Hsp70 nucleotide exchange factor Fes1 is essential for ubiquitin-dependent degradation of misfolded cytosolic proteins

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Protein quality control systems protect cells against the accumulation of toxic misfolded proteins by promoting their selective degradation. Malfunctions of quality control systems are linked to aging and neurodegenerative disease. Folding of polypeptides is facilitated by the association of 70 kDa Heat shock protein (Hsp70) molecular chaperones. If folding cannot be achieved, Hsp70 interacts with ubiquitylation enzymes that promote the proteasomal degradation of the misfolded protein. However, the factors that direct Hsp70 substrates toward the degradation machinery have remained unknown. Here, we identify Fes1, an Hsp70 nucleotide exchange factor of hitherto unclear physiological function, as a cytosolic triaging factor that promotes proteasomal degradation of misfolded proteins. Fes1 selectively interacts with misfolded proteins bound by Hsp70 and triggers their release from the chaperone. In the absence of Fes1, misfolded proteins fail to undergo polyubiquitylation, aggregate, and induce a strong heat shock response. Our findings reveal that Hsp70 direct proteins toward either folding or degradation by using distinct nucleotide exchange factors.

Hsp110 | proteasome | triage | ubiquitin ligase | Ubr1

Cells use quality control (QC) mechanisms to counteract the cytotoxic accumulation of misfolded proteins triggered by internal damage and environmental stressors. QC mechanisms selectively recognize and target misfolded polypeptides to folding-promoting factors or remove them from the cell by degradation. Malfunction of QC mechanisms results in deleterious consequences and is linked to many pressing human maladies including neurological disorders as well as to aging (1, 2).

In eukaryotes, misfolded cytosolic or nuclear proteins are degraded by the ubiquitin-proteasome system (UPS) (3). Proteasomal degradation is induced by polyubiquitylation of the substrate, which labels the protein for degradation by the 26S proteasome. In this process, a set of enzymes is required to activate (E1 enzyme) and selectively conjugate (E2 and E3 enzymes) ubiquitin to the misfolded substrate or its growing polyubiquitin chain (4). E3 ligases involved in QC recognize structural characteristics of a variety of misfolded proteins. For example, the nuclear ubiquitin E3 ligase San1 targets misfolded proteins to proteasomal degradation by recognizing exposed hydrophobicity of misfolded proteins (5).

Molecular chaperones of the 70 kDa heat shock protein (Hsp70) class promote protein folding and prevent aggregation by binding and shielding hydrophobic peptide segments of client proteins (6, 7). The association of Hsp70 with substrate is controlled by its ATPase activity. When the Hsp70 carries ATP, substrate can bind and release with fast kinetics. ATP hydrolysis is stimulated by cochaperones of the Hsp40 class that directly associate with and deliver substrates to the Hsp70. Substrate remains associated with Hsp70 until ADP release is triggered by nucleotide exchange factors (NEFs) and ATP rebinds. As an outcome, NEFs trigger substrate release from Hsp70s.

Interaction of Hsp70 with substrates is coordinated with QC mechanisms so that misfolded proteins are triaged between folding and degradation (8–10). For example, in metazoan cells, the Hsp70-interacting E3 ligase Carboxyl-terminus of Hsp70 Interacting Protein (CHIP) induces ubiquitin-proteasome–mediated degradation of misfolded proteins (11, 12). In yeast, a functional Hsp70 system is required for ubiquitylation and proteasomal degradation of many misfolded proteins (13, 14). Three E3 ligases, Hul5, San1, and Ubr1, have been shown to ubiquitylate misfolded proteins that depend on Hsp70 for their degradation, but how Hsp70 and the E3 ligases coordinate their interactions is not known (15–19). NEFs are candidates that potentially could coordinate QC by releasing misfolded proteins from Hsp70 but up until now, their roles in this process have been undefined.

Here we report that the NEF Fes1 is a key triaging factor in protein QC that it is specifically required for targeting misfolded proteins recognized by Hsp70 to the UPS. Our results support a model in which Fes1 facilitates the selective release of misfolded proteins from the Hsp70 system, thereby promoting their destruction by the ubiquitin-proteasome machinery.

Results

Cells Lacking Fes1 Are Hypersensitive to Induced Protein Misfolding and Display a Strong and Constitutive Heat Shock Response. Fes1 is a biochemically well-characterized yeast Hsp70 NEF of the Heat shock binding protein 1 (HspBP1)-class with no assigned cell biological function (20, 21). Using a reverse genetics approach, we set out to investigate the involvement of Fes1 in QC. We constructed a *fes1Δ* strain and could confirm the previously reported sensitivity to high temperature (Fig. S1A) (22). Importantly, *fes1Δ* cells were also found to be hypersensitive to protein misfolding induced either by the toxic proline ring analog azetidine-2-carboxylic acid or the toxic arginine analog canavanine (Fig. 1A). The sensitivity of *fes1Δ* cells to distinct conditions that induce protein misfolding implicates Fes1 in QC.

To further investigate the physiological role of Fes1, we analyzed the overall protein profile of *fes1Δ* cells grown under nonstressful conditions (Yeast extract peptone dextrose, YPD, 25 °C) by SDS/PAGE. Three protein species migrating at positions between the 64- and 97-kDa size standards were markedly induced in samples from *fes1Δ* cells compared with the WT

Fig. 1. Inactivation of *FES1* results in hypersensitivity to induced protein misfolding and strong activation of the heat shock response. (*A*) *fes1Δ* strains are hypersensitive to the amino acid analogs azetidine-2-carboxylic acid (AzC, 0.1 μM) and canavanine (Can, 3.6 mM). (*B*) Total protein extracts from WT and *fes1Δ* cells grown in YPD at 25 °C separated by SDS/PAGE and stained with Coomassie Brilliant Blue. The arrows mark the positions of induced Hsp70, Hsp90, and Hsp104 (*Results*). (*C*) Western analysis of HA-epitope tagged Hsp70s Ssa1, Ssa2, Ssa3, and Ssa4 expressed from their endogenous loci in WT and *fes1Δ* cells. Protein extract gel loading was adjusted as indicated (%). (*D*) Hsf1-Myc hypo- and hyperphosphorylation status in WT and *fes1Δ* cells was monitored using a gel shift assay based on Phos-Tag. Heat shocked (HS) cells were transferred from 25 °C to 37 °C for 30 min. Standard SDS/PAGE (*Right*). (*E*) Heat shock induction in Hsp70 NEF single- and double-mutant strains analyzed as in *B* and growth phenotypes at different temperatures.

control (Fig. 1*B*). After purification, using denaturing anion exchange chromatography, these proteins were identified by MS as Hsp70 (70 kDa), Hsp90 (81 kDa), and Hsp104 (102 kDa), a finding that was confirmed by Western analysis (Fig. S1*B*). Further analysis of Stress-seventy class A (Ssa)-class Hsp70 proteins in *fes1Δ* cells revealed an expression pattern that was strikingly similar to that resulting from a strong and constitutive heat shock response in WT cells (Fig. 1*C*). Whereas *SSA2* is a constitutively expressed gene that is unresponsive to stress, *SSA1*, *SSA3*, and *SSA4* are all transcriptionally induced by heat shock factor (Hsf1) in response to cytoplasmic protein folding stress (23). This response is triggered by accumulation of misfolded proteins in the cytoplasm via a mechanism that relies on hyperphosphorylation of Hsf1 (24). We directly tested if Hsf1 was constitutively activated in *fes1Δ* cells under nonstressful growth conditions by assessing the phosphorylation status of Hsf1 using phosphoprotein gel shift analysis. This assay revealed that Hsf1 was hyperphosphorylated in the *fes1Δ* mutant in comparison with WT cells (Fig. 1*D*). Taken together, our data clearly show that the heat shock response is constitutively active in *fes1Δ* cells at nonstressful conditions, suggesting that the cells accumulate misfolded proteins in the cytoplasm.

As a supporting experiment to test whether *fes1Δ* mutants accumulate misfolded proteins, we used an aggregation reporter consisting of a fusion between highly stable GFP and thermolabile firefly luciferase that unfolds after heat shock and coaggregates with available misfolded proteins (25). Before heat shock, both WT and *fes1Δ* cells grown at 25 °C expressed luciferase-GFP evenly in the cytoplasm (Fig. S1*C*), with 9.3% and 25.0% of the cells containing luciferase-GFP aggregates, respectively. After heat shock at 37 °C for 30 min, 20.7% and 42.7% of the WT and *fes1Δ* cells, respectively, contained aggregates.

Because Fes1 represents only one of the three eukaryotic classes of Hsp70 NEFs present in the cytoplasm of yeast, we asked whether the Hsp110-class members Sse1 and Sse2 or the Bcl2-associated athanogene (BAG)-class member Snl1 have a similar or overlapping role in protecting cells against misfolded proteins (26, 27). Remarkably, we found a strong constitutive heat shock response to be a specific property of *fes1Δ* cells because none of the other mutants (*sse1Δ*, *sse2Δ*, and *snl1Δ*) displayed high-level expression of Hsp70, Hsp90, and Hsp104 (Fig. 1*E*). This is particularly striking in the light of the fact that the *sse1Δ* mutation causes a much more severe growth defect than *fes1Δ* (Fig. 1*E*). Moreover, NEF double mutants accumulated

Hsp70, Hsp90, and Hsp104 proteins to similar extents as *fes1Δ* single mutants, and none of the double mutations exacerbated the growth phenotypes of the individual mutations.

The strong heat shock response in *fes1Δ* cells prompted us to perform a series of supporting experiments to investigate the importance of the induction of heat shock proteins for *fes1Δ* cells (Fig. S2). First, we partially attenuated the activity of the essential Hsf1 by removal of the C-terminal activation domain (*hsf1ΔCT*) (28). When *fes1Δ* was combined with this mutation, the *hsf1ΔCT* mutation attenuated the induction of Hsp70 and Hsp90, which went along with a strong synthetic phenotype; the double mutant displayed extremely poor growth at 30 °C (Fig. S2 *A* and *B*). Similarly, when *fes1Δ* was combined with deletions of genes encoding either cotranslationally acting Hsp70 proteins (*ssb1Δ*, *ssb2Δ*) or the inducible Hsp70 system (*ssa1Δ*, *ssa3Δ*, *ssa4Δ*) (29) as well as a deletion of *HSP104*, which encodes the main cytoplasmic disaggregation machine in *Saccharomyces cerevisiae* (30), all resulted in severe synthetic growth defects, in particular at higher temperature (Fig. S2*C*). Together, these findings demonstrate that *fes1Δ* cells depend on and induce multiple chaperone systems to cope with the defects associated with the lack of Fes1.

Fes1 Is Specifically Required for UPS-Dependent Degradation of Misfolded Proteins. The observation that misfolded proteins accumulate in *fes1Δ* cells prompted us to ask if Fes1 is a general factor required for degradation of misfolded proteins associated with Hsp70. Initially, we took advantage of two mutant versions of the globular and compactly folded dihydrofolate reductase (DHFR) that are metabolically unstable at 30 °C (Fig. S3). The basic characterization of these DHFR mutants is presented in Figs. S3–S8. Briefly, degradation of reporter proteins (DHFR-Ura3) carrying the DHFR^mutC or DHFR^mutD modules was found to be dependent on the ubiquitin-proteasome pathway because their degradation was severely impaired by mutations in genes encoding UPS components (*uba1-ts*, *ubc4Δ ubc5Δ*, *ubr1Δ*, and *ump1Δ*). Moreover, degradation of these proteins (DHFR-Ura3) requires the Ssa-class of Hsp70 and Hsp40 (Figs. S4–S6). Pull-down experiments performed with DHFR^mutC-FLAG demonstrated that this folding-deficient protein is associated with Ssa1 and Ydj1, which are representatives of the Hsp70 and Hsp40 chaperone classes, respectively (Figs. S5 and S6). Failure to degrade DHFR^mutC leads to its accumulation as intracellular aggregates (Fig. S7). We tested if Fes1 associates with DHFR^mutC by performing immunoprecipitation. Fes1 copurified with DHFR^mutC-FLAG
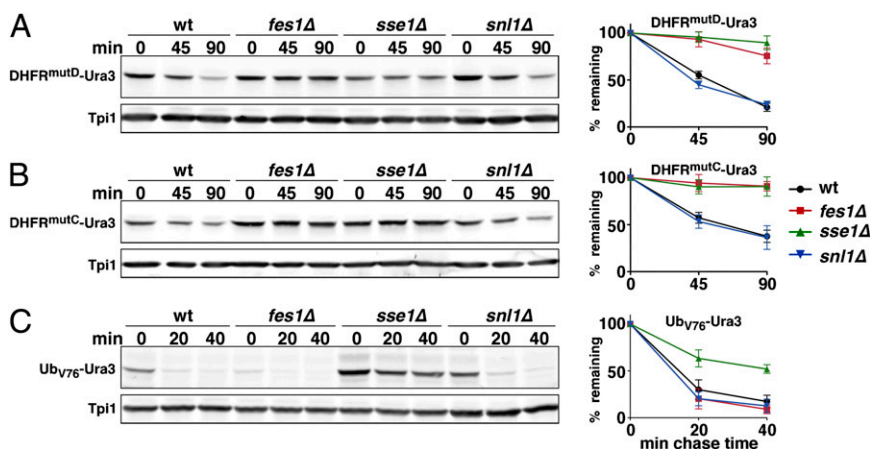
Fig. 2. Fes1 is required for UPS degradation of misfolded DHFR mutants. (*A*, *B*) Two folding-deficient variants of DHFR (DHFR^MutC-Ura3, DHFR^MutD-Ura3) fused to Ura3 were expressed from the P_CUP1 promoter (Fig. S3) in isogenic WT, *fes1Δ*, *sse1Δ*, and *snl1Δ* strains. (*C*) A third substrate (Ub-V76-Ura3) is a noncleavable ubiquitin fusion protein that is targeted by the UFD pathway. Following cycloheximide (CH) addition, extracts were prepared by glass bead lysis of cells collected at the indicated time points and analyzed by quantitative Western blot. The data represent at least three experiments with independent transformants (error bars indicate SD).

but not with the natively folded control protein DHFR-FLAG (Fig. S8*A*), indicated that Fes1 interacts specifically with misfolded DHFR. We therefore asked whether Fes1 is required for degradation of DHFR^mutC-Ura3 and DHFR^mutD-Ura3 and found that the *fes1Δ* mutation caused a drastic stabilization of both proteins (Fig. 2), which was also reflected by growth of the cells on media lacking uracil (Fig. S8*B*).

Independent evidence for a general role for Fes1 in the degradation of misfolded proteins came from the analysis of an interactor identified in a Fes1 two-hybrid screen. In this screen, we found Fes1 to interact with Hsp70 of both the Ssa class (nine isolates for Ssa1 and two for Ssa4) and the ribosome-associated Ssb class (one Ssb1 isolate) (31). Apart from Hsp70 proteins, a number of other interactors were found, including Sis1, an Hsp40 that binds directly to Hsp70 (32, 33), and an internal fragment of the mitochondrial RNA polymerase (Rpo41^T920-L1217). Importantly, two well-characterized mutations in Fes1 substituting residues specifically required for the association with Hsp70 (Fes1^A79R, R195A) abolished the interaction with both Sis1 and Rpo41^T920-L1217 (Fig. 3*A*) (21). We conclude that Fes1 interaction with both of these proteins depends on Hsp70, most likely via formation of ternary complexes. A comparison of the interacting stretch of Rpo41^T920-L1217 with the corresponding sequence (C897-I1179) in the crystal structure of human mitochondrial RNA polymerase (34) indicated that the identified segment does not represent a stable independently folding structural domain (Fig. 3*A*). Rpo41^T920-L1217 is an internal fragment of the C-terminal polymerase domain taken out of structural context wherein it is normally stabilized by numerous interactions with both the N- and C-terminal domains. Direct measurement of solubility by ultracentrifugation showed that the bulk of the Rpo41^T920-L1217 population was aggregated (Fig. 3*B*). These observations indicated that Rpo41^T920-L1217 is a misfolded polypeptide with a tendency to aggregate and therefore a likely target for ubiquitin-dependent QC mechanisms. We tested this hypothesis by monitoring the stability of Rpo41^T920-L1217 in WT and *fes1Δ* cells. Strikingly, although Rpo41^T920-L1217 was degraded in WT cells, the protein was completely stable in *fes1Δ* cells over 90 min (Fig. 3*C*). Similar to what was observed for the misfolded DHFR derivatives described above, Hsp70 function and the proteasome system was required for the degradation of Rpo41^T920-L1217 (Fig. 3*D*) (35). Inactivation of the E3 ubiquitin ligase Ubr1, which is required for the selective ubiquitylation of misfolded proteins, resulted in a stabilization of Rpo41^T920-L1217 (16, 17). Cells expressing Fes1^A79R, R195A that do not interact with Hsp70 did not support degradation of Rpo41^T920-L1217 (Fig. 3*E*). We conclude that, similar to degradation of the misfolded DHFR derivatives described above, the proteolytic targeting of the aberrant polypeptide Rpo41^T920-L1217 by the UPS depends on the

Ssa1-class of Hsp70 and its NEF Fes1. Together, our findings obtained with multiple distinct test proteins identify Fes1 as a factor essential for the degradation of misfolded cytosolic proteins.

We found that the role of Fes1 in ubiquitin-dependent proteolysis is specific for misfolded proteins because the degradation of several other known ubiquitin-dependent substrates was unaffected. Specifically, the artificial ubiquitin (Gly76Val)-fusion degradation (UFD) pathway substrate Ub_V76-Ura3 (36) is degraded normally in *fes1Δ* cells (Fig. 2*C* and Fig. S8). Similarly, the extremely fast degradation of two natural UPS substrates, the latent transcription factor Stp1 (37) and the glucose-triggered and Hsp70-dependent degradation of the gluconeogenic enzyme fructose-1,6-bisphosphatase 1 (38) proceeded at WT rates (Fig. 3*F*). In contrast to the specific effects of the *fes1Δ* mutation toward misfolded proteins, a severe reduction of the total NEF activity in the cytoplasm by the *sse1Δ* mutation (Sse1 is sevenfold more abundant than Fes1; ref. 39) resulted not only in an increased stability of folding-impaired substrates, but also of the UFD pathway substrate Ub_V76-Ura3 (Fig. 2*C* and Fig. S8). Deletion of *SNL1* had no effects on any of the substrates tested (Fig. 2 and Fig. S8). Thus, in contrast to Sse1 or Snl1, Fes1 has a dedicated and specific role in misfolded protein degradation.

Fes1 Releases Misfolded Proteins from Hsp70, Thereby Targeting Them for Ubiquitylation. An established biochemical function of NEFs is to stimulate nucleotide exchange in Hsp70, a process that results in substrate release (22, 31). We tested if Fes1 promotes the release of misfolded proteins from Hsp70 by monitoring the amount of Hsp70 that binds misfolded DHFR^MutC in *fes1Δ* cells and WT cells. We choose the Hsp70 Ssa2 for detection because it is expressed at the same constitutive level in both cell types (Fig. 1*C*). It is worthy to note that Ssa2 is competing with the higher induced levels of Ssa1, Ssa3, and Ssa4 in the *fes1Δ* strain. Strikingly, when immunoprecipitating DHFR^MutC from WT and *fes1Δ* cells and analyzing the amount of Ssa2 that copurified, we found that substantially more Ssa2 copurified with DHFR^MutC immunoprecipitated from *fes1Δ* cells than from WT cells (Fig. 4*A*) despite the higher levels of competing Ssa chaperones. Next, we investigated the involvement of Fes1 in heat shock–induced ubiquitylation (15). We predicted that misfolded proteins induced by heat treatment require Fes1 for efficient ubiquitylation. Confirming our prediction, heat shock–induced ubiquitylation was severely impaired in *fes1Δ* cells (Fig. 4*B*). In *sse1Δ* cells, in contrast, the levels of ubiquitylated proteins were already increased at the lower temperature and further increased upon heat stress. The increased amounts of ubiquitin-modified proteins in these mutants even in the absence of external stress argue strongly against a specific function of Sse1 in promoting ubiquitylation of misfolded proteins and rather point to a general

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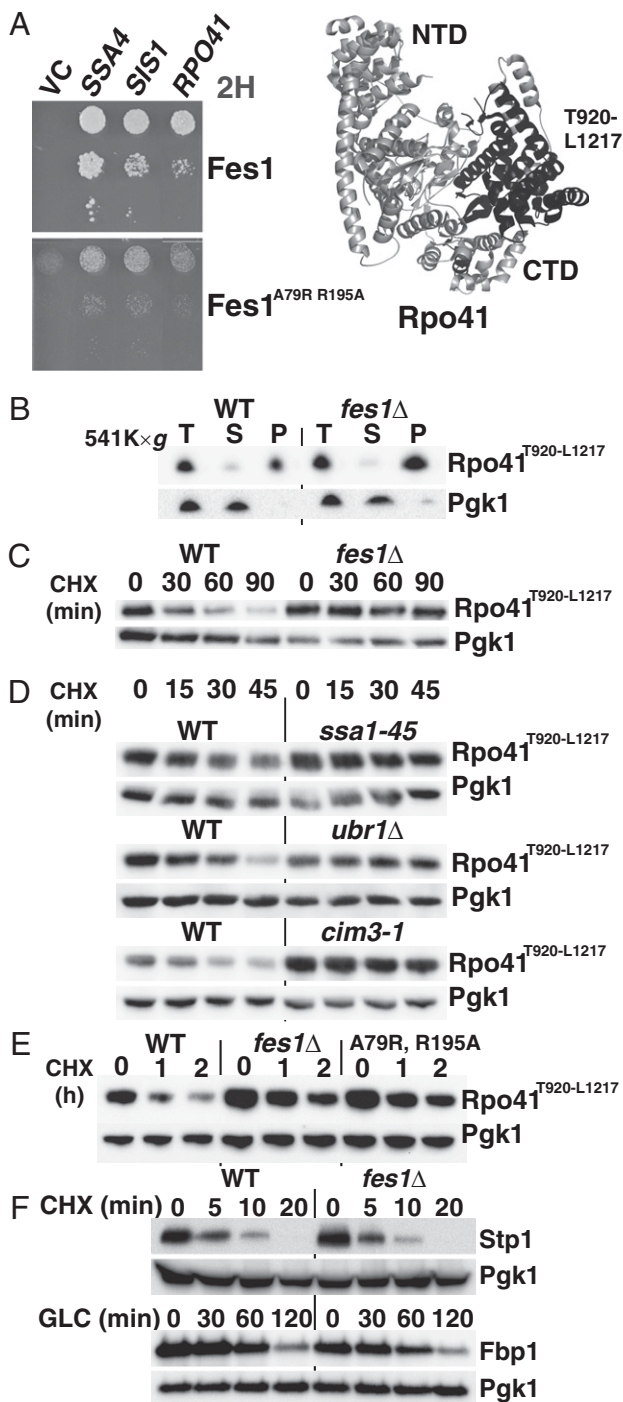
Fig. 3. Fes1 interacts with and is required for UPS degradation of Hsp70-associated misfolded protein Rpo41$^{T920-L1217}$. (*A*) Growth on medium selective for interaction of a yeast two-hybrid reporter strain expressing Fes1 or the Hsp70-binding mutant Fes1$^{A79R, R195A}$ as baits and Hsp70 (*SSA4*), Hsp40 (*SIS1*), or a fragment of *RPO41* as preys Vector control (VC). The isolated *RPO41* expresses a truncated polypeptide (T920-L1217) that corresponds to an internal region of the C-terminal domain (CTD) of mitochondrial RNA polymerase (marked in black in the crystal structure of human homolog). The N-terminal domain (NTD) is indicated. (*B*) Western analysis of total lysate (T) and supernatant (S) and pellet (P) fractions following ultracentrifugation (541,000 × g). Pgk1 functions as a soluble control protein. (*C*) Degradation of Rpo41$^{T920-L1217}$ in WT and *fes1Δ* cells after translation arrest by cycloheximide (CHX). (*D*) Degradation at a restrictive temperature (37 °C) as in *C* in strains carrying mutations in Hsp70 (*ssa1-45 ssa2Δ ssa3Δ ssa4Δ*), the proteasome (*cim3-1*) or the ubiquitin E3 ligase Ubr1 (*ubr1Δ*). (*E*) Degradation as

in *C* in strains carrying Hsp70 binding (A79R, R195A) mutations in the chromosomal *FES1* locus. (*F*) UPS degradation (CHX) of transcription factor Stp1-HA (Stp1). Induced Hsp70-dependent ubiquitin-proteasome degradation of fructose-1,6-bisphosphatase (Fbp1-HA) followed after addition of 2% glucose to Yeast extract peptone-ethanol medium.

role of Sse1 in protein folding. Finally, we directly investigated if Fes1 promotes interactions between ubiquitin ligases and their misfolded protein substrates. The ubiquitin ligase Ubr1-HA specifically copurified with misfolded DHFR^{MutD} but not with native DHFR (Fig. 4*C*). Importantly, approximately fivefold more Ubr1-HA bound the misfolded protein in purifications from WT compared with *fes1Δ* extracts. We conclude that Fes1 has an important general role in releasing misfolded proteins from Hsp70 and targeting them for ubiquitylation and proteasomal degradation.
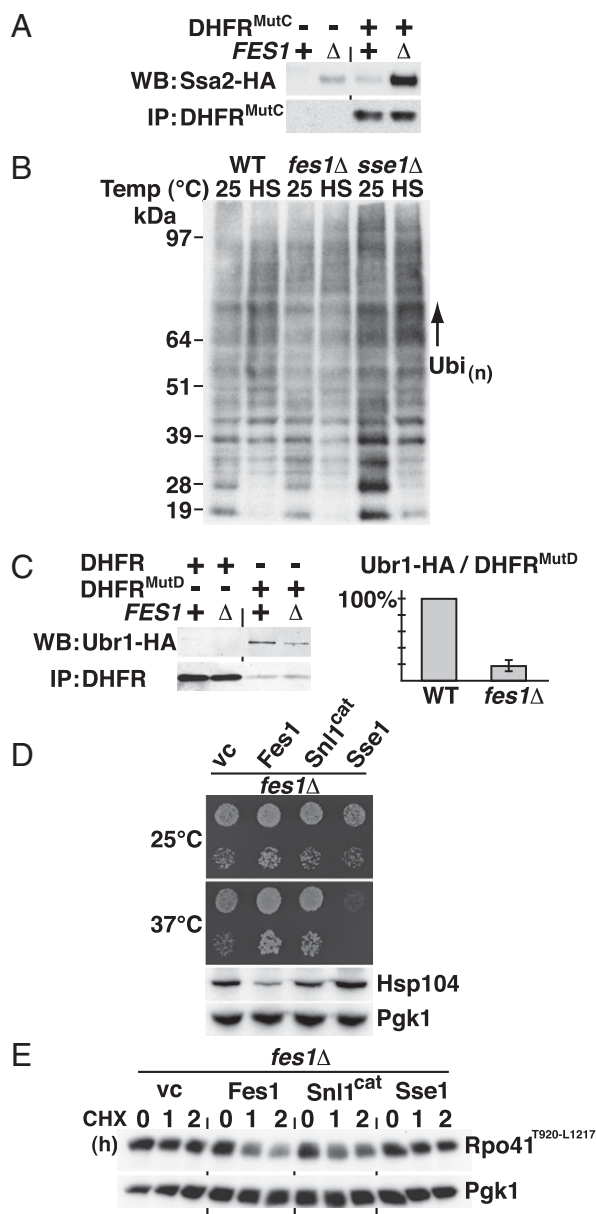
Structurally Unrelated NEF but Not Sse1 Can Partially Replace Fes1 Function. We tested if Fes1 function could be executed by a structurally unrelated Hsp70 NEF by expressing the soluble C-terminal catalytic BAG domain of Snl1 (Snl1^{Cat}) (40) and Sse1. Expression of the Snl1^{Cat} in *fes1Δ* cells resulted in partial suppression of the temperature-sensitive growth phenotype (Fig. 4*D*) as well as partial restoration of Rpo41$^{T920-L1217}$ degradation (Fig. 4*E*). In contrast, Sse1 overexpression exacerbated the temperature-sensitive growth phenotype and did not suppress the defect in Rpo41$^{T920-L1217}$ degradation. Expression of Snl1^{Cat} or Sse1 did not suppress the induced heat shock response of *fes1Δ* cells (Fig. 4*D*). The observation that Snl1^{Cat} can partially replace the function of Fes1 suggests that Fes1 supports protein degradation by a simple Hsp70 release mechanism, whereas Sse1 functions in a competing pathway.

Discussion

Based on the findings reported here, we propose a model for the role of Fes1 in cytoplasmic protein QC that integrates all available data (Fig. 5). Abundant Hsp70 associates transiently with exposed hydrophophic patches of misfolded proteins and maintains these aggregation-prone proteins soluble. Hsp70 can refold misfolded proteins in ATPase-driven cycles catalyzed by the dominant NEFs of the Hsp110 class (Sse1/Sse2). Hsp110 contributes directly to the folding process by binding hydrophobic peptide stretches of the substrate protein. Misfolded proteins resilient to Hsp70-Hsp110 refolding will undergo repetitive and futile interactions with Hsp70-Hsp110. Eventually the Hsp70-substrate complexes will encounter the NEF Fes1. Fes1 triggers nucleotide exchange of the Hsp70, which results in the release of the misfolded protein from Hsp70 and facilitates the interaction with downstream ubiquitin E3 ligases that target misfolded proteins for degradation by the UPS.

In our model, we propose that NEFs of the Hsp110 class mainly support protein folding activities of Hsp70s, whereas Fes1 targets Hsp70-associated misfolded proteins for degradation. This notion is supported by comparisons of the phenotypes of *sse1Δ* and *fes1Δ* strains (Fig. 1*E*) as well as by the observation that overexpression of the catalytic domain of Snl1 suppresses *fes1Δ*, whereas that of Sse1 does not (Fig. 4 *D* and *E*). We find that *sse1Δ* cells, in contrast to *fes1Δ* cells, are very slow growing at all temperatures, likely because removal of this abundant NEF induces a general Hsp70 activity defect. Consistent with this understanding, *sse1Δ* mutants expressing folding-impaired Ura3 reporter remain uracil auxotrophs despite the observed stabilization of these reporter proteins (Fig. S8), indicating that they accumulate as nonnatively folded proteins. Furthermore, *sse1Δ* cells do not exhibit a strong heat shock phenotype as do *fes1Δ* cells (Fig. 1*E*), but do accumulate larger amounts of ubiquitylated proteins at normal growth temperature as well as under heat stress conditions (Fig. 4*B*). In conclusion, *sse1Δ* degradation phenotypes likely reflect general

A

DHFR^MutC − − + +
FES1 + Δ | + Δ

WB: Ssa2-HA

IP: DHFR^MutC

B

WT   fes1Δ   sse1Δ
Temp (°C) 25 HS 25 HS 25 HS
kDa
97−
64−   Ubi_{(n)}
51−
39−
28−
19−

C

DHFR + + − −
DHFR^MutD − − + +
FES1 + Δ | + Δ

Ubr1-HA / DHFR^MutD
100%

WB: Ubr1-HA

IP: DHFR

WT   fes1Δ

D

vc Fes1 Snl1^cat Sse1
fes1Δ

25°C

37°C

Hsp104
Pgk1

E

fes1Δ
vc   Fes1   Snl1^cat   Sse1
CHX 0 1 2 | 0 1 2 | 0 1 2 | 0 1 2
(h)   Rpo41^{T920-L1217}
Pgk1

Fig. 4. Fes1 is required for the release of misfolded proteins from Hsp70 and their targeting to the UPS. (A) Western blot (WB) analysis of amounts of Hsp70 Ssa2-HA copurifying with DHFR^MutC-FLAG immunoprecipitated (IP) from WT and fes1Δ protein extracts. (B) Western analysis of total poly-ubiquitin levels (Ubi_{(n)}) in WT, fes1Δ, and sse1Δ cells grown at 25 °C or heat-shocked (HS) at 42 °C for 30 min. Loading was normalized to amount of cells used for denaturing protein extraction. (C) WB analysis of amounts of Ubr1-HA copurifying with DHFR^MutD-FLAG IP from WT and fes1Δ protein extracts. Quantification of the amount of Ubr1-HA copurifying with DHFR^MutD-FLAG normalized to WT (100%) (error bars represent SD of experiments performed with five independent transformants). (D) Growth of cells (fes1Δ) transformed with vector control (vc) or plasmids expressing Fes1, Snl1^cat, or Sse1. Heat shock response of each transformant was assessed by immuno-blotting (Hsp104). (E) UPS degradation after cycloheximide (CHX) of Rpo41^{T920-L1217} followed in fes1Δ cells expressing Fes1, Snl1^cat or Sse1.

malfunction of the Hsp70 system (i.e., that misfolded proteins become stabilized in sse1Δ cells because of extensive aggregation or as a consequence of abundant production of misfolded poly-peptides competing for the degradation pathways).

Biochemical differences between Hsp110 and Fes1 might explain how these two NEFs can induce such diverse fates of

Hsp70 substrates as folding and degradation. In contrast to Fes1, Hsp110 is a chaperone that recently has been shown to directly bind hydrophobic peptides (41–43). Thus, direct binding of hy-drophobic patches might help to maintain the misfolded protein soluble, help the protein to fold, and shield it from interactions with the ubiquitylation machinery. In support of the latter concept, the E3 ligase San1 directly interacts with hydrophobic patches of its misfolded protein substrates (5). Indeed, Sse1 inhibits San1-dependent ubiquitylation in vitro, corroborating the notion of direct competition for substrate binding (14). Further support for the hypothesis of qualitative activity differences between Hsp110 and Fes1 is provided by the observation that purified Hsp70-Hsp40-Sse1 can extract and refold luciferase from aggre-gates, whereas Hsp70-Hsp40-Fes1 cannot (42). When all these data are considered, a simple hypothesis emerges: Sse1 has sub-strate binding activities and promotes protein folding probably by mediating rebinding to Hsp70, whereas Fes1 is devoid of the same activities and therefore triggers release of Hsp70 substrates that are to be ubiquitylated and degraded. Our model provides the conceptual foundation for how distinct classes of NEFs reg-ulate Hsp70 function in protein folding and degradation.

Materials and Methods

Strains, Plasmids, and Growth Media. Details on constructions of strains and plasmids are available upon request. Plasmid inserts expressing various DHFR-derived test proteins used in this study are depicted in Fig. S1A. Constructs expressing DHFR-Ura3 and DHFR^mutC-Ura3 were generated and kindly pro-vided by Jörg Höckendorff. Standard media include YPD medium and am-monia-based synthetic complete dextrose (SC).

Heat Shock Protein Purification and MS Analysis. Strains were expanded in YPD medium at 25 °C and harvested at logarithmic growth phase by centrifu-gation. Cells were lysed in denaturing lysis buffer (40 mM Tris·HCl pH 7.5 1 mM EDTA 8 M urea) by bead beating. After preclearing by centrifugation (21,100 × g), the supernatant was applied onto a gravity-flow Q-Sepharose (GE Healthcare Life Sciences) column. The column was extensively washed with denaturing lysis buffer, and bound proteins were eluted using an NaCl step gradient (50-mM increments up to 600 mM NaCl) prepared in de-naturing lysis buffer. Eluted proteins were separated by SDS/PAGE followed by Coomassie Brilliant Blue staining. Protein bands were excised and sub-jected to tryptic extraction and MS analysis.

Yeast Two-Hybrid Assay. A genomic yeast plasmid library (pJG4-5) was screened for interactors (growth on SC-galactose media lacking lysine and leucine) with full-length Fes1 fused to the N terminus of the DNA-binding domain of LexA in yeast strain CAY235 (44).

Analytical Ultracentrifugation of Rpo41^{T920-L1217}. Cells expressing Rpo41^{T920-L1217}-HA were grown at 30 °C to logarithmic growth phase, harvested by cen-trifugation, and lysed in 50 mM Tris·HCl pH 7.5, 5 mM MgCl_2, 10 mM DTT, 1 mM PMSF, and 1 mM N-ethylmaleimide by bead beating. Lysates were cleared from cell debris by low-speed centrifugation (1500 × g) and deter-gents were added to the following final concentrations, 1% (vol/vol) Non-idet P-40, 1% (vol/vol) Tween 20, and 0.1% SB3-14. The solubilized lysates

Hsp110

ADP◄

ATP
Folding

Hsp70 ADP

Substrate

ADP◄

Fes1

ATP
Degradation
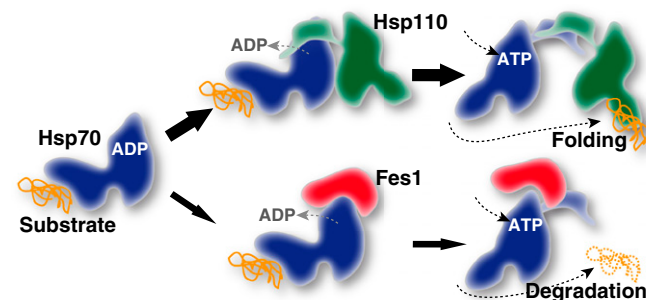
Fig. 5. Model for Hsp70 substrate triage regulated by NEFs (Discussion).

were subjected to 30 min of ultracentrifugation in a TLA 100.3 rotor (Beckman) at 100,000 rpm (541,000 × g) and analyzed by Western blotting.

Western Blot Analysis and Determination of Protein Stability. Cells were grown at 30 °C to logarithmic growth phase, and cycloheximide was added to the cultures at a final concentration of 100 mg/L. Strains carrying temperature-sensitive alleles were expanded at permissive 25 °C and incubated at restrictive 37 °C for 30 min before cycloheximide addition. Protein extracts were prepared from samples taken at the time points indicated either by glass bead lysis in ice cold Native lysis buffer (50 mM Na·Hepes pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1% (vol/vol) Triton-X100 containing Complete Protease Inhibitor Mixture (Roche)) or by NaOH and trichloroacetic acid as described previously (45). Equal amounts of proteins were separated by SDS/PAGE and analyzed by quantitative Western blotting (46) or by chemiluminescence detection (SuperSignal West Dura Extended-Duration Substrate; Pierce) and quantified using an LAS1000 system (Fuji Photo Film Co.).

Coimmunoprecipitation of Mutant DHFR and Associated Proteins. Cells were grown at 30 °C to logarithmic growth phase in SC medium supplemented with 100 or 400 μM CuSO$_4$ to induce the expression of the DHFR constructs. After harvest by centrifugation, cells were broken by glass bead lysis in either LWB150 [40 mm Hepes-KOH, pH 7.4, 150 mm KCl, 5 mm MgCl2, 5% (vol/vol) glycerol], 0.1% (vol/vol) Triton X-100) or Native lysis buffer (see previous paragraph). The lysates, precleared by centrifugation, were incubated with ANTI-FLAG M2 Affinity Gel (Sigma) for 60 min. After washing extensively with buffer, bound proteins were eluted with sample buffer or Native lysis buffer containing 3× FLAG-Peptide (200 mg/L). Coimmunoprecipitated proteins were detected by Western blotting.

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