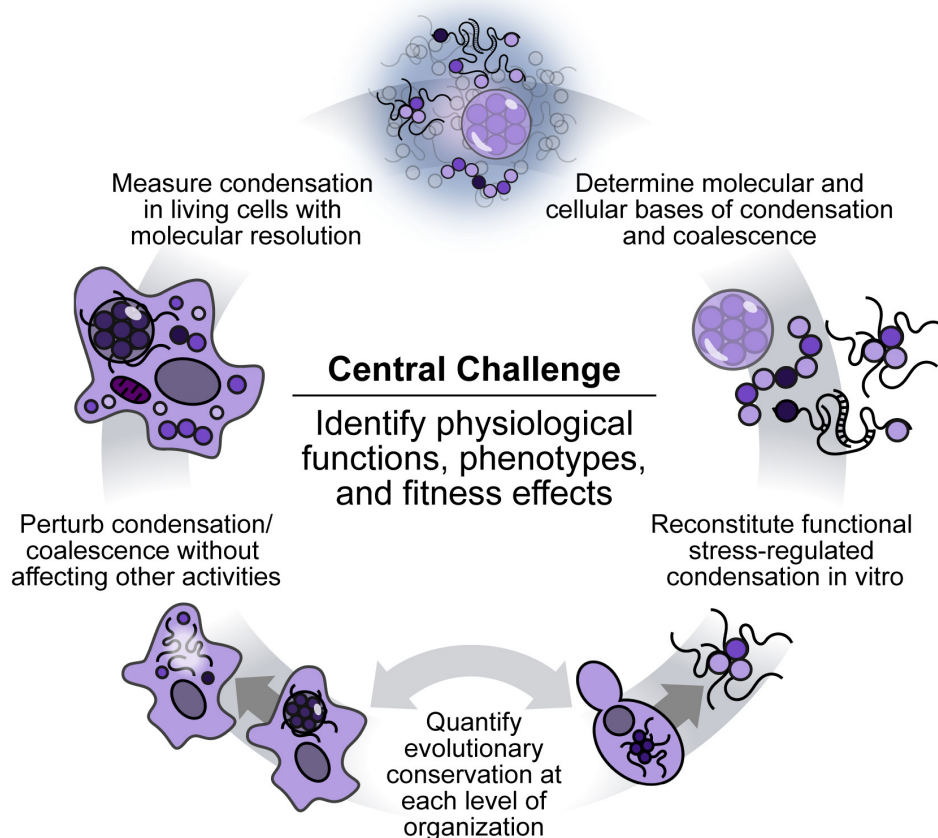
Figure 2.6: Grand challenges in the study of stress granules and stress-induced condensation

The first central challenge is to identify the functions of stress-induced condensates

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and stress granules and determine how these functions are executed. Of particular importance is the identification of fitness-related cellular phenotypes. The near-total reliance on molecular or imaging phenotypes, in the absence of function- and fitness-related phenotypes (growth, survival, differentiation, and activity), has become tolerated in ways that may hinder progress. For example, given that canonical stress granules only become microscopically visible during severe stress in some important cases [Grousl et al., 2009, Wallace et al., 2015], the reliance on microscopic methods may blind us to wide swaths of functional phenomena. In addition, the identification of a cellular phenotype would make it possible to design genetic screens that search for factors that are not just involved in focus formation but are integral to stress granule function.

Similarly, the use of inducers that robustly and reliably produce stress granules but are of uncertain physiological relevance, such as the broadly popular sodium arsenite, may have hidden disadvantages. If cells have not evolved to respond to a trigger, the cellular response is likely to lack organizational and molecular features that characterize responses to more physiological triggers such as heat, hypoxia, and osmotic shock. Even for these stresses, intensities that exceed physiological levels are in routine experimental use. Moreover, to validate a potent inducer such as sodium arsenite phenotypically against physiological inducers remains challenging until a phenotype or function of physiological stress granules is itself firmly established. Surmounting this central functional challenge will require sustained searches, a focus on physiology to match the extraordinary attention given to biophysics, and perhaps, new thinking to identify a set of standardized phenotypes for functional studies.

Surrounding this central challenge lurk many other intertwined grand challenges (Figure 2.6). Some are well established: determining the molecular bases of condensation and accumulation and measuring molecular-scale condensation in living cells. Success on the latter would allow us, for the first time, to observe all the stages of stress-triggered condensation *in vivo*, even under mild stress conditions where large canonical stress granules do not form

(Figure 2.2).

In attempting to discern the molecular determinants of condensation and stress granule formation, less discussed is the crucial difficulty—another grand challenge—of perturbing these phenomena cleanly, that is, without disrupting other activities. By analogy, study of an enzyme might involve, in order of decreasing disruption, a gene knockout, a temperature-sensitive mutation, a catalytic mutation, or development of a specific and reversible inhibitor. Despite considerable strides in this direction for stress granules (including screens for gene knockouts that disrupt stress granules [Yang et al., 2014]), at this moment, the search for clean perturbations remains almost entirely open.

In the absence of defined functions, another clear grand challenge looms: biochemical reconstitution of stress granule activities and functions. Reconstitution demonstrates the sufficiency of specific molecules and conditions to recapitulate cellular behavior. At present, all efforts have necessarily focused on reconstitution of traits without any unambiguous link to cellular fitness or adaptive function. Our situation in the stress granule field is remarkably different from historical efforts to purify specific biochemical fractions or molecules that could recapitulate an observed cellular activity.

Finally, the evolutionary conservation of stress granules provides powerful motivation for their study. However, how conserved are they? To what degree are the following conserved: specific components and stages, molecular determinants such as domains, biophysical forces, formation and dispersal pathways, regulators, and ultimate functions? Answering these questions would meet our final grand challenge (Figure 2.6). Serious efforts to use evolutionary approaches, and to move beyond a handful of model organisms, have the potential to dramatically accelerate progress in our understanding of these enigmatic structures and processes. To the extent that stress granules are not merely reliable side-effects of some other biological process, consistent contributions to cellular and organismal fitness will be the decisive factors in their preservation across the tree of life.

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These grand challenges underscore that the field of stress granule biology is at a pivotal point. As we approach the 40-year mark since stress granules were first observed in tomato plants [Nover et al., 1983], we are due to move toward a deeper understanding of stress granules. Armed with clearly defined challenges, we can tackle the fundamental unknowns that still remain. Massive parallel surges in our understanding of composition and assembly mechanisms, both cell-biologically and biophysically, appear poised to drive a positive feedback loop of research integrating studies of assembly at multiple biological scales, mechanistic studies of the impact of condensation on mRNA lifecycles, and finally, the fitness advantages that stress-induced condensation imparts.

CHAPTER 3

TRANSCRIPTOME-WIDE MRNA CONDENSATION PRECEDES STRESS GRANULE FORMATION AND EXCLUDES STRESS-INDUCED TRANSCRIPTS

This chapter has been adapted from [Glauninger et al., 2023], a manuscript in preparation, along with my co-first authors Dr. Jared Bard and Caitlin Wong Hickernell. Respective contributions: Dr. Edward Wallace completed the initial sedimentation experiment measuring transcriptome-wide condensation in *S. cerevisiae* following 42 or 46°C stress. I completed Sed-seq experiments on heat stress, azide stress, and ethanol stress. The 3 of us completed the degron-tag experiments. I designed and measured the solubility reporters. I completed the CHX, proteinase K, HAC1 RT-PCR assay Sed-seq experiments. Dr. Jared Bard completed the polysome profiling and inducible YONL construct experiments. Caitlin Wong Hickernell completed the microscopy in the work, both standard and single molecule.

3.1   Summary

Stress-induced condensation of mRNA and protein is widely conserved across eukarya, yet the function, mechanisms of formation, and how these clumps relate to massive stress granules remain largely unresolved. The release of ribosome-free mRNA following stress-induced polysome collapse is considered to be the trigger of stress granule formation by enabling various RNA-RNA and RNA-protein interactions along the body of a transcript. Here, we show mRNA condensation contextually excludes induced transcripts across diverse stresses, occurs even outside of stress, and mechanistically relates to specific interactions in competition with the translation initiation machinery. Stress-induced mRNA condensation is compositionally and mechanistically distinct from stress granule formation, implying a model that mRNA condensates are precursors which accumulate to form microscopically visible stress granules.

In stress, newly produced transcripts escape mRNA condensation and are translated, providing a simple mechanism by which cells can tune protein production to maintain homeostasis across distinct environmental challenges.

Highlights of this work:

Biochemical fractionation by sedimentation coupled with RNA-Sequencing (Sed-Seq) measures transcriptome-scale mRNA condensation during stress.

Virtually all pre-existing mRNAs condense following stress, in primarily a length-independent manner.

Stress-induced transcripts relatively escape condensation and are translationally up-regulated following heat shock.

mRNA condensation is observed outside of stress, including the well-studied endogenous gene HAC1.

Interactions in competition with translation initiation, rather than the production of ribosome-free mRNA, underlie mRNA condensation

Stress-induced mRNA condensation is context-dependent: different transcripts escape and are translationally upregulated in different stresses

Table 3.1: Highlights of advances in understanding mRNA condensation during stress

3.2 Introduction

All cells must be able to respond to changing environments to thrive. When faced with sudden maladaptive environmental changes–termed stresses, cells execute a gene expression program known as the heat shock response (HSR) [Morano et al., 2012, Gasch et al., 2000, Cotto and Morimoto, 1999]. Universally conserved across eukarya and occurring across a wide variety of stresses, ranging from physical to chemical, the HSR produces a set of molecular chaperones termed heat shock proteins [Lindquist, 1986]. Concomitant with the activation of the transcriptional HSR, stresses trigger both the clumping of mRNA and

protein into biomolecular condensates [Farny et al., 2009, Cherkasov et al., 2013, Hoyle et al., 2007, Khong et al., 2017, Protter and Parker, 2016, Nover et al., 1989, Riback et al., 2017] as well as translational reprogramming to privilege production of the heat shock proteins [Preiss et al., 2003, Lindquist, 1981, Verghese et al., 2012]. Although detailed understanding of condensation and translational reprogramming during stress is murky, it has been long proposed that stress-induced condensation could serve as a mechanism by which cells rapidly reprogram translation.

Similarly to the execution of the transcriptional HSR, stress-induced condensation of mRNA and protein occurs following exposure to a wide array of different stresses across eukarya [Wallace et al., 2015, Kramer et al., 2008, Nilsson and Sunnerhagen, 2011, Maruri-López et al., 2021]. Often studied in the context of microscopically visible foci termed stress granules (SGs), which are defined by the colocalization of poly(A)+ mRNA and specific protein markers into foci following stress, these enigmatic structures have received considerable attention since being discovered decades ago [Kedersha et al., 1999]. Although their function remains unknown [Glauninger et al., 2022], SGs have been labeled 'membraneless organelles' and considerable hypotheses have been made of their physiological roles, and more generally those of stress-induced condensates [Boeynaems et al., 2018, Brangwynne, 2013].

Stress causes global translational downregulation coupled with translational activation of transcriptionally induced mRNAs [Lindquist, 1981, Preiss et al., 2003]. Although protein condensation has been shown to promote stress message translation [Iserman et al., 2020], considerably less is known about the roles of mRNA condensation in reprioritizing translation. Stress-induced mRNA condensation has been hypothesized to play a role in housekeeping transcript storage and translational repression by sequestering mRNAs into SGs, yet these proposed functions remain controversial [Escalante and Gasch, 2021, Glauninger et al., 2022, Ivanov et al., 2019, Kedersha and Anderson, 2002, Mateju and Chao, 2022]. Contributing to the dearth of accepted roles of SGs in translational reprogramming are the

contradictory results regarding which mRNAs are enriched or depleted from SGs [Stöhr et al., 2006, Glauninger et al., 2022, Kedersha and Anderson, 2002, Khong et al., 2017].

Studies of mRNA recruitment to stress granules have not yet led to consensus about the identity of recruited mRNAs or the molecular bases of recruitment. Low-throughput work using microscopy-based approaches observed the localization of most poly(A)+ RNA to stress granules, while the stress-induced transcripts avoided recruitment [Stöhr et al., 2006, Kedersha and Anderson, 2002]. These early results have not survived more modern transcriptome-wide approaches, which have shown recruitment of a small proportion of the transcriptome and no privileged treatment of stress-induced transcripts [Khong et al., 2017, Namkoong et al., 2018]. Instead, length and translational status outside of stress dominate RNA recruitment [Khong et al., 2017, Matheny et al., 2021, 2019]. The importance of understanding the mRNA components of SGs is exemplified by the essential role that mRNA is implicated to play in SG formation.

The release of ribosome-free mRNA upon stress-induced translation shutdown is considered the 'universal trigger' of SG formation [Hofmann et al., 2021, Kedersha et al., 2000, Bounedjah et al., 2014]. The enrichment of long RNAs in SGs has been interpreted as evidence for valency- (and thus length-) dependent interactions along the body of an mRNA, such as with certain SG nucleator proteins like G3BP1/2 or RNA-RNA interactions between transcripts [Van Treeck et al., 2018, Yang et al., 2020, Guillén-Boixet et al., 2020, Sanders et al., 2020]. Yet, the SG transcriptome has been reported to be insensitive to the deletion of G3BP1/2, considered the central regulator of SG formation [Kedersha et al., 2016, Tourrière et al., 2003, Matheny et al., 2021]. Therefore, the biophysical and molecular bases of SG formation triggered by physiological stresses remain unclear.

Understanding the events and molecular interactions underlying SG formation is likely to illuminate the still-elusive functions of mRNA condensation. The model that SGs form as a consequence of promiscuous interactions by exposed, ribosome-free transcripts following

stress-induced translational collapse starkly contrasts with models that mRNA recruitment to SGs promotes translational reprogramming. Instead, this has led to the concept that SGs may represent maladaptive entanglements of RNA analogous to misfolded protein aggregates [Ripin and Parker, 2022, Tauber et al., 2020,]. Further challenges to a significant role of mRNA condensation in translational reprogramming include: 1) only 10% of mRNA is estimated to be recruited to SGs, 2) mutations which prevent SG formation do not prevent stress-induced translational shutdown, and 3) crucially, the SG transcriptome is thought to be unchanged in response to different stresses [Khong et al., 2017, Kedersha et al., 2016, Matheny et al., 2021]. It is harder to envision mRNA condensation contributing to translational reprogramming in response to an array of different stresses if the same transcripts are thought to condense irrespective of the environmental challenge. However, methodological concerns raise questions regarding the enrichment of long mRNAs in SGs, and it is possible that submicroscopic, SG-precursor condensates play a role in stress-induced translational reprogramming [Glauninger et al., 2022]. Thus, the functional roles of stress-induced RNA condensation, including potential translational reprogramming, remain an area of active research.

Which mRNAs condense, and what role does length play? To what extent is cytoplasmic mRNA condensation a stress-specific phenomenon? How does mRNA condensation relate to subsequent accumulation into SGs? And what is the function of mRNA condensation during stress? Here, using biochemical fractionation by sedimentation and RNA-sequencing (Sed-seq), we show that all pre-stress transcripts condense during stress regardless of their lengths, while stress-induced transcripts escape condensation and are robustly translated. We discover that certain endogenous transcripts are condensed before stress, only to be released upon heat shock for translational activation. mRNA condensation appears to be a distinct precursor potentiating SG formation. Although the mRNA condensation response is distinct across stresses, a surprisingly simple explanation rationalizes the differences. Follow-

ing stress exposure, newly transcribed transcripts escape condensation and are preferentially translated. Together, these results show that mRNA condensation occurs even basally outside of stress and is measurable before visible stress granules form, expanding the importance of understanding mRNA condensation for cellular physiology in and outside of stress.

3.3   Results

3.3.1   Sed-seq enables measurement of transcriptome-scale mRNA condensation

In this work, we measured condensation of RNA into heavy assemblies via biochemical fractionation by sedimentation coupled with RNA sequencing (Sed-seq) (Figure 3.1A). We collected and quantified transcript abundances in total, supernatant, and pellet fractions, and statistically estimated the proportion of each transcript in the supernatant (pSup) using Bayesian methods. Our assay is blind as to whether a pelletable RNA species localizes to a particular RNA granule, such as a SG or processing body, enabling an unbiased measurement of stress-induced RNA condensation. Additionally, we included the chelating agent EDTA to disassemble ribosomes in our lysis buffer to prevent polysome-associated mRNAs from sedimenting in addition to condensed mRNAs (Methods, [Wallace et al., 2015]). We note that after 10 minutes of heat shock Pab1-marked SGs form at 46 but not at 42°C [Wallace et al., 2015, Cherkasov et al., 2013]. Thus, to investigate stress-induced RNA condensation in both the absence and presence of SGs, we utilized heat shock at either 42 or 46°C for 10 minutes in *S. cerevisiae* as our model system.
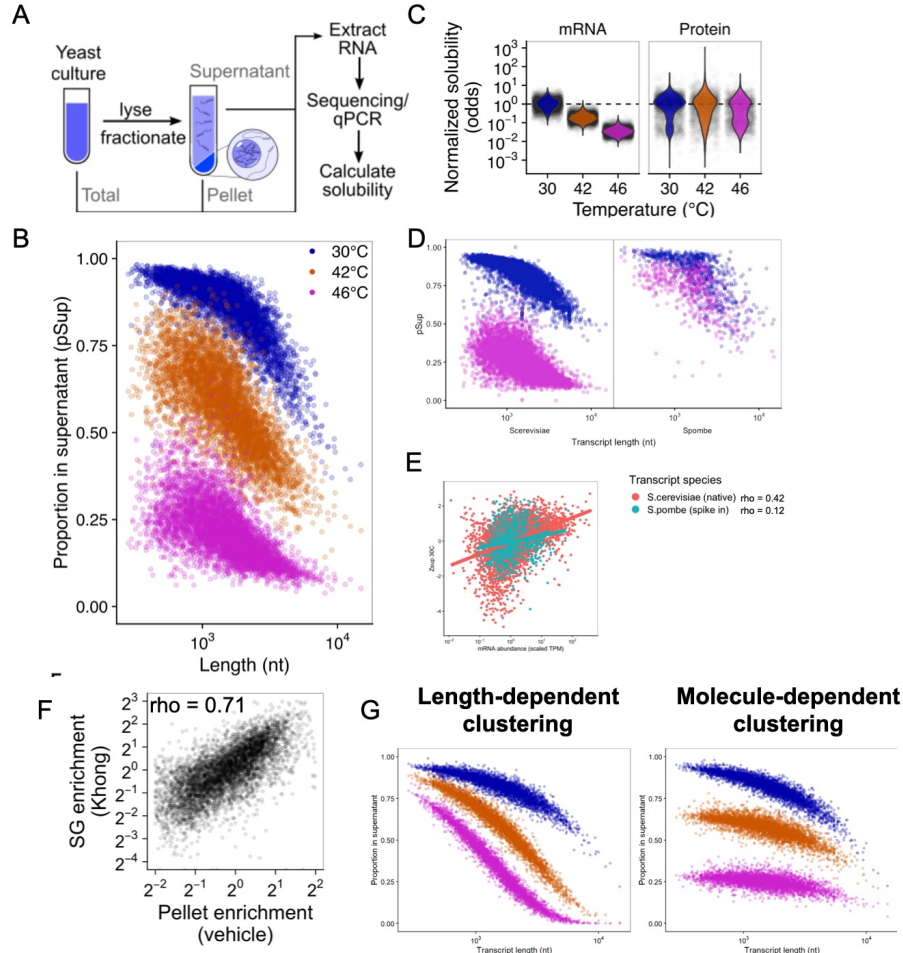
Figure 3.1: Measuring mRNA condensation under stress and non-stress conditions. A) Analysis of mRNA condensation by sedimentation and RNA-sequencing (Sed-seq) enables calculation of mRNA proportion in the supernatant (pSup) across conditions. B) Transcriptome pSup in unstressed (30°C) and temperature stressed (42/46°C, 10 min) conditions inversely correlates with length. C) In contrast to proteins—where only a subset (10%) exhibit stress-induced condensation, most mRNAs condense during temperature stress. D) Longer exogenous, spiked-in *S. pombe* mRNA possess lower pSup's, which indicates the length dependence of pelleting arises from the experimental sedimentation conditions, rather than intrinsic biology. E) Zsup 30°C analysis finds minimal correlation of relative pelleting and abundance of the spiked in *S. pombe* RNAs. F) Correlation of our vehicle pelleting to Khong 2017 SG enrichment azide suggests issues with previous, not length controlled, work. G) In silico modeling/ fitting finds a minor role of transcript length in mRNA condensation.

3.3.2   mRNA condensation of the entire pre-stress transcriptome following heat shock is proportional to the magnitude of stress

The sedimentation behavior of the transcriptome was obtained under unstressed and heat shocked conditions, and quantified as pSups for each gene across conditions (Figure 3.1B). The sedimentation of any biomolecule (including mRNA) is mass-dependent and not solely caused by condensation. For example, many long transcripts possessed pSups below 0.50—more than half the RNA ended up in the pellet—at 30°C (Figure 3.1B), a condition in which stress granules are not observed. We speculate that mRNAs sediment in part due to the huge mass of mRNP complexes. Thus, comparing sedimentation behavior between stressed and unstressed conditions is required to draw conclusions about stress-induced condensation.

Comparing the sedimentation behavior between unstressed and stressed samples leads to a few observations: 1) the pSup values of all mRNA species decreased following temperature stress (and a greater decrease is observed following the more severe 46°C stress) and 2) length-dependent sedimentation was observed under all conditions. We interpret the former observation as global condensation of the pre-existing transcriptome in response to temperature stress. Comparing the change in solubility of mRNA and protein (adapted from Wallace et al. [2015]) following temperature stress leads to a striking difference. As opposed to the protein case, where ~10% of the proteome exhibits stress-induced condensation, the entire transcriptome condenses following exposure to temperature stress (Figure 3.1C). Previous work concluded that only 10% of bulk RNA transcripts localize to SGs, and that the transcripts of less than 200 genes were >50% SG localized during arsenite stress [Khong et al., 2017]. The increased magnitude of the stress-induced pelletable condensates we observed leads us to speculate that smaller, not microscopically visible assemblies are the dominant condensed species in stress.

To further understand the observed length-dependence of sedimentation across conditions, we spiked exogenous *S. pombe* total RNA into stressed and unstressed lysate before

completing Sed-seq analysis (Figure 3.1D,E). In unstressed lysate, we observe a similar degree of sedimentation and length dependence at 30°C of both endogenous and exogenous mRNAs. In contrast, at 46°C the endogenous transcripts have decreased pSup's while the exogenous transcripts pSup's are largely unaffected. The most parsimonious explanation for these results is that the observed length dependence of pSup is unrelated to mRNA condensation but rather caused by intrinsic sedimentation differences of uncondensed mRNAs with different lengths/masses. Stress-induced condensation causes an additional decrease in pSup observed in stress conditions.

Previous work has concluded that transcript length is a major determinant of SG enrichment following azide stress in yeast [Khong et al., 2017]. Yet, this study did not include an unstressed control. Our observation of length-dependent pelleting in the absence of stress led us to ask how our unstressed pellet enrichment compared to the previously published yeast azide SG transcriptome (Figure 3.1F). With a ρ=0.71, the high correlation between our unstressed sedimentation and previous measurements of the SG transcriptome raises the question whether previous work concluding SG recruitment is length dependent was plagued by this length sedimentation artifact.

This conclusion is supported by the fact that the length-dependence of sedimentation in the stressed samples is similar to that of unstressed, where no stress granules are present (Figure 3.1B). The slight increase in the length dependence of pSup measured during stress is consistent with Matheny et al., where they find only slight enrichment of longer transcripts in the RNP granule fraction of stressed cells compared to unstressed [Matheny et al., 2019].

Simple computational simulations were completed to clarify the relative contributions of length-dependent and length-independent factors on stress-induced mRNA condensation. In one set of simulations, the likelihood of a transcript becoming crosslinked (condensed) during stress scaled with its length. On the other hand, in the length-independent case, all mRNAs had the same likelihood of crosslinking. After calculating crosslinking/ condensation,

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centrifugation was simulated using sedimentation theory (Methods and Figure 3.1G). By comparing the experimental data to these simulations, we observed that the experimental data matched more similarly to the length-independent clustering case: even the shortest messages are observed to have large pSup decreases following stress (Figure 3.1G). We thus conclude that stress-induced mRNA condensation minimally depends on transcript length.

What is the function of mRNA condensation in stress? While this initial analysis demonstrates that virtually all mRNAs condense during stress and rules out a dominant role for length-dependence in stress-induced mRNA condensation, how much specificity exists in the preferential recruitment or exclusion of certain transcripts?

3.3.3   mRNAs encoding stress response genes escape condensation during stress and are preferentially translated

Beyond the effect of length, what other factors influence the variation in mRNA sedimentation before and after stress? First, we observed a correlation between relative pSup between conditions: transcripts with greater-than-average or less-than-average pSups for their length in the unstressed control tend to maintain this relative positioning following stress (Figure 3.2A). This relationship is more easily seen when we quantify the length-independent variation in pSup's within each condition: we calculate the Z score for the amount of each mRNA in the supernatant relative to transcripts of similar length (termed Z_{sup}). Comparing the Z_{sup}'s at 30°C to those obtained at either 42 or 46°C reveals a considerable correlation in length-independent pelleting between conditions (Figure 3.2B). To identify which transcripts have the most divergent sedimentation behavior between stress and unstressed conditions, we calculate ΔZ_{sup} as the difference in Z_{sup}'s in stressed versus unstressed cells.

Certain mRNAs—those encoding the heat shock proteins—are transcriptionally induced following exposure to a wide array of different stresses. To what extent do stress-induced mRNAs condense like a typical transcript? To answer the above question, we plot ΔZ_{sup} versus
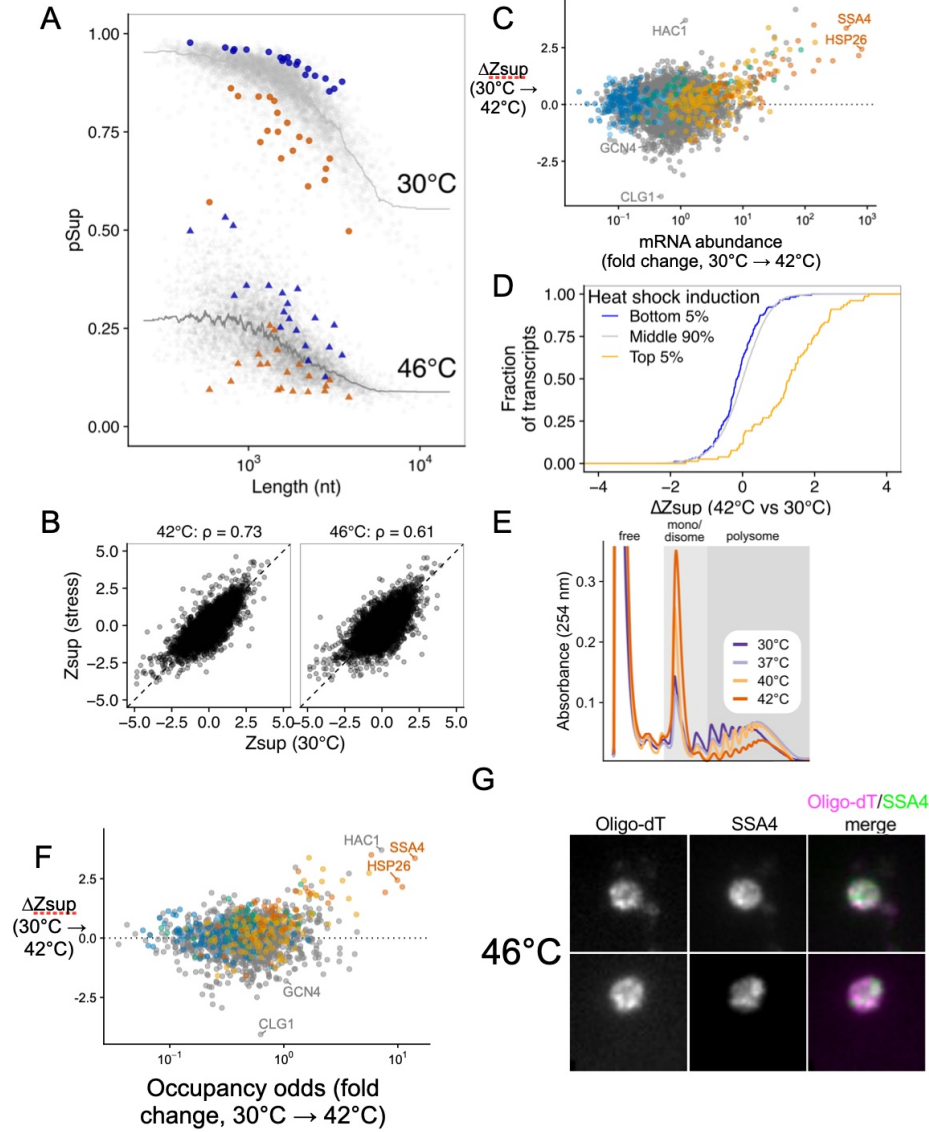
Figure 3.2: Stress-induced transcripts escape condensation following heat shock and are preferentially translated. Caption continued on next page.

mRNA abundance fold change in 42°C stress. Strikingly, transcripts that are transcriptionally induced during heat shock relatively escaped condensation (FFigure 3.2C-D). This observation was not Hsf1-specific. For example, mRNAs induced by the stress-responsive transcription factors Msn2/4 also escape condensation, proportional to their transcriptional induction. How do these biochemical condensation results connect to stress granule recruitment assayed microscopically? FISH imaging confirms that while the bulk transcriptome

Figure 3.2: Stress-induced transcripts escape condensation following heat shock and are preferentially translated. A) Relative sedimentation during stress is largely predetermined by unstressed sedimentation. Transcripts with pSup's well above (blue) or below (orange) the length-binned average trend line (grey line) during unstressed conditions (circles) maintain their relative solubilities following 46°C stress (triangles). B) Zsup 30°C correlates with Zsup 42 and 46°C. C) Fold change mRNA (20 min heat shock 42°C) correlates with ΔZsup 42°C vs 30°C (10 min), with the notable exception of HAC1, revealing that stress-induced transcripts escape condensation following temperature stress. D) ECDF plot showing stress-induced messages (top 5% fold change 42°C ← 30°C) escape RNA condensation (ΔZsup 42°C °30°C) relative to the remainder of the transcriptome E) Polysome profiling of unstressed (30°C) and stressed (37, 40, and 42°C, 20 min) yeast shows polysome collapse following temperature stress. Free, mono-/di-somal, and polysomal fractions were isolated for RNA-sequencing. F) Plot of fold change occupancy odds vs ΔZsup (42°C ← 30°C). Translation induction correlates with RNA condensation escape. HAC1 is no longer an outlier. G) FISH microscopy reveals poly(A)+ mRNA forms foci following temperature stress (46°C, 10 min), while stress-induced Ssa4 mRNA escapes SG localization.

(assayed as poly(A)+ RNA) localizes to foci, the mRNA of the heat shock gene SSA4 escapes stress granule recruitment (Figure 3.2G). These results are consistent with previous studies also utilizing low-throughput, microscopy based assays, which found that certain stress-induced transcripts encoding heat shock proteins escape stress granule recruitment [Kedersha and Anderson, 2002, Stöhr et al., 2006].

What is the functional significance of the condensation escape of stress-induced transcripts? Due to their lack of ribosome colocalization and formation in translation-limiting conditions, SGs are considered to be sites of translational arrest. To measure the translational state of the transcriptome, we utilized polysome sequencing of the free, mono-/di-some, and polysome fractions in and outside of stress to quantify ribosome occupancy (what amount of transcripts for a given gene are ribosome-associated rather than in the free fraction) (Figure 3.2E). To measure how the translational status of each mRNA is altered following heat shock, we calculate the fold change in occupancy in stress. In accordance with the global translational downregulation observed across stress, most mRNAs have an occupancy fold change below 1 (i.e. they are less ribosome associated during stress). The stress-induced

transcripts, which escape condensation, become translationally upregulated following heat shock (Figure 3.2F). The observed correlation between condensation escape and translational upregulation of the stress-induced mRNAs provides tantalizing evidence consistent with a role for mRNA condensation in translational reprogramming. But how does condensation mechanistically occur?

We note that a single transcript stands out as escaping condensation but not being transcriptionally induced: HAC1, which encodes the transcription factor controlling the unfolded protein response. We return to this apparent outlier later.

3.3.4 Widespread mRNA condensation, epitomized by HAC1, is observed outside of stress

To what extent are transcripts condensed basally? It is widely held that the increased population of ribosome-free mRNA during stress is the "universal trigger" of stress granule formation [Hofmann et al., 2021]. To address whether ribosome-free mRNA is sufficient to cause mRNA condensation in the absence of stress, we utilize the natural experiment comparing the pelleting behavior of two abundant translationally-repressed transcripts, HAC1 and GCN4. These are both well-expressed genes that are translationally silent during unstressed conditions [Weinberg et al., 2016]. If ribosome-free RNA is sufficient to trigger specific mRNA condensation even outside of stress, one might expect both HAC1 and GCN4 to be condensed during unstressed conditions, and thus be observed to have an extremely negative Z_{sup} 30°C (i.e. they sediment much more than expected based off their length). This does not align with the data.

Rather, while GCN4 sediments like a typical message of its length at 30°C, HAC1 possesses a pSup far below other transcripts of similar length (Fig 3A-B). We interpret this as HAC1 and not GCN4 being condensed under basal conditions. How can this be understood? We obtain insight from understanding how the mechanisms of translational repression differ

between the two messages. HAC1 is regulated through the presence of a base pairing interaction between its 5' untranslated region and its (uncleaved) intron, which blocks translation initiation until activation of the unfolded protein response [Sathe et al., 2015, Uppala et al., 2022, Di Santo et al., 2016]. On the other hand, GCN4 is repressed through four upstream open reading frames (uORFs) (Figure 3.3A) [Hinnebusch, 2005, Mueller and Hinnebusch, 1986]. Although the coding sequence of GCN4 is not being translated, the message itself is being initiated upon at the uORFs.

HAC1 relatively decondenses in heat shock, as its Z_{sup} increases from approximately -4 to 0 (Figure 3.3B). It is known that HAC1 is spliced and translationally activated following exposure to ER stress [Cox and Walter, 1996]. Further, HAC1 has been found to be minorly spliced after hours of growth at 39°C [Hata et al., 2022]. Could HAC1 be robustly activated following a more severe 42 or 46°C shock? To investigate this, we utilize an RT-PCR based assay measuring the relative proportions of spliced and unspliced HAC1. As a control, we demonstrate that the classic ER stress inducer DTT causes complete splicing of HAC1 (Figure 3.3C). Interestingly, we observe that HAC1 mRNA is mostly spliced following a 42°C, 10 min stress (Figure 3.3C), which suggests that the UPR becomes strongly activated during acute, severe heat shock. Comparing the relative amount of spliced and unspliced HAC1 in the supernatant and pellet fractions reveals that unspliced HAC1 mRNA possesses a lower pSup (is more enriched in the pellet fraction) than spliced HAC1 (Figure 3.3C, quantified in Figure 3.3D). Consistent with the observed splicing of HAC1, polysome profiling revealed that HAC1 mRNA is recruited to polysomes at 42°C (Figure 3.3E). This is consistent with re-analysis of published ribosome profiling datasets [Iserman et al., 2020, Gerashchenko and Gladyshev, 2014, Mühlhofer et al., 2019], confirming the translational activation of HAC1 following intense temperature stresses. Further supporting the functional activation of HAC1 in severe heat shock, we observe transcriptional induction of known HAC1-dependent UPR target genes (Figure 3.3F) [Kimata et al., 2006].
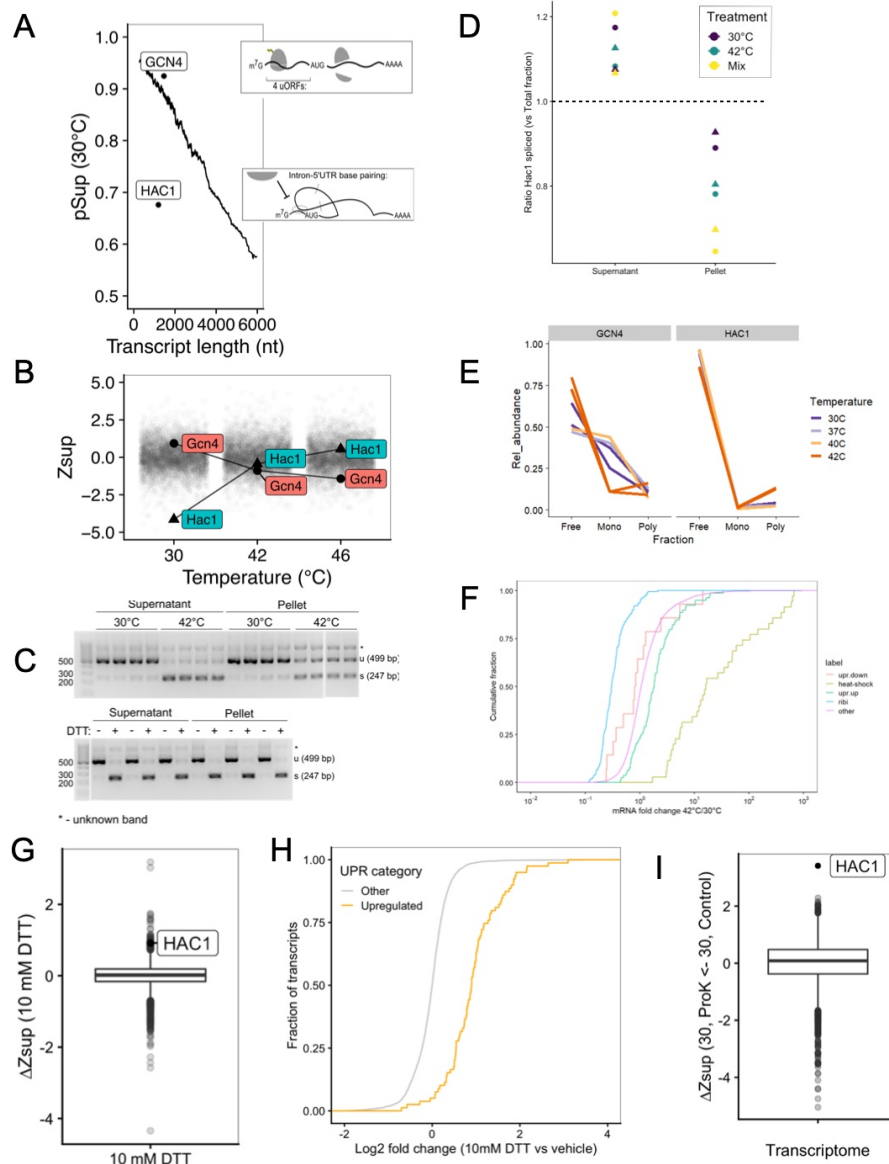
Figure 3.3: mRNA condensation is observed even outside of stress and is associated with blocked translation initiation. Caption continued on next page.

To further support that HAC1 decondensation correlates with its translational activation and UPR induction, we applied Sed-seq methodology to cells exposed to the ER stressor DTT. DTT treatment caused relative solubilization of HAC1 mRNA (ΔZ_{sup} DTT > 0.9, which is in the top 1% of all transcripts) without causing transcriptome-wide RNA condensation (Figure 3.3G). Additionally, UPR genes dependent on HAC1 for their induction were

Figure 3.3: mRNA condensation is observed even outside of stress and is associated with blocked translation initiation. A) We observe divergent pSup behavior of the basally translationally-repressed transcripts HAC1 and GCN4 during unstressed conditions. Although both mRNAs are translationally silent, HAC1 is blocked in translation initiation while the upstream open reading frames (uORFs) of GCN4 are actively translated. B) Zsup values of HAC1 and GCN4 at 30, 42, and 46°C show that HAC1 mRNA decondenses following temperature stress. C) RT-PCR measurement of HAC1 splicing status shows that HAC1 gets spliced following 42°C stress and that the unspliced isoform is relatively less soluble. D) Quantification of HAC1 splicing status shows that spliced HAC1 possesses a higher pSup than unspliced HAC1 under all conditions. E) Polysome profiling coupled to sequencing reveals that HAC1 stress-induced splicing and decondensation correlates with translational activation. F) The transcriptional UPR (based off categorization from Kimata et al) is activated during 42°C heat shock, which supports that HAC1 is functionally active as a transcription factor during temperature stress. G,H) Activating the unfolded protein response by treating with the ER stressor DTT causes HAC1 mRNA decondensation and UPR induction. I) HAC1 condensates at 30°C are dissolved by proteinase K treatment.

transcriptionally upregulated as a result of the DTT treatment (Figure 3.3H). These results lead us to conclude that HAC1 mRNA is basally condensed, and that ER stress causes its specific decondensation concomitant with its splicing and translational activation. However, what is the physical nature of the HAC1-containing condensate?

We next aimed to understand what type of interactions hold together the basal HAC1 condensate. We hypothesized that treating unstressed cellular lysate with proteinase K to degrade proteins may dissolve HAC1 condensates (Figure 3.3I). If this is true, we would expect HAC1 mRNA to become relatively solubilized compared to the remainder of the transcriptome. Consistent with this prediction, HAC1 possessed the single greatest ΔZ_{sup} (30°C, proK $\leftarrow$ control) of the entire transcriptome (Figure 3.3I). This indicates that the HAC1 condensate is held together by protein-dependent interactions, rather than RNA-mediated interactions.

In sum, these results indicate that translation initiation-blocked, unspliced HAC1 mRNA is astonishingly basally condensed and decondenses following splicing and translational activation. This provides an example of a well-studied, endogenous gene whose mRNA's trans-

lational status is linked to its condensation behavior. In conjunction with the widely appreciated observation that stress granules form under numerous conditions in which translation initiation is downregulated, this raises our key hypothesis: blocked translation initiation causes mRNA-specific biomolecular condensation.

3.3.5 Translation initiation block causes mRNA-specific and global condensation

To test whether specific condensation of a single mRNA species can be caused by blocking its translation initiation, we engineer exogenous solubility reporter constructs with variable 5'UTRs (Figure 3.4A). The 5'UTR hairpins have been adapted from previous work in which it was shown that inserting hairpins of increasing strength into a transcript's 5'UTR titratably decreases protein expression [Weenink et al., 2018]. Additionally, as a control for translational repression without blocking translation initiation, we generated two further reporters based on GCN4. The uORF reporter possesses the GCN4 5'UTR, while the uORF control reporter has the GCN4 5'UTR with 5 nucleotide substitutions that disrupt the start codons of the 4 uORFs to cause translation of the coding sequence [Mueller and Hinnebusch, 1986]. To validate the reporters, the steady state protein—by flow cytometry—and mRNA—by RNA-seq—levels were measured for each strain and used to estimate translational efficiency (protein per transcript) (Figure 3.4B). Together, these results indicate we have generated solubility reporters with various degrees of translation initiation, enabling us to ask: does blocking initiation of a transcript cause its specific condensation outside of stress? In fact, the medium and strong hairpin constructs were condensed basally without changing the pSup's of the bulk transcriptome (Figure 3.4C,D), demonstrating that blocking translation initiation causes transcript-specific mRNA condensation, even outside of stress. The pSup of the uORF reporter was comparable to that of the medium hairpin construct, but, importantly, the uORF reporter pSup was greater than that of the strong hairpin, which has a

similar degree of translational repression (Figure 3.4D). Therefore, mRNA condensation is particularly sensitive to blocking translation through initiation blocks. To expand these results, we tested to what extent global translation initiation block causes transcriptome-wide mRNA condensation.
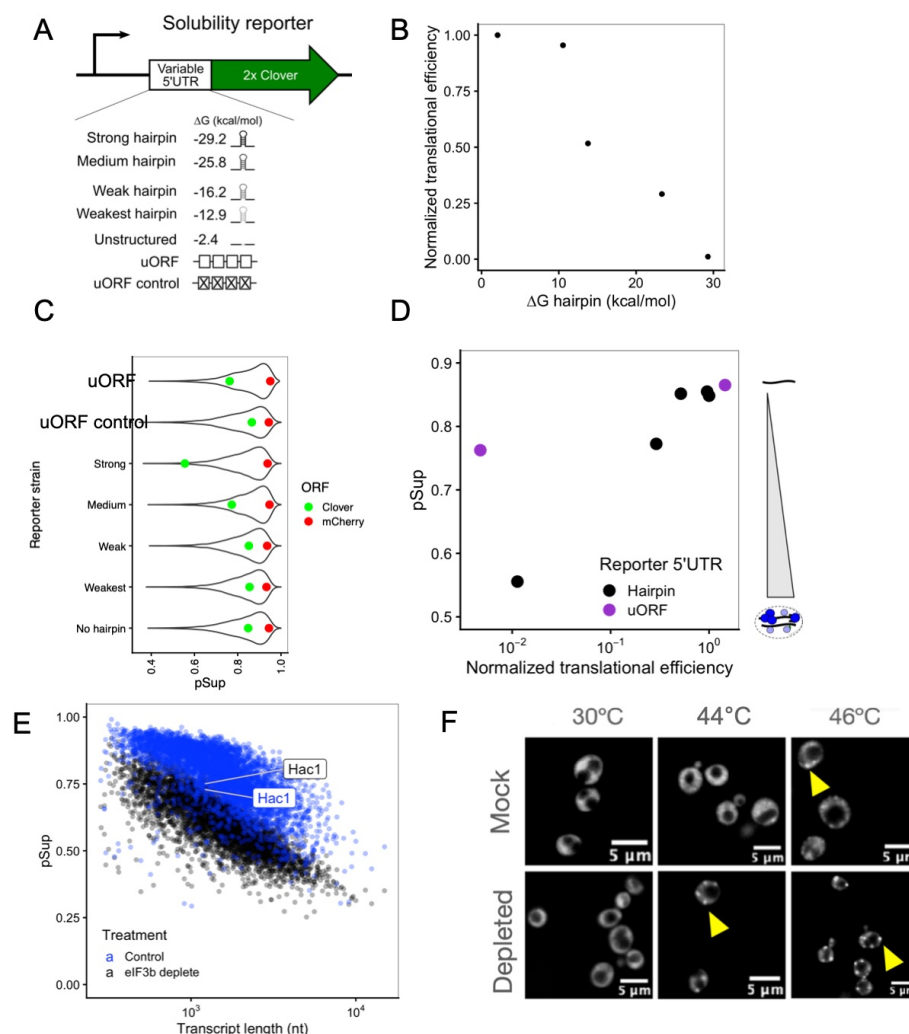
Figure 3.4: Translation initiation block causes mRNA-specific and global condensation. Caption continued on next page.

We tagged the essential initiation factor eIF3b with the auxin-induced degron system, enabling its targeted degradation to block translation initiation globally and completed Sedseq [Mendoza-Ochoa et al., 2019]. We find eIF3b degradation decreases mRNA pSup's across

Figure 3.4: Translation initiation block causes mRNA-specific and global condensation. A) Engineering solubility reporters with variable strength 5'UTR hairpins or uORFs enable testing how translation initiation block impacts RNA condensation. B) Flow cytometry measuring steady-state protein levels of the hairpin reporters shows that increasing hairpin stability decreases translational efficiency (steady state protein levels normalized by steady state mRNA levels), which is an estimate of translation initiation. Our convention is to represent increasing hairpin strength with positive ΔG values, predicted by RNAFold. C) pSup's of the solubility reporters in difference strains with mCherry control measured by Sedseq. D) Blocking translation initiation causes specific mRNA condensation, even outside of stress. Increasing hairpin strength causes condensation of the reporter transcript. uORF containing transcripts are less condensed for a given translation block. E) Depleting the essential translation initiation factor eIF3b causes transcriptome-wide mRNA condensation. Importantly, the mRNA of HAC1, which is already condensed prestress, does not have a further pSup decrease. F) Depletion of eIF3b potentiates SG formation. eIF3b depletion, although not sufficient to trigger Pab1-marked SG formation on its own, predisposes cells to form visible SGs at milder temperature shocks (44°C). More SGs are observed when compared to vehicle at traditional SG-inducing temperatures (46°C).

the transcriptome (Figure 3.4E). The HAC1 transcript, which is already basally condensed, maintained its pSup, which supports that its condensation is due to its translation initiation block. These results show that inhibiting translation initiation globally is sufficient to cause widespread mRNA condensation.

Finally, to study how mRNA condensation triggered by eIF3b depletion relates to stress granule formation, we depleted eIF3b and imaged the localization of the canonical SG-marker Pab1. It has been previously reported that pharmacalogically inhibiting the initiation factor eIF4A is sufficient to cause SG formation [Mazroui et al., 2006]. In contrast to this result, we observed that eIF3b depletion was insufficient to trigger Pab1-marked SGs under our conditions (Figure 3.4F). However, eIF3b depletion before stress caused formation of more SGs at 46°C and triggered SGs at lower temperatures at which Pab1 remained diffuse in the vehicle condition (Figure 3.4F-G). These data indicate that globally blocking translation initiation via eIF3b depletion causes widespread mRNA condensation which potentiates and intensifies stress granule formation.

3.3.6   mRNA condensates are related to, but distinct from, stress granules

To further clarify the relationship between biochemically isolatable mRNA condensates and microscopically visible SGs, we tested how blocking SG formation affects pelletable mRNA condensation. Pharmacological treatment with cycloheximide (CHX) has been widely shown to prevent stress granule formation, so we completed Sed-seq on vehicle and CHX pre-treated cells followed by temperature stress to see whether CHX blocks condensation. Because CHX acts by stalling ribosomes onto mRNAs, it was important to confirm that our sedimentation conditions were still able to release bound ribosomes from transcripts in the CHX-treated samples. We found that the sedimentation of CHX-treated samples at 30°C only caused a slight decrease in median pSup values, which indicates that polysomes are still able to be successfully dissembled following CHX treatment (Figure 3.5A,B). Thus, the Sed-seq methodology is validated for studying the CHX-dependence of stress-induced condensation. We observe that CHX pretreatment inhibits but does not prevent temperature stress-induced mRNA condensation (Figure 3.5A), demonstrating that a manipulation blocking SGs does not block condensation assayed biochemically. We conclude that, in contrast to SG formation, an increased population of ribosome-free RNA is not required for mRNA condensation, and posit that pelletable mRNA condensates are precursors to SGs.

3.3.7   Blocking translation initiation at distinct steps implicates an upstream, competitive step rather than a specific factor in initiation to cause condensation

Given the above findings, we next ask the related question of whether mechanistically blocking translation initiation at different stages has differential impacts on mRNA condensation. Depleting initiation at a certain step and observing condensation would be consistent with something upstream of the factor triggering condensation. By stalling translation initia-
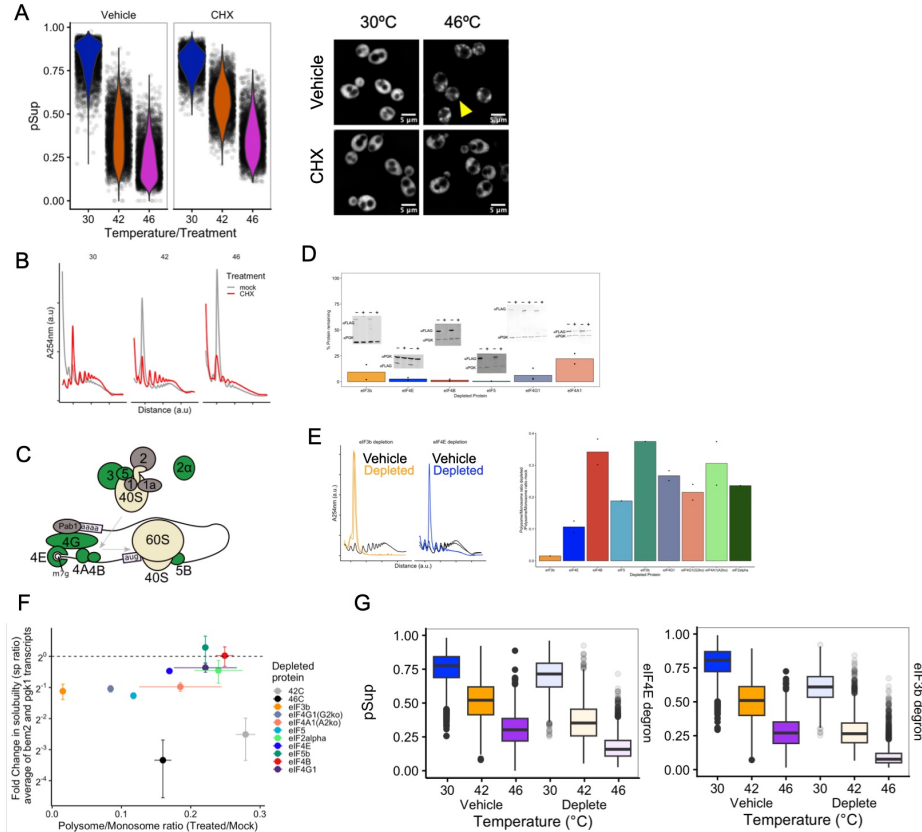
Figure 3.5: Specific interactions competing with translation initiation machinery—rather than ribosome-free RNA—cause mRNA condensation and are amplified during stress. A) Pharmacologically blocking polysome collapse reveals that, in sharp contrast to SG formation, ribosome-free mRNA is not necessary for stress-induced RNA condensation. Pretreating cells with cycloheximide (CHX) before temperature stress inhibits but does not prevent formation of biochemically-isolatable RNA condensates. B) Polysome profiling reveals CHX pre-treatment inhibits polysome collapse during heat shock. C) Diagram of translation initiation: showing which we depleted (green). D) Western blotting of strains engineered with degron tags on different translation initiation factors shows successful depletion of targeted initiation factors. E) Polysome profiling of the various degron strains reveals that initiation factor depletion causes polysome collapse, indicative of translation initiation downregulation. F) Plotting degree of translation downregulation following depletion (as measured by fold change polysome/monosome ratio (treated vs mock)) against degree of RNA condensation shows more translation initiation downregulation correlates with more condensation. Importantly, there is a threshold: some depletions cause large polysome collapse (>50%) without measurable condensation. G) Sed-seq analysis on eIF3b and eIF4E depletions followed by temperature stress. Both depletions cause mRNA condensation, which additional condensation occurring following stress. This implies stress-induced condensation is caused by more than just ribosome-free mRNA production.

tion downstream of the triggering step, a population of susceptible mRNA builds up and condenses. On the other hand, depleting initiation and observing that condensation is not triggered would imply that the condensing step occurs downstream of that factor. For example, preventing 40S scanning and observing condensation implies that the key step for triggering mRNA condensation cannot be 60S subunit joining to form 80S at the start codon, as translation initiation is blocked before that step occurs.

To stall initiation at distinct steps, we generated different yeast strains with degron tags on many key initiation factors (Figure 3.5C). Western blotting confirmed successful translation initiation factor degradation, and polysome profiling confirms that the treatments result in widespread polysome collapse across strains (Figure 3.5D,E). Depleting many initiation factors caused mRNA condensation in the absence of stress (Figure 3.5F). Because the different depletions will be differentially effective at blocking translation initiation, it is important to compare the degree of condensation to the degree of translational collapse (Figure 3.5F). These results find that, rather than implicating a specific step of initiation as responsible for mRNA condensation, there exists a general correlation between degree of translation initiation block and mRNA condensation, irrespective of which factor was depleted. Therefore, we conclude the species making an mRNA susceptible to condensation lies upstream of translation initiation and is in competition with translation initiation. The idea that something upstream of translation initiation causes mRNA condensation is not new. For example, the dominant model for SG formation is that ribosome-free mRNA is susceptible to condense. However, our model for how nontranslating mRNA mechanistically condenses is distinct, which we expand upon in the discussion. Importantly, it is clear that translation initiation block is insufficient to explain the degree of stress-induced mRNA condensation, indicating that models based purely on ribosome-free mRNA cannot explain the data.

3.3.8 Although blocking translation initiation causes mRNA condensation, the generation of translationally-stalled transcripts alone cannot explain stress-induced condensation

A key observation is that some degron-mediated initiation blocks halt translation to a much greater degree than temperature stresses while causing significantly less pelletable mRNA condensation and no visible stress granule formation (Figure 3.5F,3.4E,F). Therefore, the mRNA condensation observed in response to heat shock cannot simply be due to the associated downregulation of translation during stress. We posit that stress, in addition to generating translationally-inactive mRNA susceptible to condensation, also upregulates the condensation processes which target a translation-initiation-blocked mRNA for assembly. To further study the roles of translation dependent and independent processes on stress-induced mRNA condensation, we completed Sed-seq on eIF3b- or eIF4E-depleted samples followed by heat shock at either 42 or 46°C (Figure 3.5G). We find that the effect of either initiation depletions and temperature on mRNA condensation are additive. Translation initiation blocked yeast experience amplified transcriptome-wide mRNA condensation if they are subjected to a heat shock following depletion. This cannot be simply due to increased translational repression as a result of the heat shock, as eIF3b depletion alone causes unobservable translation, when measured by polysome profiling (Figure 3.5E,F). This is consistent with our aforementioned observations that pre-depleting eIF3b potentiates SG formation from heat shock. We therefore conclude that temperature stress causes mRNA condensation, in part, by increasing the susceptibility of nontranslating mRNA for 'sticky' assembly factors. In sum, temperature stress represses translation to generate a susceptible population of mRNA, the condensation of which is amplified by other stress-dependent processes.

3.3.9 Different mRNAs escape condensation in different stresses

To further clarify the roles of length and transcriptional induction in stress-induced mRNA condensation, we performed Sed-seq during other stresses (Figure 3.6A,B). These include treatment with sodium azide and ethanol, both robust SG inducers (Figure 3.6C). Further, azide treatment was used in early attempts to define the SG transcriptome [Khong et al., 2017]. Sedimentation profiling shows that both azide and ethanol treatments trigger transcriptome-wide mRNA condensation, with magnitudes that depend on stress intensity. Similar to our observations following exposure to heat shock, longer transcripts do not preferentially condense following ethanol or azide stresses (Figure 3.6B). Previous work has concluded that the SG transcriptomes in response to hyperosmotic and arsenite stress are "highly similar" [Matheny et al., 2021], raising the question of how distinct the condensation responses to temperature, ethanol, and azide are. Comparison of the ΔZ_{sup}'s obtained from from 42°C to those in response to 46°C, ethanol, and azide reveals that different transcripts escape RNA condensation in different stresses (Figure 3.6D). Perhaps as expected, the 2 temperature stress condensation responses are most similar (r = 0.78). In contrast, there is some similarity in the condensatome of ethanol stress (r = 0.57), and almost no relation to that experienced following azide stress (r = -0.02) (Figure 3.6D). With widely distinct condensation responses, can a common feature explain the context-dependent escape of certain transcripts across the 3 stresses?

Fascinatingly, similar to the heat shock case, the transcripts induced following azide or ethanol stress escape condensation, as measured by ΔZ_{sup} (Figure 3.6E). We reiterate that these results are surprising, given that transcriptome-wide surveys of SG recruitment have not reported the exclusion of stress-induced transcripts. Moreover, transcripts induced in a particular stress tend to escape condensation in that particular stress and not in other stresses (Figure 3.6F). Interestingly, the heat shock induced transcripts escape condensation in response to azide stress, which may reflect a shared aspect of the transcriptional
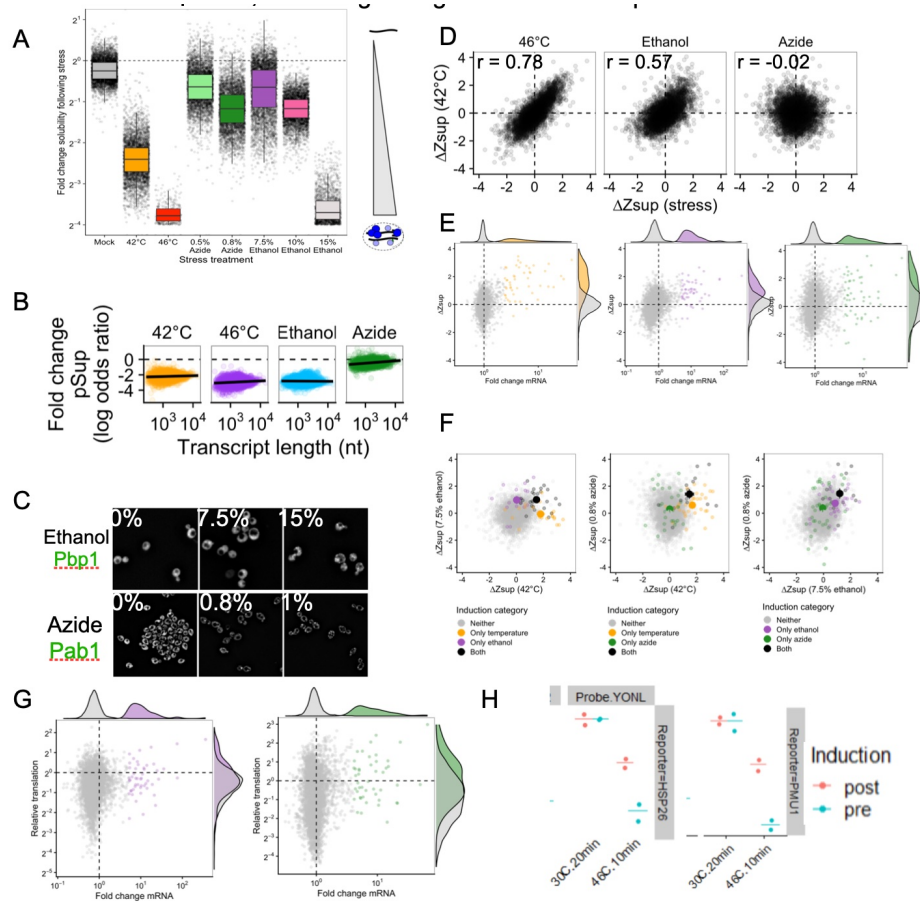
Figure 3.6: Stress-induced mRNA condensation is not length-dependent across various stresses; escape is stress-context specific, which argues against intrinsic sequence features determining mRNA condensation. A) mRNA condensation was measured in response to various stresses (temperature: orange, ethanol: blue, azide: green). B) Stress-induced RNA condensation is minimally length-dependent across a variety of stresses, including azide. C) Microscopy reveals formation of stress granules under these stress conditions. D) Stress-induced (ΔZsup's) weakly correlate across stresses E) Transcriptionally induced transcripts escape condensation (ΔZsup) across stresses. F) Transcripts which are induced in a stress tend to escape condensation in specifically that stress, unless they are induced in multiple stresses. G) Transcriptional induction correlates with translational upregulation in azide but not ethanol stress (fold change occupancy in polysome profiling). H) Newly induced exogenous, reporter transcripts are privileged to escape condensation.

responses between these stresses (Figure 3.6F). Further, just as in the heat shock case, we find that the transcriptionally induced transcripts, which escape condensation, are preferentially translated following either ethanol or azide stresses (Figure 3.6G). Our results that condensation recruitment differs across stresses argues strongly that biological context is

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a major determinant of mRNA condensation and may explain differences in translational regulation across stresses.

3.3.10   Newly transcribed mRNAs escape condensation during stress

How is the cell able to dynamically alter which mRNAs condense in response to different stresses? To ask to what extent the timing of synthesis relative to stress onset affects the condensation of an mRNA, we utilized an artificial reporter system enabling precise control of the timing of transcriptional induction. By inducing the YONL reporter either before or following the onset of stress, it can be studied whether the condensation behavior of the same transcript, by intrinsic nucleotide sequence, alters depending on if it pre-exists before stress exposure or is newly transcribed following onset. Indeed, we observed that the YONL reporter possessed a greater solubility following heat shock if it was transcriptionally induced after the onset of stress (Figure 3.6H). This is clear evidence that newly transcribed messages are privileged to escape condensation, and provides a surprisingly simple explanation for the divergent condensation behavior observed across stresses.

3.4   Discussion

What is the physiological role of mRNA condensation in and outside of stress? Which mRNAs condense during stress, and why? What is the relationship between mRNA condensation and stress granule formation? We have discovered that, across multiple stress conditions, the bulk of the transcriptome forms translationally silent condensates while stress-induced transcripts escape condensation and are robustly translated. Stress-induced mRNA condensation acts upstream of and in competition with translation initiation, and recency of transcription, rather than length—determines which mRNAs condense after stress. We find that certain messages are basally condensed, including the well-studied transcription factor HAC1, as a result of their translation initiation block. Stress-induced condensation of

mRNA is distinct from accumulation into microscopically-visible stress granules, mirroring the behavior of proteins [Wallace et al., 2015].

3.4.1 mRNA condensation into biochemically isolatable assemblies is related, yet distinct, from SG recruitment

The condensation of HAC1 and strong hairpin mRNA basally as a result of their translation initiation block clearly shows that mRNA condensation is not exclusive to stress. Observation of stress-induced mRNA condensation under conditions in which Pab1-marked SGs do not form (either the milder 42°C,10 min shock or following pharmacological block of SG formation) indicates that we are studying something similar yet non-identical to SGs. This is further supported by the fact that we see escape of transcriptionally induced transcripts, a result which is unclear in the SG literature, as conclusions that induced mRNAs escape SG recruitment from low-throughput, microscopy-based studies have not been recapitulated in recent transcriptome-wide studies [Stöhr et al., 2006, Kedersha and Anderson, 2002, Khong et al., 2017, Namkoong et al., 2018, Matheny et al., 2021]. Although we cannot rule out that sub-visible SGs are forming under our imaging conditions (a general limitation of microscopy-based approaches), that depletion of eIF3b triggers measurable condensation without observable stress granule formation further underscores this point. Given the potentiation of SG formation by eIF3b depletion and the intensification of RNA sedimentation in the more severe SG-forming 46°C stress, we posit that pelletable mRNA condensates are SG precursors (Figure 3.7). Further work is needed to clarify the relationship between pelletable assemblies and SGs. If pelletable condensation is a prerequisite for later accumulation into SGs, perturbations that prevent condensation measured biochemically are also expected to block SG formation microscopically.
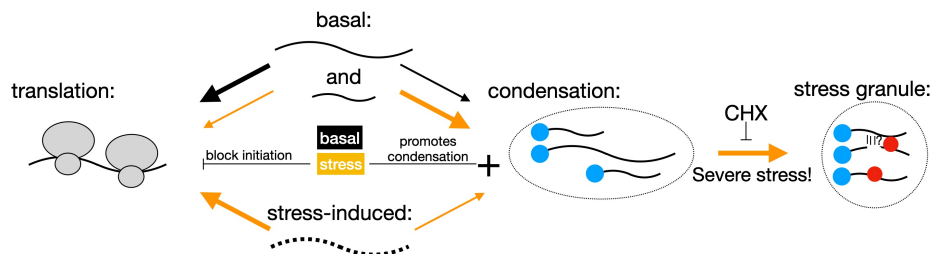
Figure 3.7: Transcriptome-wide mRNA condensation prepares the cell to focus translation on stress-induced mRNAs.

3.4.2 Rethinking the mechanism of mRNA condensation

Although SGs have been studied for decades, the mechanism by which an mRNA becomes recruited to these enigmatic structures remains unsolved. Current thinking dictates that SGs are triggered by an increase in concentration of nontranslating RNA following polysome collapse that are susceptible to intermolecular interactions, such as RNA-RNA interactions or binding by SG nucleator proteins like the well-studied G3BP1/2. Because these interactions occur along the body of the message and are more likely to occur on transcripts with more RNA available to participate, this model predicts a strong length-dependence to SG recruitment. Importantly, our finding that stress-induced mRNA condensation is length independent argues that interactions across the body of a transcript cannot determine condensation. Instead, we propose that mRNA is in competition between translation initiation and condensation, such as has been proposed for the competition between translation initiation and mRNA decay [Chan et al., 2018]. Our observation that stress-induced transcripts, irrespective of their lengths, escape condensation and are robustly translated across stresses supports this proposition.

Further, the condensation of HAC1 and strong hairpin 5'UTR mRNA outside of stress supports that mRNA condensation is primarily protein dependent and part of a specific, evolved regulatory regime. Under basal conditions, a haploid yeast cell possesses only ~15 molecules of HAC1 mRNA (out of ~30,000 total transcripts) compared to the ~3,000 mRNAs that are estimated to localize to SGs (10% of the transcriptome) in stress [Khong et al.,

2017]. It has been suggested that mRNA condensates form when the amount of ribosome-free RNA exceeds the cell's RNA chaperoning capacity, which leads to promiscuous intermolecular RNA-RNA interactions [Ripin and Parker, 2022]. In this framework, it is puzzling that much smaller numbers of specific molecules such as HAC1 or strong hairpin reporter mRNA are able to condense. This is underscored by the results that another basally ribosome-free transcript GCN4 does not condense and that HAC1 condensates are proteinase K sensitive. We speculate that the subsequent accumulation into microscopically-visible foci is mechanistically different, and may very well rely on RNA-RNA interactions. The clear differences in mRNA condensation and recruitment to SGs imply another question: do these assemblies serve distinct roles in the cell?

3.4.3   Towards the function of stress-induced condensation

The finding that condensation escape is stress-context sensitive strongly argues against current thinking that features independent of stress (such as transcript length or pre-stress translation levels) determine condensation, as these models predict enrichment of the same transcripts in SGs across stresses. Our updated model, in which different messages escape in different stresses, enables mRNA condensation to be dynamic in response to different cellular assaults. What could mRNA condensation functionally accomplish for cells? It has been long proposed that mRNA condensation could act to transiently store and repress translation of housekeeping genes to prioritize production of heat shock proteins.

eIF2α phosphorylation and condensation of translation initiation factors are two mechanisms by which stress can downregulate global cellular capacity for protein production. It is plausible this results in increased competition for the limited remaining protein synthesis machinery. To enable the rapid production of proteins encoded by stress-induced transcripts, the cell has elegantly evolved to rapidly sequester the bulk transcriptome in condensates, which simultaneously spatially separates them from ribosomes (ipso facto preventing their

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translation) and indirectly privileges stress-induced messages by reducing the competitive burden for the translation machinery. Consistent with this interpretation, our polysome sequencing data show the global translational downregulation of most transcripts coupled with the preferential translational induction of stress-induced transcripts in 42°C shock. Further, we hypothesize mRNA storage in condensates protects them from cellular nucleases, which could otherwise degrade nontranslating RNA, for later retrieval. Although the inverse correlation observed between mRNA condensation and translational activation is functionally exciting, further work is required to tease apart whether causality links the two, and, if so, in which direction (or perhaps in both) causality acts.

3.4.4   Transcripts of the Hsf1 regulon consistently escape stress-induced condensation to act as putative molecular timers for stress recovery

While different messages condense in different contexts, why has the cell evolved to induce and exclude transcripts of the Hsf1 regulon across a range of stresses such as following temperature, ethanol, and chemical insults? The Hsf1 regulon encodes molecular chaperones linked to the resumption of growth and the dissolution of SGs following stress cessation [Cherkasov et al., 2013]. Our group has previously shown that protein condensates formed following 42°C heat shock are resolubilized–not degraded–following stress [Wallace et al., 2015], and these chaperones are sufficient to dissolve physiological protein condensates formed *in vitro* [Yoo et al., 2022]. Because stress-induced condensates are made up of both mRNA and protein, it is plausible that the chaperone machinery also serves to disperse mRNA condensates, either directly or indirectly. Thus, we hypothesize that induction and condensation escape of the Hsf1 regulon is shared across stresses to time the dispersal of ubiquitously-triggered mRNA/protein clumps; they prime the cell for resumption of normal activities.

3.4.5 How do new transcripts escape stress-induced condensation?

What about newly transcribed transcripts enables their escape from condensation? Potentially, stress triggers the condensation of a "sticky" protein/complex that sweeps up preexisting mRNA upon stress induction. In this model, new mRNA escapes condensation because the "sticky" factor has already been sequestered in kinetically-trapped condensates. Although plausible, the fact that we see more condensation in the more severe 46°C stress argues that the "sticky" factor cannot be completely sequestered at 42°C. Rather, what if the active translation of stress-response messages mediates their escape?

The condensation of HAC1 and strong hairpin mRNA at 30°C are caused by their blocks in translation initiation, and we demonstrate that specific global blocks in translation initiation–we believe dependent on eIF4E-mediated interactions–are sufficient to cause transcriptome-wide mRNA condensation. Further, it is widely appreciated that the downregulation of translation initiation correlates with the condensation of RNA across a variety of stresses. Perhaps, the robust translation of the heat shock messages explains their escape from condensation. By constantly going through cycles of translation initiation, "sticky" eIF4E-dependent interactions are unable to accumulate. This sets up our positive feedback model connecting mRNA condensation escape and preferential translation.

Initially, stress-induced mRNA condensation mops up the bulk transcriptome, leaving the translational machinery primed to translate new mRNAs. The newly-transcribed stress-induced messages are produced and preferentially translated, as compared to the sequestered pre-existing messages. By being actively translated, the stress messages avoid condensing themselves. This self-reinforcing loop provides a simple explanation for the data and can be easily tested in future studies. We propose the stress-induced messages contain additional "marks" or features (such as being bound by certain proteins, possessing mRNA modifications, or having unique sequence motifs) that reinforce their privileged translation and condensation escape, although evidence demonstrating this remains to be uncovered.

3.4.6 Final thoughts

Future studies will address whether condensed mRNA is destined for retrieval and rapid translational resumption when stress ends, uncover the specific proteins and interactions contributing to RNA condensation, and dissect functional differences and the mechanistic connection between pre-microscopic condensates and large foci. Previous work supports that SG formation plays a minor role in stress-induced translational reprogramming: only a small fraction ($\sim$10%) of the transcriptome localizes to SGs, and SG formation is dispensable for translational attenuation during stress [Mateju and Chao, 2022, Khong et al., 2017, Kedersha et al., 2016]. In contrast, we suggest that widespread, microscopically hidden mRNA condensation of the pre-existing transcriptome contributes to privileged translation of new, stress-induced messages.

3.5 Methods

3.5.1 Stress treatments

Unless otherwise noted, BY4741 yeast was used in experiments. Cells were grown overnight at least 12 hours to OD600 = 0.4 with SCD before being exposed to stress. Temperature stresses were completed by centrifuging the culture and exposing the yeast pellet to either 42°C or 46°C water baths. Control cells were placed inside a 30°C incubator. Azide stresses were completed at either 0.5% w/v or 0.8% w/v for 30 min in media that was at pH 6.8. Azide was added from a 10% w/v azide stock in water. Mock treatments were completed by adding pure water at the same volume to cultures. Ethanol stresses were completed by resuspending centrifuged cell pellets in SCD with either 5%, 7.5%, 10%, or 15% ethanol for 15 min. Control cells were mock treated and resuspended in SCD.

3.5.2   Biochemical fractionation by Sed-seq

Biochemical fractionation was completed similarly to Wallace et al. [2015], with the major exception that 20,000 g for 10 min was used rather than the original 100,000g for 20 min. In short, cells were harvested by centrifugation and then lysed via cryolysis. Lysate was resuspended in soluble protein buffer (SPB: 20 mM HEPES, pH 7.4, 140 mM KCl, 2 mM EDTA, 0.1 mM TCEP, 1:200 protease inhibitor, 1:1000 Superase Inhibitor). The cells were fractionated and a total, supernatant, and pellet sample was taken. RNA was isolated using Zymo RNA extraction columns, and RNA integrity was assessed by gel. RNA was analyzed by RNA-seq or qPCR.

3.5.3   Modeling: calculation of pSup

pSup fitting was completed as in Wallace et al. [2015]. In short, a Bayesian fitting approach was used with the RSTAN package, in which the relative abundances (counts) from the total, supernatant, and pellet fractions were used to calculate mixing ratios such that $S + P = T$.

3.5.4   Confocal microscopy

Standard confocal microscopy was completed as in Wallace et al. [2015], generally using Pab1-GFP as the SG marker unless otherwise noted.

3.5.5   Auxin induced degron depletions

Auxin induced degron depletions were adapted from the approach in Mendoza-Ochoa et al. [2019]. In short, the endogenous protein of interest was genetically engineered to contain the degron tag in a strain of yeast in which the TIR1 ligase had been genetically integrated. Beta-estradiol was used to induce TIR1 expression, and then auxin was added to trigger depletion. After 2 hours of auxin exposure, cells were temperature treated and then harvested as normal.

3.5.6 Solubility reporters

Solubility reporters were engineered using the Yeast Toolkit [Lee et al., 2015]. Variable 5'UTRs were engineered depending on the construct and genetically integrated into the Leu locus. Each strain also possessed an identical copy of an mCherry construct, as a control.

Steady state protein levels were measured using flow cytometry, and steady state mRNA levels were measured by RNA-seq. Translational efficiency was estimated as steady state protein level divided by steady state RNA level.

The standard Sed-seq protocol was used to measure the condensation behavior of each strain.

CHAPTER 4

THERMODYNAMIC SPECIFICITY CONTROLS PAB1 CONDENSATION ACROSS TEMPERATURES AND ORTHOLOGS

This work builds off a project started by Dr. Ruofan Chen studying the structural dynamics of Pab1 condensates using HDX-MS [Chen et al., 2022]. My temperature-dependent condensate morphology work will be published as part of a revision of Chen et al. [2022], which is currently a preprint. The Pab1 ortholog work was completed in collaboration with Dr. Samantha Keyport Kik, who purified Pab1 protein from *S. cerevisiae*, *S. kudriavzevii*, and *K. marxianus*, and the ortholog HDX-MS data will be published in Keyport Kik et al. [2023]. I am graciously indebted to Isabelle Gagnon who purified the stickerless Pab1 RRM123 construct.

4.1 Summary

Stress-induced condensation occurs in response to a variety of environmental insults across eukarya. Yet, due to their heterogeneity and intractability towards typical structural approaches, little is known about the actual structures and formation mechanisms of condensates. Poly(A)-binding protein (Pab1 in budding yeast) is a canonical stress granule marker, whose condensation acts as a physiological stress sensor. Our recent work has found that Pab1 condenses by a sequential activation and partial unfolding mechanism of its RNA-recognition motif (RRM) domains [Chen et al., 2022]. We proposed the concept of thermodynamic specificity wherein the free energy surface of the RRM controls its activation and participation in the condensation process. Here, we show using hydrogen-deuterium exchange mass spectrometry (HDX-MS) that Pab1 condensates formed at different temperatures exhibit different degrees of RRM activation and participation in condensation. Further, Pab1

orthologs with different condensation temperatures have RRM stabilities tuned to their activation thresholds, leading to differences in the condensate. Experimental demonstration that different stress temperatures generate different condensate structures and that RRMs from Pab1 orthologs with different condensation temperatures have different activation thresholds provides additional evidence supporting the role of thermodynamic specificity in Pab1 condensation.

4.2 Introduction

4.2.1 Molecular mechanisms of stress-induced condensation

Poly-A binding protein (Pab1 in budding yeast) is a canonical stress granule marker that localizes to stress granules following exposure to a variety of different stresses [Riback et al., 2017, Wallace et al., 2015, Buchan et al., 2011, Wheeler et al., 2016]. Because purified Pab1 protein condenses autonomously *in vitro* when exposed to physiological stress conditions and its condensation has been shown to promote cellular fitness in response to prolonged stress *in vivo*, Pab1 is a physiological stress sensor [Riback et al., 2017]. Due to their heterogeneity and intractability to typical structural approaches, the molecular mechanisms and structural contacts underlying condensation have remained engimatic. However, recent exciting work using HDX-MS has uncovered the mechanism by which Pab1 condenses [Chen et al., 2022].

In contrast to many condensing systems where low complexity regions are responsible for condensation [Martin and Mittag, 2018, Lee et al., 2022], Pab1 condensation requires its folded RNA-recognition motifs (RRMs) and is only modulated by its low-complexity P domain [Riback et al., 2017]. Pab1 condenses by a partial unfolding and sequential activation mechanism, in which its RRMs partially unfold upon temperature increase into an activated, condensation-competent state to form heterogenous interactions with other activated RRMs on the same or different protomers [Chen et al., 2022]. Each RRM has a different

propensity to activate, which is governed by its thermodynamic stability, a concept termed thermodynamic specificity [Chen et al., 2022]. Not only is the concept of thermodynamic specificity exciting due to its potential broad applicability to other condensing systems, but also because it predicts different condensate morphologies in response to different severity temperature treatments.

We posit that thermodynamic specificity causes differing condensate structures when formed at different temperatures. Because each RRM has a different activation threshold, milder stresses may not be sufficient to activate the most stable RRMs, leading to a condensate with a lesser degree of crosslinking between protomers [Chen et al., 2022]. HDX-MS provides insight into the structural dynamics of a biomolecular system [Englander et al., 1972, 1996], requiring low sample quantity and applicable for study of protein assemblies [Masson et al., 2019, Ozohanics and Ambrus, 2020]. Moreover, HDX-MS has previously been used to study Pab1 condensation mechanism [Chen et al., 2022]. Therefore, HDX-MS is the ideal method to study how Pab1 condensate structure varies following different condensation temperatures.

4.2.2   Stress-induced condensation across environmental niches

Organisms have evolved to thrive in a swath of environments, ranging from exposure to extreme temperatures to harsh chemicals [Gostinčar et al., 2009, Gunde-Cimerman et al., 2003, 2009, Zeikus, 1979]. An environment that is considered "stressful" to one organism could be the optimal growth condition for another. Therefore, organisms that thrive at different temperature niches may be expected to execute their heat shock responses (HSR) at different temperatures. In fact, this has been shown to precisely be the case for three fungal species, the psychrophilic *S. kudriavzevii*, the mesophilic *S. cerevisiae*, and the thermophilic *K. marxianus* [Keyport Kik et al., 2023]. Not only do these organisms execute their HSRs at different temperatures, but their condensation responses have also evolved to occur relative to

their optimal growth temperatures. Fascinatingly, *in vitro* reconstitution of Pab1 from each fungal species found that the different condensation onset temperatures were autonomously encoded in the primary sequence of each protein.

How primary sequence encodes condensate onset temperature of Pab1 across orthologs is unknown. Furthermore, whether the mechanisms and resulting structures of orthologous Pab1 condensates are conserved is unknown. I hypothesize thermodynamic specificity underlies Pab1 condensation across orthologs. By changing the intrinsic stabilities of the RRMs, the activation thresholds for Pab1 condensation can in theory be adjusted to an organism's relative niche. Here we use HDX-MS to investigate the conservation of Pab1 condensation across species, and provide a rational, biophysical explanation as to how Nature tunes the condensation onset temperature of Pab1 across organisms.

4.2.3 Hydrogen-Deuterium Exchange Mass Spectrometry

HDX-MS is an exquisitely sensitive tool that can be applied to study challenging biomolecular systems, including protein complexes, amyloid fibrils, and Pab1 condensates [Sun et al., 2021, Kheterpal et al., 2000, Whittemore et al., 2005, Chen et al., 2022]. HDX reports on the structural dynamics of the backbone amides of a protein [Englander et al., 1972, Skinner et al., 2012]. A protein of interest is diluted into a solution of deuterium, and time points are taken to measure the uptake of deuterons over time using, for example, MS. H $\rightarrow$ D exchange occurs only if the hydrogen bond is broken and the associated amide protein is exposed to solvent. Therefore, HDX reports on both structure and dynamics of a protein sample [Masson et al., 2019].

The Linderstrøm-Lang model posits that amide protons only exchange when they are in an "open" state where they are not participating in a hydrogen bond (e.g. in an α -helix or β -sheet). Once in the open state, protons exchange with a sequence- and environment-dependent intrinsic exchange rate k_{chem} with the overall reaction given by Equation 4.1

[Englander et al., 1997]:

$$H_{closed} \underset{k_{close}}{\overset{k_{open}}{\rightleftharpoons}} H_{open} \xrightarrow{k_{chem}} D \tag{4.1}$$
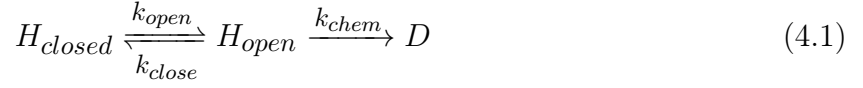
From this, the observed exchange rate k_{obs} of a proton at steady state is governed by Equation 4.2:

$$k_{obs} = \frac{k_{open}k_{chem}}{k_{open} + k_{close} + k_{chem}} \tag{4.2}$$

There are 2 typical exchange regimes. When the intrinsic exchange rate is much faster than the closing rate ($k_{chem} >> kclose$), exchange occurs whenever the hydrogen bond transiently breaks (EX1 limit). This provides kinetic information on the opening rate of breaking the hydrogen bond (Equation 4.3):

$$k_{obs} = k_{open} \tag{4.3}$$

On the other hand, if the intrinsic exchange rate is much slower than the closing rate ($k_{chem} << kclose$), exchange reports on the thermodynamic stability of the hydrogen bond (EX2 limit; Equation 4.4):

$$k_{obs} = \frac{k_{open}k_{chem}}{k_{open} + k_{close}} = k_{chem}\frac{1}{1 + K_{eq}} \tag{4.4}$$

Where K_{eq} is the equilibrium constant for breaking of the hydrogen bond. The protection factor (PF) governs the fraction of time that the hydrogen bond is broken/ exchange-susceptible and relates to the thermodynamic stability of the bond (Equation 4.5):

$$PF = 1 + K_{eq} = \frac{k_{chem}}{k_{obs}}, \text{where } K_{eq} = \exp(\frac{-\Delta G}{RT}) \tag{4.5}$$

Intrinsic k_{chem} can easily be calculated for a given peptide sequence [Bai et al., 1993],

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and mass spectrometry can easily determine whether a peptide is exchanging in the EX1 (two distinct isotopic envelopes of low and high mass with the decrease in one matched by an increase in the other envelope) or EX2 (a single mass envelope continuously shifting to increasing weight) regime [Vinciauskaite and Masson, 2023].

Following deuteration for a given amount of time, the sample is quenched by lowering the pH to ~2.5 to minimize back exchange of the labile deuteron label [Mayne, 2016, Walters et al., 2012]. In our bottom-up HDX-MS workflow, the sample is sent through a protease column for nonspecific digestion, and then peptides are gathered onto a trap column for desalting. From there, a gradient of water and acetonitrile is used to elute peptides onto an analytical column for separation before deuteration is measured by mass spectrometry (Figure 4.1).
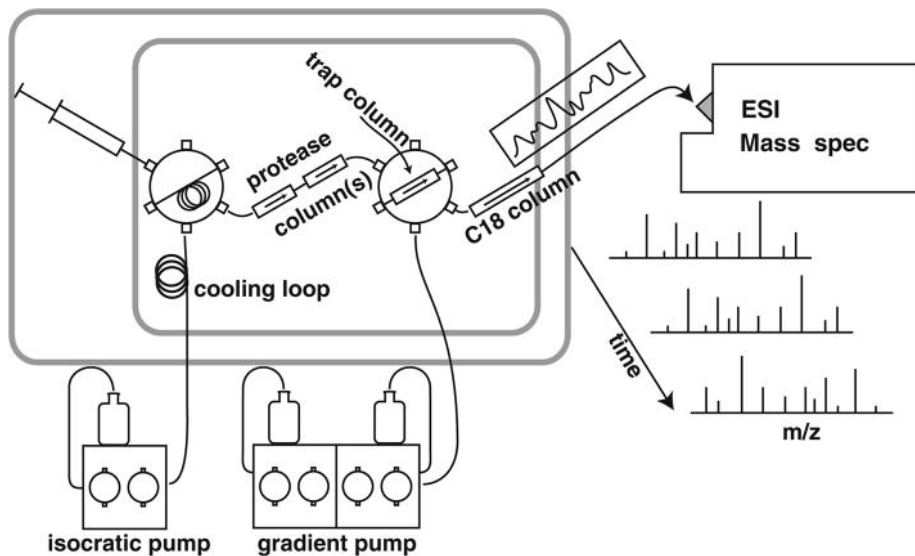
Figure 4.1: HDX-MS hardware. Our on-line HDX-MS system enables analysis of structural dynamics of Pab1 condensates. Figure from [Mayne et al., 2011].

In sum, using HDX-MS, one is able to measure the structural dynamics of Pab1 condensates across temperatures and orthologs to probe thermodynamic selectivity in condensation.

4.3   Results

4.3.1   Validating HDX-MS results against published work

Before investigating temperature- or species- dependent structural changes of Pab1 condensation, we validate our system by repeating previously published work [Chen et al., 2022]. First, MS2 spectra of monomeric Pab1 protein peptides were obtained after proteolysis and chromatography (Figure 4.1). These were used to generate a peptide map showing the primary sequence coverage. Peptides positioned across the sequence of Pab1 were identified, enabling the interrogation of structural dynamics across the protein by HDX (Figure 4.2).
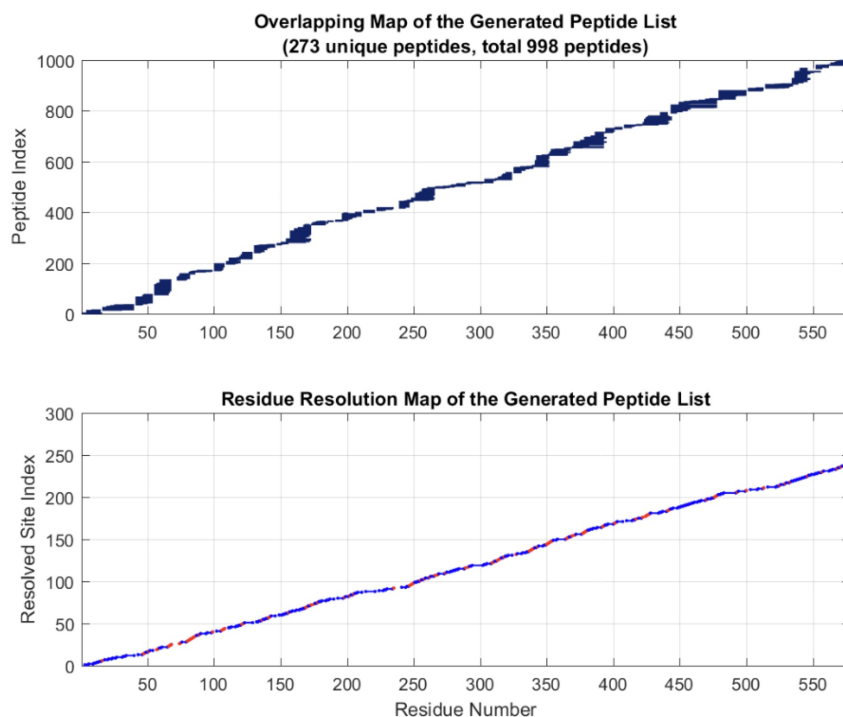
Figure 4.2: Peptide map of Pab1. Bottom-up HDX-MS of Pab1 identifies 273 unique peptides covering almost the entirety of the Pab1 primary sequence. The peptide map was generated using ExMS2 software [Kan et al., 2019].

With a working analysis pipeline, we complete HDX studies of monomeric and condensed Pab1, as has been completed by Chen et al.. Measuring uptake across different time points enables careful quantification of HDX protection and comparison between samples.

Comparison between previously published data and the work herein confirms the robustness of our HDX-MS methodology and its suitability for measuring potential differences in condensates formed under different conditions or from orthologous Pab1's. For example, our work qualitatively matches that from published data obtained under near identical conditions (Figure 4.3). Satisfyingly, the uptake of deuterium closely matches expectations from the protein's known domain organization (structure of Pab1 from both experimental measurements and computational predictions [Kozlov et al., 2002, Schäfer et al., 2019, Jumper et al., 2021]). The 5 structured domains of Pab1, RRMs 1-4 and the C-terminal domain, exhibit decreased rates of deuteration when compared to the unstructured regions including the linkers at the N and C termini and between RRMs, and the low complexity P domain. The presence of secondary structure will increase the PF—decreasing uptake rate—as in equations 4.4 and 4.5, due to the decreased fraction of protons in the H_{open} state. Further, the agreement in relative rates of uptake between folded domains (for example, deuteration rate of CTD > RRM3) further validate the approach. Overall, the new HDX-MS for Pab1 monomers closely match previously published work under similar conditions [Chen et al., 2022].

Beyond the agreement between the monomeric Pab1 samples, comparison of the differences in deuterium uptake upon condensation provides additional validation (Figure 4.4). Not only do we also observe increased dueterium uptake in the RRMs upon Pab1 condensation, but we also observe the decreased deuteration in the unstructured regions (linkers, N- and C- termini, P domain) of Pab1.

In sum, the high agreement between deuterium uptake from my and previous work [Chen et al., 2022] supports the suitability of using HDX-MS to probe potential differences in Pab1 condensates formed at different temperatures and from different species.
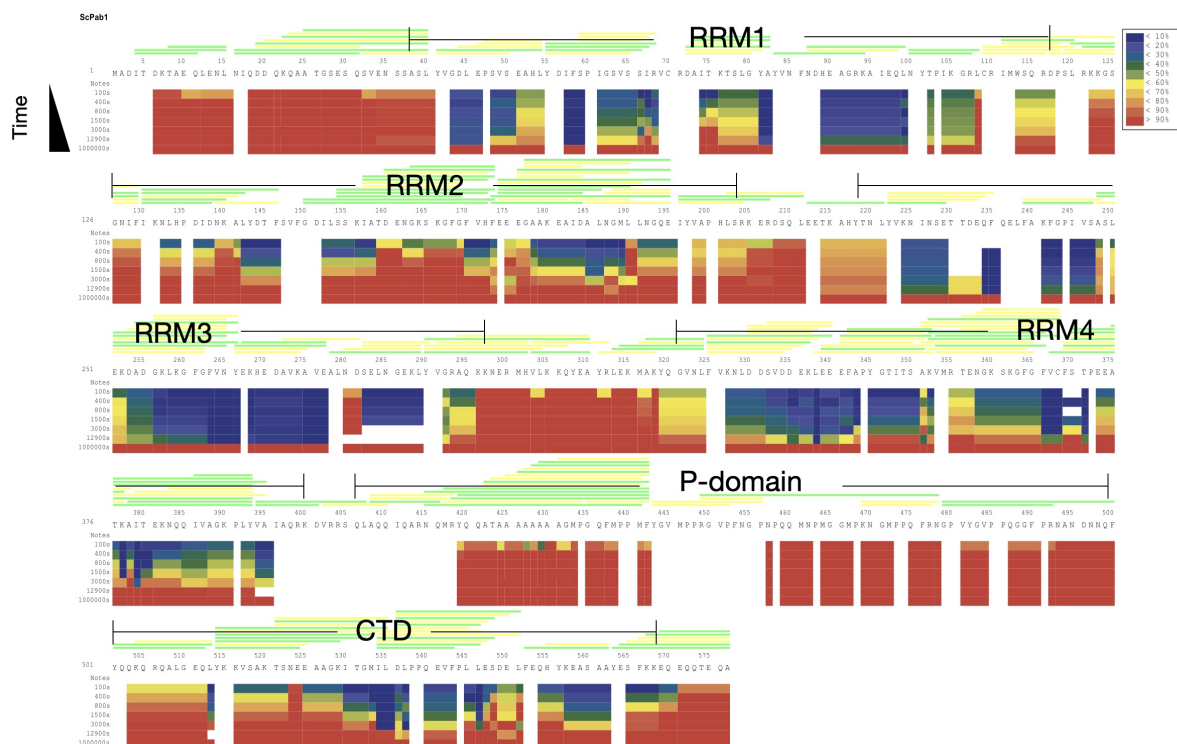
Figure 4.3: Validation of monomeric Pab1 HDX data. Qualitative agreement between previously published work [Chen et al., 2022] and the work herein supports the robustness of our methodology.
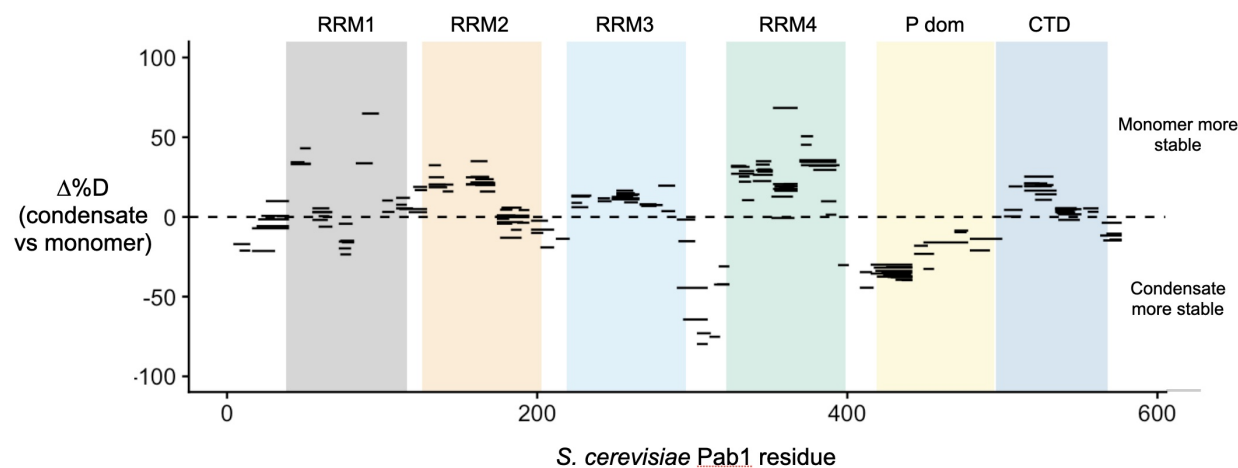
Figure 4.4: Validation of condensed Pab1 HDX data. Qualitative agreement between previously published work [Chen et al., 2022] and the work herein (shown) supports the robustness of our methodology for similarly prepared 46°C condensates. Shown is the 100s time point.

4.3.2 Putative amyloid-like crosslinks connect Pab1 protomers in the condensate and are important for condensation

Deeper analysis of our HDX data revealed the presence of sites of extreme protection in the condensate. For example, when comparing the deuteration of the condensate to the monomer under long time points (37°C, pD 8, overnight), we observe that specific regions of condensed Pab1 are protected at these extreme treatments (Figure 4.5). Because the saturated timepoint is meant to generate fully deuterated sample to control for back exchange during proteolysis and chromatography, the resistance of these regions in the condensate to exchange indicates regions of particularly high protection. The degree of protection is reminiscent of that which has been observed in the cross-β architecture of amyloid fibrils [Whittemore et al., 2005], raising the possibility that amyloid-like interactions link Pab1 protomers in the condensate. In fact, there exists clear evidence supporting the presence of cross-β and other similar structures in condensates [Peran and Mittag, 2020, Kato et al., 2012, Murray et al., 2017, Guenther et al., 2018, Hughes et al., 2018].
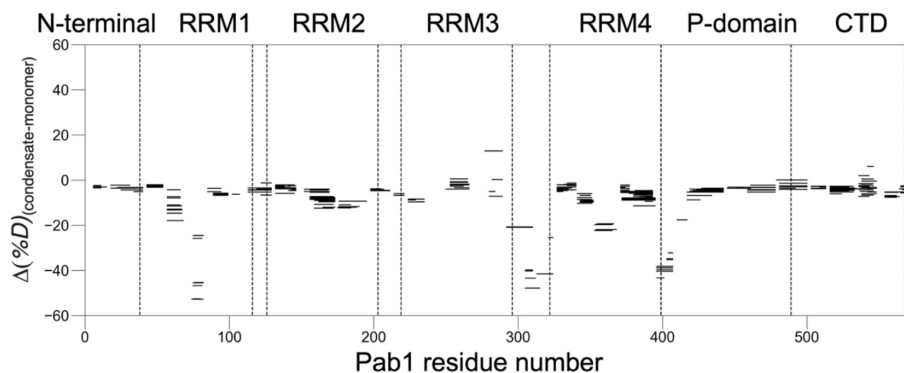
Figure 4.5: Sites of extreme protection in Pab1 condensates. Reanalysis of previous work [Chen et al., 2022] reveals that Pab1 condensates do not fully exchange (when compared to monomeric Pab1) at 3 sites (in RRM1, between RRMs 3 and 4, and after RRM4) at long exchange times (37°C, pD 8, overnight).

Further evidence supporting the existence of the crosslinkers is that these regions exhibit increased protection across all time points. Bimodal decomposition analysis shows that

the RRM1 putative linker region exists as two distinct populations: a population whose exchange is consistent with that of the monomer, and another population with virtually no observable deuterium uptake [Chen et al., 2022]. The complete recalcitrance of the region to deuturation indicates the entire stretch of approximately 10 amino acids makes essentially infinitely stable hydrogen bonds. This is consistent with a cross-β like structure, rather than that of typical reversible and promiscuous "stickers" which underlie condensation of many low complexity region systems [Wang et al., 2018]. Additionally, the relative population of the RRM1 L2 hairpin peptide in the 46°C condensate which exchanges in the slow regime is 80%, indicating that most Pab1 protomers utilize this architecture [Chen et al., 2022].

If these regions are crosslinks important for the molecular organization of Pab1 condensates, one may expect that removing them disrupts Pab1 condensation. To ask this question, I designed a "stickerless" Pab1 construct lacking the 3 putative linkers (Figure 4.6. Because two of the stickers are C terminal to RRM3, we generated a variant of Pab1 truncated directly after it. Additionally, we replaced the roughly 10 residue stretch encoding the putative RRM1 sticker with a $(GSS)_4$ linker. Because the RRM1 L2 hairpin is predominantly unstructured, we hoped the replacement with the GSS linker would be minimally perturbative. Strikingly, the "stickerless" RRM123 construct did not condense when heated under our conditions (Figure 4.6). For reference, the parent RRM123 (the first half of Pab1 protein, spanning from the N terminus to the linker following RRM3) has a condensation onset temperature of roughly 46°C. The prevention of condensation onset in the "stickerless" mutant indicates that the putative crosslinkers form important molecular interactions underlying Pab1 condensate formation.

We note the hydrodynamic baseline of 5 nm is greater than expected ($\sim$3 nm for a typical RRM123 construct). This raises the possibility that RRM1 of the stickerless RRM123 construct is partially unfolded, raising the R_h, as a result of the GSS linker insertion. Nevertheless, even if this is the case, the fact that the unfolded RRM1 does not trigger premature

RRM123 (stickerless)
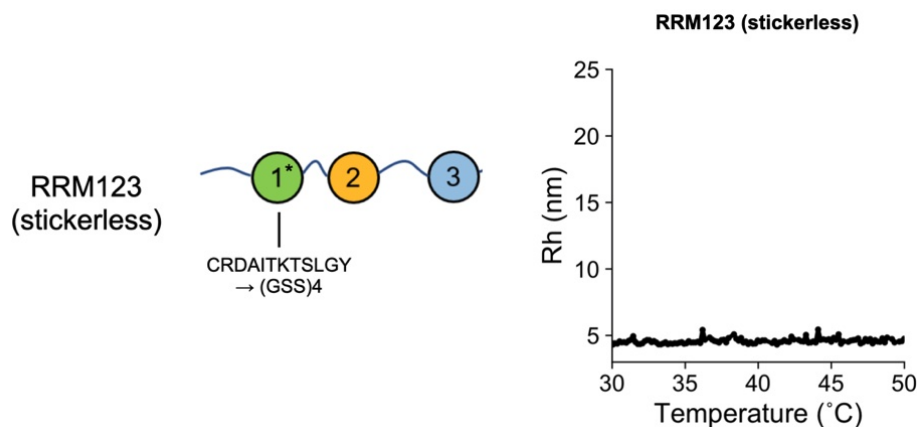
Figure 4.6: Stickerless RRM123 does not condense. The 3 putative crosslink sites are located within RRM1, in the linker between RRMs 3 and 4, and after RRM4. A Pab1 construct designed without any stickers (by truncating Pab1 directly after RRM3 and replacing sticker 1 with a GSS linker) does not condense by DLS.

condensation—given that partial unfolding is needed for Pab1 condensation activation—argues that specific contacts involving the RRM1 L2 hairpin are needed for crosslinking.

4.3.3 The hydrogen bond networks of Pab1 condensates formed at different temperatures are similar yet distinct

The thermodynamic specificity model of Pab1 condensation posits that the free energy surfaces of each RRM control the activation order for partial unfolding [Chen et al., 2022]. In this model, we predict that different condensation onset temperature treatments lead to different degrees of partial unfolding of the RRMs. For example, a decreased temperature of condensation onset may be insufficient to activate the most stable RRMs (i.e. RRM3) while still activating those with greater activation propensities. The inactivation of RRM3 would result its inability to form interacting pairs between protomers, leading to a distinct, less entwined condensate structure (Figure 4.7). If the different RRMs sense temperature independently, one expects that the decreased activation temperature would still cause activation of the remaining, more activatable RRMs, and the hydrogen bond networks of these

domains are largely unchanged.

To test this question, I prepared condensates at 39°C, 42°C, and 46°C. It is known that these temperatures are sufficient to form Pab1 condensates, as condensation onset has been observed by DLS even at 37°C [Riback et al., 2017]. However, because the kinetics of Pab1 condensation at lower temperatures are slowed [Riback et al., 2017, and data not shown], longer temperature treatments were required to obtain sufficient material for our HDX protocol. Therefore, while enough 46°C condensates could be generated from a 20 minute treatment, incubations of 3 hours at 42°C and overnight at 39 °C were completed (Figure 4.7).
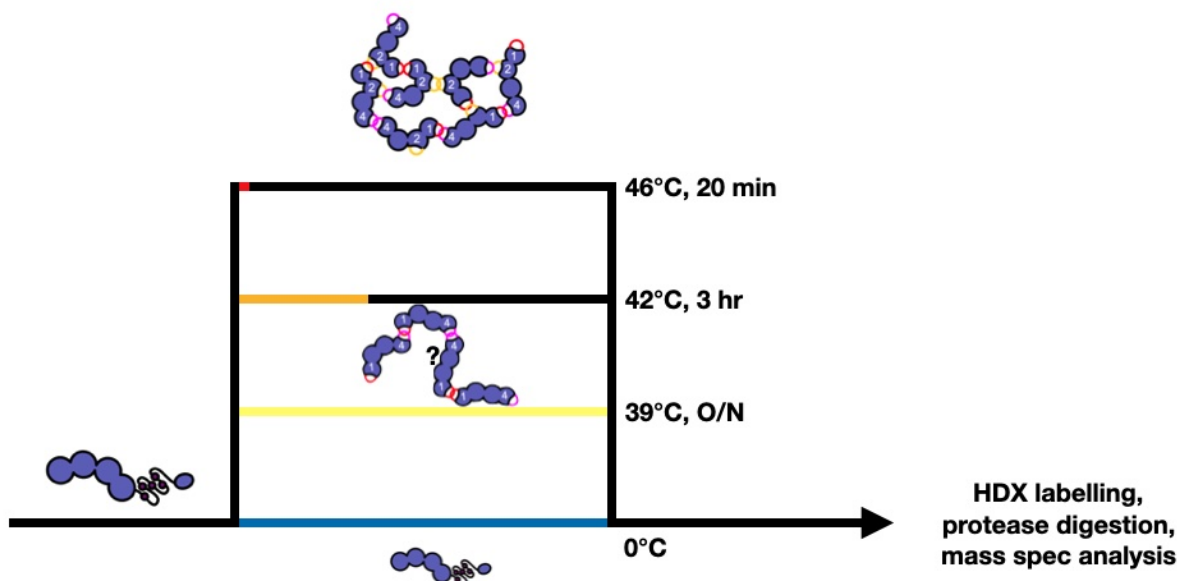
Figure 4.7: Preparation of Pab1 condensates at different temperatures. Pab1 condensates were prepared at different condensing temperatures at pH 6.5 (39°C, overnight; 42°C, 3 hours, or 46°C, 20 min). Treatment length was extended for 39°C and 42°C condensates due to slowed kinetics of assembly at lower condensation temperatures.

HDX-MS of the condensates was completed as before, and the differences in deuterium uptake compared to monomeric Pab1 were plotted against each other (Figure 4.8). In the plot, each faint dot represents the difference in uptake of a peptide in the condensate compared to the monomer at a given time point. The points are colored by domain, and summary

points for each domain (mean ± standard error) are additionally plotted.
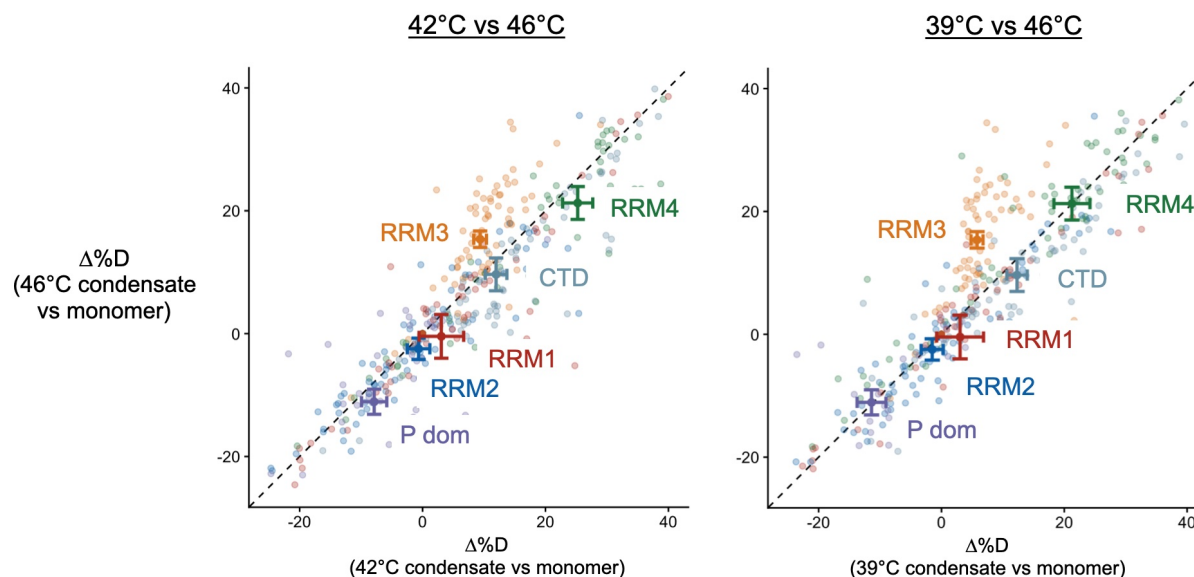
Figure 4.8: Pab1 condensates formed at different temperatures have different structures. HDX-MS finds that Pab1 condensates formed at 39°C, 42°, and 46°C have largely conserved hydrogen bond networks with distinct differences. Although structural differences for most Pab1 domains were quantitatively the same upon condensation across the different temperatures (on line), RRM3 exhibits more deuteration at higher temperatures (data displayed as uptake from unimodal fit by HDExaminer, peptides of each domain were grouped and summarized as mean ± standard error)

The first order result is that there is general agreement in the changes in deuterium uptake between different temperature condensates (Figure 4.8). Generally, there is increased deuterium uptake in the RRMs (consistent with partial unfolding) and decreased uptake in the P domain (consistent with increased structure/contacts in the condensate). We interpret this as the overall conservation of condensate structure and formation mechanism across the different temperature treatments: sequential activation and partial unfolding of the RRMs. Fascinatingly, a single domain stands out by exhibiting temperature-dependent uptake differences: RRM3.

In contrast to the other domains, RRM3 uptakes consistently more deuterium at increasing temperatures. This is consistent with it having a greater degree of partial unfolding at

higher temperatures, and precisely matches our aforementioned predictions from the thermodynamic specificity model: RRM3, by being the most stable RRM, is less activated at milder temperature treatments, which leads to less partial unfolding to the condensation-competent state and less participation in condensation. The general agreement in deuteration of the other domains argues the individual RRMs act largely independently of each other.

4.3.4 The hydrogen bond networks of Pab1 condensates formed from different yeast orthologs are largely conserved yet distinct

The condensation responses of different fungi adapted to different temperature niches are set relative to their optimal growth temperatures [Keyport Kik et al., 2023]. Further, the set of proteins which condense in response to temperature stress is largely conserved from psychrophile to thermophile [Keyport Kik et al., 2023]. In the case of Pab1, the orthologs' primary sequences control their condensation onset temperatures [Keyport Kik et al., 2023]. Yet, central questions remain: 1) To what degree is the mechanism of formation and resulting condensate structure of Pab1 orthologs conserved? 2) How does primary sequence determine condensation onset temperature? To answer these questions, we employ HDX-MS to investigate the structural dynamics of the monomers and condensates of three Pab1 orthologs. In contrast to many other structural techniques, HDX-MS can be used to study insoluble systems such as condensates, while providing residue-resolved insight into the hydrogen bond network of biomolecules in a label-free manner.

Previous work from our labs using HDX-MS to investigate the mechanism of *S. cerevisiae* Pab1 condensation found that it condenses by a mechanism of sequential activation involving partial unfolding [Chen et al., 2022]. An increase in temperature causes the RNA-recognition motif domains (RRMs) to partially unfold enabling these regions along with the linkers between them to form crosslinks between Pab1 monomers. Here, we adapt our protocol to study monomeric and condensed Pab1 from the cryophile *S. kudriavzevii*, the mesophile

S. cerevisiae, and the thermophile *K. marxianus.* HDX-MS reports on the stability of the protein's hydrogen bond network involving amide protons. Deuteration in D_2O can occur when a hydrogen bond is broken and the amide proton is exposed to solvent. By measuring D-uptake over time, HDX-MS provides a time series of the structural and thermodynamics changes that occur upon condensation. Monomeric Pab1 deuterium uptake across a range of time points for multiple peptides in each ortholog supports the suitability of our approach, by showing that we have hundreds of peptides reporting on the structural dynamics throughout the primary sequence (Figure 4.9A).

The patterns of deuteron uptake across organisms are largely conserved. This result indicates that the structure and dynamics of the monomer for the orthologous Pab1's are very similar. This is sensible, as the orthologous Pab1's are computationally predicted to have conserved structures [Jumper et al., 2021]. The regions of low deuteration in the monomer (blue) correspond to the folded domains RRM1, RRM2, RRM3, RRM4, and the CTD, while regions of high deuteration typically correspond to the unstructured P domain and various linkers.

Next, to study differences in structural dynamics of the condensates, we calculate the difference in deuterium uptake for each peptide between the condensed and monomeric states (Figure 4.9B). Numerous peptides have increased deuterium uptake in the condensate ($\Delta\%$ deuteration > 0), indicative of weaker or a reduced number of hydrogen bonds in the condensed structure for that peptide. The patterns of deuteration change are highly conserved across the Pab1 orthologs. The four RRMs undergo increased exchange across each ortholog, which is consistent with the loss of hydrogen bonded structure via partial unfolding in the condensate. The P domain in contrast has an increased level of protection consistent with it providing cross-protomer contacts in the condensate. Previously observed specific putative contact points between Pab1 protomers in RRM1, the linker between RRM3 and 4, and following RRM4 are conserved for all three species. In sum, the structural dynamics of the
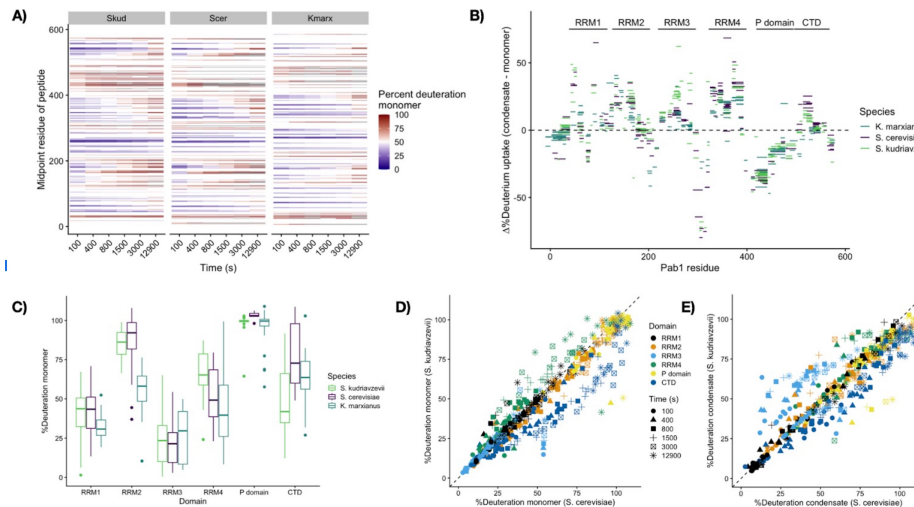
Figure 4.9: HDX-MS investigation of Pab1 ortholog monomers and condensates reveals conservation of condensate structure and mechanism. A) Pab1 ortholog peptide deuterium uptake mapped onto primary sequence. B) Woods plot comparing HDX levels after 100s of labeling betweeen the condensates and monomers indicates that partial unfolding in the RRMs in the condensate is conserved across orthologs. C) Pab1 monomer deuterium uptake at 3000 seconds reveals domain stability differences between orthologs. D) Comparison of peptide deuterium uptake in monomeric Pab1 of *S. cerevisiae* and *S. kudriavzevii*. E) Comparison of peptide deuterium uptake in condensed Pab1 of *S. cerevisiae* and *S. kudriavzevii*.

condensate are highly conserved across the Pab1 orthologs, implying a common mechanism of formation. We thus conclude that each Pab1 ortholog condenses using a shared mechanism of sequential activation and partial unfolding.

4.3.5 Differences in monomer Pab1 structural dynamics could explain the differences in condensation onset temperature among orthologs

Understanding towards how condensation temperature is encoded in the Pab1 primary sequence can be gleaned from a closer look at differences in the monomeric deuterium uptake values between species. Our previous work introduced the concept of "thermodynamic specificity," where the intrinsic free energy surfaces of the different RRMs were responsible for their sequential activation. In other words, a less stable RRM requires less energy input (lower temperature) to trigger its partial unfolding and activation. This concept can be ex-

tended to understanding differences in condensation onset temperature across species. One may expect that the onset temperature can be decreased by decreasing the stability of a Pab1 RRM to favor its activation, while stabilizing an RRM would increase the condensation onset temperature. Indeed, the monomeric HDX rates of each domain report on their respective stabilities, as an unfolding event must occur to enable proton exchange (Figure 4.9C,D). The data find that the deuteration of RRMs 1 and 3 are largely similar across the orthologs, implying their stabilities are similar. However, the *S. kudriavzevii* Pab1 ortholog possesses a more dynamic (less stable) RRM4 while *K. marxianus*'s Pab1 possesses a less dynamic (more stable) RRM2 when compared to *S. cerevisiae*.

Due to the 98% sequence identity between *S. cerevisiae* and *S. kudriavzevii* Pab1's, we can compare the deuteration of identical peptides between orthologs (Figure 4.9D). Regions of the protein with identical sequences (RRM1, RRM2, and P domain) have identical uptake in both orthologs, which supports that deuteration of each domain is largely modular. On the other hand, regions with different primary sequences (RRM3, RRM4, and CTD) can have similar or different deuteration rates. It is interesting that RRM3, which has 6 point mutants, has similar monomeric stability, while RRM4, with only one mutation (A343T), is less stable in *S. kudriavzevii*. These observations are consistent with RRM stability being linked to condensation onset temperature. We hypothesize *S. kudriavzevii* RRM4 has a lower activation threshold due to its A343T mutation, which is responsible for its lower condensation onset temperature. In contrast, we hypothesize *K. marxianus* RRM2 has a higher activation threshold, which causes its increased condensation onset temperature. Fusion Pab1 constructs swapping domains from the different temperature-niche organisms would provide an easy test to these hypotheses. In fact, engineering the 1 residue mutation A343T into *S. cerevisiae* Pab1 to swap in RRM4 of *S. kudriavzevii* decreases the condensation onset temperature by 1°C, which is ∼half of the condensation onset temperature difference between the WT proteins (Figure 4.9). Although more work is to be done, this is an encouraging

sign supporting that the monomeric HDX uptake rate informs relative activation propensity in condensation.
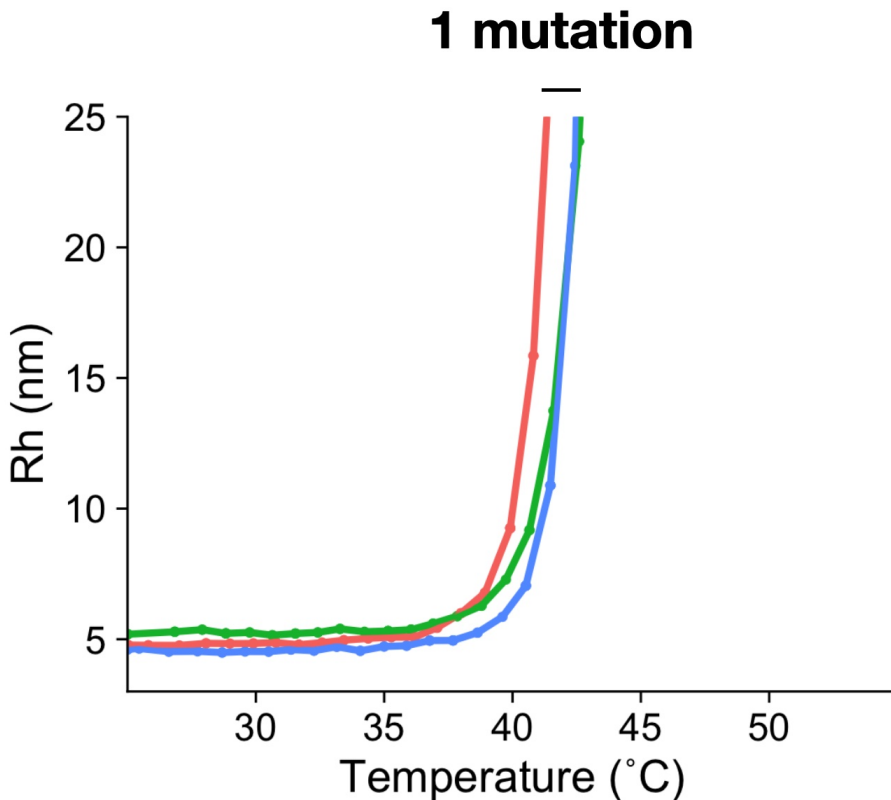
Figure 4.10: A single point mutation decreases Pab1 condensation onset temperature. Sc-Pab1 A343T (the single residue difference in RRM4 between *S. cerevisiae* and *S. kudriavzevii* (red) has a 1°C lower condensation onset temperature compared to WT ScPab1 (blue and green), which is ~half of the condensation difference between *S. cerevisiae* and *S. kudriavzevii* Pab1 proteins.

Interestingly, we find that the increased deuteration of the S. kudriavzevii RRM4 is not maintained in the condensate structure (Figure 4.8E). Instead, we find that the clearest difference between the *S. cerevisiae* and *S. kudriavzevii* condensate structural dynamics is increased deuterium uptake of RRM3 in the cold adapted yeast. This indicates a separation between the monomeric dynamics, which presumably inform the mechanism of triggering condensation onset, and the condensate dynamics, which inform the structure of the mature condensate.

Using HDX-MS, we find that Pab1 condensate structural dynamics are similar across three orthologs with widely different condensation onset temperatures. Importantly, RNA recognition motif stability correlates with condensation onset temperature. Because Pab1 has been found to condense via a sequential activation and partial unfolding mechanism, these results provide a parsimonious explanation to how Pab1 condensation can be set respective to an organism's temperature niche. In sum, our observations argue for the conservation of Pab1 condensation mechanism and structure from cryophile to thermophile. By tuning the stability—and thus activation threshold—of orthologous Pab1 RRMs, Nature enables Pab1 condensation to be triggered in response to each organism's relative stress temperature.

4.4   Discussion

4.4.1   A core architecture in Pab1 condensate structure may explain its hydrogel properties

Studying the identity and strength of the intermolecular interactions linking protomers in the condensate is critical to understand their physical states and potential functions. For example, liquid-like condensates are characterized by weaker, shorter lifetime interactions. On the other hand, more solid-like condensates would possess stronger, longer lifetime interactions [Alberti et al., 2019]. An extreme example of this is an amyloid fibril, in which irreversible aggregation leads to the assembly of a specific structure [Kato et al., 2012]. The material state of a condensate may inform potential functions. For example, a pure LLPS assembly is spontaneously reversible once the environmental conditions (temperature in the case of Pab1) return to basal because condensation is governed by the system's thermodynamics. Yet, how the disassembly kinetics compare to biological timeframe is relevant. One can imagine a more liquid-like assembly which quickly decondenses upon stress cessation. This hypothetically may be useful to the cell for immediate on off switching or temperature

sensing. In contrast, forming an irreversible solid-like assembly with specific interactions may enable the cell to create a "latch" delaying return to basal activities. It appears many condensing proteins utilize such a system, as molecular chaperones are required for rapid dissolution of stress granules [Cherkasov et al., 2013]. In fact, many proteins—including Pab1—form hydrogels upon condensing and can be understood through a phase separation coupled to percolation (PSCP) framework [Mittag and Pappu, 2022, Riback et al., 2017]. Hydrogel formation may depend on the formation of amyloid-like fibrils (or other more stable, specific interactions) within the condensate [Kato et al., 2012]. In the case of Pab1, it is unknown which interactions underlie hydrogel formation. Because of the clear functional implications of the material state of the condensate, we studied Pab1 condensates by HDX-MS to gain insight into this question.

Our identification of sites of extreme protection in Pab1 condensates provide compelling evidence for the presence of strong, specific interactions underlying Pab1 condensation. The three regions possess comparable protection to deuteration as amyloid fibrils. Further, the complete protection of the 3 regions (each residue in the peptide is recalcitrant to deuteration) is consistent with a cross-beta architecture in the condensate. Future work can be completed to deeper understand the nature of the crosslinks. For example, biochemical reconstitution of the putative crosslinks can test whether they spontaneously form fibrils. If this is the case, the partial unfolding mechanism of Pab1 condensation can be rationalized as uncaging irreversible crosslinks (the three regions are in RRM1 and adjacent to RRMs 3 and 4).

The presence of amyloid-like, specific interactions may explain the hydrogelation of Pab1. Rather than Pab1 condensation be the sum of weak, nonspecific interactions that would give rise to a reversible liquid state, the core architecture of strong interactions gives rise to an irreversible hydrogel. Is the material state of Pab1 condensates functionally important? Finding specific mutations that perturb Pab1's gelation while maintaining its ability to condense would provide a compelling test to answer this question. The mutations can be

validated through *in vitro* reconstitution, and then inserted into yeast *in vivo* to assess functional importance. If it is true that Pab1 gelation enables the cell to "set a molecular latch" for recovery, the immediate reversion of Pab1 condensation upon return to 30°C conditions may perturb cellular recovery following stress cessation. The result in Figure 4.6 that removing all 3 crosslinks completely prevents condensation provides promise that a more expansive mutational screen will provide a set of Pab1 molecules with interesting differences in material state.

4.4.2 Structure function relationship: Could different temperature condensates serve different functions?

Can Pab1 condensates formed at different temperatures have different functions? One may imagine that a yeast cell would sense a 42°C stress (for example, being ingested by a bird [Triandafillou et al., 2020]) differently than a 46°C stress (potentially damaging thermal shock). The ideal responses to these scenarios could in theory be very different (prepare to be dispersed across the globe versus enter a protective state of dormancy). If condensation is a functional aspect of the stress response, as we hypothesize, the condensation responses to these different temperature stresses could very well be distinct. Evidence supporting or refuting this would be very informative.

The result that Pab1 condensates formed at different temperatures have distinct structures is striking. It is a widely understood dogma of biology that structure leads to function. Therefore, the condensates could in theory accomplish different roles in the cell. More concretely, the difference between milder and more severe heat shock condensates is differential activation and partial unfolding of RRM3. Previous work has concluded that Pab1 release bound mRNA upon condensation [Riback et al., 2017]. This is logical, given that its RNA recognition motifs locally unfold to condense. Less activation and unfolding of RRM3 at milder temeperature shocks could preserve some of Pab1's RNA-binding capacity. This may

functionally enable Pab1 condensates formed at lower temperatures to sequester certain mRNAs in condensates while those formed at severe temperatures eject RNA. Future *in vitro* study can measure potential differences of mRNA recruitment to Pab1 condensates at different temperatures to test this hypothesis.

More broadly, the different structures of Pab1 condensates formed at different temperatures raise the possibility that other protein condensates at different treatments also possess distinct structures and potential functions.

4.4.3   How does primary sequence encode condensation onset temperature?

Understanding how condensation behavior is encoded in primary sequence is a major area of inquiry. The set of three temperature-adapted yeast studied herein provides a fantastic system to ask how primary sequence encodes condensation onset temperature. The finding that the intrinsic stabilities of the RRMs, as measured by monomeric Pab1 deuteration rate, correlates with the order of condensation onset temperature, as predicted by the thermodynamic specificity model, provides a compelling hypothesis as to how Nature has tuned Pab1 condensation temperature. The stabilities, and thus activation barriers, of Pab1 RRMs can be tuned to set condensation onset temperatures according to an organism's temperature niche. For example, a thermophilic Pab1 ortholog could evolve to increase the stabilities of its RMMs to increase the condensation onset temperature (more energy is required to trigger partial unfolding).

What is particularly interesting is that for our set of three Pab1's, Nature has relatively tuned the stabilities of a small number of RRMs, rather than adjusted all four of them. For example, in the case of *S. cerevisiae* and *S. kudriavzevii*, RRMs 1 and 2 exhibit the same primary sequence and exchange rates. On the other hand, RRMs 3 and 4 have differences in sequence but only RRM4 exhibits an increased rate of uptake in the cold-adapted yeast. Nature could have theoretically decided to make all 4 RRMs slightly more stable, yet this

HDX data suggests that Nature chose to tune the stability of a single RRM. In the same vein, although the thermophilic Pab1 has a greater sequence difference to that of the mesophile (only 69% sequence identity between Pab1 from *S. cerevisiae* and *K. marxianus*) and none of the RRMs have the same sequence, there is a dramatic difference in monomeric HDX uptake in only RRM2. This suggests that even in this case, Nature chose to dominantly tune only the stability of a single RRM.

Why may this be?

One possibility is that because the differences in temperature niche between these organisms is relatively small on the absolute Kelvin scale, minimal tuning is sufficient. That the *S. cer* Pab1 A343T mutant possessed a decreased condensation onset temperature with only a single point mutation in RRM4 from the cold-adapted yeast demonstrates that minimal tuning of RRM stability caused a measurable change in onset temperature. A separate possibility is that mutating a single RRM is a shorter evolutionary route than adjusting all four of them, and thus probabilistically more likely. Future work assaying the condensation behavior of a panel of temperature-niche fusion constructs can further clarify how condensation onset temperature is encoded into primary sequence.

4.4.4 Putative broader applicability of thermodynamic selectivity in protein condensation

Thermodynamic selectivity has proven to be a powerful framework to understand Pab1 condensation at different temperatures and from different orthologs. The concept that the intrinsic thermodynamics of a protein determines its activation for condensation is likely broadly applicable to biology. For example, many known SG proteins contain RRMs (such as TIA-1 and TDP-43 [Garnier et al., 2017, Rayman et al., 2018]), and we predict their condensation is also governed by thermodynamic selectivity. Using HDX-MS to study whether these protein systems also condense by sequential activation and partial unfolding can test

the broader applicability of thermodynamic selectivity in biology.

Also interesting is further study to uncover how heterogeneous mixtures of condensing proteins behave. If activation and partial unfolding underlie the condensation of a wide range of proteins, do they mix and form heterogenous condensates or form separate assemblies each containing an individual species of protein? The finding that Pab1 condensates contain specific crosslinks raises the possibility that it may not co-condense with other condensing proteins lacking these sequences, even if they theoretically also condense according to thermodynamic selectivity. Additionally interesting would be to examine by HDX-MS to what extent the resulting structures of Pab1 condensates are different in a heterogenous assembly.

4.5   Methods

4.5.1   Protein expression and purification

Protein purification of Pab1 constructs was completed as in Riback et al. [2017]. In short, 6xHis-tagged constructs were overexpressed in a BL21 strain of *E. coli*. After harvesting, cells were lysed by sonication on ice in buffer (20 mM HEPES, pH 6.5, 150 mM KCl, 25 mM imidazole, and protease inhibitor tablets). Lysate was clarified by centrifugation and then purified via FPLC using the following protocol: His column (keep elution), overnight TEV protease cleavage, His column (keep flowthrough), Heparin column, and sizing column. Protein concentration was measured via absorbance, using the theoretical extinction coefficient calculated by ProtParam [Gasteiger et al., 2005].

4.5.2   Dynamic light scattering

DLS measurements were completed as in Riback et al. [2017]. In short, a DynaPro NanoStar instrument was used to measure hydrodynamic radius of protein sample with a temperature

ramp of 0.25°C/min from 25°C to 50°C. Sample concentrations, unless differently noted, were 15 uM protein in 20 mM HEPES, pH 6.4, 150 mM KCl, 2.5 mM MgCl$_2$, and 1 mM TCEP. Samples were centrifuged at 20,000 g for 30 minutes prior to DLS experiments, and buffer was filtered.

4.5.3   Condensate preparation

Conensates were prepared as in Chen et al. [2022], with the exception that Pab1 was exposed to the condensing condition at either 5 uM or 60 uM. And that condensates were prepared at pH 6.5 (not pH 6.8).

Temperature condensates of *S. cer* Pab1 were prepared at either 46°C for 20 min, 42°C for 3 hr, or 39°C for overnight.

Ortholog condensates were prepared as follows: *S. cer* and *S. kud* Pab1 condensates were treated at 46°C for 20 min. *K. marx* Pab1 condensates were treated at 55°C for 10 min.

4.5.4   Hydrogen-deuterium exchange mass spectrometry

HDX labeling and LC-MS was completed as in Chen et al. [2022].

4.5.5   HDX-MS data analysis

HDX-MS data analysis was completed as in Chen et al. [2022].

CHAPTER 5

TRANSITION METAL SIGNALING IS A PUTATIVE MECHANISM TO MODULATE STRESS-INDUCED CONDENSATION

5.1 Summary

Due to their unique chemical properties, metals are essential nutrients across the tree of life. Yet, as Uncle Ben from Spider-Man wisely stated, "With great power comes great responsibility" [Lee et al., 2002], and metal ions are certainly no exception. Because excessive metals are exquisitely toxic, organisms have evolved mechanisms to tightly control their levels and locations. This generates extraordinary concentration gradients that biology harnesses for cellular signaling. Yet, underexplored is the role transition metal signaling may play in biomolecular condensation, which leads to the central aim of this chapter: To what extent has evolution harnessed transition metal signaling to control biomolecular condensation?

5.2 Introduction

5.2.1 Transition metal homeostasis is essential for life

Serving as cofactors for an estimated 25% of all proteins, metals are essential cellular nutrients [Maret, 2010, Zoroddu et al., 2019, Jomova et al., 2022]. Yet, metal cations can be exceptionally toxic to the cell through mismetalation and by catalyzing Fenton-like chemistry to generate damaging free radicals [Robinson and Glasfeld, 2020, Wardman and Candeias, 1996]. Thus, free metal concentrations are kept tightly controlled by the cell using strategies such as sequestration and transportation [Bird, 2015]. An exemplar demonstrating the importance of metal homeostasis is the concept of nutritional immunity: that at the host-pathogen interface the immune system withholds certain metals that are essential bacterial

nutrients while providing other toxic metals as a killing mechanism [Murdoch and Skaar, 2022]. Certain proteins—including transmembrane transporters, metallochaperones, and transcription factors— have evolved to control metal levels and are critical for cellular life [Finney and O'Halloran, 2003, Glauninger et al., 2018].

Underscoring the exquisite control of transition metals, a yeast cell possesses less than one molecule of free Cu(I) cation [Rae et al., 1999]. Fascinatingly, life has also evolved mechanisms by which to utilize the maintained metal concentration gradient for signalling-notably in the propagation of action potentials in nerve cells via Na^+ and K^+ cations, but also in Ca^{2+}-triggered muscle contractions [Stuart et al., 1997, Ebashi and Endo, 1968]. Nature has evolved to use metals as critical signaling molecules for essential cellular processes.

5.2.2 *Stress-induced condensation is likely functional and activates the heat shock response*

Yeast respond to heat shock in a number of ways. These include large scale translational shut off, the formation of reversible, aggregated foci visible via microscopy, and the rapid transcription and translation of a small set of heat shock proteins [Lindquist, 1986, Wallace et al., 2015, Lindquist, 1981]. The heat shock response is an ancient and universally conserved response [Wu, 1995]. Additionally, stress-induced condensation is adaptive [Riback et al., 2017], by still largely enigmatic molecular mechanisms. The correlation between formation of aggregated cytosolic assemblies composed of proteins and ribonucleic acids and the translational reprogramming upon the HSR has led to the hypothesis that the formation of these assemblies is a critical component of this translation reprogramming [Wallace et al., 2015, Iserman et al., 2020].

In support of this, many translation initiation factors required for traditional cap-dependent translation (including eIF3, eIF4, eIF5) aggregate during heat stress [Wallace et al., 2015, Iserman et al., 2020]. Additionally, preexisting cellular mRNAs are also localized to these

aggregates during induction of stress [Lee and Seydoux, 2019]. On the contrary, ribosomal proteins and other initiation factors required for cap-dependent translation remain in the soluble fraction [Wallace et al., 2015]. This would be consistent with the sequestration of preexisting, non-stress responsive mRNAs to allow for the prioritized translation of stress messages in a cap-independent manner. In fact, evidence supports the necessity of cap-independent translation for yeast survival of starvation stress [Gilbert et al., 2007]. This view postulates stress-induced condensation functionally causes translational reprogramming during stress. In support of this hypothesis, previous work has shown that mutations inhibiting the ability of the core stress granule marker polyA-binding protein (Pab1) to phase separate in response to temperature increase results in reduced viability for yeast during heat stress [Riback et al., 2017].

Separately, protein condensates are thought to be endogenous substrates of molecular chaperones [Ali et al., 2022, Triandafillou et al., 2020, Yoo et al., 2022, Begovich and Wilhelm, 2020, Snead and Gladfelter, 2019, Kroschwald et al., 2018]. Because activation of the Hsf1 regulon is controlled by the chaperone titration model [Voellmy and Boellmann, 2007, Zheng et al., 2016], formation of condensates may even be signals of stress to mount a heat shock response.

5.2.3   Modulation of condensation by metalation

Phase separation is a physical phenomenon resulting in demixing of proteins such as oil and water [Alberti, 2017]. Despite being hotly researched in recent years, discovery of physiological functions of condensation has proven to be challenging [Hyman et al., 2014, Shin and Brangwynne, 2017, Holehouse and Pappu, 2018]. Much like cation signaling, condensation upon stress promises to be an attractive putative signaling mechanism: enabling rapid remodeling of the cellular environment in response to a stimulus. Because condensation has been shown to be functionally important in adaptation to temperature stress in *S. cerevisiae*

[Riback et al., 2017], we utilize it as an excellent model system for studying the molecular details of the functional role of condensation.

The role of metals in condensation is an area that has been under-explored in the literature. The possibility of transition metal modulation of phase separation is attractive for a multitude of reasons. First, cations are known to be critical players in the heat shock response. pH drop during heat stress is necessary for induction of heat stress response proteins under conditions of glucose withdrawal [Triandafillou et al., 2020]. Moreover, metals play a necessary role in virtually all essential cellular processes, so it is likely they are involved in the stress-induced condensation. Finally, because a quarter of proteins are estimated to bind metals[Maret, 2010], it is reasonable that certain condensing proteins possess specific metal binding sites which may modulate condensation. Alternatively, transition metals may act nonspecifically through dielectric effects.

Disregulated metal homeostasis has been linked to the progression of neurogenerative, protein-misfolding diseases [Lee et al., 2018, Kawahara et al., 2001]. Previous work has shown that the yeast homologue to the temperature-sensitive TRP channel family—which is a general cation transporter—is involved in the cellular response to osmotic stress [Denis and Cyert, 2002]. The homologs in the pathogenic fungi *Aspergillus fumigatus* and *Candida albicans* have been shown to be important for pathogenesis [De Castro et al., 2014, Yu et al., 2014]. Finally, transition metals have been shown to help trigger condensation of different proteins in separate contexts (including ones with similar RRM domain architecture) [Rayman et al., 2018, Du and Chen, 2018, Garnier et al., 2017]. Further, it is known that zinc levels increase during oxidative stress of mammalian cells [Rayman et al., 2018], raising the possibility of the role of metal signaling in stress response. I hypothesize that cytosolic free metal levels are altered upon heat stress in yeast, and that this directly modulates phase separation of Pab1, which affects the cell's functional ability to respond. In pursuit of this, I have completed preliminary work showing that Zn^{2+} modulates the induction of aggregation

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by Pab1 *in vitro*.

5.3 Results

5.3.1 Pab1 is predicted to possess metal binding sites and its condensation is promoted by Zn^{2+}

To begin this exploratory project, the Pab1 sequence was bioinformatically analyzed to predict putative cation binding sites. To do this in an unbiased manner, we employed IonCom, which uses complementary *ab initio* and template-based approaches to predict binding sites for 13 different metals [Hu et al., 2016]. Interestingly, many Zn(II) binding sites were predicted by the software: of 577 total residues in *S. cerevisiae* Pab1, 25 of them were predicted to participate in Zn(II) binding pockets (Figure 5.1). These predicted sites are located in many domains of Pab1, from RRM1-4 to the CTD. Particularly exciting is the putative binding site in the RRMs, as their activation and partial unfolding is known to trigger Pab1 condensation [Chen et al., 2022].

Motivated by this initial suggestion, we incubated Pab1 with varying amount of Zn(II) and measured its temperature-induced condensation by dynamic light scattering (DLS). DLS measures the hydrodynamic radius of biomolecules in solution and has been previously used to measure temperature-induced condensation [Stetefeld et al., 2016, Riback et al., 2017]. Our DLS approach provides a measure of condensation onset, and we observe that increasing amounts of Zn(II) caused a dramatically earlier condensation onset temperature (where the hydrodynamic radius raises up) (Figure 5.2A). At the highest concentration treatment (100 uM Zn(II)), we observed an elevated R_h baseline, indicating that some assembly occurs even without temperature increase. As a negative control to ensure the Pab1 preparation was not loaded with metals from, a Pab1 sample was measured after incubation with EDTA, a powerful chelating agent of multivalent metals, to ensure any potential residual metals from

Predicted Binding Residue(s):
E46 R68 Y81 A91 A95 H134 K164 H172 H201 E206 D255 K257 K259
N265 D271 G365 C368 E373 E374 N383 N523 E541 E553 H555 E558
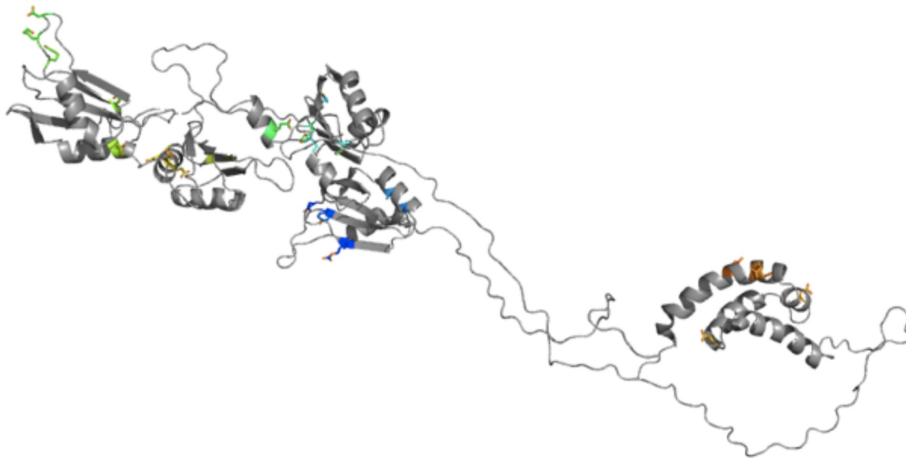
Figure 5.1: Pab1 is bioinformatically predicted to possess numerous Zn^{2+} binding sites. Of 577 residues in Pab1, 25 of them were predicted to bind Zn^{2+}. Predicted using IonCom software: Hu et al. [2016].

the protein purification were not interfering [Oviedo and Rodríguez, 2003]. That the EDTA treatment did not perturb condensation onset suggests that the protein is free of potentially interfering contaminants. In sum, Zn(II) promotes Pab1 condensation. A downside of DLS is that it measures the initial onset of condensation, so a complementary bulk assay would be helpful in teasing out the role of Zn(II) in condensation activation.

To this end, we complete biochemical fractionation methodology to study bulk Pab1 condensation. Sedimentation has been used to isoalate large assemblies, including Pab1 condensates [Cherkasov et al., 2013, Riback et al., 2017]. We utilize this approach to study how Zn(II) affects bulk Pab1 condensation (Figure 5.2B). After incubating Pab1 with different concentrations of Zn(II), Pab1 was held at either 30 or 46°C for 20 minutes before being separated by centrifugation to collect supernatant (soluble Pab1) and pellet (condensed Pab1) fractions. Bulk condensation is measured by running an SDS-PAGE gel of the fractions. If more Pab1 protein is in the pellet and less is in the supernatant, we conclude greater condensation. As can be seen, the tested concentrations of Zn(II) does not cause measurable
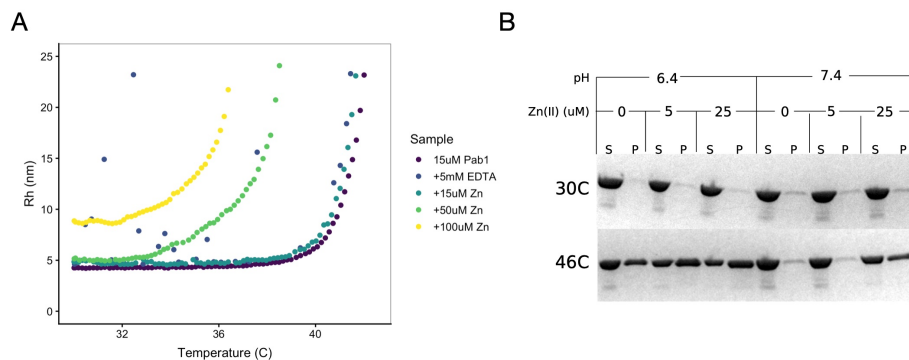
Figure 5.2: Zn^{2+} promotes Pab1 condensation. A) Addition of Zn^{2+} decreased the condensation onset temperature of Pab1, as measured by DLS. Interestingly, 100uM Zn^{2+} treatment caused an increased starting baseline, indicating Zn^{2+} causes Pab1 assembly without increased temperature. B) Biochemical fractionation measures bulk condensation by sedimentation. Increased Zn^{2+} concentration promotes additional condensation following 46°C treatment. (S: supernatant, P: pellet)

condensation at 30°C, but promotes condensation in temperature stress (Figure 5.2B). Two different pH values were chosen (acidity promotes Pab1 condensation). pH 6.4 is sufficient to cause measurable condensation of Pab1 without Zn(II), yet addition of Zn(II) caused increased Pab1 pelleting. In contrast, at pH 7.4 apo-Pab1 does not condense under our conditions. However, addition of 25 uM Zn(II) causes measurable Pab1 condensation. Together, Zn(II) promotes both onset and bulk amount of Pab1 condensation. Is this effect specific to Zn(II), or does addition of any cationic transition metal promote Pab1 condensation?

5.3.2   Pab1 likely contains a specific Zn^{2+} binding site

To address the specificity of the Zn(II) effect, we incubated Pab1 protein with a panel of different metals before completing fraction methodology. Of the 8 multivalent cations, only Zn(II) and Cu(II) promoted Pab1 condensation following temperature treatment (Figure 5.3). We note that promotion by Cu(II) incubation was inconsistently observed when the experiment was repeated (data not shown), and we are thus hesitant to make strong claims regarding the effect of Cu(II) on Pab1 condensation. Interestingly, the Cu(II) treatment—

see cutout in Figure 5.3—resulted in the formation of Pab1 assemblies that were resistant to resolubilization by SDS-PAGE analysis, a feature uniquely observed following Cu(II) treatment.
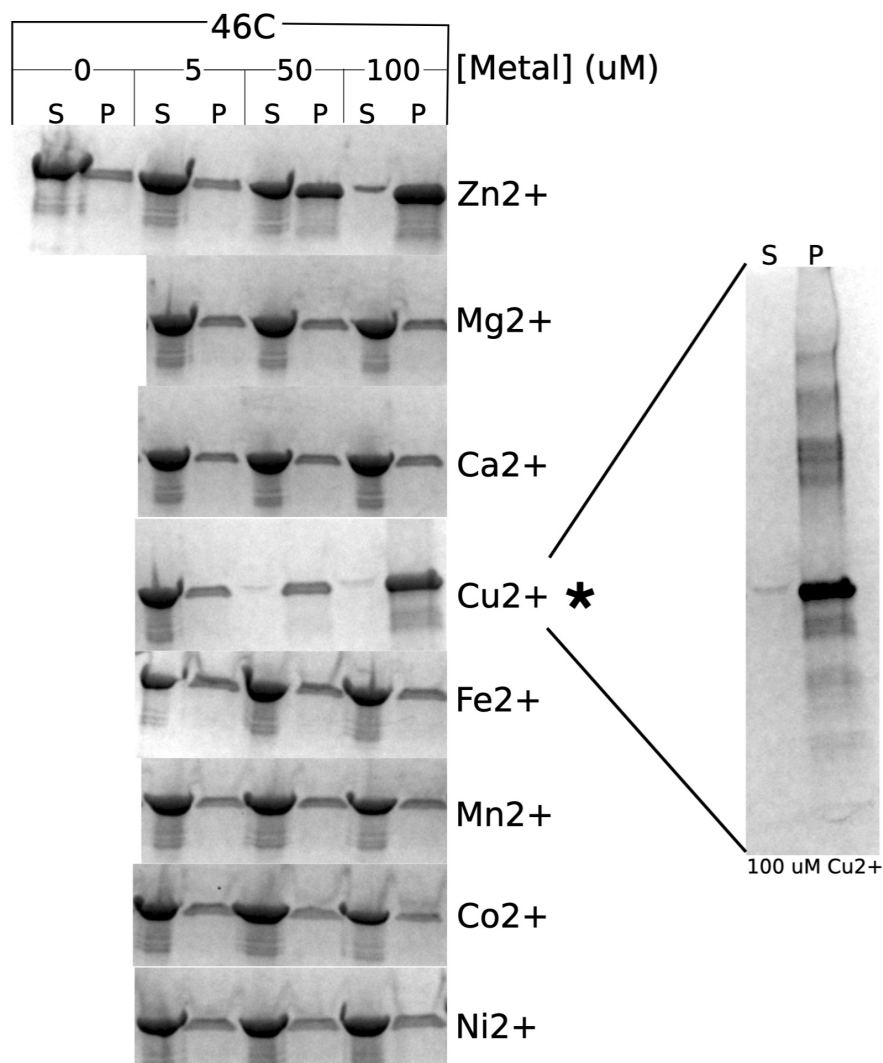
Figure 5.3: Zn^{2+} specifically promotes Pab1 condensation. Pelletable species consistent with condensate formation do not ubiquitously form in response to treatment with a panel of divalent cations. Zn(II) specifically promotes condensation. We note that Cu(II) incubation also promoted Pab1 condensation; however, this effect is inconsistently observed in technical replicates, indicating we do not fully understand its effect on Pab1 condensation. The Cu(II) cutout shows the abnormal presence of higher order Pab1 species in the pellet following Cu(II)-promoted condensation, which were uniquely resistant to dissolution by SDS-PAGE as compared to other condensates.

The finding that Zn(II) specifically promotes Pab1 condensation is parsimonious with the aforementioned bioinformatic predictions, and argues for the existence of a specific Zn(II) binding site in Pab1. To gain further insight into the nature of this binding site, we utilized a mutagenesis approach (Figure 5.4). In addition to studying the WT Pab1 protein, 3 mutants were studied: 1) RRM123- is a truncated version of Pab1 containing RRMs 1 through 3; 2) RRM3 is more severe truncation only containing the lone RRM3; and 3) H123 is a mutant of full length Pab1 in which its histidine residues have been mutated to the next most common residue in its sequence alignment.
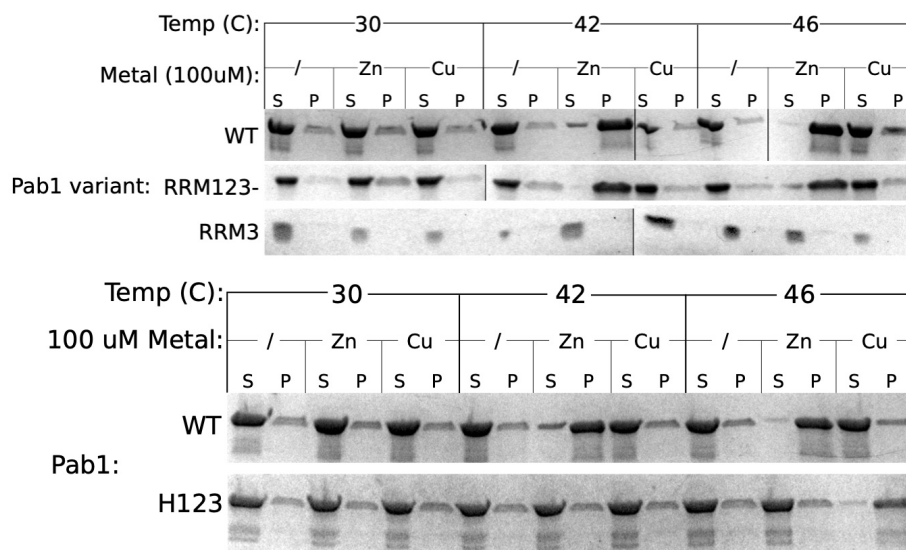
Figure 5.4: The Zn^{2+}-binding site is likely located in RRM 1-3 and contains a histidine residue. Biochemical fractionation of 4 Pab1 variants, the identity of which are explained in the paragraph directly above, was completed following metal incubation and temperature exposure. Protein purification of RRM123-, RRM3, and H123 constructs was completed by Dr. Ruofan Chen and is graciously acknowledged.

The combination of 2 results suggests the Zn(II) binding site requires a histidine located in RRM 1, 2, or 3. First, RRM123- condensation is promoted by Zn(II) at 42 and 46°C. Thus, a Zn(II) binding site exists within RRM123. Next, Zn(II) does not promote H123 variant condensation at 42 or 46°C. Thus, mutation of a histidine in one of these RRMs prevented Zn(II) binding. Separately, NMR peak shifts upon addition of 2mM Zn(II) of

Pab1 RRM3 indicates that RRM3 binds Zn(II) (Figure 5.5).
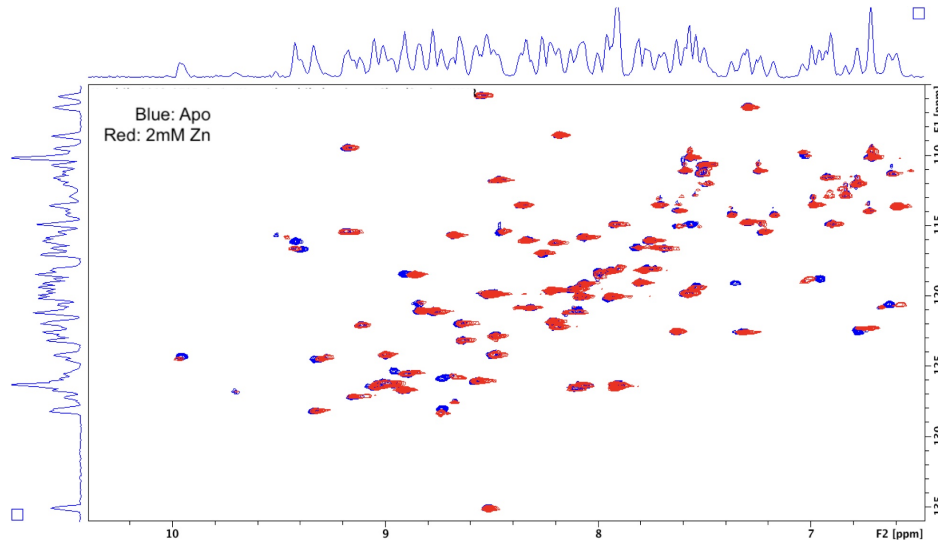
Figure 5.5: Pab1 RRM3 binds Zn^{2+}. Incubating isotopically labeled Pab1 RRM3 with Zn(II) causes noticable peak shifts by NMR. NMR spectrum was not assigned. Protein purification by Dr. Ruofan Chen and NMR help by Dr. Joseph Sachleben are graciously acknowledged.

Whether there exists multiple or a single binding site cannot be determined from these experiments. Additionally, whether Zn(II) bridges different RRMs intermolecularly or binds within a single RRM cannot be determined. Together, Pab1 contains a specific Zn(II) binding site which promotes its condensation.

5.4   Discussion

5.4.1 Zn^{2+} signaling in Pab1 stress-induced condensation is plausible but must still be tested

The work herein showing the presence of a specific Zn(II) binding site in Pab1 that promotes its condensation is exciting, yet much is still to be done. A role for Zn(II) in Pab1 condensation *in vivo* is plausible, yet remains to be tested. Future work should be completed to further characterize the Zn(II) binding site in Pab1. X-ray absorption spectroscopy (XAS)

can inform the structure of the binding site [Ortega et al., 2012]. Separately, HDX-MS on Zn(II)-induced Pab1 condensates can test to what extent the condensed structure differs when triggered by Zn(II), and can potentially inform binding residues. Identifying the binding pocket is important because it enables minimally perturbative mutational study to test whether it is functionally relevant *in vivo*. Further, one could see whether the binding pocket is evolutionarily conserved across orthologs, which would further support its functional importance.

Additionally, the question of the relevance of zinc signalling in heat shock in budding yeast must still be addressed. Zinc is an essential cellular nutrient, and an ever growing body of work supports that it is an important signalling molecules for biology [Liang et al., 2016]. It is known to play an important role in stress tolerance in yeast, suggesting the notion is at least plausible [Zhao and Bai, 2012]. Additionally, zinc deficiency is intrinsically stressful to cells, as it causes an Msn2/4 transcriptional response [Gauci et al., 2009]. Yet, it is unknown whether a pool of labile zinc is released in heat shock in yeast. In other systems, release of labile Zn(II)—termed the zinc wave—can serve as a secondary messenger in signalling [Yamasaki et al., 2007]. Analogous fluorescence-based microscopic approaches can ask whether heat shock in budding yeast releases a labile population of Zn(II) for putative signalling.

5.4.2 *Potential roles of Pab1 condensation in response to Zn^{2+} stress?*

Zn(II)-triggered Pab1 condensation may play a role outside of heat shock. For example, it is well appreciated that acute metal exposure is stressful to the cell, and Zn(II) causes a characteristic transcriptional response [Hosiner et al., 2014]. Because we have demonstrated that high levels of zinc are sufficient to cause Pab1 condensation even in the absence of elevated temperature exposure, perhaps Pab1 senses acute zinc stress. Testing whether cellular fitness of a strain of yeast encoding a Zn(II)-binding deficient Pab1 mutant is perturbed after

acute zinc stress is a simple test of this question.

5.4.3 Zinc importance in the stress response separate from Pab1

Zn(II) signalling can theoretically have a wide impact on the cell beyond simply affecting Pab1 condensation. As an illustrative example, Ydj1 is an Hsp40 chaperone that is involved in the dispersal of condensates [Yoo et al., 2022]. Ydj1 is an attractive candidate for involvement in Zn(II) sensing, as it possesses a zinc finger-like region containing 2 zinc binding domains [Fan et al., 2005]. Mutants to one of these zinc binding domains exhibit temperature-sensitive growth defects in budding yeast, and this zinc binding domain was determined to be neeeded for Ydj1 to synergistically interact with Hsp70 to prevent protein aggregation [Fan et al., 2005]. Therefore, Zn(II) is relevant in playing an important role in the dispersal of stress-induced condensates.

Beyond any role Zn(II) could serve cytoplasmically, it is plausible that Zn(II) signalling acts in the nucleus during heat shock. It has been shown that a zinc wave occurs in the nucleoli of mammalian cells during temperature stress [Pirev et al., 2010]. The nucleolus is the site of ribosome biogenesis [Hadjiolov, 2012] and condensing proteins in yeast are enriched in nucleolar proteins [Wallace et al., 2015], which makes this a question worth following up on.

5.4.4 Widespread role of Zn(II) condensation in disease?

Could Zn(II)-triggered condensation be a widespread phenomenon relevant to disease? Our work localized the Zn(II)-binding site to the RRMs of Pab1. Because several classic stress granule proteins possess RRM domains, it is worth investigation whether their RRMs also possess condensation-triggering Zn(II) binding sites. In fact, several of these proteins are known to bind zinc via their RRM. For example, Zn^{2+} binds His94 and His96 in RRM2 of TIA-1 [West et al., 2022], and Zn^{2+} binds the RRM2 of TDP-43 of [Golovin et al., 2020].

In the case of TDP-43, Zn(II) binding causes its aggregation [Garnier et al., 2017]! It is not obvious why a domain responsible for RNA binding would simultaneously evolve Zn(II) binding sites in multiple proteins, and is an interesting question worthy of future study.

Zn(II) homeostasis is relevant to disease. For example, problems with the metal's homeostasis are associated with neurodegeneration. This is thought to in part be caused by excess metal promoting amyloid aggregation [Szewczyk, 2013]. To underscore the importance of this to health, chelator therapy has been used as a drug approach to reduce the free levels of zinc and other metals as an attempt to treat Alzheimer's disease [Lannfelt et al., 2008, Lee et al., 2004, Trombley et al., 1998].

5.5 Methods

5.5.1 Protein expression and purification

Protein purification of Pab1 constructs was completed as in Riback et al. [2017]. In short, 6xHis-tagged constructs were overexpressed in a BL21 strain of *E. coli*. After harvesting, cells were lysed by sonication on ice in buffer (20 mM HEPES, pH 6.5, 150 mM KCl, 25 mM imidazole, and protease inhibitor tablets). Lysate was clarified by centrifugation and then purified via FPLC using the following protocl: His column (keep elution), overnight TEV protease cleavage, His column (keep flowthrough), Heparin column, and sizing column. Protein concentration was measured via absorbance, using the theoretical extinction coefficient calculated by ProtParam [Gasteiger et al., 2005].

5.5.2 Dynamic light scattering

DLS measurements were completed as in Riback et al. [2017]. In short, a DynaPro NanoStar instrument was used to measure hydrodynamic radius of protein sample with a temperature ramp of 0.25°C/min from 25°C to 50°C. Sample concentrations, unless differently noted, were

15 uM protein in 20 mM HEPES, pH 6.4, 150 mM KCl, 2.5 mM MgCl$_2$, and 1 mM TCEP. Samples were centrifuged at 20,000 g for 30 minutes prior to DLS experiments, and buffer was filtered.

5.5.3 Biochemical fractionation by centrifugation

Biochemical fractionation by centrifugation was completed as in Riback et al. [2017]. In short, purified protein (typically at 5 uM) was incubated at the given temperature for 10 minutes. Samples were buffered in 20 mM HEPES, 150 mM KCl, 2.5 mM MgCl$_2$, and 1 mM TCEP. Samples were centrifuged at 10,000 g for 10 minutes. Fractions of the supernatants were taken, and the pellet was washed twice with clean buffer before being resuspended in Laemmli buffer. Finally, samples were analyzed by SDS-PAGE.

5.5.4 NMR

NMR was completed as in [Chen et al., 2022]. In short, NMR spectra were taken using a Bruker AVANCE IIIHD 600 or a Bruker AVANCE III 500 NMR spectrometer. Protein at > 150 uM was examined at 20 mM HEPES, pH 6.8, 150 mM KCl, 2.5 mM MgCl$_2$, 1 mM TCEP.

CHAPTER 6

FINAL CONCLUSIONS

6.1   Summary

In sum, this thesis has provided insights into the stress-induced condensation of mRNA and protein. We first provided an overview of recent results and major questions in the field of stress-induced condensation. We raised a series of grand challenges, which relate to the general problem of studying a cellular phenomena without widely-established functions, to be overcome in the coming years.

Next, we interrogated stress-induced mRNA condensation using budding yeast as a model organism. We found that most of the transcriptome condenses across stresses in a largely length-independent manner. We found that newly transcribed transcripts escape condensation across a variety of distinct stresses, and posited a model that the mRNA condensation enables the cell to focus translation on stress response transcripts. Finally, we studied the relationship between mRNA condensates isolated biochemically and microscopically visible stress granules, concluding that SG formation is a downstream event from initial, functional condensation.

This thesis also examined more deeply the molecular details of Pab1 condensation. By studying the structural dynamics of Pab1 condensates formed at different temperatures and from different orthologs using HDX-MS, we found that the concept of "thermodynamic specificity" underlies Pab1 condensation at each of these conditions. We also uncover a putative core architecture of crosslinks between Pab1 protomers within the condensate, which may be required for Pab1 condensation and could explain its hydrogel properties.

Finally, we examined the possibility that transition metal signalling could play a role in the stress-induced condensation response. We found that Pab1 possesses a specific Zn(II) binding site that promotes its condensation. Although future work is needed to test the

relevance of Zn(II) signalling in the stress response, transition metal signalling is an exciting possibility by which the cell may tune condensation behavior to adapt to environmental insults.

6.2 Future directions

6.2.1 What is the mechanism of mRNA condensation?

Our work posits a model in which mRNA condensation is mediated by proteins in a transcript-length independent manner. Future work should aim to identify which protein factors are involved. This can be studies by completing a pulldown experiment on a condensed mRNA, isolating the attached proteins, and quantifying which proteins are enriched by mass spectrometry. The basally-condensed, strong hairpin reporter provides a perfect exogenous transcript for study. Using the weakest hairpin reporter as a negative control will help isolate enriched proteins. Because of the drastic increase in mRNA condensation in stress that cannot be explained by translation downregulation alone, it will also be of interest to repeat these studies in stress to see whether additional proteins act. Once putative protein factors are identified, knockout studies can be completed to test whether they are necessary and sufficient for mRNA condensation.

6.2.2 What is the function of mRNA condensation?

Although the work herein connects mRNA condensation to preferential translation in stress, we have not established a causal relationship between condensation escape and enhanced translation. This is the central question in the stress-induced mRNA condensation field and must be pursued. If we are successfully able to identify protein factors required for mRNA condensation, we should be able to make perturbations to these proteins which promote or inhibit mRNA condensation and see whether the preferential translation is enhanced or lost.

A separate approach would be utilizing *in vitro* reconstitution. mRNA condensates can be added to an *in vitro* translation system to study how the condensed nature of the transcript affects its ability to be translated.

6.2.3 To what extent do specific interactions control Pab1 condensation?

The finding that three regions of Pab1 exhibit large protection factors in the condensate and could represent amyloid-like crosslinks between protomers is fascinating and deserves further interrogation. Although imperfect, testing whether Pab1 condensates bind the commonly used amyloid dyes Thioflavin T and Congo red would inform the physical nature of the crosslinks. Further, studying Pab1 condensate structure via X-ray Footprinting and Mass Spectrometry (XF-MS) would provide insight into condensate structure complementary to that obtained by HDX-MS. In comparison to HDX-MS, which informs the stability and solvent accessibility of backbone amide hydrogen bonds, XF-MS assays side chain solvent accessibility [Gupta, 2019].

Beyond obtaining a deeper understanding of the structural nature of the putative crosslinks, additional mutational work is warranted to gain a greater understanding of their functional importance to the condensation process. That the stickerless Pab1 RRM123 construct did not condense at the assayed temperatures indicates the putative stickers are important in condensation. However, the higher-than-expected hydrodynamic radius baseline of the construct suggests that the mutation destabilized RRM1. Thus, further work can test the condensation behavior of a stickerless full length and properly folded Pab1 construct. It is also of interest to generate constructs in which subsets of the three putative stickers have been deleted. These approaches can be complemented by bioinformatic work to study to what extent the putative stickers are conserved evolutionarily.

A separate aspect to be tested is how the putative stickers affect the reversibility of Pab1 condensates. I hypothesize the specific, stable crosslinks contribute to the irreversible hydro-

gelation of Pab1 following condensation. It would be exciting to generate a stickerless Pab1 construct with rapid decondensation kinetics. Perhaps, this construct would not require the molecular chaperone system for its disaggregation following stress cessation. The construct could additionally be genetically integrated *in vivo* to test the biologic importance of the material state of Pab1 condensates.

6.2.4 To what extent do transition metals modulate condensation?

Although the *in vitro* studies in this work make a compelling argument that Zn^{2+} signalling may modulate the Pab1 condensation response *in vivo*, whether this is biologically relevant in the cellular stress response remains to be seen. Studying the stress-induced condensation response of yeast treated with heat shock in Zn(II)-depleted media could inform this question. Another simple approach would be to concretely identify the Pab1 Zn(II)-binding sites so that they can be mutationally perturbed. A distinct question is whether Pab1 condenses in response to physiological Zn(II) stress *in vivo*.

A separate approach worthy of investigation is completing a proteome-wide investigation of the metal-dependence of condensation using an *in vitro* lysate system, which has been previously used in the lab. Heat shocking yeast lysate that has been resuspended in distinct buffers supplemented with different metals or chelators can see how transition metals affect the condensation of the proteome in an unbiased manner. I hypothesize that the condensation of numerous proteins will be modulated by transition metals. Targets of interest can then be deeply studied *in vitro*, such as has been done with Pab1 herein.

Transition metal homeostasis is known to play a key role at the host-pathogen interface in a process termed nutritional immunity. Therefore, it would be of interest to study how transition metal signalling could play a role in condensation of pathogenic fungi. In theory, rather than a stress, protein condensation could be a mechanism by which a pathogenic fungus senses it has reached the host environment and must prepare for infection.

6.3 Final thoughts

Although the lack of broadly established functions for stress-induced condensation has plagued the field, precisely this dearth in basic understanding makes it such an exciting area to work in. I am optimistic that further study, which carefully interrogates the molecular details underlying condensation by iteratively combining *in vivo* and *in vitro* reconstitution approaches, will uncover the physiological roles played by this universally conserved phenomenon.

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