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Unrestrained AMPylation targets cytosolic chaperones and activates the heat shock response

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Protein AMPylation is a conserved posttranslational modification with emerging roles in endoplasmic reticulum homeostasis. However, the range of substrates and cell biological consequences of AMPylation remain poorly defined. We expressed human and *Caenorhabditis elegans* AMPylation enzymes—huntingtin yeast-interacting protein E (HYPE) and filamentation-induced by cyclic AMP (FIC)-1, respectively—in *Saccharomyces cerevisiae*, a eukaryote that lacks endogenous protein AMPylation. Expression of HYPE and FIC-1 in yeast induced a strong cytoplasmic Hsf1-mediated heat shock response, accompanied by attenuation of protein translation, massive protein aggregation, growth arrest, and lethality. Overexpression of Ssa2, a cytosolic heat shock protein (Hsp)70, was sufficient to partially rescue growth. In human cell lines, overexpression of active HYPE similarly induced protein aggregation and the HSF1-dependent heat shock response. Excessive AMPylation also abolished HSP70-dependent influenza virus replication. Our findings suggest a mode of Hsp70 inactivation by AMPylation and point toward a role for protein AMPylation in the regulation of cellular protein homeostasis beyond the endoplasmic reticulum.

AMPylation | FIC protein | chaperones | proteostasis | HSP70

How complex organisms maintain homeostasis in the midst of internal and environmental stress is a fundamental question in biology. The stability of the proteome is essential to maintain cellular processes and contributes to organismic health and lifespan. Cellular protein homeostasis (proteostasis) is continuously challenged by a variety of stressors that trigger protein misfolding and aggregation (1). Aging and age-associated diseases progressively increase the accumulation of misfolded, damaged, and aggregated proteins, thus interfering with numerous biological processes (2).

To overcome proteotoxicity, cells are equipped with compartment-specific stress responses that provide protection through transcriptional, translational, and posttranslational regulation of protein degradation and protein folding (3). The mitochondrial (mt) unfolded protein response (UPR^{mt}), as well as the endoplasmic reticulum (ER) unfolded protein response (UPR^{ER}), regulate chaperone function and protein degradation pathways within these organelles (4). The heat shock response (HSR) controls extensive heat shock protein (Hsp) chaperone networks throughout the cell and is essential to survive acute stress (5). Together, these responses provide the cell with the capacity to react to and endure various stresses, while maintaining proteostasis.

Hsps are involved in all branches of cellular stress responses that support protein folding (6). The mitochondrion-resident mtHsp40 and mtHsp70 proteins ensure protein homeostasis within this critical organelle, whereas the ER-resident Hsp70-family chaperone BiP/Grp78 refolds unfolded and misfolded proteins within the ER and helps remove and degrade terminally damaged proteins from the ER. In addition to their direct involvement in protein folding, individual or complexed Hsps inhibit or inactivate stress-response regulators, including the UPR^{ER} stress sensors IRE1 and PERK, and the transcriptional regulator of the HSR, heat shock factor 1 (HSF1), in negative feedback loops (7, 8).

Recent work on BiP's function in ER homeostasis identified a major role for a particular posttranslational modification, AMPylation, in the regulation of BiP's ATPase and chaperone activity (9–11). Protein AMPylation involves the transfer of AMP from ATP to a Ser or Thr side chain and is carried out by enzymes that contain a Fic (filamentation-induced by cyclic AMP) domain (Fic proteins), an evolutionarily conserved protein family present in both bacteria and metazoans, but lacking in fungi and plants (12, 13). In prokaryotes, Fic proteins are often associated with toxin–antitoxin systems, such as the VbhT-VbhA pair encoded by *Bartonella schoenbuchensis*, leading to modification of Gyrase and Topoisomerase IV (14, 15). Several human pathogens are equipped with Fic-domain effector proteins that covalently AMPylate and inactivate small GTPases of the Rho and Rab family in their respective host cells (16, 17). Eukaryotic Fic proteins AMPylate a variety of molecular targets, including BiP, core histones, and translation elongation factors that contribute to the regulation of the UPR^{ER}, innate immunity, and perception of light (10, 11, 18, 19). Nevertheless, our knowledge of the range of substrates and consequences of AMPylation remains incomplete.

Here we examine heterologous expression of Fic proteins in *Saccharomyces cerevisiae*, which lacks endogenous protein AMPylation. In addition to activating the UPR, we find that expression of active *Homo sapiens* huntingtin yeast-interacting protein E (HYPE) or *Caenorhabditis elegans* FIC-1 in yeast results in the functional ablation of cytosolic chaperone pools with concomitant induction of a strong Hsf1-mediated HSR. Furthermore, we observed massive protein aggregation and inhibition of translation, eventually causing growth

Significance

The stability of the proteome is essential to cellular and organismic health and lifespan. To maintain proteostasis, cells are equipped with a network of chaperones that support folding of nascent proteins, as well as refolding of unfolded or misfolded proteins. Aging and age-associated diseases progressively increase the accumulation of misfolded, damaged, and aggregated proteins, thus taxing the chaperoning machinery to its limits. Here, we describe how AMPylation of cytosolic heat shock proteins (HSP) leads to a collapse of proteostasis, the induction of a strong heat shock response, inhibition of translation, as well as the formation of protein aggregates. AMPylation-mediated inhibition of HSP70 may represent a strategy for targeted ablation of this chaperone.

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arrest and lethality. In vitro, both FIC-1 and HYPE covalently AMPylated cytosolic Hsp40, Hsp70, and Hsp90. Overexpression of Ydj1 and Ssa2, a cytosolic Hsp40/Hsp70 pair, rescued growth of *S. cerevisiae*. Expression of active HYPE in human cells confirmed AMPylation-dependent interference with the chaperoning network involved in protein aggregation and the induction of the Hsf1-dependent HSR. Our findings identify a trigger that can cause collapse of the cellular chaperoning machinery and activate Hsf1, mediated by AMPylation of cytosolic chaperones. Protein AMPylation may thus regulate proteostasis beyond the ER and present a target for intervention.

Results

Protein AMPylation in *S. cerevisiae* Results in Growth Arrest and Cell Death. To explore the consequences of protein AMPylation in an unbiased manner, we introduced enzymes that carry out this modification into the budding yeast *S. cerevisiae*, a eukaryote that lacks endogenous AMPylation machinery. We expressed a set of Fic domain-containing proteins in yeast under the control of a galactose-inducible promoter. This set included *Vibrio parahaemolyticus* VopS, *C. elegans* FIC-1, and *H. sapiens* HYPE, as well as mutant versions with increased or impaired AMPylation activity (14, 16). Upon galactose induction, cells that express active FIC-1 (E274G) and—to a lesser extent—active HYPE (E234G), showed attenuated cell growth (Fig. S1 *A*–*C*). In contrast, expression of wild-type or activity-impaired mutants (FIC-1 H404A, HYPE H363A) did not diminish growth. Moreover, expression of a mutant of FIC-1 that retains the ability to auto-AMPylate but cannot AMPylate other substrates (FIC-1 E274G/H404A) was also benign (Fig. S1*B*). Overexpression of VopS, a potent bacterial Fic protein known to modify small GTPases (16), did not affect cell viability (Fig. S1*D*). To exclude copy number variation as being responsible for these findings, we engineered yeast strains that carry galactose-inducible FIC-1 or mutants thereof as single-copy genomic integrations. Whereas growth rates were indistinguishable when grown on dextrose, induction of FIC-1 (E274G) resulted in impaired growth and eventual growth arrest in liquid culture (Fig. 1*A*). Expression of FIC-1 (E274G) upon galactose induction was verified by immunoblotting (Fig. S1*E*). To test whether the growth impairment was reversible or lethal, we transferred cells grown for 3 h in galactose into repressive dextrose-containing media and monitored cell growth. Cells that express active FIC-1 (E274G) failed to recover from growth arrest upon promoter shut-off, indicating that in *S. cerevisiae* expression of FIC-1 (E274G) and the level of AMPylation associated with it is lethal (Fig. 1*B* and Fig. S1*F*).

AMPylation Triggers the HSR in *S. cerevisiae*. Given that AMPylation has been reported to target BiP and modulate ER homeostasis in mammalian cells (9–11), we wondered whether the toxicity of FIC-1 (E274G) seen in yeast could be attributed to ER stress. We introduced a fluorescent reporter of the unfolded protein response (UPRE-GFP) into strains bearing estradiol-inducible FIC-1 and HYPE genes. Upon ER stress, the transcription factor Hac1 activates the UPRE-GFP reporter, a signal that can be quantified by flow cytometry. For comparison, we also introduced reporters of cytosolic stress responses, including the HSR (HSE-YFP), activated by the transcription factor Hsf1, and the general stress response (STRE-GFP), activated by the transcription factors Msn2/4. FIC-1, FIC-1 (E274G), and HYPE (E234G) did induce the UPRE-GFP reporter in the presence of estradiol, but did so very weakly, and not significantly more than they induced the STRE-GFP reporter (Fig. S2 *A* and *B*). In marked contrast, expression of both FIC-1 (E274G) and HYPE (E234G) robustly induced the HSE-YFP reporter (Fig. 2*A*).

To test whether AMPylation not only promoted HSE-YFP reporter activation but also induced a genome-wide HSR, we performed RNA deep sequencing (RNA-seq) to compare cells

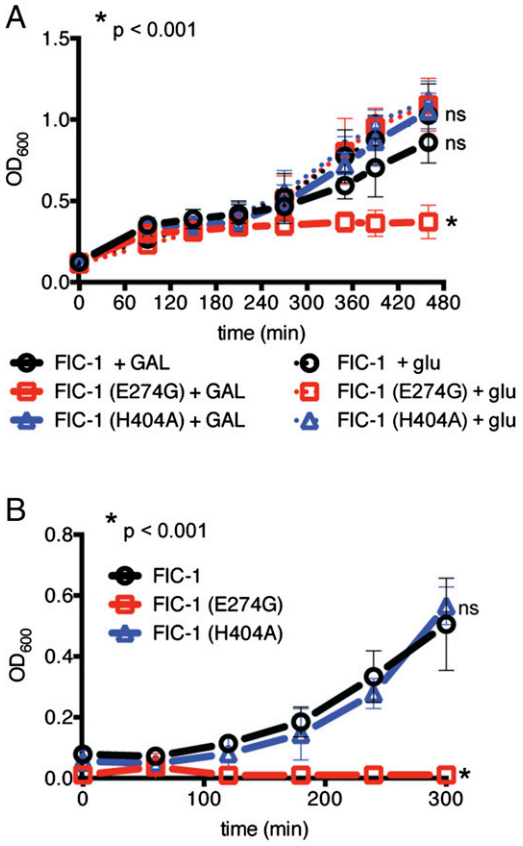

Fig. 1. Overexpression of *C. elegans* FIC-1 (E274G) in *S. cerevisiae* is lethal. (*A*) Time courses of *S. cerevisiae* inducibly expressing genomically integrated FIC-1 genes grown in repressive (glu) or inductive (GAL) conditions. (*B*) Growth of *S. cerevisiae* inducibly expressing genomically integrated FIC-1 genes in repressive (glu) condition following induction for 3 h in (GAL) medium. *P* values calculated using repeated measure ANOVA tests; ns, not significant ($P > 0.05$). Data shown represents average of six independent experiments.

induced to express FIC-1 (E274G) for 2 h to cells heat-shocked at 39 °C for 30 min. We found that the two transcriptomes were highly correlated ($r = 0.81$). Gene ontology (GO) analysis of the set of 91 genes that were induced ≥fourfold by both heat shock and FIC-1 (E274G) showed a strong enrichment for the GO term "protein folding" ($P < 10^{-10}$). In contrast, there was no enrichment for biological processes or molecular functions in the set of 41 genes that were induced by FIC-1 (E274G) but not by heat shock. Expression of FIC-1 (E274G) thus mimics the response to elevated temperature (Fig. 2*B* and Fig. S2*C*). Among the most strongly up-regulated genes were canonical HSP genes (e.g., *SSA4*, *SSE2*, *HSP10*, *HSP42*, *HSP82*); the most down-regulated set included ribosomal protein genes (RPGs; e.g., *RPL31B*, *RPL21A*, *RPS22A*, *RPS12*, *RPS31*) (Fig. S2 *D* and *E*). A comparison by RNA-seq of induction of FIC-1 (E274G) over time with a heat shock time course showed stable up-regulation of HSPs and down-regulation of RPGs in both conditions (Fig. 2*C*). Global gene expression remained correlated as well (Fig. S2*C*). However, AMPylation induced even larger maximal fold-changes in transcript levels than did heat shock: AMPylation is thus an even stronger inducer of the HSR than elevated temperature (Fig. 2*C*).

To determine whether the down-regulation of RPGs attenuated translation, we performed a ^{35}S-methionine/cysteine labeling experiment. Expression of FIC-1 (E274G) decreased global translation (Fig. 2*D*) and modified the overall translation profile, the most pronounced change being the appearance of a strong

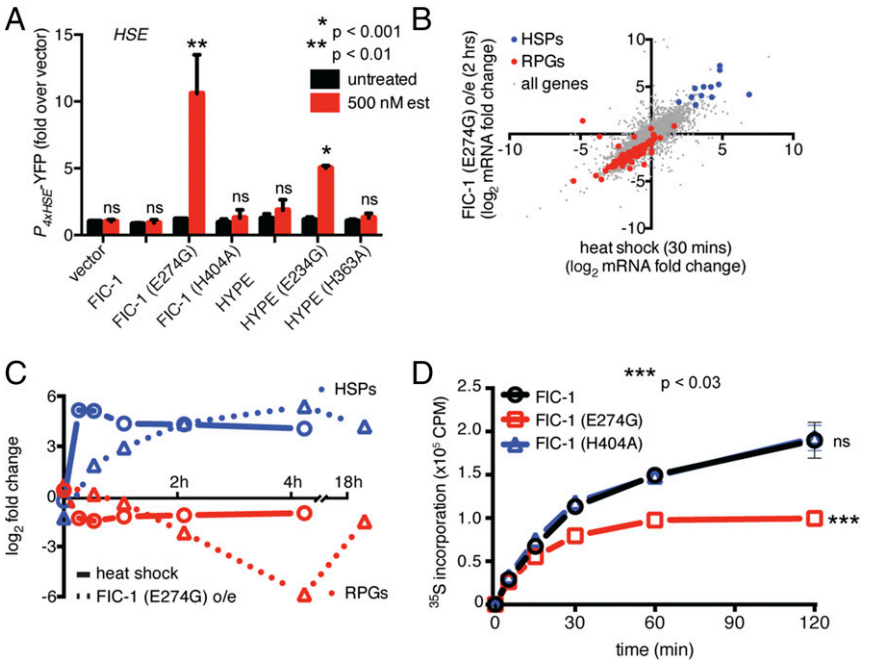

Fig. 2. Protein AMPylation induces a strong HSR in *S. cerevisiae*. (*A*) Induction of P_{4xHSE}YFP heat shock reporter in the presence of distinct Fic proteins. Results are normalized to empty vector controls. (*B*) Comparison of global changes in transcript levels induced by overexpression of FIC-1 (E274G) or heat shock. HSP genes are highlighted in blue; RPGs are colored in red. (*C*) Time-resolved analysis of gene transcription during heat shock (solid lines) or in the presence of FIC-1 (E274G) (dashed lines). HSPs are highlighted in blue, RPGs are colored in red. (*D*) Protein translation in the presence of FIC-1 proteins as monitored by ^{35}S-Cys/^{35}S-Met incorporation. Counts are OD-normalized to account for different growth behaviors of individual strains. FIC-1 (E274G) expression was induced at timepoint −2 h when starting cell starvation. *P* values calculated using repeated-measure ANOVA tests (*D*) or Student's *t* test (*A*); ns, not significant ($P > 0.05$).

band running at ∼70 kDa, likely representing Hsp70 family proteins, (Fig. S2*F*). In summary, FIC-1 (E274G) expression induces a strong HSR, accompanied by up-regulation of HSPs and attenuation of protein translation.

HYPE (E234G) Triggers a HSR in Human Cells and Promotes the Formation of HSF1 Foci in the Nucleus. Because AMPylation is an artificial stressor for yeast, we turned our attention to human cells, which encode the endogenous AMPylation enzyme HYPE, to determine the wider validity of the yeast results. To this end, we performed RNA-seq on HeLa cells that ectopically express active HYPE (E234G) and compared its transcriptome to that of untransfected controls. Expression of HYPE (E234G) induced both UPR^{ER} target genes as well as HSF1 target genes. The set of genes up-regulated ≥fourfold was enriched for the GO terms "stress response," "chaperone," and "response to unfolded protein" (Fig. 3 *A*–*C* and Fig. S3*A*). We then focused on the apparent activation of the HSF1-mediated cytosolic stress response and used an HSF1 reporter cell line that contains a GFP cassette driven by consensus heat shock elements (HSE-GFP) (20), analogous to the HSE-YFP reporter used in yeast. Transfection of this reporter line with HYPE (E234G) triggered induction of GFP, whereas transfection with wild-type HYPE, AMPylation-deficient HYPE (H363A), or a vector containing only mCherry failed to do so (Fig. S3*B*). Next, as an independent readout, we monitored HSF1 clustering into nuclear stress granules, the subnuclear foci that are a hallmark of HSF1 activation in human cells (21). Indeed, expression of HYPE (E234G) induced HSF1 to form subnuclear clusters that were similar to those formed in heat-shocked cells (Fig. 3*D*; representative images shown in Fig. S3*C*). By contrast, expression of wild-type HYPE, HYPE (H363A), or mCherry did not trigger HSF1 clustering (Fig. 3*D* and Fig. S3*C*). Thus, RNA-seq, the HSE-GFP reporter, and HSF1 immunofluorescence all indicate that HYPE (E234G) activated HSF1 and the HSR in human cells.

To assess protein translation in the presence of HYPE (E234G), we cotransfected HeLa cells with mCherry and various HYPE constructs and measured mCherry intensity after 24 h. HYPE (E234G) led to a marked decrease in mCherry expression, compared with wild-type HYPE or AMPylation-deficient HYPE (H363A) (Fig. S3*D*). Metabolic ([^{35}S]methionine/cysteine) labeling of de novo synthesized proteins in transfected HeLa cells confirmed HYPE (E234G)-mediated attenuation of mCherry expression and showed additional changes in the protein translation profile (Fig. S3*E*). Notably, just as in yeast cells expressing FIC-1 (E274G), cells that express HYPE (E234G) produced an additional 70-kDa protein, presumably representing Hsp70 family members (Fig. S3*E*).

FIC-1 (E274G) and HYPE (E234G) Disrupt Cytosolic Proteostasis. HSF1 and the HSR are activated when the cytosolic chaperoning machinery fails and unfolded or misfolded proteins accumulate and aggregate. To determine whether AMPylation impairs proteostasis, we imaged yeast and human cells that express aggregation reporters. In yeast, we imaged Hsp104-YFP—a disaggregase that forms discrete foci marking aggregated proteins in stressed cells—upon induction of FIC-1 (E274G). Indeed, FIC-1 (E274G)—but not wild-type FIC-1 or AMPylation-deficient FIC-1 (H404A)—triggered Hsp104-YFP to form foci, indicating that the function of cytosolic chaperones had been compromised (Fig. 4*A*). Next, we tagged FIC-1 (E274G) at its C terminus with YFP, induced its expression with estradiol, and imaged live cells. We observed localization to the perinuclear and cortical ER as demonstrated by the partial signal overlap with Ire-1–mCherry, but also to juxtamembranous and cytosolic puncta that were distinct from the ER (Fig. 4*B*, *Upper*). Indeed, cells coexpressing FIC-1 (E274)-YFP and mKate2-Ssa2 (a strictly cytosolic Hsp70 chaperone) showed colocalization of the two proteins (Fig. 4*B*, *Lower*), suggesting that a fraction of FIC-1 (E274G) is present in the cytosol of *S. cerevisiae*. Subcellular fractionation of FIC-1 (E274G)-expressing animals confirmed that a small amount of

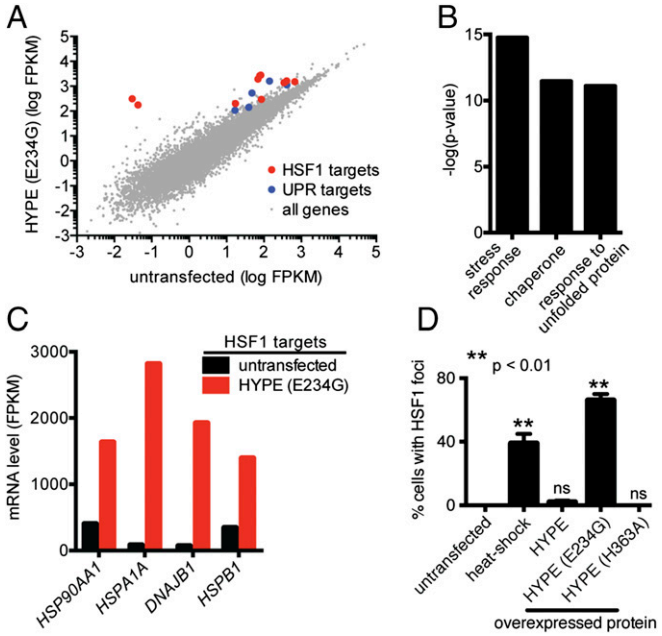

Fig. 3. HYPE (E234G) triggers a HSR in human cells. (*A*) Genome-wide mRNA expression levels (fragments per kilobase of transcript per million mapped reads, FPKM) as measured by RNA-seq in untransfected HeLa cells and in the presence of HYPE (E234G). Red dots are example HSF1 target genes; blue dots are example UPR target genes. (*B*) GO analysis of mRNA enrichments upon expression of HYPE (E234G). (*C*) HSF1 target gene mRNA expression levels in untransfected HeLa cells and in the presence of HYPE (E234G). (*D*) Quantification of HYPE (E234G)-induced nuclear spec formation. Bars represent means of at least three independent experiments. *P* values calculated using Student's *t* test (*A*); ns, not significant ($P > 0.05$).

FIC-1 (E274G) is present in the cytosol (Fig. S4*A*). To further support our hypothesis that FIC-1 (E274G) is also active in the cytoplasm, we generated nanobodies [VHHs (variable domain of the heavy immunoglobulin chain)] specific for FIC-1. Two unrelated FIC-1–specific nanobodies (VHH), $VHH_{FIC\text{-}1\_4}$ and $VHH_{FIC\text{-}1\_19}$, were isolated and shown to bind to FIC-1 but not its human ortholog HYPE (Fig. S4*B*). Variation of the VHH:FIC-1 (E274G) ratios inhibited target AMPylation in vitro in a dose-dependent manner, whereas the presence of inhibitory HYPE-specific VHH_1 did not affect FIC-1 (E274G) activity (Fig. S4*C*). Cytosolic expression of these FIC-1–specific VHHs in yeast showed that both $VHH_{FIC\text{-}1\_4}$ and $VHH_{FIC\text{-}1\_19}$ markedly improved growth when synthesis of FIC-1 (E274G) was induced (Fig. S4*D*). Coexpression of an irrelevant VHH (VHH_7, a class II MHC-specific VHH) failed to rescue growth (Fig. S4*D*). This finding supports our hypothesis that FIC-1 (E274G)-promoted modification of cytosolic targets is the cause for the observed phenotypes in yeast.

In human cells, we used a destabilized mutant firefly luciferase (FlucDM-GFP) that clusters in stressed cells and loses its luciferase activity (22). We cotransfected HeLa cells with HYPE constructs and FlucDM-GFP and monitored reporter clustering as well as luciferase activity. We observed that the presence of HYPE (E234G) induced aggregation of the FlucDM-GFP and showed a significant reduction in luciferase activity—similar to cells coexpressing an aggregation-prone poly-glutamine construct—whereas wild-type HYPE or HYPE (H363A) did not (Fig. 4*C*). Together, these results demonstrate that AMPylation compromises proteostasis and triggers the formation of cytosolic protein aggregates in both yeast and human cells.

FIC-1 (E274G) and HYPE (E234G) Modify Hsp40, Hsp70, and Hsp90 in Vitro. The cytosolic chaperones Hsp40, Hsp70, and Hsp90 support protein folding and are thought to repress HSF1. Because AMPylation activates HSF1, we wondered whether Hsp40, Hsp70, and Hsp90 might be substrates of FIC-1 (E274G) and HYPE (E234G). To test this, we expressed and purified recombinant *C. elegans* HSP-1 (cytosolic Hsp70 ortholog), HSP-3 (BiP ortholog), and DAF-21 (cytosolic Hsp90), as well as human

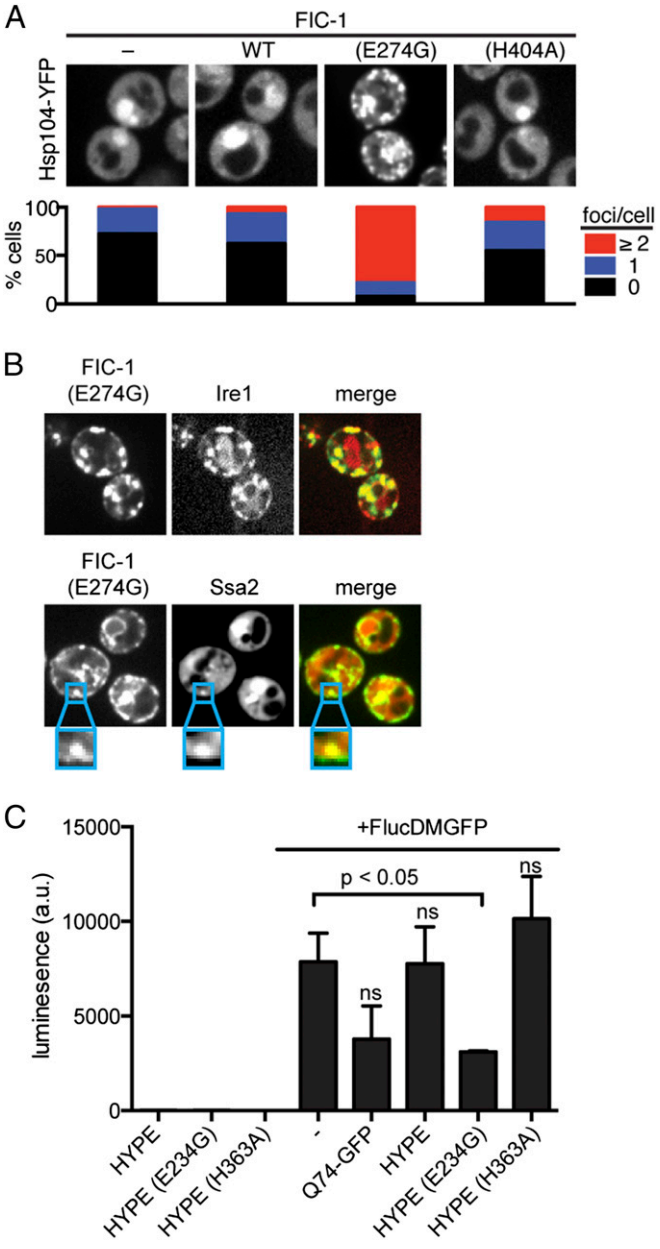

Fig. 4. AMPylation induces cytosolic protein aggregation in *S. cerevisiae* (magnification: 100×; enlargements are an additional 2× magnified) and human cells and AMPylation enzymes partially localize to the cytosol. (*A*) Fluorescence microscopy of yeast cells expressing Hsp104-YFP to mark protein aggregates in the presence and absence of expression of FIC-1, FIC-1 (E274G), or FIC-1 (H404A) in *S. cerevisiae*. Quantification of the number of Hsp104-YFP aggregates/cell in the absence or presence of FIC-1 (E274G) is shown below. At least 50 cells were imaged in each condition. (*B*) Fluorescence microscopy images of subcellular localization of FIC-1 (E274G)-YFP, SSa2-mKate2 and Ire-1–mCherry. (*Upper*) Cells coexpressing FIC-1 (E274G)-YFP and Ire-1–mCherry; (*Lower*) cells coexpressing FIC-1 (E274G)-YFP and SSa2-mKate2. (*C*) HYPE (E234G) induces protein aggregation in HeLa cells via quantification of luciferase activity of the FlucDMGFP reporter. Q74-GFP is a polyglutamine protein that causes protein aggregation. *P* values calculated using Student's *t* test (*A*); ns, not significant ($P > 0.05$).

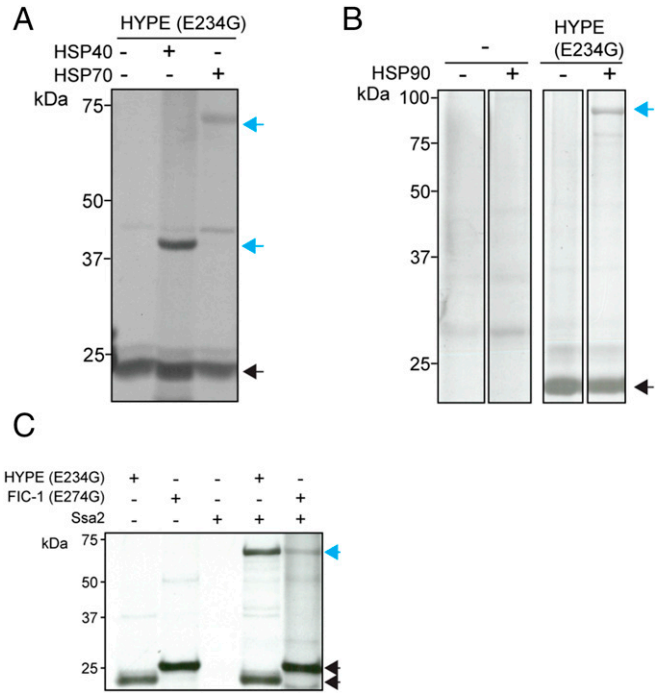

Fig. 5. FIC-1 (E274G) and HYPE (E234G) AMPylate Hsp40, Hsp70, Hsp90, and Hsf-1 in vitro. In vitro AMPylation reaction using α^{33}P-ATP as nucleotide substrate to monitor AMPylation of Hsp70, Hsp40 (*A*), and HSP90 (*B*) by HYPE (E234G), as well as *S. cerevisiae* Hsp70 (SSA2) by FIC-1 E274G and HYPE E234G (*C*). Solid black arrows depict auto-AMPylated enzymes [HYPE (E234G), FIC-1 (E274G)]; cyan arrows depict AMPylated target proteins.

Hsp40 and Hsp70 and performed in vitro AMPylation assays. Because recombinant wild-type AMPylases (FIC-1, HYPE) function only poorly in vitro (9, 11, 14, 17, 23), we performed all experiments using only the constitutively active versions (FIC-1 E274G, HYPE E234G). FIC-1 (E274G) efficiently AMPylated HSP-1, HSP-3, and DAF-21 (Fig. S5*A*), whereas no detectable binding of α^{33}P-ATP to HSP-1, HSP-3, or DAF-21 was observed. Similarly, HYPE (E234G) AMPylated Hsp40, Hsp70, and Hsp90 (Fig. 5 *A* and *B*). We also tested whether the two enzymes could modify nonendogenous substrates. Although HYPE (E234G) AMPylated *C. elegans* proteins HSP-1, HSP-3, and DAF-21, FIC-1 (E274G) was unable to AMPylate human Hsp70 or Hsp90, but efficiently AMPylated human Hsp40 (Fig. S5 *B–D*). Furthermore, both enzymes catalyzed AMPylation of recombinant yeast Ssa2, the major cytosolic Hsp70 protein found in yeast (Fig. 5*C*). Similar reactions using a tetratricopeptide repeat (TPR)-domain–containing version of FIC-$1_{aa134–508}$ (E274G) as AMPylase confirmed that Hsp40, HSP-1, HSP-3, and Ssa2, but not Hsp70, were modified by FIC-$1_{aa134–508}$ (E274G) (Fig. S5*E*). To map the sites of AMPylation on the identified HYPE targets (Hsp40, Hsp70), we subjected the modified proteins to mass spectrometry. We identified multiple sites of modification on both Hsp40 and Hsp70 (Fig. S5 *E–G*). The modified residues clustered into well-defined subdomains of the chaperones: Hsp70 was modified on five threonine residues in the nucleotide binding (ATPase) domain, whereas Hsp40 was predominantly AMPylated on six residues in its C-terminal portion, with an additional single site N-terminal to the J domain (Fig. S5*H*). In summary, these results show that HYPE and FIC-1 can efficiently modify a number of cytosolic chaperones, potentially altering their function.

FIC-1 (E274G) AMPylates Cytosolic Hsp70 in Vivo. By analogy to the established finding that ER-localized Fic proteins AMPylate the Hsp70 chaperone BiP, we next tested whether cytosolic Hsp70 could also be AMPylated in vivo. To this end, we analyzed the *S. cerevisiae* model and examined time-resolved samples from cells that overexpress FIC-1 (E274G) by immunoblot. Consistent with the increase in translation of a 70-kDa protein observed by [^{35}S]methionine/cysteine incorporation (Fig. S2*F*), probing the membrane with an Hsp70-specific antibody showed that intracellular Hsp70 levels increased over time (Fig. 6*A*, *Upper*). To determine whether Hsp70 is AMPylated, we reprobed the membrane with a Thr-AMP–specific polyclonal antibody. Indeed, the Thr-AMP antibody demonstrated that Hsp70 is AMPylated in *S. cerevisiae* (Fig. 6*A*, *Lower*). In contrast, similar experiments performed for yeast cells that express either FIC-1 or FIC-1 H404A failed to show up-regulation of cytoplasmic HSP70s or their AMPylated equivalents (Fig. S6*A*). These results confirm that only expression of FIC-1 (E274G) triggers a HSR accompanied by elevated levels of cytosolic HSP70 proteins. Cytosolic Hsp70 may thus be a major target of FIC-1 (E274G) in yeast.

Cytosolic Hsp70 Detoxifies FIC-1 (E274G) Overexpression in Yeast. Our data fit a model in which AMPylation of cytosolic chaperones causes a failure of cytosolic proteostasis, thus activating HSF1 and inducing the HSR. A prediction of this model is that additional chaperones should attenuate these effects. To test this prediction, we integrated additional copies of Ssa2 (Hsp70) and Hsc82 (Hsp90) into the genome of the *S. cerevisiae* strain that expresses FIC-1 (E274G) under the control of the estradiol-inducible promoter (Fig. 6*B* and Fig. S6*B*). Ssa2 partially suppressed the consequences of overexpression of FIC-1 (E274G) by improving growth in the presence of estradiol, yet without reducing intracellular FIC-1 (E274G) levels (Fig. S6*C*). Moreover, additional Hsc82 had no effect on cell viability (Fig. S6*B*), whereas coexpression of Ydj1 [a yeast Hsp40 ortholog that improves the efficiency of Ssa2's chaperone activity (24)] and Ssa2 further improved growth in the presence of FIC-1 (E274G) (Fig. 6*B*). Coexpression of Hsc82 with Ssa2 or with Ssa2 and Ydj1 antagonized the growth rescue afforded by these chaperones and reduced fitness in the presence of FIC-1 (E274G) (Fig. 6*B*). Hsp90 may therefore stabilize or potentiate the AMPylation activity of FIC-1 (E274G). In addition to suppressing the growth phenotype, Ssa2 also abrogated the breakdown in the cytosolic chaperoning machinery triggered by expression of FIC-1 (E274G), as evidenced by the decrease in Hsp104-YFP foci (Fig. 6*C* and Fig. S6*D*). Overexpression of FIC-1 (E274) in *S. cerevisiae* thus results in inactivation of cytosolic chaperones, especially Hsp70, thus disrupting proteostasis.

AMPylation of Hsp70 Alters Its Cellular Localization Dynamics. Heat shock triggers Hsp70 to partially relocalize to the nucleus (25). Although wild-type Hsp70 shows a dynamic influx–efflux pattern, the chaperoning-impaired mutant Hsp70 K71E is almost completely immobilized and absent from the nucleus following heat shock (25). To directly monitor Hsp70 function in mammalian cells, we tested whether AMPylation would change the subcellular redistribution of Hsp70 during heat shock. We used GFP-Hsp70 and GFP-Hsp70 K71E constructs to monitor their localization in the presence of HYPE (E234G). HeLa cells expressing HYPE, HYPE (E234G), or HYPE (H363A), together with GFP-Hsp70 or GFP-Hsp70 K71E were analyzed by fluorescence microscopy. In the absence of heat stress, less than 5% of cells contained a substantial fraction of GFP-Hsp70 in the nucleus (Fig. 7*A* and Fig. S7). Upon heat shock, ∼30% of cells cotransfected with wild-type HYPE or HYPE (H363A) showed GFP-Hsp70, but not GFP-Hsp70 K71E, relocalization to the nucleus. In contrast, cells coexpressing HYPE (E234G) and GFP-Hsp70 showed no relocalization, mimicking GFP-Hsp70 K71E (Fig. 7*A* and Fig. S7). We conclude that AMPylation interferes with Hsp70 localization dynamics during stress.

AMPylation Disrupts Influenza Virus Replication and Infectivity. To examine the consequences of AMPylation-induced interference with Hsp70 function, we investigated the effects of AMPylation on influenza virus infection in 293T cells, an HSP70-dependent process. Influenza virus RNA polymerase activity requires nuclear

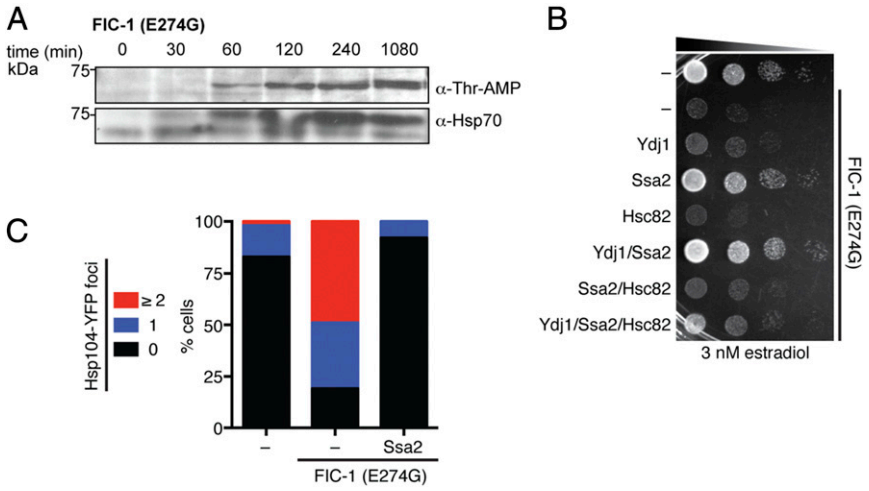

Fig. 6. FIC-1 (E274G) AMPylates cytosolic Hsp70 in vivo and Hsp70 suppresses FIC-1 (E274G)-mediated toxicity. (*A*) Increase in in vivo Hsp70 AMPylation following expression of FIC-1 (E274G) in S. *cerevisiae*. Samples were collected at indicated time intervals, OD-normalized, and probed with indicated antibodies. (*B*) Yeast growth test upon coexpression of FIC-1 (E274G) and Ydj2, Ssa2, and Hsc82. (*C*) Quantification of protein aggregation in the presence of FIC-1 (E274G) or FIC-1 (E274G) together with Ssa2.

localization of the viral ribonucleoproteins (vRNPs), a process that relies on active Hsp70 shuttling from the cytoplasm into the nucleus (26). We therefore hypothesized that AMPylation of Hsp70 would result in a reduction of vRNP nuclear localization and activity. We tested RNA polymerase activity in a minigenome transcription/replication assay where transient expression and nuclear localization of the vRNP complex components (NP, PB2, PB1, and PA) results in EGFP expression (27). As controls, we used VHHs

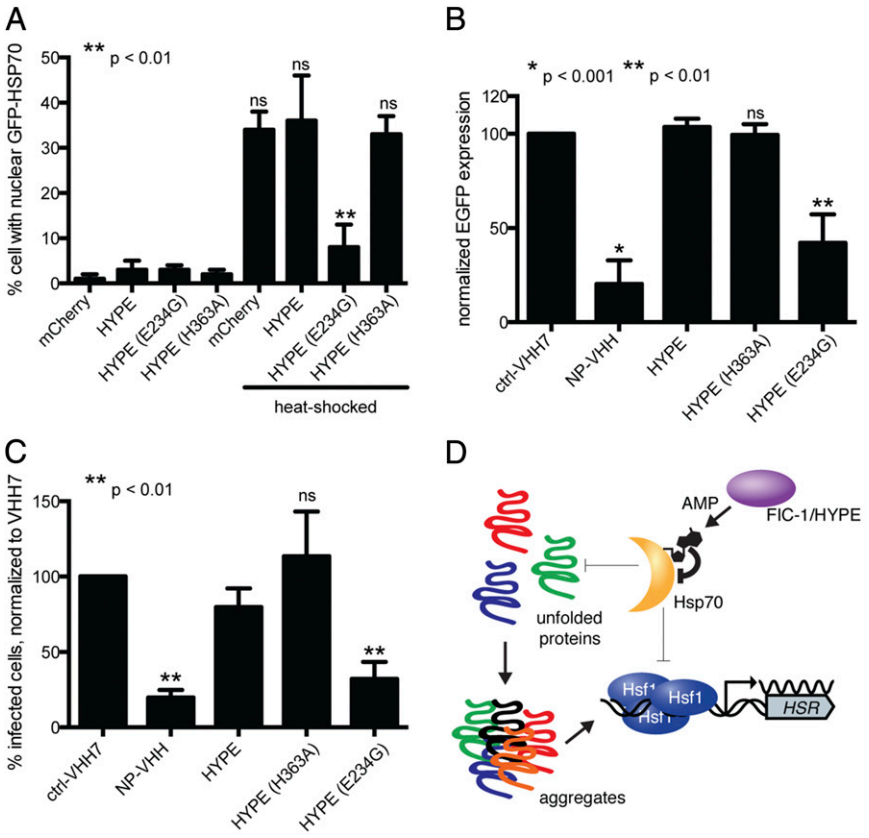

Fig. 7. AMPylation of Hsp70 alters its ATPase activity and cellular dynamics. (*A*) Quantification of GFP-Hsp70 nuclear localization. Averages from at least three replicas with more than 100 cells per sample analyzed in each experiment are shown. (*B* and *C*) Hyper-AMPylation inhibits influenza virus polymerase activity and replication. Influenza minigenome assay to monitor vRNP polymerase activity in host cell nucleus (*B*) and influenza infection assay (*C*). Data normalized to nonrelevant VHH-7–expressing cells. Average of three independent replicas including SD shown here. (*D*) Schematic model depicting how AMPylation might trigger the activation of a HSR in eukaryotic cells. *P* values calculated using Student's *t* test (*A*); ns, not significant ($P > 0.05$).

previously demonstrated to inhibit (anti-NP) or not affect (anti-class II MHC) assembly of vRNPs and virus replication, respectively (28). Indeed, although coexpression of HYPE or HYPE (H363A) together with the vRNP components had no effect on GFP synthesis, HYPE (E234G) reduced GFP expression, indicating impaired polymerase activity (Fig. 7*B*). HYPE (E234G) also efficiently attenuated viral infection as assessed by nucleoprotein levels 5 h postinfection (Fig. 7*C*). In summary, these results show that AMPylation can disrupt influenza transcription and replication through inhibition of cytosolic HSP70.

Discussion

Cells inevitably suffer exposure to various stressful cues that challenge protein homeostasis. Evolutionarily conserved stress responses help overcome and limit the damage imposed by stress. In this work, we uncovered a mode of induction of one such critical stress-response pathway, the HSF1-driven HSR. We found that Fic protein-mediated AMPylation leads to disrupted proteostasis and activation of HSF1 in yeast and human cells. In yeast, this response is associated with irreversible damage that culminates in cell death. Importantly, the toxicity associated with AMPylation in yeast can be suppressed by overexpression of cytosolic Hsp70, further implicating disrupted proteostasis as the root of the growth defect in general and supporting the notion that AMPylation impairs cytosolic Hsp70 function. However, Hsp70 could suppress toxicity by generally promoting protein folding without being the consequential AMPylation target.

Introduction of heterologous AMPylation enzymes into *S. cerevisiae* produced catastrophic consequences. The irreversible toxicity imposed by AMPylation on yeast host underscores the potency of this modification and its potential to wreak havoc on cellular homeostasis. In our model for the consequences of AMPylation (Fig. 7*D*), we propose that AMPylation inhibits Hsp70 activity, leading to protein misfolding and aggregation. Increased protein aggregation not only sequesters diverse chaperones, removing them from the general pool required to support folding of nascent proteins, but may also limit the availability of diverse essential factors that fall victim to growing aggregates. HSF1 senses the dearth of available chaperones and induces expression of the HSR, only to have one of its major targets, Hsp70, continue to be inhibited. Moreover, a breakdown of the cytosolic chaperoning machinery is coupled to inhibition of de novo protein synthesis, another process essential for growth. Thus, unrestrained AMPylation generates a perfect storm that disrupts both protein synthesis and folding, and undermines the effectiveness of the heat shock feedback loop by continuing to inhibit Hsp70.

The concept of modulating the HSF1-mediated HSR has been explored as a potential route for the treatment of neurodegenerative diseases, viral infections, and diverse cancers (29, 30). Although the detailed mechanisms of how protein aggregation-associated neurodegenerative diseases develop remain unclear, there is a large body of evidence suggesting a strong tie to the Hsp40/Hsp70/Hsp90 protein-folding machinery as well as the HSR and UPR^{ER} (31). Because hyper-AMPylation disrupts proteostasis in both the ER and cytosol, modulation of HYPE activity may boost the homeostatic capacity of these compartments and counteract protein aggregation, potentially offering a novel avenue toward ameliorating these conditions. In the realm of viral infections, we show that active HYPE can prevent transcription and replication functions essential for propagation of influenza by inhibition of Hsp70 nuclear localization and activity. As such, induction of AMPylation may be an effective antiviral strategy. Finally, most cancer cells have elevated Hsp70 and Hsp90 levels that enable them to resist dysregulation of protein homeostasis during tumorigenesis or anticancer therapies, thus enhancing cancer cell survival and tumor growth (32). Harnessing AMPylation to inhibit Hsp70 activity could be a powerful and broad-spectrum anticancer therapy that may synergize profoundly with Hsp90 inhibitors to cripple cancer's proteostatic support system.

Although the role of AMPylation in regulating stress responses via modification of Hsp70-class chaperones has been reported in several studies, it mostly confined the function of AMPylation to tuning the activity of the UPR^{ER} by altering the activity of the ER-resident Hsp70 chaperone BiP (9–11). However, subcellular localization of metazoan Fic proteins remains a matter of debate. Whereas several studies suggested that HYPE localizes to the ER and the adjacent nuclear envelope, we recently provided evidence for the presence of endogenous *C. elegans* FIC-1 in the cytoplasm (19). Furthermore, global AMPylation studies in mammalian cells showed that the plurality of modified targets are cytosolic (33). When expressed in yeast, FIC-1 colocalized both with ER and cytosolic markers. Cytosolic expression of inhibitory VHHs partially prevented FIC-1 (E274G)-associated cell death, suggesting that the enzyme is active in the cytosol, too. These data support a broader localization of FIC-1 and HYPE that includes both the ER and the cytosol, allowing AMPylation to modulate proteostasis in both of these compartments. Further efforts are required to work out the details of whether or not endogenous HYPE AMPylates cytosolic targets under certain stress conditions.

Although overexpression of FIC-1 (E274G) and HYPE (E234G) in yeast results in comparable outcomes, there are differences as well. FIC-1 (E274G) promotes far more pronounced phenotypes than does HYPE (E234G). In contrast, HYPE (E234G) in vitro AMPylates Ssa2 more efficiently than FIC-1 (E274G). However, whereas both enzymes modify similar targets in yeast, the residues involved and the extent to which the individual HSPs are AMPylated may vary. We also cannot exclude the possibility of targets uniquely AMPylated by either FIC-1 (E274G) or HYPE (E234G). Indeed, although target pools for FIC-1 (E274G) and HYPE (E234G) show substantial overlap, some targets are modified only by one of these AMPylases (19). Whether the functions of FIC-1 and HYPE are completely identical or show slight differences in the processes they regulate in worm and man, respectively, is an open question.

Overall, our work describes a mechanism for the simultaneous inactivation of Hsp70 and the activation of a robust HSR in the absence of heat stress. This mechanism depends on posttranslational protein AMPylation by Fic proteins. We are struck by the observation that Fic proteins can directly modify the major cellular chaperones, Hsp40, Hsp70, and Hsp90, suggesting a critical role for AMPylation enzymes in modulating the proteostasis network. By deploying Fic proteins to different compartments, cells—and genetic engineers—can modulate proteostasis and stress response pathways beyond the ER.

Materials and Methods

Yeast Growth Experiments. Synthetic defined yeast media with raffinose and complete amino acid supplemental mixture (SR-CSM), synthetic defined yeast media with dextrose and complete amino acid supplemental mixture (SD-CSM), or yeast extract with peptone and dextrose (YPD) (5 mL) was inoculated with respective strains and grown overnight at 30 °C. The next day, cultures were diluted in intended growth medium to OD_{600} = 0.1–0.3 and continuously cultured at room temperature or 30 °C. For growth-curve experiments, samples were taken in duplicate or triplicate for each measured time point. For agar-plate experiments, cells were plated as a dilution series on respective plates.

Yeast Stress Reporter Assays. Reporter constructs to assay for activation of the HSR (HSE), the general STRE and the UPRE consisted of GFP (STRE, UPRE constructs) or YFP (HSE constructs) with a series of 4× HSE, 4× STRE, or 4× UPRE sequences in a crippled *CYC1* promoter combined in a single integrating vector backbone (34). Constructs were integrated into a wild-type (W303a) yeast strain background at the *leu2* locus. To enable estradiol-mediated induction of FIC-1 and HYPE constructs, a chimeric, hormone-responsive transcriptional activator (GEM), consisting of the Gal4 DNA binding domain, the human estrogen receptor ligand binding domain, and the activation domain from the yeast transcription factor Msn2 (35), was transformed into the reporter strains and integrated into the *his3* locus. Subsequently, the three reporter/GEM strains were transformed with 2-μ plasmids encoding the FIC-1 and HYPE constructs under the control of the

GAL1 promoter and transformants were selected on synthetic defined-URA (SD-URA) plates. For assays, cells were grown from single colonies in SD-URA overnight, diluted into fresh media, and then left untreated or treated with 500 nM estradiol to induce the constructs for 4 h at 30 °C. Fluorescent reporters were measured by flow cytometry in a BD LSRFortessa equipped with a high-throughput sampler. Data were analyzed using FlowJo.

VHH Generation, Purification, and Evaluation. VHH generation, purification, and evaluation was performed as described in ref. 18. To test VHH–FIC-1 interaction in solution, ~100 μg of recombinant $HYPE_{aa187–437,}$ or FIC-$1_{aa258–508}$ was incubated with 100 μg VHH–5-carboxytetramethylrhodamine (TAMRA) at 4 °C for 1 h and analyzed on a Superdex S75 10/300 GL size-exclusion column. Absorbance at 280 nm (proteins) as well as at 545 nm (TAMRA) was recorded to assess the occurrence of specific interactions as evidenced by coelution of FIC-$1_{aa258–508}$ and a candidate VHH that results in overlapping peak maxima at both 280 nm and 545 nm.

Subcellular Fraction of Yeast Cells. Cells were lysed with a coffee grinder (36), resuspended in 1 mL PBS, and centrifuged at 300 × *g* for 5 min to remove unlysed cells. Cleared total cell lysate was transferred into a fresh tube and centrifuged at 13,000 × g for 15 min to pellet the insoluble fraction (ER, nuclei, protein aggregates, and so forth). The soluble (cytosolic) fraction was transferred into a fresh tube and samples were analyzed by immunoblotting.

Cell Culture. HeLa and HEK cells were cultured in DMEM supplemented with 10% (wt/vol) FBS.

Immunoblotting and Immunofluorescence Staining, in Vitro AMPylation Assays. Samples were treated, visualized, and analyzed exactly as described in ref. 18. For analysis of nuclear Hsf-1 foci formation and Hsp70-GFP localization, at least 10 randomly chosen frames from 3 independent samples were chosen and assessed by eye. Table S1 lists all antibodies used in this study. In vitro AMPylation assays were performed and analyzed as described previously (18).

Plasmid Construction. Primers used for plasmid constructions are listed in Table S2. Plasmids were routinely cloned using Gibson cloning (37).

Yeast [^{35}S]cysteine/Methionine Pulse-Labeling Experiment. Yeast cells were grown in SR-CSM overnight at 25 °C. The next morning, cells were diluted to OD_{600} = 0.2 in SR-CSM without Cys/Met and incubated at 25 °C for 3 h. All cultures were then supplemented with EasyTag Express ^{35}S Protein Labeling Mix (Perkin-Elmer) and 2% galactose to allow and induce transgene expression. Samples were collected at indicated time points postinduction. For cpm analysis (to quantify total ^{35}S-Cys/Met incorporation, as shown in Fig. 2*D*), 200 μL OD-normalized culture was mixed with 200 μL 10% trichloroacetic acid (TCA) on ice and boiled for 15 min. Next, 20 μL of each sample were transferred to a 1-cm^2 filter paper, air-dried, washed twice in individual containments with 10 mL 5% TCA, rinsed once with acetone, and air-dried overnight. The next morning, filters were transferred into scintillation counter vials containing 5 mL Opti-Flour (Perkin-Elmer) and analyzed. Samples were collected at indicated time points at least in duplicate. For autoradiographical visualization (to qualitatively assess changes in protein expression profiles as shown in Fig. S2*E*), 1 mL from the same culture was centrifuged and resulting pellets were resuspended in 30 μL PBS, boiled for 15 min, supplemented with 6× SDS-sample buffer, and boiled again for 20 min. Samples were then normalized for radioactive content before loading as described above.

HeLa ^{35}S-Pulse Labeling Experiment. Cells were grown in six-well plates, transfected with Lipofectamine2000 (Life Technologies) according to the manufacturer's instructions, and incubated for 24 h. Cells were starved in cysteine/methionine-free medium for 3 h, supplemented with EasyTag Express ^{35}S Protein Labeling Mix (Perkin-Elmer) for 15 min, and collected on ice. Total count normalization was done by scintillation counting, as described above.

HSE-Reporter Assays, Cell Viability Assays, and FACS Analysis. HSE-reporter cells were kindly provided by Susan Lindquist, Whitehead Institute of Biomedical Research, Cambridge, MA, and an assay was performed as described previously (20). For cell viability analysis, treated cells grown in six-well plates were trypsinized and stained with LIVE/DEAD Fixable Violet Dead Cell Stain according to the manufacturer's instructions (Life Technologies).

Data acquisition was performed on a BD LSR II (BD Biosciences) using CellQuest Pro (BD Biosciences) software. Data were analyzed with FlowJo (Tree Star).

RNA-seq. For yeast RNA-seq, 5 mL of cells were grown to OD_{600} = 0.5 at 30 °C and either left untreated, heat-shocked at 39 °C for 30 min or FIC-1 (E274G) was induced with 500 nM estradiol for 2 h. Cells were spun and pellets were snap frozen in liquid N_2 and stored at –80 °C. Pellets were thawed on ice, and total RNA was purified via phenol/chloroform separation using phase lock tubes (5 prime) followed by ethanol precipitation, as described previously (35). For HeLa cells overexpressing mutants of HYPE, 10^7 cells were pelleted and stored at –80 °C. Total RNA was extracted using a RNeasy kit (Qiagen). Total RNA samples were submitted to the Whitehead Genome Technology Core where polyA + RNA was purified, fragmented, and sequencing libraries were prepared with barcoding. Samples were multiplexed in a single lane of an Illumina Hi-SEq. 2500 and deep sequencing was performed. Reads were assigned by the barcode to the appropriate sample.

Data were processed using a local version of the Galaxy suite of next-generation sequencing tools. Reads were groomed and aligned to the SacCer3 *S. cerevisiae* reference genome or the Hg19 reference human genome using Tophat, transcripts were assembled and quantified using Cuffflinks, and fold-changes were computed using Cuffdiff (38).

GO analysis was performed using Gorilla (39) and redundant GO terms were removed with REVIGO using a similarity threshold of 0.5 (40).

Protein Purification. Purification of $HYPE_{aa187–437,}$ $HYPE_{aa187–437}$ (E234G), $HYPE_{aa187–437}$ (H363A), FIC-$1_{aa258–508,}$ FIC-$1_{aa258–508}$ (E274G), FIC-$1_{aa258–508}$ (H404A), FIC-$1_{aa134–508}$ (E274G), Hsp-1, and Hsp-3 has been described previously (19); Hsp40, Hsp70 and DAF-21 were purified accordingly. Human Hsp90 was purchased from Sigma.

VHH–HYPE Interaction Tests. Approximately 100 μg of recombinant $HYPE_{aa187–437,}$ or FIC-$1_{aa258–508}$ was incubated with 100 μg VHH–TAMRA at 4 °C for 1 h and analyzed on a Superdex S75 10/300 GL size-exclusion column. Absorbance at 280 nm (proteins) as well as at 545 nm (TAMRA) was recorded.

Minigenome Assay and Influenza Infection. For the minigenome assay, 293T cell were transfected with pCAGGS plasmids encoding for the viral NP,PB2, PB1, and PA proteins and pPolI-EGFP to provide an influenza model genome encoding for EGFP. HYPE variants or single-domain antibodies (VHHs) as positive and negative controls were coexpressed from pCDNA and pCAGGS plasmids, respectively. EGFP levels were measured 24 h posttransfection by flow cytometry.

For influenza A infection assays we transfected 293T cells with plasmids encoding for HYPE variants or VHHs. Twenty-four hours posttransfection, we infected the cells with influenza A/WSN/33 at a multiplicity of infection of 5 for 5 h. Cells were then trypsinized, fixed in 4% PFA, permeabilized with 0.1% saponin, and stained with an influenza nucleoprotein-specific VHH (αNPVHH1) (41) covalently coupled to Alexa647. Infected cells were quantified by flow cytometry.

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