Sequestration of Highly Expressed mRNAs in Cytoplasmic Granules, P-Bodies, and Stress Granules Enhances Cell Viability

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Abstract

Transcriptome analyses indicate that a core 10%–15% of the yeast genome is modulated by a variety of different stresses. However, not all the induced genes undergo translation, and null mutants of many induced genes do not show elevated sensitivity to the particular stress. Elucidation of the RNA lifecycle reveals accumulation of non-translating mRNAs in cytoplasmic granules, P-bodies, and stress granules for future regulation. P-bodies contain enzymes for mRNA degradation; under stress conditions mRNAs may be transferred to stress granules for storage and return to translation. Protein degradation by the ubiquitin-proteasome system is elevated by stress; and here we analyzed the steady state levels, decay, and subcellular localization of the mRNA of the gene encoding the F-box protein, UFO1, that is induced by stress. Using the MS2L mRNA reporter system UFO1 mRNA was observed in granules that colocalized with P-bodies and stress granules. These P-bodies stored diverse mRNAs. Granules of two mRNAs transported prior to translation, ASH1-MS2L and OXA1-MS2L, docked with P-bodies. HSP12 mRNA that gave rise to highly elevated protein levels was not observed in granules under these stress conditions. ecd3, pat1 double mutants that are defective in P-body formation were sensitive to mRNAs expressed ectopically from strong promoters. These highly expressed mRNAs showed elevated translation compared with wild-type cells, and the viability of the mutants was strongly reduced. ecd3, pat1 mutants also exhibited increased sensitivity to different stresses. Our interpretation is that sequestration of highly expressed mRNAs in P-bodies is essential for viability. Storage of mRNAs for future regulation may contribute to the discrepancy between the steady state levels of many stress-induced mRNAs and their proteins. Sorting of mRNAs for future translation or decay by individual cells could generate potentially different phenotypes in a genetically identical population and enhance its ability to withstand stress.

Introduction

High throughput yeast microarray studies indicate that the mRNA abundance of a common core of 10–15% of the yeast genome is modulated by a variety of different environmental challenges such as DNA damage, heat, oxidative, osmotic, heavy metal, and salt stress [1]. This response, known as the environmental stress response (ESR) represents a network of interlinked functions that preserves cell integrity [2,3]. A hallmark of the ESR is a down-regulation of protein synthesis genes and an up-regulation of genes that encode chaperones and genes involved in protein degradation [4].

The ubiquitin-proteasome system is the major pathway for regulated protein degradation in the cell. In most cases proteins targeted to the proteasome are covalently linked to chains of ubiquitin by a cascade of E1, ubiquitin activating- and E2 ubiquitin conjugating enzymes, and an E3 ubiquitin ligase [5]. The large family of Skp1-Cdc53-F-box protein (SCF) ubiquitin ligase complexes regulates growth and cell cycle progression in all eukaryotes [6]. In yeast about seventeen different F-box proteins recruit degradation substrates to the SCF complex. Of all the F-box proteins, transcription only of UFO1 is highly induced by DNA damage and arsenate stress [four- and sixfold respectively 7,8]. UFO1 may function in maintenance of genome stability as in the absence of Pif1 helicase ufo1Δ mutants show a 74-fold increase in gross chromosomal rearrangements [9]. This role is consistent with the key function of SCFUfo1 in degradation of the mating switch Ho endonuclease [10–13], and the translesion DNA polymerase, Rad23 [14]. However, despite the robust induction of transcription of UFO1 mRNA in response to stress, ufo1Δ mutants do not display enhanced sensitivity to arsenate [8] or UV [15] compared with their isogenic wild types. Functional profiling in yeast shows that this is a widespread phenomenon as deletion mutants of many genes highly induced by a particular stress do not exhibit enhanced sensitivity to the specific stress (reviewed by [16]).

The dynamics of the transcriptome in response to changing conditions is mainly determined by the balance between transcription and mRNA decay and in many instances functionally related genes show a negative correlation between transcription and decay [17,18]. Genome-wide proteomics revealed that only ca. 70% of the steady state protein levels can be attributed to mRNA abundance, indicative of translational regulation [19–23].
Furthermore the lifecycle of mRNA molecules is complex and involves dynamic changes in subcellular localization to distinct cytoplasmic bodies. These include processing bodies (PBs) that are rich in mRNA decay enzymes such as mRNA decapping enzymes (the Dcp1/Dcp2 heterodimer and its activator, Dhh1), Xrn1 5’-3’ exonuclease, and repressors of translation [24–27]. The protein composition of PBs suggests that they are centers of mRNA degradation [28–30]. Under stress conditions mRNAs are also present in Stress Granules (SGs) that form when initiation of translation is impaired. SGs contain primarily translation initiation factors and may therefore serve as storage centers from which the mRNAs can be returned to the polysomes for translation [27,29,31–34]. Despite their apparently distinct roles several lines of evidence suggest a functional relationship between PBs and SGs. First, genetic studies show that formation of yeast SGs depends on biogenesis of PBs [27]. Second, PBs and SGs are often colocalized suggesting that mRNAs may be transferred from one body to the other [27]. Third, yeast deficient for the decapping activators, Pat1 and Dhh1, are blocked in both repression of translation and in PB formation [30,35]. Finally, recent studies link transcription with both mRNA nuclear export, decay, and translation: two subunits of the RNA Pol II holoenzyme transcription complex, Rpb4 and Rpb7, escort the mRNA transcripts from the nucleus to the cytoplasm where they physically interact with subunits of the PBs [36,37]; these subunits are important for coordination of mRNA synthesis with decay [18]. Additional PB-associated proteins, yeast Dhh1 and its Xenopus ortholog, shuttle between the nucleus and cytoplasm [38,39]. Thus the discrepancy between transcript and protein level could be attributable to the complex regulatory mechanisms of the mRNA lifecycle.

Here we report analysis of the lifecycle of UFO1 mRNA whose expression is induced by several kinds of stress, yet the null mutant shows no enhanced sensitivity to these stresses. We compared UFO1 mRNA lifecycle with that of the heat shock gene, HSP12 that represents a different paradigm of regulation. HSP12 is regulated by the ESR and encodes a membrane protein important for preserving membrane organization under stress conditions [40]. Induction of HSP12 mRNA is slow [41], but the protein attains a high cellular level after stress. We measured induction of UFO1 and HSP12 mRNAs and the stability of their mRNAs after stress by quantitative real time PCR (qRT-PCR). We also compared their steady state protein levels in response to different stresses. Furthermore to dissect the UFO1 mRNA lifecycle we followed the mRNA molecules in single cells by tagging genomic UFO1 with bacteriophage MS2L sequence for detection with the viral capsid protein fused to GFP [42,43]. As further reference mRNAs we used MEA2 mRNA that is constitutively expressed at a high level from a strong promoter [24,44], the low copy mRNAs of ASH1 and OXA1 that are localized to the bud [42] and the mitochondrial [43], respectively, prior to their translation. We visualized the subcellular localization of these mRNA molecules in single cells that expressed fluorescent subunits of the PBs and SGs [27,31,45] and found that the highly expressed UFO1 and MEA2 mRNAs enter PBs. Genetic analysis using mutants unable to form PBs showed that cell viability is strongly reduced when UFO1, MEA2 or HSP12 genes are expressed at a high level from the GAL promoter. Our interpretation is that sequestration in PBs plays a major role in preserving cell viability. We suggest that the ability to store highly abundant mRNAs in PBs for future regulation is a key facet of the stress response allowing individual cells to sort mRNAs for decay or translation. This mechanism has the potential to facilitate acquisition of a variety of different phenotypes in a genetically identical population enhancing its ability to withstand the stress.

**Results**

Expression of UFO1 mRNA in cell populations in response to stress

We followed UFO1 expression using qRT-PCR on mRNA extracted from cells at different times after treatment with arsenate, H2O2, or UV irradiation. UFO1 is under repression during normal growth conditions [46] and treatment with arsenate led to a fourfold increase in the level of UFO1 mRNA after 15 minutes that stayed high for at least one hour during which the cells were assayed. Treatment with H2O2 gave a threefold increase in mRNA level after 15 minutes followed by a decrease back to the basal level of the untreated control from the 30 minute time point. After UV irradiation the UFO1 transcript level increased fourfold after 15 minutes and remained high for one hour (Figure 1A). The mRNA abundance is dependent upon the relative rates of transcription and decay, and when we examined the decay of UFO1 mRNA in response to the above stresses we observed stabilization of UFO1 mRNA in response to UV. The half-life of UFO1 mRNA was ca. 7 minutes in untreated cells and in cells treated with arsenate or H2O2, but extended to almost 30 minutes after UV irradiation (Figure 1B). Ufo1 protein levels are very low in untreated control from the 30 minute time point. After UV irradiation the UFO1 transcript level increased fourfold after 15 minutes and remained high for one hour (Figure 1A). The mRNA abundance is dependent upon the relative rates of transcription and decay, and when we examined the decay of UFO1 mRNA in response to the above stresses we observed stabilization of UFO1 mRNA in response to UV. The half-life of UFO1 mRNA was ca. 7 minutes in untreated cells and in cells treated with arsenate or H2O2, but extended to almost 30 minutes after UV irradiation (Figure 1B). Ufo1 protein levels are very low in untreated cells [47], however, by using 10-fold the amount of cells we could observe the protein after arsenate, H2O2, or UV stress (Figure 1C). GFP-Ufo1 protein was stabilized in cells treated with arsenate or H2O2, but has the same half-life after UV irradiation as in the untreated control cells (Figure S1). Thus the Ufo1 protein could reflect accumulation during the prolonged arsenate or H2O2 treatments, but in contrast in UV-irradiated cells could be newly translated from the stabilized UFO1 mRNA. The UFO1 promoter has sequence elements similar to those regulated by the Yap1 oxidative stress- and Pdr1 pleiotropic drug response transcription factors. Untreated mutant cells showed a reduced basal level of UFO1 mRNA expression in yap1Δ and pdr1Δ mutants compared with wild type cells (Figure 1D) and there was no induction of UFO1 mRNA expression in response to arsenate, H2O2,
and UV stresses indicating that induction of UFO1 mRNA is Yap1- and Pdr1-dependent (Figure 1E).

Detection of UFO1 mRNA in single cells in response to stress

Within a population of cells of identical genotype the response of individual cells to stress can differ [48,49]. Therefore to test the response of single cells we tagged the UFO1 ORF with MS2L DNA and expressed the coat protein (CP) that binds the MS2L RNA as a GFP-fusion protein [43]. To ensure the specificity of detection of UFO1 mRNA by the CP\textsuperscript{GFP} fusion protein we induced CP expression in logarithmic cells with SC glucose medium lacking methionine for one hour. Subsequently, both wild type control and UFO1-MS2L cells were treated with arsenate, H\textsubscript{2}O\textsubscript{2}, or UV.
irradiation and observed after 30 minutes by confocal microscopy. In the control cells the CP-GFP protein was visible diffusely throughout the cytoplasm both before and after each stress treatment whereas in response to arsenate, H₂O₂, and UV the UFO1-MS2L cells showed pronounced cytoplasmic granules ranging in number from zero to five (Figure 2A). Cessation of the treatment by incubation in fresh medium led to their disappearance (Figure 2B). No granules were observed after 30 minutes in treated UFO1-MS2L, yap1Δ or UFO1-MS2L, pdr1Δ mutant cells (Figure 2C) similar to the qRT-PCR results (Figure 1B).

Time course of granule appearance after stress
Accumulation of the granules was gradual and not all the cells in the population responded within the same time frame and to the same extent. We therefore quantified 150–200 cells for each treatment by defining three different classes of granules per cell: zero, 1–2 (few), or >3 (multiple). In response to arsenate after 30 minutes we observed an increase in the number of cells with a few or multiple granules followed by an increase in cells with a few granules over the 90-minutes of the experiment (Figure 3A); H₂O₂ led to an increase in cells with a few and with multiple granules after 30 minutes. By 90 minutes there was a slight increase in cells without granules and a slight decrease in the number of cells with 1–2 granules; the relative number of cells with multiple granules was unaltered (Figure 3B). After UV-irradiation UFO1-MS2L-CP-GFP granules were visible after 15 minutes; this included cells with a few granules, but mostly with multiple granules. The relative number of cells with a few granules increased during the first hour and then decreased; the number of cells with multiple granules decreased from 30 minutes post-irradiation until the end of the experiment (Figure 3C).

Characterization of the UFO1 mRNA granules
To determine whether the granules that appeared after stress treatment of UFO1-MS2L cells correspond to PBs or SGs we tested for colocalization of the UFO1 mRNA with the PB marker protein, Dcp1RFP, and with the SG marker protein, eIF4ERFP, by mating UFO1-MS2L cells with PB- or SG-tagged strains [31]. Neither UFO1-MS2L granules nor PBs or SGs were visible in untreated cells growing in glucose medium (Figure 4A); cells in SC medium without glucose showed a single red fluorescent PB or SG, however, there was no induction of UFO1 mRNA in response to glucose deprivation (Figure 4B) and [31]. After 30 minutes of arsenate or H₂O₂ treatment of the glucose-deprived cells, we observed induction of UFO1 mRNA granules and these colocalized with both the PB and SG marker proteins (Figure 4C and 4D, respectively). Indeed control cells that coexpressed the PB marker protein, Dhh1GFP, and the SG marker protein, eIF4ERFP, showed overlap between the two types of granules after arsenate treatment (Figure 4E).

Two distinct highly abundant mRNAs, UFO1 and MFA2, colocalize to the same PBs
The colocalization of stress-induced UFO1 mRNA with proteins associated with PBs and SGs together with the overlap of these bodies with one another after stress (Figure 4) suggest that these granules may house highly expressed mRNAs. To determine whether more than one mRNA species is present in the same granule we used a second well-characterized mRNA tagging system in which U1A binding sites are inserted into the 3′-UTR of the mRNA of interest and coexpressed with the U1A-GFP RNA-binding protein [44]. Wild type cells were transformed with the plasmid for producing U1A-GFP protein alone, or cotransformed with pMFA2-U1A and the above plasmid. Expression of MFA2 is regulated by the strong constitutive GPD (glyceraldehyde-3-
phosphate dehydrogenase) promoter [24,44] and reaches a high steady state level. As both the U1A and the CP proteins are fused to GFP, we changed the marker of the CPGFP protein to mCherry so that we could detect each mRNA species separately in the same cells. Control untreated or arsenate stressed cells that expressed only U1AGFP protein showed no granule formation. Untreated cotransformants of pMFA2-U1A and UFO1-MS2L showed multiple MFA2-U1A granules, but no granules attributable to UFO1-MS2L mRNA. After arsenate treatment UFO1-MS2L mRNA granules bound by red CPmCherry were observed and these colocalized with the MFA2-U1A granules. This result indicates that multiple mRNA species are found in the same PB (Figure 5).

Low-abundance mRNAs ASH1-MS2L and OXA1-MS2L partially colocalize with PBs and SGs
To determine whether all mRNA granules correspond to PBs we assayed two further mRNAs that are specifically localized prior to their translation: ASH1-MS2L mRNA that is transported from the mother cell to the bud [42], and OXA1-MS2L mRNA that is localized to the mitochondria [43]. The PB marker Edc3mCherry was produced in cells that expressed ASH1-MS2L or OXA1-MS2L together with pCP-MS2L-GFPx3 for detection of their mRNAs. The cells were treated with the above stresses and imaged after 30 minutes with the confocal microscope. Both ASH1-MS2L and OXA1-MS2L mRNAs showed a similar response to the stress treatments. A small number of untreated cells showed at most a few Edc3mCherry-marked PBs, however, most cells lacked CPGFP granules of either ASH1-MS2L or OXA1-MS2L mRNAs. After 30 minutes with arsenate, H2O2, or glucose deprivation, we observed CP GFP granules corresponding to ASH1-MS2L (Figure 6A) or OXA1-MS2L (Figure 6B) mRNAs. These granules did not fully colocalize with PBs as did UFO1 mRNA, however, they were often docked, partially colocalized (overlapping), or appeared in the same cells with the PB marker protein Edc3mCherry, but at a distinct location in the same cell (Figure 6C).

Highly expressed Hsp12 protein is encoded by a mRNA not observed in mRNA granules
Both whole-genome microarray experiments [7,8] and our qRT-PCR data (Figure 1) indicate that UFO1 steady state mRNA levels are elevated in response to stress. However, it is only by using 10-fold the amount of cells compared with our standard protocols that we are able to detect Ufo1GFP protein after any of the stresses applied. We therefore examined the fate of the mRNA of HSP12, the protein of which is highly expressed in response to stress [40]. We treated cells with arsenate, H2O2, UV, glucose deprivation, 37°C or NaCl and analyzed induction of the protein by Western blotting. Hsp12GFP protein was induced after all the treatments, particularly after 37°C (Figure 7A and Figure S2). To examine mRNA localization we fused the MS2L tag to the HSP12 ORF and treated the cells with the same stresses. In contrast to UFO1-MS2L, we did not detect granules in HSP12-MS2L cells and the CP-GFP signal remained diffuse throughout the cytoplasm (Figure S3 and [50]). The basal level of HSP12 mRNA in untreated cells was higher than that of UFO1 (Figure 7B); however, the relative increase of HSP12 steady state mRNA level after stress was lower than the high induced...
levels of UFO1 mRNA in these samples (cf. Figure 7C and Figure 1A). The elevation of HSP12 mRNA level results from increased transcription as HSP12 mRNA is slightly destabilized after arsenate, whereas after H2O2, and UV treatments the stability is similar to the untreated cells with a half-life of ca. 10 minutes in both untreated and stressed cells (Figure 7D). There was no stabilization of the HSP12 mRNA after UV irradiation as observed for UFO1 mRNA (Figure 1B).

High-level expression of mRNA affects the viability of mutants unable to form PBs

To determine what role sequestration of UFO1 and of MEA2 mRNAs in PBs may have we examined the viability of cells defective in the formation of PBs under two different conditions: (a) exposure to arsenate, H2O2, UV, or glucose deprivation, and (b) high level expression of UFO1 or HSP12 from the GAL promoter, and of MEA2 from the strong GPD promoter. We used a

**Figure 4. UFO1-MS2L mRNAs induced by stress colocalize with subunits of PBs and SGs.** A. UFO1-MS2L cells at A600 = 0.5 that produce PB marker protein, Dcp1EFP, or the SG marker, elf4EFP, were transferred to fresh SC 2% glucose medium without methionine for 1 hour. B. Cells as in A, deprived for glucose for 30 minutes. C. Cells as in B, treated for 30 minutes with 1 mM arsenate, or D. 8.8 mM H2O2. E. Cells at A600 = 0.5 that produce both the PB marker protein, Dhh1GFP, and elf4EFP, treated with 1 mM arsenate for 30 minutes in the presence or the absence of glucose.

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single pat1Δ mutant defective in a mRNA decapping enzyme and two different double mutants: edc3Δ, pat1Δ mutants defective in both PB and SG formation, and edc3Δ, lsm4Δ mutants that have reduced PB and SG formation. (Edc3 enhances mRNA decapping [51] and Lsm4 is a subunit of a heptameric complex involved in mRNA decay [27]). The edc3Δ and lsm4Δ single mutants are able to form PBs under conditions of glucose deprivation, however, in the absence of Edc3, there is a requirement for the C-terminal prionlike domain of Lsm4 for PB formation [52]. To visualize granule formation isogenic wild type, pat1Δ, edc3Δ, pat1Δ, and edc3Δ, lsm4Δ cells were transformed to express the PB marker, Dcp2Cherry and the SG marker protein, Pab1GFP, untreated or treated with 1 mM arsenate for 30 minutes. A. Control wild type cells at A600 = 0.5 producing U1AGFP untreated or treated with 1 mM arsenate for 30 minutes. B. wild type UFO1-MS2L cells at A600 = 0.5 expressing pMFA2-U1A, with their respective RNA-binding proteins, Dcp2Cherry and U1AGFP, untreated or treated with 1 mM arsenate and stained with Hoechst 33342 at a final concentration of 2.5 μg/mL for 30 minutes.

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Figure 5. UFO1-MS2L and MFA2-U1A mRNAs are sequestered in the same PBs after arsenate stress. A. Control wild type cells at A600 = 0.5 producing U1AGFP untreated or treated with 1 mM arsenate for 30 minutes. B. wild type UFO1-MS2L cells at A600 = 0.5 expressing pMFA2-U1A, with their respective RNA-binding proteins, Dcp2Cherry and U1AGFP, untreated or treated with 1 mM arsenate and stained with Hoechst 33342 at a final concentration of 2.5 μg/mL for 30 minutes.

Discussion

We analyzed the functional consequence of accumulation of high levels of mRNA in yeast cells under different stress conditions and identified stations in the lifecycle of mRNAs that could explain the discrepancy between transcript abundance and protein level. Our data suggest that the ability to store highly abundant mRNAs in PBs for future regulation is a key facet of the stress response as it allows individual cells to sort mRNAs for decay or translation. A prototype of this kind of regulation is induction of UFO1 mRNA under stress conditions. Our qRT-PCR experiments show that steady state levels of UFO1 are elevated in cells stressed by arsenate, H2O2, or UV. This observation is compatible with microarray data [8,17,53]. The Yap1 and Pdr1 dependence of UFO1 transcription suggests that UFO1 is part of the early stress response that includes genes that encode several heat shock proteins [54]. Transcriptome studies have shown that production and degradation of mRNA are often coordinated [18]. However, in our experiments even though the steady state UFO1 mRNA level was elevated after each stress, the mRNA lifecycle was different for UV, an acute stress, compared with arsenate and H2O2 treatments. Arsenate did not affect UFO1 mRNA stability, and after H2O2 there was a very slight destabilization of the mRNA. In contrast UV irradiation led to an elevation of UFO1 mRNA there was no marked change in the stability of UFO1 mRNA after arsenate, H2O2 or UV. This observation is compatible with microarray data [8,17,53]. The Yap1 and Pdr1 dependence of UFO1 transcription suggests that UFO1 is part of the early stress response that includes genes that encode several heat shock proteins [54]. Transcriptome studies have shown that production and degradation of mRNA are often coordinated [18]. However, in our experiments even though the steady state UFO1 mRNA level was elevated after each stress, the mRNA lifecycle was different for UV, an acute stress, compared with arsenate and H2O2 treatments. Arsenate did not affect UFO1 mRNA stability, and after H2O2 there was a very slight destabilization of the mRNA. In contrast UV irradiation led to an elevation of the steady state UFO1 mRNA level that remained high due to stabilization of the mRNA. In contrast to UFO1 mRNA there was no marked change in the stability of HSP12 mRNA after arsenate, H2O2 or UV. A general stabilization of mRNAs expressed from the GAL promoter in response to UV was reported by [55] who did not observe mRNA stabilization after starvation, heat or osmotic stress. UV irradiation did not lead to disassembly of polysomes, however, the mRNAs were no longer associated with the polysomes but accumulated in cytoplasmic granules without losing their polyA tails suggesting they were not designated for decay [55].
By increasing the yeast extract concentration tenfold we were able for the first time to detect Ufo1 protein expressed from genomic UFO1 after arsenate, H2O2, and UV. The protein steady state level was very low in untreated cells (1% that of a-tubulin) but after these stresses showed a 10-fold elevation. Examination of the Ufo1 protein half-life after arsenate and H2O2 showed that the protein was stabilized thus the higher level can be attributed to lack of proteasomal degradation. In contrast UV irradiation did not affect the half-life of Ufo1 protein and in light of Galliard’s observation that polysomes are not disassembled after UV irradiation, our interpretation is that the elevated steady state level of Ufo1 protein is due to renewed translation of the stabilized UFO1 mRNA. This is compatible with the proposed role for Ufo1 in maintenance of genome stability [9] and may be indicative of a remodeling of the genome during recovery. These steady state Ufo1 protein values are comparable to results of a study in which we expressed the bacterial reporter genes, luxA and luxB, in yeast from the UFO1 promoter [46]. Luciferase activity was elevated 10-fold in response to 40 mJ/cm2 UV whereas arsenate or H2O2 led to a threefold elevation of enzyme activity. Direct measurement of UFO1 transcription here by qRT-PCR shows that all three stress treatments lead to very similar elevation of transcription suggesting that luciferase activity may have been affected by prolonged treatment with arsenate or H2O2. HSP12 represents a different paradigm of regulation in that the basic steady state mRNA level was higher in unstressed cells and fold-elevation of steady state level was less than for UFO1. However, the salient difference was in the cellular amounts of each protein in untreated cells: steady state Hsp12 protein levels are two orders of magnitude those of Ufo1 (Figure S3) and [47]. This may be due to differences in translatability of HSP12 mRNA that encodes a protein of 107 amino acids compared with UFO1 whose gene product has 668 residues [56]. Ribosome profiling has shown that translation efficiency can differ 100-fold between different genes with shorter genes having a higher ribosome density [22]. Nuclear export is an important regulatory step for molecular chaperones [57]; in addition the 5'-untranslated region (5'-UTR) plays an important role in determining translation efficiency of HSP12 mRNA in Aspergillus oryzae [58,59] and Arabidopsis [60].

Cells with the UFO1-MS2L reporter showed granules after stress; these granules correspond to UFO1-MS2L mRNA molecules bound to the fluorescent capsid protein, CP<sup>ΔPP</sup>. Granules were only visible when the genomic UFO1 ORF was fused to MS2L excluding the possibility they were comprised of aggregated

**Figure 6. ASH1 and OXA1 mRNA granules interact with PBs.** A. ASH1-MS2L cells at A<sub>600</sub> = 0.5 with pCP-MS2L-GFPx3 and the PB marker, Edc3<sup>ΔCherry</sup>, either untreated, treated with 1 mM arsenate or with 8.8 mM H<sub>2</sub>O<sub>2</sub> for 30 minutes or transferred to SC without glucose for 30 minutes. Merge x5 represents 5 times enlargement of selected granules indicated with white arrows in the whole cells. B. OXA1-MS2L cells treated as in A, and visualized by confocal microscopy. C. Histograms of ASH1-MS2L cells or D. OXA1-MS2L cells, showing percentages of overlapping, docked, or distinct granule types in a population of cells untreated, treated with 1 mM arsenate or with 8.8 mM H<sub>2</sub>O<sub>2</sub> for 30 minutes or stressed in SC without glucose (n = >100 cells).
Moreover, after cessation of arsenate stress, the granules disappeared; this could be indicative of decay or return to the polysomes. There was a similar decrease of cells with granules after H2O2 treatment that could be due to cellular responses to reactive oxygen species [3]. UFO1-MS2L granules were not visible in UFO1-MS2L, yap1Δ or pdr1Δ mutants that lack the transcription factors shown by our qRT-PCR results to be essential for induction of UFO1 by stress. Therefore the granules are indeed UFO1-MS2L mRNA bound to CPGFP. Individual cells showed considerable variation both in their response time and in their number of granules as observed in many other systems. Genes involved in the stress response are expressed with a high level of cell-to-cell variation, stochastic noise attributed to epigenetic factors [61–64]. This is considered to enhance the ability of the population to survive adverse conditions by enabling them to sample multiple phenotypes [16,62].

Colocalization of the UFO1-MS2L mRNA granules with the PB and SG marker proteins, Dcp1GFP and eIF4ERFP, respectively, indicated that the UFO1-MS2L mRNA is sequestered in PBs and SGs. SGs were only visible in the absence of glucose [31] and this necessitated incubation of the cells in SC medium without glucose prior to stress treatment in these experiments. The mRNAs of two highly expressed genes - UFO1-MS2L induced by arsenate stress, and MFA2-U1A constitutively expressed from the strong GPD promoter - colocalized to the same PBs indicating that these granules house multiple copies of diverse mRNAs. Using protein markers for PBs and SGs we found that these two bodies colocalize after stress consistent with a role for PBs as a sorting station for future regulation of mRNAs for decay or storage [28,65].

Our data indicate clear differences in the lifecycles of UFO1 and HSP12 mRNAs. UFO1 transcription was elevated three- to fourfold in response to arsenate, H2O2, or UV and the UFO1 mRNA was present in PBs and SGs. In contrast, HSP12 mRNA levels that were double those of UFO1 mRNA in untreated cells were elevated at most twofold after these stresses. Furthermore under these conditions in which we and [40] observed strong induction of the Hsp12 protein we did not observe granules corresponding to HSP12-MS2L mRNA. The half-life of UFO1 mRNA was not affected by arsenate and H2O2 stresses, but the mRNA was stabilized after UV irradiation; none of these stress treatments affected the stability of HSP12 mRNA. On the protein level we could only observe Ufo1GFP protein in cells with genomic UFO1-GFP after stress by taking 10-fold the number of cells for analysis; in contrast genomic Hsp12 GFP protein was easily detectable in untreated cells and was elevated between two- and fivefold in cells treated with H2O2, 37°C, and NaCl using our standard experimental protocols. Our genetic analysis suggests that sequestration of highly expressed mRNAs in PBs is an important mechanism for survival. In edc3Δ, pat1A mutants that are unable to form PBs we observe elevated protein levels compared with the isogenic wild type cells. mRNA decay rates are not affected in these mutants and our interpretation is that the mRNAs lose their regulation and enter the polysome fraction. Here again we observe a difference between

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**Figure 7. Induction of Hsp12 protein and mRNA, and mRNA decay after stress.** A. WB of protein produced from genomic HSP12-GFP in response to stress. B. UFO1 and HSP12 mRNA levels in untreated cells analyzed by qRT-PCR. mRNA levels were normalized to ACT1. C. Induction of HSP12 mRNA by stress. Wild type cells at A600 = 0.5, treated with 1 mM arsenate, 8.8 mM H2O2, irradiated with 40 mJ/cm² UV or shifted from 30°C to 37°C for 40 minutes. Aliquots were collected at the times indicated and analyzed by qRT-PCR. D. HSP12 mRNA decay. pGAL-HSP12 was expressed in hsp12Δ mutants by overnight induction with 2% galactose. Next morning cells at A600 = 0.5 were untreated, or stressed with 1 mM arsenate or 8.8 mM H2O2 for 30 minutes, or irradiated with 40 mJ/cm² UV. The cells were washed and transferred to SC medium with 4% glucose. Samples were collected immediately after addition of glucose and at the times indicated and analyzed by qRT-PCR. mRNA levels were normalized to ACT1 and to time 0 (untreated log cells). doi:10.1371/journal.pgen.1002527.g007
Figure 8. Ectopic high level gene expression affects viability of mutants unable to form PBs and SGs. A. Visualization of wild type, pat1Δ, edc3Δ, pat1Δ or edc3Δ, lsm4Δc cells expressing the PB marker Dcp2mCherry and the SG marker Pab1GFP untreated or exposed for 30 minutes to 1 mM arsenate or 8.8 mM H2O2, irradiated with 40 mJ/cm² UV, or incubated in SC medium without glucose for 30 minutes. B. Viability of wild type, pat1Δ; edc3Δ, pat1Δ or edc3Δ, lsm4Δc cells untreated (Unt) or treated with 1 mM arsenate, 8.8 mM H2O2 or irradiated with 40 mJ/cm² UV analyzed by the spot test viability assay. C. Wild type, pat1Δ; edc3Δ, pat1Δ or edc3Δ, lsm4Δc cells expressing empty pGAL-vector (YCp), pGAL-GFP-UFO1, pGAL-HSP12 or MFA2-U1A (pRP1193) were grown in SC medium with 2% glucose or induced in 2% galactose medium overnight, diluted and regrown in the same media to A600 = 0.5 for spot test analysis on SC plates with 2% glucose or 2% galactose, respectively. D. WB analysis of w.t or edc3Δ, pat1Δ cells expressing UFO1, HSP12 or MFA2 from the GAL promoter. Glu (noninducing conditions) and Gal (inducing). The intensities of each protein band were normalized to the α-tubulin loading control using ImageJ [79]. E. Comparison of UFO1 and HSP12 mRNA decay. pGAL-GFP-UFO1 or pGAL-HSP12 was expressed in w.t or edc3Δ, pat1Δ cells by overnight induction with 2% galactose. Next morning cells at A600 = 0.5 were washed and transferred to SC medium with 4% glucose. Samples were collected immediately after addition of glucose and at the times indicated and analyzed by qRT-PCR. mRNA levels were normalized to ACT1 and to time 0 (untreated log cells).

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the PB-associated UFO1 and MEA2 mRNAs and HSP12 mRNA: UFO1 and MEA2 mRNAs show a six- to eightfold elevation of steady state protein levels, respectively, in the edc3Δ, pat1Δ mutants compared with Hsp12 that shows a threefold elevation of protein level compared with wild type cells. Overexpression of all three genes affects the viability of the edc3Δ, pat1Δ mutants. Pat1 and Dih1 function in the coordination of translation and PB formation acting as repressors of translation and enhancers of PB formation [30,35]. The nonregulated elevated translation we observe in the edc3Δ, pat1Δ mutants may exert its effect on viability through depletion of translation factors required for maintenance of basic essential cell functions. Fold-elevation of HSP12 mRNA levels in response to stress is lower than that of UFO1 and the high levels of HSP12 mRNA produced from the GAL promoter may create a requirement for their sequestration in PBs to maintain the balance between storage, decay, and translation. In addition the high level of HSP12 mRNA could lead to a depletion of essential Hsp-specific regulatory factors and affect biosynthesis of molecular chaperones crucial for withstanding the stress [66,67].

It is not clear whether the lifecycle of every mRNA species involves a sojourn in PBs. Transcripts of housekeeping genes could go straight to the polysomes [18,29], or a certain fraction of transcripts, depending on the environmental conditions, could be imprinted with the RNA polymerase II associated Rpb4-Rpb7 heterodimer for sojourn and future sorting in PBs [18]. This heterodimer recruits mRNA transcripts from the nucleus to the cytoplasm and physically interacts with the PB proteins, Pat1 and Len2, prior to being reimported into the nucleus [36,68]. Besides nuclear export [69], Rpb4 and Rpb7 regulate other stages of the mRNA lifecycle such as exit from PBs [70], and 5' to 3' and 3' to 5' decay [36,68]. Moreover even though Ash1-Ms2L and Oxa1-Ms2L CP-EGFP granules did not show the full colocalization observed for UFO1 and MEA2 mRNAs we did observe docking of their granules with PBs, similar to the dynamic docking interaction reported for dendritically localized mRNAs in Drosophila neurons [71]. Transport granules share some factors with PBs and this may allow reciprocal transfer of mRNA and proteins between them [28].

We present a diagram that summarizes putative alternative lifecycles for mRNA based on the sample of genes studied here (Figure 9). The ability to store UFO1 and other mRNAs in PBs and SGs provides a mechanism for individual cells to regulate their future sorting into pathways for decay or translation. Under normal growth conditions mRNAs could be translated immediately (a), or could pass through PBs that could lead to delayed translation (b); or direction of the mRNA for decay (c). Under stress conditions pre-existing mRNAs undergo enhanced translation as we propose for HSP12 mRNA (α), or are retracted from the polysomes (β) for future sorting for storage (γ) or decay (δ). Furthermore, after stress mRNAs can shuttle between PBs and SGs (ε) from where they can return to translation (α). mRNA lifecycles under normal growth conditions have blue arrows, stress conditions are indicated with brown arrows. A more comprehensive description of the mRNA lifecycle, particularly of mRNA decay, can be found in [80].

![Figure 9. Pathways for mRNA under normal and stress conditions.](image)

**Yeast strains**

Strain genotypes are listed in Table 1. Strain yMK1366 has genomic dIF4E fused to RFP as a marker for PBs, and strain yMK1307 has genomic sIF4E fused to RFP as a marker for SGs [31]. Strain yRP1600 is pat1Δ; strain yRP1752 has double deletions of edc3Δ, pat1Δ [72]; and strain yRP2338 has double deletions, edc3Δ, lowAΔ [52], yap1Δ or pdr1Δ deletion strains were constructed by homologous recombination [73] with a PCR fragment that encodes an auxotrophic URA3 marker gene amplified from YCpGAL [74] flanked with 40 bps for targeting, using the primer pairs yap1::URA3F/R to delete YAP1, and pdr1::URA3F/R to delete PDR1 (Table 2). The deletions were confirmed using the primer pair YAPI5’F for yap1Δ and PDR15’F for pdr1Δ with the reverse primer URA3midR complementary to the middle of the URA3 gene.

**Materials and Methods**

**Yeast strains**

Strain genotypes are listed in Table 1. Strain yMK1366 has genomic dCP1 fused to RFP as a marker for PBs, and strain yMK1307 has genomic sIF4E fused to RFP as a marker for SGs [31]. Strain yRP1600 is pat1Δ; strain yRP1752 has double deletions of edc3Δ, pat1Δ [72]; and strain yRP2338 has double deletions, edc3Δ, lowAΔ [52], yap1Δ or pdr1Δ deletion strains were constructed by homologous recombination [73] with a PCR fragment that encodes an auxotrophic URA3 marker gene amplified from YCpGAL [74] flanked with 40 bps for targeting, using the primer pairs yap1::URA3F/R to delete YAP1, and pdr1::URA3F/R to delete PDR1 (Table 2). The deletions were confirmed using the primer pair YAPI5’F for yap1Δ and PDR15’F for pdr1Δ with the reverse primer URA3midR complementary to the middle of the URA3 gene.

**Fusion of genomic UFO1 and heat-shock gene, HSP12, to the MS2L cassette**

To the UFO1 or HSP12 ORFs we fused a PCR-amplified cassette comprising 12 MS2L-CP binding sites and the *Schizosaccharomyces pombe* his5+ selectable marker between two copies of the lambda phage loxP sequence from plasmid pLoxHis5MS2L [43]. Forty nts of homology to UFO1 or HSP12 were introduced into each primer for site-specific integration of the PCR cassette. The entire loxP::Sphis5+::loxP::MS2L PCR cassette was integrated after the STOP codon of UFO1 using the primers UFO1endF and UFO13’UTRTR, and at the end of the HSP12 ORF using primers HSP12endF and HSP123’UTRTR. UFO1 cassette integration was confirmed by colony PCR amplification using the primer pair, UFO1ORFF/UFO13’UTR and of HSP12 with the primer pair, HSP12ORFF/HSP123’UTR. Subsequently the *S. pombe* his5+
marker was excised with Cre recombinase by transforming positive colonies with plasmid pSH47 that encodes Cre recombinase under the GAL promoter [43]. Transformants were isolated on glucose plates and transferred to galactose medium for excision of the MFA2 mRNA expressed from pRP1193 [24], both are regulated by the LoxP followed immediately by the Cre recombinase by transforming positive transformants were isolated on glucose plates and transferred to galactose medium for excision of the MFA2 gene. This yielded histidine auxotrophic colonies in which MFA2 ORF was verified by sequencing the PCR product amplified with the primers used to check cassette integration. pCP-MS2L mCherry was then transformed into UFO1-MS2L yeast and treated with arsenate for confirmation of a red signal. Subsequently plasmids pRP1193 [24] and pRP1187 [44] for expression and visualization of MFA2-UTR mRNA were cotransformed into the cells for treatment and confocal microscopy.

Plasmids

pDH1H1-GFP [45] and pRP1574 that encodes EDC3-mCherry [27] were used as markers of PBs. pRP1658 that encodes both PAB1-GFP and DCP2-mCherry was used to provide markers for PBs and SGs [44]. pRP1187 encoding U1A-GFP [44] was used to detect MFA2 mRNA expressed from pRP1193 [24], both are regulated by the GDP constitutive promoter. pCP-MS2L-GFPx3 [43] and pCP-MS2L-mCherry (below) were used to detect mRNAs with MS2L binding loops; pGAL-GFP-UF01 [13], pGAL-HSP12 [75] and pBM123 (YcpGAL) [74] were used in growth analysis studies. pGAL-GFP-UF01 [13], pGAL-HSP12 [75] and pGAL-MFA2 [75] were used in WB analysis in Figure S8D.

pCP-MS2L-mCherry plasmid construction: pCP-MS2L-mCherry was constructed by homologous recombination in vivo by replacing the first GFP ORF in pCP-MS2L-GFPx3 with a PCR product that encodes mCherry. The primer pair MS2L-mChF/mCh-GFP2R was designed with homologous ends to MS2L and the third copy of GFP. The reverse primer has a STOP codon after the mCherry ORF so that only mCherry will be expressed fused to the CP. The mCherry PCR product together with pCP-MS2L-GFPx3 were transformed into wild type yeast for recombination in vivo. pCP-MS2L-mCherry was extracted from yeast and amplified in E. coli for verification of replacement of GFP with mCherry by PCR and sequencing using primer pair Check-MS2LF/Check-mChR. The pCP-MS2L-mCherry plasmid was then transformed into UFO1-MS2L yeast and treated with arsenate for confirmation of a red signal. Subsequently plasmids pRP1193 [24] and pRP1187 [44] for expression and visualization of MFA2-UTR mRNA were cotransformed into the cells for treatment and confocal microscopy.

**Growth conditions**

Yeast cultures were grown overnight at 30°C in a rotary thermoshaker at 120 rpm in synthetic minimal medium (SC) supplemented with the appropriate amino acids and carbon source [76]. Next morning cultures were diluted to A600 = 0.1, regrown to early exponential phase, A600 = 0.5, and treated as detailed below.

**Arsenate and H2O2.** For arsenate treatment cells at A600 = 0.5 in SC glucose medium were incubated with 1 mM Na2HAsO4 for H2O2 we diluted the 30% stock solution 1:1000 to a final concentration of 8.8 mM. Aliquots were collected by brief centrifugation at the times indicated in the figures.

**UV irradiation.** Cells at A600 = 0.5 were irradiated with 40 mJ/cm² UV on a transilluminator and aliquots were collected as above.

**Glucose deprivation.** Cells at A600 = 0.5 were collected by brief centrifugation, washed in fresh SC medium without glucose and incubated at 30°C for 30 minutes in fresh SC medium without glucose. Cells were collected by brief centrifugation and visualized by confocal microscopy.

---

**Table 1. Yeast strains.**

| Yeast strains | Genotype | Reference |
|----------------|-----------|-----------|
| BY4741 | MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 | Euroscarf |
| BY4739 | MATa, leu2Δ0, lys2Δ0, ura3Δ0, YML088w::kanMX4 | Euroscarf |
| yRP840 | MATa his4Δ339 leu2-3,112 trp1 ura3-52 cup1::LEU2/PGK1p/GFApG | (5) |
| BY4742 | MATa, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, YFL014w::kanMX4 | Euroscarf |
| W303 | MATa, ade2, can1, his3, leu2, trp1, ura3 | (9) |
| UFO1-GFP | MATa, H5+, leu2Δ0, met15Δ0, ura3Δ0 | Invitrogen |
| UFO1-M52L | MATa, ade2, can1, his3, leu2, trp1, ura3 | This study |
| UFO1-M52L | MATa, ade2, can1, his3, leu2, trp1, ura3 | This study |
| UFO1-M52L, yap1Δ | MATa, ade2, can1, his3, leu2, trp1, ura3, yap1::URA3 | This study |
| UFO1-M52L, pdr1Δ | MATa, ade2, can1, his3, leu2, trp1, ura3, pdr1::URA3 | This study |
| elf4ε-RFP | MATa, ADE2, his3Δ11,15, leu2-3, 112, trp1-1, ura3-1, CDC33-RFP::NAT | (7) |
| DCP1-RFP | MATa, ade2, his3Δ11, 15, leu2-3, 112, trp1-1, ura3-1, DCP1-RFP::NAT | (7) |
| pat1Δ | MATa, his4, leu2, cup1::LEU2PM, trp1, ura3, pat1::LEU2 | (6) |
| edc3A, pat1Δ | MATa, trp1, leu2, ura3, edc3::NEO, pat1::LEU2, cup1::LEU2/PGK1p/GFApG | (8) |
| edc3A, lsm4Δc | MATa, leu2, trp1, ura3, lys2, his4, cup1::LEU2/PGK1p/GFApG, Lsm4::NEO, edc3::NEO | (2) |
| yap1Δ | MATa, ade2, can1, his3, leu2, trp1, ura3, yap1::URA3 | This study |
| pdr1Δ | MATa, ade2, can1, his3, leu2, trp1, ura3, pdr1::URA3 | This study |
| HSP12-GFP | MATa, leu2Δ0, met15Δ0, ura3Δ0, GFP(S65T)–His3MX | (3) |
| HSP12-M52L | MATa, ade2, can1, his3, leu2, trp1, ura3 | This study |
| ASH1-M52L | MATa, his3Δ11, leu2Δ0, met15Δ0, ura3Δ0, ASH1::loxP·M52L::ASH13’UTR | (1) |
| OXA1-M52L | MATa, his3Δ11, leu2Δ0, met15Δ0, ura3Δ0, OXA1::loxP·M52L::OXA13’UTR | (4) |

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Osmotic stress. Cells at $A_{600} = 0.5$ were incubated in 0.5 M NaCl for 30 minutes prior to visualization by confocal microscopy.

Heat shock. Cells at $A_{600} = 0.5$ were shifted from 30°C to 37°C for 40 minutes prior to visualization by confocal microscopy.

Growth analysis

For over-expression experiments YCpGAL [74], pGAL-GFP-UFO1 [13], pRP193 encoding MFA2-U1A-MS2L [24] or pGAL-HSP12 [75] were transformed into wild type or mutant yeast. Cells were grown overnight in SC with 2% glucose or induced with 2% galactose, diluted to $A_{600} = 0.1$ in the appropriate media, and regrown to $A_{600} = 0.5$. At this stage viability was assayed using a spot test with 5 μl drops of 10-fold serial dilutions plated on the appropriate medium. Plates were incubated at 30°C for 3 days and scanned.

Detection of UFO1-MS2L or HSP12-MS2L mRNA

UFO1-MS2L or HSP12-MS2L cells were transformed with pCP-MS2L-GFP3 that encodes the MS2L-CP fused to three tandem repeats of GFP or with pCP-MS2L-mCherry (above), both expressed from the inducible MET25 promoter [42]. Transformed cells at $A_{600} = 0.5$ were incubated in SC medium with 2% glucose but lacking methionine for 1 hour prior to treatment with stress agents as above and visualized by confocal microscopy.

Quantitative real-time PCR (qRT–PCR)

Total RNA was extracted using a MasterPure Yeast RNA Purification kit (Epicentre) according to the manufacturer’s protocol. The amount of total RNA extracted from the cells was measured by absorbance at 260 nm. First-strand cDNA was synthesized from 1 μg total RNA using a blend of RNA primers (random hexamers and anchored oligodT 3:1 (v/v)) and Verso Enzyme mix following the manufacturer’s instructions (Thermo Scientific Verso cDNA Kit). A final concentration of 20 ng/μl of synthesized cDNA was used as template for the PCR reaction. qRT-PCR was performed using Thermo-Start DNA Polymerase, UFO1 primers (UFO1-RTF and UFO1-RTR), HSP12 primers (HSP12-RTF and HSP12-RTR), or ACT1 primers (ACT1-RTF and ACT1-RTR) and an ABsolute Blue QPCR SYBR Green ROX Mix, as follows: denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, for 30 cycles. Appropriate non-RT and non-template controls were included in each PCR reaction, and dissociation analysis was performed at the end of each run to confirm the specificity of the reaction. Primer efficiency for UFO1, HSP12 and ACT1 mRNAs was determined (Figure S5). Data were analyzed using the comparative Ct method: $\delta\delta C_t = (\delta C_t}_{sample} - (\delta C_t}_{reference}$. Here $\delta C_t}_{sample}$ is the Ct value for each treated sample normalized to the endogenous housekeeping gene Actin and $\delta C_t}_{reference}$ is the Ct value for the calibrator (untreated cell) also normalized to Actin (Figure 1). A simple $\delta C_t$ calculation (sample normalized to Actin) was used in Figure 7B.

mRNA decay

pGAL-GFP-UFO1 or pGAL-HSP12 were expressed in a61A or hsp12Δ mutants, respectively, by overnight induction with 2% galactose. Next morning cells were diluted to $A_{600} = 0.1$, and regrown to $A_{600} = 0.5$, then treated with the indicated stresses. The

| Table 2. Primer list. |
|-----------------------|
| yap1::URA3F | GCCACCCAAAAGTCTAAGAAAGAATGTTTTCCATAAACCATGTCGAAGACTACATAATTAGGAAGCTGCTG |
| yap1::URA3R | CATTATGAAAGAATGTTTTCCATAAACCATGTCGAAGACTACATAATTAGGAAGCTGCTG |
| pdr1::URA3F | CACTTGCTACATATATCATCAGGCGAGGACATGTCGAAGACTACATAATTAGGAAGCTGCTG |
| pdr1::URA3R | TTTGGGCGAGGACATGTCGAAGACTACATAATTAGGAAGCTGCTG |
| YAP1 S' F | CGGAAAGCCGAGTAAACGAC |
| PDR1 S' F | GCAGGAAAGCAGTAAACGAC |
| URA3midR | CTTCCACCGATGCTCTTTGAGCAATAAGCC |
| UFO1endF | ACAATGTTGCTGAGTCCACATGTCGAAGACTACATAATTAGGAAGCTGCTG |
| HSP12endF | TGAATCTGTTGCTGAGTCCACATGTCGAAGACTACATAATTAGGAAGCTGCTG |
| UFO1'UTR | ATAAATATTAAATCATGCTCTCAGTTAAATCTTGAATCT |
cells were washed and transferred to SC with 4% glucose. Samples were collected immediately after glucose addition and at the times indicated and analyzed by qRT-PCR.

Coexpression of MS2L-tagged mRNAs with markers of PB or SGs
Cells expressing DCP1-RFP or elF4E-RFP were mated with the Ufo1-MS2L strain and the diploids were transformed with pMS2L-CP-GeP3. ASh1-MS2L or OXA1-MS2L cells were transformed with the PB marker plasmid pRP1374 [27] that encodes EDC3-mCherry. All strains were treated with stress as described in the relevant figures and visualized by confocal microscopy. To study coexpression of PBs and SGs yeast strains elF4E-RFP [31] transformed with pDHH1-GFP [45] or wild type transformed with pRP1650 [27] were treated as indicated in the relevant figures and visualized by confocal microscopy.

Microscopy
All images were acquired with an Olympus FV1000 laser-scanning confocal microscope using the ×60 objective lens. The fluorescence was excited with 543 nm for the red fluorescent markers and 405 nm for GFP. For the coexpression experiments sequential screening was used to avoid overlapping. Images are representative of three independent experiments. For the time course experiments images of untreated cells and those exposed to stress (1 mM arsenate, 8.8 mM H2O2, or 40 mJ/cm2 UV) were subjected to quantitative analysis by defining 3 different classes of cells with zero, 1–2, or 3 or more granules. 150–200 cells were assayed for each treatment at each time point. Hoechst staining was used for cell nuclei.

Induction of protein expression in yeast
For expression from the GAL1 promoter, yeast cells were grown overnight in 2% galactose medium. GFP-tagged protein was observed using a Nikon fluorescence microscope fitted with GFP-specific filter set: dichromic 505 nm, excitation 450–490 nm, emission (low pass) 515 nm (Nikon).

Western blot analysis
For Western blot analysis cells were grown overnight in 2% glucose medium, or for induction from the GAL1 promoter with 2% galactose. Next morning the cells were diluted to A600 = 0.1 and regrown to A600 = 0.5 for TCA precipitation [77] and Western blotting [78].

Supporting Information
Figure S1 Half-life of elF4E-RFP in w.t. cells in response to stress. elF4E-RFP protein half-life was determined by expressing pGAL-GeP-UFO1 in w.t. cells either untreated or exposed to arsenate, H2O2, UV, or heat shock as described in the Material and Methods. Cells were grown overnight in 2% galactose medium, diluted to A600 = 0.1 and regrown to A600 = 0.5. Cycloheximide was added to 10 μg/ml and glucose to 4% at the zero time point and equal aliquots of cells were collected at each time point for TCA precipitation and Western blot analysis. α-tubulin was used as a loading control and the membranes were incubated with anti-GFP and anti-α-tubulin antibodies.

Figure S2 Comparison of Ufo1 and Hsp12 protein levels in wild-type cells. Wild-type cells with GFP-tagged genomic UFO1 or HSP12, untreated or exposed to arsenate, H2O2, UV, starvation, heat shock, or salt stress. The Ufo1GFP and Hsp12GFP protein levels were normalized to the α-tubulin loading control of the same sample using ImageJ [79] from the anti-GFP Western blots presented in Figure 1C and Figure 7A.

Figure S3 Microscopic analysis of Hsp12GFP protein and of HSP12-MS2L mRNA under different stress conditions. A. Cells at A600 = 0.5 with genomic HSP12-GFP for visualization of Hsp12GFP protein were treated for 30 minutes with 1 mM arsenate, 8.8 mM H2O2, UV-irradiated with 40 mJ/cm2, transferred to SC medium without glucose, shifted from 30°C to 37°C, or incubated in 0.5 M NaCl. B. HSP12-MS2L cells at A600 = 0.5 with CPGFP protein for visualization of HSP12 mRNA were treated with the same stresses as in A.

Figure S4 Relative protein levels in edc3A, pat1A mutants compared with wild type. The intensity of the protein bands in the WB in Figure 3D was calculated using ImageJ [79] and normalized to the α-tubulin loading control of the same sample. The normalized values for non-induced (glucose) w.t. and edc3A, pat1A mutant and induced (galactose) w.t. and edc3A, pat1A mutant are presented in the histogram and accompanying Table. The two right-hand columns indicate the fold induction for each protein in the edc3A, pat1A mutant compared with w.t. under noninducing and inducing conditions.

Figure S5 Standard curves for primer pairs used for qRT–PCR. Different amounts of cDNA (1, 50, 100, 150, and 200 ng) prepared as described in the Materials and Methods were assayed in triplicate in qRT-PCR reactions using the primer pairs in Table 2. The results (Ct) were plotted as a function of the log cDNA concentration and the efficiency of each primer pair (E) was calculated using the formula: $E = (10^{-1/\text{slope}}) \times 100$. The efficiencies are: $UFO1 = 99.25$, $HSP12 = 98.84$, $ACT1 = 98.03$.

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Author Contributions
Conceived and designed the experiments: AL DR. Performed the experiments: AL. Analyzed the data: AL DR. Contributed reagents/materials/analysis tools: AL DR. Wrote the paper: AL DR.

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