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ADAPTIVE STRESS RESPONSE THROUGH A FLEXIBLE CONDENSATE CASCADE

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ABSTRACT

Cells must respond to a broad, ever-changing spectrum of environmental insults, yet cannot afford a dedicated signaling pathway for every possible stress. This dissertation asks how a single stress-response system achieves both flexibility and selectivity, using the yeast heat shock response (HSR) as a model.

At its core, this work establishes that HSR activation proceeds through a flexible condensate cascade: heat shock drives sequential condensation of orphan ribosomal proteins, the co-chaperone Sis1, the chaperone Hsp70, the transcription factor Hsf1, the Mediator coactivator, and RNA polymerase II. Five mechanistically distinct proteotoxic stresses, heat shock, arsenite, the proline analog AZC, the ribosome biogenesis inhibitor DZA, and hyperosmotic NaCl, each engage this cascade, but do so at different entry points and with different efficiencies, converging on Sis1 condensation as a shared, obligate bottleneck. Despite broadly overlapping condensation of the chaperone-sensing machinery across all five stresses, only heat shock and arsenite productively activate HSR transcription, showing that condensation of the sensing arm is necessary but not sufficient for output. Transcriptional activation is instead gated downstream, at the level of Hsf1 and the transcriptional machinery itself. Nuclear depletion of Sis1 further demonstrates that, rather than acting as an activating relay, Sis1 functions as a rheostatic repressor of Hsf1 whose influence is titrated in proportion to stress severity — allowing the chaperone network to respond flexibly to diverse insults while insulating the core transcriptional program from spurious activation. This convergent-cascade architecture reframes the HSR not as a pleiotropic responder to any proteotoxic insult, but as a selectively gated program that grants heat and other proteostasis-collapsing stresses privileged access while still sensing a much broader range of chemically distinct challenges.

This convergent-cascade model builds on and extends a foundational finding: that the downstream, transcription-proximal half of the HSR condensate cascade itself assembles in

an ordered, stepwise fashion rather than through concerted, all-or-none recruitment. Live-cell imaging and transcriptomics show that Hsf1 first clusters upon partial release from Hsp70; phosphorylation and further Hsp70 dissociation then permit the coactivator Mediator to partition into these clusters; and Mediator-bound Hsf1 assemblies finally recruit RNA polymerase II. This hierarchical assembly produces graded, rather than switch-like, transcriptional output, and fully assembled condensates drive an adaptive three-dimensional reorganization of the yeast genome that is dispensable for transcription itself but confers a measurable fitness benefit — establishing condensate architecture, and not merely condensate presence, as a regulated determinant of cellular behavior, and providing the mechanistic scaffold onto which the upstream cascade is built.

Finally, this dissertation asks whether the logic of pathway-level prioritization scales up to how cells triage many simultaneous environmental demands. Profiling single-cell transcriptomes across twenty combinatorial environments — varying carbon source, nutrient richness, osmolarity, temperature, and reactive oxygen species — and resolving this data with a spectral mutual-information method reveals that environmental information is organized hierarchically across the transcriptome: variation attributable to carbon source dominates the shallowest modes of transcriptional variation, followed in order by nutrient richness, osmolarity, temperature, and oxidative stress. This statistical hierarchy predicts, and is borne out by, physiological prioritization: under combined heat and osmotic stress, cells preferentially mount the osmotic-stress program and suppress the heat-shock response, a prioritization encoded within individual cells rather than emerging only at the population level. Mechanistically, this prioritization is implemented through differential translation initiation — osmotic stress collapses polysomes more severely than heat shock, preventing the accumulation of orphan ribosomal proteins that would otherwise sequester Sis1 and Hsp70 away from Hsf1 — directly linking environmental priority routing to the same condensate cascade described above, and suggesting that a common molecular vocabulary underlies both

pathway-level and organism-level stress prioritization.

CHAPTER 1

INTRODUCTION

1.1 The history of the heat shock response

The heat shock response (HSR) as described by the Pincus lab is the stress response pathway in yeast that enables the cell to survive a period of elevated temperature and return to normal functioning upon the removal of the insult. The HSR pathway as a whole is also highly conserved across life. From single celled organisms all the way to humans, many of the pathway's components and their respective relationships display similar or identical behaviors. Because of this, its study is vital in the understanding of a variety of diseases. In humans, when Hsf1 activity is diminished, there is a resulting build-up of protein aggregates that can lead to neurodegenerative diseases such as Parkinson's, Alzheimer's, ALS, and others [Ankar and Sistonen, 2011]. On the other hand, excessive Hsf1 activity leads to overactivity of the HSR often resulting in cancers [Mendillo et al., 2012]. Understanding the role of individual components in the stress response has led to and promises to provide even greater understanding of the cell's response to not only heat stress but stress in general (Fig. 1.1).

This rapid and robust response pathway gives the cell incredible adaptive capability. The key players of this response are the transcription factor Hsf1 and its chaperone and co-chaperone Hsp70 and Sis1 respectively. Under non heat shock response conditions, Hsf1 is bound by Hsp70, with the assistance of Sis1, in an inactive state [Krakowiak et al., 2018]. Upon heat shock (HS), Hsp70 and Sis1 are titrated away to other clients. In particular, one set of clients is the population of newly synthesized ribosomal proteins. The reason for their vulnerability has to do with a larger disruption in ribosome biogenesis and will be discussed later in this chapter. The end result of this titration, however, is the release of Hsf1 to function as a transcription factor. In this role, it strongly regulates the transcription of a

suite of genes important to the cell's survival of HS, including Hsp70, thereby regulating its own activity in a feedback loop. This transcription regulation occurs via the sequential recruitment of various other transcriptional machinery resulting in the assembly of HSR condensates. This partnership between Hsf1 and its chaperones serves as the basis for a highly regulated and highly effective stress response.

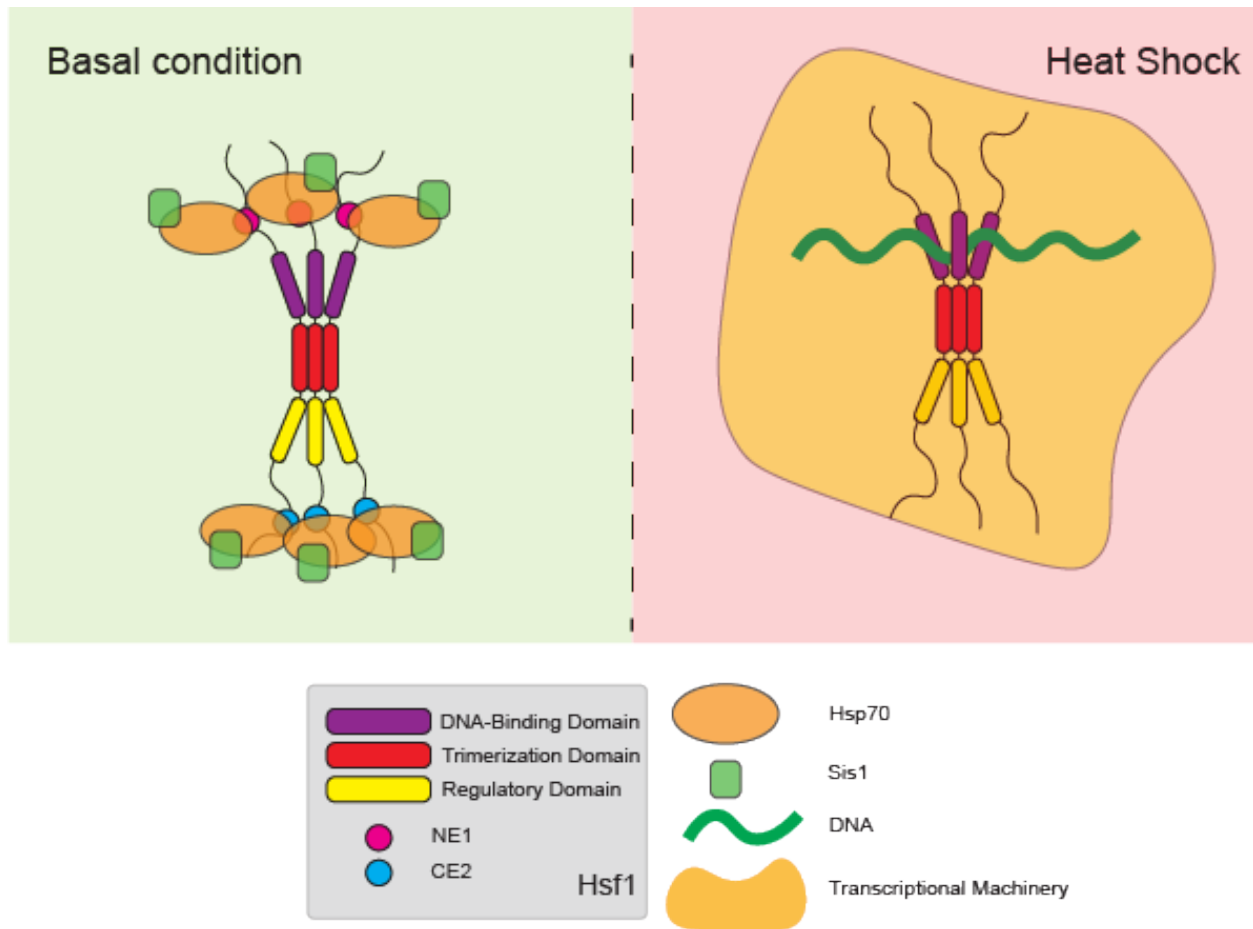


Figure 1.1: Structure and interactions of Hsf1 in basal (Non-Heat Shock) and Heat Shock conditions within the nucleus

Previous work in the lab has primarily focused on the role of Hsf1 specifically in HS conditions. However, as we have come to understand more about this response, we have seen that factors such as the disruption of ribosome biogenesis are also seen in other stresses. It therefore stands to reason that variable stress likely variably activates components of the HSR pathway. The ability of cells to use a response pathway in various conditions is key to

conservation of energy and resources in times of stress. The focus of my work has been to identify certain stresses that may share HSR machinery, and to interrogate the extent and degree of overlap between their individual responses.

1.2 Biomolecular condensates as a transient organizational method

The cell is adept at isolating specific functions or events to individualized compartments within it. This process is crucial for efficiency and specificity. It enables the cell to create environments that are incredibly unique to individual processes, such as transport in the golgi network or degradation in lysosomes. Many of the components for these processes cannot function in the greater cellular environment while various components that exist outside of these compartments could not function within them. The most well-known of these specialized compartments are of course the organelles. Without organelles, the cell would have far fewer abilities at its disposal.

A newly emerging class of specialized compartments known as biomolecular condensates are receiving increased attention [Banani et al., 2017]. Organelles serve constant dedicated functions within the cell and as such are generally membrane bound to keep their contents separate from the greater environment. This provides incredible protection and efficiency for the processes that occur within them. However, this formality in their construction leaves little room for adaptation in the presence of stress or variable environments. The cell's solve for this are biomolecular condensates.

The formal definition for these temporary constructions is membrane-less structures formed through specific clustering of various components [Banani et al., 2017]. They often form in response to a transient need of the cell to adapt to an environmental change. In the instance of this need, components designed to address the change arrange into concentrated regions that can perform a specific transient function. A well-known example for

this type of assembly is the nucleolus that exists within the nucleus and changes shape and function or can disappear nearly completely in the presence of stress or cell division. Components of biomolecular condensates include various proteins and nucleic acids that interact via multivalent interactions, weaker than those of more permanent assemblies of or within membrane bound organelles. This gives such condensations heterogeneity and dynamicism which together contribute to an overall flexibility in form and function. Molecules within in these condensates can filter in and out with the local environment as needed, and the entire assembly itself can interact with other cellular components such as DNA, other organelles, and foreign intruders to the cell.

The bonds that hold these assemblies together, while weak, pose the threat of aggregation, as many components consist of hydrophobic intrinsically disordered regions either as a primary function or as a byproduct of an induced stress. To combat this, one of the main classes of participant in biomolecular condensates are chaperone and co-chaperone proteins that promote movement and inhibit aggregation. This serves a dual purpose of mixing the components of the condensate to ensure that they can find their intended targets and to prevent a permanent aggregation. This latter role is especially important in maintaining their transient property as their reversibility upon the removal of stress or return to basal conditions is crucial for the health of the cell and the conservation of resources. I will detail how each of these properties play a role in both heat shock and overall stress response throughout the work presented here.

1.3 Sequential assembly of heat shock response condensates

The heat shock response, as we define it in our lab, consists of the formation of two sets of biological condensates. One is those formed from the accumulation of Hsp70 and Sis1 at newly synthesized ribosomal proteins at the nuclear periphery. The other, which will be described in this section, is the formation of HSR transcriptional condensates at the DNA

within the nucleus. The latter has been recently determined to form in a highly regulated sequential manner [Chowdhary et al., 2022, 2025]. Hsf1 consists of a coiled coil leucine zipper trimerization domain, a DNA binding domain, and a regulatory domain flanked on either side by intrinsically disordered regions (IDRN and IDRC). Under basal conditions, Hsf1 exhibits small levels of transcription factor activity at proteostasis regulatory genes. During this time, a much larger portion of the population is bound up by Hsp70. Hsp70 binds Hsf1 at regions within the IDRs termed NE1, and CE2 which, while far from the DNA binding domains, results in an allosteric repression of these regions when bound [Krakowiak et al., 2018, Peffer et al., 2019]. This occurs because of the large relative size of Hsp70 compared to the crucial domains of Hsf1 and conformational changes that block the interactions both with the DBD and trimerization domain with their targets. Upon heat shock, Hsp70 is titrated away freeing the Hsf1. Once freed, Hsf1 is allowed to form trimers and target DNA via the DNA binding domain. When this is allowed to occur, the rest of the necessary transcriptional machinery is recruited to the DNA as well, and interestingly, the genome undergoes a 3D rearrangement concurrent with a drastic change in transcriptional output. A body of work spearheaded by Surabhi Chowdhary in the lab has detailed the process of HSR condensation that occurs from this point [Chowdhary et al., 2022, 2025].

Previously, two models existed for the formation of HSR condensates. The first was a spontaneous assembly of all transcriptional machinery at once at the DNA that ultimately resulted in condensates once all components had arrived. The other was a sequential model whereby Hsf1 bound, and this binding then recruited one component before recruiting the next resulting in the final condensed state. Chowdhary’s work has definitively shown the latter result to be true [Chowdhary et al., 2025].

A series of rapamycin induced anchoring away of each major component of the transcriptional machinery was conducted to prove not only the presence of a sequential recruitment model but also the order of recruitment itself [Chowdhary et al., 2025]. The key components

analyzed were Hsf1, the Mediator complex, and RNA Polymerase II. Without anchoring away, these components have been shown via fluorescent microscopy, to produce colocalized condensates upon heat shock. The first step was to anchor away Hsf1, artificially removing it from the nucleus upon rapamycin treatment. When Hsf1 is removed from the system, condensation of both mediator and RNA Pol II is eliminated even in the presence of heat shock, indicating that they rely on the presence of Hsf1 to cluster. The next step was to anchor away one of the primary subunits of mediator (Med15). In this case, upon heat shock, Hsf1 clustering was still observed, but Pol II clustering was eliminated. Also of note, was the lack of 3D genome reorganization. Finally, upon anchoring away of Pol II during heat shock, clustering of Hsf1 and Med15 were still observed although there was still a lack of 3D genome reorganization. This showed Pol II's location in the sequence as downstream of Hsf1 and Med15 clustering.

Only in the presence of all three components did we see concordant clustering and 3D genome reorganization. Taken together this data presents a model of sequential recruitment of components (HS $\rightarrow$ Hsf1 $\rightarrow$ Med15 $\rightarrow$ Pol II) that ultimately leads to transcriptional condensates with genome reorganization capability [Chowdhary et al., 2025] Figure 1.2).

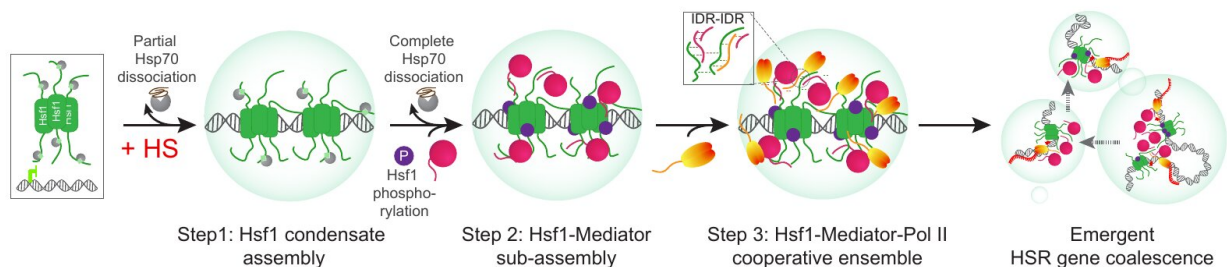


Figure 1.2: **Hsf1 activation during stress**

1.4 Transcriptional regulation through HSR condensates

Once Hsf1 is freed from Hsp70 to its fully active state as a transcription factor, it targets a very specific cohort of genes. As mentioned in the previous section, its DNA binding domain

(DBD) targets specific sequences of DNA referred to as heat shock elements or HSEs. Each HSE consists of at least three sequential inverted repeats of the bases NGAAN where N can be any nucleotide. There are over 70 of these HSE sites throughout the yeast genome. Under basal conditions, Hsf1 has been shown to be active at 18 of these sites [Solís et al., 2016]. This group generally consists of chaperone proteins that assist in the folding and development of mature proteins. Removal of Hsf1 from the nucleus under basal conditions shows the presence of protein aggregation and a significant drop in growth [Solís et al., 2016].

Hsf1 while essential under basal conditions in yeast, sees its activity greatly intensified under heat shock conditions, hence the name, as it activates HSR genes. Upon heat shock, Hsf1 makes its way to an additional suite of genes that manage the cell's proteostasis network and help the cell to survive the stress and rapidly recover. These genes include a host of heat shock specific and non-specific chaperones. Examples include Hsp70, Hsp90, Hsp26, and Hsp42 [Solís et al., 2016]. Hsp70 will be discussed in depth later in this chapter, but as a whole, this group of proteins serve to prevent aggregation of other proteins that have a higher likelihood of misfolding and aggregation in heat shock as well as refolding denatured proteins. Hsp104, specifically, acts as a protein disaggregase alongside Hsp70. It also upregulates several proteins involved in the protein degradation system such as Ubi4 and Cdc48 [Solís et al., 2016]. This enables the cell to rapidly and efficiently degrade proteins that cannot otherwise be handled by the chaperone network and could cause damage to the cell if not disposed of.

Activation of Hsf1 during heat shock enables the cell to rapidly respond to stress, shifting its resources in a matter of seconds from prioritizing growth and reproduction to maintaining proteostasis and surviving this particular insult. Importantly, it also manages its own activity, creating a feedback loop with Hsp70. As discussed, Hsp70 binds Hsf1 to inactivate it. The gene for Hsp70 itself is one of the primary targets of Hsf1 in heat shock [Krakowiak et al., 2018]. Thus, the longer that the stress proceeds, the more Hsp70 are produced. These

additional Hsp70 can then address protein accumulations throughout the cell as needed. As this extra volume of Hsp70 saturates the cell and its various clients, there will then be sufficient molecules to once again inhibit Hsf1. In this way, Hsf1 directly tunes its own activity preventing run away heat shock response behavior and setting the stage for a rapid recovery and reset at the moment the stress is removed. Ultimately this response is tightly regulated and highly specific, giving the cell the best chance at survival in sub-optimal environments.

1.5 Orphaned ribosomal protein condensate assembly in response to heat

One of the most immediate effects of stress on the cell, as in heat shock, is the repression of ribosome biogenesis as the cell transitions from prioritizing growth to prioritizing survival. This is carried out via transcriptional reduction of ribosomal RNAs (rRNAs). However, due to the large demand of ribosome production under basal conditions, a great volume of ribosomal proteins are already being translated when this halt occurs. Additionally, translation is only brought to a halt at higher levels of heat stress than is transcription alteration. To craft a mature ribosome, the cell requires both these ribosomal proteins and rRNAs. Without rRNAs it is left with a surplus of newly synthesized ribosomal proteins unable to be assembled into mature ribosomes. Importantly, these ribosomal proteins are highly aggregate prone and pose great danger to the cell if not sequestered [Ali et al., 2023].

This is where the partners of Hsf1, its chaperone Hsp70, and corresponding co-chaperone Sis1 step in. As these orphaned ribosomal proteins (oRPs) begin to accumulate, Sis1 is recruited [Ali et al., 2023]. Sis1 itself is a J-domain protein that stimulates Hsp70 ATP-ase activity. When Hsp70 is ATP bound it is in an open state, rapidly associating and dissociating from its clients. When ADP bound it clamps down or closes on these clients. Sis1 in facilitating ATP-ase activity, drives Hsp70's strong binding to clients. It then follows that as Sis1 is recruited to oRPs, Hsp70 will no longer remain tightly bound to Hsf1. Instead,

it will be encouraged to bind tightly to its other clients, particularly those in close proximity to Sis1. Hsp70 itself is less of a specific chaperone and more of a broad actor. It has some specific sequence preferences, but more importantly it targets hydrophobic regions of proteins such as on the IDRs of Hsf1 or on the inappropriately exposed interior portions of mis-folded or unfolding proteins. This makes oRPs a perfect Hsp70 target. Therefore, Sis1 relocalization to oRPs results in consistent Hsp70 presence, in turn freeing Hsf1 to behave in the formation of HSR condensates (Fig. 1.3).

Once present among oRPs, Hsp70 and Sis1 serve to churn the ribosomal proteins, keeping them from aggregating. In their presence, accumulations of oRPs display classic biomolecular condensate behavior. They are physically distinct from their surroundings and display dynamicism via fission and fusion. Time-lapse 3-D lattice light-sheet imaging showed these behaviors in a strain with fluorescently tagged Sis1, Hsp70, and Rpl26a, a representative ribosomal protein [Ali et al., 2023]. Importantly, it also displayed constant signal from the tagged Rpl26a, indicating that these proteins are in fact stable throughout the sequestration process rather than being degraded.

The specific necessity of Hsp70 in this suite of behavior was evaluated by removing it from the system. In cell lysate assays, the removal of Hsp70 via Hsp70 inhibitor VER-155008, saw accumulations of RPs formed where Sis1 was still recruited, but their behavior differed dramatically [Ali et al., 2023]. Rather than dynamic condensates, solid and irregular aggregates of oRPs were observed indicating Hsp70's role in keeping these condensates liquid like and dynamic. It also identified Sis1's activity as upstream of Hsp70 as expected.

When removing Sis1 from the system, its role as the key for reversibility was revealed. Depletion of Sis1 from the nucleus via a rapamycin anchor-away system was utilized to analyze the behavior of oRP condensates in the absence of its chaperones [Ali et al., 2023]. In non-treated cells, tagged ribosomal proteins accumulate at the nucleolar periphery upon heat shock and rapidly disperse throughout the cytosol upon return to standard tempera-

ture. Removal of Sis1, on the other hand, saw the accumulation of oRPs under heat shock, but when heat shock was removed, they could no longer disperse to the cytosol. This reversibility is key for not only the prevention of potentially hazardous aggregates, but also in the conservation of cellular resources. Protein translation is incredibly energy intensive, and ribosomal protein production accounts for a significant portion of overall translation occurring in the cell. To simply degrade newly synthesized ribosomal proteins under stress would be a massive waste of resources, energy, and time, at a period when the cell has none of those to spare. The importance of maintaining oRPs throughout stress cannot be understated.

A model of oRP condensate behavior was therefore constructed, where the accumulation of oRPs in the presence of heat shock recruits Sis1 which in turn recruits Hsp70, the presence of which keep the condensates liquid-like, dynamic, and reversible [Ali et al., 2023].

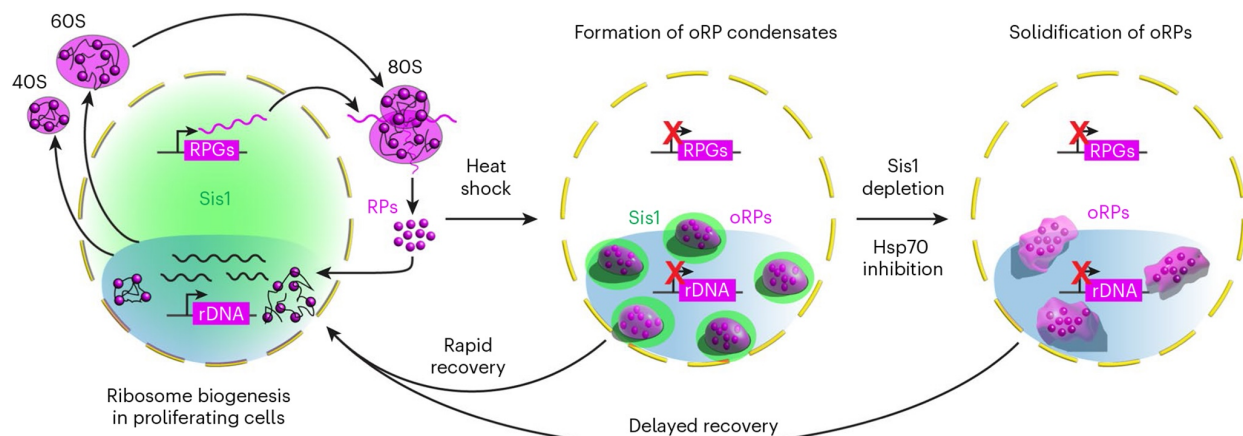


Figure 1.3: Model of orphaned ribosomal protein condensate assembly upon heat shock. [Ali et al., 2023]

1.6 Ribosome biogenesis as the cornerstone of stress response

The cell must take the management of oRPs under stress so seriously because one of the primary functions of any living cell is to produce ribosomes. Ribosomes after all are responsible for the production of all proteins in the cell and therefore the rate of ribosome production is directly related with growth rate and cell health. In fact, some might even claim that

ribosome production is the actual driving force behind evolution and every other instance of adaptation is a fortunate byproduct serving the ultimate goal of making more ribosomes. While this view may be extreme, it does point to the idea that the management of ribosome biogenesis is a fundamentally important function of the cell. Its rate is finely tuned based on environmental cues that impact cellular health.

As mentioned above, this regulation takes the form of sequestration of oRPs into condensates upon heat shock, leading ultimately to changes in transcription via HSR condensates [Ali et al., 2023]. However, heat shock is far from the only stress that a cell can face. A whole suite of toxins or environmental conditions are known to impact ribosome biogenesis either directly or indirectly and are used to do so in various experimental methods. Some of these stresses include exposure to arsenite, diazaborine (DZA), and L-azetidine-2-carboxylic acid (AzC). To start, arsenite leads to a host of cellular stresses. It triggers oxidative stress by disrupting the electron transport chain, binding to dithiol-containing subunits of Complex I and III, uncoupling oxidative phosphorylation. The result of this uncoupling is a reactive oxygen species (ROS) cascade as electrons leak out of the ETC leading to the creation of large amounts of ROS which damage lipids across the cell in various membranes and disrupt overall cell health. Not only this, but it activates the HOG MAPK cascade. The result of this activation is a stress response similar to the HSR where specific transcriptional programs are upregulated, in this case focused on growth and proliferation genes like Msn2 and Msn4. Also, directly related to ribosome biogenesis, it binds cystine residues within zinc-fingers in various proteins leading to protein misfolding and disrupting key elements of transcriptional machinery. This leads to overall disruption of ribosome biogenesis.

Introduction of AzC has a more specific but highly impactful effect on the cell. AzC functions as a toxic analog to proline. It is therefore indistinguishable from proline to prolyl-tRNA synthetase and will compete with proline for charging onto the tRNA. Proline, as an amino acid, is incredibly important for tertiary structure formation of proteins. Its 5-

membered ring makes it the perfect shape for producing kinks in amino acid chains that are utilized in the formation of tertiary structures such as α -helices and β -sheets. AzC only has a 4-membered ring and as such cannot replicate these structures. This results in mature proteins that are misfolded and generally non-functional. These proteins begin to aggregate much the same way oRPs do and the result is the recruitment of stabilizing and sequestration chaperones to prevent damage to the cells. This behavior mimics closely the formation of oRP condensates and makes it a likely activator of some or all of the stress response condensate assemblies.

Finally, DZA acts almost as a direct inhibitor of ribosome biogenesis. When introduced, it binds specifically to the second ATPase domain of a protein called Drg1p preventing its ability to perform ATP hydrolysis [Loibl et al., 2014]. Drg1p in normal conditions is responsible for removing shuttling factors such as Rlp24 from the pre 60S particle of the ribosome. Rlp24 directs the transport of the particle from the nucleus to the nuclear pore/cytoplasm where it can assemble into a mature ribosome after removal of Rlp24 and other shuttling factors. DZA blocks this removal, leaving Rlp24 and all subsequent shuttling factors bound to the 60S particle [Loibl et al., 2014]. The outcome of this is two-fold. The first is that, without mature 60S subunits available, 43S pre-initiation complexes accumulate on mRNA transcripts, unable to join with a 60S subunit to form a translation-competent ribosome. Additionally, Rlp24 and the other shuttling factors trapped on the pre-60S particle cannot return to the nucleus to complete their normal shuttling cycle, causing ribosomal proteins to build up in the nucleus awaiting processing. This leads to the build-up of ribosomal proteins in the nucleus awaiting processing and shuttling away from the nucleus. Ultimately, the result is a breakdown of ribosome biogenesis and the build-up of ribosomal proteins in an event that we predicted to have much similarity with the condensation of oRPs under heat shock.

I also explored the introduction of osmotic shock via heightened NaCl exposure as an

orthogonal stress pathway. Much previous work in our lab by Dr. Annisa Dea has studied the effects of osmotic stress in yeast. She identified routes of prioritization of various concurrent stresses in the cell, concluding that osmotic shock is generally prioritized over other stress responses. When such a stress is placed on the cell, the HOG MAPK pathway is activated directly, similar to what is seen in arsenite stress. This results in a host of transcriptional changes again centered around Msn2/4 genes and their related cohort [Gasch et al., 2000]. This stress was selected as one that operates largely outside of the observed HSR to serve as a comparison point for these more ribosome biogenesis centered stress factors.

1.7 Two condensates under heat shock

The large bodies of work detailed in the previous sections create the idea of a set of condensate consequences in the presence of heat shock. Upon infliction of stress, Hsp70 is recruited, via Sis1, away from Hsf1. Now separate, they can go on to aid in the formation of two distinct biomolecular condensates at different areas of the nucleus [Ali et al., 2023, Chowdhary et al., 2022]. While both result from the same initial heat shock, the one managed by Hsp70 helps the cell wait out the stress and prevents damage, and the one managed by Hsf1 rapidly addresses the stress itself, altering the cell's activity via transcription. Based on this idea, it seems that the activity of Sis1 could be said to influence the formation and behavior of both of these incredibly important stress response activities. It then stands to reason that other stresses or environmental conditions that impact Sis1 localization would in turn impact Hsp70 localization, Hsf1 activity, and overall activation of the heat shock response.

The goal of the work that will be outlined in this thesis was to explore these ideas and to split them into two large questions. The first: Is there a direct link between these two condensates at either end of the heat shock response that could constitute a sort of condensate cascade? That is, is the formation or presence of oRP condensates directly related to that of HSR condensates? The second question: If so, is this cascade activated

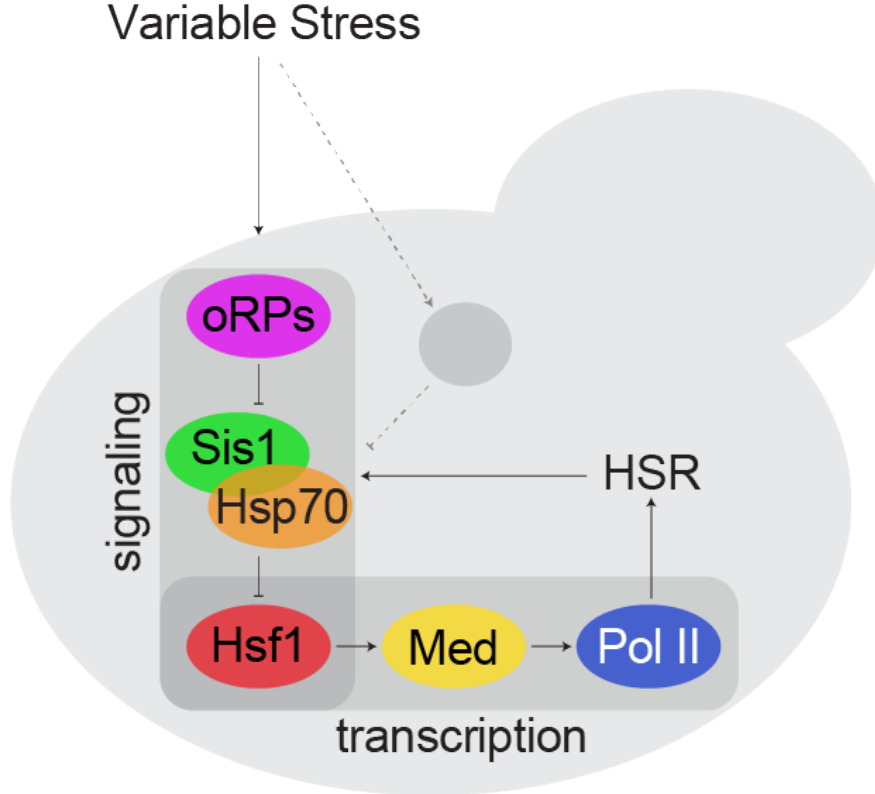


Figure 1.4: Proposed model for overlapping condensate response to stress

in other stresses and to what extent? We know of other stresses that can impact ribosome biogenesis and therefore likely Sis1 behavior. In those stresses, do we see similar physical and genetic changes as compared to what we see in a standard heat shock?

To address the physical changes, I performed a series of imaging assays using spinning-disk confocal microscopy where I analyzed the behavior of fluorescently labeled components of the heat shock response, Sis1, Hsp70, Hsf1, mediator (Med15), and RNA Polymerase II (Pol II) under the suite of stresses described in the previous section. In each stress, I looked for changes in localization and behavior of the HSR components. Along with each stress, I also evaluated their behavior in the presence of cycloheximide (CHX). CHX serves to halt translation elongation. It does this by mimicking an uncharged tRNA lodging itself in the E site of the ribosomal machinery. This prevents eEF2 translocation and ultimately results in stalling of translation. The effect of this is the prevention of oRP formation.

By incubating cells with CHX before adding an additional stress, there will be no need to sequester orphaned ribosomal proteins because they simply will not form in the first place. This method is used to split the two halves of the HSR. Any condensation that I see of Sis1 and Hsp70 will indicate condensation clients other than oRP condensates. Furthermore, any condensation of Hsf1, Med15, or Pol II will be the result of these alternate condensates recruiting Sis1 and Hsp70 in a similar manner to oRPs.

Additionally, each stress was also applied in strains in which Sis1 can be sequestered away from the nucleus by anchoring it to the rapamycin, as detailed in the section on oRP condensate formation. When done in non-stress conditions, we see an increase in HSR activation due to the inability of Hsp70 to actively bind Hsf1 in the absence of Sis1 [Feder et al., 2021, Alford et al., 2021]. By observing the behavior of Hsp70 and Hsf1 in this strain with and without anchoring Sis1 away and with and without the variable stresses, I was able to directly question the behavior of Hsp70 without the influence of Sis1. That is, its affinity for clients other than Hsf1 in various stresses, indicative of the presence of other stress response condensates or protein aggregations. The outcomes of all of these experiments are discussed in chapter 3, examining the convergent condensate cascade.

1.8 Transcriptional changes of the heat shock response

The physical changes observed in heat shock preempt a massive change in transcriptional output as the cell rapidly adapts to this new environment. Using RNA-seq, one can see the highly specific transcriptional program controlled by Hsf1 in the presence of heat shock. Upon heat shock, around 1000 genes see some form of transcriptional change [Gasch et al., 2000]. The majority of these genes actually face downregulation as managed by the repressive Environmental Stress Response (rESR) [Gasch et al., 2000]. This is a larger transcriptional program that responds to stress more broadly. These genes include those necessary for ribogenesis, as made evident previously in this chapter. Among those upregulated are of course

the Hsf1 targets as well as proteins related to induced Environmental Stress Response (iESR) that manage things like metabolism, protein degradation, and oxidative stress defense [Gasch et al., 2000]. The more than 40 genes associated with Hsf1 activity see some of the largest changes in expression with some looking at a more than ten-fold increase in mRNA output during stress [Solís et al., 2016]. By analyzing RNA-seq data from cells under stress, one can craft a picture of the cell’s transcriptional state and survival mechanism priorities in specific environments.

In my work, I performed RNA-seq not only in heat shock, but in the entire suite of stress conditions outlined previously in this chapter. This enabled me to link physical changes in the condensation conditions that resulted from each stress directly with their transcriptional output. Additionally, I looked at each stress alone and after a rapamycin induced anchor away of Sis1 to observe how much of each transcriptional change was due to oRP condensate production and how much came from outside sources. In doing so, I was able to construct a model explaining how various stresses converged onto a shared condensate cascade and to what extent they activated this cascade compared to heat shock alone. This model shapes our understanding of how the cell responds to unique stresses with conserved mechanisms of heat shock to conserve resources and gene space.

1.9 Conclusion

Taken together, the work described in this thesis craft the idea of a flexible condensate cascade that serves to insulate the heat shock response from variable stresses. The cell is able to utilize a common set of physical and transcriptional responses in various environments. However, only specific stresses such as arsenite are able to activate a partial transcriptional response, and only heat shock is able to produce the full transcriptional response. This idea highlights the cell’s efficiency and robustness in its adaptation to stress. By using a core set of stress response components the cell can quickly and effectively adapt to a swath

of insults with minimal resources and prioritize those resources for specific responses. We also found that the behavior of Sis1 serves to differentiate the cell's response to various insults. It's showed strong condensation in all stresses, but its localization and used pool of molecules is dependent on the individual stress, being recruited to distinct clients for each. While this does free Hsf1 to act to initiate condensate activity in all stresses, we only see full activation in heat. Sis1 therefore tunes the activity of Hsf1 distinctly in each stress. It serves to buffer the HSR by maintaining nuclear Sis1 to a state that prevents full condensate activation outside of HS. Additionally, we see a complete decoupling of condensate behavior from transcriptional output. This shifts our thinking of stress signal integration in the HSR from graded and inclusive to a more binary process that buffers against full activation with a defined threshold or set of conditions necessary to induce a full response. Rather than a promiscuous program that responds equally to a variety of stresses, this program in fact differentiates between like stresses and responds according and specifically. This specificity is a key component of survival in all life. Understanding the link between the individual condensate reactions and the way that they are activated in variable environments helps us to better understand this efficiency mechanism. It also promises to inform study of a greater, interconnected stress response network. Understanding this network will help us to understand life, evolution, and even death.

CHAPTER 2

EMERGENT 3D GENOME REORGANIZATION FROM THE STEPWISE ASSEMBLY OF TRANSCRIPTIONAL CONDENSATES

Author Contributions to This Chapter

The work in this chapter is from a manuscript posted as a bioRxiv preprint [Chowdhary et al., 2025]. Conceptualization of the project was carried out by Surabhi Chowdhary and David Pincus. Methodology, investigation, and resources were contributed by Surabhi Chowdhary, Sarah Paracha, and myself, with formal analysis led by Surabhi Chowdhary. The original draft was written by Surabhi Chowdhary and David Pincus, and all authors contributed to review and editing. My specific contributions consisted of several rounds of imaging and analysis and I assisted in the overall conceptualization and editing of the paper.

Transcriptional condensates are clusters of transcription factors, coactivators, and RNA Pol II associated with high-level gene expression, yet how they assemble and function within the cell remains unclear. Here we show that transcriptional condensates form in a stepwise manner to enable both graded and three-dimensional (3D) gene control in the yeast heat shock response. Molecular dissection revealed a condensate cascade. First, the transcription factor Hsf1 clusters upon partial dissociation from the chaperone Hsp70. Next, the coactivator Mediator partitions following further Hsp70 dissociation and Hsf1 phosphorylation. Finally, Pol II condenses, driving the emergent coalescence of HSR genes. Molecular analysis of a series of Hsf1 mutants revealed graded, rather than switch-like, transcriptional activity. Separation-of-function mutants showed that condensate formation can be decoupled from gene activation. Instead, fully assembled HSR condensates promote adaptive 3D genome reconfiguration, suggesting a role of transcriptional condensates beyond gene activation.

2.1 Introduction

Gene regulation is a fundamental process that underpins growth and development, and disrupted gene control is the basis for most human diseases. 3D genome organization and biomolecular condensates are key determinants of eukaryotic gene regulation, and mutations in either are causally linked to cancer, neurodegeneration, and developmental disorders [Pei et al., 2024, Shin and Brangwynne, 2017, Lupianez et al., 2015]. Biomolecular condensates are self-organizing, membrane-free macromolecular assemblies that selectively partition specific biomolecules [Banani et al., 2017]. Compartmentalization of transcriptional machinery in biomolecular condensates at specific genomic loci is associated with robust expression of cell identity, proliferation, development, and stress-induced genes [Boija et al., 2018, Sabari et al., 2018, Cho et al., 2018, Lu et al., 2020, Zamudio et al., 2019, Wei et al., 2020, Chowdhary et al., 2022, Zhang et al., 2022].

Transcriptional condensates are hypothesized to involve cooperative association of sequence-specific transcription factors, coactivators, and RNA Polymerase II (Pol II) [Boija et al., 2018, Sabari et al., 2018, Cho et al., 2018, Zamudio et al., 2019, Tuttle et al., 2021, Boehning et al., 2018, Nair et al., 2019, Sharp et al., 2022, Hnisz et al., 2017], yet how they are assembled is unclear. There are two plausible models for how transcriptional condensates may form, each with potentially distinct functional implications (Figure 2.1A). The first is the concerted recruitment model, wherein constituent biomolecules are recruited in a non-stoichiometric and synergistic manner, and a network of multivalent interactions among the various components reinforces the spontaneous assembly of the condensate (Figure 2.1A). These low-affinity, multivalent interactions among domains with compatible surface properties are commonly attributed to intrinsically disordered regions (IDRs), which are frequently present in the constituent proteins [Lyons et al., 2023, Van Der Lee et al., 2014, Posey et al., 2018]. Also embedded within this network of disordered interactions are allosteric regulatory interactions between the structured domains of the proteins, which may proscribe complex

kinetic control [Soutourina, 2018, Robinson et al., 2015, Rengachari et al., 2021, Richter et al., 2022]. An alternative model of condensate assembly is the sequential recruitment model, which entails a hierarchical and ordered recruitment process, where each component condenses in a stepwise fashion (Figure 2.1A). The extent to which the assembly and activity of transcriptional condensates differentially depend on cooperative interactions among multiple components versus specific pairwise interactions is, however, unclear. Unravelling the process of condensate assembly, capturing the real-time dynamics, and accounting for the complexity arising from regulatory mechanisms requires investigation of condensates in living cells.

The eukaryotic heat shock response (HSR) provides an ideal experimental system for the functional analysis and molecular characterization of transcriptional condensates. First, the HSR is an evolutionarily conserved transcriptional program that controls the expression of molecular chaperones and other components of the protein homeostasis (proteostasis) network [Vinayachandran et al., 2018, Pincus et al., 2018, Garde et al., 2024, Duarte et al., 2016, Mahat et al., 2016, Pincus, 2020, Morimoto, 1998]. Second, the HSR involves transcriptional control through condensates [Kainth et al., 2021, Dea and Pincus, 2024]. In *S. cerevisiae* (budding yeast), Heat Shock Factor 1 (Hsf1), the master transcription activator of the HSR, forms biophysically dynamic subnuclear clusters with Mediator (Med15) and Pol II (Rpb3) (Figure 2.1B) [Chowdhary et al., 2022]. These clusters are also observed in mammalian cells [Zhang et al., 2022]. Hsf1 clusters in yeast constantly rearrange (intermix) and form at canonical Hsf1 target/HSR gene and mRNA loci, marking them as sites of active nascent mRNA production [Chowdhary et al., 2022]. Third, the HSR provides the opportunity to characterize the functional regulation of the condensates. Hsf1 transcriptional condensates (aka HSR condensates) involve interactions between multiple transcriptional components via their IDRs and structured domains that are susceptible to post-translational modifications [Chowdhary et al., 2022]. Lastly, the HSR provides the opportunity to dissect the

dynamics of a physiological condensate assembly process over time. Hsf1 condensates in yeast are inducible and reversible as they rapidly form (demix) and dissolve (remix) during heat shock, with the kinetics closely paralleling HSR gene activation and attenuation [Chowdhary et al., 2022]. Moreover, HSR condensates promote the coalescence of chromosomally unlinked HSR genes, reorganizing the yeast 3D genome during stress [Chowdhary et al., 2022, 2017, 2019].

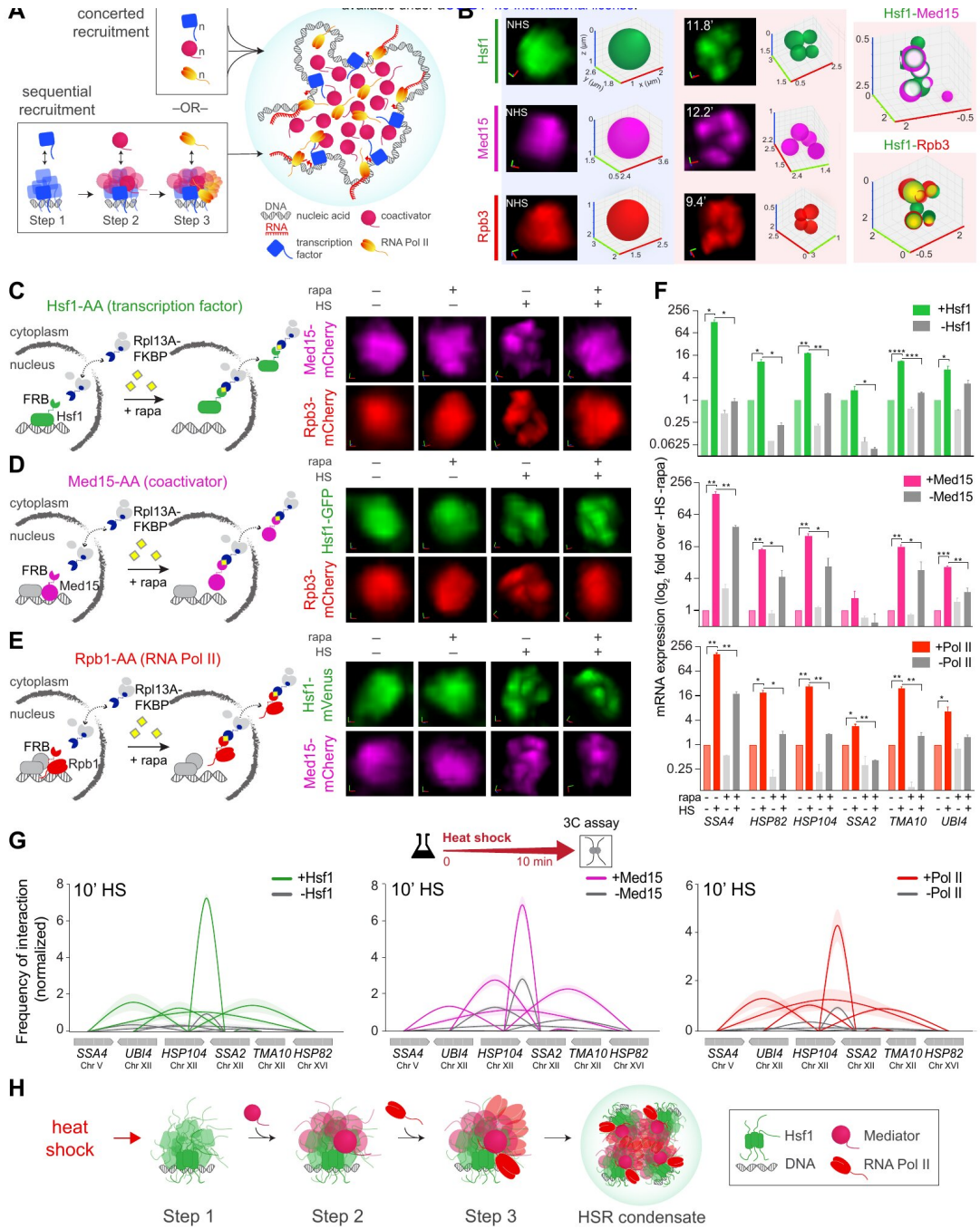


Figure 2.1: Ordered recruitment of Hsf1, Mediator, and Pol II in the HSR condensates.

(A) Models of transcriptional condensate assembly. Concerted recruitment (top) predicts nonstochastic, simultaneous recruitment of constituent biomolecules, driving the spontaneous assembly of the condensate. The stepwise recruitment model (bottom) posits an

(Figure 2.1 continued)

orderly cascade of events, whereby each component is serially recruited, resulting in the gradual assembly of the condensate. **(B)** Left and middle: Representative 3D images of live yeast cells expressing either Hsf1-mVenus, Med15-mCherry, and Rpb3-mCherry before (non-heat shock) and following heat shock (39°C) for the times indicated. Shown are the zoomed-in 3D volumetric renderings of the nuclei of the three individual cells. x (red), y (green) and z (blue) axes are indicated. The Hsf1, Med15, Rpb3 nuclear foci are depicted as 3D bubble charts. Right: 3D bubble chart depiction of the colocalization of Hsf1 foci with Med15 or Rpb3 at 10 min heat shock. The colocalization data are derived from cells co-expressing Hsf1-mVenus with Med15-mCherry or Rpb3-mCherry, as shown in [Chowdhary et al., 2022]. **(C)** Live cell images of Med15-mCherry or Rpb3-mCherry nuclear localization in Hsf1-Anchor Away (AA) cells under control (-rapa) or Hsf1-depleted (+rapa) conditions, in presence or absence of heat shock (10 min-HS). **(D)** same as C, except Hsf1-GFP and Rpb3-mCherry nuclear localization in Med15-AA cells. **(E)** same as C, except Hsf1-mVenus and Med15-mCherry nuclear localization in Rpb1-AA cells. **(F)** mRNA expression of HSR genes in Hsf1-AA, Med15-AA, and Rpb1-AA strains heat shocked for 10 min (or not), as measured by RT-qPCR. Depicted are means + SD; n=2, qPCR=4. ****P <0.0001; ***P <0.001; **P <0.01; *P <0.05 (calculated using multiple unpaired t-tests). **(G)** Intergenic contacts (solid arcs) between indicated HSR gene pairs determined by TaqI-3C in 10-min HS conditions in presence or absence of Hsf1 (left), Med15 (middle), Rpb1 (right). Shown are means +/- SD (SD, arc shading); n=2, qPCR=4. **(H)** Model of stepwise recruitment of Hsf1, Mediator, and RNA Pol II in the HSR condensates. Step 1, Hsf1 independently clusters upon heat shock; step 2, Mediator clusters in the presence of Hsf1; step 3, Pol II clusters in the presence of Hsf1 and Mediator.

Here, we resolve how HSR condensates assemble *in vivo*. We provide experimental evidence that HSR condensates conform to a stepwise assembly process. Using live cell imaging and transcriptomics we show that the stepwise assembly of condensates enables the graded activation of HSR gene transcription, rather than a switch-like response. We identify separation-of-function mutants of Hsf1 that fail to promote Pol II clustering yet induce HSR gene activation. Notably, the absence of Pol II condensates virtually eliminates intergenic contacts among HSR genes, and the lack of Pol II condensates and intergenic interactions is associated with a fitness cost, underscoring the unique and adaptive role of Pol II condensates in the spatial gene control that extends beyond gene activation. We propose that the serial assembly of HSR condensates enables cells to dynamically fine-tune HSR gene expression, and the emergent properties of the fully assembled HSR condensates adaptively reconfigure

the 3D genome for promoting cellular fitness during stress.

2.2 Ordered assembly of HSR condensates during heat shock

To dissect how HSR condensate assembly occurs in living cells, we used the rapamycin-based “anchor away” (AA) system [Haruki et al., 2008] to conditionally deplete Hsf1, the Mediator subunits Med15 and Ssn3/Cdk8, and the largest RNA Pol II subunit Rpb1 from individual yeast nuclei [Pincus et al., 2018, Chowdhary et al., 2019, Anandhakumar et al., 2016, Solís et al., 2016]. We first derived strains in the Hsf1-AA background, with either Med15 or Rpb3 endogenously tagged with mCherry, then monitored Med15 or Rpb3 clustering in live cells in the presence or absence of nuclear Hsf1 (Figure 2.1C). Under nonstress conditions, both Med15 and Rpb3 remain diffusely localized in the nucleus, even in the absence of nuclear Hsf1. Following a brief heat shock, both Med15 and Rpb3 formed clusters, but not when Hsf1 was depleted from the nucleus, suggesting that Hsf1 is required for Mediator and Pol II condensate formation.

Next, we generated strains with Hsf1-GFP and Rpb3-mCherry in the Med15-AA background and monitored Hsf1 or Rpb3 clustering in the presence or absence of nuclear Med15 (Figure 2.1D). While both Hsf1 and Rpb3 formed clusters upon heat shock in the presence of nuclear Med15, only Hsf1 could cluster upon nuclear depletion of Med15, and Rpb3 remained diffuse. This suggests that heat shock-activated Hsf1 can form condensates independently of Mediator, and that Mediator is necessary for Pol II condensate formation. By contrast, in the Ssn3/Cdk8-AA strain, both Hsf1 and Rpb3 formed clusters upon heat shock in the presence and absence of nuclear Ssn3/Cdk8 (Figure 2.2A). Thus, the tail subunit of the Mediator complex, Med15, which has been previously implicated in directly associating with Hsf1 [Kim and Gross, 2013], is required for Pol II condensate formation upon heat shock, but the reversibly associated kinase module containing Ssn3/Cdk8 is dispensable, aligning with their distinct roles in HSR gene activation [Anandhakumar et al., 2016].

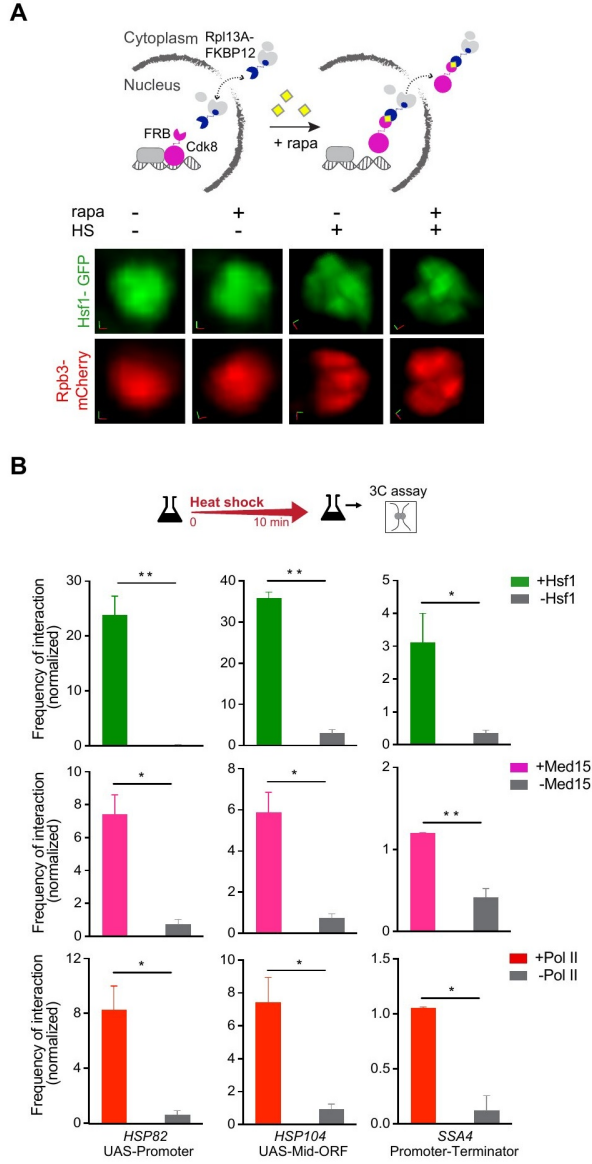


Figure 2.2: **Ssn3/Cdk8 anchor away and intragenic 3C interactions, related to Figure 2.1.** (A) Live cell images of Rpb3-mCherry (RNA Pol II subunit) or Hsf1-GFP nuclear localization in Cdk8/Ssn3-Anchor Away (AA) cells under control (-rapa) or Cdk8/Ssn3-depleted (+rapa) conditions, before and after heat shock (10 min-HS). Shown are the zoomed-in 3D volumetric renderings of the nuclei. x (red), y (green) and z (blue) axes are indicated. (B) Intragenic interactions within representative Hsf1 target genes under 10 min-heat shock conditions in cells with nuclear depletion of Hsf1, Mediator (Med15), and Pol II (Rpb3), as determined by TaqI-3C. Shown are means + SD; n=2, qPCR=4. **P <0.01; *P <0.05 (calculated using unpaired t-test).

Finally, we derived Hsf1-GFP and Med15-mCherry strains in the Rpb1-AA background and monitored Hsf1 or Med15 clustering in the presence or absence of nuclear Rpb1 (Figure 2.1E). Both Hsf1 and Med15 formed clusters in the presence and absence of nuclear Rpb1, suggesting that Pol II is dispensable for Hsf1 and Mediator condensate assembly. Collectively, these data suggest that the assembly of HSR condensates follow an orderly cascade of events, where Hsf1 initially clusters in response to heat shock, then recruits Mediator to condense, and the Hsf1-Mediator subassembly in turn recruits Pol II to condense (Figure 2.1H).

Functionally, the nuclear depletion of Hsf1, Med15, and Rpb3 all reduced transcriptional induction of HSR target genes during heat shock (Figure 2.1F). Likewise, depletion of each of the factors reduced the frequency of both intragenic and intergenic interactions between representative Hsf1 target/HSR genes (Figures 2.1G, 2.2B), a key property of HSR gene expression measured by a highly sensitive chromosome conformation capture assay TaqI-3C [Chowdhary et al., 2019, 2020]. Together, the imaging and molecular assays in the AA strains reveal that, although the cell biological assembly of HSR condensates proceeds via discrete steps, the molecular functions of stress-induced gene activation and 3D genome reorganization require assembly of all the components.

2.3 Hsf1 clustering upon Hsp70 release

Since Hsf1 can independently cluster, we hypothesized that the mechanisms central to regulating Hsf1 activity would also influence Hsf1 clustering. In particular, the chaperone Hsp70 directly binds to Hsf1 to repress Hsf1 activity, while phosphorylation of IDRs on Hsf1 positively regulates Hsf1 transcriptional activity [Zheng et al., 2016].

The chaperone Hsp70 binds to Hsf1 to repress its activity [Peffer et al., 2019, Krakowiak et al., 2018]. AlphaFold Multimer was recently shown to accurately predict interactions of structured regions with IDRs [Evans et al., 2021, Omid et al., 2024]. As Hsf1 is known

to form a trimer and Hsp70 may exist as a dimer in eukaryotes [Takakuwa et al., 2019, Malinverni et al., 2015, Rabindran et al., 1993, Sorger and Nelson, 1989], we performed de novo modeling of Hsf1 and Hsp70 in a 3:2 stoichiometry to predict their interactions. Without specific training on complexes of Hsf1 and Hsp70, AlphaFold2 Multimer [Jumper et al., 2021] predicted that Hsp70 interacts with Hsf1 via two distinct binding sites, the N-terminal element 1 (NE1) located in the N-terminal IDR and the conserved element 2 (CE2) in the C-terminal IDR (Figure 2.3A, B). This is consistent with the previous finding that mutations in either or both NE1 or CE2 short linear motifs disrupt Hsp70 binding to Hsf1. As a consequence of disrupted Hsp70 binding, Hsf1 activity is de-repressed, leading to increased basal expression of HSR genes [Peffer et al., 2019, Krakowiak et al., 2018].

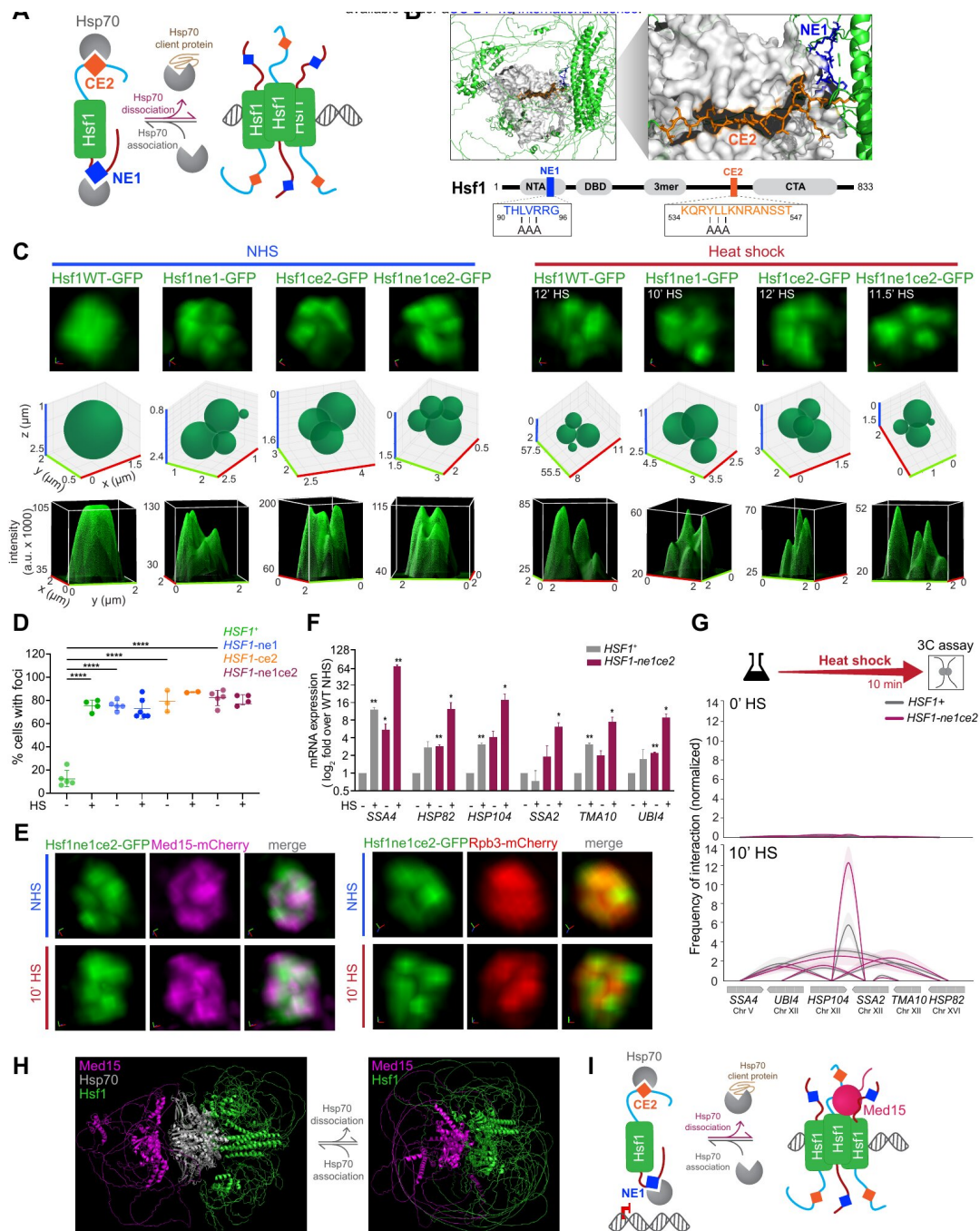


Figure 2.3: Hsf1 and Mediator cluster upon Hsp70 release.

(A) Schematic of Hsp70 regulation of Hsf1, highlighting the N-terminal element 1 (NE1) and the conserved element 2 (CE2)—short linear motifs where Hsp70 binds to repress Hsf1. Hsf1 is activated upon sequestration of Hsp70 by its client proteins. (B) Top: AlphaFold2 Multimer model of the Hsf1-Hsp70 structure (3:2 stoichiometry), showing interactions be-

the Hsf1 NE1 and CE2 regions and Hsp70. This is not an experimentally determined structure. The predicted structures of Hsf1 (cartoon representation), Hsp70 (surface), and the NE1/CE2 sites (stick) are visualized using PyMOL. Bottom: Hsf1 domain map. NTA, N-terminal activation domain; DBD, DNA binding domain; 3mer, trimerization domain; NE1, N-terminal element 1 (blue); CE2, conserved element 2 (orange); CTA, C-terminal activation domain. Boxes: NE1 and CE2 sequences and alanine substitutions in the Hsf1-ne1AAA and Hsf1-ce2AAA mutants. **(C)** Top row: 3D rendered live-cell images of Hsf1-GFP nuclear localization of wild type (WT) and Hsf1-ne1, Hsf1-ce2, and Hsf1-ne1ce2 cells under non-heat shock conditions (NHS) and heat shock (HS) conditions at times indicated. Middle row: 3D bubble charts displaying foci assignments for cells shown in the top row. Bottom row: 3D surface plots of signal intensity. **(D)** Percentage of cells with >2 Hsf1 foci per live cell quantified by FindFoci for WT and Hsf1 mutant strains under NHS and 10 min-HS conditions. Shown are means $\pm$ SD; $n = 50\text{--}65$ cells/condition. **** $P < 0.0001$. P values were calculated by ANOVA followed by Tukey's post hoc analysis. **(E)** Live imaging of cells co-expressing Hsf1-ne1ce2-GFP with Med15-mCherry or Rpb3-mCherry. **(F)** mRNA expression of representative HSR genes measured by RT-qPCR in WT and Hsf1-ne1ce2 double mutant in NHS and 10 min-HS conditions. Shown are means $\pm$ SD; $n = 2$, qPCR=4. ** $P < 0.01$; * $P < 0.05$ (calculated using multiple unpaired t-tests). **(G)** Intergenic interactions between indicated Hsf1 target gene pairs in WT and Hsf1-ne1ce2 mutant under NHS and 10 min-HS conditions. Shown are means $\pm$ SD; $n = 2$, qPCR=4. **(H)** AlphaFold2 Multimer modeling of Hsf1, Hsp70, and Med15 interactions visualized in PyMOL. Hsp70 binding to Hsf1 is predicted to sterically occlude Med15 binding to Hsf1. **(I)** Schematic of steric hinderance by Hsp70 binding at Hsf1 to inhibit Mediator recruitment.

To determine whether binding of Hsp70 to Hsf1 regulates Hsf1 clustering and HSR condensate formation in cells, we generated mutants of Hsf1 disrupting NE1 and CE2 alone and in combination, tagged them with GFP, and expressed them from the endogenous *HSF1* promoter as the only source of Hsf1 in the cell. Live cell imaging revealed that, in contrast to the diffuse nuclear localization of wild type Hsf1, all three mutants—Hsf1-ne1, Hsf1-ce2, and Hsf1-ne1ce2—constitutively cluster into a similar number of subnuclear foci and in a similar fraction of unstressed cells, phenocopying wild type Hsf1 during heat shock. The clusters of Hsf1 mutants persisted during heat shock (Figures 2.3C, D, 2.4A). The Hsf1-ChIP experiments showed no increase in basal DNA binding at HSR genes or the non-native HSE-YFP locus (Figure 2.4B, C), suggesting that the constitutive clustering of Hsf1 is not associated with increased DNA binding of Hsf1 mutants.

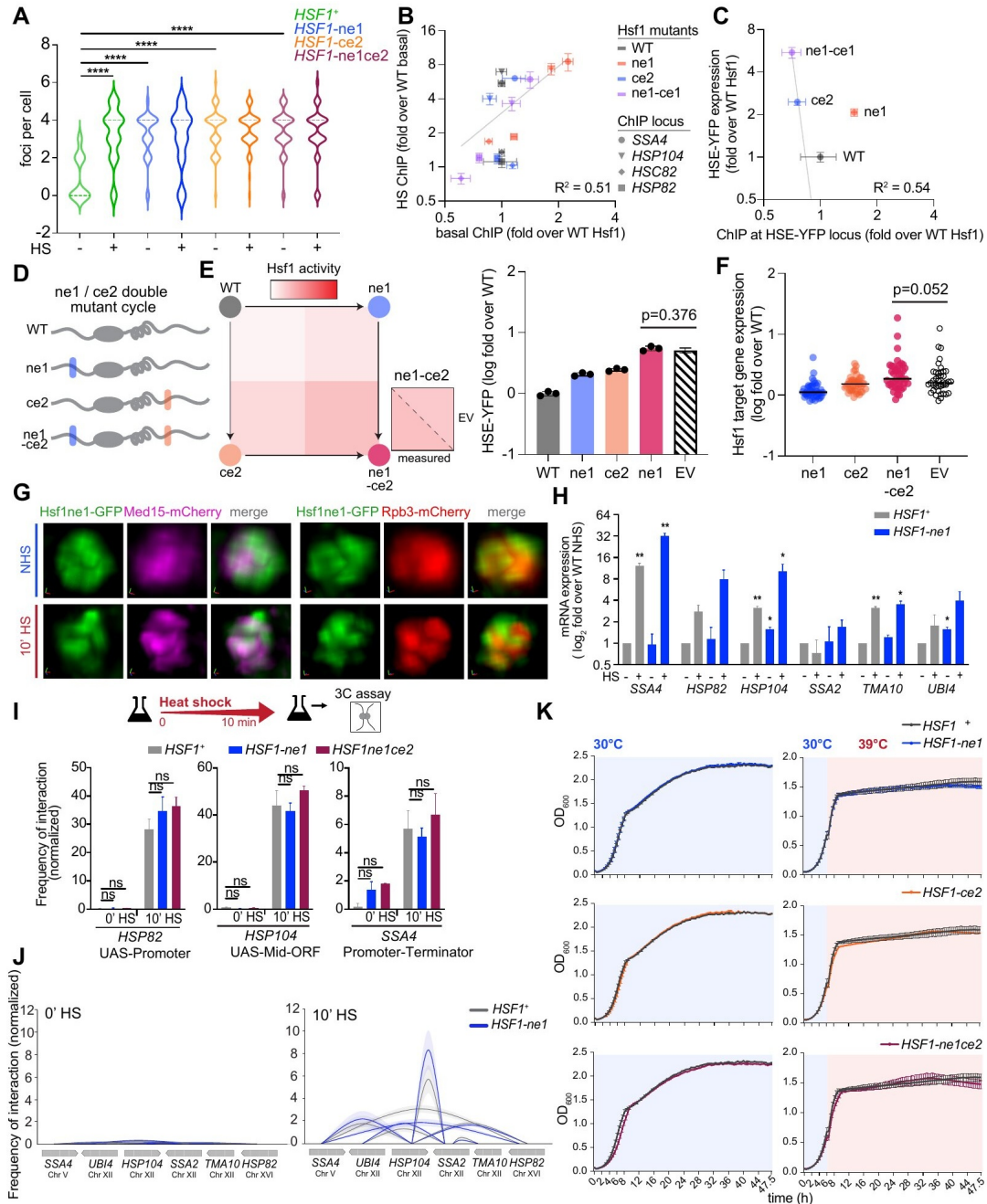


Figure 2.4: Cellular, molecular, and growth phenotypes of Hsp70 binding mutants, related to Figure 2.3.

(A) Quantification of the number of Hsf1 nuclear foci in WT, Hsf1-ne1, Hsf1-ce2, and Hsf1-ne1ce2 cells under non-heat shock (NHS) and 10 min heat shock (HS) conditions. Foci were detected and quantified using the automated FindFoci algorithm in Fiji; 50-65 cells

(Figure 2.4 continued)

were evaluated per condition for each cell type. ****P < 0.0001. P values were calculated by ANOVA followed by Tukey's post hoc analysis. **(B)** Hsf1 occupancy in WT, Hsf1-ne1, Hsf1-ce2, and Hsf1-ne1ce2 cells at endogenous Hsf1 target genes. The Hsf1 ChIP signal is shown as fold change relative to WT under basal conditions versus 15 min heat shock. n=2; qPCR=6. **(C)** Hsf1 ChIP enrichment at the HSE-YFP reporter locus. Hsf1 ChIP signals are plotted against reporter expression levels (fold change relative to WT). n=2; qPCR=6. **(D)** Schematic of the double mutant cycle for Hsf1-ne1 and Hsf1-ce2. **(E)** Left: HSE-YFP levels in WT, Hsf1-ne1, Hsf1-ce2, and Hsf1-ne1ce2 mutants shown as a heat map normalized to minimal and maximal Hsf1 activity in the dynamic range of the assay. Shown is the comparison between the measured HSE-YFP expression in the double mutant (Hsf1-ne1ce2) and the expected value (EV) calculated from the single mutants. Right: Quantitative analysis of HSE-YFP reporter activity in the NE1-CE2 double mutant cycle. P value was calculated using an unpaired t-test; n=3. **(F)** Double mutant cycle analysis for NE1 and CE2 Hsp70 binding sites using published RNA-seq data [Peffer et al., 2019]. P value was calculated using a paired t-test. **(G)** 3D rendered live-cell images of cells co-expressing Hsf1-ne1-GFP with Med15-mCherry or Rpb3-mCherry. **(H)** mRNA expression of representative HSR genes measured by RT-qPCR in WT and Hsf1-ne1 mutant under NHS and 10 min-HS conditions. Shown are means + SD; n=2, qPCR=4. **P < 0.01; *P < 0.05 (calculated using multiple unpaired t-tests). **(I)** Intragenic interactions within representative HSR genes under NHS and 10 min-HS conditions in WT, Hsf1-ne1, and Hsf1-ne1ce2 double mutant, as determined by TaqI-3C. Shown are means + SD; n=2, qPCR=4. ns (not significant), P > 0.05. P values were calculated by ANOVA followed by Tukey's post hoc analysis. **(J)** TaqI-3C analysis of intergenic contacts (solid arcs) among HSR genes in WT and Hsf1-ne1 cells under NHS and 10-min HS conditions. Shown are means +/- SD (SD, arc shading); n=2, qPCR=4. **(K)** Quantitative growth curves of WT and Hsf1 mutants at 30°C and 39°C (n=2; q=6).

Furthermore, using the previously published RNA-seq datasets [Peffer et al., 2019] and a single cell fluorescent reporter assay for Hsf1 activity in the mutants with individual and combined disruptions of NE1 and CE2, and leveraging the genetic interaction theory as previously applied to the HSR [Brandman et al., 2012, Alford et al., 2021], we derived that the NE1 and CE2 sites regulate Hsf1 independently of one another (Figure 2.4D-F). Together, these results indicate that Hsf1 can cluster immediately following the partial release of Hsp70, and that the presence or absence of one Hsp70 binding site has no bearing on either the ability of Hsp70 to bind to the other site or Hsf1's ability to cluster upon dissociation of Hsp70.

2.4 Hsf1 and Mediator cluster upon complete dissociation of Hsp70

In our AA experiments, we demonstrated that Hsf1 is necessary for Med15 and Pol II condensate formation upon heat shock. To ask whether the constitutively clustered Hsf1 alone could drive the clustering of Mediator and Pol II, we derived strains with either Med15 or Rpb3 endogenously tagged with mCherry, set in the background of the Hsf1-GFP mutants with disrupted Hsp70 binding sites (Figures 2.3E, 2.4G) [Chowdhary et al., 2022]. While Hsf1 was constitutively clustered in all mutants, Med15 was diffuse in the Hsf1-ne1 and Hsf1-ce2 single mutants but appeared constitutively clustered in the Hsf1-ne1ce2 double mutant. In contrast, Rpb3 remained diffuse in all mutant strains in nonstress conditions. Upon heat shock, however, all the mutants displayed clustering of both Med15 and Rpb3, similar to wild type Hsf1.

Thus, the microscopically similar Hsf1 clusters are in fact molecularly distinct: Hsf1 with a single mutated Hsp70 binding site clusters without Mediator or Pol II; Hsf1 with complete disruption of Hsp70 binding clusters along with Mediator but not Pol II; and the heat shock-activated Hsf1 co-condenses with both Mediator and Pol II. While basal expression of many, if not all, HSR genes, was elevated in the Hsf1-ne1 and Hsf1-ne1ce2 mutants, each exhibited a substantial increase in inducible transcriptional activity, with the double mutant showing highest expression levels (Figures 2.3F, 2.4H).

We asked if the constitutive clusters of Hsf1 mutants could drive interactions among HSR genes. Using TaqI-3C, we observed that single or double mutation of the Hsp70 binding sites did not result in any significant intergenic interactions among HSR genes. However, consistent with imaging results showing that heat shock induces full assembly of HSR condensates and high-level transcriptional activity, Hsf1 mutants showed a marked increase in both intra- and intergenic interactions among HSR genes in response to heat shock (Figures 2.3G, 2.4I, J). Thus, while the release of Hsp70 from Hsf1 and the constitutive clustering of either Hsf1

or Med15 alone are not sufficient to drive Pol II clustering or the emergence of intergenic interactions, the Hsf1 mutants retain the ability to restore wild-type functions during heat shock. This recovery is mirrored by the lack of fitness consequences of the mutants at elevated temperature (Figure 2.4K).

While Hsp70 is bound to Hsf1, it prevents Hsf1 clustering, which inhibits Mediator clustering. This suggests that interactions between Hsf1 and Hsp70, and between Hsf1 and Med15, are mutually exclusive. Consistent with this, AlphaFold2 Multimer modeling of Hsf1, Hsp70, and Med15 (3:2:1 stoichiometry) and Hsf1 and Med15 (3:1 stoichiometry) predicts steric occlusion of Med15 by Hsp70 (Figure 2.3H). Although these structural predictions with Med15 may not fully represent the functional organization in cells, which would also involve the larger Mediator subassembly, our findings suggest that interactions between Hsf1 and Hsp70 may preclude Hsf1 from engaging with Mediator under basal conditions (Figure 2.3I).

Taken together, our data show that Hsf1 can form clusters independently of Mediator and Pol II. The initial clustering of Hsf1 requires at least partial dissociation from its repressor, Hsp70. To partition Mediator within clusters, Hsf1 must fully unleash both its activation domains by completely dissociating from Hsp70. Hsf1-Mediator subassembly would then allow recruitment of Pol II upon heat shock, leading to full assembly of HSR condensates.

2.5 Regulation of Hsf1 and Mediator clustering by phosphorylation

Although complete Hsp70 dissociation is sufficient to drive Hsf1 and Med15 into clusters, additional regulation is required for Pol II clustering and HSR gene coalescence. Hsf1 is phosphorylated upon activation (Figure 2.5A) [Anckar and Sistonen, 2011, Joutsen and Sistonen, 2019], and a number of kinases have been implicated in Hsf1 regulation under different conditions across eukaryotes [Cotto et al., 1996, Hietakangas et al., 2003, Holmberg et al., 2001, Kline and Morimoto, 1997, Xia et al., 1998, Yamamoto et al., 2007]. Yeast Hsf1

can be phosphorylated on nearly 75 sites (Figure 2.5B), and mimicking phosphorylation is sufficient to constitutively activate Hsf1, suggesting positive regulatory potential [Zheng et al., 2016].

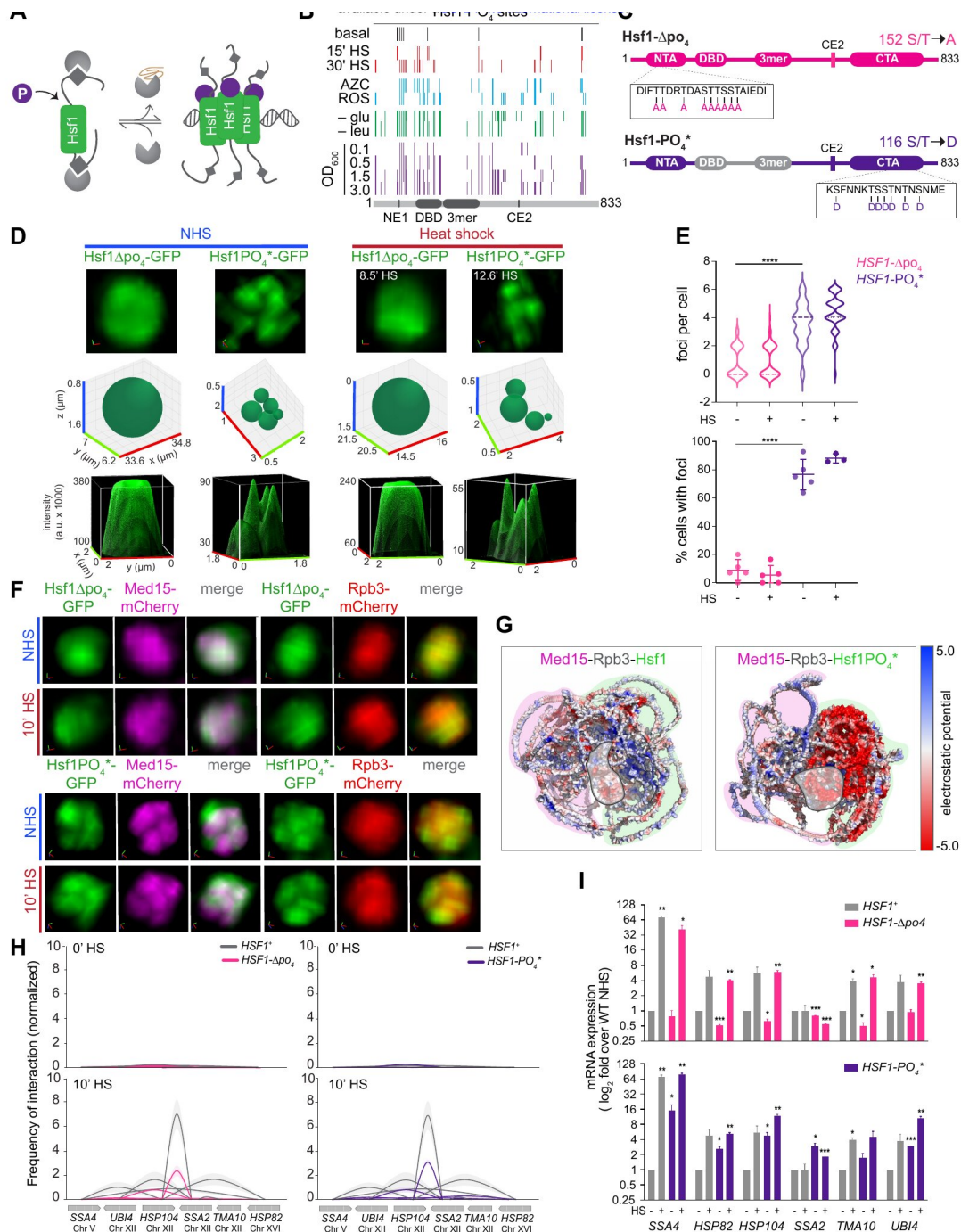


Figure 2.5: Phospho-regulation of Hsf1 and Mediator clusters.

(Figure 2.5 continued)

(A) Schematic of Hsf1 phosphorylation upon activation. **(B)** Phosphorylation sites observed in various stress and non-stress conditions shown along the primary sequence of Hsf1. HS, heat shock; AZC, Azetidine-2-carboxylic acid (1 mM); ROS, oxidative stress induced by menadione (125 μ M); -glu, glucose depletion; -leu, leucine depletion. Data are derived from previously published Hsf1-3xFLAG-V5 IP/MS experiments [Zheng et al., 2016]. **(C)** Domain architecture maps of the phospho-null (Hsf1- Δ po4) and phospho-mimetic (Hsf1-PO4*) mutants. Hsf1- Δ po4 has all 152 possible phosphorylation sites replaced with alanine to prevent phosphorylation. Hsf1-PO4* has 116 phosphorylation sites in the IDRs replaced with aspartate to mimic constitutive phosphorylation. **(D)** Live imaging of Hsf1-GFP nuclear localization in Hsf1- Δ po4 and Hsf1-PO4* cells in NHS and HS conditions. Top row, 3D volumetric renderings; middle, 3D bubble charts; bottom, 3D surface plots of signal intensity. **(E)** Quantification of the number of Hsf1 nuclear foci per live cell (top) and the percentage of cells with >2 Hsf1 foci (bottom) in Hsf1- Δ po4 and Hsf1-PO4* cells under NHS and 10 min-HS conditions. Shown are means $\pm$ SD; n=50-60 cells/condition. ****P < 0.0001. P values were calculated by ANOVA followed by Tukey's post hoc analysis. **(F)** Top: Live imaging of cells co-expressing Hsf1- Δ po4-GFP with Med15-mCherry or Rpb3-mCherry under NHS and HS conditions. Bottom: same as top, except Hsf1-PO4*-GFP cells are shown. **(G)** AlphaFold2 Multimer models of wild type Hsf1 or Hsf1-PO4* in complex with Med15 and Rpb3 (3:1:1 stoichiometry) predict that phospho-mimetic residues disrupt charge block interactions of Rpb3 with Hsf1-Med15. The electrostatic surface potentials were determined using the Adaptive Poisson-Boltzmann Solver (APBS) [Baker et al., 2001] and visualized in PyMOL. **(H)** TaqI-3C analysis of intergenic contacts among HSR target genes in WT, Hsf1- Δ po4, and Hsf1-PO4* cells under NHS and HS conditions. Shown are means $\pm$ SD; n=2, qPCR=4. **(I)** mRNA expression measured by RT-qPCR of representative HSR genes in WT, Hsf1- Δ po4, and Hsf1-PO4* cells under NHS and 10 min-HS conditions. Depicted are means $\pm$ SD; n=2, qPCR=4. ***P < 0.001; **P < 0.01; *P < 0.05 (calculated using multiple unpaired t-tests).

We tested the role of Hsf1 phosphorylation in HSR condensate formation by utilizing a phospho-null mutant of Hsf1 (Hsf1- Δ po4), in which alanine substitutions were made at all 152 potential phosphorylation sites, and a phospho-mimetic mutant (Hsf1-PO4*) with aspartate substitutions at 116 phosphorylation sites in the IDRs, as previously described (Figure 2.5C) [Zheng et al., 2016]. When tagged with GFP, Hsf1- Δ po4 and Hsf1-PO4* displayed opposing nuclear distribution patterns: Hsf1- Δ po4 remained persistently diffuse, even upon heat shock, while Hsf1-PO4* was constitutively condensed, displaying a similar number of foci and fraction of cells with foci to wild type Hsf1 during heat shock (Figures 2.5D, E, 2.3D, 2.4A).

Since Hsf1- Δ po4 failed to cluster, both Med15 and Rpb3 showed diffuse nuclear localization patterns despite heat shock (Figure 2.5F). This confirms that Hsf1 clustering is upstream of and necessary for the clustering of Mediator and Pol II. In contrast, in Hsf1-PO4* cells, Med15 was constitutively clustered along with Hsf1-PO4*, while Rpb3 remained diffuse, even upon heat shock (Figure 2.5F). These results indicate that phosphorylation is both necessary and sufficient for the clustering of Hsf1 and Med15, but not Pol II.

Consistent with this interpretation, AlphaFold2 Multimer modeling of Hsf1 with Med15 and Rpb3 revealed compatible charge block interactions between the IDRs of wild type Hsf1 and Med15 that form a potential composite docking site for Rpb3, which is precluded by the extended acid patches in the IDRs of Hsf1-PO4* formed by the phospho-mimetic residues (Figure 2.5G). Thus, while Med15 is condensed in both Hsf1-ne1ce2 and Hsf1-PO4* cells, Rpb3 clusters upon heat shock in cells expressing Hsf1-ne1ce2 but not Hsf1-PO4* (Figures 2.3E and 2.5F). Although these structural models of isolated subunits of Mediator and Pol II with Hsf1 may not be representative of the interaction sites of the fully assembled complexes in cells, we interpret these results to suggest that phosphorylation of Hsf1 may modulate charge blocks in the IDRs that determine the affinity of Hsf1 for different surfaces on Mediator, which could potentially expose different surfaces for interaction with Pol II.

We next examined the intergenic interactions between HSR genes in Hsf1 phospho-mutants. Despite their opposing condensate phenotypes, both Hsf1- Δ po4 and Hsf1-PO4* displayed similar intergenic interaction frequencies: neither showed interactions under basal conditions, and both showed substantially reduced intergenic interactions during heat shock compared to wild type Hsf1 (Figure 2.5H). Surprisingly, Hsf1-PO4* cells showed elevated expression of key HSR genes both under basal conditions and upon heat shock (Figure 2.5I, bottom), despite the lack of Pol II condensates. Moreover, Hsf1- Δ po4 cells, also lacking Pol II condensates, induced expression of representative HSR genes during heat shock to levels comparable to wild type Hsf1 (Figure 2.5I, top). Thus, both Hsf1- Δ po4 and Hsf1-PO4* rep-

resent separation-of-function mutants demonstrating that Pol II condensates are dispensable for gene activation and have roles in 3D genome remodeling.

2.6 Differential epistatic interactions impinge on condensate formation and gene activation

Since disruptions to the Hsp70 binding sites and hyper-phosphorylation of Hsf1 resulted in constitutive clustering of Hsf1 and Med15, but not Pol II, we hypothesized that combining these mutations could synergistically reprogram Hsf1 to recruit Pol II to condense in the absence of heat shock. To test this, we generated a series of combinatorial mutants of Hsf1 with disruptions to one or both Hsp70 binding sites, either in the $\Delta po4$ or PO4* background (Figure 2.6A).

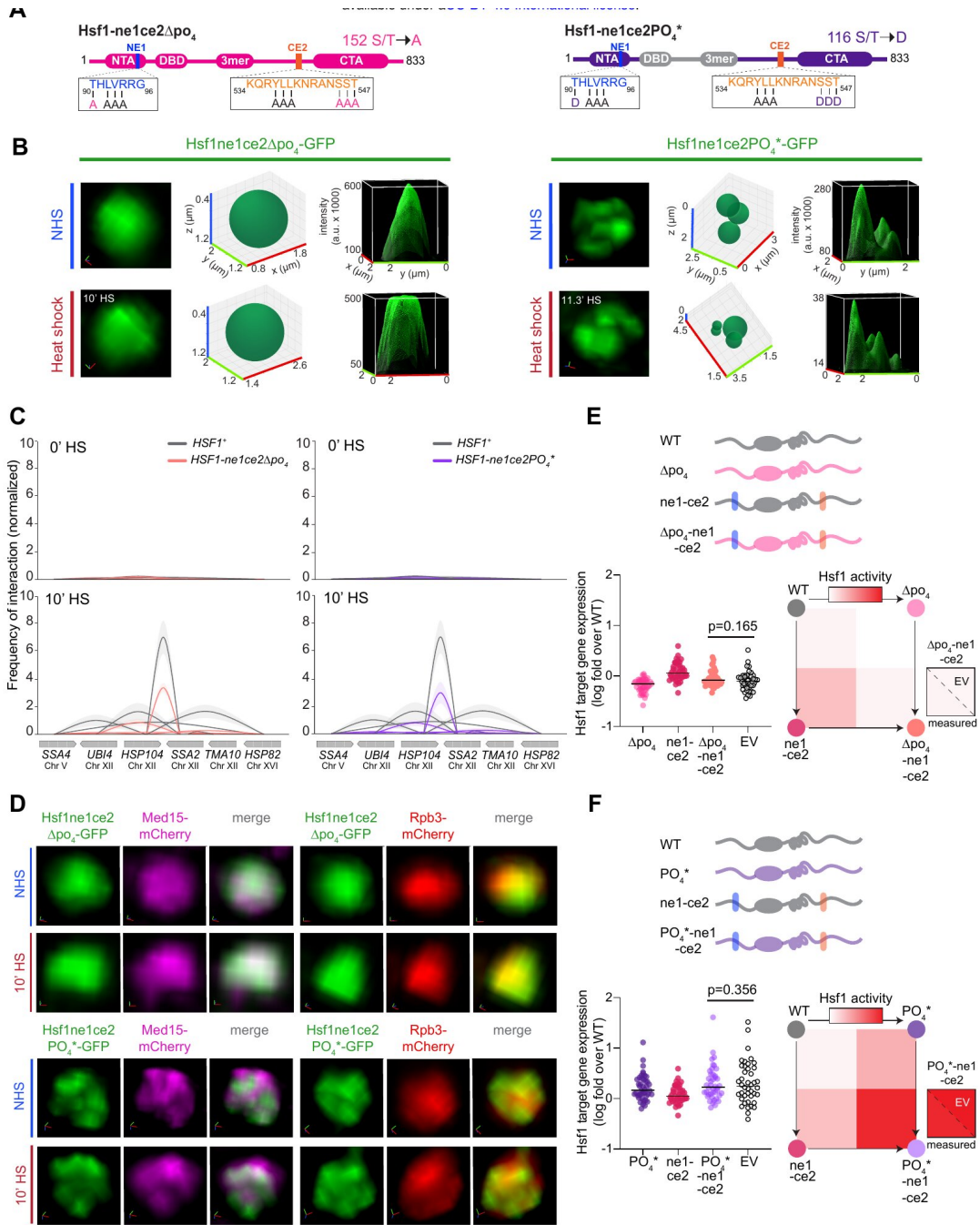


Figure 2.6: Differential epistatic control of condensate formation and transcriptional activity.

(A) Domain architecture maps of the Hsf1-ne1ce2 Δ po4 and Hsf1-ne1ce2PO4* combinatorial mutants. (B) Live imaging of Hsf1-GFP nuclear localization in Hsf1-ne1ce2 Δ po4 and Hsf1-ne1ce2PO4* cells in NHS and HS conditions. For each mutant, left panels show 3D

(Figure 2.6 continued)

volumetric renderings; middle, 3D bubble charts; and right, 3D surface plots. **(C)** TaqI-3C analysis of intergenic contacts among HSR target genes in WT, Hsf1-ne1ce2 Δ po4, and Hsf1-ne1ce2PO4* cells under NHS and HS conditions. Shown are means $\pm$ SD; n=2, qPCR=4. **(D)** Top: Live imaging of cells co-expressing Hsf1-ne1ce2 Δ po4-GFP with Med15-mCherry or Rpb3-mCherry under NHS and HS conditions. Bottom: same as top, except Hsf1-ne1ce2PO4*-GFP cells are shown. **(E)** Top: schematic of the double mutant cycle for Hsf1-ne1ce2 and Hsf1- Δ po4. Bottom left: mRNA expression of HSR genes measured by RNA-seq in the double mutant cycle for Hsf1-ne1ce2 and Hsf1- Δ po4. Bottom right: depiction as heat map normalized to minimal and maximal activity in the dynamic range of the assay. Shown is the comparison of the measured mRNA expression level in the double mutant with the expected value (EV) calculated from the single mutants (P value calculated using a paired t-test). For each pairwise test, n=2. **(F)** As in E, except the double mutant cycle for Hsf1-ne1ce2 and Hsf1-PO4* is shown.

Live cell imaging and molecular assays revealed that the phospho-mutant phenotypes were dominant to the Hsp70 binding site mutants. Similar to the Hsf1- Δ po4 mutant, Hsf1-ne1 Δ po4, Hsf1-ce2 Δ po4, and Hsf1-ne1ce2 Δ po4 all showed diffuse nuclear localization under both basal and heat shock conditions (Figures 2.6B, 2.7A). The Hsf1-ne1ce2 Δ po4 failed to drive HSR gene coalescence upon heat shock (Figure 2.6C), consistent with the absence of Pol II clustering (Figure 2.6D). Like the Hsf1-PO4* mutant, the Hsf1-ne1-PO4*, Hsf1-ce2-PO4*, and Hsf1-ne1ce2PO4* mutants clustered along with Med15 (Figure 2.6B, D, 2.7B), both in presence and absence of heat shock, and were unable to drive Pol II clustering or heat shock-dependent intra- and inter-genic interactions among HSR genes (Figures 2.6C, D, 2.7C). Thus, rather than synergistic interactions, the combined mutations displayed epistatic interactions: in terms of Hsf1 and Med15 condensate formation, mutations to the Hsf1 phosphorylation sites mask the effects of mutations to the Hsp70 binding sites.

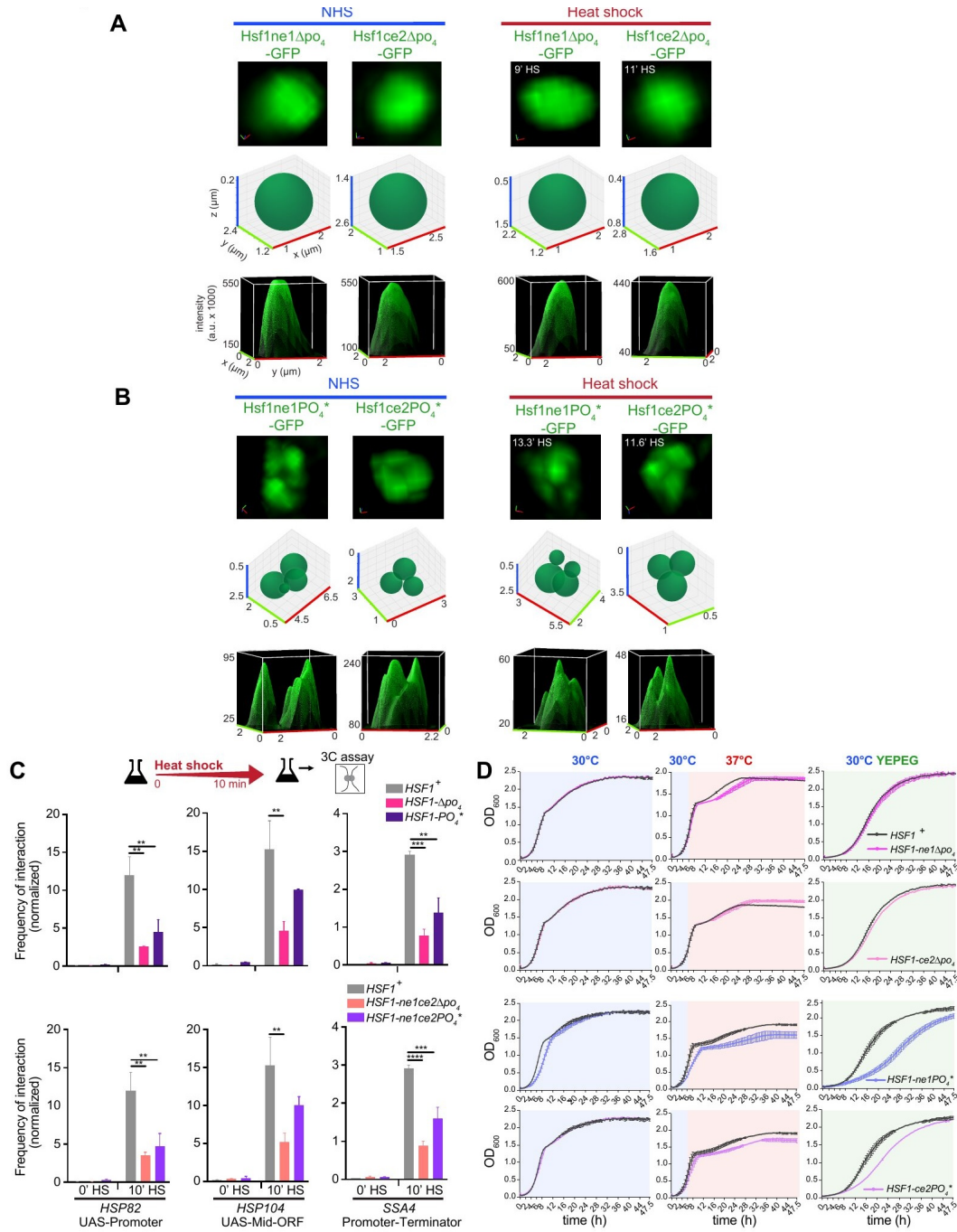


Figure 2.7: Cellular, molecular, and growth phenotypes of Hsf1 phospho-mutants, related to Figures 2.5 and 2.6.

(A) Top row: Representative 3D live-cell images of Hsf1-Δpo4-GFP combinatorial mutants under NHS and HS conditions for the times indicated. Shown are the zoomed-in 3D volumetric renderings of the yeast nuclei. x (red), y (green) and z (blue) axes are indicated. Middle

(Figure 2.7 continued)

row: 3D bubble chart depiction of Hsf1 nuclear foci in cells shown in the top row. Bottom row: 3D surface plots displaying signal intensity in cells in top row. **(B)** As in A, except images of Hsf1-PO4*-GFP combinatorial mutants are shown. **(C)** Intragenic interactions within representative HSR genes under NHS and 10 min-HS conditions in WT, Hsf1- Δ po4, Hsf1-PO4*, Hsf1-ne1ce2 Δ po4, and Hsf1-ne1ce2PO4*, as determined by TaqI-3C. Shown are means + SD; n=2, qPCR=4. ****P <0.0001; ***P <0.001; **P <0.01. P values were calculated by ANOVA followed by Tukey's post hoc analysis. **(D)** Growth curves of Hsf1 combinatorial mutants cultured at 30°C in glucose media, 37°C (mild stress) in glucose media, and 30°C in ethanol/glycerol (YPEG) media (moderate stress). The quantitative growth assays were performed with two biological and six technical replicates of each cell type per condition.

To quantify the genetic interactions between the various Hsf1 mutations at the level of transcriptional activity, we performed double mutant cycle analyses with the Hsf1-ne1ce2 mutant and the phospho-mutants [Horovitz, 1996]. We measured mRNA expression levels of all 42 HSR genes in Hsf1-ne1ce2, Hsf1- Δ po4, Hsf1-PO4*, Hsf1-ne1ce2 Δ po4, and Hsf1-ne1ce2PO4* cells, and we observed that the expression levels of the double mutants (Hsf1-ne1ce2 Δ po4 and Hsf1-ne1ce2PO4*) across HSR genes were statistically indistinguishable from the expected values of the linear combination of the expression levels in the single mutants (Hsf1-ne1ce2, Hsf1- Δ po4, and Hsf1-PO4*) (Figure 2.6E, F). This indicates that, in contrast to condensate formation, there is no measurable epistasis between the mutations at the level of Hsf1 transcriptional activity. Thus, while the effects of mutations in the Hsf1 phosphorylation sites overpower the impact of disruptions to the Hsp70 binding sites on condensate behavior, both converge to regulate Hsf1's transcriptional activity independently. The differential epistatic relationships at the level of condensate formation and transcriptional output provides further evidence that the process of condensate formation is distinct from gene activation.

2.7 Graded HSR gene expression from the stepwise assembly of HSR condensates

Despite the ability to genetically separate condensate formation from transcription, WT cells do couple the processes during heat shock. What functional role in the transcriptional activation process do HSR condensates play? Our data so far indicate that the assembly of transcriptional condensates in the HSR proceeds through a series of separable steps rather than as a single concerted event. This suggests that each step in the HSR condensate assembly process may correspond to a progressive increase in HSR transcriptional activity.

Indeed, RNA-seq analysis revealed a graded increase of the basal mRNA expression, spanning an order of magnitude, across the allelic series of Hsf1 mutants (Figure 2.8A). Principal component analysis, which supported this graded increase along PC2, clustered the mutants into groups that aligned with their condensate assembly phenotypes (Figure 2.8B). These results suggest that the HSR can operate as a rheostat, dynamically tuning Hsf1 activity via modulation of its propensity to assemble condensates (Figure 2.8C).

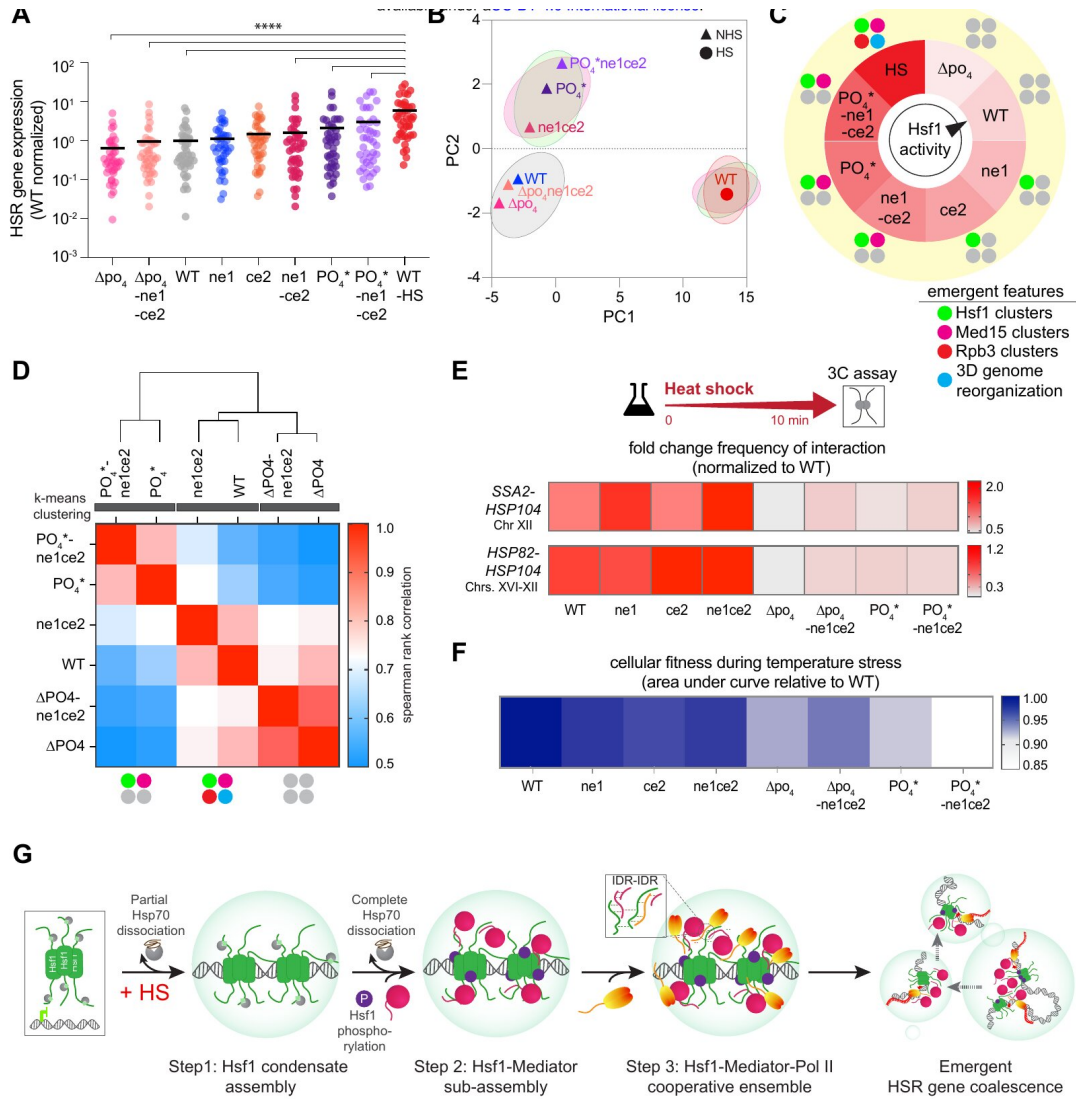


Figure 2.8: Emergent 3D genome reorganization and fitness during stress.

(A) mRNA expression levels of HSR genes measured by RNA-seq across the allelic series of Hsf1 mutants under basal conditions compared to wild type in basal (WT) and 10 min-heat shock (WT-HS) conditions. Expression is plotted as fold change relative to the WT mean basal expression. For each pairwise test, $n=2$. **** $P < 0.0001$ (calculated using paired t-

(Figure 2.8 continued)

test). The data points for Hsf1-ne1 and Hsf1-ce2 mutants were derived from [Peffer et al., 2019]. **(B)** Principal component analysis (PCA) of RNA-seq datasets of the allelic series of Hsf1 mutants under basal conditions and of wild type in non-heat shock and 10 min-heat shock conditions. The cell-type groups are displayed as per their condensate phenotypes: green oval, cell types that can cluster Hsf1; magenta, Med15; red, Rpb3; and grey, none. **(C)** Model of Hsf1 activity as a transcriptional rheostat. **(D)** Spearman rank correlation matrix of RNA-seq datasets of wild type and Hsf1 mutants. Fold changes (10 min HS/NHS) of normalized gene counts were used to determine the optimal number of clusters grouped by k-means and hierarchical clustering. The analysis was performed with two biological replicates (n=2) for each cell type. **(E)** Fold change (10 min HS/NHS) interaction frequencies between representative chromosomally linked and unlinked HSR gene pairs for WT and allelic series of Hsf1 mutants, as measured by TaqI-3C. n=2, qPCR=4. **(F)** Integrated cell growth across the allelic series of Hsf1 mutants relative to WT during temperature stress. The quantitative growth assay was performed with two biological and six technical replicates of each cell type. **(G)** Model of stepwise condensate assembly and emergent 3D genome remodeling. Step 1 involves the initial assembly of Hsf1 condensates upon partial dissociation of Hsp70. Step 2 follows with the formation of Hsf1-Mediator subassembly upon complete Hsp70 dissociation and Hsf1 phosphorylation. Step 3 culminates in the formation of a cooperative ensemble constituting Hsf1, Mediator, and Pol II. Multiple such ensembles intermix to drive the spatial repositioning of HSR genes, promoting cellular fitness during stress.

2.8 A specialized role of Pol II condensates in 3D genome organization and cellular fitness

The separation-of-function mutants of Hsf1 that fail to promote Pol II clustering can still induce high-level HSR gene activity, suggesting that Pol II condensates may be more involved in fine-tuning or sustaining, rather than initiating, gene activation. Instead, Pol II condensates are linked to HSR gene repositioning in response to stress.

K-means clustering analysis of the heat shock-inducible mRNA expression revealed three distinct clusters that aligned with the ability of cells to form Pol II condensates. Group 1, comprising WT-Hsf1 and Hsf1-ne1ce2, can drive clustering of Pol II along with Hsf1 and Mediator upon heat shock. Group 2, which includes Hsf1-PO4* and Hsf1-ne1ce2PO4*, lacks clustering of Pol II, but not of Hsf1 and Mediator. Group 3, which constitutes Hsf1- Δ po4 and Hsf1-ne1ce2 Δ po4, fails to drive the clustering of Pol II, and of Hsf1 and Mediator as

well (Figure 2.8D).

Interestingly, mutants that fail to cluster Pol II (Groups 2 and 3) show a substantial reduction in intergenic interactions between HSR genes, whereas those that can cluster Pol II (Group 1) exhibit robust intergenic interactions (Figure 2.8E). Additionally, mutant cells lacking the ability to form Pol II condensates grow slower at elevated temperatures than WT and the mutants that can cluster Pol II (Figures 2.8F, 2.7D). These results suggest that the inability to support Pol II condensate assembly and spatial gene repositioning during heat shock is associated with reduced fitness under acute stress, underscoring an adaptive function of Pol II condensates in 3D genome reorganization during stress.

2.9 Discussion

Here, we elucidate the process underlying the assembly of transcriptional condensates in the yeast HSR. Based on our live cell investigations, we present a model in which condensates assemble sequentially. This model provides a framework for understanding the graded and tunable gene control typical of dynamic cellular environments. The prevailing view in the literature—largely derived from *in vitro* reconstitution studies—is that condensates spontaneously assemble once constituent biomolecules reach concentrations above a critical threshold. Our results indicate instead that HSR condensate assembly follows an ordered, stepwise process, wherein each key component serially condenses. This process is highly responsive to various layers of regulation, enabling precise, tunable, robust, and reversible control of HSR gene expression.

In response to stress, the transcription activator Hsf1 forms clusters upon releasing the chaperone Hsp70; partial dissociation is sufficient to initiate clustering (Figure 2.3C). Following phosphorylation and complete dissociation from Hsp70, Hsf1 partitions the coactivator Mediator into clusters (Figures 2.3E, 2.5F), likely by unmasking interaction surfaces. The Hsf1-Mediator sub-assembly then sequentially recruits Pol II into clusters. Building

on these and the previous observations in the HSR, we propose that HSR gene regulation unfolds as follows (Figure 2.8G): Upon heat shock and partial release of Hsp70, Hsf1 trimers cooperatively bind to multiple Heat Shock Elements (HSEs) within the upstream activating sequences (UASs) of HSR genes [Erkine et al., 1999], likely priming the ordered assembly of Hsf1 clusters. Phosphorylation of Hsf1 and further dissociation from Hsp70 promote non-stoichiometric—and potentially additional direct allosteric—interactions between Hsf1 and Mediator. This composite interaction surface of the Hsf1-Mediator sub-complex then facilitates the recruitment of Pol II to pre-initiation complexes at the core promoters of HSR genes. The low complexity IDRs of Hsf1, Mediator, and Pol II likely participate in multivalent interactions, helping stabilize the ordered cooperative ensemble. Many of these ensembles then come together to drive the coalescence of HSR genes across the genome.

Beyond establishing a conceptual framework for the HSR in eukaryotes, our discovery of the stepwise recruitment process offers critical new insights into broader mechanisms of gene activation. First, our findings help resolve the longstanding conflict over whether Mediator is recruited at the UASs/enhancers or the core promoters of genes, and whether Pol II helps recruit Mediator or vice versa. Cryo-EM studies of pre-initiation complexes (PICs) have captured Mediator at core promoters [Rengachari et al., 2021, Robinson et al., 2016], while ChIP-seq analyses have detected substantial Mediator binding at enhancer/UAS regions, particularly for HSR genes [Chowdhary et al., 2022, Anandhakumar et al., 2016]. This apparent discrepancy may be explained by UAS-promoter looping, a phenomenon that HSR genes actively engage in during heat shock [Chowdhary et al., 2017, 2019]. Alternatively, Mediator may first be recruited at the UAS by the transcription activator and then facilitate the recruitment of Pol II to the core promoter. Our findings, based on conditional depletion approaches and in an allelic series of Hsf1 mutants, show that Mediator recruitment into condensates by the transcription activator Hsf1 occurs upstream of, and is necessary for, the recruitment and clustering of Pol II. Our results corroborate with and provide a cell biological

manifestation of a recent single-molecule study of a synthetic gene construct, which showed that Mediator binds to the UAS-associated transcription activator independently of the core promoter, and that this binding is necessary for the subsequent recruitment of Pol II into the PIC complex [Zhou et al., 2024] and its activation [Palacio and Taatjes, 2025].

Second, our findings provide new insights on the function of Pol II condensates in gene activation. Pol II condensates are shown to be associated with highly expressed mammalian genes and are hypothesized to be involved in transcriptional bursting, co-transcriptional splicing, and in the regulation of transcription initiation [Wei et al., 2020, Boehning et al., 2018, Du et al., 2024, Guo et al., 2019]. Studies in live cells have shown that Pol II condensates often colocalize with Mediator subunits or transcription activators [Cho et al., 2018]. By employing an inducible system, nuclear factor depletion strategies, and various Hsf1 mutants to study HSR condensate assembly, we were able to decouple the formation of Pol II condensates from the assemblies/co-assemblies of Mediator and transcription activators, which has allowed us to selectively parse out and focus on the specific role of Pol II condensates.

The adaptive significance of Pol II condensates appears to be linked to 3D genome reorganization. Our data suggest that Pol II condensates specifically enable intergenic interactions among HSR genes, as the mutants of Hsf1 that are incapable of driving Pol II clustering exhibit impaired 3D genome remodeling and reduced fitness under stress (Figure 2.8). This indicates that Pol II condensates enhance cellular adaptation, potentially by optimizing the spatial proximity and subnuclear localization of the HSR regulon. The Hsf1 target genes are shown to be targeted to the nuclear pore complexes (NPCs) [Brickner et al., 2012, Sumner and Brickner, 2022], which may facilitate efficient export of stress-induced mRNAs, coordinating nuclear transcription with cytoplasmic translation [Zid and O’Shea, 2014, Zander et al., 2016]. Additionally, heat shock and other stresses are known to induce cytoplasmic acidification [Yoo et al., 2019, Triandafillou et al., 2020]. These pH shifts may influence the

behavior of biomolecules and promote formation of cytosolic stress granules, the adaptive condensates of mRNAs and translation initiation factors implicated in post-transcriptional gene control [Glauninger et al., 2022, 2024, Cairo et al., 2024, Iserman et al., 2020, Riback et al., 2017, Altamirano and Alberti, 2024]. Stress-associated pH changes may serve as a unifying signal that synchronizes transcriptional activation in the nucleus with post-transcriptional control in the cytoplasm.

Mechanistically, what drives Pol II condensate formation and HSR gene coalescence remains unclear. Beyond possible biophysical regulation, such as pH-sensitive conformational changes in Hsf1, Mediator, and/or Pol II, other factors involved in chromatin organization as well as mRNA processing and export likely contribute [Kainth et al., 2021, Rubio et al., 2024]. Stress-induced transcriptional attenuation, which involves the repression of transcription factor controlling genes involved in growth and proliferation, may also help liberate Pol II to condense [Rawat et al., 2021, Sawarkar, 2022]. Moreover, the mRNA transcripts emerging from the Hsf1 target genes may contribute to Pol II condensation. HSR mRNAs have been shown to form “transperons”—ribonucleoprotein particles containing mRNAs from multiple co-regulated genes [Nair et al., 2021, 2022]. The rapid activation of the HSR resulting in multiplexing of mRNAs may promote Pol II condensate assembly. This would not only facilitate rapid nuclear export of the HSR mRNAs but also prevent RNA buildup, which may function as biophysical negative feedback, dispersing the condensed transcriptional machinery [Zander et al., 2016, Henninger et al., 2021].

The ordered assembly mechanism for HSR condensates we describe enables graded control of HSR gene expression, allowing the HSR to function as a rheostat rather than a binary switch (Figure 2.8C). This nuanced regulation may be crucial for adaptation to varying stress intensities and durations. By contrast, 3D genome reorganization—linked to Pol II condensate assembly—acts as a switch. Under conditions when the magnitude or duration of stress exceeds a certain threshold, Pol II condensates may enable cells to maximize the adaptive

capacity of the transcriptional response. The stepwise assembly mechanism we observed for HSR condensates in yeast could potentially apply to transcriptional condensates in mammalian cells, enabling graded control of transcriptional responses as the cellular signaling environment changes in development, disease, or environmental adaptation. Indeed, recent observations suggest that 3D genome organization in the context of transcriptional regulation and Pol II interaction with super-enhancer condensates are both dynamic and transient processes [Du et al., 2024, Brückner et al., 2023]. More specifically in the context of the HSR, intergenic interactions among Hsf1 target genes and transperon formation among the target mRNAs was recently reported to be conserved in human cells, indicating that 3D genome remodeling is a conserved feature of the HSR across eukaryotes [Pataki and Gerst, 2024]. These parallels suggest an ancient and extensive role for condensate-mediated genome organization in the eukaryotic lineage.

2.10 Materials and Methods

Yeast Strains

To tag Med15 and Rpb3 with mCherry, PCR amplicons containing the mCherry coding sequence and homology to the 3'-ends of either MED15 or RPB3 were amplified from the plasmid pFA6a-mCherry-hphMX6. For tagging Hsf1 with mEGFP, the PCR amplicon with homology to the 3'-end of HSF1 was amplified from the plasmid pFA6a-mEGFP-hphMX6. These amplicons were then transformed into their respective haploid strains for in-frame genomic integration. The plasmid pNH604-HSF1pr-HSF1-GFP was used as a template for site-directed mutagenesis to introduce NE1AAA, CE2AAA, and double mutations through QuickChange PCR. The resulting plasmids, pNH604-HSF1pr-HSF1-ne1AAA-GFP, pNH604-HSF1pr-HSF1-ce2AAA-GFP, and pNH604-HSF1pr-HSF1-ne1AAAce2AAA-GFP, were then linearized with Pme I (New England Biolabs) and transformed into the strain DPY034 for integration at the TRP1 locus to obtain strains DPY1804, DPY1805, and DPY1806. The loss of parental HSF1 plasmid was confirmed by growth on 5-FOA media. Plasmids pNH604-HSF1pr-HSF1 Δ po4-GFP, pNH604-HSF1pr-HSF1PO4*-GFP, pNH604-HSF1pr-HSF1 Δ po4ne1AAA-GFP, pNH604-HSF1pr-HSF1 Δ po4ce2AAA-GFP, pNH604-HSF1pr-HSF1 Δ po4ne1AAAce2AAA-GFP, pNH604-HSF1pr-HSF1PO4*ne1AAA-GFP, pNH604-HSF1pr-HSF1PO4*ce2AAA-GFP, and pNH604-HSF1pr-HSF1PO4*ne1AAAce2AAA-GFP were linearized with Fse I (New England Biolabs) and transformed in the strain DPY034 for integration at the TRP1 locus. The loss of parental HSF1 plasmid was confirmed by growth on 5-FOA media, yielding strains SCY028, DPY1940, SCY033, SCY034, SCY035, SCY036, SCY037, and SCY038. A complete list of strains is provided in Table 2.1. PCR primer sequences are provided in Table 2.2.

Table 2.1: Yeast strains used in this chapter.

Strain name	Genotype	Reference/Source
DPY001	<i>MATα ADE2 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1</i>	[Chowdhary et al., 2022]
DPY032	DPY001; <i>HSF1-mVenus::HIS3</i>	[Chowdhary et al., 2022]
SCY001	DPY001; <i>MED15-mCherry::hphMX6</i>	[Chowdhary et al., 2022]
SCY002	DPY001; <i>RPB3-mCherry::hphMX6</i>	[Chowdhary et al., 2022]
SCY003	DPY032; <i>MED15-mCherry::hphMX6</i>	[Chowdhary et al., 2022]
SCY004	DPY032; <i>RPB3-mCherry::hphMX6</i>	[Chowdhary et al., 2022]
BY4741	<i>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	[Chowdhary et al., 2019]
BY4742	BY4741; <i>MATα</i>	[Chowdhary et al., 2019]
BY4742-AA	<i>MATα tor1-1 fpr1Δ RPL13A-FKBP12::NAT MET15⁺ lys2Δ ura3Δ his3Δ leu2Δ</i>	[Chowdhary et al., 2019]
BY4742-HSF1-AA	BY4742-AA; <i>HSF1-FRB-yEGFP::KAN-MX</i>	[Chowdhary et al., 2019]

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(Table 2.1 continued)

Strain name	Genotype	Reference/Source
SCY018	BY4742-HSF1-AA; <i>MED15-mCherry::hphMX6</i>	This study
SCY019	BY4742-HSF1-AA; <i>RPB3-mCherry::hphMX6</i>	This study
HHY212	<i>MATa tor1-1 fpr1::loxP-LEU2-loxP RPL13A-2xFKBP12::loxP-TRP1-loxP ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1</i>	[Anandhakumar et al., 2016]
YM116	HHY212; <i>MED15-FRB::HIS3</i>	[Anandhakumar et al., 2016]
YM117	YM116; <i>LEU2</i> and <i>TRP1</i> excised with Cre recombinase	[Anandhakumar et al., 2016]
SCY020	YM117; <i>HSF1-mEGFP::hphMX6</i>	This study
SCY021	YM117; <i>RPB3-mCherry::hphMX6</i>	This study
YM100	HHY212; <i>LEU2</i> and <i>TRP1</i> excised with Cre recombinase	[Anandhakumar et al., 2016]
YM114	YM100; <i>CDK8-FRB::HIS3</i>	[Anandhakumar et al., 2016]
SCY022	YM114; <i>HSF1-mEGFP::hphMX6</i>	This study
SCY023	YM114; <i>RPB3-mCherry::hphMX6</i>	This study
yFR1324	<i>MATα ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11,15 ura3-1 tor1-1 fpr1::NAT RPL13A-2xFKBP12::TRP1 RPB1-FRB::KAN-MX</i>	[Chowdhary et al., 2019]
SCY024	yFR1324; <i>HSF1-mVenus::hphMX6</i>	This study
SCY025	yFR1324; <i>MED15-mCherry::hphMX6</i>	This study

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(Table 2.1 continued)

Strain name	Genotype	Reference/Source
DPY182	DPY001; <i>hsf1Δ::KAN HSF1pr-HSF1-GFP::TRP1</i>	[Chowdhary et al., 2022]
DPY034	DPY001; <i>hsf1Δ::KAN; pRS316-HSF1</i>	[Chowdhary et al., 2022]
DPY1804	DPY034; <i>HSF1pr-HSF1-ne1AAA-GFP::TRP1</i> without pRS316-HSF1	This study
DPY1957	DPY1804; <i>MED15-mCherry::hphMX6</i>	This study
SCY026	DPY1804; <i>RPB3-mCherry::hphMX6</i>	This study
DPY1805	DPY034; <i>HSF1pr-HSF1-ce2AAA-GFP::TRP1</i> without pRS316-HSF1	[Chowdhary et al., 2022]
SCY012	DPY1805; <i>MED15-mCherry::hphMX6</i>	[Chowdhary et al., 2022]
SCY013	DPY1805; <i>RPB3-mCherry::hphMX6</i>	[Chowdhary et al., 2022]
DPY1806	DPY034; <i>HSF1pr-HSF1-ne1AAAce2AAA-GFP::TRP1</i> without pRS316-HSF1	This study
DPY1959	DPY1806; <i>MED15-mCherry::hphMX6</i>	This study
DPY1963	DPY1806; <i>RPB3-mCherry::hphMX6</i>	This study
SCY027	DPY034; <i>HSF1pr-HSF1Δpo4-GFP::TRP1</i>	This study
SCY028	SCY027 without pRS316-HSF1	This study
SCY029	SCY028; <i>MED15-mCherry::hphMX6</i>	This study
SCY030	SCY028; <i>RPB3-mCherry::hphMX6</i>	This study

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(Table 2.1 continued)

Strain name	Genotype	Reference/Source
DPY1940	DPY034; <i>HSF1pr-HSF1PO4*-GFP::TRP1</i> without pRS316-HSF1	This study
SCY031	DPY1940; <i>MED15-mCherry::hphMX6</i>	This study
SCY032	DPY1940; <i>RPB3-mCherry::hphMX6</i>	This study
SCY033	DPY034; <i>HSF1pr-HSF1PO4*-ne1AAA-GFP::TRP1</i> without pRS316-HSF1	This study
SCY034	DPY034; <i>HSF1pr-HSF1PO4*-ce2AAA-GFP::TRP1</i> without pRS316-HSF1	This study
SCY035	DPY034; <i>HSF1pr-HSF1PO4*-ne1AAAce2AAA-GFP::TRP1</i> without pRS316-HSF1	This study
SCY036	DPY034; <i>HSF1pr-HSF1Δpo4-ne1AAA-GFP::TRP1</i> without pRS316-HSF1	This study
SCY037	DPY034; <i>HSF1pr-HSF1Δpo4-ce2AAA-GFP::TRP1</i> without pRS316-HSF1	This study
SCY038	DPY034; <i>HSF1pr-HSF1Δpo4-ne1AAAce2AAA-GFP::TRP1</i> without pRS316-HSF1	This study
SCY039	SCY035; <i>MED15-mCherry::hphMX6</i>	This study
SCY040	SCY035; <i>RPB3-mCherry::hphMX6</i>	This study
SCY041	SCY038; <i>MED15-mCherry::hphMX6</i>	This study
SCY042	SCY038; <i>RPB3-mCherry::hphMX6</i>	This study
DPY144	DPY001; <i>HSE-YFP::LEU2</i>	[Krakowiak et al., 2018]
DPY1750	DPY144; <i>hsf1Δ::KAN HSF1pr-HSF1-FLAG::TRP1</i>	This study
DPY1747	DPY1750; <i>HSF1pr-HSF1-ne1AAA-FLAG::TRP1</i>	This study
DPY1748	DPY1750; <i>HSF1pr-HSF1-ce2AAA-FLAG::TRP1</i>	This study
DPY1749	DPY1750; <i>HSF1pr-HSF1-ne1AAAce2AAA-FLAG::TRP1</i>	This study

Table 2.2: Primers used for construction of yeast strains.

Primer Name	Sequence (5' → 3')
<i>Primers used in construction of strains: SCY018, SCY025, DPY1957, DPY1959, SCY029, SCY031, SCY039, SCY041</i>	
Fw Med15-mCherry-hphMX6	G TTCAGAACAAATTCAATGTATGGGATTGGAATAATTGGACAAGTGCTACTCCAGCTGAAGCTTCGTAC
Rv Med15-mCherry-hphMX6	C AAACGAAGTAACTTCAAAAGTATCAAAAGTATGGAAACTTCAAATGTCCGCATAGGCCACTAGTG
<i>Primers used in construction of strains: SCY019, SCY021, SCY023, SCY026, DPY1963, SCY030, SCY032, SCY040, SCY042</i>	
Fw Rpb3-mCherry-hphMX6	A TGCATCTCAAATGGGTAATACTGGATCAGGAGGGTATGATAATGCTTGGCCAGCTGAAGCTTCGTAC
Rv Rpb3-mCherry-hphMX6	T TCGGTTTCGTTCACTTGTTTTTTTTCCTCTATTACGCCCACTTGAGAACCGCATAGGCCACTAGTG
<i>Primers used in construction of strains: SCY020 and SCY022</i>	
Fw Hsf1-mEGFP-hphMX6	A GGACCCGACAGAGTACAACGATCACCGCCTGCCCAAACGAGCTAAGAAACCAGCTGAAGCTTCGTAC
Rv Hsf1-mEGFP-hphMX6	A CTATATTAAATGATTATATACGCTATTTAATGACCTTGCCCTGTGTACCGCATAGGCCACTAGTG
<i>Primers used in construction of strains: SCY036 and SCY038</i>	
Fw Hsf1 Δ po4-ne1AAA-GFP_QC	T CATGATGCTAGAAAACCAGCTGCTGCTGCTCATGCAGCAGCAAGAGGTGCTCCATTGGGTATTTATCAAGC
Rv Hsf1 Δ po4-ne1AAA-GFP_QC	G AGCAGCAGCAGCTGGTTTTCTAGCATCATGAA
<i>Primers used in construction of strain SCY037</i>	
Fw Hsf1 Δ po4-ce2AAA-GFP_QC	G GTGCACCTATAAGACCGTACAAGCAAAGGGCTGCTGCTAAGAATCGTGCAAACGCGGCTGCAGCTGCAGAG
Rv Hsf1 Δ po4-ce2AAA-GFP_QC	C TTGTACGGTCTTATAGGTGCACCAGCATTGTTATTTCG

Culture Conditions

For microscopy, cells were grown at 30°C in SDC (synthetic dextrose complete) media to early-log density ($A_{600} = 0.4-0.5$). For the Anchor Away experiments, rapamycin (LC Laboratories) was added at a final concentration of 1 $\mu\text{g}/\text{ml}$ to early log cultures ($A_{600} = 0.3-0.35$) grown at 30°C in SDC. BY4742-HSF1-AA cells were incubated at 30°C for ~ 90 min, yFR1324, YM114, and YM117 for ~ 60 min, in presence of rapamycin before imaging [Chowdhary et al., 2019, Anandhakumar et al., 2016]. For 3C, RT-qPCR, and RNA-seq analyses, cells were grown at 30°C in YPD (yeast extract-peptone-dextrose) to mid-log density ($A_{600} = 0.65-0.8$). A portion of the culture was maintained at 30°C as the non-heat-shocked (NHS) sample, while the remaining culture was subjected to an instantaneous 30°C to 39°C thermal upshift for the indicated duration (heat-shocked (HS) sample). For the Anchor Away 3C and RT-qPCR experiments, rapamycin was added to early log cultures ($A_{600} = 0.35-0.4$). Cells were grown to mid-log density at 30°C in YPD in the presence of rapamycin as described above. For the quantitative growth assay, cells were grown to stationary phase at 30°C in YPD media. The saturated cultures were diluted to a uniform cell density of $A_{600}=0.15$ and transferred to a 48-well microtiter plate. Cells were then grown either at 30°C for 48 h, or at 30°C for 6 h followed by a shift to 37°C for 42 h, as indicated. For flow cytometry, cells were grown to log phase at room temperature (25°C) in SDC media. The cells were then transferred to a 96-well microtiter plate and incubated at 30°C for 1 h following the addition of cycloheximide at a final concentration of 50 $\mu\text{g}/\text{ml}$.

Fluorescence Microscopy

Fluorescence live-cell imaging was performed as previously described [Chowdhary et al., 2022]. Briefly, cells were grown at 30°C in SDC to early log density of $A_{600} = 0.5$. An aliquot of cells was immobilized on a concanavalin A-coated glass bottom dish, and fresh SDC media was added before imaging. Images were acquired on Leica TCS SP5 II STED-CW

laser scanning confocal microscope (Leica Microsystems) equipped with a GaAsP hybrid detector, with STED mode disabled. Heat shock at 39°C was applied by heating the objective (HCX PL APOCS 63x/1.4 oil UV) with a Biopetechs objective heater system and controlling the temperature in the incubator chamber enclosing the microscope. Argon laser was used at lines 488 and 514 nm for excitation of GFP and mVenus, while mCherry was excited either at 561 nm or by using the HeNe laser at 594 nm. For the Anchor Away experiments, the SDC media in the dish was replaced with SDC supplemented with rapamycin at a final concentration of 1 $\mu\text{g}/\text{ml}$, and the cells were imaged at room temperature ($\sim 25^\circ\text{C}$) or heat-shocked at 39°C for the indicated times. All images were acquired in xyz scan mode, capturing 8 planes along the z-axis with a 0.25 μm interplanar distance. For dual-color live-cell imaging, fluorophores were sequentially scanned in two channels, with scanning modes switched on between lines to minimize bleed-through and inter-channel crosstalk. The images were deconvolved using the YacuDecu function, which applies the Richardson-Lucy algorithm to calculate theoretical Point Spread Functions (PSFs) (<https://github.com/bnorthan>) [Rueden et al., 2017]. The PSFs were computed based on the imaging parameters for each condition. Custom Fiji plugins were used for basic image processing, such as to colorize, split or merge channels, create composites, and adjust the brightness [Schindelin et al., 2012]. 3D rendering and visualization were performed using the ClearVolume plugin in Fiji [Royer et al., 2015].

Chromosome Conformation Capture (3C)

TaqI-3C was performed as previously described [Chowdhary et al., 2022, 2017, 2019, 2020]. Briefly, cells were grown to mid-log density ($A_{600} = 0.8$) at 30°C, and either maintained at 30°C or heat-shocked at 39°C for 10 min, followed by crosslinking with formaldehyde (1% final concentration). For 3C assays involving conditional depletion by Anchor Away, cells were pre-treated with rapamycin as described above, heat-shocked at 39°C for 10 min (or not), and then crosslinked. Crosslinked cells were harvested and subjected to glass bead lysis

in FA lysis buffer (50 mM HEPES pH 7.9, 140 mM NaCl, 1% Triton X-100, 0.1% Sodium deoxycholate, 1 mM EDTA, 1 mM PMSF) by vortexing at 4°C for two 20-min cycles. A 10% fraction of the chromatin lysate was digested with 200 U of Taq I (New England Biolabs) at 60°C for 7 h. Taq I was heat-inactivated at 80°C for 20 min. The digested chromatin was centrifuged, and the pellet was resuspended in 100 μ l of 10 mM Tris-HCl (pH 7.5). The Taq I-digested chromatin was diluted 7-fold, and 10,000 cohesive end units of Quick T4 DNA ligase (New England Biolabs) were added for proximity ligation at 25°C for 2 h. The ligated sample was treated with DNase-free RNase (Sigma Aldrich) at 37°C for 20 min, followed by Proteinase K (Invitrogen) at 65°C for 12 h. The 3C DNA template was then extracted using phenol-chloroform and precipitated with ethanol. The 3C interaction frequencies were quantified by qPCR on a CFX Real-Time PCR system (BioRad) using Power SYBR Green PCR master mix (Fisher Scientific). Normalization controls were applied to account for: (i) variations in primer pair efficiencies; (ii) primer dimer background; (iii) differences in 3C template recovery; and (iv) ensuring a ligation-dependent 3C signal. The sequences of 3C primers used in this study are provided in Table 2.3. Detailed algorithms for calculating normalized 3C interaction frequencies are outlined below and in [Chowdhary et al., 2020].

Table 2.3: Forward (F) primers used for TaqI-3C.

Name	Sequence (5' → 3')
<i>UBI4</i> F+524	GTAAGCAGCTAGAAGATGGTAGAACC
<i>HSP104</i> F-63	AGGCATTGTAATCTTGCCTCAATTC
<i>HSP104</i> F+1550	CCCTTGATGCTGAACGTAGATATG
<i>SSA2</i> F+198	AGGTAACAGAACCACTCCATCTTTC
<i>SSA2</i> F+1368	TCTCTACTTATGCTGACAACCAACC
<i>TMA10</i> F+811	ATGCAAAAACACTTCCCAGAATAG
<i>SSA4</i> F+198	GCCTTCTTATGTGGCTTTTACTGAC
<i>SSA4</i> F+2255	ATAAGAAAGTCATCGCCAAACAAC
<i>HSP82</i> F-290	CCTCTCTCAACACAGTAATCCATAAAC
<i>HSP82</i> F+740	AATTAGTCGTCACCAAGGAAGTTG
<i>HSP82</i> F+1445	GCCAGAACACCAAAAAGAACATCTAC
<i>ARS504</i> F (internal control)	GTCAGACCTGTTTCCTTTAAGAGG

Reverse Transcription-qPCR (RT-qPCR)

RT-qPCR was performed as described previously [Chowdhary et al., 2022]. Briefly, cells were cultured to a mid-log density ($A_{600} = 0.8$) at 30°C and either maintained at 30°C or heat-shocked at 39°C for 10 min. Transcription was terminated by adding 20 mM sodium azide. For RT-qPCR experiments involving conditional depletion by Anchor Away, cells were pre-treated with rapamycin, heat-shocked at 39°C for 10 min (or not), and then treated with sodium azide. Cells were harvested and lysed using glass beads in TRIzol (Invitrogen) and chloroform for 10 min at 4°C. Total RNA was precipitated with ethanol. A fraction of total RNA (~20 μ g) was treated with RNase-free DNase I (New England Biolabs) at 37°C for 15 min, followed by heat-inactivation of DNase I at 75°C for 10 min. RNA was purified using the RNA Clean and Concentrator kit (Zymo Research). 2 μ g of purified

RNA and random hexamers were used with the Superscript IV first-strand synthesis system (Invitrogen) for preparing cDNA. The cDNA reaction mix was diluted 2-fold, and 2 μ l of the diluted cDNA template was used for qPCR. Relative cDNA levels were quantified using the $\Delta\Delta$ Ct method [Chowdhary et al., 2017]. qPCR signal from SCR1 Pol III transcript was used as a normalization control to account for variations in cDNA recovery. The sequences of primers used for RT-qPCR analysis are provided in Table 2.4.

Table 2.4: Primers used for RT-qPCR.

Name	Sequence (5' $\rightarrow$ 3')
<i>HSP104</i> F+1922	TTAGCTAATCCAAGGCAACCAG
<i>HSP104</i> ORF R+1922	ACCTTCATCGTACCCGACATAAC
<i>HSP82</i> F+1838	ACATGGAAAGAATCATGAAGGCTC
<i>HSP82</i> R+1838	AAGTCCTTGACAGTCTTGTCTTGAG
<i>SSA4</i> F+1539	ATCTACTGGGTAAATTTGAGTTGAGC
<i>SSA4</i> R+1539	CTAGCTGATTCTTAGCTTGAACACG
<i>SSA2</i> F+1905	CTAAATTGTACCAAGCTGGTGGTG
<i>SSA2</i> R+1905	AATACAGAGGAAAGCAAAAGTAAAC
<i>TMA10</i> F+159	ACGAAGCAAAGTCTAACCCTAAAG
<i>TMA10</i> R+159	TTTCATTGTTTTGGGAGTTAGAGC
<i>UBI4</i> F+524	GTAAGCAGCTAGAAGATGGTAGAACCC
<i>UBI4</i> R+524	TGAATTTTCGACTTAACGTTGTCTG
<i>SCR1</i> F	CTCCACCTTCACCGCTGTTAG
<i>SCR1</i> R	AAATATGGTTCAGGACACACTCC

RNA Sequencing

Cells were cultured to a mid-log density ($A_{600} = 0.8$) at 30°C and either maintained at 30°C or heat-shocked at 39°C for 10 min. Transcription was terminated by adding 20 mM

sodium azide. Cells were harvested by centrifugation, then lysed in YR Digestion buffer with Zymolyase using the YeaStar RNA kit (Zymo Research). Total RNA was isolated as per the YeaStar RNA kit manual and purified using the RNA Clean and Concentrator kit with in-column DNase I treatment (Zymo Research). 50 μ g of the purified total RNA and oligo d(T)25-coupled paramagnetic beads were used with NEBNext High Input Poly(A) mRNA Isolation Module (New England Biolabs) to enrich for the poly(A)+ RNA. The quality, yield, and integrity of the total RNA and polyA- enriched RNA were evaluated on Bioanalyzer with the RNA 6000 Pico kit (Agilent Technologies). 100 ng of purified poly(A) mRNA was used for cDNA library preparation using the NEBNext Ultra™ II Directional RNA Library Prep kit, and libraries were barcoded using NEBNext Multiplex Oligos for Illumina (Index Primers Sets 3-4). The average fragment length, concentration, and quality of the cDNA library were analyzed with Bioanalyzer using the High Sensitivity DNA kit (Agilent Technologies). The libraries were paired-end deep sequenced on the Illumina NovaSeqX platform (NovaX-10B-300-(PE150)) at the University of Chicago Functional Genomics Core Facility (RRID:SCR_019196). For RNA-seq data analysis, see below.

Quantitative Growth Assay

Cells were grown to saturation in YPD media at 30°C. The overnight grown cultures were then diluted to a density of A600=0.15 and transferred to a 48-well microtiter plate. The plates were incubated on the SPECTROstar Nano absorbance microplate reader (BMG LABTECH), equipped with a full spectrum UV/vis spectrometer and orbital shaking. For growth kinetic measurements in basal conditions, the plates were incubated at 30°C with shaking for 48 h, with absorbance readings taken every 30 min at 600 nm. For measurements at elevated temperature, plates were first incubated at 30°C for 6 h, then at 37°C for additional 42 h. Absorbance measurements, protocol set-up, and data acquisition were managed using the MARS data analysis software.

Flow Cytometry

For the reporter assays, cells were grown to mid-log phase in SDC media at 25°C. A 50 μ l aliquot of cells was transferred into a 96-well microtiter plate containing 50 μ l of SDC supplemented with cycloheximide at a final concentration of 50 μ g/ml and incubated at 30°C for 1 h to allow maturation and folding of the reporter. HSE-YFP reporter activity was measured using BD Fortessa HTS 4-15 benchtop analyzer at the University of Chicago Cytometry and Antibody Technology Core Facility (RRID: SCR_017760). The data were analyzed using FlowJo.

Chromatin Immunoprecipitation (ChIP)

250 ml culture of cells were grown to an OD 600 of 0.8 in YPD media, heat-shocked at 39°C for 15 min (or maintained at 30°C), then crosslinked with 1% formaldehyde. Cells were filtered and then cryolysed in lysis buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% Sodium deoxycholate, 1 mM EDTA, 2 mM PMSF, and 250 μ g/ml cOmplete™, EDTA-free Protease Inhibitor Cocktail) at 4°C. The chromatin lysate was sonicated to an average fragment size of ~250 bp using Biorupter Pico (Diagenode). A fraction of the sonicated chromatin was incubated with anti-FLAG M2 magnetic beads (Sigma Aldrich) for 16 h at 4°C. The beads were sequentially washed with lysis buffer, high salt buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 1% Triton X-100, 0.1% Sodium Deoxycholate, 1 mM EDTA), and wash buffer (10 mM Tris pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% Sodium Deoxycholate, 1 mM EDTA). Chromatin was eluted from the beads with 50 μ l of 3X-FLAG peptide. RNA and proteins were removed by treatment with DNase-free RNase (final concentration of 200 μ g/ml; incubation at 37°C for 1 h) and Proteinase K (final concentration of 50 μ g/ml; incubation at 60°C for 16 h). The ChIP template was extracted using phenol-chloroform and precipitated in the presence of ethanol. ChIP occupancy was quantified by qPCR. The ChIP DNA quantities were determined by interpolation from a standard curve generated

with genomic DNA. The qPCR signal for each primer combination was normalized to the corresponding signal from the input DNA to account for variations in DNA template recovery.

Foci Counting and Characterization

Hsf1, Med15 and Rpb3 foci were detected and quantified in 3D live-cell images using the automated FindFoci plugin in ImageJ [Herbert et al., 2014], as described previously [Chowdhary et al., 2022]. Briefly, a region of interest was defined in each cell’s nucleus, and foci were identified across all z planes (z=8). We trained the FindFoci algorithm using the optimizer function in the plugin submenu to predict optimum parameter settings for foci assignment. The following parameters were applied to all images in this study: (a) background, one standard deviation above mean; (b) search method, above background with an optimal value of 0.3; and (c) merge option, relative above background with peak parameter of 0.2, and minimum size of 1. We used the x, y, z position coordinates and sizes of peak intensity regions to create 3D bubble charts in MATLAB (MATLAB, 2021. version 9.11.0.1873467 (R2021b), Natick, MA: The MathWorks, Inc.). For visualizing images as 3D surface plots, a 10×10 pixel square was marked around the nucleus, and representative planes for each cell were displayed as 3D surface plots using the Interactive 3D Surface Plot plugin in ImageJ (contributing author: Kai Uwe Barthel, Germany). The plot parameters for scale, rotation, smoothing, and lightning were adapted for optimal display of 3D surface plots, with consistent settings applied to both non-heat shock and heat shock conditions.

RNA-seq Analysis

Adapter sequences were trimmed from raw reads using TrimGalore v0.6.10 (<https://github.com/FelixKrueger/TrimGalore>) in paired-end mode (`-paired`) with default settings. The fasta file of *S. cerevisiae* reference genome sequence (SacCer3) and its corresponding annotation gtf file were downloaded from NCBI. Genome indices were generated

using STAR v2.7.11b (`-runMode genomeGenerate -sjdbOverhang 100`) [Dobin et al., 2013]. Trimmed FASTQ files were used to map the reads to the *S. cerevisiae* reference genome with STAR v2.7.11b (`-outSAMtype BAM Unsorted -alignIntronMin 10 -alignIntronMax 5000 -alignMatesGapMax 2000 -quantMode GeneCounts -genomeSAindexNbases 10`), generating BAM files. These BAM files were sorted and indexed using SAMtools v1.19.2 [Li et al., 2009]. The raw counts of genes were extracted from the sorted BAM files using HT-seq v2.0.4 (`-f bam -m intersection-strict -s reverse`) [Putri et al., 2022]. The raw counts were then normalized using DESeq2 v1.34.0 [Love et al., 2014]. The normalized gene counts were used for principal component analysis (PCA). K-means clustering, hierarchical clustering, and Spearman rank correlation were performed using the Morpheus portal (<https://software.broadinstitute.org/morpheus/>). For k-means clustering, the normalized fold changes (HS/NHS) of gene counts were used to determine the optimal number of clusters through the elbow method. The hierarchical clustering dendrogram was created based on a $(1 - \text{Spearman correlation})$ distance matrix.

Quantification of 3C

The interaction frequencies determined by TaqI-3C were calculated as previously described [Chowdhary et al., 2022, 2017, 2019, 2020]. The percent digestion efficiency was calculated by amplifying a region across the Taq I restriction site using a pair of convergent primers (sequences provided in Table 2.5). The digestion efficiency was determined for each primer combination using the formula:

Table 2.5: Reverse (R) primer used for percent digestion determination in TaqI-3C.

Name	Sequence (5' → 3')
<i>UBI4</i> R+524	TGAATTTTCGACTTAACGTTGTCG
<i>HSP104</i> R-63	ATCGTTAGAGCCCTTTCTGTAAATTG
<i>HSP104</i> R+1550	CCACATTTTGGATCATGGAGTTG
<i>SSA2</i> R+198	GCTTCATATCACCTTGGACTTCTG
<i>SSA2</i> R+1368	TTCAATTTGTGGGACACCTCTTG
<i>TMA10</i> R+811	CCGGTTATAGGACCCTTATTGATG
<i>SSA4</i> R+198	TTTACGTCCGATCAGACGCTTAG
<i>SSA4</i> R+2255	GTGTTAAACTCCGGTCAAAGAAAC
<i>HSP82</i> R-290	GAAGGACCTGGTTGGTATTAAGATG
<i>HSP82</i> R+740	AATGCTTAACGTACAATGGGTCTTC
<i>HSP82</i> R+1445	ATTCATCAATTGGGTCCGTCAAG
<i>ARS504</i> R (internal control)	CATACCCTCGGGTCAAACAC

$$\% \text{ digestion} = 100 - \frac{100}{2^{[(Ct_R - Ct_{ARS504})^{DO} - (Ct_R - Ct_{ARS504})^{UND}]}} \quad (2.1)$$

Here, Ct_R is the cycle threshold (Ct) quantification of the digested only (DO) or undigested (UND) templates, and Ct_{ARS504} the cycle threshold quantification of the *ARS504* locus (a region lacking a Taq I site).

For measurement of normalized frequency of intragenic or intergenic interactions, the Ct values of digested only (DO_{3C}) and ligated (Lig_{3C}) templates from the crosslinked chromatin, as well as the digested (DO_{gDNA}) and ligated (Lig_{gDNA}) genomic DNA, were used in the following formula:

$$\text{Normalized Frequency of Interaction} = \frac{\frac{\left(2^{-\Delta C_{t_{Lig3C}} / ARS504_{Lig3C}}\right) / \left(2^{-\Delta C_{t_{DO3C}} / ARS504_{DO3C}}\right)}{(\text{Digestion site 1}) \times (\text{Digestion site 2})}}{\frac{\left(2^{-\Delta C_{t_{Lig_{gDNA}}} / ARS504_{Lig_{gDNA}}}\right) / \left(2^{-\Delta C_{t_{DO_{gDNA}}} / ARS504_{DO_{gDNA}}}\right)}{(\text{Digestion site 1}) \times (\text{Digestion site 2})}} \quad (2.2)$$

- (i) Ct values were calculated by subtracting Ct (no-template) from those of Lig_{3C} , DO_{3C} , or gDNA templates.
- (ii) $2^{-\Delta C_{t_{ARS504}}}$ represents fold-over signals normalized to the $ARS504$ locus.
- (iii) Ligation-dependent signals (LDS) are determined as the ratio of fold-over normalized signals of Lig_{3C} and DO_{3C} templates $\left(2^{-\Delta C_{t_{Lig3C}} / ARS504_{Lig3C}}\right) / \left(2^{-\Delta C_{t_{DO3C}} / ARS504_{DO3C}}\right)$; also applies to the gDNA control.
- (iv) LDS values were corrected for any variation in Taq I digestion efficiencies between sites 1 and 2.
- (v) Normalized Frequency of Interaction is the ratio of ligation-dependent signals from the 3C and gDNA control templates after correcting for differences in the digestion efficiencies.

Quantification of Genetic Interactions

To quantify genetic interactions between various Hsf1 mutations, we measured Hsf1 activity using single cell fluorescent reporter and bulk RNA sequencing (RNA-seq), and we leveraged the genetic interaction theory as previously applied to the HSR [Brandman et al., 2012, Alford et al., 2021]. To this end, we assessed whether the various mutations alter Hsf1 activity independently or have interdependent effects using double mutant cycles [Horovitz,

1996]. Based on the expression level of a reporter or target gene in wild type cells and two single mutants, we calculated the expected value of the expression level in the double mutant. If the two mutations act independently, the expected expression level in the double mutant would be the sum of the log fold changes of the individual single mutants relative to wild type. If the measured expression level diverges from the expected value, the effects of the mutations have some degree of interdependence.

Statistical Tests

Student's t test (two-tailed) was used to calculate statistical significance between all pairwise comparisons (assuming parametric distributions). In Figures 2.3D, 2.5E, 2.4A and I, and 2.7C, one-way ANOVA followed by Tukey's post hoc analysis were used. Each pairwise comparison is done with means of two independent biological samples (n=2) +SD. n.s., $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

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Declaration of Interests

The authors declare no competing interests.

CHAPTER 3

CHAPERONE CONDENSATES BUFFER THE HEAT SHOCK RESPONSE AGAINST PLEIOTROPIC INPUTS

3.1 Author Contributions to this chapter

The work in this chapter is from a manuscript in the final stages of submission. Conceptualization of the project was carried out by myself and David Pincus. I performed the majority of the experimentation and analysis. This included experimental design, imaging, RNA-seq, and data analysis. I also contributed greatly to the quantification pipeline design used for the analysis of all images. This final pipeline was fleshed out and completed by David Pincus, Asif Ali, and Abani Neferkara. Surabhi Chowdhary also contributed to the representative imaging and analysis for the figures. The majority of the FRET experimentation was carried out by Jenny Krystosek with analysis completed by herself and David Pincus. Sean Martin also completed experiments and analysis for an HSE-YFP reporter in each stress. Finally, the manuscript construction and overall structure was designed by myself, David Pincus, and Jenny Krystosek.

3.2 Abstract

Stress response pathways can be specific, dedicated to one stimulus, or pleiotropic, funneling distinct perturbations into a shared program. The heat shock response (HSR), the Hsf1-driven transcriptional program, has been linked to numerous stresses, suggesting it is pleiotropic. Here we find that high amplitude HSR activation is specific to heat shock in budding yeast. Heat shock activates the HSR through a condensate cascade in which orphan ribosomal proteins condense with Sis1 and Hsp70, titrating these chaperones away from their repressive interactions with Hsf1, triggering Hsf1 condensation with the transcrip-

tional machinery, and activating HSR genes. Other stressors likewise drive Sis1 and Hsp70 condensation, redeploying Sis1 to stimulus-specific subcellular sites. However, chaperone condensation is not sufficient to activate the HSR. Sis1 and Hsp70 condense under conditions in which the HSR remains inactive, and Sis1 depletion increases HSR activation under all conditions except heat shock. These results suggest that chaperone condensates buffer the HSR, setting a threshold for activation and imparting specificity.

3.3 Introduction

Maintaining protein folding homeostasis (proteostasis) is one of the most fundamental challenges facing living cells [Hartl et al., 2011, Hipp et al., 2019]. The heat shock response (HSR) is a conserved transcriptional program that regulates expression of molecular chaperones [Lindquist, 1986, Richter et al., 2010]. Despite its name, the HSR is activated not only by elevated temperature but by a broad spectrum of perturbations including oxidative damage, misincorporation of amino acid analogs, ethanol, nutrient depletion, mitochondrial import stress, and ribosome biogenesis inhibitors [Ankar and Sistonen, 2011, Gomez-Pastor et al., 2018, Pincus, 2020]. This apparent pleiotropy raises a basic question: does every stress reported to induce the HSR engage it to the same extent, or is high amplitude activation reserved for specific stimuli?

In budding yeast, the transcription factor Hsf1 drives HSR gene expression as an essential nuclear protein whose activity is regulated post-translationally [Sorger and Pelham, 1988b, Wiederrecht et al., 1988]. Under basal conditions, Hsf1 is held inactive by the chaperone Hsp70 (Ssa1/2) [Shi et al., 1998, Zheng et al., 2016, Krakowiak et al., 2018, Masser et al., 2019, Ciccarelli and Andréasson, 2024]. Upon stress, Hsp70 is titrated away from Hsf1 by competing substrates including newly synthesized polypeptides [Baler et al., 1992, Tye and Churchman, 2021], allowing Hsf1 to become hyperphosphorylated, oligomerize, and activate transcription [Sorger, 1990, Ankar and Sistonen, 2011]. Hsf1 activation is accompanied by

transcriptional condensate formation. Upon heat shock, assembly of Hsf1 with the Mediator coactivator and RNA Pol II into nuclear clusters at target-gene loci coincides with transcriptional bursting and reorganization of the 3D genome [Chowdhary et al., 2019, 2022, Zhang et al., 2022].

Whether this condensation drives transcription, however, is uncertain. Hsf1 forms condensates and restructures the 3D genome under ethanol stress before and separably from target gene induction [Rubio et al., 2024, Mohajan et al., 2025], and separation-of-function Hsf1 mutants fail to nucleate Pol II condensates yet nonetheless activate HSR genes to wild type levels [Chowdhary et al., 2025]. In mammalian cells, HSF1 condensate formation has been found to be anti-correlated with chaperone output, with transcription tracking foci resolution rather than formation [Gaglia et al., 2020], and stress-specific HSF1 condensates can be transcriptionally productive or inert depending on context [Dudley et al., 2026]. Forced formation of synthetic transcription factor droplets can be neutral or inhibitory for transcription [Trojanowski et al., 2022]. Together these observations argue that condensate formation *per se* is neither required nor sufficient for transcription.

A parallel line of investigation has revealed a connection between ribosome biogenesis (RiBi) and the HSR. RiBi is highly sensitive to stress and shuts down rapidly upon heat shock and other proteotoxic insults [Warner, 1999, Albert et al., 2019]. This generates a pool of orphan ribosomal proteins (oRPs)—newly synthesized subunits that cannot assemble into ribosomes and are consequently aggregation-prone. Stress-triggered condensation does not necessarily represent indiscriminate damage and is increasingly recognized as specific, adaptive, and evolutionarily tuned [Wallace et al., 2015, Riback et al., 2017]. Consistent with this paradigm, oRPs are sequestered into reversible chaperone-associated condensates at the nucleolar periphery upon heat shock [Ali et al., 2023, 2024]. The J-domain co-chaperone Sis1 is recruited to oRP condensates and, along with Hsp70, away from the nucleoplasm, thereby de-repressing Hsf1 to initiate the HSR [Feder et al., 2021, Garde et al., 2023].

Here we combine single-cell imaging, a quantitative FRET biosensor of Sis1 self-association, transcriptomics, and acute nuclear depletion of Sis1 to dissect how the yeast HSR discriminates among stresses. We establish that heat shock activates the HSR through a multistep condensate cascade in which oRPs condense at the nucleolar periphery with Sis1 and Hsp70, releasing Hsf1 to condense with Mediator and RNA Pol II at HSR genes. By contrast, across five chemically distinct stresses, although condensation of Sis1 and Hsp70 occurs broadly, high amplitude HSR transcription does not; full HSR activation is specific to heat shock, with moderate activation also observed in arsenite. Chaperone condensation is thus decoupled from transcriptional output. Depleting nuclear Sis1 increased HSR activation in all conditions except heat shock, suggesting that only heat shock quantitatively depletes Sis1 nuclear availability. These results reframe stress signal integration in the HSR from graded and inclusive to a more switch-like process with a threshold set by nuclear chaperone availability. We propose that chaperone condensation buffers the HSR, imparting specificity to an otherwise promiscuous program.

3.4 Results

3.4.1 Heat shock initiates a condensate cascade from orphan ribosomal proteins to RNA polymerase II

Heat shock halts ribosome biogenesis while translation continues, generating orphan ribosomal proteins (oRPs) that accumulate at the nucleolar periphery and nucleate early events of the heat shock response (HSR) [Feder et al., 2021, Ali et al., 2023]. To monitor this in single cells, we pulse-labeled newly synthesized ribosomal proteins with HaloTag chemistry and imaged them in cells co-expressing the nucleolar marker Nsr1-mScarlet. Newly made Rpl26a-Halo was efficiently incorporated into ribosomes and exported to the cytosol in unstressed cells but accumulated in discontinuous peri-nucleolar rings upon heat shock (Fig. 1, B and

C). We then imaged each downstream step of the HSR cascade under basal conditions, under heat shock alone, heat shock with cycloheximide (CHX) to block translation, and heat shock with 1,6-hexanediol (1,6-HD), which can dissolve some liquid-like condensates [Kroschwald et al., 2017]. We tagged each of the following with fluorescent proteins at their endogenous loci (see methods): the co-chaperone Sis1, the chaperone Hsp70 (Ssa1), the transcription factor Hsf1, the Mediator subunit Med15, and RNA polymerase II (Rpb3) (Fig. 1, D to F). We imaged and segmented >100 cells in 3D in each condition across multiple replicates throughout this study.

(Figure 3.1 continued)

conditions. Scale bar, 5 μm . (C) Quantification of oRP accumulation (Wilcoxon rank-sum test). (D–F) Representative images of Sis1, Hsp70 (Ssa1), Hsf1, Med15, and Pol II (Rpb3) under HS, HS + 50 $\mu\text{g/ml}$ CHX, and HS + 5% 1,6-HD. Scale bars, 5 μm . (G) Condensation index (ratio of brightest 5% voxels to the mean fluorescence) for each protein under unstressed, HS, HS + CHX, and HS + 1,6-HD conditions. Small points, individual cells; diamonds, replicate means. *P*-values from Wilcoxon rank-sum tests are indicated.

Rather than segmenting individual condensates, which would require setting arbitrary thresholds for each protein in each condition, we defined a generic, unitless "condensation index" that we could apply across proteins, fluorophores, and conditions. The condensation index is defined as the ratio of the brightest 5% of voxels in each fluorescence channel to the average fluorescence for each 3D-segmented cell computed from Cellpose-segmented images via the CellQuant pipeline (Fig. S1; see methods) [Stringer et al., 2021, Neferkara et al., 2026]. Although the condensation index is an imperfect metric, it can be applied across proteins without bespoke analysis and is agnostic to the localization, shape, and intracellular heterogeneity of the different condensates, enabling coarse grained comparisons across proteins and conditions.

CellQuant: Cellpose single-cell segmentation of RNA Pol II

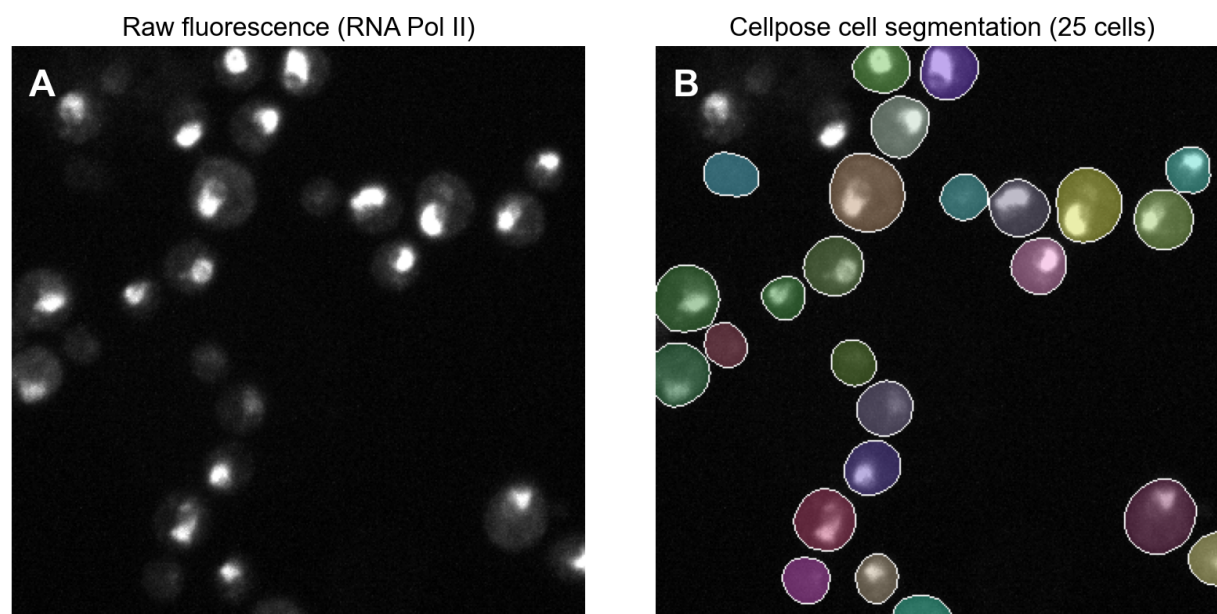


Figure 3.2: Single-cell segmentation with the CellQuant pipeline.

(Figure 3.2 continued)

Representative field of cells expressing endogenously tagged RNA polymerase II (Rpb3). **(A)** Raw fluorescence shown as a maximum intensity projection. **(B)** Whole-cell segmentation by Cellpose [Stringer et al., 2021]: each detected cell is assigned a distinct label (random colors) and its boundary outlined in white. All condensation-index measurements in this study were computed cell-by-cell from CellQuant segmentations of images acquired identically across proteins and stress conditions (see methods). Scale bar, 5 μm .

All five proteins showed increased condensation index values upon heat shock (Fig. 1 G), and CHX suppressed condensation of every protein, consistent with oRPs and other newly synthesized proteins as the initiating signal [Tye and Churchman, 2021, Dea and Pincus, 2024]. 1,6-HD dissolved Sis1, Hsf1, Med15, and Pol II condensates, and for Hsf1, Med15, and Pol II 1,6-HD drove the condensation index below the unstressed baseline as the proteins leaked into the cytoplasm, an artifact of dissolution of the nuclear pore complex we previously reported [Chowdhary et al., 2022]. Hsp70 was the exception as its condensation was insensitive to 1,6-HD, perhaps due to tight ADP-state binding to client proteins [Craig and Marszalek, 2017, Faust et al., 2020]. These results are consistent with a signaling pathway in which heat shock engages a condensate cascade, $\text{oRP} \rightarrow \text{Sis1} \rightarrow \text{Hsp70} \rightarrow \text{Hsf1} \rightarrow \text{Med15} \rightarrow \text{Pol II}$, in which each step is sensitive to CHX.

Importantly, the causal chain in this signaling cascade was formally established in previous work. oRPs are required for Sis1 to localize to the nucleolar periphery and for full activation of HSR genes upon heat shock, as demonstrated via depletion of the ribosomal protein gene transcriptional activator Ifh1 [Ali et al., 2023]. Sis1 re-localization is required for HSR activation upon heat shock, as demonstrated via ectopic expression of NLS-Sis1 [Feder et al., 2021]. Hsp70 binding to Hsf1 inhibits Hsf1 condensation under nonstress conditions, as demonstrated by point mutations in Hsf1 [Chowdhary et al., 2022]. Finally, upon heat shock, Hsf1 is required for Med15 condensation, Med15 is required for Pol II condensation, and Pol II is dispensable for condensation of both Hsf1 and Med15, as demonstrated using conditional depletion alleles [Chowdhary et al., 2025, Male et al., 2026]. Taken together,

these results demonstrate that a functional condensate cascade relays nucleolar RiBi stress to activate the HSR upon heat shock.

3.4.2 Diverse stresses engage the upstream cascade

We next asked whether four mechanistically unrelated stresses activate the same cascade. We used sodium arsenite, a metalloid oxidant; the proline analog azetidine-2-carboxylic acid (AZC); the specific 60S RiBi inhibitor DZA; and hyperosmotic NaCl. We chose concentrations and timepoints as utilized in previous studies [Tye et al., 2019, Dea et al., 2026, Alford et al., 2021]. First, we observed that oRP accumulation was selective. DZA, which stalls 60S ribosomal subunit maturation [Loibl et al., 2014, Pertschy et al., 2007], drove accumulation of Rpl26a comparably to heat shock. By contrast, arsenite and AZC did not, and NaCl showed low level oRP accumulation (Fig. 2, B and C).

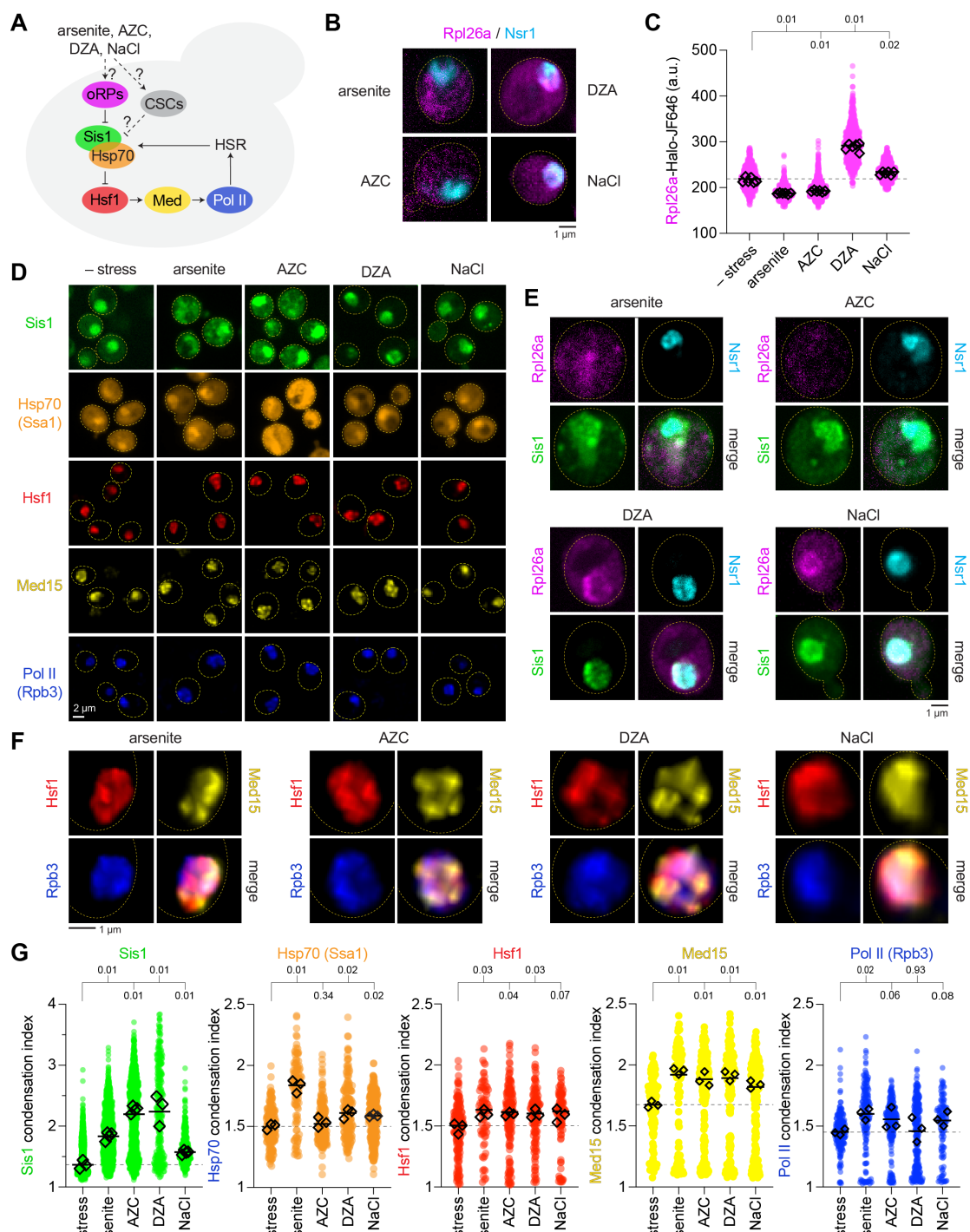


Figure 3.3: Diverse stresses engage the upstream cascade but decouple at distinct steps.

(A) Model of stress inputs converging on the cascade. CSC: condition-specific client. (B) Representative images of newly synthesized Rpl26a (magenta) and Nsr1 (cyan) under unstressed, 1 mM arsenite for 1 hr, 5 mM AZC for 1 hr, 25 μ g/ml DZA for 1 hr, and 1 M NaCl

(Figure 3.3 continued)

for 30 min conditions. Scale bar, 5 μm . (C) Quantification of oRP accumulation across stresses. *P*-values from Wilcoxon rank-sum tests are indicated. (D–F) Representative images of Sis1, Hsp70, Hsf1, Med15, and Pol II under each stress. Scale bars, 5 μm . (G) Condensation index for each protein under unstressed, arsenite, AZC, DZA, and NaCl conditions. Small points, individual cells; diamonds, replicate means. *P*-values from Wilcoxon rank-sum tests are indicated.

Sis1 condensation was induced by all four stresses, and to levels surpassing heat shock in AZC and DZA (Fig. 2 G). Moreover, the subcellular distribution of condensed Sis1 varied across stresses (Fig. 2, D–F), consistent with its redeployment to stimulus-specific sites. However, the coupling between adjacent steps in the cascade broke down in a stress-specific manner. Following AZC treatment, Sis1 condensed maximally yet Hsp70 condensation was unchanged from baseline, although its total levels were increased (Fig. S2). Med15 condensed under all stresses, but Pol II largely failed to condense in the chemical stresses. Thus, each stress appears to distinctly disturb the proteostasis network and differentially engage the transcriptional machinery.

3.4.3 *Chaperone condensation does not predict HSR transcriptional output*

If condensation of Sis1 were sufficient to activate the HSR, all five stresses should drive HSR transcription. However, RNA-seq across all conditions showed that only heat shock and arsenite induced HSR target genes above the unstressed baseline (Fig. 3, A; Fig. S3), while AZC, DZA, and NaCl failed to significantly activate the HSR transcriptional program (Fig. 3 B). Heat shock and arsenite induction of the HSR genes was diminished by CHX, whereas the weak responses to AZC, DZA, and NaCl were CHX-insensitive. Principal component analysis separated the transcriptomes along two orthogonal axes. Stress identity mapped onto PC1 and CHX treatment to PC2 (Fig. 3 C).

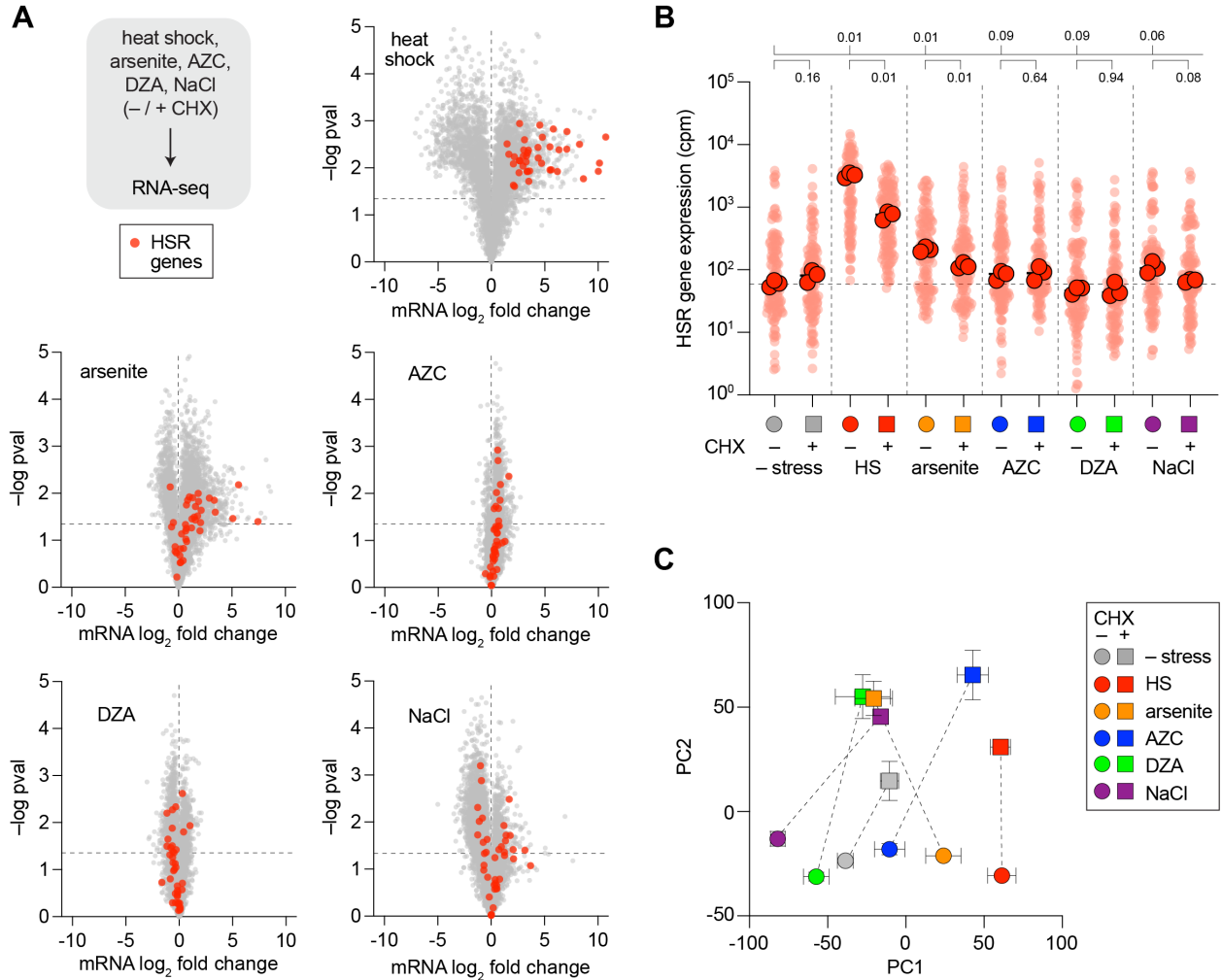


Figure 3.4: HSR activation is limited to HS and arsenite.

(A) Volcano plots of RNA-seq $\log_2$ fold change versus significance for each stress, with HSR target genes highlighted (red). (B) HSR target-gene expression (cpm) under unstressed, HS, arsenite, AZC, DZA, and NaCl conditions, each $\pm$ CHX. Small points, individual genes; large points, biological replicate means; dashed line, unstressed baseline. Two-tailed t-test P -values of replicate means indicated. (C) Principal component analysis of the RNA-seq profiles. Symbols, condition means; error bars, SD; dashed lines connect $\pm$ CHX pairs.

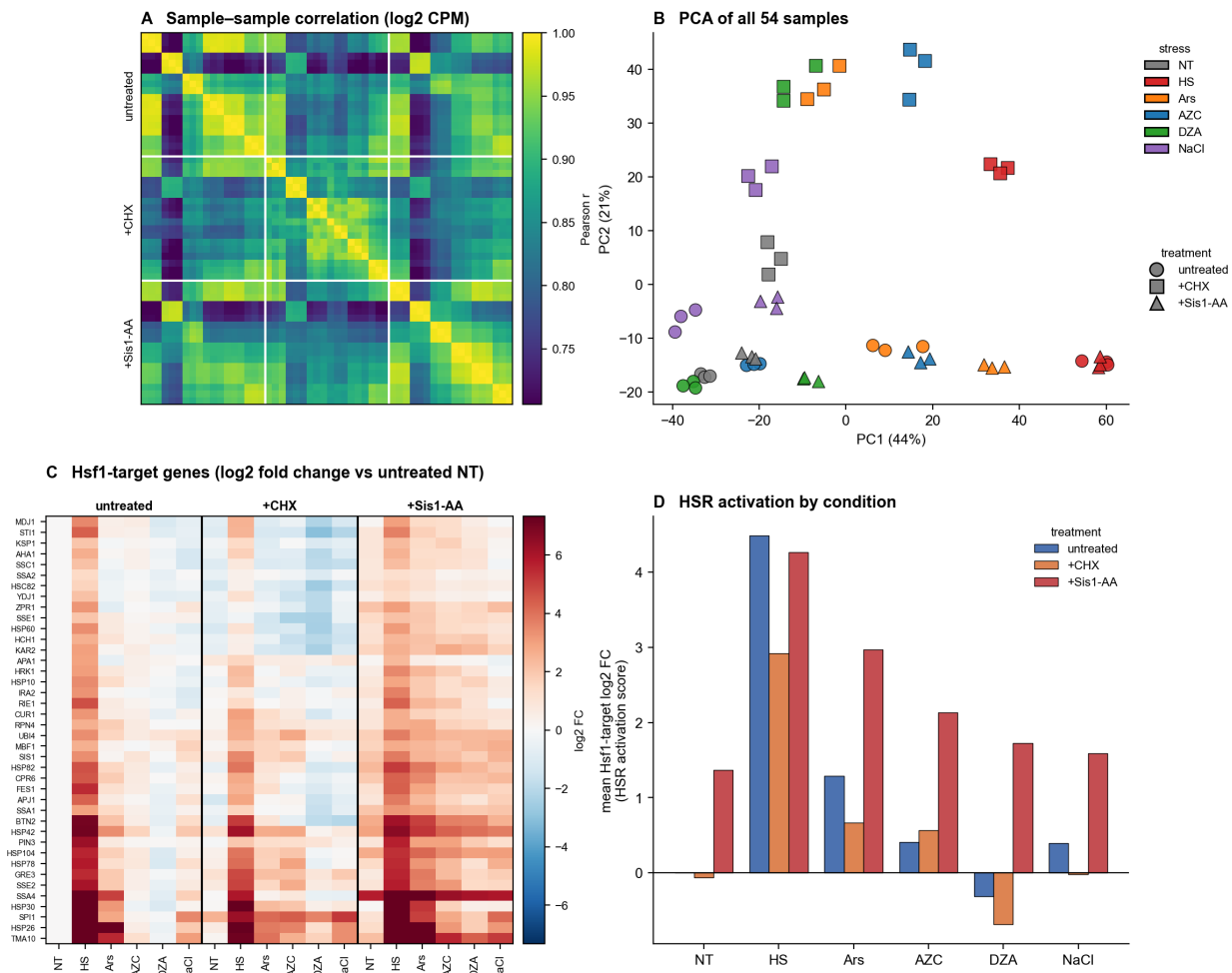


Figure 3.5: Overview of the RNA-seq datasets.

(A) Pearson correlation between all 54 samples computed on log₂ CPM, ordered by treatment block (untreated, +CHX, +Sis1 anchor-away) and by stress; the block-diagonal structure confirms reproducibility among biological triplicates. (B) Principal-component analysis of all 54 samples (top 2000 most variable genes); color denotes stress and symbol denotes treatment. (C) Expression of Hsf1 target genes (rows) across the 18 conditions (mean of three replicates), shown as log₂ fold change relative to untreated NT and clustered by gene. (D) HSR activation score (mean log₂ fold change across the Hsf1 targets) for each stress, grouped by treatment.

Notably, no single upstream readout tracked the separation of HS and arsenite—which induce HSR genes—from AZC, DZA, and NaCl—which do not. DZA resulted in accumulated 60S precursors but did not result in HSR activation; arsenite induced HSR activation without accumulating oRPs; and AZC drove the strongest Sis1 condensation of any stress yet

mounted a weak transcriptional response. Chaperone condensation appears to be broadly induced across conditions, yet full HSR activation is specific to heat shock.

3.4.4 A FRET biosensor enables orthogonal quantification of Sis1 condensation across conditions

The condensation index scores spatial heterogeneity rather than condensation *per se*. We therefore built an orthogonal, cell-based biophysical assay to quantify Sis1 self-association directly. We tagged Sis1 with the photoconvertible fluorophore mEos3.2 [Zhang et al., 2012] and adapted the DAmFRET principle [Khan et al., 2018, Posey et al., 2021, Miller et al., 2023]: partial photoconversion generates a mixed donor–acceptor pool whose intermolecular FRET reports Sis1-Sis1 proximity independently of visible puncta. The sensor behaved as designed. Photoconversion lowered donor signal, raised acceptor signal, and increased FRET in UV-exposed cells (Fig. 4, B and C), providing a quantitative measure of Sis1 self-proximity.

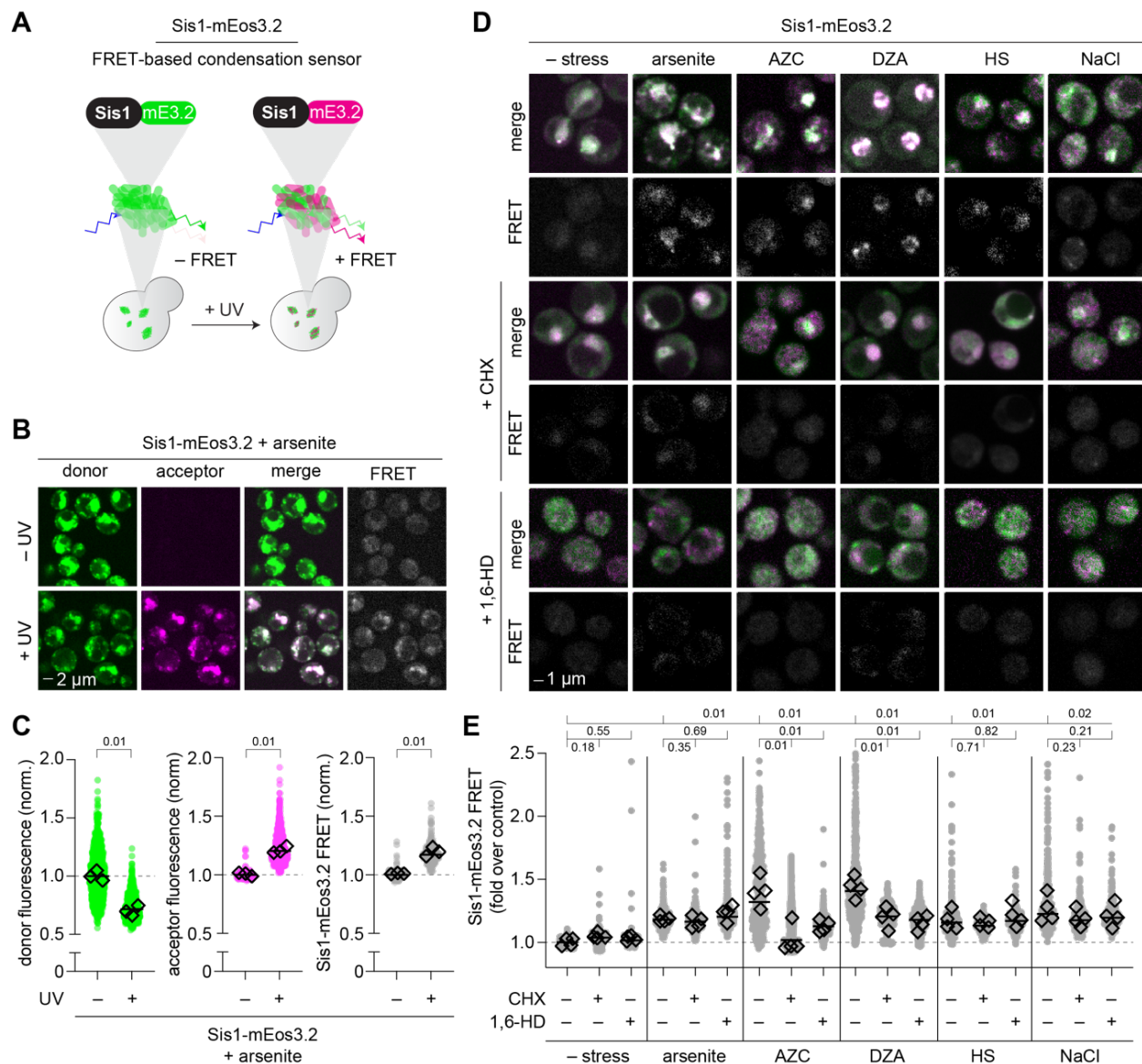


Figure 3.6: **A** FRET biosensor quantifies Sis1 self-association across stresses.

(A) Design of the Sis1-mEos3.2 DAMFRET sensor: partial photoconversion generates an intramolecular donor (green)-acceptor (magenta) pool whose FRET reports Sis1 self-proximity independent of visible foci. (B) Representative donor, acceptor, and FRET images $\pm$ UV. Scale bar, 5 μ m. (C) Donor, acceptor, and FRET signal in UV-exposed versus unexposed cells. (D) Representative FRET images across unstressed, arsenite, AZC, DZA, HS, and NaCl conditions, each $\pm$ CHX or 1,6-HD. (E) FRET quantification across all conditions. Two-tailed t-test *P*-values of replicate means indicated.

Applied across the perturbation panel, FRET signal increased above the unstressed baseline under every stress and paralleled what we observed using the condensation index (Fig. 4,

D and E). Non-photoconverted ($-UV$) controls acquired in parallel showed low signal across conditions (Fig. S4). Thus, by an independent method, we find that Sis1 increases its self-proximity in response to these stimuli. Consistent with the condensation index, the FRET signal was differentially sensitive to CHX and 1,6-HD, indicating that the biophysical character of Sis1-marked condensates and their dependence on ongoing translation varies with the stress.

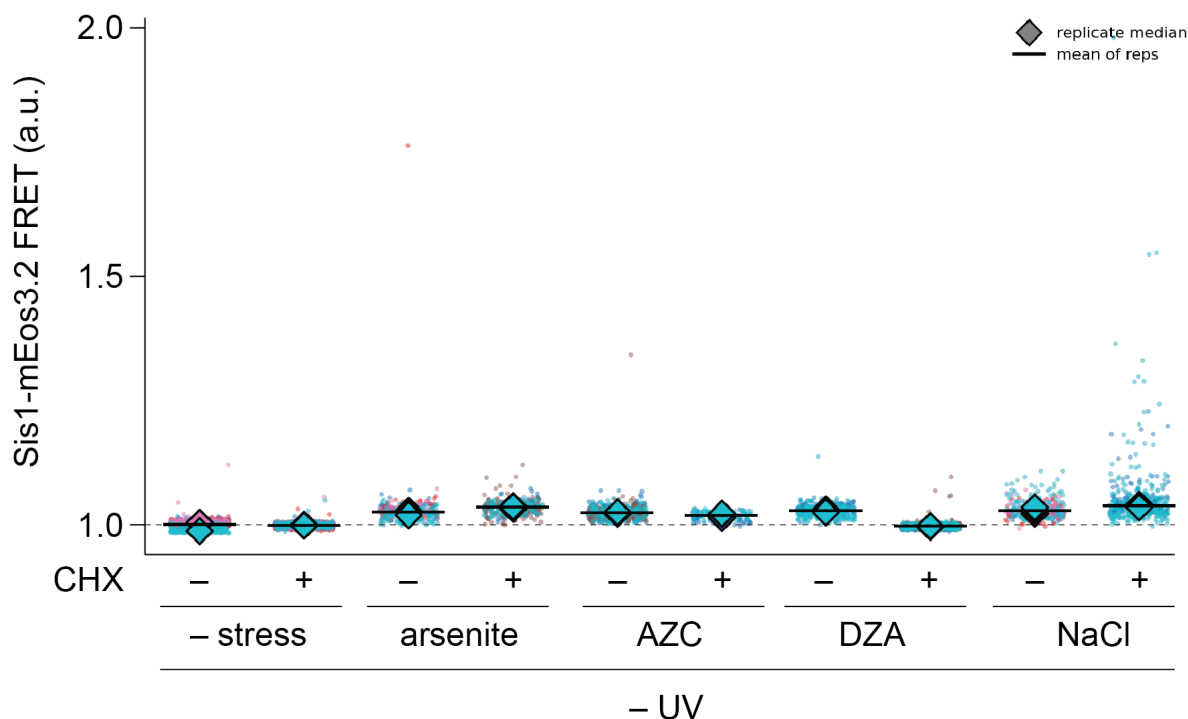


Figure 3.7: **Non-photoconverted ($-UV$) controls for the Sis1-mEos3.2 FRET measurements.**

FRET signal in Sis1-mEos3.2 cells that were *not* UV-irradiated ($-UV$), acquired in parallel with the $+UV$ data in Fig. 4 across the stress panel: unstressed ($-$ stress), arsenite, AZC, DZA, and NaCl, each without and with 50 $\mu\text{g}/\text{ml}$ cycloheximide (CHX). Without the 405 nm photoconversion pulse no acceptor pool is generated, so these samples report only background bleed-through and thus set the floor of the assay. FRET remains flat near this baseline in every condition, confirming that the stress-dependent signal in Fig. 4E reflects photoconversion-dependent Sis1 self-association. Superplot: small points, individual cells; diamonds, per-replicate medians; black bars, mean of replicate medians.

3.4.5 *Nuclear Sis1 buffers HSR output under sub-saturating stress*

The disconnect between Sis1 condensation and transcription prompted us to test the function of Sis1 directly. We used the anchor away (AA) system [Haruki et al., 2008] to conditionally deplete nuclear Sis1 as we have previously [Feder et al., 2021, Ali et al., 2023]. In the rapamycin-resistant Sis1-AA strain, rapamycin recruits Sis1-FRB to FKBP12-tagged Rpl13A on cytosolic ribosomes, rapidly clearing Sis1 from the nucleus (Fig. 5 A). Rapamycin treatment reduced 3D colocalization of Sis1 with both Hsf1 and Hsp70 (Fig. 5, B), confirming depletion. However, depleting nuclear Sis1 did not abolish downstream condensation. Hsf1 and Hsp70 still condensed, and in several conditions condensed more (Fig. 5, D and E). Instead, nuclear Sis1 depletion amplified HSR gene expression under basal conditions, arsenite, AZC, DZA, and NaCl (Fig. 5, F and G). Heat shock was the sole exception: anchoring away Sis1 did not further activate the HSR and modestly reduced it (Fig. 5 G). Nuclear Sis1 therefore acts as a repressor of Hsf1 [Feder et al., 2021] whose influence is inversely proportional to stress severity; it is regulatory in the unstressed and sub-saturating regimes, and dispensable once heat shock drives the response to saturation.

(Figure 3.8 continued)

$\pm$ rapamycin. Small points, individual cells; diamonds, replicate means. P -values from Wilcoxon rank-sum tests are indicated. **(F)** Principal component analysis of RNA-seq from Sis1-AA cells across stresses $\pm$ rapamycin. Symbols, condition means; error bars, SEM. **(G)** HSR target-gene expression (cpm) in Sis1-AA cells $\pm$ rapamycin under each condition. Two-tailed t-test P -values of replicate means indicated.

3.4.6 *Condensation of the transcriptional machinery gates HSR output*

To quantitatively assess the relationship between condensation of the signaling pathway components and HSR activation across conditions, we plotted the condensation index of each protein against average HSR target gene induction across the panel of stresses (Fig. 6, A-F). Consistent with the decoupling of Sis1 and HSR activation across stressors, Sis1 condensation was weakly anti-correlated with induction, and Hsp70 condensation was statistically uncorrelated. By contrast, condensation of the transcriptional machinery tracked HSR output: Hsf1 condensation correlated with HSR induction, as did Mediator and Pol II. Arraying the readouts condition-by-condition makes the pattern explicit that Sis1 and Hsp70 condensation are elevated across stresses irrespective of output, whereas HSR gene activation occurs only when condensation propagates through to Pol II (Fig. 6 G).

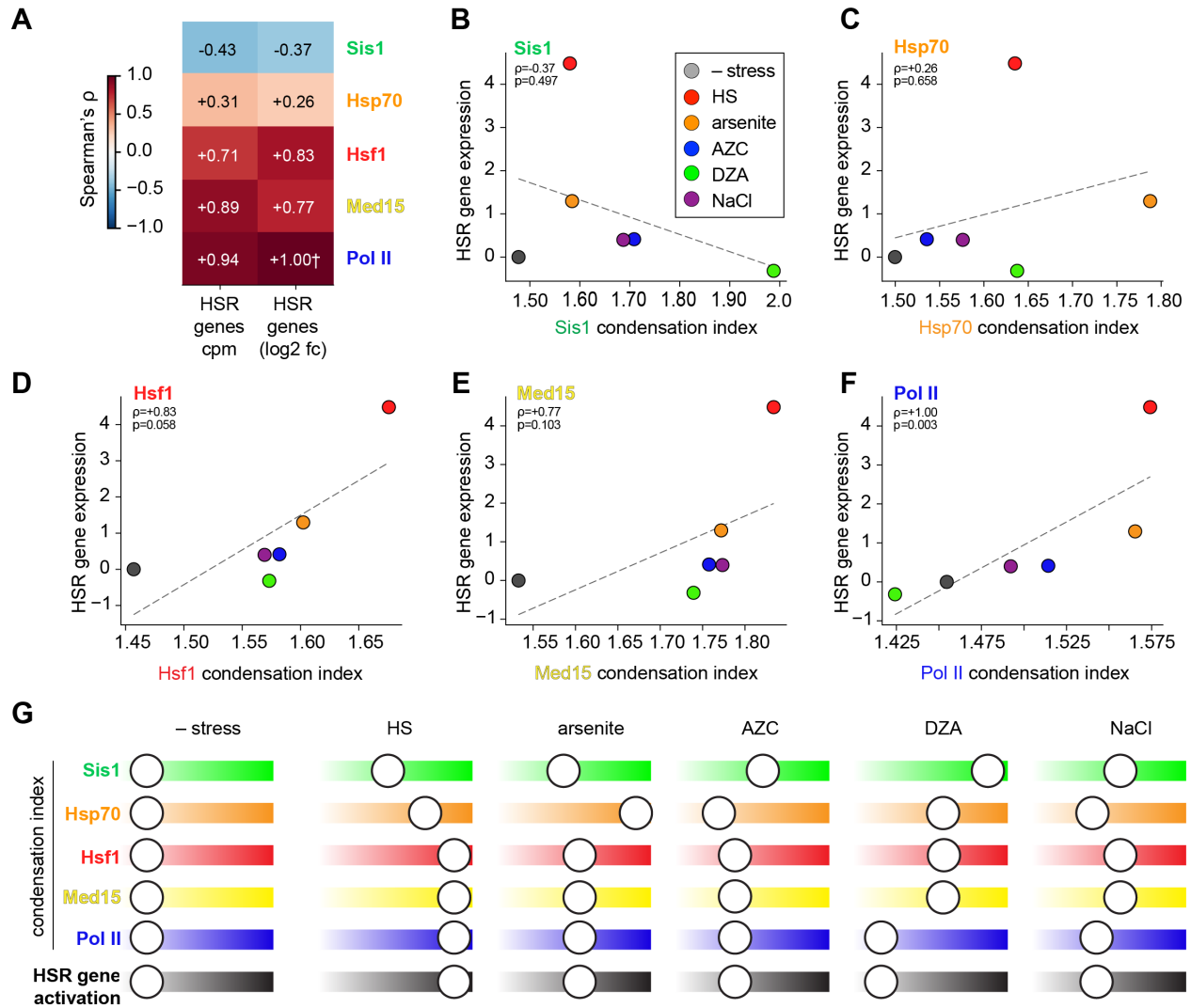


Figure 3.9: Chaperone condensation is uncorrelated with HSR activation.

(A) Heatmap of Spearman's ρ between each protein's condensation index and HSR target-gene expression, computed against both cpm and log₂ fold change, across stress conditions. (B–F) Condensation index versus HSR log₂ fold change for Sis1, Hsp70, Hsf1, Med15, and Pol II. Each point is one condition, colored by stress; dashed line, linear fit; Spearman's ρ and P inset. †, perfect rank concordance. (G) Summary slider schematic. For each protein row, the marker position along the gradient bar denotes the mean condensation index, from low (left) to high (right); the bottom row shows HSR gene activation on the same scale.

3.5 Discussion

Despite its name, the heat shock response has been associated with numerous stresses, suggesting it is pleiotropic [Ankar and Sistonen, 2011, Gomez-Pastor et al., 2018, Pincus, 2020]. However, here we find that, although the upstream chaperone machinery responds to every stress we tested, high amplitude HSR transcription is specific to heat shock. Heat shock drives a cascade of biomolecular condensates: oRPs $\rightarrow$ Sis1 $\rightarrow$ Hsp70 $\rightarrow$ Hsf1 $\rightarrow$ Mediator $\rightarrow$ Pol II. By contrast, chemically distinct stresses trigger Sis1 condensation without efficiently propagating to the transcriptional machinery. This mirrors work in which Hsf1 condensates form before and separably from transcription [Rubio et al., 2024, Mohajan et al., 2025] and can be genetically uncoupled from gene activation [Chowdhary et al., 2025].

We propose that chaperone condensation buffers the HSR. Nuclear Hsp70, with Sis1, represses Hsf1 [Garde et al., 2023, 2024], and condensation of Sis1 onto stress-specific substrates can either deplete or buffer this repressive capacity, apparently depending on the location, duration, and severity of the stress. In this way, what matters is which chaperone pool a stress engages, not only how much each chaperone condenses: a stress that deploys Sis1 onto substrate sites without depleting the nuclear repressive pool would be effectively absorbed by the buffer.

Biochemical and genetic evidence is consistent with this repressive buffer architecture. Hsp70 represses Hsf1 through conserved, direct bipartite contacts [Krakowiak et al., 2018, Peffer et al., 2019], and a genome-wide screen identified Sis1 as the strongest basal repressor of the HSR, acting with Hsp70 to gate Hsf1 [Alford et al., 2021]. Different stresses also reduce the cellular chaperone capacity to different extents [Alford and Brandman, 2018]. These reporter-based assays, however, register more chemical stresses as robust activators than the RNA-seq in this study. Indeed, we reproduced this discrepancy: an Hsf1-driven fluorescent reporter is strongly induced by AZC where the endogenous genes are only modestly induced (Fig. S5) [Zheng et al., 2018]. A long-lived reporter likely integrates Hsf1 activity that never

reaches, or rapidly subsides from, high amplitude endogenous transcription; earlier RNA-seq timepoints might thus have captured stronger activation. Nevertheless, at the timepoints we chose, chaperone condensation and HSR activation are uncoupled.

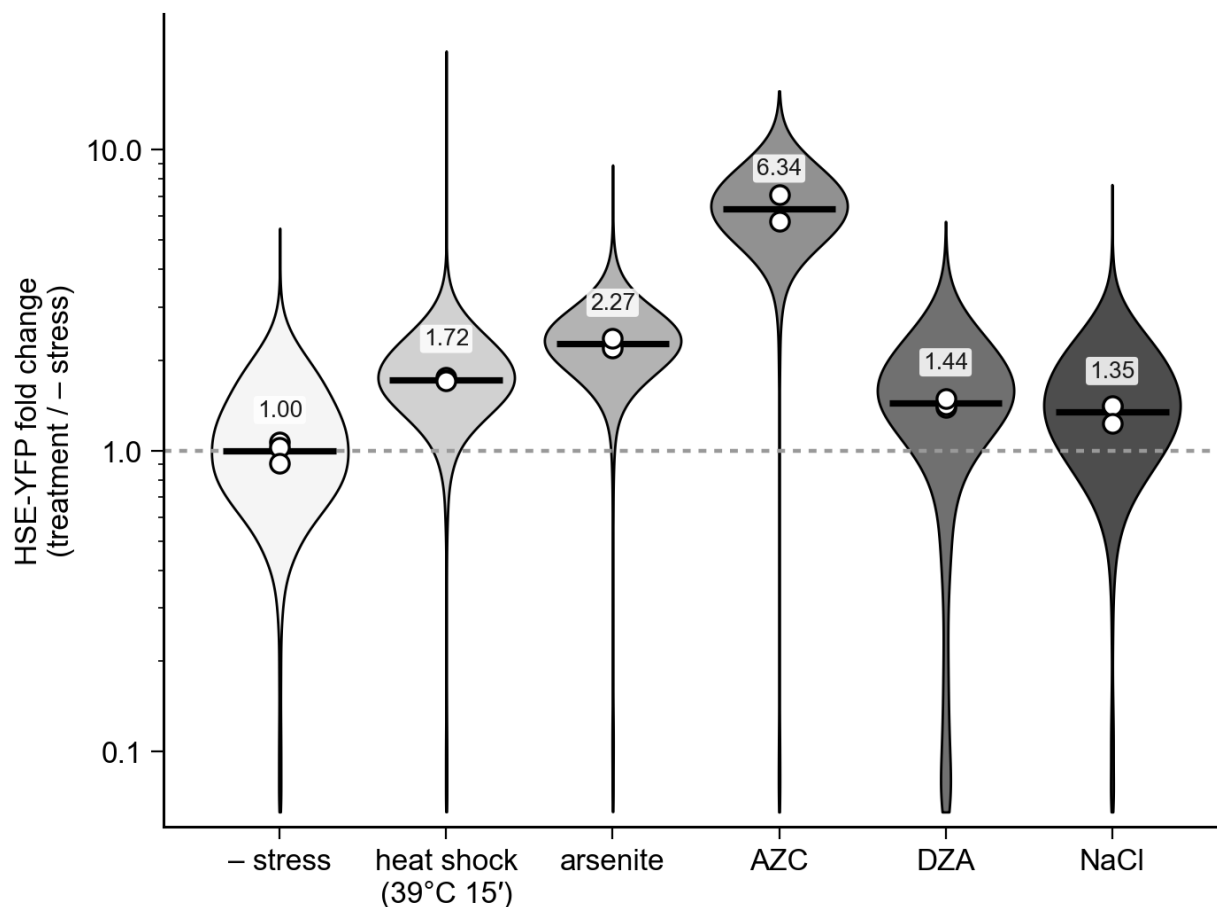


Figure 3.10: **HSE-YFP** reporter registers **AZC** as a strong **HSR** inducer.

Flow cytometry of cells carrying an HSE-YFP transcriptional reporter (10,000 cells per condition). Per-cell reporter fluorescence (FITC), normalized to side scatter, is expressed as fold change relative to the median of unstressed (– stress) cells and plotted on a log scale. Violins, single-cell distributions pooled from three biological replicates; black bars, population medians (values above each violin); open circles, per-replicate medians; dashed line, no change. Stress conditions: heat shock, 39 °C, 15 min; arsenite, 1 mM, 1 h; AZC, 5 mM, 1 h; DZA, 25 μ g/mL, 1 h; NaCl, 1 M, 30 min.

What substrates recruit Sis1 under each stress? Under heat shock, oRPs are a primary client [Ali et al., 2023], but substrates in other conditions are uncharacterized. DZA inhibits Drg1, blocking release of late 60S biogenesis factors and stalling assembly intermediates at

the nuclear pore [Loibl et al., 2014, Pertschy et al., 2007]; these intermediates, rather than free oRPs, are the likely recruitment signal. AZC, a proline analog incorporated at proline codons, produces misfolded proteins because proline uniquely constrains backbone dihedral angles and is enriched in disordered regions, so its substitution may promote aberrant helix formation. Arsenite oxidizes protein cysteines, forming disulfide-linked aggregates and inactivating metal-coordinating proteins, and impairs mitochondrial import [Richter et al., 2010], potentially orphaning mitochondrial proteins that recruit Sis1 as oRPs do [Ali et al., 2024]. Ssa1 (Hsp70) is itself a thiol sensor whose cysteine modification releases Hsf1 through a route genetically separable from heat [Wang et al., 2012, Santiago and Morano, 2022, Morano et al., 2012]; arsenite may thus activate the HSR partly by chemically disabling the chaperone buffer—a plausible reason it is the one chemical stress in the panel to drive substantial transcription. NaCl drives water efflux and crowding that push proteins past their solubility limits [Alberti and Hyman, 2021] and reversibly constrict nuclear pore complexes [Zimmerli et al., 2021], which could expose FG-nucleoporin substrates for Sis1, paralleling DNAJB6 (a human Sis1 homolog) surveilling them at the NPC [Kuiper et al., 2022, Prophet and Schlieker, 2022].

More broadly, the proteostasis network spatially sorts client proteins into distinct condensates—e.g., JUNQ/INQ, the insoluble protein deposit (IPOD), and cytosolic Q-bodies (CytoQ)—via factors such as Hsp42 and Btn2 along with the Hsp70 system [Kaganovich et al., 2008, Escusa-Toret et al., 2013, Miller et al., 2015, Sontag et al., 2017]. Because such sequestration also shields Hsp70 from client overload [Specht et al., 2011, Ho et al., 2019], the Sis1 condensates we describe may intersect with these spatial proteostasis hubs.

Chaperone substrates may not fully explain why heat shock activates the HSR so strongly, because heat can also be read out directly by both Hsf1 and the genome. Purified human HSF1 undergoes a cooperative transition to its DNA-binding trimer near heat shock temperatures [Hentze et al., 2016, Kmiecik and Mayer, 2022], and HSF1 homologs undergo

temperature-triggered phase separation with a set point tuned to each species' physiological temperature [Ren et al., 2025], raising the possibility that yeast Hsf1 also directly condenses in response to temperature. The chromatin template may be heat-sensitive as well. Heat shock drives rapid, transcription-independent nucleosome loss across HSR loci [Petesch and Lis, 2008], and the strongest heat-induced accessibility gains genome-wide fall at stress transcription factor binding sites, Hsf1 among them [Shivaswamy et al., 2008]; strong acidic activation domains like those on Hsf1 can decondense chromatin even when transcription is blocked [Tumbar et al., 1999], and in plants heat-induced nucleosome depletion is maintained as chromatin memory [Brzezinka et al., 2016]. The nucleolus also sequesters a megabase of DNA [Németh et al., 2010, van Koningsbruggen et al., 2010] and is a dominant architectural hub of the yeast genome [Duan et al., 2010], yet remodels under heat shock, relocating chromatin and epigenetic regulators [Azkanaz et al., 2019, Frottin et al., 2019] in ways that could shift HSR gene accessibility without input from Hsf1. Over a billion years of recurrent thermal stress, the genome fold may itself have retained a "soft mode" that preferentially exposes HSR genes as temperature rises [Russo et al., 2026]—a structural memory of heat that, alongside a thermosensory Hsf1 and the chaperone buffer, could render the response specifically sensitive to heat.

In summary, the heat shock response is not as pleiotropic as its inducer list implies. Chemically diverse stresses converge on condensation of Sis1 and Hsp70, but this condensation is decoupled from transcription and buffers rather than triggers the HSR. We propose that high amplitude activation is reserved for stresses that surpass the buffer and propagate through Hsf1 to the transcriptional machinery, with chaperone condensation enforcing a spatial threshold that imparts specificity to a potentially promiscuous program.

3.6 Materials and Methods

3.6.1 *Yeast strains and plasmids*

All experiments were performed in *Saccharomyces cerevisiae* strain W303 (*MATa*, *ade2-1*, *ura3-1*, *his3-11,15*, *trp1-1*, *leu2-3,112*, *can1-100*) or derivatives thereof. Fluorescent protein fusions were constructed by integration at endogenous loci using standard homologous recombination [Meurer et al., 2018]. The Sis1-mEos3.2 reporter strain was generated by replacing the *SIS1* stop codon with mEos3.2 [Zhang et al., 2012]. The Sis1 anchor-away (Sis1-AA) strain was constructed in the RPL13A-FKBP12 background by fusion of the FRB domain to the Sis1 C-terminus [Haruki et al., 2008]. A complete list of strains is provided in Table S1.

Table 3.1: Yeast strains and plasmids used in this study.

Strain	Description	Relevant genotype (modifications to W303 MATa)	Figure(s)
DPY2167	Orphan-RP / nucleolar imaging / Sis1	<i>SIS1-mVenus::HIS</i> , <i>Rpl26a-Halo</i> (<i>CRISPR</i>), <i>mScarlet-Nsr1</i> (<i>CRISPR</i>)	Fig. 1, 2
DPY1846	Halo-Ssa1 (Hsp70)	<i>SIS1-mVenus::HIS3</i> , <i>NSR1-mScarI::kanMX</i> , <i>HaloTag-SSA1::LEU</i>	Fig. 1, 2; S2
DPY2266	Hsf1 / Med15 / Rpb3 imaging	<i>HSF1-mVenus::HIS3</i> , <i>MED15-mCherry::hphMX</i> , <i>RPB3-mTFP1::kanMX</i>	Fig. 1, 2; S2
DPY2122	Sis1-mEos3.2 FRET biosensor	<i>SIS1-mEos3.2::kanMX</i>	Fig. 4; S4
DPY2162	Sis1 anchor-away + Hsf1	<i>tor1-1</i> , <i>fpr1Δ::NAT</i> , <i>RPL13A-2×FKBP12::TRP1</i> , <i>SIS1-FRB-GFP::HIS3</i> , <i>HSF1-mKate::URA3</i> , <i>HaloTag-SSA1::LEU2</i>	Fig. 5
DPY1298	Sis1 anchor-away (RNA-seq)	<i>tor1-1</i> , <i>fpr1Δ::NAT</i> , <i>RPL13A-2×FKBP12::TRP1</i> , <i>SIS1-FRB-GFP::HIS3</i>	Fig. 3, 5
DPY144	HSE-YFP reporter	<i>HSE-mVenus::LEU2</i>	Fig. S5

3.6.2 Growth conditions and stress treatments

Cells were grown in synthetic complete (SC) medium or YPD at 25°C to mid-log phase ($\text{OD}_{600} \approx 0.4\text{--}0.8$). Stress conditions: heat shock (HS), 39°C for 15 min; arsenite, 1 mM NaAsO_2 for 1 hr; AZC, 5 mM for 1 hr; DZA, 25 $\mu\text{g}/\text{ml}$ for 1 hr; NaCl, 1 M for 30 min. Where indicated, CHX was added at 50 $\mu\text{g}/\text{ml}$ 15 min prior to stress; 1,6-HD at 5% (w/v) for 10 min before fixation or live imaging [Kroschwald et al., 2017]. Rapamycin was used at 1 $\mu\text{g}/\text{ml}$ for anchor-away experiments [Haruki et al., 2008].

3.6.3 Fluorescence microscopy and CellQuant analysis

Cells were imaged live or after mild fixation with 1% paraformaldehyde for 5 min on a Marianas SoRa spinning disc confocal with a 100 $\times$ high-NA oil immersion objective. Condensation index was quantified using our CellQuant image analysis pipeline (Fig. S1; [Stringer et al., 2021, Neferkara et al., 2026]). Condensation index is computed as the ratio of fluorescence intensity in the brightest 5% of voxels to the mean cellular fluorescence for each 3D-segmented cell, providing a cell-autonomous, threshold-free measure of protein redistribution. Colocalization (Pearson’s r) was calculated within nuclear or whole-cell masks as appropriate. Images were analyzed using Fiji [Schindelin et al., 2012]. Raw images and processed datasets are deposited at Zenodo (DOI: 10.5281/zenodo.21417933).

3.6.4 *Sis1-mEos3.2* FRET measurements

The *Sis1-mEos3.2* FRET sensor was designed based on the DAmFRET approach [Khan et al., 2018, Posey et al., 2021, Miller et al., 2023]. Cells expressing *Sis1-mEos3.2* were UV-irradiated on the microscope with a 1 s pulse of a 405 nm laser at 30% power to photoconvert a subset of molecules from green (donor) to red (acceptor). FRET was measured by acceptor-sensitized emission upon donor excitation, normalized to total acceptor fluorescence to correct for variable conversion efficiency [Lakowicz, 2006]. FRET values were normalized

to the mean value of unstressed cells on each imaging day; the AZC condition was acquired as a separate imaging batch and normalized per-day accordingly.

3.6.5 *RNA-seq and analysis*

Total RNA was extracted by heating with 1% SDS in Trizol, followed by phase separation by centrifugation and column purification. Three-prime end mRNA sequencing was performed by Plasmidsaurus. Reads were aligned to *sacCer3* using STAR and quantified with featureCounts. Differential expression analysis used DESeq2 [Love et al., 2014]. HSR genes were defined as those induced ≥ 2 -fold ($p_{\text{adj}} < 0.05$) by heat shock relative to unstressed cells. PCA used variance-stabilizing-transformed counts. RNA-seq data are deposited at GEO (GSE339192).

3.6.6 *Statistical analysis*

Two statistical tests were used depending on the unit of comparison. For single-cell measurements (condensation index, FRET, colocalization), where each condition comprises hundreds of cells across multiple biological replicates, comparisons used the two-sided Wilcoxon rank-sum test on individual-cell values, with replicate means shown as large symbols for reference. For RNA-seq target-gene expression and FRET summary comparisons, where each condition is represented by only the biological replicate means themselves ($n = 3$ per condition), comparisons used the two-tailed t-test on replicate means, since sample sizes are too small for a nonparametric rank test to be informative. $P \leq 0.05$ was considered significant in both cases; exact P-values are shown on the relevant plots.

3.7 Acknowledgments

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The authors declare no competing interests.

CHAPTER 4

A HIERARCHICAL ARCHITECTURE ORGANIZES CELLULAR ADAPTATION TO COMPLEX ENVIRONMENTS

Author Contributions to This Chapter

The work in this chapter is from a manuscript in preparation [Dea et al., 2026]. Annisa Dea performed the bulk of experimentation and analysis described in the paper. Valeryia Aksianiuk assisted in the establishment of early experimental conditions and scRNA-seq analysis. The information-theoretic framework used in this paper was first used in the Raman lab and adapted for use here with the help of Ben Doran. I assisted in imaging for various conditions analyzed in this paper as well as editing and final review.

4.1 Introduction

Living systems persist in environments that can change unpredictably. Thus, survival depends on the capacity to adapt (e.g. ‘adaptability’)—a systems-level property emerging from interacting molecular components and regulatory layers expressed as a coherent, organized phenotypic logic [Anderson, 1972, Jacob and Monod, 1961, Balch et al., 2008, Morimoto, 2011, Lindquist, 1986]. Previous work on cellular adaptation has mostly examined the effects of single environmental perturbations. From this approach a useful organizing picture has emerged in which cells harbor a shared, global environmental stress response layered with condition-specific transcriptional programs [Gasch et al., 2000, Gasch and Werner-Washburne, 2002, Berry and Gasch, 2008, Brauer et al., 2008, Capaldi et al., 2008]. However, cells rarely experience stresses in isolation. Natural environments vary along multiple axes, and combined perturbations can interact, imposing competing or synergistic physiological demands. These observations raise a key question: in complex environments, is adaptability

governed by a stereotyped organizing structure, or does it fragment into context-specific programs that must be re-interpreted for each distinct environment?

Addressing this question has been challenging as the space of possible environments is combinatorially expansive, and responses to combined perturbations can be non-additive reflecting nonlinear interactions across regulatory layers [McClean et al., 2007, Hao and O’shea, 2012, Vidal et al., 2013]. Moreover, similar physiological challenges can be resolved through multiple molecular routes, while distinct stresses can converge on shared bottlenecks—obscuring common structure when analyses focus on individual genes or individual conditions. Thus, it is difficult to determine whether responses across complex environments respect an underlying molecular logic or are fundamentally high-dimensional and idiosyncratic.

Here, we test whether responses across complex environments exhibit shared structure. We chose *S. cerevisiae* as our model system because its molecular biology is extensively mapped, it can be robustly cultured across diverse conditions, and it is supported by a mature genetic toolkit for perturbation and manipulation. We profiled yeast single-cell transcriptomes across 20 environments, varying carbon source, nutrient richness, osmolarity, temperature, and reactive oxygen species—thereby sampling combinations of well-studied individual stresses [Gasch et al., 2000, Brewster and Gustin, 2014, Anckar and Sistonen, 2011, Ma, 2013, Kourtis and Tavernarakis, 2011, de Nadal et al., 2011, Dea and Pincus, 2024]. Rather than analyzing each condition in isolation, we treated cellular transcriptomes measured across all environments as a single ensemble and interrogated whether there were statistical patterns of transcriptional variation that organize adaptive responses. Our results revealed a hierarchical structure of adaptation where variation in transcriptional responses was ordered by sensitivity to carbon source first, then nutrient richness, osmolarity, temperature, and finally reactive oxygen species. This hierarchy suggested that some environmental features limit the range of responses available to others, effectively gating how cells respond

in combined environments. We experimentally validated these predictions within individual cell populations. Further, we found that this prioritization logic manifested mechanistically within single cells, emerging from differential regulation of translation initiation and synthesis of ribosomal proteins.

4.2 Experimental Design: Sampling Multi-Dimensional Environmental Space

We grew haploid *S. cerevisiae* cells in different environments in which we varied carbon source (glucose or ethanol + glycerol), media composition (yeast extract with peptone (YP) or synthetic complete (SC)), temperature (30°C or 37°C), osmolarity (isotonic, KCl, or sorbitol), and reactive oxygen species (ROS) (none, H₂O₂, or diamide) (Fig. 4.1). We selected 20 combinations of these environmental cues that resulted in a broad range of observed growth rates (Fig. 4.2). We grew cultures to maximal logarithmic phase growth rate in each of these environments to allow cells to adapt physiologically. We then harvested cells for single-cell transcriptional profiling (scRNA-seq); in each environment, we sequenced $\geq 1,000$ cells with ≥ 500 genes expressed (Fig. 4.3) [Gasch et al., 2017, Nadal-Ribelles et al., 2019, Jariani et al., 2020].

The 20 environments were selected to sample combinations of well-characterized cellular perturbations while spanning a broad range of observed growth rates (Table 4.1). Quantitative QC metrics from Cell Ranger for all 20 ancestral-strain libraries are provided in Table 4.2.

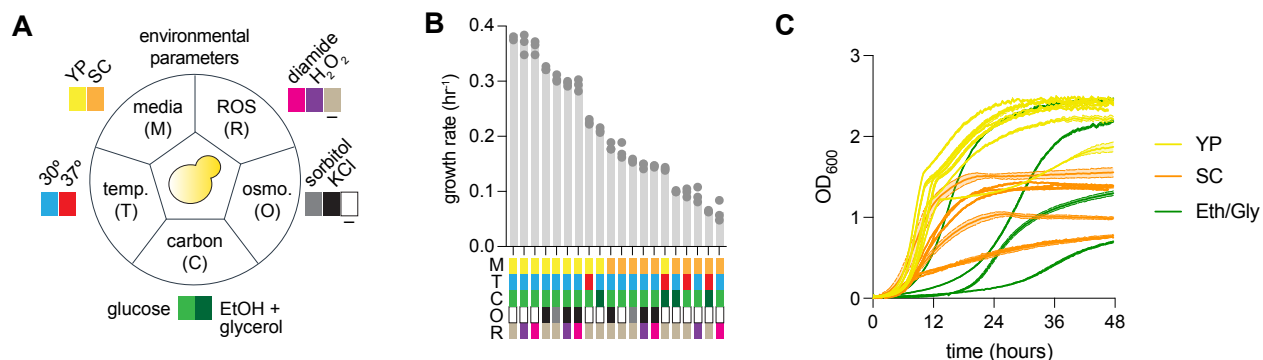


Figure 4.1: Experimental design. (A) Schematic of the perturbation space: carbon source (glucose vs. ethanol/glycerol), media richness (YP vs. SC), osmolality (isotonic, KCl, sorbitol), temperature (30°C vs. 37°C), and reactive oxygen species (none, H₂O₂, diamide). Twenty combinations were selected to span a broad range of physiological conditions. (B) OD₆₀₀ growth curves of cells grown in the 20 environmental conditions collapsed. (C) Waterfall plot of steady-state growth rates. Steady-state growth confirms that transcriptional responses reflect adapted states rather than acute stress.

Table 4.1: The 20 complex environments used for transcriptional profiling and growth assays. YP = yeast extract + peptone (rich); SC = synthetic complete (defined). All conditions at 30°C unless otherwise noted.

#	Label	Media	Carbon source	Temp.	Osmolyte	ROS
1	YPD-30	YP	2% glucose	30°C	—	—
2	YPD-37	YP	2% glucose	37°C	—	—
3	YPD-KCl	YP	2% glucose	30°C	500 mM KCl	—
4	YPD-sorbitol	YP	2% glucose	30°C	500 mM sorbitol	—
5	YPD-diamide	YP	2% glucose	30°C	—	50 μ M diamide
6	YPD-H ₂ O ₂	YP	2% glucose	30°C	—	50 μ M H ₂ O ₂
7	YPD-KCl-diamide	YP	2% glucose	30°C	500 mM KCl	50 μ M diamide
8	YPD-KCl-H ₂ O ₂	YP	2% glucose	30°C	500 mM KCl	50 μ M H ₂ O ₂
9	SDC-30	SC	2% glucose	30°C	—	—
10	SDC-37	SC	2% glucose	37°C	—	—
11	SDC-KCl*	SC	2% glucose	30°C	500 mM KCl	—
12	SDC-sorbitol	SC	2% glucose	30°C	500 mM sorbitol	—
13	SDC-diamide	SC	2% glucose	30°C	—	50 μ M diamide
14	SDC-H ₂ O ₂	SC	2% glucose	30°C	—	50 μ M H ₂ O ₂
15	SDC-KCl-diamide	SC	2% glucose	30°C	500 mM KCl	50 μ M diamide
16	SDC-KCl-H ₂ O ₂	SC	2% glucose	30°C	500 mM KCl	50 μ M H ₂ O ₂
17	YPEG-30	YP	2% EtOH/2% glycerol	30°C	—	—
18	YPEG-37	YP	2% EtOH/2% glycerol	37°C	—	—
19	SCEG-30	SC	2% EtOH/2% glycerol	30°C	—	—
20	SCEG-37 [†]	SC	2% EtOH/2% glycerol	37°C	—	—

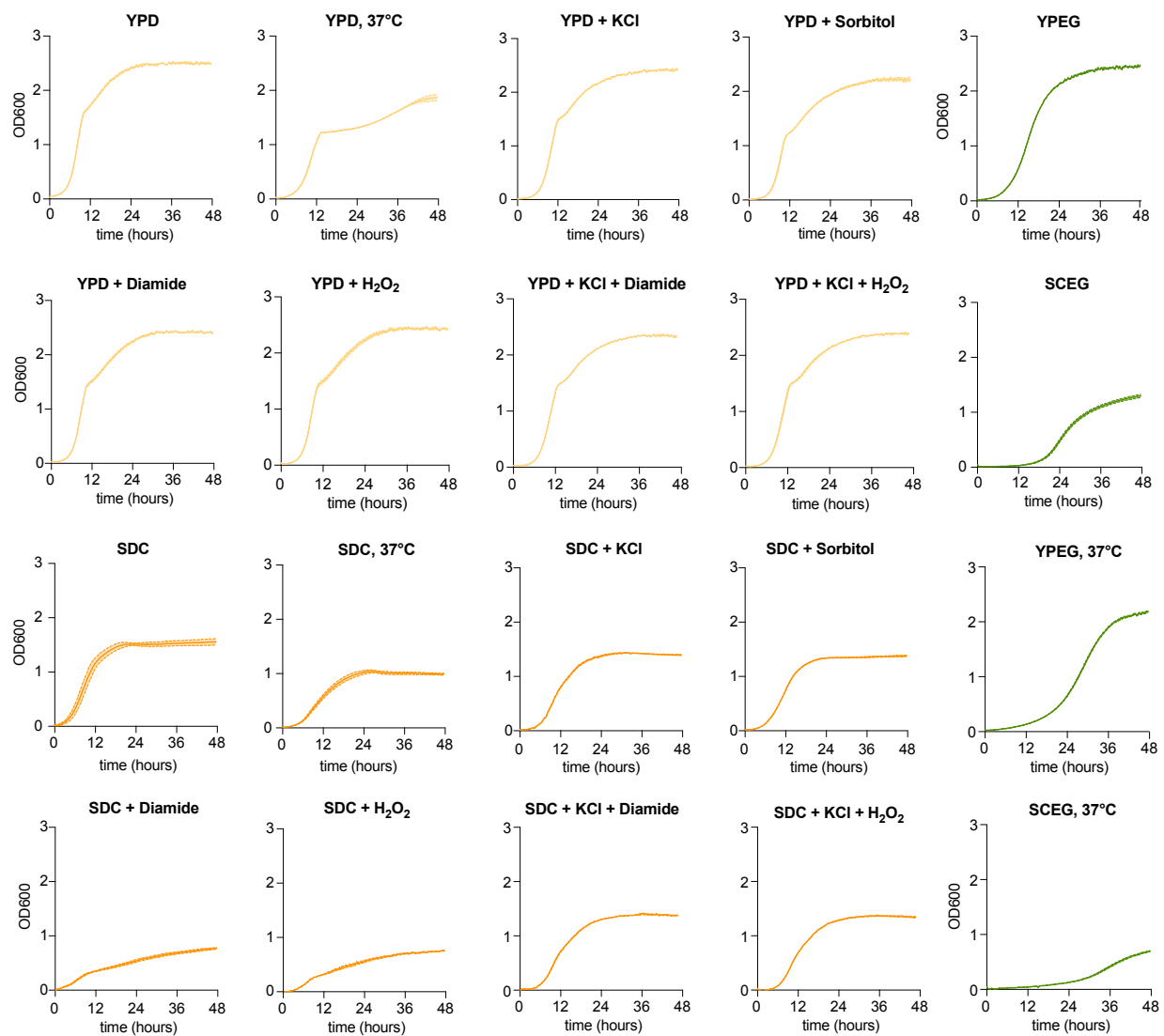


Figure 4.2: Growth characterization across 20 environments. Full growth curve panel for all 20 conditions, colored by media type.

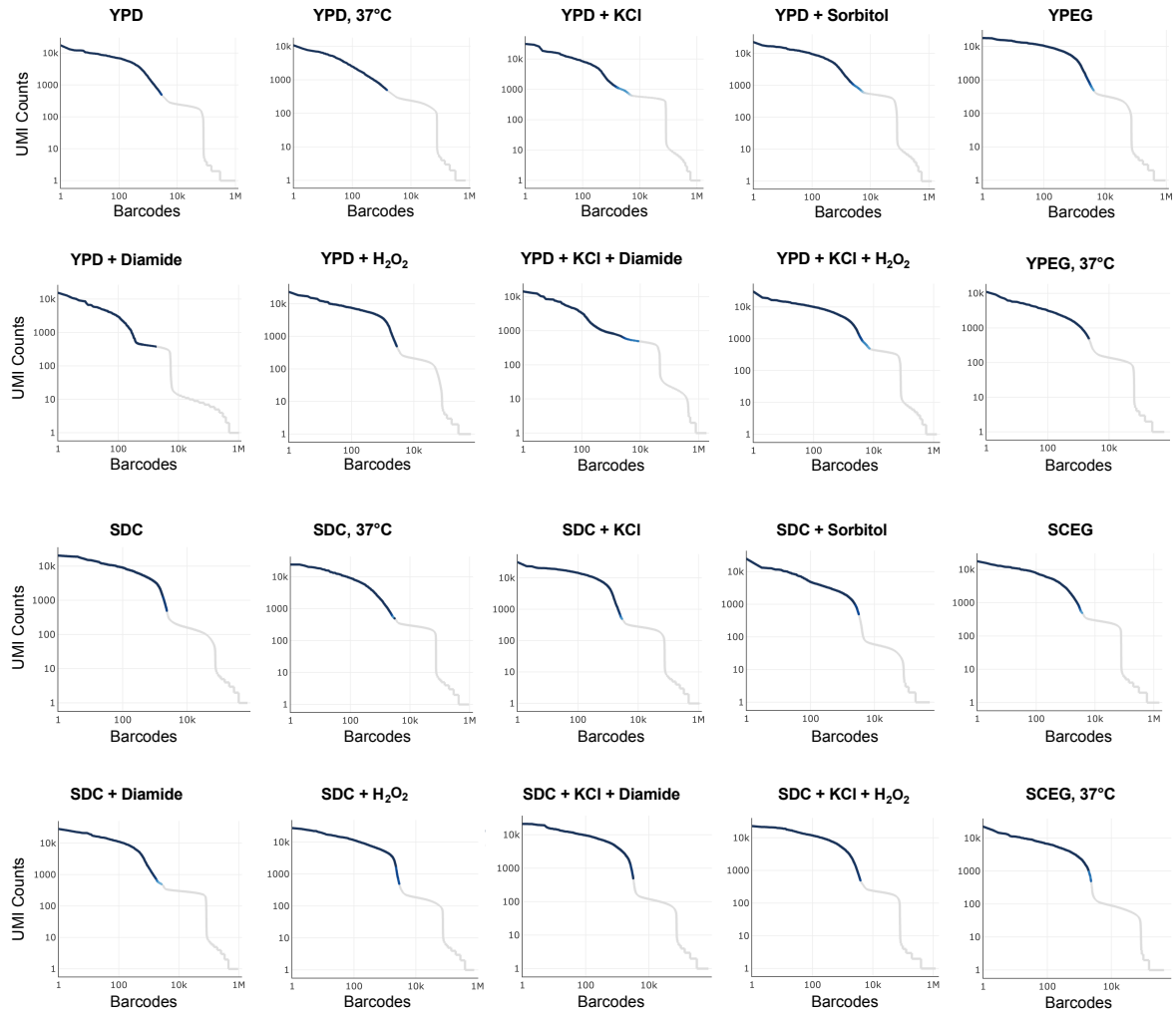


Figure 4.3: scRNA-seq quality control across 20 libraries. Barcode rank plots thresholds for all 20 conditions.

Table 4.2: Illumina and Cell Ranger QC summary for all 20 ancestral-strain scRNA-seq libraries. Sat. = library saturation; Q30 = fraction of bases with Phred score ≥ 30 ; Map Genome/Txome = fraction of reads mapped to genome/transcriptome; Med. = median per cell.

Condition	Reads	Valid BC (%)	Sat. (%)	Q30 (%)	Map Genome (%)	Map Txome (%)	Cells	Med. Genes	Med. UMI
SCEG-30	160M	97.3	66.0	95.6	75.4	53.5	3,353	902	1,688
SCEG-37	160M	96.7	80.9	96.0	64.1	39.9	2,212	1,172	2,416
SDC	145M	97.4	79.5	94.8	78.8	60.1	2,313	1,219	3,119
SDC-37	172M	97.3	70.0	95.5	75.4	52.4	2,767	902	1,429
SDC-diamide	146M	97.4	59.4	95.7	74.4	51.7	2,056	844	1,620
SDC-H ₂ O ₂	158M	97.4	69.4	95.2	76.3	51.9	2,813	1,362	4,314
SDC-KCl	169M	98.0	72.2	94.8	85.2	67.3	2,756	1,348	3,271
SDC-KCl-diamide	147M	97.7	77.5	95.4	82.9	59.4	3,191	1,303	2,958
SDC-KCl-H ₂ O ₂	175M	97.4	69.7	95.0	79.5	57.7	3,844	1,206	2,644
SDC-sorbitol	132M	97.6	85.4	95.7	78.5	54.3	3,123	939	1,701
YPD	149M	97.1	73.6	95.8	75.9	57.7	2,844	744	1,180
YPD-37	142M	96.5	74.7	93.3	68.5	46.7	1,534	522	828
YPD-diamide	140M	97.7	93.5	95.4	82.2	62.2	1,896	311	416
YPD-H ₂ O ₂	164M	97.1	79.1	95.7	76.3	57.8	2,862	1,217	2,506
YPD-KCl	159M	97.9	46.1	95.8	81.8	59.5	2,846	653	1,262
YPD-KCl-diamide	142M	97.3	61.6	94.6	79.6	56.6	6,343	358	590
YPD-KCl-H ₂ O ₂	163M	97.8	54.0	95.7	85.4	60.7	5,440	960	1,968
YPD-sorbitol	130M	97.6	41.0	95.6	81.7	60.3	4,236	680	1,286

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(Table 4.2 continued)

Condition	Reads	Valid BC (%)	Sat. (%)	Q30 (%)	Map Genome (%)	Map Txome (%)	Cells	Med. Genes	Med. UMI
YPEG-30	160M	97.7	67.4	95.1	81.0	58.3	3,659	1,078	2,178
YPEG-37	115M	96.5	75.6	95.3	65.7	40.9	2,260	675	1,113

Uniform manifold approximation and projection (UMAP) revealed separation of cells into clusters based on the environment in which they were grown (Fig. 4.4). Cells grown in ethanol + glycerol were fully separated from cells grown in glucose, and cells grown in hyperosmotic environments largely separated from isotonic cells, but variation in other environmental categories was less apparent in the UMAP visualization.

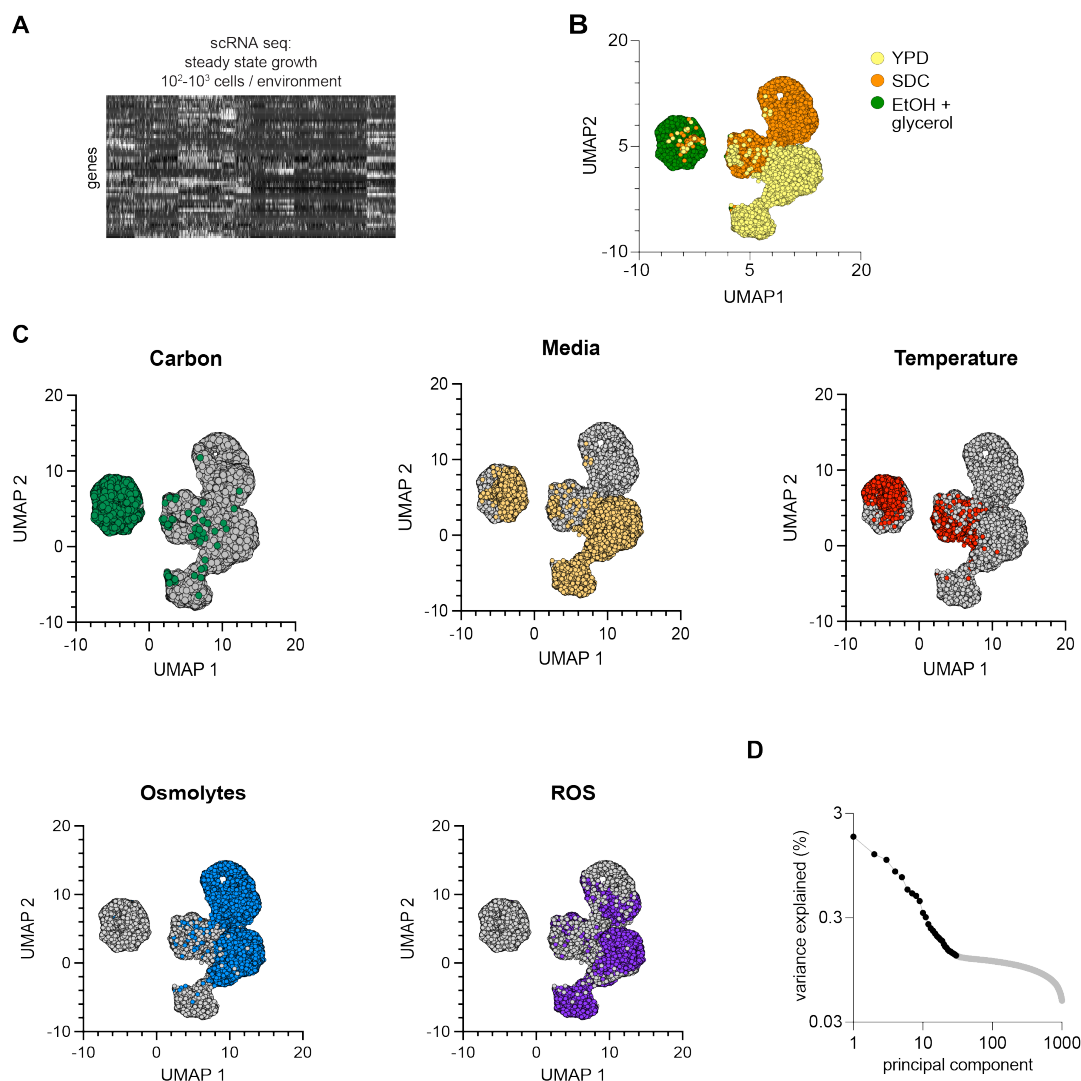


Figure 4.4: UMAP visualization of single-cell transcriptomes across 20 environments. (A) Full UMAP colored by media type. (B–F) Side-by-side panels colored by each individual environmental variable. Carbon source drives the dominant separation; osmolarity, media, temperature, and ROS create sub-structure within carbon clusters but are partially obscured in the 2D embedding.

We next interrogated the statistical structure of transcriptional variation across all cells and environments using singular value decomposition (SVD) — a method that decomposes high-dimensional datasets into orthogonal modes of variation ordered by statistical scale. Formally, SVD factors the expression matrix as $\mathbf{X} = \mathbf{U}\mathbf{\Sigma}\mathbf{V}^\top$, where $\mathbf{U}$ contains the left singular vectors (LSVs, equivalent to principal components), $\mathbf{\Sigma}$ is a diagonal matrix of singular values ordered by variance captured, and $\mathbf{V}^\top$ encodes gene loadings. In this representation, the first principal component (PC1) captures the largest scale of coordinated variation in the data, PC2 the second largest, and so on, producing a spectrum of PCs (‘eigenspectrum’) that describes how transcriptional variability is distributed across scales. We computed the eigenspectrum of the full expression matrix (cells $\times$ genes) and found that the top 30 PCs together captured 11.59% of the total transcriptional variance. Importantly, the variance explained by these PCs decreased approximately linearly with PC rank (Fig. 4.4). Recent work has suggested that such scaling behavior indicates the presence of structured biological signals across multiple statistical scales rather than a small number of dominant programs [Zaydman et al., 2022, Doran et al., 2025]. This observation suggested that meaningful transcriptional variation might extend well beyond the shallowest PCs.

To determine what biological information was encoded across these scales, we asked how environmental cues mapped onto different regions of the eigenspectrum. Each cell contributes to each PC to a certain degree, and each cell was part of a population exposed to a set of environmental stresses defining its complex environment (carbon source, osmolarity, temperature, media composition, and ROS stress). Standard dimensionality-reduction visualizations such as UMAP compress information across PCs and therefore obscure how information is distributed across statistical scales. To instead directly interrogate the eigenspectrum, we applied a technique called SCALES (Spectral Correlation Analysis of Layered Environmental Signals) [Zaydman et al., 2022, Doran et al., 2025]. SCALES quantifies how biological meta-data maps onto different spectral layers of high-dimensional datasets in a purely data-driven

manner, using mutual information (MI) as the measure of dependence between transcriptional structure and environmental identity. Intuitively, MI captures whether knowing a cell’s environmental context reduces uncertainty about the cell’s transcriptional state in a particular spectral window: high MI means environment strongly predicts transcription in that window; zero MI means the two are independent.

Briefly, SCALES partitions the LSVs into overlapping windows of three consecutive components and, for each window w , computes pairwise cell–cell correlations in spectral space (Fig. 4.5). It then calculates the mutual information between the distribution of these correlations $\mathbf{C}_w$ and a binary label E_{env} indicating whether each cell pair shares the same environmental attribute:

$$I(\mathbf{C}_w; E_{\text{env}}) = \sum_c \sum_e p(c, e) \log_2 \frac{p(c, e)}{p(c)p(e)}, \quad (4.1)$$

where $p(c, e)$ is the joint probability and $p(c)$, $p(e)$ are marginal probabilities. To make MI values directly comparable across environmental variables, we normalized them to cumulative distribution functions:

$$\text{MI}_{\text{CDF}}(w, \text{env}) = \frac{\sum_{w'=0}^w I(\mathbf{C}_{w'}; E_{\text{env}})}{\sum_{w'=0}^W I(\mathbf{C}_{w'}; E_{\text{env}})} \in [0, 1], \quad (4.2)$$

with $W = 40$ the final spectral window. The output of SCALES is therefore an MI_{CDF} profile across PC windows for each environmental variable: a steep rise at shallow windows indicates that information is concentrated in the dominant modes of transcriptional variation, while a gradual rise indicates information distributed across deeper spectral scales.

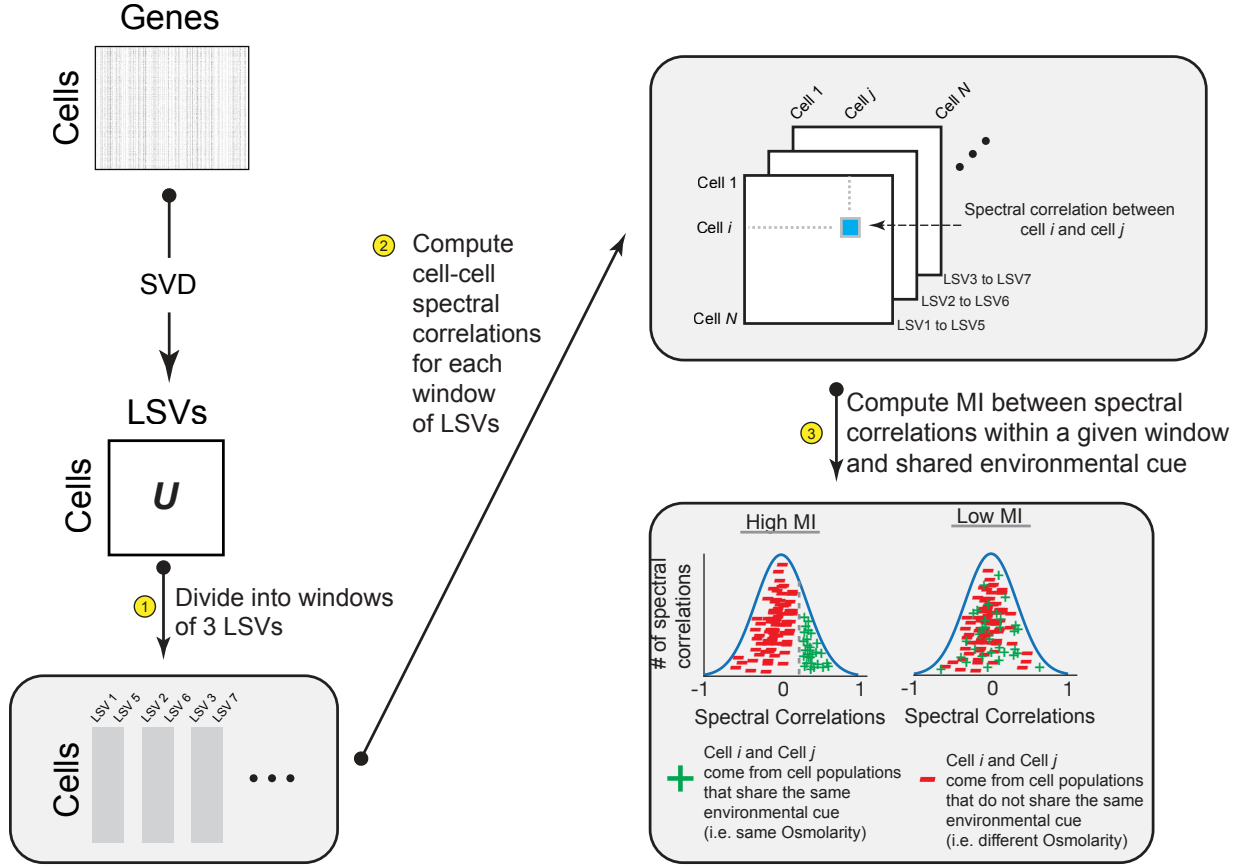


Figure 4.5: The SCALES analytical pipeline. Singular value decomposition of the log-normalized expression matrix yields left singular vectors (LSVs). LSVs are partitioned into sliding windows of three consecutive components. Within each window, pairwise cell–cell spectral correlations are computed. Mutual information is calculated between the distribution of spectral correlations and shared environmental labels of cell pairs. The resulting MI(CDF) profile across PC windows reveals where environmental information is encoded across statistical scales [Zaydman et al., 2022, Doran et al., 2025].

Applying SCALES to sequential windows of PCs revealed two key features of transcriptional organization across the environments we studied. First, the shallowest PCs contained partial information about multiple environmental variables. Inspection of the genes with the highest loadings on these components showed that PC1 was enriched for glycolytic and gluconeogenic enzymes reflecting carbon source identity (e.g., *ENO2*, *PDC1*, *ADH1*), and PC2 for canonical stress-response genes involved in metabolism (e.g., *HSP12*, *HSP26*, *SSA1*). Thus, the leading modes collectively captured a broad, condition-agnostic transcriptional shift re-

sembling the canonical environmental stress response (ESR) [Gasch et al., 2000, Gasch and Werner-Washburne, 2002, Brauer et al., 2008]. Second, each environmental cue was resolved within distinct regions of the eigenspectrum spanning shallow to deeper statistical scales. Carbon source information appeared within the shallowest PCs, followed sequentially by media composition, osmolarity, temperature, and ROS stress (Fig. 4.6). Importantly, shuffled controls in which environmental labels were permuted across cells produced mutual information values well below those of the true data across all PC windows, confirming that the observed hierarchy reflects genuine environmental signal rather than statistical noise. In other words, when viewed through the decoder of SVD, the ensemble of transcriptomes resolved into a spectrally ordered representation of environmental variables, with different cues occupying distinct statistical scales.

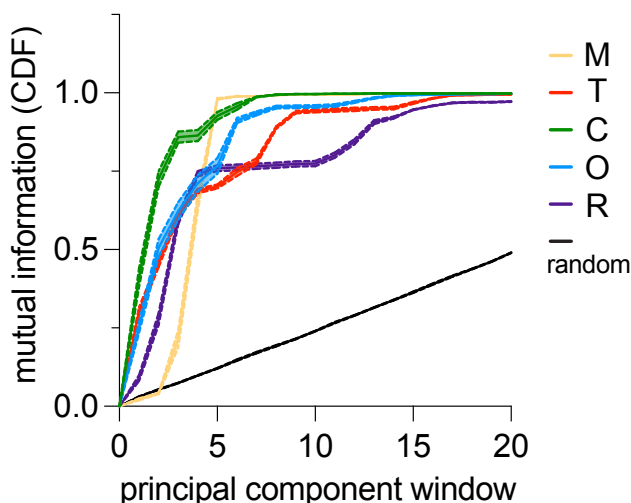


Figure 4.6: Hierarchical encoding of environmental information across the eigenspectrum. Mutual information cumulative distribution functions (MICDFs) for the five environmental variables. Carbon source information saturates in the shallowest PCs, followed sequentially by media composition, osmolarity, temperature, and reactive oxygen species. Shuffled controls (gray) confirm that the observed hierarchy reflects genuine environmental signal. The shallowest PCs jointly capture a condition-agnostic signature corresponding to the canonical environmental stress response (ESR) [Gasch et al., 2000].

The genes with the highest loadings on the first five principal components are listed in Table 4.3, confirming that PC1 is dominated by glycolytic enzymes (carbon source identity)

while deeper PCs capture progressively more environment-specific programs.

Together, these analyses revealed that transcriptional adaptation is distributed across separable spectral scales: a shared ESR-like program dominates the leading modes of variation, while distinct environmental cues emerge in progressively deeper components in an ordered manner. This structure suggested that environmental information is hierarchically organized within the yeast transcriptome. However, these analyses were performed on populations that had already reached steady-state growth in each environment. We therefore next asked whether this statistical hierarchy reflects a physiological hierarchy that emerges during the dynamics of adaptation.

Table 4.3: Top 10 genes by absolute loading for each of the first five principal components from SVD of the ancestral scRNA-seq dataset. Positive and negative loadings indicate opposing directions of transcriptional variation along each PC.

PC1			PC2		PC3	
#	Gene	Loading	Gene	Loading	Gene	Loading
1	<i>TDH3</i>	−0.116	<i>INO1</i>	+0.104	<i>INO1</i>	+0.148
2	<i>CCW12</i>	−0.108	<i>RPL9A</i>	−0.091	<i>HSP82</i>	−0.134
3	<i>PDC1</i>	−0.103	<i>RPS9B</i>	−0.081	<i>ZRT1</i>	+0.131
4	<i>ENO2</i>	−0.101	<i>GAP1</i>	+0.081	<i>CYC1</i>	−0.114
5	<i>FBA1</i>	−0.098	<i>RPS22A</i>	−0.080	<i>MET6</i>	+0.114
6	<i>CDC19</i>	−0.090	<i>MET6</i>	+0.079	<i>PDC5</i>	+0.112
7	<i>CWP2</i>	−0.085	<i>ARG4</i>	+0.079	<i>HSP60</i>	−0.112
8	<i>21S_RRNA</i>	−0.083	<i>RPS31</i>	−0.076	<i>SHM2</i>	+0.111
9	<i>GPM1</i>	−0.083	<i>FIT2</i>	+0.075	<i>HSP10</i>	−0.103
10	<i>TPI1</i>	−0.082	<i>ZRT1</i>	+0.075	<i>SSA1</i>	−0.097

PC4			PC5	
#	Gene	Loading	Gene	Loading
1	<i>OLI1</i>	−0.214	<i>PDC5</i>	+0.137
2	<i>21S_RRNA</i>	−0.197	<i>ENO2</i>	+0.136
3	<i>15S_RRNA</i>	−0.160	<i>TDH3</i>	+0.122
4	<i>CWP2</i>	−0.131	<i>21S_RRNA</i>	+0.120
5	<i>FIT3</i>	−0.122	<i>RIB4</i>	+0.114
6	<i>CCW12</i>	−0.119	<i>GPM1</i>	+0.112
7	<i>FIT2</i>	−0.114	<i>PDC1</i>	+0.110
8	<i>INO1</i>	−0.096	<i>CDC19</i>	+0.107
9	<i>TDH3</i>	−0.090	<i>HTB1</i>	+0.105
10	<i>AI1</i>	−0.088	<i>FBA1</i>	+0.101

4.3 The Statistical Hierarchy Predicts and Is Reflected in Physiological Prioritization

We focused experiments on yeast populations adapting to changes in osmolarity and temperature — a combination of cues we did not assay within our initial dataset. Because osmolarity ranked above temperature in our statistical hierarchy, our model suggested that under combined stress, cells would mount the osmotic-stress program preferentially over the heat-shock program (HSR) (Fig. 4.7A).

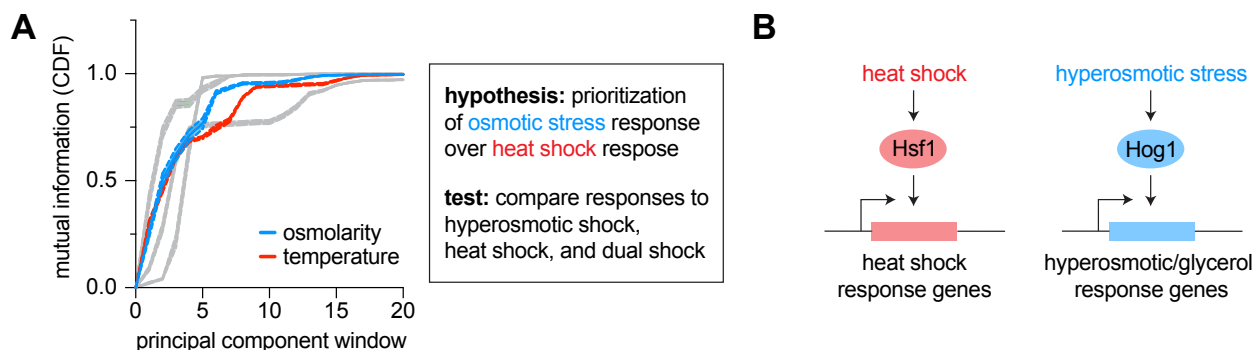


Figure 4.7: Environmental hierarchy predicts that responses to osmotic stress will be prioritized over heat shock. (B) MICDF of environmental conditions with the positions of temperature and osmolarity cues highlighted. (B) Transcriptional response to heat shock is under the control of master regulator Hsf1. Transcriptional response to osmotic stress is under the control of Hog1.

To test this hypothesis, we transcriptionally profiled cell populations via bulk RNA-seq while systematically varying the order and combination of osmotic and temperature challenges. We grew cells to exponential phase in rich media at 30°C and: (i) left them untreated; (ii) heat shocked them at 39°C; (iii) osmotically shocked them at 0.8 M NaCl; or (iv) shocked them with both 39°C and 0.8 M NaCl. Compared to heat shock only, genes associated with the heat shock response (HSR target genes) showed significantly lower induction in the dual stress condition (Fig. 4.8). By contrast, the genes associated with osmotic shock (HOG target genes) were induced nearly to the same extent in hyperosmotic shock and in the dual stress condition. Moreover, the canonical HSR target gene *HSP104*

showed the same induction in the dual stress as in osmotic shock, while the canonical HOG target gene *STL1* showed substantially increased induction in the dual stress compared to heat shock. Thus, osmotic stress prevented heat stress from inducing *HSP104*, but heat stress did not prevent osmotic stress from inducing *STL1*. Ribosomal protein genes (RPGs) were repressed under both heat and hyperosmotic stress, but their expression level in the dual stress more closely resembled hyperosmotic shock than heat shock. Across HSR targets, HOG targets, and RPGs, the measured expression levels in the dual stress condition deviated from the expected value of expression levels if heat shock and osmotic shock influenced gene expression independently. Considering the whole yeast transcriptome, the dual stress condition was better correlated with the osmotic stress condition ($r=0.83$) than with heat shock ($r=0.45$).

As an orthogonal method to measure induction of the HSR and HOG pathway, we measured the fluorescence of specific reporters [Zheng et al., 2016, Stewart-Ornstein et al., 2012, Hao and O’shea, 2012]. The HSR reporter exhibited minimal induction in dual stress compared to heat shock (Fig. 4.9). By contrast, the HOG reporter was robustly induced in the dual stress condition. When cells were given time to initiate the HSR prior to osmotic stress, subsequent addition of osmoshock still caused HSE-RFP fluorescence to plateau. HSR inhibition was dose-dependent on both KCl concentration and temperature. These results were consistent with our bulk transcriptional profiling data, with prioritization of the HOG pathway even more apparent in the reporter assays. Overall, these data demonstrate environmental epistasis between heat and osmotic shock at the level of gene expression, with the hyperosmotic shock program showing prioritization over the heat shock program — a result consistent with the statistical hierarchy we observed.

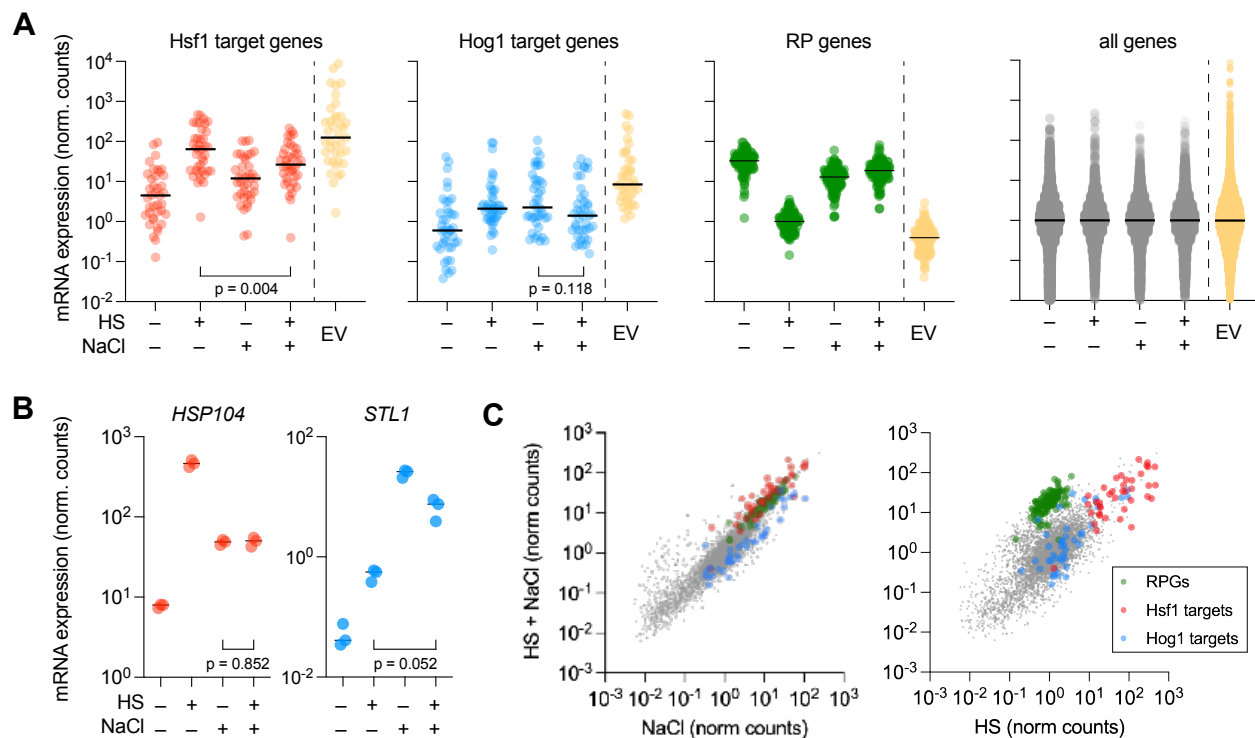


Figure 4.8: Transcriptional epistasis between heat and osmotic stress. (A) Expression of Hsf1 targets, Hog1 targets, RPGs, and all genes across four conditions ($-/+$ heat shock $\times$ $-/+$ NaCl). The dual stress transcriptome correlates more strongly with osmotic shock ($r = 0.83$) than with heat shock ($r = 0.45$). (B) The canonical HSR target gene *HSP104* and HOG target gene *STL1* illustrate asymmetric suppression: osmotic stress prevents heat-induced *HSP104* expression, but heat does not prevent osmotic induction of *STL1*.

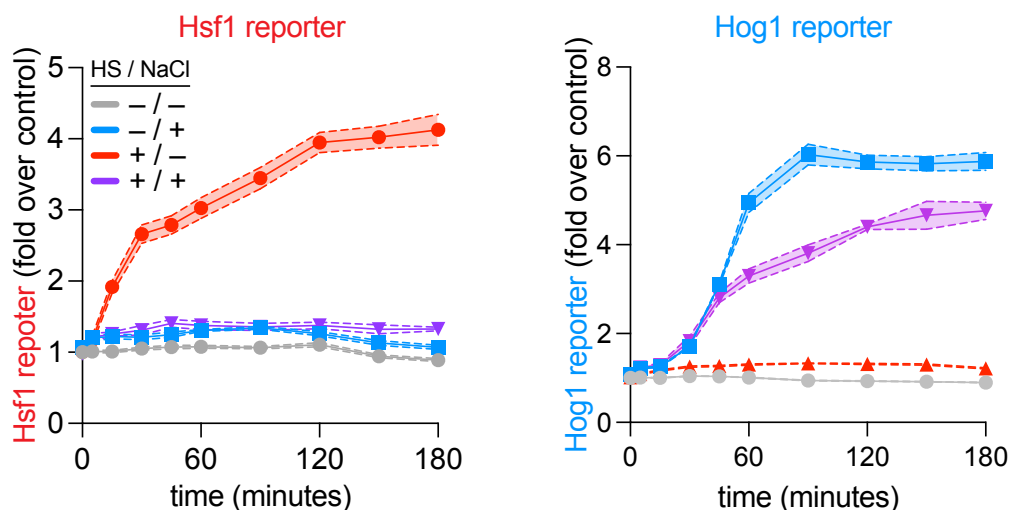
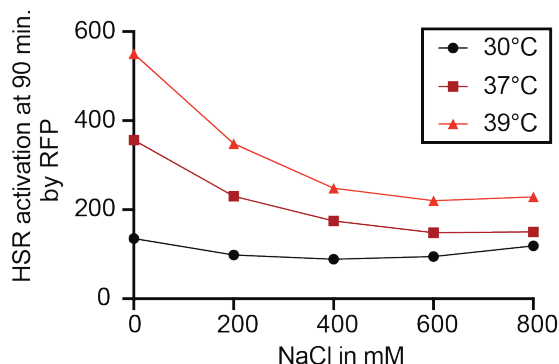
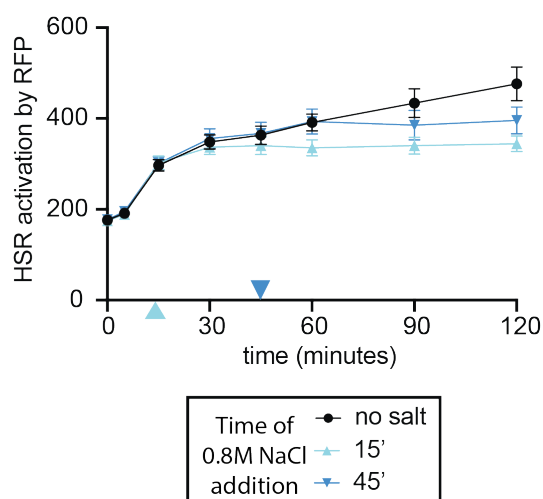
A**B****C**

Figure 4.9: Reporter validation of pathway prioritization. (A) HSR (RFP) and HOG (GFP) reporter time courses across stress conditions. (B) Transcriptome-wide scatter plots: dual stress versus each single stress. (C) Time-of-addition experiments: NaCl suppresses HSR whether added before or after heat shock, confirming that prioritization is robust to stress order.

Next, we extended our validation of the statistical hierarchy an additional layer. The statistical hierarchy suggested that cells would prioritize their response to alternative carbon source over both the HSR and HOG pathways [Visser et al., 1990, Merico et al., 2007]. Therefore, we used the reporter assays to test this by repeating the environmental epistatic

cycle ($-/+$ heat shock, $-/+$ osmotic shock) with cells growing either in glucose or ethanol + glycerol. Cells grown in ethanol + glycerol showed reduced output of both the HSR and HOG pathways compared to cells grown in glucose (Fig. 4.10). Thus, the output of the HSR in response to heat shock depended both on carbon source and osmolarity, and the output of the HOG pathway depended on carbon source. These results illustrated a three-way environmentally-associated transcriptional epistasis consistent with the prioritization scheme suggested by the statistical hierarchy.

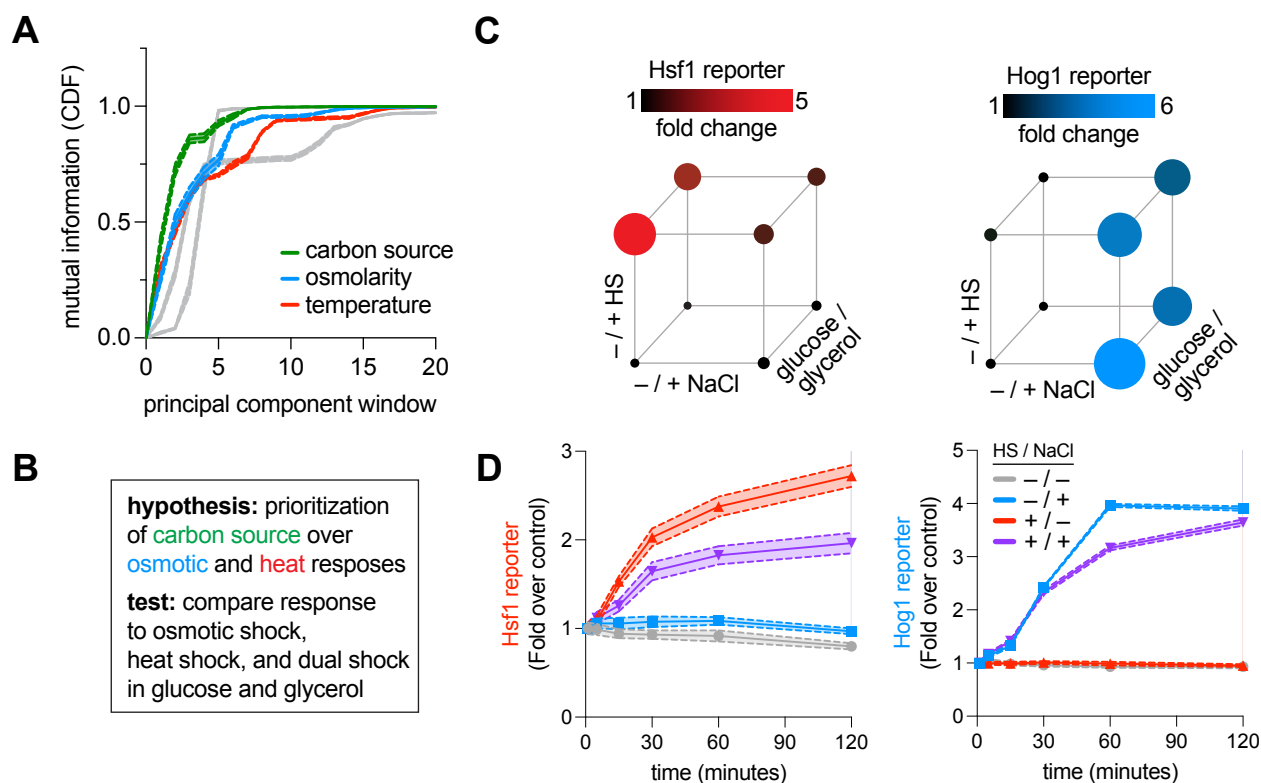


Figure 4.10: Three-way environmental epistasis consistent with the spectral hierarchy. (A) HOG and HSR reporter outputs across all stress combinations in glucose vs. ethanol/glycerol. (B) Contingency cube visualization. Carbon source reduces output of both pathways, establishing its dominance as the shallowest layer of the hierarchy — consistent with its position in the SCALES eigenspectrum.

Overall, we found that the hierarchy derived from statistical analysis conducted across an ensemble of adapted yeast populations was reflected in how individual cell populations deploy stress responses when individual stressors are combined. Motivated by these results,

we next asked whether this prioritization logic was encoded at the biological scale of single cells — as opposed to emerging at a population level — and if so, what molecular mechanisms allow one environmental cue to suppress adaptation to another cue in combined conditions.

4.4 Differential Translation Initiation as an Environmental Priority Router

The prioritization of adaptation to osmoshock over heat shock suggested the existence of a cross-pathway inhibition mechanism [McClean et al., 2007, Madhani and Fink, 1997, Hao et al., 2013]. The master regulators of both pathways, Hog1 and Hsf1, accumulate in the nucleus upon activation [Brewster and Gustin, 2014, Hao and O’shea, 2012, Zheng et al., 2016, Sorger and Pelham, 1988a]. Using yeast strains in which the only copies of Hog1 and Hsf1 are tagged in the genome with fluorescent proteins, we imaged cells across the conditions of heat shock, osmoshock, and dual stress (Fig. 4.11). Hog1 nuclear localization increased upon both osmotic shock and dual heat and osmotic shock, albeit to a lesser extent in the dual stress [Muzzey et al., 2009, Vidal et al., 2013]. In contrast, Hsf1 nuclear localization increased upon heat shock but not in the dual stress condition [Zheng et al., 2016]. Thus, osmotic stress prevented heat-shock-dependent nuclear enrichment of Hsf1, implicating upstream regulation of Hsf1 in the cross-pathway inhibition mechanism.

Our results motivated a natural question: why is nuclear localization of Hsf1 dependent on the presence or absence of osmotic stress? In the absence of stress, it is known that the chaperone Hsp70 and co-chaperone Sis1 prevent Hsf1 from oligomerizing, binding DNA, and recruiting requisite transcriptional machinery for adaptation to heat shock [Krakowiak et al., 2018, Zheng et al., 2016, Garde et al., 2023, Ali et al., 2023, Garde et al., 2024]. Heat shock and other stressors disrupt ribosome biogenesis and pre-rRNA processing, leading to the accumulation of unassembled “orphan” ribosomal proteins (oRPs) [Ali et al., 2023, Tye et al., 2019, Albert et al., 2019]. oRPs condense and recruit Sis1 and Hsp70 to the nucleolus

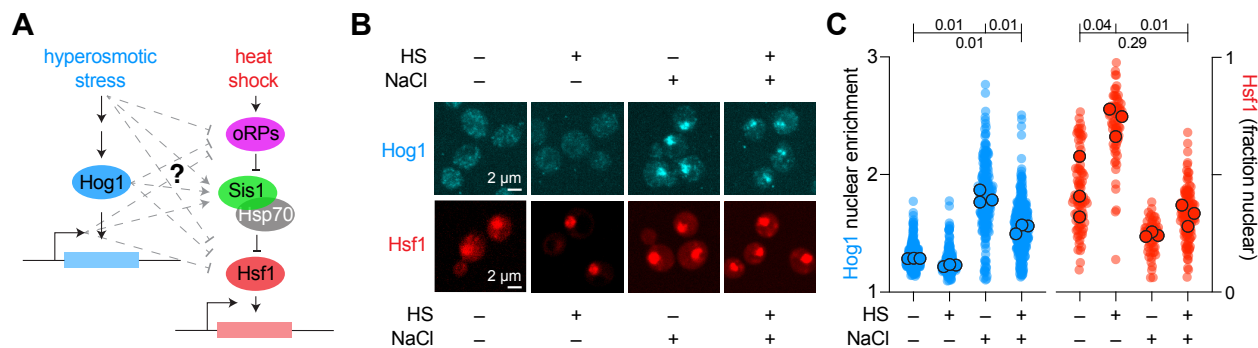


Figure 4.11: Single-cell pathway prioritization. (A) Fluorescence imaging of Hog1-GFP and Hsf1-RFP nuclear localization across the four conditions ($-/+$ heat shock $\times -/+$ NaCl). (B) Single-cell quantification of nuclear enrichment. Prioritization is encoded within individual cells, not via population averaging: under dual stress, cells robustly activate Hog1 but fail to enrich Hsf1 in the nucleus.

and away from Hsf1, leaving Hsf1 free to activate the HSR genes [Ali et al., 2023, Feder et al., 2021, Frottin et al., 2019]. We therefore hypothesized that osmotic shock may interact with the capacity for oRPs to recruit Sis1 and Hsp70 away from Hsf1. To test this idea, we used HaloTag-based pulse labeling of Rpl26a, a ribosomal protein of the large subunit, to monitor oRP accumulation [Ali et al., 2023]. We imaged newly synthesized Rpl26a in cells also expressing fluorescently tagged Sis1 and the nucleolar marker Nsr1 across the heat shock, osmoshock, and dual stress conditions (Fig. 4.12). Pulse-labeled Rpl26a showed increased nucleolar localization upon heat shock but not in dual heat and osmotic shock. Likewise, the increase in co-localization of Sis1 with pulse-labeled Rpl26a upon heat shock was abrogated in the dual stress condition [Feder et al., 2021, Klaips et al., 2020]. These data demonstrated that osmotic shock prevented accumulation of oRPs and co-localization of Sis1 upon heat shock, explaining why HSR induction is reduced in the dual stress compared to heat shock.

We next interrogated why osmoshock prevented accumulation of oRPs while heat shock did not. Production of ribosomal proteins requires ongoing protein synthesis, and full activation of the HSR requires that cells be actively translating proteins prior to heat shock [Tye and Churchman, 2021, Ali et al., 2023]. We assessed global protein synthesis by monitoring polyribosome (polysome) abundance — clusters of ribosomes simultaneously translating a

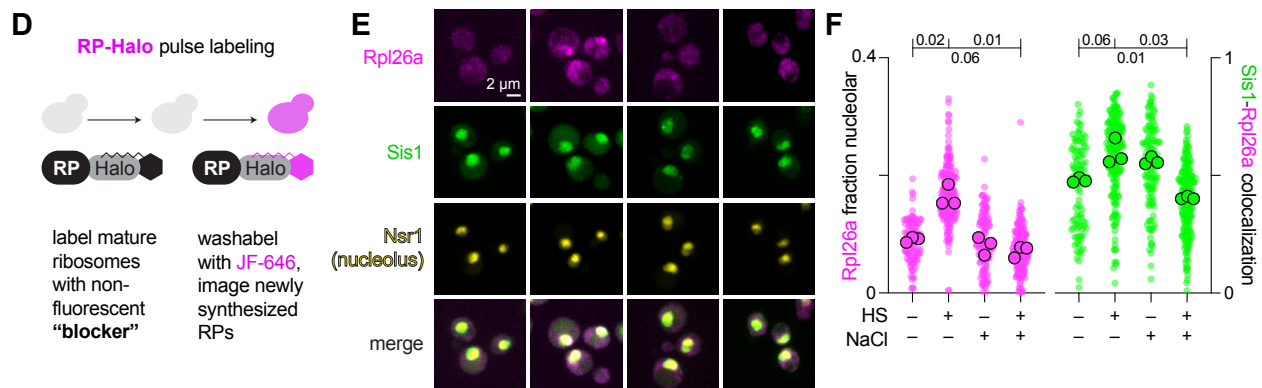


Figure 4.12: Osmotic shock short-circuits the condensate cascade. (A) RP-Halo pulse labeling scheme: pre-existing ribosomes are blocked, and newly synthesized Rpl26a is labeled with JF-646. (B) Imaging of Rpl26a, Sis1, and the nucleolar marker Nsr1 across conditions. (C) Quantification of Rpl26a nucleolar fraction and Sis1–Rpl26a colocalization. Under osmotic shock and dual stress, oRP condensation at the nucleolus is prevented, explaining the upstream suppression of HSR observed in Figure 4.11. Connects mechanistically to the condensate cascade framework of Chapter 3.

single mRNA, whose levels reflect the rate of translation initiation — across the cycle of heat and osmoshock [Aboulhoda et al., 2017]. Compared to unstressed cells, all three stress conditions resulted in reduced polysome and increased monosome peaks, indicating that these stresses result in an inhibition of translation initiation (Fig. 4.13) [Costa-Mattioli and Walter, 2020, Iserman et al., 2020, Cherkasov et al., 2013, Sonenberg and Hinnebusch, 2009]. However, although doubly shocked cells showed a complete collapse of polysomes, osmotically shocked cells retained low levels of light polysomes, and heat shocked cells retained low levels of both light and heavy polysomes. The ratios of polysome to monosome peaks show that the dual stress condition more closely resembles osmotic shock than heat shock. Together, these results demonstrate the mechanism for crosstalk inhibition between the HOG pathway and the HSR: osmotic shock results in the collapse of polysomes, preventing the accumulation of oRPs and other newly synthesized proteins, thereby leaving Sis1 and Hsp70 available to continue to repress Hsf1 from activating the HSR (Fig. 4.14) [Ali et al., 2023, Krakowiak et al., 2018, Garde et al., 2024].

Taken together, our findings demonstrate that prioritization is not merely a population-

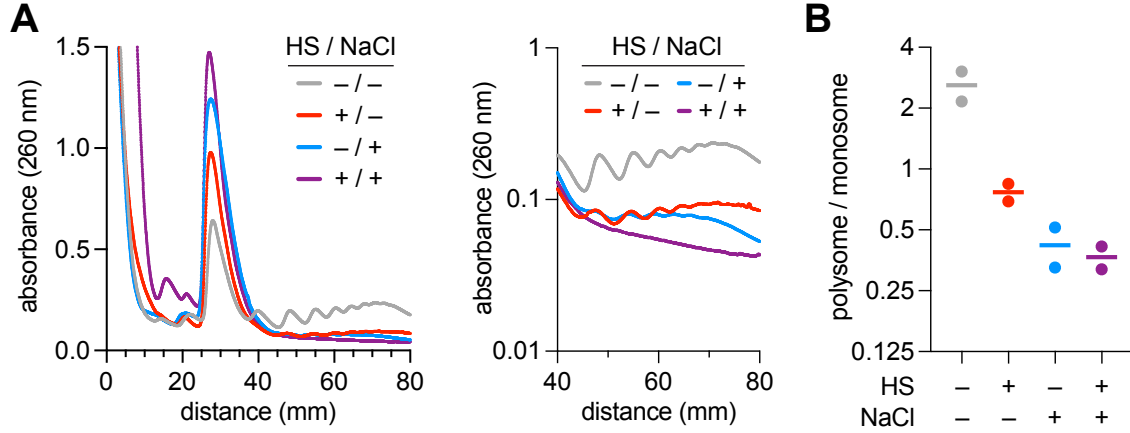


Figure 4.13: Differential translational collapse routes pathway priority. (A) Sucrose gradient polysome profiles across the four conditions. (B) Polysome-to-monosome ratios. The hierarchy of translational collapse (osmotic > heat) explains the hierarchy of adaptive suppression: more severe collapse prevents oRP accumulation and prevents HSR activation.

level averaging phenomenon but a property molecularly encoded within single cells. Under dual stress, individual cells show robust Hog1 activation but diminished Hsf1 nuclear enrichment. The associated reduction in oRP accumulation and stronger collapse of polysomes in dual stress illustrates differential translation initiation as a control point that can gate pathway output [Costa-Mattioli and Walter, 2020, Pakos-Zebrucka et al., 2016, Iserman et al., 2020]. Although we focused on a particular set of stress programs, our findings offer mechanistic resolution for how the statistical hierarchy of adaptive prioritization is directly instantiated at the level of single cells.

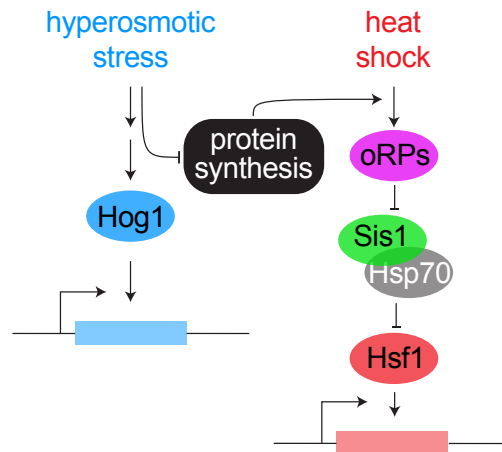


Figure 4.14: Mechanistic model integrating the priority-routing logic. Polysome collapse under osmotic stress prevents oRP accumulation, retains Sis1 and Hsp70 on Hsf1, and suppresses HSR activation. Translation initiation acts as the molecular router that implements the physiological hierarchy observed in Figs. 4.8–4.10 and the statistical hierarchy of Fig. 4.6.

4.5 Materials and Methods

Yeast strains

All strains used in this chapter are in the W303 background (*MATa*; *leu2-3,112*; *trp1-1*; *can1-100*; *ura3-1*; *ade2-1*; *his3-11,15*) and are listed in Table 4.4.

Table 4.4: Yeast strains used in this chapter. All strains are in the W303 background. FP = fluorescent protein; LEU = integrated at *LEU2* locus.

Strain	Genotype	Description	Figures
DPY144	<i>4×HSE-Venus::LEU2</i>	HSE reporter	3.8, 3.9, 3.10
—	<i>LEU2::HSE-mApple; TRP1::pHOR2-GFP</i>	HSR/HOG dual reporter	3.9, 3.10
—	<i>Hog1-[FP]; Hsf1-[FP]</i> (endogenous tags)	Hog1/Hsf1 localization	3.11
—	<i>Sis1-Venus::HIS3; Nsr1-mScarletI::KAN; Rpl26a-HaloTag</i>	oRP/Sis1 colocalization	3.12
DPY1846	<i>Sis1-mVenus::HIS3; Nsr1-mScarletI::KAN; ssa1pr-HALO-ssa1ORF::LEU</i>	Ssa1-HaloTag imaging	3.12
DPY731	<i>TDH3pr-mKate2</i>	mKate2 reference strain	competition assays

Unless otherwise indicated, cells were grown at 30°C with shaking at 200 rpm in yeast extract–peptone–dextrose (YPD) medium. Overnight cultures were grown to saturation, diluted to an initial optical density (OD₆₀₀) of 0.05 in the appropriate media, and allowed to grow to mid-logarithmic phase prior to experimentation. For stress or perturbation experiments, cells were transferred into condition-specific media for dilution as described below.

Selection of environmental stressors

We selected stress conditions that perturb distinct and well-characterized cellular programs in budding yeast while remaining within sublethal, physiologically interpretable ranges. Comparison of rich medium (YP) and defined synthetic complete (SC) medium probes the degree of nutrient buffering and biosynthetic demand. Temperature shift from 30°C (optimal

growth) to 37°C induces a defined heat stress response and challenges proteostasis, membrane fluidity, and ribosome biogenesis. Carbon source substitution from fermentable D-glucose to non-fermentable glycerol/ethanol (2%/2%) enforces respiratory metabolism. Osmotic stress was induced using 500 mM sorbitol (non-ionic osmolyte) and 500 mM KCl (ionic osmolyte) to distinguish general osmotic pressure effects from ion-specific stress signaling. Oxidative stress was imposed using diamide (50 μ M), which preferentially oxidizes thiol groups, and hydrogen peroxide (50 μ M), which generates reactive oxygen species. Together, these perturbations span nutrient, proteotoxic, metabolic, osmotic, ionic, and redox axes, enabling comparison of cellular responses across qualitatively distinct types of environmental stress.

Stress time course via fluorescent reporter assays

Fluorescent transcriptional reporters were integrated at the *LEU* locus and expressed under the control of HSE or pHor2 to report on heat shock response or osmotic stress activity, respectively. Three biological replicates of each strain were serially diluted 5 times for a final dilution of 1:125 and grown in synthetic dextrose media overnight at room temperature. When cells reached mid-log phase, 520 μ L of each replicate was transferred to microcentrifuge tubes and aerated by shaking in a thermomixer at 30°C for one hour. Cells were then heat-shocked, osmotically shocked, or doubly shocked. Cells were heat-shocked by transferring the microcentrifuge tube into a preheated thermomixer set to 39°C. Cells were osmotically shocked by adding 125 μ L of 4 M NaCl SDC to a final concentration of 800 mM NaCl. At each time point, 50 μ L of cells was transferred to a 96-well plate of SDC at 50 μ g/mL final concentration cycloheximide. After time course collection, the plate was incubated at 30°C for 1 hour to promote fluorescent reporter maturation before cytometry.

Flow cytometry

HSE-mApple and pHor2-GFP reporter levels in heat shock, osmotic shock, and double shock time courses were measured with a BD Fortessa HTS 4-15 benchtop cytometer at the University of Chicago Cytometry and Antibody Technology Facility (RRID: SCR_017760). Fluorescent measurements of 10,000 cells per replicate were performed using the 488-525 FITC fluorescence filter (pHor2-GFP) and 561-PE Dazzle (HSE-mApple). Raw fluorescence values were normalized by side scatter in FlowJo, and median fluorescence value was calculated for each of the three replicates.

Quantitative growth assay

Yeast strains were grown overnight at 30°C in YPD. In the morning, cells were diluted to OD₆₀₀ of 0.05 in one of the 20 selected environmental configurations. 600 μ L of culture was loaded into 48-well plates and growth dynamics were measured using a Spectrostar Nano plate reader (BMG Labtech). OD₆₀₀ measurements were recorded every 20 minutes for 48 hours with continuous shaking. Maximum growth rates were estimated by fitting a linear model to the log-transformed OD₆₀₀ values during exponential growth.

Fixed-cell imaging

Constructs containing fluorescently tagged Hog1, Hsf1, Sis1, and Rpl26a were used for imaging experiments. Cells were grown at 30°C with 200 rpm shaking overnight. In the morning, cells were diluted to OD₆₀₀ of 0.1 and grown in the same conditions for 4 hours to reach log phase. Cells were then heat-shocked, osmotically shocked, or doubly shocked. Cells were heat-shocked by transferring 200 μ L of cells into a preheated microcentrifuge in a thermomixer set to 39°C. Cells were osmotically shocked by adding 100 μ L of 4 M NaCl SDC to 400 μ L of cells to a final concentration of 800 mM NaCl. Reactions were terminated by addition of paraformaldehyde (16% stock) to a final concentration of 1% and fixed for 5 minutes

at room temperature. Fixation was quenched by addition of glycine (1.25 M stock) to a final concentration of 100 mM. Cells were immediately plated onto Concanavalin A coated imaging 96-well plates for immobilization. Cells were then washed and imaged in KPIS media (1.2 M sorbitol, 0.1 M potassium phosphate, pH 7.5). Imaging was performed using a SoRa Marianas spinning disk equipped with a 100 $\times$ /NA 1.3 oil objective and Yokogawa camera at the University of Chicago Integrated Light Microscopy Core (RRID: SCR_019197). Image processing and single-cell quantification were performed using CellQuant [Neferkara et al., 2026].

Newly synthesized ribosome imaging

For HaloTag labeling experiments, 100 μ L of log-phase culture was transferred to an Eppendorf tube and incubated in a thermomixer at 30°C with 1,200 rpm shaking for 1 hour. For Rpl26a pulse-chase experiments, pre-existing ribosomes were blocked by adding 1 μ L of 10 mM HaloTag blocker (100 $\times$ stock) to 100 μ L of culture (final 100 μ M) and incubated for 15 minutes. Cells were pelleted by centrifugation for 30 seconds at room temperature, washed twice with 100 μ L SDC to remove excess blocker, and resuspended in 20 μ L SDC.

For stress and HaloTag dye labeling, 20 μ L of cells was transferred into pre-prepared tubes containing 5 μ L of 5 μ M JF-646 HaloTag ligand in SDC with the indicated stress conditions. Stress conditions included: no stress (tube at 30°C), osmotic stress (4 M NaCl SDC in tube), heat stress (tube pre-warmed to 39°C), and combined heat and osmotic stress (4 M NaCl SDC pre-warmed to 39°C). For heat-containing conditions, tubes were equilibrated at 39°C for at least 30 minutes prior to cell addition to ensure uniform temperature. Heat shock response (HSR) dynamics were assayed on the 2–10 minute timescale, whereas Hog1 pathway dynamics were assayed at 15–30 minutes. Cell preparation and imaging were performed as described above for fixed-cell imaging.

Single-cell RNA sequencing

Cells were grown overnight in YPD to saturation, diluted to an OD₆₀₀ of 0.05 in condition-specific media, and harvested at mid-log phase. Mid-log phase was verified by growing the cells in the Spectrostar Nano plate reader and monitoring for previously measured log-phase OD and time. Cells were harvested via filtration and washed twice with PBS to remove remaining media before collection with a cell scraper. Cells were zymolyase-treated [Jariani et al., 2020] prior to droplet generation. Libraries were generated using the Chromium Single Cell 3' Kit v3.1 (10x Genomics) according to the manufacturer's protocol. Sequencing was performed on a NovaSeq 6000 at the University of Chicago Functional Genomics Facility (RRID: SCR_019196).

Bioinformatic processing for single-cell RNA sequencing

Sequencing reads were processed using Cell Ranger 7.1.0 (10x Genomics) with `--expect-cells 2000`, `--localcores 8`, and `--localmem 64`. Reads were aligned to the *S. cerevisiae* S288C reference genome assembly R64-1-1 (Ensembl; INSDC assembly GCA_000146045.2) with gene annotation imported from the *Saccharomyces* Genome Database (SGD). Gene-level UMI counts were obtained from the filtered feature-barcode matrices output by Cell Ranger. All downstream analyses were performed in Python using Scanpy [Wolf et al., 2018]. Quality control and filtering were performed as follows: cells were retained if they expressed between 200 and 4,000 genes and had at least 300 total UMI counts; genes expressed in fewer than 10 cells were excluded. Gene expression counts were normalized to 10,000 counts per cell and log-transformed. The log-normalized full-gene matrix was frozen (`adata.raw`) before downstream subsetting, so that SVD for SCALES analysis could operate on unscaled data (see below). Highly variable genes (HVGs) were then selected and expression values were scaled to unit variance across cells; a scanpy PCA on the scaled HVG matrix was used for UMAP visualization only and is distinct from the SVD used for SCALES (Listing 4.1).

```

1 # QC thresholds (applied to filtered CellRanger output)
2 MIN_GENES, MAX_GENES, MIN_COUNTS = 200, 4000, 300
3
4 # 1. Compute per-cell QC metrics directly from sparse matrix
5 adata.obs["n_genes"] = (X > 0).sum(axis=1) # genes detected
6 adata.obs["total_counts"] = X.sum(axis=1) # total UMI
7
8 # 2. Filter cells and genes
9 sc.pp.filter_cells(adata, min_genes=MIN_GENES)
10 sc.pp.filter_cells(adata, max_genes=MAX_GENES)
11 sc.pp.filter_cells(adata, min_counts=MIN_COUNTS)
12 sc.pp.filter_genes(adata, min_cells=1)
13
14 # 3. Normalize and log-transform
15 sc.pp.normalize_total(adata, target_sum=1e4) # library-size
16 # normalize
17 sc.pp.log1p(adata) # log(counts + 1)
18
19 # 4. Freeze log-norm full-gene matrix BEFORE HVG subsetting and
20 # scaling.
21 # SCALES SVD reads adata.raw.X --- log-norm, unscaled, all
22 # genes.
23 # z-scoring before SVD would equalize variance and distort the
24 # spectrum.
25 adata.raw = adata
26
27 # 5. Select highly variable genes, scale, and run PCA (for UMAP

```

```

    only)
24 sc.pp.highly_variable_genes(adata, min_mean=0.0125, max_mean=3.0,
25                             min_disp=0.5)
26 adata = adata[:, adata.var.highly_variable].copy()
27 sc.pp.scale(adata, max_value=10)
28 sc.tl.pca(adata, n_comps=50, svd_solver="arpack") # UMAP input;
    NOT used by SCALES

```

Listing 4.1: scRNA-seq preprocessing pipeline (`preprocess.py`). QC thresholds, normalization, and the critical decision to freeze `adata.raw` before scaling so that the SVD for SCALES operates on unscaled log-normalized counts. Full source: <https://github.com/annisa-dea/complex-environments>.

To characterize the statistical structure of transcriptional adaptation across environments, we applied singular value decomposition (SVD) to the log-normalized, library-size-normalized gene expression matrix. Critically, SVD was applied to the unscaled matrix stored in `adata.raw` — preserving the natural variance structure of the transcriptome — not the scaled HVG matrix used for UMAP. This yields left singular vectors (LSVs) ordered by transcriptional variance captured, analogous to principal components.

Information-theoretic foundations

The SCALES framework rests on mutual information (MI) as a measure of statistical dependence between transcriptional structure and environmental identity. Before describing the pipeline, I briefly review the information-theoretic concepts that motivate this choice.

For a discrete random variable X with probability mass function $p(x)$, the *Shannon entropy* quantifies the average uncertainty in X :

$$H(X) = - \sum_x p(x) \log_2 p(x), \quad (4.3)$$

measured in bits when the logarithm is base 2. Entropy is maximized when X is uniformly

distributed (maximum uncertainty) and zero when X is deterministic. The *joint entropy* of two variables X and Y is defined analogously over their joint distribution $p(x, y)$:

$$H(X, Y) = - \sum_x \sum_y p(x, y) \log_2 p(x, y). \quad (4.4)$$

Mutual information measures the reduction in uncertainty about X obtained by observing Y (or equivalently, the reduction in uncertainty about Y obtained by observing X). It can be expressed in three equivalent forms:

$$I(X; Y) = H(X) + H(Y) - H(X, Y) \quad (4.5)$$

$$= H(X) - H(X | Y) \quad (4.6)$$

$$= \sum_x \sum_y p(x, y) \log_2 \frac{p(x, y)}{p(x)p(y)}. \quad (4.7)$$

The third form (Eq. 4.7) is the Kullback–Leibler divergence between the joint distribution $p(x, y)$ and the product of marginals $p(x)p(y)$, providing a direct interpretation: MI quantifies how far the joint distribution deviates from independence. $I(X; Y) = 0$ if and only if X and Y are statistically independent, and $I(X; Y) > 0$ otherwise. MI is symmetric ($I(X; Y) = I(Y; X)$), non-negative, and invariant under invertible reparametrization of either variable [Cover and Thomas, 2006].

This last property motivates the use of MI in SCALES. The spectral correlations $\mathbf{C}_w$ depend on arbitrary choices in the pipeline (sign of singular vectors, scaling conventions, choice of unscaled vs. scaled SVD), and any sensible measure of environmental information should be invariant to such choices. Linear measures such as Pearson correlation or canonical correlation analysis would conflate genuine dependence with these arbitrary representational choices, and would also fail to detect nonlinear dependences between transcriptional structure and environmental identity. MI captures dependence of any functional form, making it the appropriate measure for asking whether a spectral window encodes environmental infor-

mation without committing to a specific functional relationship [Kinney and Atwal, 2014, Cover and Thomas, 2006].

Spectral correlation analysis across the eigenspectrum

To quantify which environmental variables were encoded within specific regions of the PC spectrum, we used the Spectral Correlation Analysis of Layered Environmental Signals (SCALES) method [Zaydman et al., 2022, Doran et al., 2025], implemented in the `spipy` package (aramanlab/spipy). The full pipeline proceeds in four stages.

Singular value decomposition. The expression matrix is decomposed as

$$\mathbf{X} = \mathbf{U} \mathbf{\Sigma} \mathbf{V}^\top, \quad (4.8)$$

where $\mathbf{X} \in \mathbb{R}^{n \times g}$ is the log-normalized expression matrix (n cells, g genes), $\mathbf{U} \in \mathbb{R}^{n \times r}$ contains the LSVs ordered by variance captured, $\mathbf{\Sigma} \in \mathbb{R}^{r \times r}$ is a diagonal matrix of singular values $\sigma_1 \geq \sigma_2 \geq \dots \geq \sigma_r > 0$, and $\mathbf{V}^\top \in \mathbb{R}^{r \times g}$ contains the right singular vectors encoding gene loadings. Z-scoring before SVD would equalize variance across genes and distort the spectral decomposition, so SVD was applied to the unscaled matrix as described above.

Spectral windowing. Rather than analyzing each component individually, we partitioned the LSV matrix into overlapping spectral windows

$$\mathbf{U}_w = \mathbf{U}_{:, w:w+N_{\text{WIN}}}, \quad (4.9)$$

where $w \in \{0, 1, \dots, W-1\}$ is the window index, $N_{\text{WIN}} = 3$ is the window width, and $W = 40$ is the total number of windows. Within each window, the corresponding singular values $\mathbf{\Sigma}_w = \text{diag}(\sigma_w, \sigma_{w+1}, \sigma_{w+2})$ weight the LSVs to produce singular-value-weighted cell

embeddings:

$$\mathbf{W}_w = \mathbf{U}_w \boldsymbol{\Sigma}_w \in \mathbb{R}^{n \times N_{\text{WIN}}}. \quad (4.10)$$

Cell-cell spectral correlations. For each window we then computed the matrix of pair-wise Pearson correlations between cells in the windowed embedding:

$$\mathbf{C}_w = \text{Pearson}(\mathbf{W}_w) \in \mathbb{R}^{n \times n}. \quad (4.11)$$

This step is implemented as `sp.calc_spcorr_mtx(U, s, range(w, w+N_WIN))` in `spipy` and yields a cell-by-cell correlation matrix specific to the variation captured in window w .

Mutual information against environmental labels. To test whether spectral correlations contained information about environmental cues, we constructed binary environmental concordance labels for each cell pair:

$$E_{\text{env}}[i, j] = \begin{cases} 1 & \text{if cells } i \text{ and } j \text{ share the same value for env,} \\ 0 & \text{otherwise.} \end{cases} \quad (4.12)$$

The condensed upper-triangular vectors of $\mathbf{C}_w$ and E_{env} were then passed to the empirical MI estimator:

$$\text{MI}(w, \text{env}) = I(\mathbf{C}_w; E_{\text{env}}), \quad (4.13)$$

implemented as `sp.empiricalMI_masked(u_corr_sq, env_corr)`. The estimator decomposes MI as $I(\mathbf{C}_w; E_{\text{env}}) = H(\mathbf{C}_w) - \frac{N_{\text{in}}}{N} H(\mathbf{C}_w \mid \text{in}) - \frac{N_{\text{out}}}{N} H(\mathbf{C}_w \mid \text{out})$, where $H(\cdot)$ denotes binwidth-corrected differential entropy estimated by 1D histogram binning of the spectral correlation values, and N_{in} and N_{out} are the counts of cell pairs that share or do not share the environmental attribute. With $K = 100$ bins and $\sim 10^7$ cell pairs per fold, the estimator operates in the regime where finite-sample bias is negligible relative to cross-condition

signal [Paninski, 2003].

Cumulative distribution and cross-fold averaging. Per-window MI values were converted to a cumulative MI profile and normalized to a CDF:

$$\text{MI}_{\text{cum}}(w, \text{env}) = \sum_{w'=0}^w \text{MI}(w', \text{env}), \quad \text{MI}_{\text{CDF}}(w, \text{env}) = \frac{\text{MI}_{\text{cum}}(w, \text{env})}{\text{MI}_{\text{cum}}(W, \text{env})}. \quad (4.14)$$

A steep rise of MI_{CDF} at shallow windows indicates that information about a particular environmental variable is concentrated in the top principal components (i.e., dominates the transcriptional hierarchy); a shallow, gradual rise indicates that information is distributed across deeper spectral scales.

To obtain robust MI estimates and quantify uncertainty, the SVD and MI computation were performed independently across $N_{\text{FOLDS}} = 6$ stratified folds of the data. Stratification ensured that the distribution of environmental conditions was preserved within each fold. Per-fold MI_{CDF} curves were averaged across folds, and the cross-fold standard error was reported as a shaded band on each curve. To confirm that observed MI values reflected genuine environmental signal rather than statistical noise, true MI curves were compared against null distributions generated by random permutation of environmental labels across cells (per-condition shuffle). True MI_{CDF} curves rose steeply from zero, while shuffled null curves remained flat or rose gradually (Fig. 4.6), confirming that the observed hierarchy reflects genuine environmental signal. The pipeline is implemented in `micdf.py` (Listing 4.2); all scripts are available at <https://github.com/annisa-dea/complex-environments>.

```

1 # Parameters
2 N_FOLDS, N_WINDOW, N_WINDOWS = 6, 3, 40
3
4 # --- Step 1: stratified k-fold split ---
5 skf = StratifiedKFold(n_splits=N_FOLDS, shuffle=True, random_state

```

```

=12345)
6 for fold_i, (_, test_idx) in enumerate(skf.split(X, np.zeros(
    n_cells))):
7     X_fold = X[test_idx, :]          # log-norm full-gene counts for
    this fold
8
9     # --- Step 2: SVD on unscaled log-norm matrix ---
10    U, s, Vt = np.linalg.svd(X_fold, full_matrices=False)
11    # U: cells x components (left singular vectors = cell
    embeddings)
12    # s: singular values; Vt: components x genes (gene loadings)
13
14    # --- Step 3: compute MI per spectral window ---
15    raw_mi = np.zeros((N_WINDOWS, n_envs))
16    for w in range(N_WINDOWS):
17        window = range(w, w + N_WINDOW)
18        # Pairwise cell-cell Pearson correlations in singular-
    value-weighted space
19        u_corr = calc_spcorr_mtx(U, s, window)          # cells x
    cells
20        u_corr_sq = squareform(u_corr, checks=False)    #
    condensed upper triangle
21
22    for env in ENV_LIST:
23        labels = adata_fold.obs[env].values
24        # Binary same/different label matrix for this
    environment
25        same_label = squareform(labels[:, None] == labels,

```

```

checks=False)
26         raw_mi[w, env] = empiricalMI_masked(u_corr_sq,
same_label)
27
28     # --- Step 4: prepend zero row and normalize to CDF ---
29     cumulative_mi = np.vstack([np.zeros(n_envs), np.cumsum(raw_mi,
axis=0)])
30     micdf_fold      = cumulative_mi / cumulative_mi[N_WINDOWS]      #
scale to [0, 1]
31
32 # --- Step 5: average across folds ---
33 mean_micdf = np.mean(micdf_per_fold, axis=0)
34 ste_micdf  = np.std(micdf_per_fold, axis=0) / N_FOLDS      # STE =
std / n_folds

```

Listing 4.2: MICDF computation pipeline (micdf.py calling spipy.core). The outer loop iterates over 6 stratified folds; the inner loops iterate over spectral windows and environmental labels. Normalization converts per-window MI values to a CDF before averaging across folds. Full source: <https://github.com/annisa-dea/complex-environments>.

Bulk RNA sequencing

For bulk RNA-seq experiments, cells were grown overnight in YPD, diluted to an OD₆₀₀ of 0.05 in condition-specific media, and harvested at mid-log phase (OD₆₀₀ = 0.4–0.6, approximately 5 hours post-dilution) by centrifugation. Cell pellets were washed and resuspended in cryopuck buffer (30% RNase-free glycerol, 20 mM HEPES-KOH pH 7.5, 120 mM KCl, 2 mM EDTA, 1 U/μL SUPERase•In RNase inhibitor), then flash-frozen dropwise into liquid nitrogen to form frozen beads. Beads were cryomilled using a mixer mill at 30 Hz for 1.5 minutes, repeated 5 times with liquid nitrogen rechilling between cycles, to produce cryopowder.

RNA was extracted by adding 350 μ L TRI Reagent directly to cryopowder, followed by incubation at room temperature for 5 minutes. RNA was purified using the Zymo Direct-zol RNA Miniprep Kit (R2050) according to the manufacturer’s protocol, including on-column DNase I digestion. RNA was eluted in 30–50 μ L RNase-free water and stored at -80°C . Library preparation and sequencing were performed at the University of Chicago Functional Genomics Facility (RRID: SCR_019196). Libraries were sequenced on an Illumina NovaSeq 6000.

Raw reads were aligned to the *S. cerevisiae* reference genome (sacCer3) using Bowtie2. Aligned reads were coordinate-sorted and indexed using SAMtools. Read counts were assigned to genes using **featureCounts** with gene annotations from the sacCer3 GTF. Count matrices were generated using both gene ID and gene name identifiers. Alignment and assignment rates were computed per sample from SAMtools **flagstat** output and the **featureCounts** summary file.

Polysome profiling

Polysome profiling was performed as previously described [Aboulhouda et al., 2017] with minor modifications. Cells were grown to mid-log phase in the appropriate media, then subjected to the indicated stress conditions. Immediately prior to harvesting, cycloheximide was added to a final concentration of 100 $\mu\text{g}/\text{mL}$ to stabilize polysomes. Cells were harvested by centrifugation at $3,000 \times g$ for 3 min at 4°C , washed once with lysis buffer (20 mM Tris-HCl pH 7.4, 140 mM KCl, 5 mM MgCl_2 , 100 $\mu\text{g}/\text{mL}$ cycloheximide, 1 mM DTT, 1% Triton X-100), and lysed by vortexing with glass beads. Lysates were clarified by centrifugation at $10,000 \times g$ for 10 min at 4°C . Equal amounts of clarified lysate (by A_{260}) were loaded onto 10–50% (w/v) sucrose gradients prepared in gradient buffer (20 mM Tris-HCl pH 7.4, 140 mM KCl, 5 mM MgCl_2 , 100 $\mu\text{g}/\text{mL}$ cycloheximide). Gradients were centrifuged in an SW41 Ti rotor at 35,000 rpm for 2.5 h at 4°C . Fractions were collected using a Brandel gradient

fractionation system with continuous monitoring of absorbance at 260 nm. Polysome-to-monosome (P/M) ratios were calculated from the integrated areas under the polysome and 80S monosome peaks.

Data and code availability

Raw sequencing data (scRNA-seq and bulk RNA-seq) for this chapter have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE327265. Processed count matrices, metadata, and analysis notebooks are available at <https://doi.org/10.5281/zenodo.19273161>. Custom Python scripts for image analysis and quantification are available at the same repository. Scripts for SCALES (MICDF) generation are available at <https://github.com/annisa-dea/complex-environments>.

CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

5.1 Summary

SUMMARY

5.2 Conservation of cellular stress machinery

Throughout this dissertation, I have investigated the converging set of machinery that cells use to respond to variable stresses. In any natural environment, cells will be exposed to a wide variety of stresses, and in order to respond and survive these insults, they must have a varied set of adaptation programs. However, it would be impossible to have a unique stress response for each individual stress. Biology constantly seeks to reduce, reuse, and recycle as a competitive result of evolution. I have shown in my work a flexible stress response mechanism that a cell can deploy to handle variable stresses in specific manners with overlapping components and pathways. Additionally, the odds that a cell will experience each stress in its life independently and sequentially are negligible so the cell must prioritize its response to multiple concurrent insults in a way that maximizes survival outcomes by utilizing these convergent pathways.

5.3 Sequential model for heat shock response condensate assembly

In chapter 2, our work set up one of these key pathways in the form of HSR condensates. We determined that in heat shock specifically, Hsf1 trimers becomes freed from Hsp70 [Krakowiak et al., 2018, Feder et al., 2021]. After this it is able to bind to DNA. The formation of mature HSR condensates can only occur once this has happened, and this formation occurs in a specific step-wise manner [Chowdhary et al., 2025]: first mediator is

recruited, followed by RNA Polymerase II, and finally, once all components are recruited, we see 3D reorganization of the genome [Chowdhary et al., 2022, 2025]. This reorganization is not required for the majority of transcriptional output change that we see, but we theorize that it is a mechanism to bring the resulting mRNA to the nuclear pore to maximize rapid translation of the proteins crucial for survival in heat shock.

Prior to this work, the components of this response were known but their method of assembly was not [Chowdhary et al., 2022]. One competing model other than sequential assembly was a spontaneous model, where each component assembled on the HSR in a more independent manner and only in the presence of each component do we finally see the transcriptional output changes associated with a robust HSR. This sequential model is an incredible discovery on its own [Chowdhary et al., 2025] and also lays the groundwork for the idea of an overlapping stress response pathway. That is, if there is a direct sequence for the formation of HSR condensates, it would be more efficient for the cell to utilize common components to respond to variable stresses. Rather than having full distinct stress response pathways for every unique insult, it can sense variable stresses with common agents to address each stress individually.

5.4 The heat shock response as a flexible condensate cascade

The establishment of this sequential model of HSR condensate formation, along with other work in the lab regarding orphaned ribosomal protein formation [Ali et al., 2023], set the stage for the crux of my thesis work: investigating the idea of a flexible and overlapping stress response condensate cascade [Dea and Pincus, 2024]. In chapter 3, I explored the idea that heat as an insult activates both ends of this condensate formation, and in fact their direct linkage via the behaviors of Sis1, Hsp70, and Hsf1 [Feder et al., 2021, Ali et al., 2023]. Upon heat shock, Sis1 is recruited to the orphaned ribosomal proteins collecting at the nuclear pore to form orphaned ribosomal protein condensates [Ali et al., 2023]. This recruitment leads

to a decreased affinity of Hsp70 for Hsf1 due to the removed ATPase-facilitating activity of Sis1 [Feder et al., 2021, Krakowiak et al., 2018]. Hsp70 is thus sequestered to locations where Sis1 is present such as the newly forming oRP condensates [Ali et al., 2023]. This then frees Hsf1 to form HSR condensates as discussed in chapter 2 [Chowdhary et al., 2022, 2025]. In my work, I was able to interrogate the idea that these disparate condensates are actually linked directly via the behavior and localization of Sis1. Microscopy of fluorescently labeled components of the heat shock response showed that stress conditions that alter the localization of Sis1 result in various levels of activation of HSR condensate and oRP condensate formation. Furthermore, recruitment of Sis1 away from the nucleus, either totally via a rapamycin induced anchor away or in response to an alternate stress such as arsenite, still results in the, albeit diminished, activation of the HSR condensates. Therefore, these condensates are directly linked via Sis1 localization and full, successful activation of both oRP and HSR condensates as well as full transcriptional output rely on this behavior, organizing into a condensate cascade.

The linkage of these two ends of the HSR, the versatility of Sis1, the breadth of its clients, and previous work suggesting the HSR’s participation in other stress inputs [Anckar and Sistonen, 2011, Gomez-Pastor et al., 2018, Pincus, 2020] made this condensate cascade a likely candidate to function at least in part in various conditions [Dea and Pincus, 2024]. One of the key triggers for the re-localization of Sis1 is disruption of ribosome biogenesis [Ali et al., 2023, Feder et al., 2021]. Sis1 itself is adept at preventing the aggregation of aberrant proteins and keeping them dynamic [Klaips et al., 2020, Ali et al., 2023]. Ribosome biogenesis, in particular, when disrupted, often results in collections of aberrant proteins, either orphaned ribosomal proteins in the case of heat shock, or misfolded/unfolded proteins when exposed to toxins such as AzC and arsenite [Tye et al., 2019]. By exposing cells to stresses that perturb ribosome biogenesis such as AzC, arsenite, and diazaborine, I determined the exact extent to which each of these stresses activates this condensate cascade. Each alternate stress

was able to activate this condensate cascade to a varied degree. However, none of them were able to fully activate the cascade as we see in heat shock. In AzC exposure, the cell sees an influx of misfolded proteins, as AzC is incorrectly incorporated as a proline residue, disrupting the tertiary folding process of α -helices and β -sheets and increasing the likelihood of organization in intrinsically disordered regions. These proteins begin to build up, and in an effort to prevent damage to the cell, they are sequestered in a process dependent on Sis1 [Ali et al., 2023, Klaips et al., 2020]. This sequestration of Sis1 is enough to decrease Hsp70 occupancy on Hsf1 [Feder et al., 2021, Krakowiak et al., 2018], ultimately leading to partial activation of HSR condensates as seen by microscopy in chapter 3. However, I saw very minimal transcriptional response.

Arsenite on the other hand causes a host of responses in the cell as it puts stress on multiple systems at once. One of these stresses is the binding of cysteine residues within zinc-fingers, resulting in widescale protein misfolding. This is yet another target of Sis1 [Klaips et al., 2020]. In this instance we see a more significant activation of HSR condensates via microscopy. Additionally, I saw a modest transcriptional response more akin to what we see in heat shock, but still significantly diminished. While some of this transcriptional output can be attributed to activation of the HOG pathway [Brewster and Gustin, 2014, Capaldi et al., 2008], many of the genes that saw increases in transcription are consistent with activation of the HSR. Diazaborine exposure results in the build-up of pre-processed and assembled ribosomal proteins within the nucleus similar to what is seen of oRPs during heat shock [Tye et al., 2019]. I saw through my experiments that this was indeed enough to activate modest condensation behavior. However, the transcriptional response to diazaborine was essentially non-existent compared to what we see in heat shock.

5.5 Conclusions

All these results together allowed me to craft a model of a flexible condensate cascade. That is, disparate stress conditions ultimately converge onto a conserved bottleneck sensor, Sis1. The cell uses a suite of stress responses such as the sequestration of orphaned, mis-folded, or otherwise dangerous proteins via Sis1 and Hsp70 activity, as well as the condensation of transcriptional machinery on the DNA of relevant genes in the presence of Hsf1. However, it seems that rather than activation of the HSR, this condensation of HSR condensate components may serve to buffer the cell against HSR activation in alternate stresses. Upon insult to the cell that disrupts protein homeostasis, Sis1 is recruited to ailing proteins that are mis-folded, unfolded, or newly synthesized. The exact character and localization for these ailing proteins varies from stress to stress. Sis1 is able to target each and in doing so, variably activates the condensate cascade but never to its full potential. Rather it seems that condensation of Hsf1 in this case is intended to sequester Hsf1 and minimize its activity. In heat shock, we see Sis1's strong localization to oRPs. This is enough to strongly recruit Hsp70 and fully free Hsf1 to form HSR transcriptional condensates and fully activate the HSR. In our alternate stresses however, the recruitment of Sis1 is to unique stress response condensates or clients in each stress. Arsenite stress sees sufficient recruitment of Sis1 in a manner similar to Hsf1 to see modest activation of the HSR both transcriptionally and in condensates. In AzC and diazaborine stress however, the recruitment of Sis1 is only enough for a very modest activation of HSR condensate formation and little to no HSR transcriptional activation. Taken together, this creates a model of stress response where machinery and behavior is in fact shared, as in Sis1 and HSR condensate formation respectively, but the full extent and output of each response is specific to the individual insult. Only upon full heat shock do I see full HSR transcriptional character.

This raises the question of the specific conditions that are necessary to activate the full HSR, differentiating it from other stresses. Hsf1 itself may serve as an inherent thermosensor.

This idea is not new and has been proven in human Hsf1 Hentze et al. [2016], Kmiecik and Mayer [2022]. On the other hand it may be the genome itself behaving differently in different temperatures. Perhaps, chromatin begins to unfold and/or rearrange in the presence of heat without chaperone assistance as a way to increase HS target gene accessibility for a more rapid transcriptional change. This could be the result of a form of soft chromatin memory over billions of years of evolution that has been documented in other organisms Brzezinka et al. [2016]. Or perhaps it is the rearrangement of the nucleolus that precipitates this increased access to HS target genes. Regardless of the method, it is clear that the heat itself is required for full HSR activation, and without it, Sis1 sequestration during stress results rather in the buffering of a full HSR through HS component condensation.

This overlap of responses is efficient not only in the utilization of shared machinery between stresses and lending specificity to variable insults, but for incredibly real instances of concurrent stresses. It is unlikely that the cell will encounter each stress in its existence one at a time with proper time to respond to each stress before the next occurs. It must therefore be able to handle multiple insults at one time. A shared response network is thus a potentially essential mechanism when it comes to managing cellular resources [de Nadal et al., 2011, Morimoto, 2011]. The work discussed in chapter 4 follows the idea that the cell will prioritize different stresses over others when faced with more than one at once. Rather than equally responding to each stress by activating multiple response pathways, it reserves the majority of its resources and response bandwidth for what it deems the most pressing or most dangerous stress. This further highlights the importance of having a convergent condensate cascade. If the cell were to face two stresses it could potentially address both using a shared or overlapping set of mechanisms.

Chapter 4 shows the extensive work done to determine this behavior. When exposed to dual stresses, the cell will prioritize one over the other in terms of transcriptional output. By comparing several pairs of concurrent stresses, we were able to identify a hierarchy of stress

response. Essentially, a ranking of responses was established from most to least prioritized. This provided great insight into the evolutionary pressure that might have led to the cell's preference for one stress response over another, or perhaps simply notes the greater threat to the cell. That is, if one stress is non-ideal but survivable it would make sense for the cell to prioritize response to a stress that is more immediately dangerous [McClean et al., 2007, Hao and O'shea, 2012].

Along this exploration of evolutionary space, chapter 4 showed what can happen to a cell when exposed to a prolonged single stress (~ 3000 generations of NaCl pressure). Intriguingly, cells in this line accumulated a set of adaptations that led to a positive growth adaptation in a high salt environment. These cells no longer exhibited such severe growth defects in salt over time. One downside, however, was that they seemed to trade this adaptation for overall stress maneuverability. Their overall stress response seemed to collapse making them much more vulnerable to alternate stresses than their parent strain.

The work covered in this thesis provides a model for a highly efficient and adaptive stress response pathway. Variable stresses variably activate a flexible condensate cascade where several components of the well described HSR can be utilized to either respond transcriptionally to stresses like heat and arsenite or buffer against a HSR in stresses such as AzC and diazaborine. This sets the stage for further exploration of the efficiency of cellular response. The open questions discussed at the end of this work pose a very interesting and worthwhile path of discovery. To understand cellular stress response in its entirety will provide not only endless insight into human disease states, but into cellular processes involved in every stage of life and even to death itself. Stress and life's resulting adaptations are the driving forces behind life and evolution, and to understand these processes is to understand life itself.

5.6 Future Directions

The future directions for my work are as numerous as there are stresses that a cell may encounter. Before moving on to additional stresses however, I think it would be very worthwhile to further explore the suite of stresses examined in chapter 3. To start, examining the Sis1 targets for all three alternate stresses would give great insight into why the cell may be activating the condensate cascade in the first place. It could also characterize the behavior and localization of Sis1 and Hsp70 themselves which would give more information about cellular stress response as a whole and why these individual stress response pathways may share components and behavior. I envision a set of immunoprecipitation experiments where Sis1 and Hsp70 are both pulled down with their clients in these variable stresses and analyzed via methods such as mass spectrometry. Once these full stress pathways have been mapped with relation to the HSR, I could then begin to explore other stresses.

A much broader approach to this idea would be to take a host of the most common environmental stresses that yeast are likely to encounter and examine the overlaps between their stress responses. In such an ambitious exploration, it would make most sense to start with a transcriptional measurement, using RNA-seq to explore how each stress alters the cell's transcriptional output. One could then group like responses and try to determine why these groupings may occur. At this point the experimental approach would split, designing specific assays to test the overlap between individual physical changes the cell may see in terms of each stress. Ultimately, it may become possible to relate a wide array of stresses into a relatively small number of overlapping stress responses that share machinery and have similar transcriptional outputs. This kind of work would likely span many sets of experiments and would no doubt produce an extensive body of work. However, I believe the results could shape our understanding of redundancy and efficiency in the cell.

Of course, much work needs to be done in identifying the mechanism the heat produces in cell to create the full HSR. Methods that explore the chromatin availability both pre and

post HS would show the accessibility of HSR target genes in this unique stress. This could also be achieved via the placement of these genes outside of canonical sites on the genome in a plasmid to reduce their chromatin dependence. Observing the state of the genome pre and post nucleolar rearrangement would also be incredibly worthwhile. Finally it could be some external factor that notices heat and interacts with the components described here. A promising candidate is small non-coding RNAs (snoRNAs) that have been shown recently in my lab to be inherently temperature sensitive (unpublished). The mystery of HS remains, but this work brings us closer to understanding the full picture.

Additionally, I would be very interested to explore the overlap of the work presented in chapters 3 and 4. Primarily, addressing the question of how concurrent stresses activate convergent stress pathways. In chapter 4, the primary focus was transcriptional output. Essentially the state of the transcriptome under concurrent stresses and whether or not it more closely relates to one stress over the other. Based on the results garnered in chapter 3, I saw that physical changes in the cell do not necessarily correspond to a change in transcription. Therefore, I would be very interested to see what physical changes occur in the cell such as condensation and genome rearrangement in the presence of dual or multiple stresses. Do these changes also collapse as seen in the transcriptome to prioritize one stress, or do we perhaps see partial activation of one or more overlapping pathways? Additionally, can compounding stress of similar affect result in stronger physical changes when applied at the same time? Do we see higher levels of HSR condensates or do multiple condition-specific condensates such as those regulated by Sis1 in the AzC and arsenite conditions lead to a heightened physical change?

APPENDIX A

ATTEMPT AT A FULL IN-VITRO RECONSTITUTION OF THE HEAT SHOCK RESPONSE

The initial work that I set out to do for my time in the Pincus lab was the full in-vitro reconstitution of the heat shock response. At the start of my work, the stepwise assembly of HSR condensates was not yet known [Chowdhary et al., 2025]. We understood HSR condensates to assemble at the DNA in times of heat stress. We also knew of the major components involved, and these are the very same I analyzed later in my thesis work. These, of course, consisting of Hsf1, Hsp70, mediator, RNA Polymerase II, and Sis1. Importantly, exciting work was appearing around the direct interactions of Hsf1 and Hsp70 [Krakowiak et al., 2018, Feder et al., 2021]. Dr. Sarah Paracha in our lab was identifying the binding sites for Hsp70 within the Hsf1 intrinsically disordered regions. These were determined to be the NE1 and CE2 sites [Feder et al., 2021]. However, some outstanding questions remained. I was interested in the method of assembly of the condensates, the relative quantities of each component present in the condensates, the relative affinities of each component for each other component in the presence of the entire system and in different environments, and the method of disassembly of condensates upon the removal of stress. To answer all of these questions and potentially more, I coalesced my ideas into the fully in-vitro reconstitution of the heat shock response. This would have enabled me to finely tune the entire system and observe the interactions of the various HSR condensate components directly.

A.1 Protein purification and in-vitro binding assays

The first step for an in-vitro analysis such as this one was to isolate each of the individual components. DNA sequences were an ideal first step as they are trivial to synthesize and a key component of HSR condensates. Without DNA there are no condensates. DNA sequences

consisting of 8 inverted repeats of HSE elements were designed before being synthesized and ordered through IDT. HSEs consist of multiple inverted repeats of the pentameric sequence nGAAn [Yamamoto et al., 2005].

From here, it became necessary to obtain each of the protein components of the HSR via protein recombination, starting with Hsf1 and Hsp70. At the time, Hsf1 was understandably held as the crux of the HSR and its relationship with Hsp70 determines the fate of the HSR [Krakowiak et al., 2018]. For Hsp70, a strain of yeast with Ssa2, one of four cellular Hsp70 molecules expressed, tagged with 6 histidine residues was obtained from Allan Drummond's lab. The 6x-His tag on this protein is important because histidine residues contain an imidazole ring. This makes them the perfect target for Nitrilotriacetic acid (NTA) nickel beads. The imidazole ring of the histidine residues are able to covalently bind with nickel ions that have been bound to the chelating agent NTA [Hochuli et al., 1988]. Washing first with a low imidazole concentration solution washes away proteins that may form weaker bonds with the nickel ions. Then a final wash with high imidazole, outcompetes the bound histidine residues of the tagged protein. This process is known as immobilized metal affinity purification [Hochuli et al., 1988]. By flash freezing cells expressing a 6x-His tagged protein with liquid nitrogen, lysing them via rapid shaking and physical insult, and running the resulting solution over a column of Ni-NTA beads, before finally washing and eluting with excess imidazole, I was able to purify Ssa1 molecules away from the rest of the cell for further experimentation.

Unfortunately, at this stage I tried to replicate this process with Hsf1 and began to encounter issues. Hsp70 being a highly organized and relatively small (44 kDa) protein, as is beneficial for chaperones, made it an ideal candidate for the notoriously difficult process of protein purification. Hsf1, on the other hand, is a relatively large (93 kDa) protein and is flanked on either terminal by large intrinsically disordered regions [Anckar and Sistonen, 2011]. These factors together make it a difficult protein for purification and previous work

has struggled to do so. I nevertheless attempted this via the 6x-His tagging method and was met with decidedly mixed results. Via extensive trial and error (see: 12 months of work), I was able to regularly purify significant quantities of Hsf1 product. Verification of the product via HPLC, however, failed to show single elution peaks consistent with a pure product or even multiple regular peaks consistent with mixed populations of dimers, trimers, oligomers, etc. Rather, I saw messy groups at no easily discernable sizes which could have indicated degraded or partial proteins or perhaps contamination of other bound proteins. Additionally, when ran with a standard protein ladder, the bulk of the peaks were at weights much higher than the known weight of Hsf1. However, these messy groups did have a non-insignificant amount of regularity between runs and the method itself is robust against contamination, indicating that I was indeed purifying Hsf1 and these irregular peaks likely pointed to some aggregation behavior of the Hsf1 itself.

A.2 Hsf1 product binds DNA at low levels

Nevertheless, I pushed forward with some of my proposed experiments, evaluating the behavior of this “purification” product in the presence of both the purified Hsp70 and synthesized HSE containing DNA. While attempting to troubleshoot the purification process of Hsf1, I started to interrogate the behavior of the Hsf1 product. Via a series of competition assays where I combined the Hsf1 product with DNA both with and without HSEs I was able to determine that this product was able to bind DNA, and does so specifically with only DNA that contained HSEs [Yamamoto et al., 2005]. Once this was confirmed, I sought to determine the specific affinity of this Hsf1 product for DNA, measured as a dissociation constant (K_D) via anisotropy. This experiment produced a K_D of 2 μ M. In general, a K_D of between 1–100 nM is expected for transcription factors with their target DNA [Stormo, 2000]. With the K_D I determined being much weaker than what one might expect, this result pointed to imperfection in the purification process. Either the product I had produced was less

functional in some way such as purifying only incomplete pieces of Hsf1, there remained some contamination in the purification process, or considering the size distribution from the HPLC analysis, the Hsf1 was aggregating in such a way that diminished its ability to bind DNA effectively.

A.3 Hsf1 product interacts with Hsp70

One additional path that I pursued despite not having ideal Hsf1 product was a competition assay with Hsf1, Hsp70, and HSE DNA. These experiments functioned similar to the DNA binding assays, where I incubated Hsf1 with both Hsp70 and DNA in various configurations. In the case of Hsf1 pre-bound to Hsp70, I observed a decreased affinity of Hsf1 for HSE containing DNA. This mimicked the binding inhibition we see in vivo when Hsf1 is bound by Hsp70 [Krakowiak et al., 2018, Feder et al., 2021]. This was a very promising result and set the stage for future experiments where the relative ratios of this relationship could be quantified.

A.4 DNA Affinity Chromatography

A brief attempt was made to further purify the Hsf1 product using DNA affinity chromatography. I bound HSE containing DNA to magnetic beads with the hope that the Hsf1 product I had purified contained a mixed population of functional and non-functional Hsf1. The idea would be to allow functioning Hsf1 to bind the beads before washing away non-functional Hsf1. I could then elute the functional Hsf1 off of the beads. Ultimately, this left me with miniscule amounts of product and HPLC of this new product showed a very similar set of peaks to that of the original product. This further pointed to the aggregation of Hsf1 outside of the cell into something that would not behave as a stable monomer or trimer for proper in-vitro analysis.

A.5 AlphaFold analysis of Hsf1 structure and Discussion

While I had been able to produce some interesting results regarding the behavior of this Hsf1 product that were in line with what was known about the HSR in yeast, it was becoming clear that a full replication of this network in-vitro was unlikely to be possible given the time and scope of my thesis. The final nail in the coffin for my time on this project came from the increased rise in popularity of AlphaFold. The full structure of Hsf1 was still unknown at the time of my experiments. Extensive previous work had found intense difficulty in identifying the full structure of Hsf1 via protein crystallography, likely due to the intrinsically disordered regions that provided such grief for my experiments [Anckar and Sistonen, 2011]. AlphaFold, an AI based system developed by Google’s Deepmind, was developed using extensive deep learning techniques that make it incredibly good at predicting a protein’s structure based on its amino acid sequence alone [Jumper et al., 2021]. Not only this but it is able to determine likely interaction behaviors when given multiple proteins or proteins and nucleic acids [Jumper et al., 2021]. A simple analysis of the sequence of Hsf1 as both a monomer and a trimer gave very interesting results that explained much of the difficulties I and my fellow researchers have faced in the effort to isolate Hsf1.

AlphaFold analysis of Hsf1 predicted a structure where, independent of outside interference, the trimerization domains will function as intended, bringing three Hsf1 molecules into a trimer [Jumper et al., 2021] (Fig. A.1). The DNA binding domains will then rest outside of this core, and finally, the entire core will be surrounded in an almost armadillo-like shell of the intrinsically disordered regions. This shell likely acts in two ways that created difficulty for my experiments. The first being the protection of the DBDs from the environment, preventing DNA from gaining access to them. The second has to do with their physical character. The IDRs being disordered means that they have exposed hydrophobic regions that make them sticky so to speak for other hydrophobic molecules [Anckar and Sistonen, 2011]. This means that these trimers are likely aggregating. The IDRs of each trimer may

be interacting with the IDRs of adjacent trimers. Apparently, to such a degree that they cannot be pulled apart via HPLC.

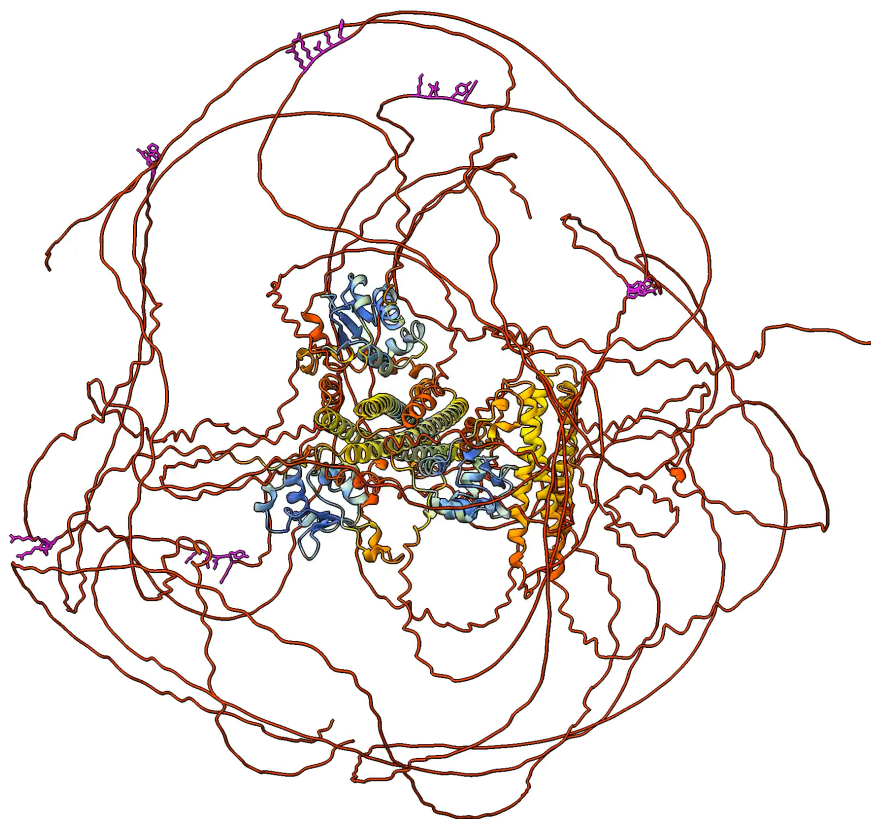


Figure A.1: **AlphaFold predictive structure for Hsf1 trimer**

This result, while devastating to my experiments, was very interesting in and of itself and gave us much insight into the behavior and activity of Hsf1 and the overall HSR. We now have a much clearer picture of Hsf1 as a trimer. We also understand the structure of each monomer and how it likely interacts with Hsp70, DNA, and transcriptional machinery [Feder et al., 2021, Chowdhary et al., 2022]. While I could not answer the questions I posed at the beginning of this line of work, it set the stage for my future work in the lab and my understanding of the HSR. I pose additional methods for Hsf1 purification involving the pull-down of Hsf1 with DNA directly or perhaps cell-free lysate assays where Hsf1 is not so fully removed from the context of the cell. In time, this proposal deserves further study and

still promises to reveal interesting and novel ideas about the interaction of components of the highly consequential heat shock response.

APPENDIX B

HSF1 AS AN INHERENT PH SENSOR

At about the time that I shelved the in-vitro reconstitution project, a very interesting paper had come out detailing the biological use of histidine residues as inherent pH sensors [Gutierrez et al., 2022]. A histidine residue is a unique amino acid in protein structures. The presence of an imidazole ring in its side chain makes it useful for the construction of kinks in the tertiary structure α -folds and β -sheets. Additionally, this imidazole ring contains two nitrogen atoms that give the ring the function of either a proton donor or proton acceptor depending on the environmental pH [Gutierrez et al., 2022]. At neutral pH, as in the physiological pH of the cell during basal conditions, only one of the nitrogen atoms of the imidazole ring is protonated, giving the residue an overall neutral charge. However, the pKa of the second nitrogen is between 6–7, meaning that only a modest drop in pH results in protonation of this second nitrogen, giving the histidine an overall positive charge of 1 [Gutierrez et al., 2022]. Doing a sequence analysis of Hsf1 showed an enrichment of histidine residues compared to overall sequence space of the average protein, specifically within the N-terminal disordered region. Even more interesting is that one of these residues resides directly in the NE1 site responsible for Hsp70 binding and inactivation of Hsf1 [Feder et al., 2021]. Another key piece of information is that upon heat shock, the overall pH of the cell is known to drop, becoming more acidic and importantly dropping below the pKa of the second nitrogen in histidine’s imidazole ring [Triandafillou et al., 2020]. All of these factors together produced the very interesting hypothesis that perhaps this inherent pH sensing ability of Hsf1 is what enables the molecule to sense heat shock and respond to it rapidly and directly.

B.1 Hsf1 histidine mutant design and rationale

The histidine containing NE1 site of Hsf1 is where Hsp70 is known to bind Hsf1 to keep it inactive [Feder et al., 2021]. The exact mechanism for how Hsp70 disrupts Hsf1 when it is functioning as a transcription factor within HSR condensates during heat shock is still unknown [Chowdhary et al., 2022]. We hypothesized that this variable character of Hsf1 in response to pH of the cell could explain this mechanism. Perhaps when the cell has properly responded to heat stress or when the stress is removed, the pH of the nuclear environment returns to neutral [Triandafillou et al., 2020], de-protonating the histidine residues of Hsf1 and increasing its affinity for Hsp70 allowing for inactivation. To test this thought, I designed a suite of Hsf1 mutant strains of yeast (Table B.1). I replaced each of the five histidine residues within the N-terminal IDR of Hsf1 with alternate amino acids. For the first strain, I wanted to mimic Hsf1’s state in non-heat shock conditions, that is all of the histidine sites would be constitutively neutral. I did this by substituting asparagine for each histidine. To mimic a fully heat shocked state, I substituted each histidine for arginine, a positively charged amino acid. Additionally, as a control, I went the other direction and substituted each histidine for aspartic acid. This amino acid is negatively charged, and while histidine would not become fully deprotonated to mimic this charge until a pH of around 14, we thought it an interesting control and proof of concept. This suite was designed to test not only the interaction of Hsp70 with the NE1 site but also to evaluate if overall charge of the IDR played a role, as each substitution swung the charge of the IDR by 5. To specifically target just the NE1 site and therefore Hsp70’s direct role [Feder et al., 2021], I made an additional 3 mutants where only the histidine within the NE1 site was substituted.

The idea behind these mutations was that they would impact the degree of responsiveness to heat shock or perhaps inhibit it altogether. If Hsf1 relies on a pH change to alter the charge or character of one or more histidine residues within its N-terminal IDR to properly respond to stress [Triandafillou et al., 2020, Gutierrez et al., 2022], then the effects of these

Histidine Substitution(s)	AA Replacement	Net Charge Change
All 5	Asparagine	Neutral
All 5	Arginine	+5
All 5	Aspartic Acid	-5
NE1 Only	Asparagine	Neutral
NE1 Only	Arginine	+1
NE1 Only	Aspartic Acid	-1

Table B.1: Histidine replacement strains and their charges

substitutions could potentially be predicted based on their natural state in specific conditions. That is to say that a constitutively neutral set of substitutions, using asparagine to mimic a non-heat shocked state could see a decrease in sensitivity to heat shock. If Hsp70 has increased affinity for the NE1 site when it is neutrally charged [Krakowiak et al., 2018, Feder et al., 2021], it may remain bound to Hsf1 more frequently during heat shock, diminishing the HSR. Alternatively, it may fall off at the same rate, but interfere with HSR condensates during HS, prematurely dampening the response or leading to a more rapid turn off upon removal of stress. On the other hand, substituting positively charged arginine could decrease Hsp70’s affinity for the NE1 site under basal conditions, leading to at least a partially constitutively active HSR or interfering with the shut off of the response when heat shock is removed. This is because it no longer has a strong enough affinity for Hsf1 to disrupt the HSR condensates [Chowdhary et al., 2022]. Finally, the substitution with constitutively negative aspartic acid could further exacerbate this effect leading to an even more heightened response both basally and in times of stress. It is also just as possible that it could have some unforeseen effect that would inform further study.

Before getting started with these mutants, I did a round of sequence analysis using AlphaFold to predict the structures and arrangements of the IDR that could result from such substitutions [Jumper et al., 2021]. The results of this step were mixed but did seem to indicate that these substitutions would impact the exact configuration of the IDR and could potentially interfere with the positioning of Hsp70 at the NE1 site [Feder et al., 2021]. This

exciting prediction gave us confidence to proceed to experimental results.

B.2 Experimental characterization of Hsf1 histidine mutants and Discussion

Overall, I wanted to test the effects of these substitutions on the three key characteristics of the heat shock response: condensate formation, transcriptional activity, and growth. For condensate formation, I needed a way to visualize the Hsf1 molecules in the cell and did this by appending an mVenus tag to each mutant. Then, using confocal spinning-disk microscopy I imaged each 5-substitution mutant under heat shock and non-heat shock conditions. It appeared that each Hsf1 mutant was able to form condensates under heat shock and remained essentially diffuse throughout the nucleus in non-heat shock conditions, consistent with the behavior of wild-type Hsf1 [Chowdhary et al., 2022]. There were some indications that potentially the arginine (+) mutant saw some interruption of condensate formation, but quantification dismissed that pretense. This was not an immediate end to the experiments, as the imaging that I conducted was only at a single time point and did not adequately assess the dynamics of condensate formation that would be necessary to make any concrete observations of condensate formation timing.

Concurrent with the imaging, I did a series of spot assays and growth assays on each of my mutants. The set up for the spot assays was relatively simple. I created a serial dilution for each strain and did spot assays on both YPD and YPG (YPD with glycerol substituted for glucose) and for each media at both 30°C and 37°C. The idea here was to test a result seen in previous work in the lab, where mutants that compromise Hsf1's activity in heat shock of course see diminished growth in heat shock conditions but also see even more diminished growth in YPG at heat shock temperatures as the cell has to juggle multiple stress inputs (heat and carbon source) [Solís et al., 2016]. We used a negative control here of a Δ NTA strain where the entire N-terminal activation domain had been removed.

This strain sees diminished transcriptional activation and therefore growth in heat shock conditions as it is unable to fully recruit the transcriptional machinery necessary for a full, robust response [Chowdhary et al., 2025].

For the growth assays, I used the same set of strains and ran growth curves on them, again in both YPD, YPG, and in heat shock and non-heat shock (Fig. B.1). This was done by growing them in triplicate and measuring their O.D. 600 every 30 minutes over the course of 48 hours in each condition. The results from both the growth and spot assays were mixed, but ultimately after several trials the variance between trials collapsed to a fairly conclusive result that substitution of histidine residues does not significantly impact growth. We saw nowhere near the reduction that we saw for the Δ NTA strain in heat shock and ultimately saw no significant difference between growth of the parent strain and the mutants in any of the conditions.

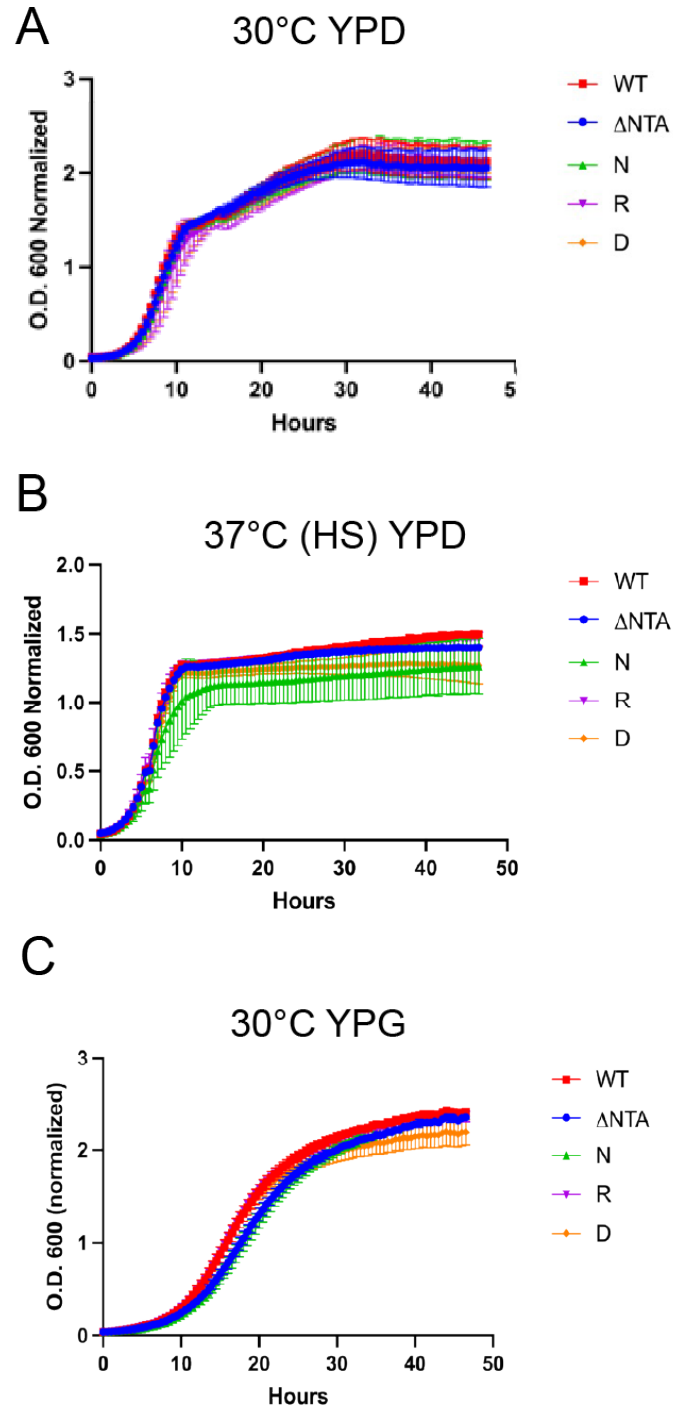


Figure B.1: Growth curves for histidine replacement mutants in NHS, HS, and alternate media conditions

While the previous data were not a strong indication for the role of pH sensitivity in Hsf1's HSR activity, all hope was not lost, as I still had the final and arguably most impor-

tant characteristic of a healthy response to test: transcriptional output. To test the bulk transcriptional output of each of these mutants under heat shock as compared to the parent strain, I created yet another set of mutants. In this case I included a sequence with YFP downstream of a series of HSEs [Yamamoto et al., 2005]. This method has been extensively used in my lab to test the degree of the HSR, using the fluorescence of the HSE-YFP as a readout for activity [Krakowiak et al., 2018]. Essentially, the more active the HSR, the more the active Hsf1 is on genomic DNA containing HSEs, and ultimately the more fluorescence we see. I analyzed the set of strains that each contained HSE-YFP and each of my substitution mutants, a Δ NTA mutant, or the parent strain of WT Hsf1 expressed on a plasmid (Fig. B.2). In each case I measured fluorescence in both heat shock and non-heat shock using flow cytometry. As with the previous suite of experiments, the fluorescence output of each mutant much more closely matched that of the parent strain than of the Δ NTA strain. The interpretation of this experiment was that the charge or presence of histidine at the NE1 site does not meaningfully impact the cell's transcriptional response to stress.

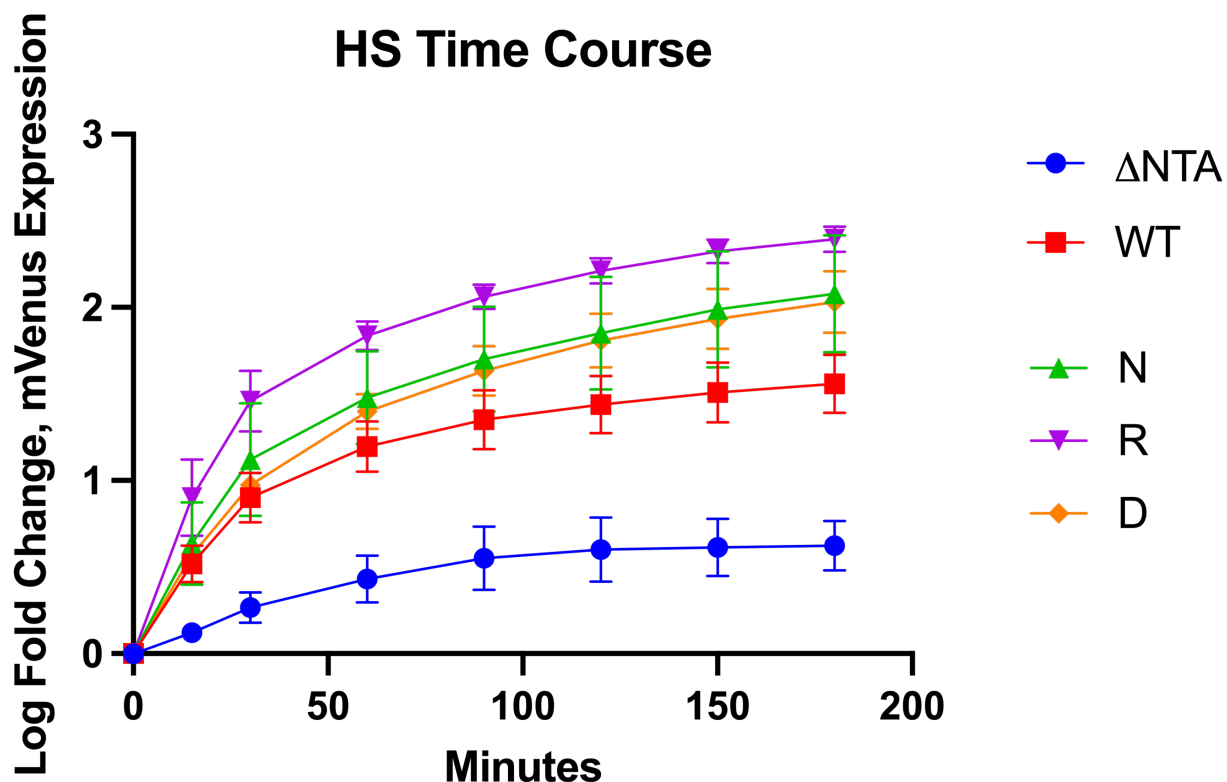


Figure B.2: HSE-YFP expression over time under heat shock for histidine mutants

This collection of experiments did not unfortunately show a relationship between nuclear pH and Hsf1 activity. However, the experimental methods here were robust and finely tuned and conclusively make this point. While no longer a strong case for further study, I do feel that they provide a keen insight into the character of Hsf1 and constitute a novel result. They left open the question of what it is specifically that tips off the cell to a heat shock that produces such a rapid and robust response [Triandafillou et al., 2020]. Brand new work in the lab is aiming to answer this question through the behavior of small nucleolar RNAs (sno-RNAs). It seems that they may have inherent temperature sensing capabilities that serve to set off a chain reaction, triggering the full HSR. In the search for truth about the order of the natural world, there will ultimately be dead-ends, but the study of such paths is crucial to find and trust in the right direction.

REFERENCES

- S. Aboulhouda, R. Di Santo, G. Therizols, and D. Weinberg. Accurate, Streamlined Analysis of mRNA Translation by Sucrose Gradient Fractionation. *Bio Protoc*, 7(19), 2017. doi:10.21769/BioProtoc.2573.
- B. Albert, I. C. Kos-Braun, A. K. Henras, C. Dez, M. P. Rueda, X. Zhang, O. Gadal, M. Kos, and D. Shore. A ribosome assembly stress response regulates transcription to maintain proteome homeostasis. *eLife*, 8, 2019. doi:10.7554/eLife.45002.
- S. Alberti and A. A. Hyman. Biomolecular condensates at the nexus of cellular stress, protein aggregation disease and ageing. *Nature Reviews Molecular Cell Biology*, 22:196–213, 2021. doi:10.1038/s41580-020-00326-6.
- B. D. Alford and O. Brandman. Quantification of hsp90 availability reveals differential coupling to the heat shock response. *Journal of Cell Biology*, 217:3809–3816, 2018. doi:10.1083/jcb.201803127.
- B.D. Alford, E. Tassoni-Tsuchida, D. Khan, J.J. Work, G. Valiant, and O. Brandman. Reporterseq reveals genome-wide dynamic modulators of the heat shock response across diverse stressors. *Elife*, 10, 2021. doi:10.7554/eLife.57376.
- A. Ali, S. Paracha, and D. Pincus. Preserve or destroy: orphan protein proteostasis and the heat shock response. *Journal of Cell Biology*, 223:e202407123, 2024. doi:10.1083/jcb.202407123.
- Asif Ali, Rania Garde, Olivia C. Schaffer, Jared A. M. Bard, Kabir Husain, Samantha Keyport Kik, Kathleen A. Davis, Sofia Luengo-Woods, D. Allan Drummond, Allison H. Squires, and David Pincus. Adaptive preservation of orphan ribosomal proteins in chaperone-dispersed condensates. *Nature Cell Biology*, 25:1691–1703, 2023. doi:10.1038/s41556-023-01253-2.
- C.D. Altamirano and S. Alberti. Surviving the heat: the role of macromolecular assemblies in promoting cellular shutdown. *Trends in Biochemical Sciences*, 2024.
- J. Anandhakumar, Y.W. Moustafa, S. Chowdhary, A.S. Kainth, and D.S. Gross. Evidence for multiple mediator complexes in yeast independently recruited by activated heat shock factor. *Mol Cell Biol*, 36:1943–1960, 2016. doi:10.1128/MCB.00005-16.
- Julius Anckar and Lea Sistonen. Regulation of HSF1 function in the heat stress response: implications in aging and disease. *Annual Review of Biochemistry*, 80:1089–1115, 2011. doi:10.1146/annurev-biochem-060809-095203.
- P. W. Anderson. More is different. *Science*, 177(4047):393–6, 1972. doi:10.1126/science.177.4047.393.

- M. Azkanaz, A. Rodríguez López, B. de Boer, W. Huiting, P.-O. Angrand, E. Vellenga, H. H. Kampinga, S. Bergink, J. H. A. Martens, J. J. Schuringa, and V. van den Boom. Protein quality control in the nucleolus safeguards recovery of epigenetic regulators after heat shock. *eLife*, 8:e45205, 2019. doi:10.7554/elife.45205.
- N.A. Baker, D. Sept, S. Joseph, M.J. Holst, and J.A. McCammon. Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc Natl Acad Sci U S A*, 98:10037–10041, 2001. doi:10.1073/pnas.181342398.
- W. E. Balch, R. I. Morimoto, A. Dillin, and J. W. Kelly. Adapting proteostasis for disease intervention. *Science*, 319(5865):916–9, 2008. doi:10.1126/science.1141448.
- R. Baler, W. J. Welch, and R. Voellmy. Heat shock gene regulation by nascent polypeptides and denatured proteins: hsp70 as a potential autoregulatory factor. *Journal of Cell Biology*, 117:1151–1159, 1992. doi:10.1083/jcb.117.6.1151.
- Salman F. Banani, Hyun O. Lee, Anthony A. Hyman, and Michael K. Rosen. Biomolecular condensates: organizers of cellular biochemistry. *Nature Reviews Molecular Cell Biology*, 18:285–298, 2017. doi:10.1038/nrm.2017.7.
- David B. Berry and Audrey P. Gasch. Stress-activated Genomic Expression Changes Serve a Preparative Role for Impending Stress in Yeast. *Molecular Biology of the Cell*, 19(11):4580–4587, 2008. doi:10.1091/mbc.e07-07-0680.
- M. Boehning, C. Dugast-Darzacq, M. Rankovic, A.S. Hansen, T. Yu, H. Marie-Nelly, D.T. McSwiggen, G. Kokic, G.M. Dailey, P. Cramer, et al. Rna polymerase ii clustering through carboxy-terminal domain phase separation. *Nat Struct Mol Biol*, 25:833–840, 2018. doi:10.1038/s41594-018-0112-y.
- A. Boija, I.A. Klein, B.R. Sabari, A. Dall’Agnese, E.L. Coffey, A.V. Zamudio, C.H. Li, K. Shrinivas, J.C. Manteiga, N.M. Hannett, et al. Transcription factors activate genes through the phase-separation capacity of their activation domains. *Cell*, 175:1842–1855 e1816, 2018. doi:10.1016/j.cell.2018.10.042.
- O. Brandman, J. Stewart-Ornstein, D. Wong, A. Larson, C.C. Williams, G.W. Li, S. Zhou, D. King, P.S. Shen, J. Weibezahn, et al. A ribosome-bound quality control complex triggers degradation of nascent peptides and signals translation stress. *Cell*, 151:1042–1054, 2012. doi:10.1016/j.cell.2012.10.044.
- M. J. Brauer, C. Huttenhower, E. M. Airoidi, R. Rosenstein, J. C. Matese, D. Gresham, V. M. Boer, O. G. Troyanskaya, and D. Botstein. Coordination of growth rate, cell cycle, stress response, and metabolic activity in yeast. *Mol Biol Cell*, 19(1):352–67, 2008. doi:10.1091/mbc.e07-08-0779.
- J. L. Brewster and M. C. Gustin. Hog1: 20 years of discovery and impact. *Sci Signal*, 7(343):re7, 2014. doi:10.1126/scisignal.2005458.

- D.G. Brickner, S. Ahmed, L. Meldi, A. Thompson, W. Light, M. Young, T.L. Hickman, F. Chu, E. Fabre, and J.H. Brickner. Transcription factor binding to a dna zip code controls interchromosomal clustering at the nuclear periphery. *Developmental cell*, 22: 1234–1246, 2012.
- K. Brzezinka, S. Altmann, H. Czesnick, P. Nicolas, M. Gorka, E. Benke, T. Kabelitz, F. Jähne, A. Graf, C. Kappel, and I. Bäurle. Arabidopsis forgetter1 mediates stress-induced chromatin memory through nucleosome remodeling. *eLife*, 5:e17061, 2016. doi:10.7554/elife.17061.
- D.B. Brückner, H. Chen, L. Barinov, B. Zoller, and T. Gregor. Stochastic motion and transcriptional dynamics of pairs of distal dna loci on a compacted chromosome. *Science*, 380:1357–1362, 2023.
- L.V. Cairo, X. Hong, M.B. Müller, P. Yuste-Checa, C. Jagadeesan, A. Bracher, S.-H. Park, M. Hayer-Hartl, and F.U. Hartl. Stress-dependent condensate formation regulated by the ubiquitin-related modifier urm1. *Cell*, 187:4656–4673. e4628, 2024.
- A. P. Capaldi, T. Kaplan, Y. Liu, N. Habib, A. Regev, N. Friedman, and E. K. O’Shea. Structure and function of a transcriptional network activated by the MAPK Hog1. *Nat Genet*, 40(11):1300–6, 2008. doi:10.1038/ng.235.
- V. Cherkasov, S. Hofmann, S. Druffel-Augustin, A. Mogk, J. Tyedmers, G. Stoecklin, and B. Bukau. Coordination of translational control and protein homeostasis during severe heat stress. *Curr Biol*, 23(24):2452–62, 2013. doi:10.1016/j.cub.2013.09.058.
- W.-K. Cho, J.-H. Spille, M. Hecht, C. Lee, C. Li, V. Grube, and I.I. Cisse. Mediator and rna polymerase ii clusters associate in transcription-dependent condensates. *Science*, 361: 412–415, 2018.
- S. Chowdhary, A.S. Kainth, and D.S. Gross. Heat shock protein genes undergo dynamic alteration in their three-dimensional structure and genome organization in response to thermal stress. *Mol Cell Biol*, 37, 2017. doi:10.1128/MCB.00292-17.
- S. Chowdhary, A.S. Kainth, D. Pincus, and D.S. Gross. Heat shock factor 1 drives intergenic association of its target gene loci upon heat shock. *Cell Rep*, 26:1828 e15, 2019. doi:10.1016/j.celrep.2018.12.034.
- S. Chowdhary, A.S. Kainth, and D.S. Gross. Chromosome conformation capture that detects novel cis- and trans-interactions in budding yeast. *Methods*, 170:416, 2020. doi:10.1016/j.ymeth.2019.06.023.
- S. Chowdhary, A.S. Kainth, S. Paracha, D.S. Gross, and D. Pincus. Inducible transcriptional condensates drive 3d genome reorganization in the heat shock response. *Molecular Cell*, 82:4386–4399.e4387, 2022. doi:10.1016/j.molcel.2022.10.013.

- Surabhi Chowdhary, Sarah Paracha, Lucas Dyer, and David Pincus. Emergent 3D genome reorganization from the stepwise assembly of transcriptional condensates. *Nature*, 2025. doi:10.1101/2025.02.23.639564. bioRxiv preprint.
- M. Ciccarelli and C. Andréasson. Protein misfolding releases human HSF1 from HSP70 latency control. *Journal of Molecular Biology*, 436:168740, 2024. doi:10.1016/j.jmb.2024.168740.
- M. Costa-Mattioli and P. Walter. The integrated stress response: From mechanism to disease. *Science*, 368(6489), 2020. doi:10.1126/science.aat5314.
- J.J. Cotto, M. Kline, and R.I. Morimoto. Activation of heat shock factor 1 dna binding precedes stress-induced serine phosphorylation. *Evidence for a multistep pathway of regulation. J Biol Chem*, 271:3355–3358, 1996.
- Thomas M. Cover and Joy A. Thomas. *Elements of Information Theory*. Wiley-Interscience, Hoboken, NJ, 2nd edition, 2006. ISBN 978-0-471-24195-9.
- E. A. Craig and J. Marszalek. How do J-proteins get Hsp70 to do so many different things? *Trends in Biochemical Sciences*, 42:355–368, 2017. doi:10.1016/j.tibs.2017.02.007.
- Eulàlia de Nadal, Gustav Ammerer, and Francesc Posas. Controlling gene expression in response to stress. *Nature Reviews Genetics*, 12(12):833–845, 2011. doi:10.1038/nrg3055.
- Annisa Dea and David Pincus. The Heat Shock Response as a Condensate Cascade. *Journal of Molecular Biology*, 436(14):168642, 2024. doi:10.1016/j.jmb.2024.168642.
- Annisa Dea, Yongqing Lan, Benjamin A. Doran, Asif Ali, Maya G. Igarashi, Lucas Dyer, Valeryia Aksianiuk, David Pincus, and Arjun S. Raman. Physiological architecture and evolutionary origins of cellular adaptability. Manuscript in preparation, 2026.
- A. Dobin, C.A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, and T.R. Gingeras. Star: ultrafast universal rna-seq aligner. *Bioinformatics*, 29:15–21, 2013. doi:10.1093/bioinformatics/bts635.
- Benjamin A. Doran, Robert Y. Chen, Hannah Giba, Vivek Behera, Bidisha Barat, Anitha Sundararajan, Huaiying Lin, Ashley Sidebottom, Eric G. Pamer, and Arjun S. Raman. Subspecies phylogeny in the human gut revealed by co-evolutionary constraints across the bacterial kingdom. *Cell Systems*, 16(2), 2025. doi:10.1016/j.cels.2025.01.001.
- M. Du, S.H. Stitzinger, J.-H. Spille, W.-K. Cho, C. Lee, M. Hijaz, A. Quintana, and I.I. Cissé. Direct observation of a condensate effect on super-enhancer controlled gene bursting. *Cell*, 187:331–344. e317, 2024.
- Z. Duan, M. Andronescu, K. Schutz, S. McIlwain, Y. J. Kim, C. Lee, J. Shendure, S. Fields, C. A. Blau, and W. S. Noble. A three-dimensional model of the yeast genome. *Nature*, 465:363–367, 2010. doi:10.1038/nature08973.

- F.M. Duarte, N.J. Fuda, D.B. Mahat, L.J. Core, M.J. Guertin, and J.T. Lis. Transcription factors gaf and hsf act at distinct regulatory steps to modulate stress-induced gene activation. *Genes Dev*, 30:1731–1746, 2016. doi:10.1101/gad.284430.116.
- Jeffrey N. Dudley, Joel E. Berends, Chayan K. De, Tongchen He, Giovana B. Veronezi, Benedict Abdon, Anjali Sengar, Matthias C. Truttmann, Mats Ljungman, Lanbo Xiao, Srinivas Ramachandran, and Sethuramasundaram Pitchaiya. Divergent condensates tune transcriptional responses during stress. *bioRxiv*, page 2026.02.12.705659, 2026. doi:10.64898/2026.02.12.705659.
- A.M. Erkin, S.F. Magroan, E.A. Sekinger, and D.S. Gross. Cooperative binding of heat shock factor to the yeast hsp82 promoter in vivo and in vitro. *Mol Cell Biol*, 19:1627–1639, 1999.
- S. Escusa-Toret, W. I. M. Vonk, and J. Frydman. Spatial sequestration of misfolded proteins by a dynamic chaperone pathway enhances cellular fitness during stress. *Nature Cell Biology*, 15:1231–1243, 2013. doi:10.1038/ncb2838.
- R. Evans, M. O’Neill, A. Pritzel, N. Antropova, A. Senior, T. Green, A. Žídek, R. Bates, S. Blackwell, and J. Yim. Protein complex prediction with alphafold-multimer. *bioRxiv*, 2021. bioRxiv. doi: 10.1101/2021.10.04.463034.
- O. Faust, M. Abayev-Avraham, A. S. Wentink, M. Maurer, N. B. Nillegoda, N. London, B. Bukau, and R. Rosenzweig. HSP40 proteins use class-specific regulation to drive HSP70 functional diversity. *Nature*, 587:489–494, 2020. doi:10.1038/s41586-020-2906-4.
- Z. A. Feder, A. Ali, A. Singh, J. Krakowiak, X. Zheng, V. P. Bindokas, D. Wolfgeher, S. J. Kron, and D. Pincus. Subcellular localization of the J-protein Sis1 regulates the heat shock response. *Journal of Cell Biology*, 220, 2021. doi:10.1083/jcb.202005165.
- F. Frotin, F. Schueder, S. Tiwary, R. Gupta, R. Korner, T. Schlichthaerle, J. Cox, R. Jungmann, F. U. Hartl, and M. S. Hipp. The nucleolus functions as a phase-separated protein quality control compartment. *Science*, 365(6451):342–347, 2019. doi:10.1126/science.aaw9157.
- G. Gaglia, R. Rashid, C. Yapp, G. N. Joshi, C. G. Li, S. L. Lindquist, K. A. Sarosiek, L. Whitesell, P. K. Sorger, and S. Santagata. HSF1 phase transition mediates stress adaptation and cell fate decisions. *Nature Cell Biology*, 22:151–168, 2020. doi:10.1038/s41556-019-0458-3.
- R. Garde, A. Singh, A. Ali, and D. Pincus. Transcriptional regulation of Sis1 promotes fitness but not feedback in the heat shock response. *eLife*, 12, 2023. doi:10.7554/eLife.79444.
- R. Garde, A. Dea, M.F. Herwig, A. Ali, and D. Pincus. Feedback control of the heat shock response by spatiotemporal regulation of hsp70. *Journal of Cell Biology*, 223, 2024.

- A. P. Gasch and M. Werner-Washburne. The genomics of yeast responses to environmental stress and starvation. *Funct Integr Genomics*, 2(4-5):181–92, 2002. doi:10.1007/s10142-002-0058-2.
- A. P. Gasch, F. B. Yu, J. Hose, L. E. Escalante, M. Place, R. Bacher, J. Kanbar, D. Ciobanu, L. Sandor, I. V. Grigoriev, C. Kendzierski, S. R. Quake, and M. N. McClean. Single-cell RNA sequencing reveals intrinsic and extrinsic regulatory heterogeneity in yeast responding to stress. *PLoS Biol*, 15(12):e2004050, 2017. doi:10.1371/journal.pbio.2004050.
- Audrey P. Gasch, Paul T. Spellman, Cheryl M. Kao, Orna Carmel-Harel, Michael B. Eisen, Gisela Storz, David Botstein, and Patrick O. Brown. Genomic expression programs in the response of yeast cells to environmental changes. *Molecular Biology of the Cell*, 11(12):4241–4257, 2000. doi:10.1091/mbc.11.12.4241.
- H. Glauninger, C.J. Wong Hickernell, J.A.M. Bard, and D.A. Drummond. Stressful steps: Progress and challenges in understanding stress-induced mrna condensation and accumulation in stress granules. *Mol Cell*, 82:2544–2556, 2022. doi:10.1016/j.molcel.2022.05.014.
- H. Glauninger, J.A.M. Bard, C.J.W. Hickernell, E.M. Airoidi, W. Li, R.H. Singer, S. Paul, J. Fei, T.R. Sosnick, E.W.J. Wallace, and D.A. Drummond. Transcriptome-wide mrna condensation precedes stress granule formation and excludes stress-induced transcripts. bioRxiv, 2024. bioRxiv. doi: 10.1101/2024.04.15.589678.
- R. Gomez-Pastor, E. T. Burchfiel, and D. J. Thiele. Regulation of heat shock transcription factors and their roles in physiology and disease. *Nature Reviews Molecular Cell Biology*, 19:4–19, 2018. doi:10.1038/nrm.2017.73.
- Y.E. Guo, J.C. Manteiga, J.E. Henninger, B.R. Sabari, A. Dall’Agnese, N.M. Hannett, J.H. Spille, L.K. Afeyan, A.V. Zamudio, K. Shrinivas, et al. Pol ii phosphorylation regulates a switch between transcriptional and splicing condensates. *Nature*, 572:543–548, 2019. doi:10.1038/s41586-019-1464-0.
- J. Ignacio Gutierrez, Gregory P. Brittingham, Yonca Karadeniz, Kathleen D. Tran, Arnob Dutta, Alex S. Holehouse, Craig L. Peterson, and Liam J. Holt. SWI/SNF senses carbon starvation with a pH-sensitive low-complexity sequence. *eLife*, 11:e70344, 2022. doi:10.7554/eLife.70344.
- Nan Hao and Erin K O’shea. Signal-dependent dynamics of transcription factor translocation controls gene expression. *Nature structural & molecular biology*, 19(1):31–39, 2012.
- Nan Hao, Bogdan A Budnik, Jeremy Gunawardena, and Erin K O’Shea. Tunable signal processing through modular control of transcription factor translocation. *Science*, 339(6118):460–464, 2013.
- F. U. Hartl, A. Bracher, and M. Hayer-Hartl. Molecular chaperones in protein folding and proteostasis. *Nature*, 475(7356):324–332, 2011. doi:10.1038/nature10317.

- H. Haruki, J. Nishikawa, and U.K. Laemmli. The anchor-away technique: rapid, conditional establishment of yeast mutant phenotypes. *Mol. Cell*, 31:925–932, 2008. doi:10.1016/j.molcel.2008.07.020.
- J.E. Henninger, O. Oksuz, K. Shrinivas, I. Sagi, G. LeRoy, M.M. Zheng, J.O. Andrews, A.V. Zamudio, C. Lazaris, and N.M. Hannett. Rna-mediated feedback control of transcriptional condensates. *Cell*, 184:207–225. e224, 2021.
- N. Hentze, L. Le Breton, J. Wiesner, G. Kempf, and M. P. Mayer. Molecular mechanism of thermosensory function of human heat shock transcription factor hsf1. *eLife*, 5:e11576, 2016. doi:10.7554/elife.11576.
- A.D. Herbert, A.M. Carr, and E. Hoffmann. Findfoci: a focus detection algorithm with automated parameter training that closely matches human assignments, reduces human inconsistencies and increases speed of analysis. *PLoS One*, 9:e114749, 2014. doi:10.1371/journal.pone.0114749.
- V. Hietakangas, J.K. Ahlskog, A.M. Jakobsson, M. Hellesuo, N.M. Sahlberg, C.I. Holmberg, A. Mikhailov, J.J. Palvimo, L. Pirkkala, and L. Sistonen. Phosphorylation of serine 303 is a prerequisite for the stress-inducible sumo modification of heat shock factor 1. *Mol Cell Biol*, 23:2953–2968, 2003.
- M. S. Hipp, P. Kasturi, and F. U. Hartl. The proteostasis network and its decline in ageing. *Nature Reviews Molecular Cell Biology*, 20(7):421–435, 2019. doi:10.1038/s41580-019-0101-y.
- D. Hnisz, K. Shrinivas, R.A. Young, A.K. Chakraborty, and P.A. Sharp. A phase separation model for transcriptional control. *Cell*, 169:13–23, 2017. doi:10.1016/j.cell.2017.02.007.
- C.-T. Ho, T. Grousl, O. Shatz, A. Jawed, C. Ruger-Herreros, M. Semmelink, R. Zahn, K. Richter, B. Bukau, and A. Mogk. Cellular sequestrases maintain basal hsp70 capacity ensuring balanced proteostasis. *Nature Communications*, 10:4851, 2019. doi:10.1038/s41467-019-12868-1.
- Erich Hochuli, Werner Bannwarth, Hansjorg Döbeli, Reiner Gentz, and Dieter Stüber. Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. *Nature Biotechnology*, 6(11):1321–1325, 1988. doi:10.1038/nbt1188-1321.
- C.I. Holmberg, V. Hietakangas, A. Mikhailov, J.O. Rantanen, M. Kallio, A. Meinander, J. Hellman, N. Morrice, C. MacKintosh, R.I. Morimoto, et al. Phosphorylation of serine 230 promotes inducible transcriptional activity of heat shock factor 1. *The EMBO journal*, 20:3800–3810, 2001.
- A. Horovitz. Double-mutant cycles: a powerful tool for analyzing protein structure and function. *Folding and Design*, 1:R121–R126, 1996.

- C. Iserman, C. Desroches Altamirano, C. Jegers, U. Friedrich, T. Zarin, A.W. Fritsch, M. Mittasch, A. Domingues, L. Hersemann, M. Jahnelt, et al. Condensation of ded1p promotes a translational switch from housekeeping to stress protein production. *Cell*, 181: 818–831 e819, 2020. doi:10.1016/j.cell.2020.04.009.
- F. Jacob and J. Monod. Genetic regulatory mechanisms in the synthesis of proteins. *J Mol Biol*, 3:318–56, 1961. doi:10.1016/s0022-2836(61)80072-7.
- Abbas Jariani, Lieselotte Vermeersch, Bram Cerulus, Gemma Perez-Samper, Karin Voordeckers, Thomas Van Brussel, Bernard Thienpont, Diether Lambrechts, and Kevin J Verstrepen. A new protocol for single-cell RNA-seq reveals stochastic gene expression during lag phase in budding yeast. *elife*, 9:e55320, 2020.
- J. Joutsen and L. Sistonen. Tailoring of proteostasis networks with heat shock factors. *Cold Spring Harb Perspect Biol*, 11, 2019. doi:10.1101/cshperspect.a034066.
- John Jumper, Richard Evans, Alexander Pritzel, Tim Green, Michael Figurnov, Olaf Ronneberger, Kathryn Tunyasuvunakool, Russ Bates, Augustin Židek, Anna Potapenko, Alex Bridgland, Clemens Meyer, Simon A. A. Kohl, Andrew J. Ballard, Andrew Cowie, Bernardino Romera-Paredes, Stanislav Nikolov, Rishub Jain, Jonas Adler, Trevor Back, Stig Petersen, David Reiman, Ellen Clancy, Michal Zielinski, Martin Steinegger, Michalina Pacholska, Tamas Berghammer, Sebastian Bodenstein, David Silver, Oriol Vinyals, Andrew W. Senior, Koray Kavukcuoglu, Pushmeet Kohli, and Demis Hassabis. Highly accurate protein structure prediction with AlphaFold. *Nature*, 596(7873):583–589, 2021. doi:10.1038/s41586-021-03819-2.
- D. Kaganovich, R. Kopito, and J. Frydman. Misfolded proteins partition between two distinct quality control compartments. *Nature*, 454:1088–1095, 2008. doi:10.1038/nature07195.
- A.S. Kainth, S. Chowdhary, D. Pincus, and D.S. Gross. Primordial superenhancers: heat shock-induced chromatin organization in yeast. *Trends in cell biology*, 31:801–813, 2021. doi:10.1016/j.tcb.2021.04.004.
- Tarique Khan, Tejbir S. Kandola, Jianzheng Wu, Shriram Venkatesan, Ellen Ketter, Jeffrey J. Lange, Alejandro Rodríguez Gama, Andrew Box, Jay R. Unruh, Malcolm Cook, and Randal Halfmann. Quantifying nucleation in vivo reveals the physical basis of prion-like phase behavior. *Molecular Cell*, 71:155–168.e7, 2018. doi:10.1016/j.molcel.2018.06.016.
- S. Kim and D.S. Gross. Mediator recruitment to heat shock genes requires dual hsf1 activation domains and mediator tail subunits med15 and med16. *J Biol Chem*, 288:12197–12213, 2013. doi:10.1074/jbc.M112.449553.
- Justin B. Kinney and Gurinder S. Atwal. Equitability, mutual information, and the maximal information coefficient. *Proceedings of the National Academy of Sciences*, 111(9):3354–3359, 2014. doi:10.1073/pnas.1309933111.

- C. L. Klaips, M. H. M. Gropp, M. S. Hipp, and F. U. Hartl. Sis1 potentiates the stress response to protein aggregation and elevated temperature. *Nat Commun*, 11(1):6271, 2020. doi:10.1038/s41467-020-20000-x.
- M.P. Kline and R.I. Morimoto. Repression of the heat shock factor 1 transcriptional activation domain is modulated by constitutive phosphorylation. *Mol Cell Biol*, 17:2107–2115, 1997.
- S. W. Kmiecik and M. P. Mayer. Molecular mechanisms of heat shock factor 1 regulation. *Trends in Biochemical Sciences*, 47:218–234, 2022. doi:10.1016/j.tibs.2021.10.004.
- Nikos Kourtis and Nektarios Tavernarakis. Cellular stress response pathways and ageing: intricate molecular relationships. *The EMBO journal*, 30(13):2520–2531, 2011.
- Joanna Krakowiak, Xu Zheng, Nikit Patel, Zoë A. Feder, Jayamani Anandhakumar, Kendra Valerius, David S. Gross, Ahmad S. Khalil, and David Pincus. Hsf1 and Hsp70 constitute a two-component feedback loop that regulates the yeast heat shock response. *eLife*, 7:e31668, 2018. doi:10.7554/eLife.31668.
- S. Kroschwald, S. Maharana, and A. Simon. Hexanediol: a chemical probe to investigate the material properties of membrane-less compartments. *Matters*, 2017. doi:10.19185/matters.201702000010.
- E. F. Kuiper, P. Gallardo, T. Gebauer, S. Mattijssen, M. P. Bagelaar, D. S. Verbeek, D. K. Bhatt, C. T. Dekker, A. Kortholt, et al. The chaperone DNAJB6 surveils FG-nucleoporins and is required for interphase nuclear pore complex biogenesis. *Nature Cell Biology*, 24:1584–1594, 2022. doi:10.1038/s41556-022-01010-x.
- J. R. Lakowicz. *Principles of Fluorescence Spectroscopy*. Springer, 3rd edition, 2006. doi:10.1007/978-0-387-46312-4.
- H. Li, B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, R. Durbin, and S. Genome Project Data Processing. The sequence alignment/map format and samtools. *Bioinformatics*, 25:2078–2079, 2009. doi:10.1093/bioinformatics/btp352.
- S. Lindquist. The heat-shock response. *Annual Review of Biochemistry*, 55:1151–1191, 1986. doi:10.1146/annurev.bi.55.070186.005443.
- Martina Loibl, Ingrid Klein, Michael Prattes, Christian Schmidt, Lisa Kappel, Gerlinde Zisser, Anna Gungl, Emma Krieger, Brigitte Pertschy, and Helmut Bergler. The drug diazaborine blocks ribosome biogenesis by inhibiting the AAA-ATPase Drg1. *Journal of Biological Chemistry*, 289(7):3913–3922, 2014. doi:10.1074/jbc.M113.536110.
- M.I. Love, W. Huber, and S. Anders. Moderated estimation of fold change and dispersion for rna-seq data with deseq2. *Genome Biol*, 15:550, 2014. doi:10.1186/s13059-0140550-8.

- Y. Lu, T. Wu, O. Gutman, H. Lu, Q. Zhou, Y.I. Henis, and K. Luo. Phase separation of taz compartmentalizes the transcription machinery to promote gene expression. *Nat Cell Biol*, 22:453–464, 2020. doi:10.1038/s41556-020-0485-0.
- D.G. Lupianez, K. Kraft, V. Heinrich, P. Krawitz, F. Brancati, E. Klopocki, D. Horn, H. Kayserili, J.M. Opitz, R. Laxova, et al. Disruptions of topological chromatin domains cause pathogenic rewiring of gene-enhancer interactions. *Cell*, 161:1012–1025, 2015. doi:10.1016/j.cell.2015.04.004.
- H. Lyons, R.T. Veettil, P. Pradhan, C. Fornero, N. De La Cruz, K. Ito, M. Eppert, R.G. Roeder, and B.R. Sabari. Functional partitioning of transcriptional regulators by patterned charge blocks. *Cell*, 186:327–345 e328, 2023. doi:10.1016/j.cell.2022.12.013.
- Q. Ma. Role of nrf2 in oxidative stress and toxicity. *Annu Rev Pharmacol Toxicol*, 53:401–26, 2013. doi:10.1146/annurev-pharmtox-011112-140320.
- Hiten D. Madhani and Gerald R. Fink. Combinatorial Control Required for the Specificity of Yeast MAPK Signaling. *Science*, 275(5304):1314–1317, 1997. doi:10.1126/science.275.5304.1314.
- Dig Bijay Mahat, Hector H. Salamanca, Fabiana M. Duarte, Charles G. Danko, and John T. Lis. Mammalian heat shock response and mechanisms underlying its genome-wide transcriptional regulation. *Molecular Cell*, 62(1):63–78, 2016. doi:10.1016/j.molcel.2016.02.025.
- G. Male, S. Mohajan, L. S. Rubio, and D. S. Gross. Mediator tail subunits hierarchically couple transcriptional condensates to gene activation and genome organization. *bioRxiv*, 2026. doi:10.64898/2026.04.21.719956. bioRxiv preprint.
- D. Malinverni, S. Marsili, A. Barducci, and P. De Los Rios. Large-scale conformational transitions and dimerization are encoded in the amino-acid sequences of hsp70 chaperones. *PLoS computational biology*, 11:e1004262, 2015.
- A. E. Masser, W. Kang, J. Roy, J. M. Kaimal, J. Quintana-Cordero, M. R. Friedländer, and C. Andréasson. Cytoplasmic protein misfolding titrates Hsp70 to activate nuclear Hsf1. *eLife*, 8, 2019. doi:10.7554/eLife.47791.
- Megan N. McClean, Areez Mody, James R. Broach, and Sharad Ramanathan. Cross-talk and decision making in MAP kinase pathways. *Nature Genetics*, 39(3):409–414, 2007. doi:10.1038/ng1957.
- Marc L. Mendillo, Sandro Santagata, Martina Koeva, George W. Bell, Rong Hu, Rulla M. Tamimi, Ernest Fraenkel, Tan A. Ince, Luke Whitesell, and Susan Lindquist. HSF1 drives a transcriptional program distinct from heat shock to support highly malignant human cancers. *Cell*, 150(3):549–562, 2012. doi:10.1016/j.cell.2012.06.031.
- Annamaria Merico, Pavol Sulo, Jure Piškur, and Concetta Compagno. Fermentative lifestyle in yeasts belonging to the *Saccharomyces* complex. *The FEBS journal*, 274(4):976–989, 2007.

- M. Meurer, Y. Duan, E. Sass, I. Kats, K. Herbst, B. C. Buchmuller, V. Dederer, F. Huber, D. Kirrmaier, M. Stefl, et al. Genome-wide C-SWAT library for high-throughput yeast genome tagging. *Nature Methods*, 15:598–600, 2018. doi:10.1038/s41592-018-0045-8.
- S. B. M. Miller, C.-T. Ho, J. Winkler, M. Khokhrina, A. Neuner, M. Y. H. Mohamed, D. L. Guilbride, K. Richter, M. Lisby, E. Schiebel, A. Mogk, and B. Bukau. Compartment-specific aggregates direct distinct nuclear and cytoplasmic aggregate deposition. *The EMBO Journal*, 34:778–797, 2015. doi:10.15252/emboj.201489524.
- Tayla Miller, Jianzheng Wu, Shriram Venkatesan, Alejandro Rodriguez Gama, and Randal Halfmann. DAMFRET measures saturating concentrations and toxicities of protein phase transitions in vivo. *Molecular Biology of the Cell*, 34:br7, 2023. doi:10.1091/mbc.E22-10-0458.
- S. Mohajan, L. S. Rubio, and D. S. Gross. Nuclear basket proteins nup2 and mlp1 drive heat shock-induced 3d genome restructuring downstream of transcriptional activation. *Journal of Biological Chemistry*, 301:110568, 2025. doi:10.1016/j.jbc.2025.110568.
- K. A. Morano, C. M. Grant, and W. S. Moye-Rowley. The response to heat shock and oxidative stress in *saccharomyces cerevisiae*. *Genetics*, 190:1157–1195, 2012. doi:10.1534/genetics.111.128033.
- R. I. Morimoto. The heat shock response: systems biology of proteotoxic stress in aging and disease. *Cold Spring Harb Symp Quant Biol*, 76:91–9, 2011. doi:10.1101/sqb.2012.76.010637.
- R.I. Morimoto. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev.*, 12:3788–3796, 1998.
- D. Muzzey, C. A. Gomez-Urbe, J. T. Mettetal, and A. van Oudenaarden. A systems-level analysis of perfect adaptation in yeast osmoregulation. *Cell*, 138(1):160–71, 2009. doi:10.1016/j.cell.2009.04.047.
- Mariona Nadal-Ribelles, Saiful Islam, Wu Wei, Pablo Latorre, Michelle Nguyen, Eulàlia de Nadal, Francesc Posas, and Lars M Steinmetz. Sensitive high-throughput single-cell RNA-seq reveals within-clonal transcript correlations in yeast populations. *Nature Microbiology*, 4(4):683–692, 2019.
- R.R. Nair, D. Zabezhinsky, R. Gelin-Licht, B.J. Haas, M.C. Dyhr, H.S. Sperber, C. Nussbaum, and J.E. Gerst. Multiplexed mRNA assembly into ribonucleoprotein particles plays an operon-like role in the control of yeast cell physiology. *Elife*, 10, 2021. doi:10.7554/eLife.66050.
- R.R. Nair, E. Pataki, and J.E. Gerst. Transperons: RNA operons as effectors of coordinated gene expression in eukaryotes. *Trends Genet*, 2022. doi:10.1016/j.tig.2022.07.005.

- S.J. Nair, L. Yang, D. Meluzzi, S. Oh, F. Yang, M.J. Friedman, S. Wang, T. Suter, I. Al-shareedah, A. Gamliel, et al. Phase separation of ligand-activated enhancers licenses cooperative chromosomal enhancer assembly. *Nat Struct Mol Biol*, 26:193–203, 2019. doi:10.1038/s41594-019-0190-5.
- Abani Neferkara, Asif Ali, and David Pincus. CellQuant: a vibecoder’s guide to image analysis. *bioRxiv*, 2026. doi:10.64898/2026.03.09.710634.
- A. Németh, A. Conesa, J. Santoyo-Lopez, I. Medina, D. Montaner, B. Péterfia, I. Solovei, T. Cremer, J. Dopazo, and G. Längst. Initial genomics of the human nucleolus. *PLoS Genetics*, 6:e1000889, 2010. doi:10.1371/journal.pgen.1000889.
- A. Omid, M.H. Møller, N. Malhis, J.M. Bui, and J. Gsponer. AlphaFold-multimer accurately captures interactions and dynamics of intrinsically disordered protein regions. *Proceedings of the National Academy of Sciences*, 121:e2406407121, 2024.
- Karolina Pakos-Zebrucka, Izabela Koryga, Katarzyna Mnich, Mila Ljubic, Afshin Samali, and Adrienne M Gorman. The integrated stress response. *EMBO reports*, 17(10):1374–1395, 2016.
- M. Palacio and D.J. Taatjes. Real-time visualization of reconstituted transcription reveals rna polymerase ii activation mechanisms at single promoters. *bioRxiv*, 2025. bioRxiv. doi: 10.1101/2025.01.06.631569.
- Liam Paninski. Estimation of entropy and mutual information. *Neural Computation*, 15(6): 1191–1253, 2003.
- E. Pataki and J.E. Gerst. Conservation of mrna operon formation in the control of the heat shock response in mammalian cells. *bioRxiv*, 2024. bioRxiv. doi: 10.1101/2024.10.05.616786.
- S. Pfeffer, D. Goncalves, and K.A. Morano. Regulation of the hsf1-dependent transcriptome via conserved bipartite contacts with hsp70 promotes survival in yeast. *J Biol Chem*, 294: 12191–12202, 2019. doi:10.1074/jbc.RA119.008822.
- G. Pei, H. Lyons, P. Li, and B.R. Sabari. Transcription regulation by biomolecular condensates. *Nature Reviews Molecular Cell Biology*, pages 1–24, 2024.
- B. Pertschy, C. Saveanu, G. Zisser, J. Griesenbeck, C. Preisinger, P. Tchelidze, C. Toloczyki, W. Rossmanith, R. Fournier, A. Albert, et al. Diazaborine treatment of yeast cells inhibits maturation of the 60S ribosomal subunit. *Molecular and Cellular Biology*, 27:6243–6255, 2007. doi:10.1128/MCB.00392-07.
- S. J. Petesch and J. T. Lis. Rapid, transcription-independent loss of nucleosomes over a large chromatin domain at hsp70 loci. *Cell*, 134:74–84, 2008. doi:10.1016/j.cell.2008.05.029.
- D. Pincus. Regulation of hsf1 and the heat shock response. *Adv Exp Med Biol*, 1243:41–50, 2020. doi:10.1007/978-3-030-40204-4_3.

- D. Pincus, J. Anandhakumar, P. Thiru, M.J. Guertin, A.M. Erkin, and D.S. Gross. Genetic and epigenetic determinants establish a continuum of hsf1 occupancy and activity across the yeast genome. *Mol Biol Cell*, 29:3168–3182, 2018. doi:10.1091/mbc.E1806-0353.
- A.E. Posey, A.S. Holehouse, and R.V. Pappu. Phase separation of intrinsically disordered proteins. *Methods in Enzymology*, pages 1–30, 2018.
- Ammon E. Posey, Kiersten M. Ruff, Jared M. Lalmansingh, Tejbir S. Kandola, Jeffrey J. Lange, Randal Halfmann, and Rohit V. Pappu. Mechanistic inferences from analysis of measurements of protein phase transitions in live cells. *Journal of Molecular Biology*, 433:166848, 2021. doi:10.1016/j.jmb.2021.166848.
- S. M. Prophet and C. Schlieker. Atypical nuclear envelope condensates linked to neurological disorders reveal nucleoporin-directed chaperone activities. *Nature Cell Biology*, 24:1561–1574, 2022. doi:10.1038/s41556-022-01001-y.
- G.H. Putri, S. Anders, P.T. Pyl, J.E. Pimanda, and F. Zanini. Analysing high-throughput sequencing data in python with htseq 2.0. *Bioinformatics*, 38:2943–2945, 2022. doi:10.1093/bioinformatics/btac166.
- S.K. Rabindran, R.I. Haroun, J. Clos, J. Wisniewski, and C. Wu. Regulation of heat shock factor trimer formation: role of a conserved leucine zipper. *Science*, 259:230234, 1993.
- P. Rawat, M. Boehning, B. Hummel, F. Aprile-Garcia, A.S. Pandit, N. Eisenhardt, A. Khavaran, E. Niskanen, S.M. Vos, J.J. Palvimo, et al. Stress-induced nuclear condensation of nelf drives transcriptional downregulation. *Mol Cell*, 81:1013–1026 e1011, 2021. doi:10.1016/j.molcel.2021.01.016.
- Q. Ren, L. Li, L. Liu, J. Li, C. Shi, Y. Sun, X. Yao, Z. Hou, and S. Xiang. The molecular mechanism of temperature-dependent phase separation of heat shock factor 1. *Nature Chemical Biology*, 21:831–842, 2025. doi:10.1038/s41589-024-01806-y.
- S. Rengachari, S. Schilbach, S. Aibara, C. Dienemann, and P. Cramer. Structure of the human mediator–rna polymerase ii pre-initiation complex. *Nature*, 594:129–133, 2021.
- J.A. Riback, C.D. Katanski, J.L. Kear-Scott, E.V. Pilipenko, A.E. Rojek, T.R. Sosnick, and D.A. Drummond. Stress-triggered phase separation is an adaptive, evolutionarily tuned response. *Cell*, 168:1028–1040 e1019, 2017. doi:10.1016/j.cell.2017.02.027.
- K. Richter, M. Haslbeck, and J. Buchner. The heat shock response: life on the verge of death. *Molecular Cell*, 40:253–266, 2010. doi:10.1016/j.molcel.2010.10.006.
- W.F. Richter, S. Nayak, J. Iwasa, and D.J. Taatjes. The mediator complex as a master regulator of transcription by rna polymerase ii. *Nature Reviews Molecular Cell Biology*, 23:732–749, 2022.

- Philip J. Robinson, Michael J. Trnka, David A. Bushnell, Ralph E. Davis, P.-J. Mattei, Alma L. Burlingame, and Roger D. Kornberg. Structure of a complete mediator-rna polymerase ii pre-initiation complex. *Cell*, 166:1411–1422.e1416, 2016.
- P.J. Robinson, M.J. Trnka, R. Pellarin, C.H. Greenberg, D.A. Bushnell, R. Davis, A.L. Burlingame, A. Sali, and R.D. Kornberg. Molecular architecture of the yeast mediator complex. *Elife*, 4:e08719, 2015. doi:10.7554/eLife.08719.
- L.A. Royer, M. Weigert, U. Gunther, N. Maghelli, F. Jug, I.F. Sbalzarini, and E.W. Myers. Clearvolume: open-source live 3d visualization for light-sheet microscopy. *Nat Methods*, 12:480–481, 2015. doi:10.1038/nmeth.3372.
- L.S. Rubio, S. Mohajan, and D.S. Gross. Heat shock factor 1 forms nuclear condensates and restructures the yeast genome before activating target genes. *Elife*, 12:RP92464, 2024.
- C.T. Rueden, J. Schindelin, M.C. Hiner, B.E. DeZonia, A.E. Walter, E.T. Arena, and K.W. Eliceiri. ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics*, 18:529, 2017. doi:10.1186/s12859-017-1934-z.
- C. J. Russo, K. Husain, R. Ranganathan, D. Pincus, and A. Murugan. Simple biological controllers drive the evolution of soft modes. *Proceedings of the National Academy of Sciences*, 123(17):e2523032123, 2026. doi:10.1073/pnas.2523032123.
- B.R. Sabari, A. Dall’Agnese, A. Boija, I.A. Klein, E.L. Coffey, K. Shrinivas, B.J. Abraham, N.M. Hannett, A.V. Zamudio, J.C. Manteiga, et al. Coactivator condensation at super-enhancers links phase separation and gene control. *Science*, 361, 2018. doi:10.1126/science.aar3958.
- A. Santiago and K. A. Morano. Oxidation of two cysteines within yeast hsp70 impairs proteostasis while directly triggering an hsf1-dependent cytoprotective response. *Journal of Biological Chemistry*, 298:102424, 2022. doi:10.1016/j.jbc.2022.102424.
- R. Sawarkar. Transcriptional lockdown during acute proteotoxic stress. *Trends Biochem Sci*, 47:660–672, 2022. doi:10.1016/j.tibs.2022.03.020.
- J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods*, 9:676–682, 2012. doi:10.1038/nmeth.2019.
- P.A. Sharp, A.K. Chakraborty, J.E. Henninger, and R.A. Young. Rna in formation and regulation of transcriptional condensates. *Rna*, 28:52–57, 2022.
- Y. Shi, D. D. Mosser, and R. I. Morimoto. Molecular chaperones as HSF1-specific transcriptional repressors. *Genes & Development*, 12:654–666, 1998. doi:10.1101/gad.12.5.654.
- Y. Shin and C.P. Brangwynne. Liquid phase condensation in cell physiology and disease. *Science*, 357, 2017. doi:10.1126/science.aaf4382.

- S. Shivaswamy, A. Bhinge, Y. Zhao, S. Jones, M. Hirst, and V. R. Iyer. Dynamic remodeling of individual nucleosomes across a eukaryotic genome in response to transcriptional perturbation. *PLoS Biology*, 6:e65, 2008. doi:10.1371/journal.pbio.0060065.
- Eric J. Solís, Jai P. Pandey, Xu Zheng, Dexter X. Jin, Piyush B. Gupta, Edoardo M. Airolidi, David Pincus, and Vladimir Denic. Defining the essential function of yeast Hsf1 reveals a compact transcriptional program for maintaining eukaryotic proteostasis. *Molecular Cell*, 63(1):60–71, 2016. doi:10.1016/j.molcel.2016.05.014.
- Nahum Sonenberg and Alan G. Hinnebusch. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell*, 136(4):731–745, 2009. doi:10.1016/j.cell.2009.01.042.
- E. M. Sontag, R. S. Samant, and J. Frydman. Mechanisms and functions of spatial protein quality control. *Annual Review of Biochemistry*, 86:97–122, 2017. doi:10.1146/annurev-biochem-060815-014616.
- P. K. Sorger. Yeast heat shock factor contains separable transient and sustained response transcriptional activators. *Cell*, 62:793–805, 1990. doi:10.1016/0092-8674(90)90123-V.
- P. K. Sorger and H. R. Pelham. Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. *Cell*, 54(6):855–64, 1988a.
- P. K. Sorger and H. R. B. Pelham. Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. *Cell*, 54:855–864, 1988b. doi:10.1016/S0092-8674(88)91219-6.
- P.K. Sorger and H.C.M. Nelson. Trimerization of a yeast transcriptional activator via a coiled-coil motif. *Cell*, 59:807–813, 1989.
- J. Soutourina. Transcription regulation by the mediator complex. *Nature reviews Molecular cell biology*, 19:262–274, 2018.
- S. Specht, S. B. M. Miller, A. Mogk, and B. Bukau. Hsp42 is required for sequestration of protein aggregates into deposition sites in *saccharomyces cerevisiae*. *Journal of Cell Biology*, 195:617–629, 2011. doi:10.1083/jcb.201106037.
- Jacob Stewart-Ornstein, Jonathan S Weissman, and Hana El-Samad. Cellular noise regulons underlie fluctuations in *Saccharomyces cerevisiae*. *Molecular cell*, 45(4):483–493, 2012.
- Gary D. Stormo. DNA binding sites: representation and discovery. *Bioinformatics*, 16(1):16–23, 2000. doi:10.1093/bioinformatics/16.1.16.
- C. Stringer, T. Wang, M. Michaelos, and M. Pachitariu. Cellpose: a generalist algorithm for cellular segmentation. *Nature Methods*, 18:100–106, 2021. doi:10.1038/s41592-020-01018-x.
- M.C. Sumner and J. Brickner. The nuclear pore complex as a transcription regulator. *Cold Spring Harbor Perspectives in Biology*, 14:a039438, 2022.

- J.E. Takakuwa, Nitika, L.E. Knighton, and A.W. Truman. Oligomerization of hsp70: current perspectives on regulation and function. *Frontiers in Molecular Biosciences*, 6:81, 2019.
- C.G. Triandafillou, C.D. Katanski, A.R. Dinner, and D.A. Drummond. Transient intracellular acidification regulates the core transcriptional heat shock response. *Elife*, 9, 2020. doi:10.7554/eLife.54880.
- J. Trojanowski, L. Frank, A. Rademacher, N. Mücke, P. Grigaitis, and K. Rippe. Transcription activation is enhanced by multivalent interactions independent of phase separation. *Molecular Cell*, 82:1878–1893.e10, 2022. doi:10.1016/j.molcel.2022.04.017.
- T. Tumbar, G. Sudlow, and A. S. Belmont. Large-scale chromatin unfolding and remodeling induced by vp16 acidic activation domain. *Journal of Cell Biology*, 145:1341–1354, 1999. doi:10.1083/jcb.145.7.1341.
- L.M. Tuttle, D. Pacheco, L. Warfield, D.B. Wilburn, S. Hahn, and R.E. Klevit. Mediator subunit med15 dictates the conserved "fuzzy" binding mechanism of yeast transcription activators gal4 and gcn4. *Nat Commun*, 12:2220, 2021. doi:10.1038/s41467-021-22441-4.
- B. W. Tye and L. S. Churchman. Hsf1 activation by proteotoxic stress requires concurrent protein synthesis. *Molecular Biology of the Cell*, 32:1800–1806, 2021. doi:10.1091/mbc.E21-01-0014.
- B. W. Tye, N. Commins, L. V. Ryazanova, M. Wuhr, M. Springer, D. Pincus, and L. S. Churchman. Proteotoxicity from aberrant ribosome biogenesis compromises cell fitness. *Elife*, 8, 2019. doi:10.7554/eLife.43002.
- R. Van Der Lee, M. Buljan, B. Lang, R.J. Weatheritt, G.W. Daughdrill, A.K. Dunker, M. Fuxreiter, J. Gough, J. Gsponer, and D.T. Jones. Classification of intrinsically disordered regions and proteins. *Chemical reviews*, 114:6589–6631, 2014.
- S. van Koningsbruggen, M. Gierliński, P. Schofield, D. Martin, G. J. Barton, Y. Ariyurek, J. T. den Dunnen, and A. I. Lamond. High-resolution whole-genome sequencing reveals that specific chromatin domains from most human chromosomes associate with nucleoli. *Molecular Biology of the Cell*, 21:3735–3748, 2010. doi:10.1091/mbc.e10-06-0508.
- Simon E Vidal, David Pincus, Jacob Stewart-Ornstein, and Hana El-Samad. Formation of Subnuclear Foci Is a Unique Spatial Behavior of Mating MAPKs during Hyperosmotic Stress. *Cell Reports*, 3(2):328–334, 2013. doi:10.1016/j.celrep.2013.01.022.
- V. Vinayachandran, R. Reja, M.J. Rossi, B. Park, L. Rieber, C. Mittal, S. Mahony, and B.F. Pugh. Widespread and precise reprogramming of yeast protein-genome interactions in response to heat shock. *Genome Res*, 2018. doi:10.1101/gr.226761.117.
- Wiebe Visser, W ALEXANDER Scheffers, WILMA H Batenburg-van der Vegte, and JOHANNES P van Dijken. Oxygen requirements of yeasts. *Applied and environmental microbiology*, 56(12):3785–3792, 1990.

- E. W. J. Wallace, J. L. Kear-Scott, E. V. Pilipenko, M. H. Schwartz, P. R. Laskowski, A. E. Rojek, C. D. Katanski, J. A. Riback, M. F. Dion, A. M. Franks, E. M. Airoidi, T. Pan, B. A. Budnik, and D. A. Drummond. Reversible, specific, active aggregates of endogenous proteins assemble upon heat stress. *Cell*, 162:1286–1298, 2015. doi:10.1016/j.cell.2015.08.041.
- Y. Wang, P. A. Gibney, J. D. West, and K. A. Morano. The yeast hsp70 ssa1 is a sensor for activation of the heat shock response by thiol-reactive compounds. *Molecular Biology of the Cell*, 23:3290–3298, 2012. doi:10.1091/mbc.e12-06-0447.
- J. R. Warner. The economics of ribosome biosynthesis in yeast. *Trends in Biochemical Sciences*, 24:437–440, 1999. doi:10.1016/S0968-0004(99)01460-7.
- M.T. Wei, Y.C. Chang, S.F. Shimobayashi, Y. Shin, A.R. Strom, and C.P. Brangwynne. Nucleated transcriptional condensates amplify gene expression. *Nat Cell Biol*, 22:1187–1196, 2020. doi:10.1038/s41556-020-00578-6.
- G. Wiederrecht, D. Seto, and C. S. Parker. Isolation of the gene encoding the *S. cerevisiae* heat shock transcription factor. *Cell*, 54:841–853, 1988. doi:10.1016/S0092-8674(88)91197-X.
- F. Alexander Wolf, Philipp Angerer, and Fabian J. Theis. SCANPY: large-scale single-cell gene expression data analysis. *Genome Biology*, 19(1):15, 2018. doi:10.1186/s13059-017-1382-0.
- W. Xia, Y. Guo, N. Vilaboa, J. Zuo, and R. Voellmy. Transcriptional activation of heat shock factor hsf1 probed by phosphopeptide analysis of factor 32p-labeled in vivo. *J Biol Chem*, 273:8749–8755, 1998.
- A. Yamamoto, J. Ueda, N. Yamamoto, N. Hashikawa, and H. Sakurai. Role of heat shock transcription factor in *saccharomyces cerevisiae* oxidative stress response. *Eukaryot Cell*, 6:1373–1379, 2007. doi:10.1128/EC.00098-07.
- Akira Yamamoto, Yasuhiro Mizukami, and Hiroshi Sakurai. Identification of a novel class of target genes and a novel type of binding sequence of heat shock transcription factor in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, 280(12):11911–11919, 2005. doi:10.1074/jbc.M411256200.
- H. Yoo, C. Triandafillou, and D.A. Drummond. Cellular sensing by phase separation: Using the process, not just the products. *J Biol Chem*, 294:7151–7159, 2019. doi:10.1074/jbc.TM118.001191.
- A.V. Zamudio, A. Dall’Agnese, J.E. Henninger, J.C. Manteiga, L.K. Afeyan, N.M. Hannett, E.L. Coffey, C.H. Li, O. Oksuz, B.R. Sabari, et al. Mediator condensates localize signaling factors to key cell identity genes. *Mol Cell*, 76:753–766 e756, 2019. doi:10.1016/j.molcel.2019.08.016.

- G. Zander, A. Hackmann, L. Bender, D. Becker, T. Lingner, G. Salinas, and H. Krebber. mrna quality control is bypassed for immediate export of stress-responsive transcripts. *Nature*, 540:593–596, 2016. doi:10.1038/nature20572.
- M. A. Zaydman, A. S. Little, F. Haro, V. Aksianiuk, W. J. Buchser, A. DiAntonio, J. I. Gordon, J. Milbrandt, and A. S. Raman. Defining hierarchical protein interaction networks from spectral analysis of bacterial proteomes. *Elife*, 11, 2022. doi:10.7554/eLife.74104.
- H. Zhang, S. Shao, Y. Zeng, X. Wang, Y. Qin, Q. Ren, S. Xiang, Y. Wang, J. Xiao, and Y. Sun. Reversible phase separation of hsf1 is required for an acute transcriptional response during heat shock. *Nat Cell Biol*, 24:340–352, 2022. doi:10.1038/s41556-022-00846-7.
- M. Zhang, H. Chang, Y. Zhang, J. Yu, L. Wu, W. Ji, J. Chen, B. Liu, J. Lu, Y. Liu, et al. Rational design of true monomeric and bright photoactivatable fluorescent proteins. *Nature Methods*, 9:727–729, 2012. doi:10.1038/nmeth.2021.
- X. Zheng, J. Krakowiak, N. Patel, A. Beyzavi, J. Ezike, A.S. Khalil, and D. Pincus. Dynamic control of hsf1 during heat shock by a chaperone switch and phosphorylation. *Elife*, 5, 2016. doi:10.7554/eLife.18638.
- X. Zheng, A. Beyzavi, J. Krakowiak, N. Patel, A. S. Khalil, and D. Pincus. Hsf1 phosphorylation generates cell-to-cell variation in hsp90 levels and promotes phenotypic plasticity. *Cell Reports*, 22(12):3099–3106, 2018. doi:10.1016/j.celrep.2018.02.083.
- D.H. Zhou, J. Jeon, N. Farheen, L.J. Friedman, J. Kondev, S. Buratowski, and J. Gelles. Mechanisms of synergistic mediator recruitment in rna polymerase ii transcription activation revealed by single-molecule fluorescence. *bioRxiv*, 2024. *bioRxiv*. doi:10.1101/2024.12.10.627625.
- B.M. Zid and E.K. O’Shea. Promoter sequences direct cytoplasmic localization and translation of mrnas during starvation in yeast. *Nature*, 514:117–121, 2014. doi:10.1038/nature13578.
- C. E. Zimmerli, M. Allegretti, V. Rantos, S. Lenz, G. Karius, G. Vergani, Y. E. Murray, P. A. Penczek, J. Kosinski, and M. Beck. Nuclear pores dilate and constrict in cellulose. *Science*, 374:eabd9776, 2021. doi:10.1126/science.abd9776.