

ARTICLE

Feedback control of the heat shock response by spatiotemporal regulation of Hsp70

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Cells maintain homeostasis via dynamic regulation of stress response pathways. Stress pathways transiently induce response regulons via negative feedback loops, but the extent to which individual genes provide feedback has not been comprehensively measured for any pathway. Here, we disrupted the induction of each gene in the *Saccharomyces cerevisiae* heat shock response (HSR) and quantified cell growth and HSR dynamics following heat shock. The screen revealed a core feedback loop governing the expression of the chaperone Hsp70 reinforced by an auxiliary feedback loop controlling Hsp70 subcellular localization. Mathematical modeling and live imaging demonstrated that multiple HSR targets converge to promote Hsp70 nuclear localization via its release from cytosolic condensates. Following ethanol stress, a distinct set of factors similarly converged on Hsp70, suggesting that nonredundant subsets of the HSR regulon confer feedback under different conditions. Flexible spatiotemporal feedback loops may broadly organize stress response regulons and expand their adaptive capacity.

Introduction

Stress response pathways enable cells to adapt to environmental changes and survive. Stress responses deactivate processes that are no longer adaptive and induce new gene expression programs for survival and growth in the new conditions. However, to avoid overshooting adaptation, the stress response must also efficiently turn off. It is unclear how response dynamics are tuned by the induction of individual downstream target genes. How and to what extent do stress responses integrate feedback from their suite of effectors?

The heat shock response (HSR) is an ancient, conserved, and prototypical stress response pathway under the control of the master regulator Hsf1 in eukaryotes (Joutsen and Sistonen, 2019; Pincus, 2020). When an environmental stressor or internal dysfunction causes an excess of newly synthesized/unfolded, misfolded, or mistargeted proteins to accumulate in the cytosol or nucleus, Hsf1 transcriptionally induces molecular chaperones and other factors involved in protein folding, disaggregation, and degradation (Kainth et al., 2021; Solis et al., 2016). Once protein homeostasis (proteostasis) is restored, molecular chaperones become available to bind and deactivate Hsf1. Previously, the heat shock-induced, Hsf1-dependent transcriptome was characterized in *Saccharomyces cerevisiae* using genomic and chemical genetic methods, revealing a compact set of 42 target genes that both show increased Hsf1 occupancy in their

enhancer region and are dependent on Hsf1 for their transcription during heat shock (Pincus et al., 2018). Here, we ask how the transcriptional induction of these 42 individual genes—collectively referred to as the HSR regulon—determines the dynamics of Hsf1 activity.

In addition to addressing basic questions of adaptive regulation of stress response pathways, understanding how cells dynamically control Hsf1 activity is relevant to human health. Indeed, Hsf1 misregulation in both directions—either too much or too little activity—is associated with disease. In aggressive cancers, Hsf1 is often hyperactivated or overexpressed due to Hsf1 locus amplification (Alasady and Mendillo, 2020). This increased Hsf1 activity not only induces Hsp90 and other chaperones to support the folding of oncoproteins but also drives a cancer-specific gene expression program that supports malignancy both in the tumor cells and the supporting microenvironment (Dai et al., 2007; Mendillo et al., 2012; Santagata et al., 2011, 2013; Scherz-Shouval et al., 2014). On the other hand, in neurodegenerative disorders, aggregates of proteins remain unresolved and are thought to sequester chaperones, hampering general cellular processes and triggering further protein aggregation (Balch et al., 2008; Sala et al., 2017). As such, loss of Hsf1 has been implicated in Huntington's disease, and increasing Hsf1 activity has been proposed as a therapeutic avenue for

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neurodegenerative disease with broad potential (Gomez-Pastor et al., 2017, 2018; Neef et al., 2011). Thus, resolving how Hsf1 activity is tuned in healthy cells may inform these disease mechanisms.

Mechanistically, Hsf1 is regulated by an Hsp70-based negative feedback loop (Garde et al., 2023; Krakowiak et al., 2018). The chaperone Hsp70 directly binds and represses Hsf1 in the nucleoplasm under non-stress conditions (Masser et al., 2019; Zheng et al., 2016). Upon heat shock, Hsp70 dissociates from Hsf1 and is targeted to protein condensates in the cytoplasm and nucleolar periphery via its cofactor, the J-domain protein Sis1 (Ali et al., 2023; Feder et al., 2021). This leaves Hsf1 free so that it can itself form active transcriptional condensates and induce its target genes, including multiple Hsp70 paralogs (Chowdhary et al., 2019, 2022). Induction of Hsp70 is required for Hsf1 deactivation, and within 15 min of heat shock, Hsf1 is rebound by Hsp70, and Hsf1 transcriptional activity is repressed again (Krakowiak et al., 2018; Masser et al., 2019). Thus, Hsf1 is dynamically tuned via its direct interactions with Hsp70. While these precedents were established in yeast, the same mechanisms have been shown to be largely conserved in mammalian cells (Kmiciek et al., 2020; Kmiciek and Mayer, 2022; Zhang et al., 2022; Ciccarelli and Andréasson, 2024).

Immediately upon heat shock, prior to or coincident with induction of the HSR regulon, the translation initiation machinery and mRNAs form reversible condensates known as stress granules (Cherkasov et al., 2013; Glauninger et al., 2022; Tauber et al., 2020; Yang et al., 2020). Stress granules are regulated by chaperones including Hsp70, J-domain proteins, Hsp104, small heat shock proteins, and Hsp90—all of which are Hsf1 targets (Kolhe et al., 2023; Mateju et al., 2017; Yoo et al., 2022). In addition to protein–RNA condensates, protein-only condensates and secretory vesicles also recruit these same chaperones during heat shock (Babazadeh et al., 2019; Escusa-Toret et al., 2013; Miller et al., 2015a, 2015b; Sontag et al., 2017, 2023). Therefore, though Hsp70-based negative regulation is the only direct Hsf1 regulation known, other targets may influence Hsf1 directly or indirectly by regulating the localization of the chaperone machinery to the various stress-induced condensates sublocalized to regions of the cytosol or nucleus.

Here, we collected $>10^7$ single-cell fluorescence measurements and $>10^4$ growth measurements to comprehensively dissect feedback regulation in the HSR. First, we characterized the transcriptional dynamics of each HSR target gene during heat stress in *S. cerevisiae*. Next, we disrupted the transcriptional induction of each target gene by deleting the 9–25 bp Hsf1 binding region in the upstream regulatory region via CRISPR/Cas9-mediated genome editing and observed how impaired induction of each target influences global output of the HSR and growth at elevated temperature. Additionally, we repeated the regulon-wide screen in response to ethanol rather than heat shock. Finally, using mathematical modeling and live cell imaging, we demonstrated that the feedback architecture of the HSR is remarkably simple: a core feedback loop controlling the expression of Hsp70 is reinforced by an auxiliary feedback loop—comprised of condition-specific subsets of the HSR regulon—controlling the interaction of Hsp70 with cytosolic

condensates and thereby regulating Hsp70 nuclear localization. Such a flexible feedback hierarchy that converges to control both expression and subcellular localization of key effectors may broadly characterize stress response regulons.

Results

Regulon-wide measurement of HSR gene expression dynamics

Previously, nascent transcript sequencing coupled to Hsf1 depletion and genome-wide Hsf1 ChIP-seq revealed that 42 target genes are directly bound by Hsf1 and dependent on Hsf1 for their transcription upon heat shock (Fig. 1 A) (Pincus et al., 2018). To establish the transcriptional dynamics of the HSR regulon at single cell resolution, we measured the expression of each Hsf1 target gene in 10^4 single cells at 10 time points over a 4-h heat shock time course. To this end, we generated a library in which we tagged each Hsf1 target gene in the genome at the 3' end with a P2A ribosome skip sequence followed by mScarlet (Fig. 1 B and Table S1). The polycistronic mRNAs expressed in the 42 reporter strains all have the same 3' untranslated regions, so differences in fluorescence signal should reflect differences in transcription more than mRNA stability. Consistent with previous RNA-seq experiments (Feder et al., 2021; Pincus et al., 2018; Solis et al., 2016), the expression level of Hsf1 target genes varies by three orders of magnitude across the regulon (Fig. 1 C). Regulon-wide basal expression level measured by mScarlet correlates well with transcript measured by sequencing ($r = 0.82$, Fig. S1 A). Upon heat shock, all target genes were induced over the time course with magnitudes ranging from $<10\%$ to >8 -fold (Fig. 1 D and Fig. S1 B).

We applied principal component analysis (PCA) to identify modes of variation across the dataset. Remarkably, 95% of the total variance was explained by the first principal component. Given the three orders of magnitude range expression, we found PC1 to be associated with basal mScarlet expression levels (Fig. 1 E). Previously, we showed that basal expression across the regulon is determined by a combination of the biochemical affinity of Hsf1 for each binding site and the accessibility of the binding site in the chromatin landscape (Pincus et al., 2018), so we already have insight into the molecular basis for this mode of variation. To focus on the variation in the dynamics, we performed PCA on the induced expression dataset (basal level-normalized). Plotting the resulting PC1 against PC2 reveals a semicontinuous manifold that corresponds to the time it takes for each gene to reach its half-maximum induction (Fig. 1 F). Upon further analysis, we observed an apparent tradeoff between the time to half-max induction and the maximum rate of induction (Fig. 1 G). These data imply that there is a continuum of gene expression profiles driven by Hsf1, ranging from genes that turn on rapidly, robustly, and transiently to those that turn on slowly, weakly but in a sustained fashion.

Regulon-wide screen for HSR feedback regulators during heat shock

To determine which Hsf1 target genes are feedback regulators of the HSR, we created two additional regulon-wide libraries by deleting the empirically determined Hsf1 binding sites—known

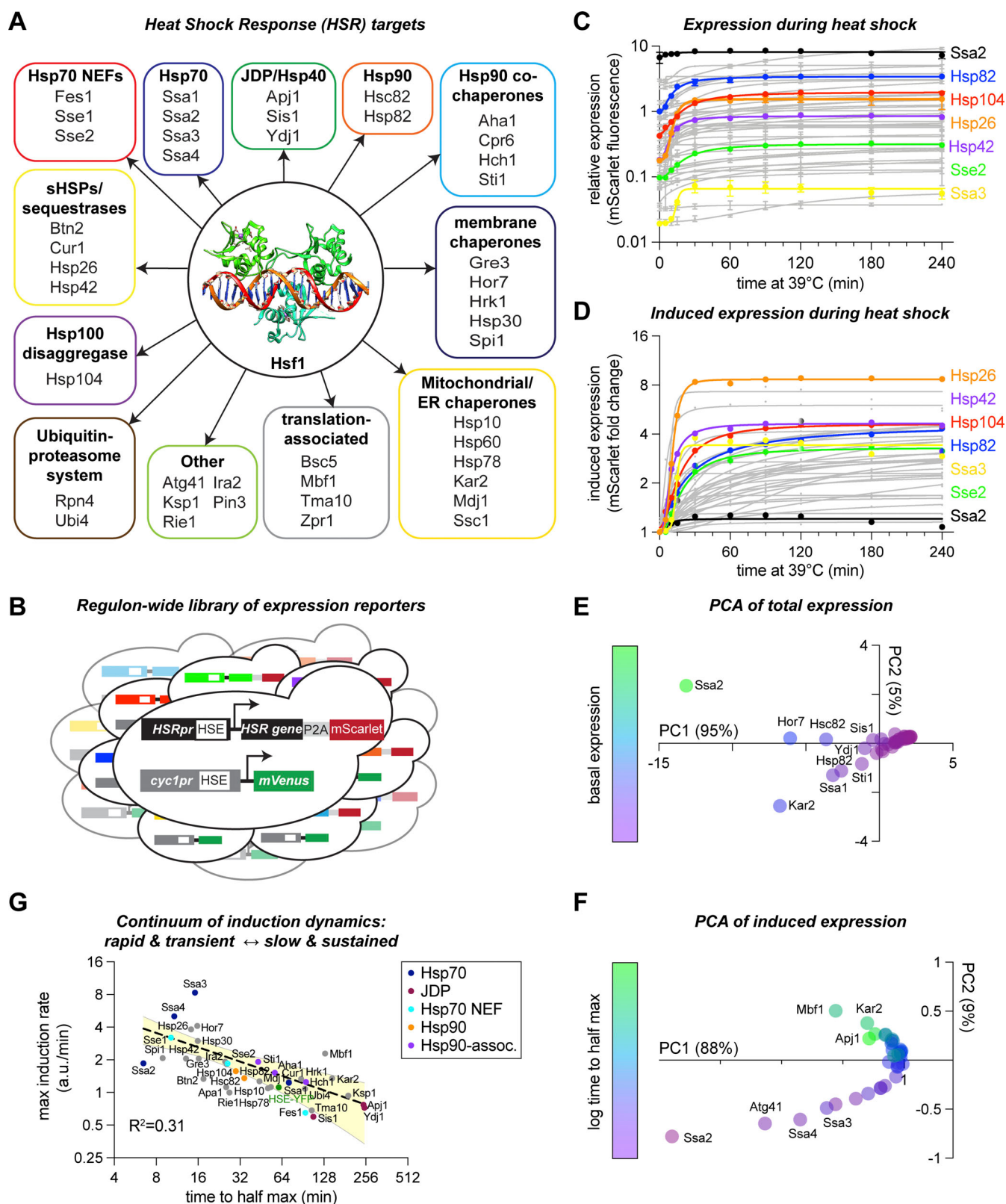


Figure 1. Regulon-wide measurement of HSR gene expression dynamics. (A) 42 genes are directly bound by Hsf1 and dependent on Hsf1 for their transcription. (B) Schematic of gene expression reporter strategy. P2A-mScarlet is fused to each gene in the HSR regulon for measurement in single cells. (C) mScarlet levels over a heat shock time course for all Hsf1 target genes, measured by flow cytometry. Each data point represents the mean of three biological replicates, connecting lines represent the non-linear curve fit for each time trace. (D) Data from C replotted to highlight fold change mScarlet during heat shock. (E) Principal component analysis (PCA) of the time course data in C showing coordinates of each gene in the first two PCs and color-coded by expression level. (F) PCA of the normalized data in D showing coordinates of each gene in the first two PCs and color coded by the log of the time to half max. (G) Maximum induction rate plotted versus time to half maximum expression calculated with parameter estimates from the nonlinear curve fits of induced expression. The yellow shaded area represents 95% CI on the bounds of the fit.

Garde et al.

HSR feedback screen

Journal of Cell Biology

3 of 16

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as heat shock elements (HSEs)—in the endogenous regulatory region of each target gene with scarless CRISPR/Cas9-mediated genome editing (see Materials and methods) (Fig. 2 A) (Pincus et al., 2018).

First, we generated HSE deletion (Δ HSE) strains in the mScarlet reporter library used above and quantified the extent to which each Δ HSE mutation alters the expression of the linked gene throughout the heat shock time course. We successfully generated Δ HSE strains for 39/42 genes in the HSR regulon. We were unable to obtain HSE deletions in the regulator regions of *AHA1*, *STII*, or *KSPI*, likely for technical rather than biological reasons. Among the set of Hsf1 target genes, *HSP26* is unique in having two distinct clusters of HSEs rather than a single Hsf1 binding peak. Simultaneous disruption of both sites nearly abrogated mScarlet induction, while individual disruption of each of the sites resulted in differential induction dynamics, separately impairing rapid and sustained induction (Fig. 2 B). Across the HSR regulon, HSE deletion reduced induction during heat shock for all genes except *HSP30*, *HOR7*, and *YDJ1* (Fig. 2 C). Residual heat shock-induced expression of these genes may be due to undefined *cis*-elements and/or transcription factors. Basal expression was also reduced for many genes, consistent with prior reports that Hsf1 drives constitutive expression of a subset of its target genes (de Jonge et al., 2017; Solís et al., 2016). However, there was little correlation between the reduction in basal mScarlet levels due to mutation of the HSE and overall activation of the HSR during heat shock as measured by a reporter ($R^2 = 0.08$) (Fig. S1 C).

Next, we constructed a second Δ HSE strain library in which each gene retains its endogenous 3' UTR to test the consequences of disrupting transcriptional induction without perturbing mRNA stability. The library also harbors a synthetic reporter of Hsf1 activity (HSE-YFP) integrated into the genome to serve as a standardized measurement of HSR activity in single cells (Zheng et al., 2016, 2018). As with the mScarlet reporters, we measured HSE-YFP levels in the Δ HSE library over 4 h of heat shock in individual cells by flow cytometry (Fig. 2 D). HSE-YFP levels varied fourfold across the mutants, but most mutants had HSE-YFP values after 4-h heat shock that fall within the wild type reproducibility range, suggesting—with the caveat that many Δ HSE mutants show incomplete loss of expression—that the induction of most Hsf1 targets neither directly regulates nor indirectly affects Hsf1 activity during heat shock (Fig. 2 E and Fig. S2 A).

We previously engineered a strain, *Hsp70 Δ FBL*, lacking Hsf1-dependent induction of the four genes encoding cytosolic Hsp70 in yeast (*ssa1/2/3/4*) that showed a pronounced defect in deactivating the HSE-YFP reporter following sustained heat shock (Krakowiak et al., 2018). None of the Δ HSE mutants approached the increased HSE-YFP levels we observed in *Hsp70 Δ FBL* (Fig. 2 E). However, six Δ HSE mutants showed significantly increased and three showed significantly reduced HSE-YFP levels compared with wild type after 4 h of heat shock ($P < 0.05$, two-tailed *t* test). The three mutants with reduced induction, *ssa2 Δ HSE*, *sis1 Δ HSE*, and *hsc82 Δ HSE*, all had increased basal HSE-YFP levels (Fig. 2 F and Fig. S2 B). The increased basal levels account for their reduced induction, consistent with previous reports that

these factors function as basal repressors (Alford et al., 2021; Brandman et al., 2012), indicating that these are not positive feedback regulators. By contrast, *fes1 Δ HSE*, *ubi4 Δ HSE*, *gre3 Δ HSE*, *pin3 Δ HSE*, *hsp42 Δ HSE*, and *hsp30 Δ HSE* showed elevated HSE-YFP levels after 4 h of heat shock. Except for *hsp30 Δ HSE*, in which the mScarlet reporter is still induced during heat shock (Fig. 2 C), these Δ HSE mutants are candidate negative feedback mutants.

Assessment of functional redundancy among key chaperone families

The three mutants with elevated HSE-YFP levels under basal conditions, *ssa2 Δ HSE*, *hsc82 Δ HSE*, and *sis1 Δ HSE*, encode members of the cytosolic Hsp70, Hsp90, and JDP chaperone families, respectively. In the HSR regulon, three additional genes encode cytosolic Hsp70 (*SSA1*, *SSA3*, and *SSA4*), one additional gene encodes Hsp90 (*HSP82*), and two additional genes encode JDPs (*APJ1* and *YDJ1*). Chaperones of each family may have redundant functions that can be revealed with multiple mutations; Hsp70 provides a demonstrative case. The relative expression level and induction dynamics of each Hsp70 paralog as measured by mScarlet levels span the full range of the library with the levels of *Ssa3* < *Ssa4* < *Ssa1* < *Ssa2* (Fig. 3 A, top). While none of the single Δ HSE mutants are candidate negative feedback regulators, *Hsp70 Δ FBL*—which lacks induction of all four paralogs—shows sustained and elevated HSE-YFP levels following heat shock (Fig. 3 A, bottom). Thus, induction of Hsp70 is required for HSR deactivation, establishing Hsp70 as a bona fide negative feedback regulator (Garde et al., 2023; Krakowiak et al., 2018).

Like the Hsp70 paralogs, the two genes encoding Hsp90, *HSC82*, and *HSP82*, also differ in their expression dynamics. While the basal expression of *Hsc82* > *Hsp82*, their expression levels converge over the heat shock time course (Fig. 3 B, top). To disrupt Hsp90 induction without disturbing its basal expression, we fused *HSC82* to the constitutive *TDH3* promoter matching the combined basal level of both paralogs in a strain deleted for the endogenous copies, generating *Hsp90 Δ FBL*. As opposed to *Hsp70 Δ FBL*, *Hsp90 Δ FBL* cells were able to deactivate the HSR upon sustained heat shock (Fig. 3 B, bottom). As such, Hsp90 does not fulfill the criteria of a negative feedback regulator of the HSR.

Due to our previous result demonstrating that *Sis1* is not a feedback regulator or the HSR (Garde et al., 2023), we wondered whether the other cytosolic JDPs encoded in the HSR regulon could be providing feedback. *Ydj1* and *Sis1* show comparable basal and induced expression levels, while *Apj1* is expressed an order of magnitude lower (Fig. 3 C, top). Previously, we engineered a strain expressing the only copy of *SIS1* from the *SUP35* promoter to disrupt induction upon heat shock while maintaining its high basal levels (Garde et al., 2023). We employed a similar promoter swapping strategy to set the expression of *Ydj1* near its basal levels in wild type cells by expressing it from the *RHO1* promoter (Fig. S3, A and B). Since *Apj1* is negligibly expressed under basal conditions, the Δ HSE mutant sufficed (Fig. S3, C and D). All these individual induction mutants showed wild type-like HSE-YFP induction profiles over a heat shock time course (Fig. 3 C, middle). Next, we disrupted the induction of

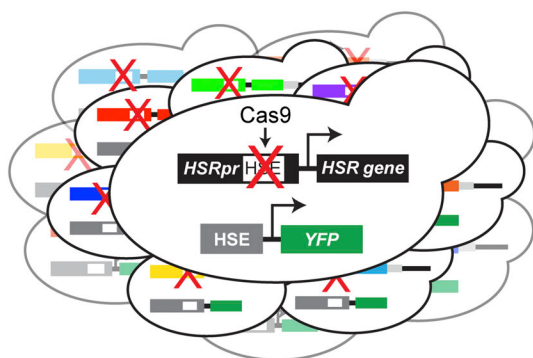
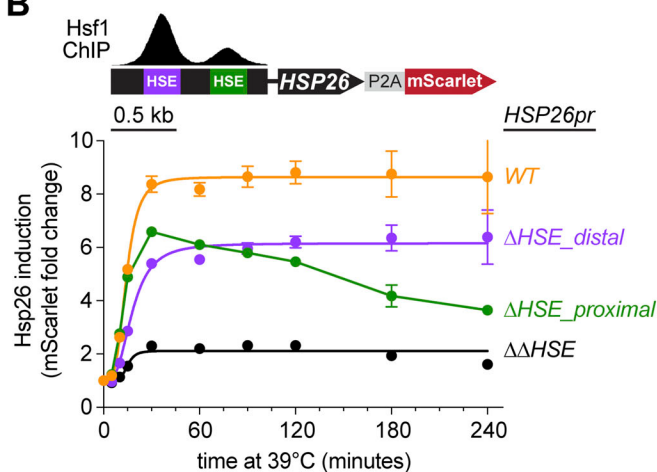
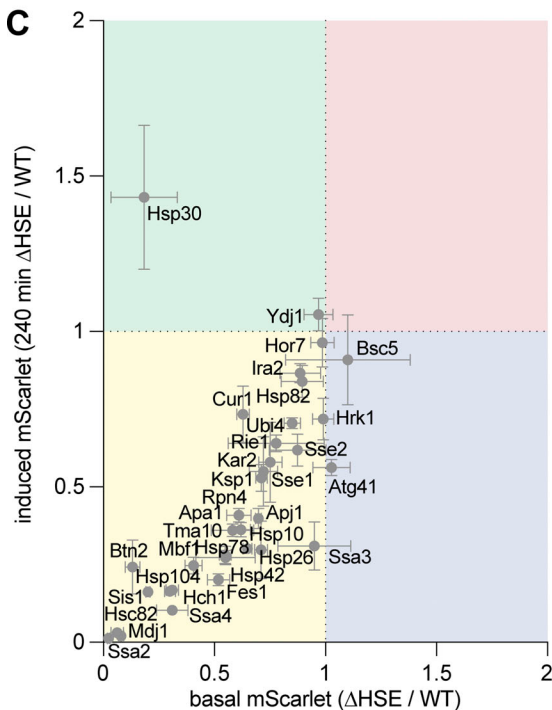
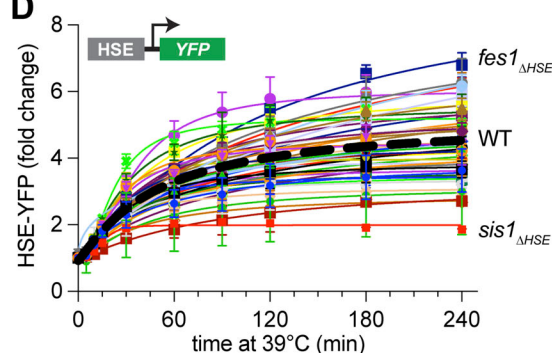
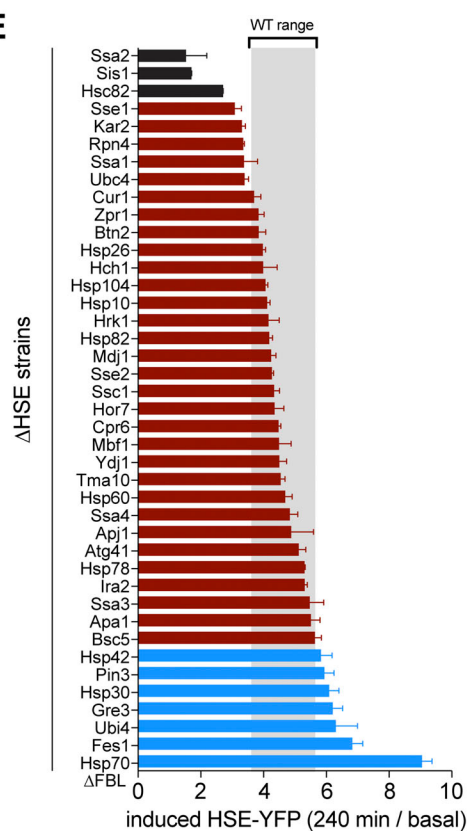
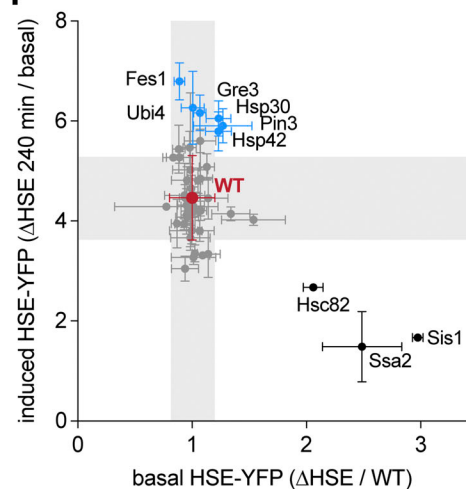
A Regulon-wide library of Δ HSE mutants**B****C****D****E****F**

Figure 2. **Regulon-wide screen for HSR feedback regulators during heat shock.** (A) Schematic of the library of Δ HSE mutants expressing the synthetic reporter of the HSR (HSE-YFP). (B) Top: Schematic of the modified *HSP26* locus with Hsf1 ChIP-seq peaks from 5 min of heat shock shown over the HSEs.

Bottom: Effect of single and double disruption of the HSEs in the *HSP26* promoter on the expression of P2A-mScarlet reporter over a heat shock time course. **(C)** mScarlet levels in each Δ HSE mutant relative to its wild type counterpart under plotted in basal conditions versus 4 h of heat shock. Each data point represents the mean and standard deviation of three biological replicates for both Δ HSE strain and wild type counterpart. **(D)** HSE-YFP fold change over a heat shock time course for all Δ HSE mutants, measured by flow cytometry. Dashed black line represents WT average. Each data point represents the mean and standard deviation of three biological replicates. **(E)** HSE-YFP fold change after 4 h of heat shock for each Δ HSE mutant and the Hsp70 Δ FBL mutant. WT range in gray represents the range of HSE-YFP levels across 20 biological replicates of WT. Cyan and black bars show significant outliers from the 95% CI. Bars show the mean of three biological replicates, error bars the standard deviation. **(F)** HSE-YFP level after 4 h of heat shock versus levels basal conditions for each Δ HSE mutant. Each data point represents the mean and standard deviation of three biological replicates. WT range in gray represents the mean and standard deviation of 20 biological replicates.

these JDPs in all pairwise combinations and all three at once. Like the single mutants, the double induction mutants—and even the triple mutant termed JDP Δ FBL—all induced HSE-YFP over a heat shock time course comparable to wild type (Fig. 3 C, bottom). These data indicate that induction of cytosolic JDPs is dispensable for feedback regulation of the HSR.

While initially characterizing the strains to perform these experiments, we observed that, in contrast to the *apj1* Δ HSE strain and the triple JDP Δ FBL strain, complete deletion of the gene encoding Apj1 had a pronounced effect on HSR dynamics. In *apj1* Δ cells, HSE-YFP levels are modestly elevated under basal conditions and induced and sustained during heat shock at substantially elevated levels (Fig. 3 D). Thus, while Apj1 is not a negative feedback regulator of the HSR, it is a negative regulator; its presence at basal levels is required for deactivation of the HSR. So, while JDPs are not feedback regulators, two different JDPs negatively regulate the HSR: Sis1 under basal conditions and Apj1 during sustained heat shock.

Growth measurements of HSR induction mutants at elevated temperature

Since only a small fraction of the HSR regulon confers negative feedback on the pathway during heat shock, we hypothesized that the induction of additional targets would be important for fitness at elevated temperatures. Notably, we previously found that Hsp70 Δ FBL—which has a strong HSR feedback phenotype—grows comparably to wild type at elevated temperatures, while induction of Sis1—which is dispensable for feedback regulation of the HSR—has a fitness defect during the diauxic shift, indicating that feedback and fitness can be uncoupled (Garde et al., 2023). To determine whether Δ HSE mutants have altered growth during heat shock, we measured quantitative growth curves for each mutant in the library relative to wild type cells in control and elevated temperature growth regimes (Fig. 4 A). Nine induction mutants had reduced maximal growth rates at 37°C relative to wild type, those affecting expression of Ydj1, Apj1, Gre3, Pin3, Ubi4, Ira2, Apa1, Hsp30, and Fes1 (Fig. 4 B). In addition, these mutants along with induction mutants of Sis1 and the mitochondrial chaperones showed growth phenotypes in the late stage of growth corresponding to the diauxic shift (Fig. 4 A). Of the nine mutants with reduced log phase growth, five were also candidate negative feedback regulators as defined by the HSE-YFP reporter assay (Fig. 4 C). Of all the induction mutants, only *hsp42* Δ HSE showed increased Hsf1 activity without a fitness defect, like Hsp70 Δ FBL. Thus, additional members of the HSR regulon confer fitness at

elevated temperatures. However, more than half the HSR induction mutants display neither feedback nor fitness defects.

Subcellular localization of chaperones in select induction mutants

To determine whether the mutants with both feedback and fitness phenotypes—*fes1* Δ HSE, *ubi4* Δ HSE, *pin3* Δ HSE, and *gre3* Δ HSE—display hallmarks of altered proteostasis, we imaged Hsp104-mKate, which marks cytosolic condensates upon heat shock (Krakowiak et al., 2018). We quantified the fraction of Hsp104-mKate localized to condensates in single cells under basal conditions and following 60 min of heat shock, a time point in which the feedback loops have been activated and the cells have largely restored proteostasis. In wild type cells, Hsp104-mKate is diffuse in the cytosol under basal conditions, and by 60 min of heat shock, <15% of Hsp104 remains condensed in the average cell (Fig. 5 A). In contrast, all four of the induction mutants with feedback and fitness phenotypes also showed elevated Hsp104 condensation under basal conditions. Three of them, *fes1* Δ HSE, *ubi4* Δ HSE, and *pin3* Δ HSE, also displayed large Hsp104-mKate condensates after 60 min of heat shock. These imaging data revealed that cytosolic proteostasis is disrupted in these mutants, suggesting that these factors impinge on Hsf1 activity indirectly.

Since Hsp104 cooperates with Hsp70 and its co-chaperones to disperse substrates (Tipton et al., 2008; Yoo et al., 2022), we hypothesized that the sustained cytosolic Hsp104 foci we observed would correspond to an increase in cytosolic localization of the key regulators of the HSR—Hsp70 and Sis1. This could result in decreased localization of Hsp70 and Sis1 to the nucleus leading to de-repression of Hsf1 and activation of the HSR. To test this, we generated a *fes1* Δ HSE strain—the induction mutant with the strongest HSE-YFP phenotype—expressing Halo-Ssa1 to image Hsp70, Sis1-mVenus, and Sec61-mScarlet to mark the nuclear boundary and cell cortex. Under basal conditions in both strains, Halo-Ssa1 was diffuse in the nucleus and cytosol, and Sis1-mVenus was concentrated in the nucleus (Fig. S4 A). Upon heat shock, both Halo-Ssa1 and Sis1-mVenus localized to cytoplasmic condensates in wild type and *fes1* Δ HSE cells, but Ssa1 and Sis1 formed a greater number of condensates in *fes1* Δ HSE cells than in wild type cells and the condensates persisted longer (Fig. 5, B–D). Correspondingly, *fes1* Δ HSE cells displayed a significantly reduced fraction of Halo-Ssa1 and Sis1-mVenus localized to the nucleus than wild type cells following 60 min of heat shock. Thus, induction of Fes1 is required to restore Hsp70 localization to the nucleus during sustained heat shock. Fes1 is a

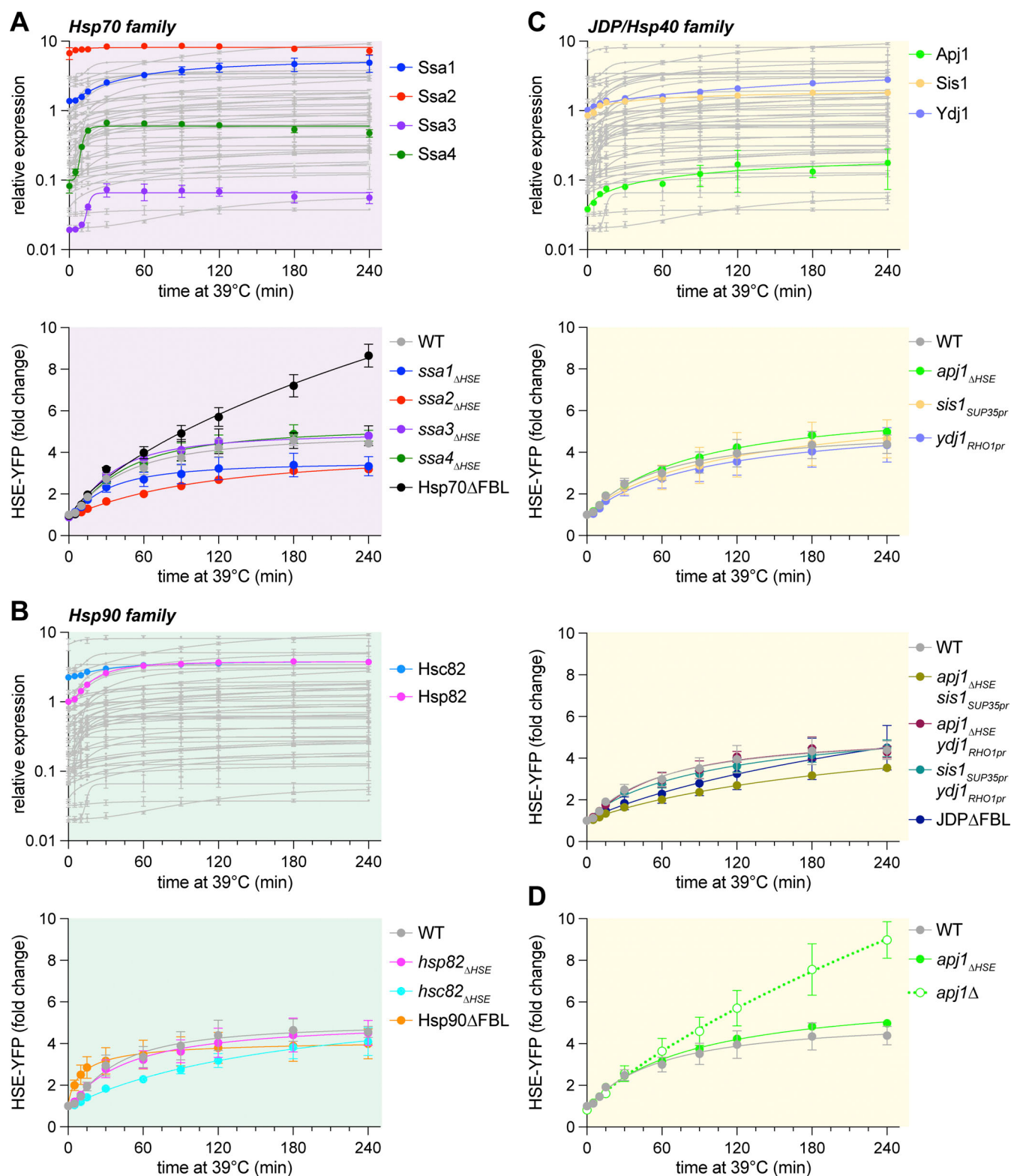


Figure 3. **Assessment of functional redundancy among Hsp70, Hsp90, and JDP families.** (A) Top: Expression of Hsp70 paralogs as measured by P2A-mScarlet over heat shock in color plotted over the full library in gray. Bottom: HSE-YFP fold change for single Hsp70 induction Δ HSE mutants and Hsp70 Δ FBL, in which none of the paralogs can be induced over a heat shock time course. Each data point represents the mean and standard deviation of three biological replicates. (B) As in A but for the Hsp90 paralogs. (C) Top as in A but for the JDPs. Middle: Individual JDP induction mutants. Bottom: Combined JDP induction mutants. (D) HSE-YFP fold change over a heat shock time course for WT, *apj1 Δ HSE*, and *apj1 Δ* . Each data point represents the mean and standard deviation of three biological replicates.

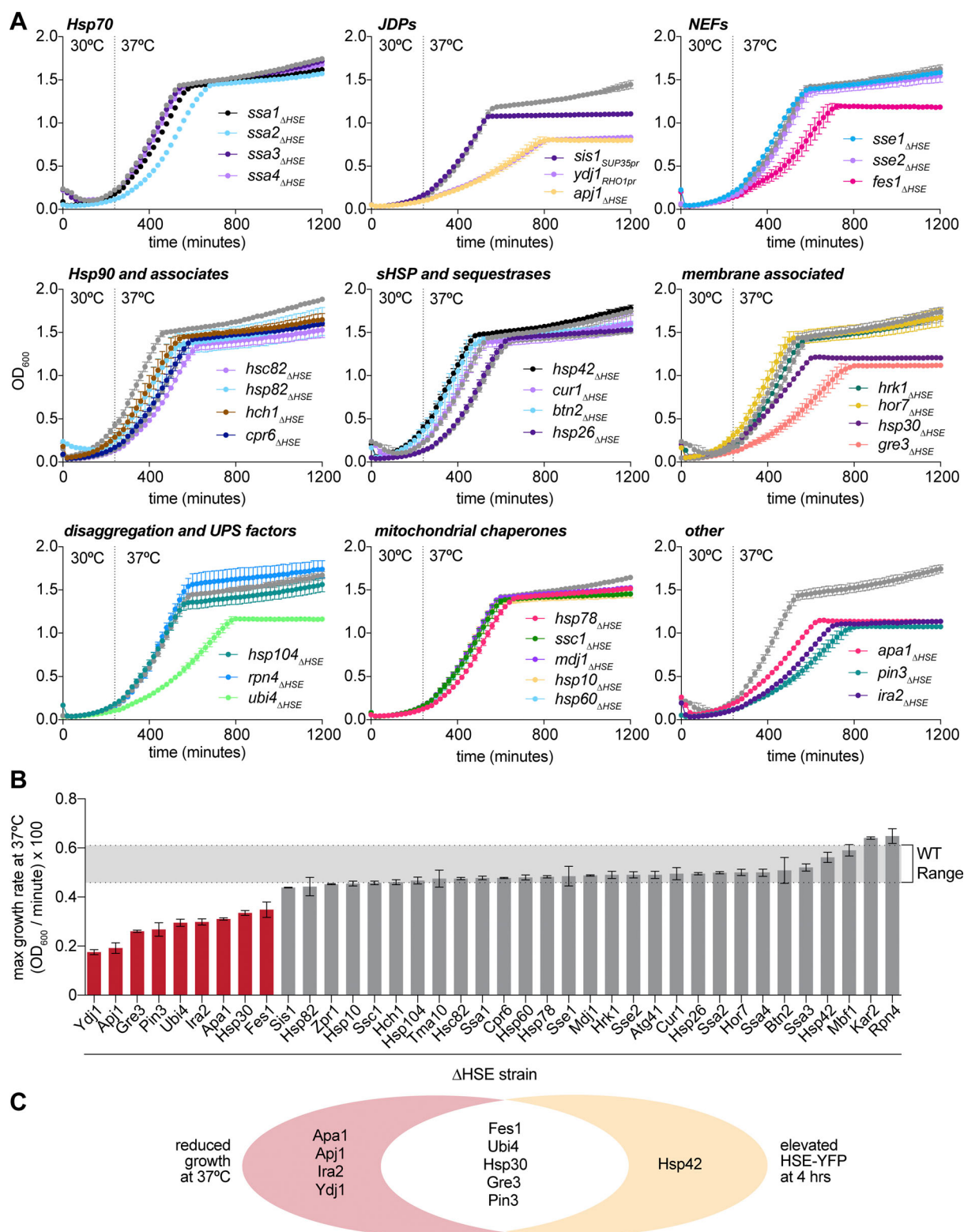


Figure 4. **Growth measurements of Δ HSE mutants.** (A) Quantitative growth assay of Hsf1 target induction mutants and WT (in gray) grown first at 30°C and switched to 37°C at the designated time, grouped by known cellular function. Each data point represents the mean optical density (OD₆₀₀) and error bars represent the standard deviation of the two biological replicates. (B) The log phase growth rate of each induction mutant (maximum derivative of the OD₆₀₀ curve). Bar height represents the mean of two biological replicates and error bar represents the range. (C) Venn diagram of Δ HSE mutants with growth and HSE-YFP phenotypes.

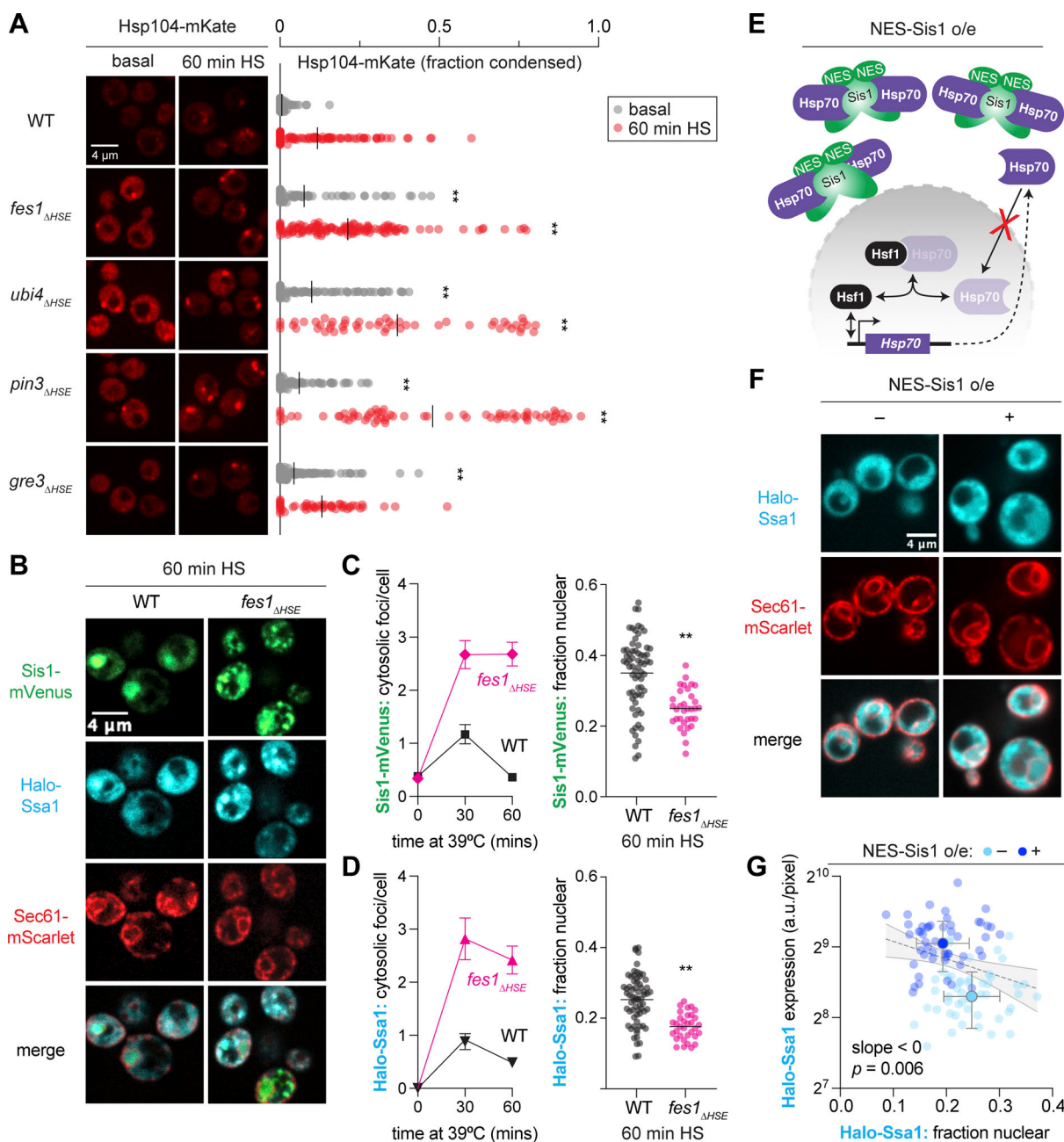


Figure 5. Subcellular localization of chaperones in select induction mutants. (A) Spinning disk confocal images of wild type and Δ HSE mutants expressing Hsp104-mKate. Scale bar is 4 μ m. The fraction of Hsp104-mKate signal condensed in each cell is quantified. Statistics: ** $P < 0.01$, two-tailed P value generated from unpaired t test. (B) Spinning disk confocal images of induction mutants expressing Halo-Ssa1 to monitor Hsp70 and Sis1-mVenus, and endogenously tagged Sec61-mScarlet to mark the nuclear boundary and cell cortex. Scale bar is 4 μ m. (C) Single cell count of condensates and quantification of nuclear localization of Sis1-mVenus during heat shock at 39°C. Each data point represents the average number of foci per cell and standard deviation of those counts for >25 cells. Statistics: ** $P < 0.01$, two-tailed P value generated from unpaired t test. (D) As in C but for Halo-Ssa1. (E) Schematic for experimental approach to reduce nuclear transport of Hsp70. Overexpression (o/e) of Sis1 tagged with a nuclear export sequence (NES) will partially sequester Hsp70 in the cytosol by virtue of strong binding interactions between Hsp70 and Sis1. (F) Cells expressing Halo-Ssa1 (Hsp70) and Sec61-mScarlet and harboring estradiol-inducible NES-Sis1 were grown in the absence or presence of 1 μ M estradiol for 12 h and imaged by spinning disk confocal microscopy. Scale bar is 4 μ m. (G) Single cell quantification of total Hsp70 levels throughout the cell (Halo-Ssa1 signal/pixel) as a function of the fraction of Hsp70 in the nucleus as defined by Sec61-mScarlet ($n = 50$ cells in each condition). Addition of estradiol shifted the distribution up and to the left, indicating that reduced nuclear Hsp70 corresponds to increased total Hsp70.

nucleotide exchange factor for Hsp70 that promotes client dissociation (Gowda et al., 2016; Masser et al., 2019), and this result suggests that Fes1 functions to release Hsp70 from cytosolic condensates to deactivate the HSR in the nucleus.

To test whether reducing the nuclear localization of Hsp70 is sufficient to increase HSR activity, we used a synthetic approach in which we overexpressed Sis1 fused to a nuclear export signal (NES). Since Sis1 binds directly to Hsp70, we reasoned that NES-

Sis1 would retain Hsp70 in the cytosol, thereby lowering the fraction of Hsp70 in the nucleus (Fig. 5 E). Since Sis1 functions as a dimer, we first demonstrated that overexpression of NES-Sis1 on top of Sis1-mVenus effectively resulted in titration of Sis1-mVenus from the nucleus to the cytosol (Fig. S4 B). Next, we imaged Halo-Ssa1 in a strain expressing Sec61-mScarlet to mark the nuclear boundary in the absence and presence of NES-Sis1 overexpression (Fig. 5 F). In this strain, we can measure both the fraction of Halo-Ssa1 in the nucleus and the total concentration of Halo-Ssa1 in each cell as a proxy for HSR activity. Upon NES-Sis1 overexpression, the fraction of nuclear Halo-Ssa1 was reduced and the total concentration of Halo-Ssa1 was increased relative to the control cells (Fig. 5 G). This result is consistent with our assumption that NES-Sis1 sequesters Hsp70 in the cytosol, though we do not directly test nuclear shuttling, and achieves the desired manipulation of reducing the fraction of Hsp70 in the nucleus. Single-cell quantification revealed a significant negative correlation between the fraction of Hsp70 in the nucleus and the total expression level of Hsp70 ($P = 0.006$). Thus, Hsp70 nuclear localization is negatively associated with total Hsp70 levels, suggesting that the fraction of Hsp70 in the nucleus is inversely proportional to HSR activity.

Mathematical model with feedback regulation of both Hsp70 expression and localization

We have refined a mathematical model of the HSR over the course of several studies (Feder et al., 2021; Garde et al., 2023; Krakowiak et al., 2018; Zheng et al., 2016). The model is based on a core two-component feedback loop, in which Hsf1 activates the expression of Hsp70 while Hsp70 represses the activity of Hsf1, which controls the dynamics of the transcriptional regulon as measured by the HSE-YFP reporter. Upon heat shock, we simulated a temperature-dependent decrease in the spontaneous folding rate of newly synthesized proteins, resulting in the accumulation of “clients” for Hsp70. Via an affinity switch that captures the titration of the JDP Sis1 away from Hsf1 by accumulated clients, Hsp70 dissociates from Hsf1, and Hsf1 induces expression of more Hsp70 until the system adapts to a new steady state (Feder et al., 2021).

To incorporate the roles of the novel feedback regulators, we first fit the model to *fes1 Δ HSE*. Architecturally, we modeled the action of Fes1 as promoting the productive release of Hsp70 from client proteins (Fig. 6, A and B). Mathematically, we modeled the *fes1 Δ HSE* strain by reducing the value of the parameter describing this productive release rate until we maximized the goodness of fit (Fig. S5). Simulation of heat shock time courses in wild type and *fes1 Δ HSE* cells quantitatively recapitulated the HSE-YFP induction dynamics we measured experimentally (Fig. 6 C). Aside from adjusting this single parameter, this updated model of the HSR required no further parameter adjustments nor any structural changes from the previous version. Indeed, tuning this same parameter also enabled us to recapitulate the dynamics of *gre3 Δ HSE*, *pin3 Δ HSE*, and *ubi4 Δ HSE* (Fig. 6, D and E), supporting the notion that these factors converge with Fes1 to enable efficient restoration of cytosolic proteostasis and Hsp70 client release. Notably, modulating this parameter failed to recapitulate the dynamic HSE-YFP response we observed in the

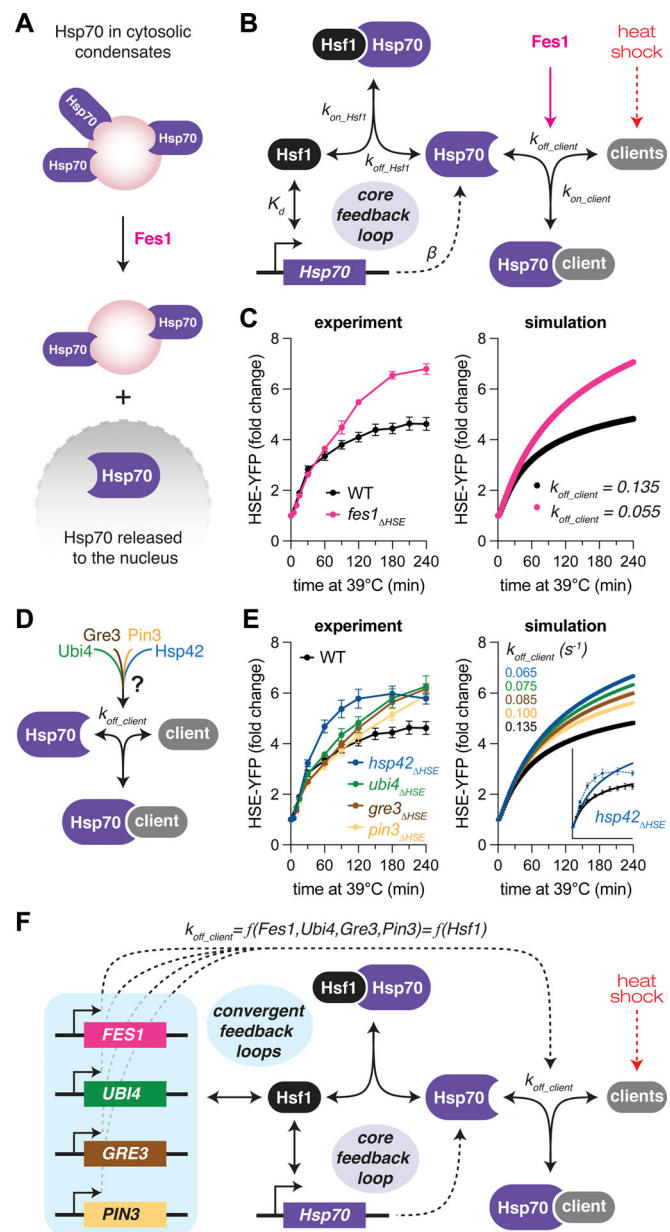


Figure 6. Model of HSR with feedback regulation of Hsp70 expression and localization. (A) Schematic of the role of Fes1 in releasing Hsp70 from cytosolic condensates. (B) Schematic of mathematical model of the HSR. (C) Experimental data and model simulations of HSE-YFP levels over heat shock time courses in wild type and *fes1 Δ HSE* cells. Experimental data points and error bars are mean and standard deviation of three biological replicates. (D) Schematic of convergence of auxiliary feedback factors on Hsp70 client release. (E) As in C but for the wild type, *ubi4 Δ HSE*, *gre3 Δ HSE*, *pin3 Δ HSE*, and *hsp42 Δ HSE*. (F) Schematic of the convergence of the auxiliary feedback factors into a single parameter in the mathematical model.

hsp42 Δ HSE mutant (Fig. 6 E), which is hyperactive at early time points relative to wild type and the other mutants. Consistent with its lack of growth phenotype, the modeling indicates that Hsp42 impinges on the HSR via a distinct mechanism compared with the other feedback mutants.

Taken together, these experimental and modeling results suggest that, except Hsp42, the additional feedback regulators

converge on the HSR by modulating the nuclear availability of Hsp70. Without any new parameters or new species, we can interpret the mathematic model in the context of the new results: the HSR is governed by a core feedback loop controlling Hsp70 expression supplemented by an auxiliary feedback loop—into which multiple targets converge—controlling Hsp70 client release and thus its subcellular localization (Fig. 6 F).

Regulon-wide screen for Hsf1 feedback regulators during ethanol stress

The HSR is activated by a wide range of stressors beyond heat shock, including ethanol, reactive oxygen species, and specific perturbations to the proteostasis network (Alford and Brandman, 2018; Alford et al., 2021; Pincus, 2020). To determine whether additional factors may participate in feedback regulation of the HSR under a different condition, we repeated the screening of the Δ HSE mutants following exposure to ethanol. In wild type cells expressing the HSE-YFP reporter, we observed dose- and time-dependent induction of the HSR as a function of ethanol concentration with half-maximal activity (EC_{50}) at 6.9% ethanol and corresponding inhibition of growth (IC_{50}) at 6.6% ethanol (Fig. 7, A and B; and Fig. S6, A and B). We settled on a dose of 8.5%—above the EC_{50} and used recently to study the HSR (Rubio et al., 2024)—to perform the time course screen of the library (Fig. 7 C). Like the results in response to heat shock, Hsp70 Δ FBL had the highest level of induction after 4 h of ethanol exposure while *ssa2 Δ HSE*, *sis1 Δ HSE*, and *hsc82 Δ HSE* all showed the lowest levels of induction due to their high basal levels (Fig. 7 D, purple bars). However, the other induction mutants that showed elevated HSE-YFP levels following heat shock were indistinguishable from wild type in response to ethanol (Fig. 7 D, red bars). Instead, four new mutants—*sscl Δ HSE*, *mdj1 Δ HSE*, *atg41 Δ HSE*, and *tma10 Δ HSE*—showed phenotypes consistent with disrupted feedback (Fig. 7 D, blue bars). Further supporting a distinct feedback network in response to ethanol and heat shock, we found that across the induction mutant library, the ratio of fold change at 4 h following heat shock and ethanol for each mutant varied substantially ($R^2 = 0.27$, Fig. 7 E). These data suggest that the auxiliary feedback loop may be comprised of condition-specific subsets of the HSR regulon.

Despite the distinct subsets of feedback regulators following heat and ethanol, we hypothesized that we could nonetheless recapitulate the altered dynamics of the ethanol-specific feedback candidates by modeling them as converging on the auxiliary feedback loop controlling Hsp70 client release. To this end, we first reconfigured the model to simulate ethanol exposure rather than heat shock. Without altering the architecture of the model or the parameters describing the core feedback loop, we accomplished this by adjusting the parameters describing the rate of client production and the interaction of clients with Hsp70 until the model could faithfully reproduce the HSE-YFP dynamics of wild type cells (Fig. 7 F). Indeed, with this model of the HSR induced by ethanol, we were able to account for the altered HSE-YFP dynamics of the ethanol-specific feedback candidates by adjusting the single parameter describing Hsp70 client release (Fig. 7 G). Given the broad range of environmental conditions that activate the HSR, additional perturbations may

likewise require the induction of distinct subsets of the HSR regulon to productively engage different clients and promote Hsp70 release (Fig. 7 H).

Discussion

In this study, we comprehensively dissected transcriptional feedback in the HSR. While negative feedback loops have long been appreciated to architecturally organize cellular stress response pathways, we present here the first regulon-wide screen of response dynamics to identify feedback regulators in an environmental stress pathway.

The major conclusion we draw is that the HSR is governed by a core negative feedback loop that determines Hsp70 expression augmented by a condition-specific auxiliary feedback loop that controls Hsp70 subcellular localization. To arrive at this understanding, we interpreted the large experimental datasets we generated within the constraints of a simple mathematical model to dissect the intricate dynamics of the two-layer feedback loop, enabling us to go beyond observation to a mechanistic understanding of the underlying processes. In cell biological terms, the results support a heuristic model in which the concentration of Hsp70 in the nucleoplasm largely determines the transcriptional output of the HSR across conditions and timescales. We anticipate that this architecture, in which the availability of a key pathway regulator is controlled via expression-level feedback and fine-tuned by spatial feedback, will characterize adaptive responses beyond the HSR.

Before screening the HSR regulon for feedback factors, we first characterized the induction dynamics of each of the 42 genes in the regulon. Analysis of the resulting dataset revealed that the HSR genes span a linear spectrum ranging from rapid/transient induction to slow/sustained induction (Fig. 1 G). Thus, while the transcription factor Hsf1 is evidently capable of regulating different genes across an expression range spanning four orders of magnitude, the one-dimensional manifold of the gene induction profiles suggests that simple underlying constraints determine the variation in expression across the regulon. Our previous finding that the level of Hsf1 binding at each gene can be predicted by the affinity of the HSE in the promoter for Hsf1 and the chromatin accessibility of the locus may provide the mechanistic explanation (Pincus et al., 2018). In the context of HSR feedback regulation, it is notable that the *SSA2/3/4* paralogs of Hsp70—components of the core negative feedback—are among the genes nearest the rapid/transient end of the induction spectrum, while *FES1* and the JDPs *SIS1*, *API1*, *YDJ1*—the strongest auxiliary feedback regulator and non-feedback regulators, respectively—are among the genes nearest the slow/sustained end (Fig. 1 G). Perhaps differential localization of HSR target genes to transcriptional condensates may contribute to the distinct dynamic induction profiles.

In addition to identifying *Fes1*, the Δ HSE mutant screen for altered HSR dynamics following heat shock revealed several other negative feedback regulators: *Ubi4*, *Gre3*, *Pin3*, and *Hsp42*. Except *hsp42 Δ HSE*, the feedback mutants exhibited reduced growth rates and disrupted cytosolic proteostasis (Fig. 4 C and Fig. 5 A). Moreover, their HSE-YFP dynamics could be

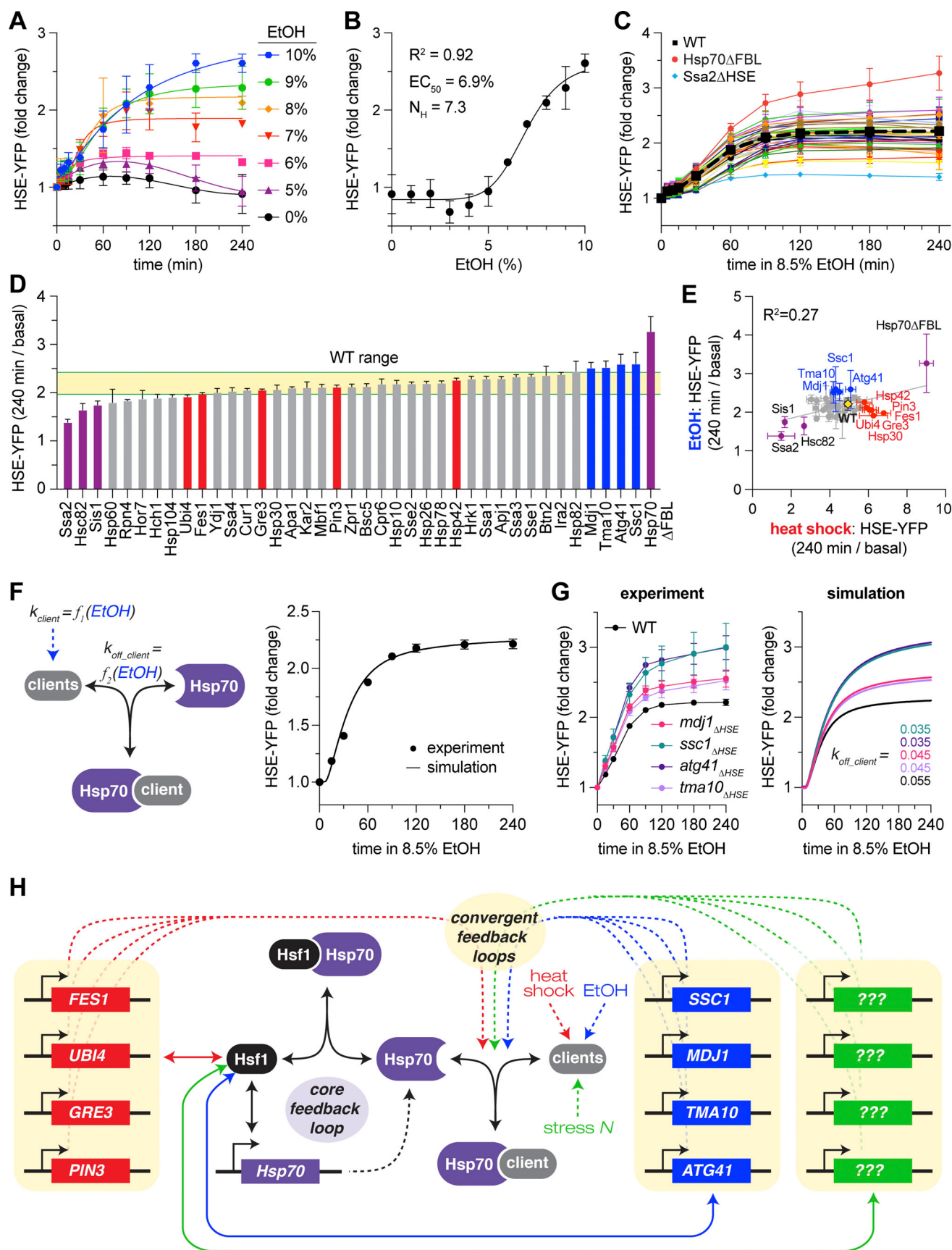


Figure 7. **Regulon-wide screen for Hsf1 feedback regulators during ethanol stress.** (A) Time-dependent dose response of the HSE-YFP reporter to ethanol (EtOH) in wild type cells. Each data point and error bar represents the mean and standard deviation of three biological replicates. 6–10% ethanol time

courses were well-fit by sigmoidal curves, but 5% and 0% had to be fit with polynomial functions. **(B)** HSE-YFP levels at 4 h were fit to a Hill function. **(C)** HSE-YFP fold change over a time course of treatment with 8.5% EtOH for all Δ HSE mutants, measured by flow cytometry. Dashed black line represents WT average. Each data point represents the mean and standard deviation of three biological replicates. **(D)** HSE-YFP fold change after 4 h of 8.5% EtOH for each Δ HSE mutant and the Hsp70 Δ FBL mutant. WT range is shown in yellow. Bars show the mean of three biological replicates, error bars the standard deviation. Purple bars show significant outliers beyond the 95% CI common to heat shock and EtOH; blue bars show outliers in EtOH; red bars show outliers in heat shock. **(E)** Scatter plot of HSE-YFP fold change after 4 h in heat shock versus EtOH for the Δ HSE mutants. **(F)** Schematic and simulation of the mathematical model re-parameterized for the HSE-YFP response to ethanol in wild type cells. **(G)** Experimental data and model simulations of HSE-YFP levels over EtOH time courses in wild type and auxiliary feedback mutant cells. Experimental data points and error bars are mean and standard deviation of three biological replicates. **(H)** Different subsets of the HSR regulon converge to function as the auxiliary feedback to control Hsp70 subcellular localization under different conditions to confer adaptive flexibility.

recapitulated by altering the value of a single parameter describing the rate of client release by Hsp70 in a mathematical model of the HSR (Fig. 6 F). For *fes1 Δ HSE*, we directly demonstrated that Hsp70 remains localized in cytosolic condensates, supporting the modeling results. Since Fes1 functions as a NEF for Hsp70, and nucleotide exchange is coupled to Hsp70 client release (Masser et al., 2019), it is intuitive why Fes1 induction during heat shock would be required for efficient liberation of Hsp70 from cytosolic clients and subsequent nuclear localization. Likewise, induction of *UBI4*, which encodes concatameric ubiquitin (Finley et al., 1987; Ozkaynak et al., 1987), can be rationalized as important for restoring cytosolic proteostasis due to its central role in the ubiquitin–proteasome system, thereby indirectly impinging on the availability of Hsp70.

The functions of Gre3 and Pin3 in the proteostasis network are less well understood. Gre3 functions as an aldose reductase that acts to convert methylglyoxal generated by glycolysis during stress to pyruvate (Aguilera and Prieto, 2001). Increased levels of methylglyoxal in *gre3 Δ HSE* cells may react with and damage cytosolic proteins, thereby sequestering Hsp70. Pin3 regulates actin nucleation and has been implicated in prion formation (Chernova et al., 2011; Madania et al., 1999). Perhaps induction of Pin3 is important for actin-dependent adaptive remodeling of cytosolic condensates that somehow serve to free Hsp70. While the details of the mechanisms remain to be resolved, it is likely that these additional feedback regulators are performing independent functions that converge to determine the availability of Hsp70 in the nucleus following heat shock.

The role of induction of Hsp42 in HSR regulation is more mysterious. Unlike the auxiliary feedback mutants—but like the core feedback mutant Hsp70 Δ FBL—*hsp42 Δ HSE* has no growth phenotype at elevated temperature. Also, relative to the auxiliary feedback mutants, the lack of induction of Hsp42 alters the dynamics of the HSE-YFP reporter at earlier time points, and the resulting time course data cannot be fit by adjusting the Hsp70 client release parameter (Fig. 6 E). Based on our results, we cannot rule out that Hsp42 acts to directly regulate Hsf1, though we have no evidence to support this.

Intriguingly, our screen for feedback mutants during ethanol stress revealed different negative feedback regulators of the HSR than it did following heat shock. Ethanol is the second largest activator of the HSR of conventional stressors (Alford et al., 2021; Rubio et al., 2024), and ethanol is physiological due to the role of yeast in wine and beer making and the use of ethanol in mitochondrial respiration. Remarkably, the ethanol-specific hits, Ssc1, Mdj1, Atg41, and Tma1, are all implicated in regulating

mitochondrial homeostasis. Ssc1 and Mdj1 encode a mitochondrially targeted Hsp70 and JDP, respectively (Craig et al., 1987; Rowley et al., 1994); Atg41 localizes to the mitochondrial surface and is required for mitophagy (Yao et al., 2015); and while Tma10 has no known function, its paralog Stf2 is known to bind and regulate the F₁F₀ mitochondrial ATP synthase (Dienhart et al., 2002; Hashimoto et al., 1984). Thus, the subset of the HSR regulon that makes up the ethanol-specific auxiliary feedback loop is comprised of genes that reflect the physiological nature of ethanol stress—namely that ethanol is a non-fermentable carbon source metabolized in the mitochondria. These results imply that without induction of mitochondrial-specific factors encoded in the larger HSR regulon, the increased stress that the mitochondria experiences in the presence of ethanol spills into the cytosol, titrating Hsp70 away from the nucleus. Consistent with this notion, the mitochondrial unfolded protein response was recently demonstrated to be mediated via titration of cytosolic Hsp70 and subsequent de-repression of Hsf1 in the nucleus (Sutandy et al., 2023).

Taken all together, we propose a model in which the HSR is dynamically regulated by a core feedback loop driven by induction of Hsp70 expression that acts regardless of the nature of the stress augmented by an auxiliary feedback loop that is condition-specific. In this view, various degrees and types of environmental stressors could produce a unique set of clients that would require a distinct subset of the HSR regulon to manage. Indeed, even different temperatures of heat shock, such as at 42°C rather than 39°C, may leverage a distinct subset of HSR target genes for feedback. These condition-specific feedback factors would then converge to enable efficient Hsp70 release back into the free cytosolic pool that can diffuse into the nucleus and repress Hsf1.

What advantages would such a two-tiered feedback architecture comprised of core and auxiliary loops confer to an adaptive response? On evolutionary timescales, this structure allows a simple, invariant network to remain fixed within a population—in this case, the Hsp70–Hsf1 negative feedback loop—while providing plasticity such that a secondary, peripheral genetic network tailored to cope with specific environmental fluctuations in any given ecological niche can evolve to fine-tune the output of the core network. In this case, the existence of two distinct subsets of condition-specific HSR effectors reflects an evolutionary past of fluctuating temperatures and carbon sources. The theoretical alternative to this mode of adaptation is a direct rewiring of the core Hsp70–Hsf1 feedback loop in new environments. This would likely render the system

susceptible to mutations that come with fitness costs in the presence of new environmental challenges, i.e., it may be a less evolvable mode of adaptation.

Extrapolating from the two conditions we tested here, we suspect that the HSR target genes not implicated in feedback during heat shock or ethanol stress may similarly converge to fine-tune Hsp70 subcellular localization in other environmental conditions experienced in the evolutionary history of budding yeast, such as nutrient, pH, and redox fluctuations. The two-tiered feedback architecture of the HSR allows for adaptive flexibility while maintaining a conserved core that was likely already present in—and may have been essential for the evolvability of—the last eukaryotic common ancestor.

Materials and methods

Strain construction

To create the library of P2A-mscarlet tagged strains, we took advantage of yeast homologous recombination by introducing a P2A-mscarlet-KAN cassette with homologous flanking ends and plated on kanamycin selective media. Successful transformants were verified by PCR and flow cytometry. To create Δ HSE induction mutants, we deleted the 9–25 bp Hsf1-binding consensus sequence by scarless CRISPR-Cas9 targeted deletion (Vyas et al., 2018). The closest match to the Hsf1 binding motif (nnTTCnnGAA) was located under the 5-min heat shock Hsf1 ChIP-seq peak from our previous study. Generally, we found one strong consensus sequence under the singular Hsf1 ChIP-peak 200–300 bp ahead of the TSS. We deleted the ChIP-verified consensus sequence in the established library of P2A-mscarlet parent lines. Cell line construction involved cloning a guide RNA, which will target the Hsf1 binding site, into an episomal, URA3-marked, Cas9-containing plasmid. The guide RNA plasmid was cotransformed with a 100-bp double-stranded repair template to repair the double-stranded break by homologous recombination, and yeast was plated on ura-selective media. HSE deletion was confirmed by Sanger sequencing. Finally, the cell lines were plated on 5-FOA selective media to expel the Cas9::ura3 plasmid. Though our P2A-mscarlet reporter strategy was tagless, it caused abnormal basal Hsf1 activity in a few lines, probably because the C terminal linker inhibited protein function. So, we created another library of Δ HSE induction mutants in our original parent line w303a; HSE-mVenus. The library of induction mutants (without the mScarlet reporter) was used in all experiments beyond the initial target gene transcription dynamics.

Heat shock and ethanol time courses

Cells were serially diluted and grown overnight on the benchtop at room temperature (25°C) in 1xSDC media. In the morning, cells were transferred to microcentrifuge tubes and aerated by shaking (1,250 RPM) at 30°C for 1 h. Centrifuge tubes were then transferred to a shaking incubator at a heat shock temperature of 39°C or the indicated concentration of ethanol was added. At each time point, 50 μ l of cells were transferred to a 96-well plate of 1xSDC at 50 μ g/ml final concentration cycloheximide on the benchtop. After the time course, the plate was incubated at 30°C

for 1 h to promote fluorescent reporter maturation before flow cytometry.

Flow cytometry

Cells were measured with the BD Fortessa HTS 4–15 benchtop analyzer at the University of Chicago Cytometry and Antibody Technology Facility. Analysis was completed in FlowJo: fluorescence excitation value for each cell was normalized by side scatter, and the median normalized fluorescence excitation value was calculated for each sample.

Quantitative growth assay

Cells were grown overnight shaking in 1xYPD at 30°C. In the morning, cells were diluted to $OD_{600} = 0.1$ in 1xYPD and transferred to a 48-well plate. Cells were grown while shaking in the SPECTROstar Nano Absorbance Plate Reader at 30°C for 4 h, then at 37°C for 20 h, and OD_{600} was measured every 20 min. The initial 4-h incubation at 30°C before heat shock yields more consistent results across biological replicates. OD_{600} of the liquid cell culture was measured every 20 min over the 24-h growth assay. Two biological replicates were measured per cell line.

Heat shock time course and imaging

Cells were grown in 2xSDC media at 30°C shaking overnight. In the morning, cells were diluted to $OD_{600} = 0.1$ and grown at 30°C shaking for 4 h to reach log phase growth. 200 μ l of cells were transferred to a microcentrifuge tube in the cell shaker at 39°C. At each time point, cells were fixed in 1% paraformaldehyde, then washed and imaged in KPIS media (1.2 M sorbitol, 0.1 M potassium phosphate, pH 7.5). Fixed cells were imaged on the Marianas Leica II Spinning Disk Confocal microscope at the University of Chicago Imaging Core. A single z-stack was captured and analyzed for each frame. ≥ 20 cells were quantified at each time point in ImageJ.

Image quantification

ImageJ was used for all image quantification. Hsp104 foci in each cell were identified using by Intermodes thresholding of each cell. Hsp70 and Sis1 foci were identified using the Triangle threshold. To determine the bounds of the nucleus, a ROI was drawn by hand based on the bounds of the nuclear membrane, marked by Sec61-mscarlet.

Mathematical modeling

Modeling was performed as described previously using the same equations (Garde et al., 2023). Best fit parameters were determined by minimizing the residual sum squared. All updated parameters are described in the text.

Online supplemental material

Fig. S1 shows HS-induced expression dynamics of the Hsf1 target genes. Fig. S2 shows Hsf1 activity dynamics after HSE deletion. Fig. S3 shows validating Ydj1 and Apj1 induction mutants. Fig. S4 shows supplemental Hsp70 and Sis1 images. Fig. S5 shows residuals of model fits. Fig. S6 shows the growth curves of cells in ethanol. Table S1 lists yeast strains used in this study. Datas S1,

S2, S3, S4, S5, S6, S7, S8, S9, S10, and S11 shows data plotted in main figures.

Data availability

The data underlying the figures are available in the published article and its online supplemental material. Unprocessed data is available from the corresponding author upon request.

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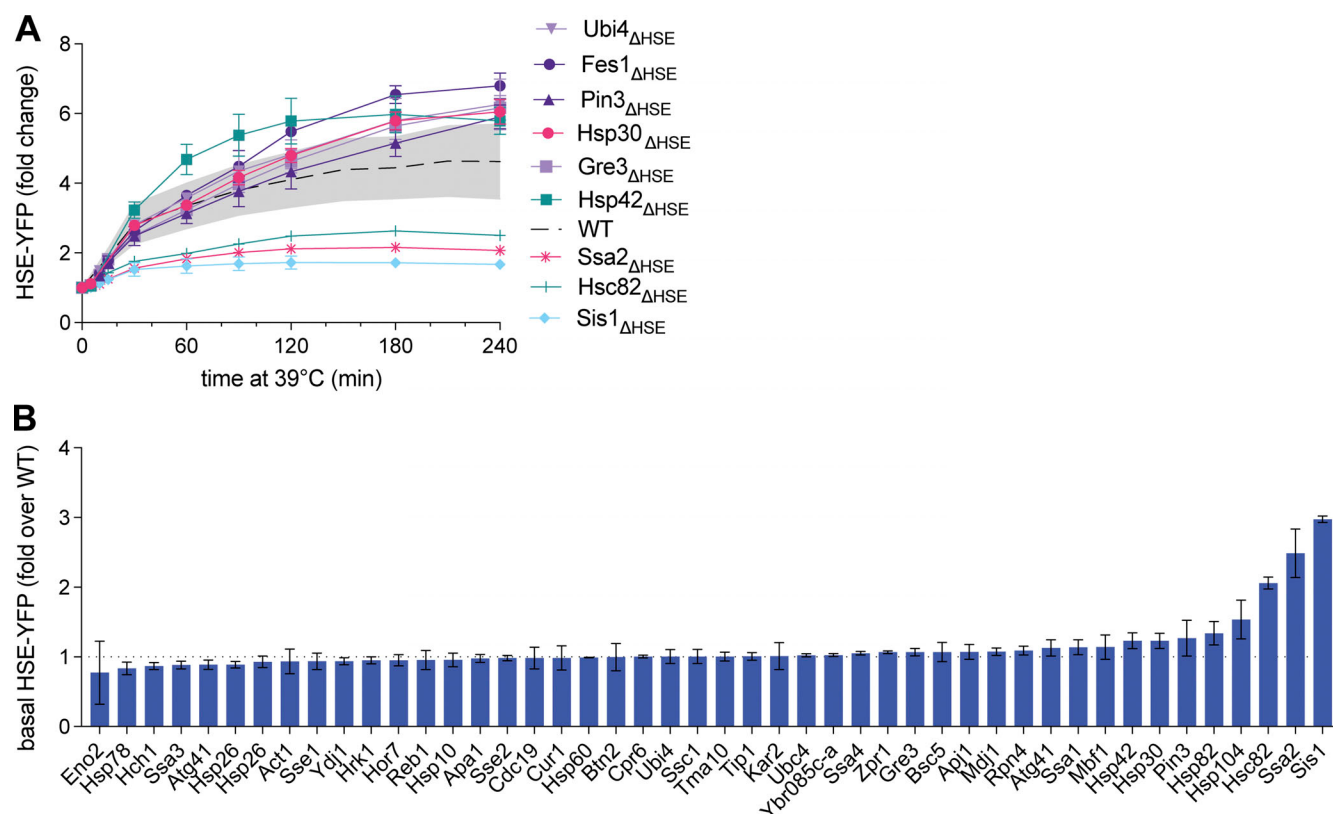


Figure S2. **Hsf1 activity dynamics after HSE deletion.** (A) Heat shock time courses for the eight Δ HSE mutants with 4-h HSE-YFP fold change outside of the statistically significant WT range. Each data point represents the mean and standard deviation of three biological replicates. Dotted line represents the average of 45 WT biological replicates and the gray shaded area represents the standard deviation of those replicates. (B) Relative non-stress levels of HSE-YFP reporter (normalized to WT) in each Δ HSE induction mutant. Bars represent the mean of three biological replicates, error bars the SD.

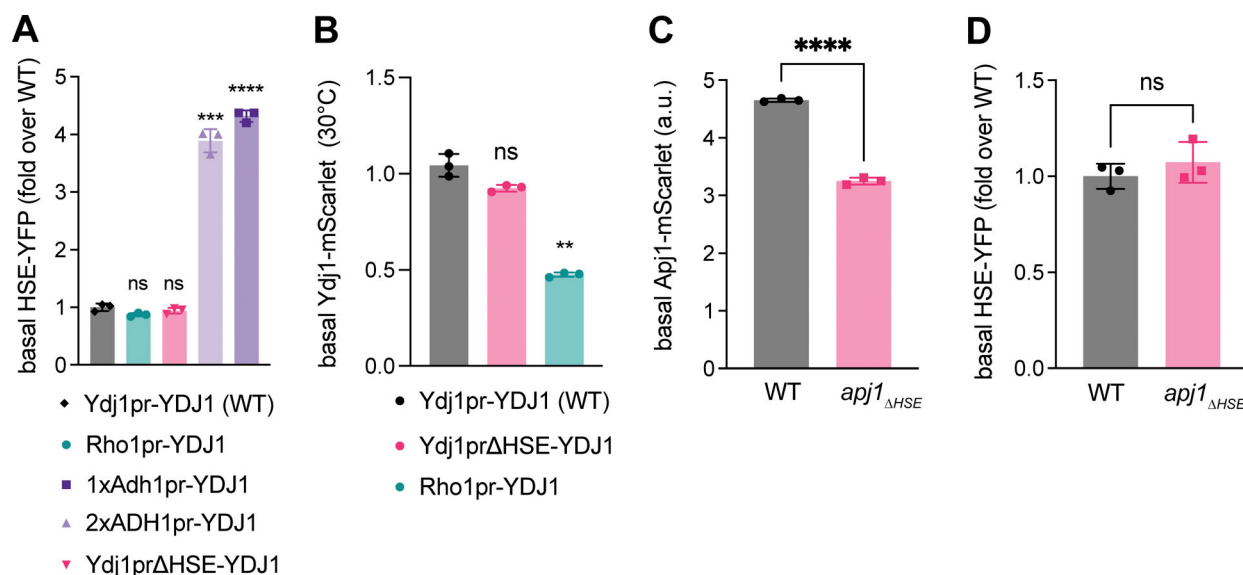


Figure S3. **Validating Ydj1 and Apj1 induction mutants.** (A) Relative basal HSE-YFP (normalized to basal HSE-YFP in WT) when Ydj1 is expressed under non-inducible promoters of various strengths. (B) Relative basal Ydj1 expression measured by mScarlet fluorescence. (C) Basal Apj1 expression measured by mScarlet fluorescence in *apj1 Δ HSE* versus WT. Statistics: P < 0.01. Bars represent the mean of three biological replicates, error bars the standard deviation. (D) Basal Hsf1 activity measured by HSE-YFP fluorescent reporter in *Apj1 Δ HSE* versus WT. Bar height represents the mean of three biological replicates, error bars represent the standard deviation. Statistics: ns is defined as P > 0.05.

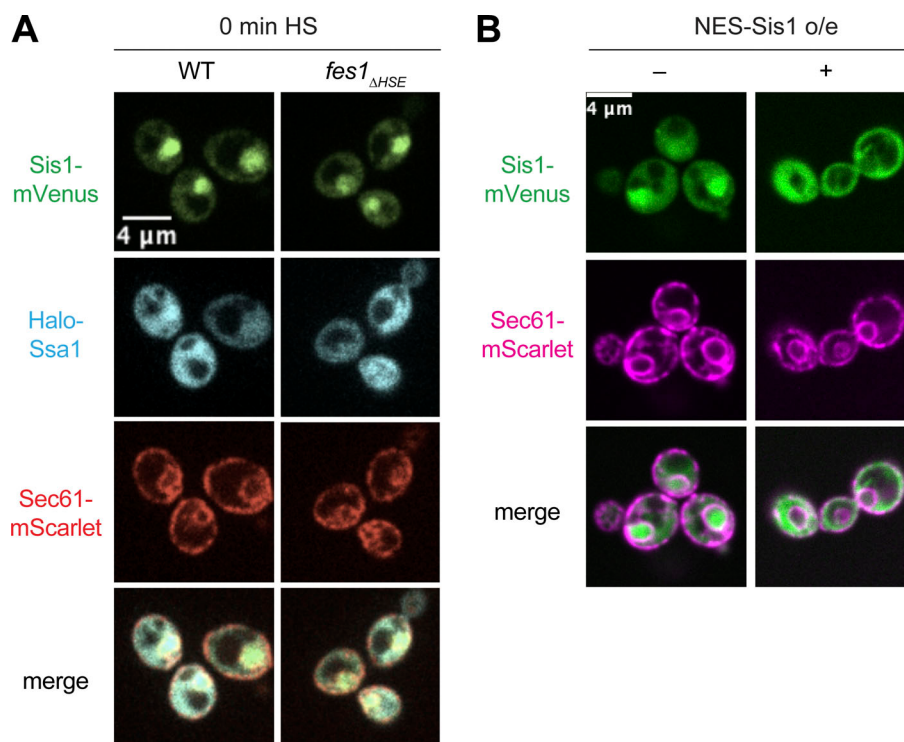


Figure S4. **Supplemental Hsp70 and Sis1 images.** (A) Images of cells expressing Sis1-mVenus, Halo-Ssa1, and Sec61-mScarlet under basal conditions in wild type and *fes1* Δ_{HSE} cells. (B) Images of cells expressing Sis1-mVenus and Sec61-mScarlet in the presence and absence of induction of NES-Sis1.

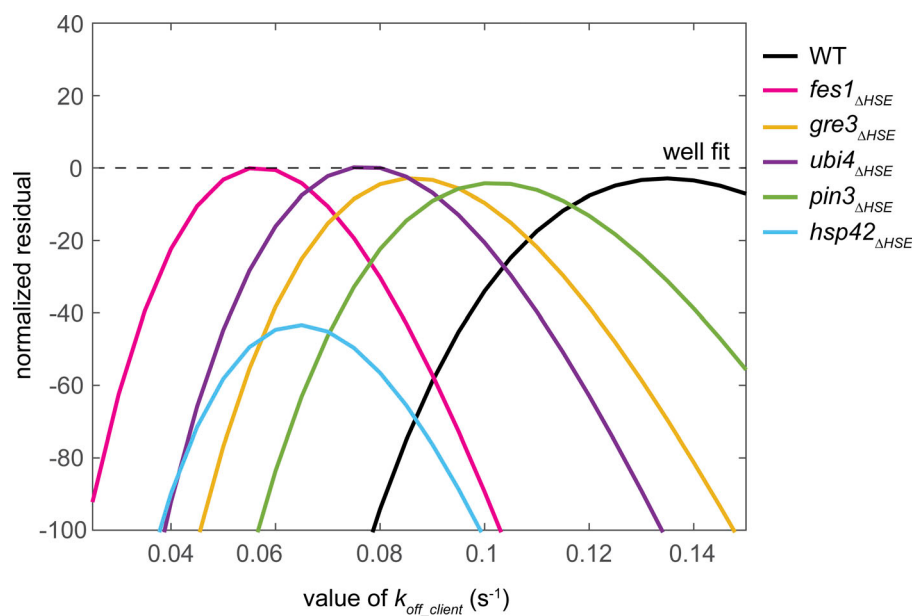


Figure S5. **Residuals of model fits.** The residual values (quantity left unfit by the model) as a function of the parameter sweeps for each Δ_{HSE} mutant and wild type.

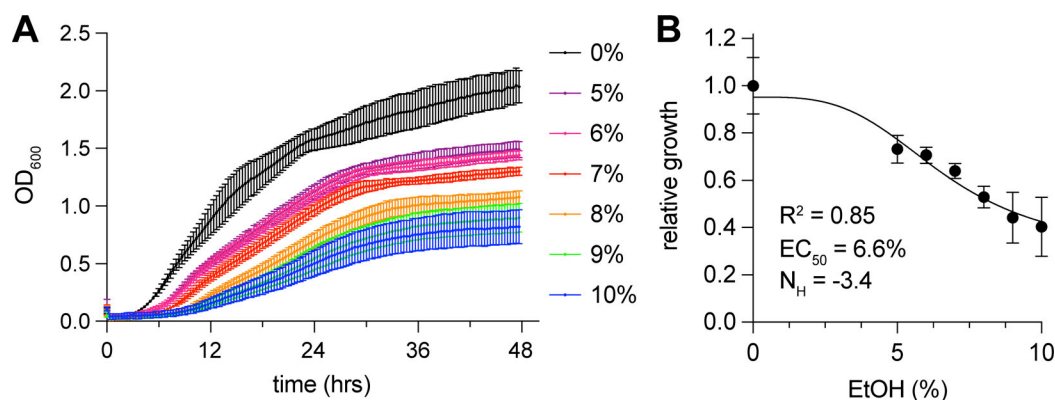


Figure S6. **Growth curves of cells in ethanol.** (A) Cells were grown at the indicated concentrations. (B) Max growth rates were fit to a Hill function.

Provided online is Table S1 and 12 Datasets. Table S1 lists yeast strains used in this study. Data S1 Data plotted in Fig. 1 C. Data S2 Data plotted in Fig. 1 D. Data S3 Data plotted in Fig. 1 E. Data S4 Data plotted in Fig. 1 F. Data S5 Data plotted in Fig. 1 G. Data S6 Data plotted in Fig. 2 A. Data S7 Data plotted in Fig. 2 F. Data S8 Data plotted in Fig. 3, relative. Data S9 Data plotted in Fig. 3, fold change. Data S10 Data plotted in Fig. 4 A. Data S11 Data plotted in Fig. 4 B.