Widespread reorganization of metabolic enzymes into reversible assemblies upon nutrient starvation

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Proteins are likely to organize into complexes that assemble and disassemble depending on cellular needs. When ≈800 yeast strains expressing GFP-tagged proteins were grown to stationary phase, a surprising number of proteins involved in intermediary metabolism and stress response were observed to form punctate cytoplasmic foci. The formation of these discrete physical structures was confirmed by immunofluorescence and mass spectrometry of untagged proteins. The purine biosynthetic enzyme Ade4-GFP formed foci in the absence of adenine, and cycling between punctate and diffuse phenotypes could be controlled by adenine subtraction and addition. Similarly, glutamine synthetase (Gln1-GFP) foci cycled reversibly in the absence and presence of glucose. The structures were neither targeted for vacuolar or autophagosome degradation nor colocalized with P bodies or major organelles. Thus, upon nutrient depletion we observe widespread protein assemblies displaying nutrient-specific formation and dissolution.

aggregation | metabolism | microscopy | proteomics | quiescence

It has been hypothesized that proteins may assemble in macromolecular "depots" in which individual components can be transiently held and released. For example, both the ribosome and tRNA multisynthetase complex (MSC) have been found to harbor components that can migrate away from the complex and in so doing acquire or regulate new functions (1). In addition, the extraordinary interconnectivity of protein interactome networks strongly suggests that many genes have multiple functions, and indeed that gene function in general can be considered to be "probabilistic," with each protein dedicated partially to a number of distinct tasks (2). Between these findings, it seems likely that many proteins will be both spatially and functionally organized into dynamic complexes that could assemble and disassemble dependent upon the needs of the cell (3).

Characterization of potential depots is especially critical when considering stationary phase [quiescent (4)] cells, which show extreme adaptations to e.g., nutrient starvation, yet must remain capable of rapid reentry into the cell cycle. Comparison of yeast cells in stationary phase cultures with those in exponentially growing cultures reveals starkly different characteristics associated with quiescence: Obvious cellular changes include cessation of proliferation; increase in mass and volume and arrest as unbudded cells; formation of a thickened cell wall (for osmotolerance and thermotolerance); condensed chromosomes (5); decreased metabolic rate and accumulation of trehalose and glycogen, which may serve in storage and protective roles against oxidative damage; and other stresses (4). Additionally, transcription is reduced by ≈3–5 times (6) and translation by ≈300-fold (7) compared with exponentially growing cells. Given the large scale remodeling of cellular processes, it has been proposed that entry into, survival in, and exit from quiescence can be regarded as a distinct developmental process termed the cell quiescence cycle (4). As with the mitotic cell cycle, passage in and out of the cell quiescence cycle ages the cell, resulting in reduced replicative capacity, but without doubling of cell number (8). Nonetheless, quiescent cells must remain poised to rapidly restart the cell cycle upon nutrient availability.

In *Saccharomyces cerevisiae*, the transition from quiescence to proliferation when nutrients become available requires a rapid, large-scale reprogramming of the metabolic and replication machinery to successfully reenter cell cycle. In support of a distinct regulatory program operating in stationary phase, mutants have been identified that are defective only in exit from quiescence but normally enter and stay viable in stationary phase (4). Also, DNA microarray analyses have shown that whereas most genes are down -regulated upon entry intro quiescence, the expression of certain transcripts is induced in quiescent cells (9, 10).

Recent studies have established that "pools" of critical cellular components may serve as reserves for reentry into cell cycle when nutrients are made available. For example, mRNAs reciprocally exchange between polysomes and cytoplasmic processing bodies (P-bodies) (11). Stationary phase yeast possess large P-bodies containing mRNAs that reenter translation when growth resumes, a process akin to maternal mRNA storage granules in higher organisms. Moreover, chromatin immunoprecipitation experiments performed with RNA polymerase II in stationary phase yeast cells demonstrate that despite global repression of transcription, RNA polymerase II is maintained upstream of hundreds of genes that were induced upon exiting stationary phase, indicating that the general transcription machinery can be held in a poised state during quiescence (10). Finally, other cellular structures such as the actin cytoskeleton also seem to undergo a quiescent phase specific transformation, whereby the highly polarized and dynamic F-actin network is reorganized into immobile, static, depolarized "actin bodies" (12). Actin bodies are seen to reassemble into F-actin upon refeeding even in the absence of de novo protein synthesis, suggesting a readily mobilizable reserve that can be used for actin patch and cable formation upon cell-cycle reentry. Most recently, An and coworkers have shown that a "purinosome" body can transiently form in tissue culture cells (21). Thus, quiescent phase cells appear rich with dynamic complexes with roles helping cells reenter the mitotic cell cycle.

In this work, we searched for more such dynamic complexes in quiescent phase yeast cells by systematically examining the subcellular locations of normally cytosolic proteins for evidence of significant reorganization after entry into stationary phase. Rather

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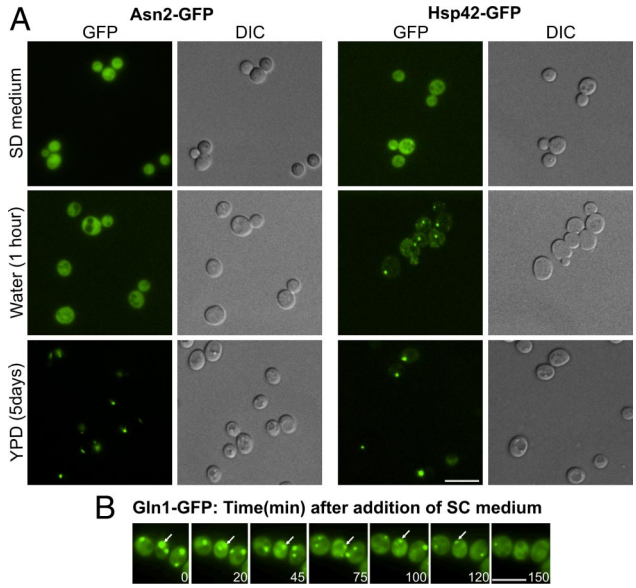

Fig. 1. Numerous cytoplasmic proteins form punctate foci after nutrient depletion. (*A*) Fluorescence microscope images of representative yeast strains expressing green fluorescent protein-tagged asparagine synthetase (Asn2-GFP) or GFP-tagged small heat shock protein molecular chaperone (Hsp42-GFP) exhibit punctate foci after nutrient depletion. Asn2-GFP and Hsp42-GFP tagged strains grown in synthetic defined minimal medium with essential nutrients (SD) (*Upper*) (1 h of growth after transfer from overnight growth in synthetic complete medium) show only diffuse cytoplasmic expression of tagged proteins; transferring strains instead to water (1 h) induces formation of punctate foci in Hsp42-GFP strains, but only to a minor extent in Asn2-GFP strains, indicating differences in induction conditions. In contrast, replicate strains grown to stationary phase in rich medium (YPD) for 5 days (*Lower*) show extensive punctate cytoplasmic foci. (*B*) Punctate formation is reversible, shown with time-lapse images of single cells expressing GFP-tagged glutamine synthetase (Gln1-GFP). After growth in rich medium to stationary phase, resulting in foci formation, addition of synthetic complete (SC) medium at time $t = 0$ results in dissolution of punctate foci within ≈120 min (arrows indicate the same punctate body over time). (Scale bar: 10 μm.)

than identifying isolated cases of such complexes, we find instead that a surprisingly large proportion of metabolic proteins form reversible macroscopic foci inside quiescent cells. Formation can be observed by multiple independent techniques, using both tagged and untagged proteins. Formation and dissolution of these foci can be controlled by availability of specific metabolites, with multiple mechanisms of formation and dissolution apparent. Thus, metabolite-specific, reversible protein assemblies appear to be a new and perhaps widespread phenomenon in cell biology.

Results and Discussion

To search for previously undetected, potentially dynamic protein complexes, we used a cell microarray technique (13) to examine changes in the localization of the GFP-tagged yeast strain collection (14) under nutrient-depleted conditions. An examination of the ≈800 strains containing GFP tagged cytosolic proteins grown to stationary phase [quiescence (4)] revealed a surprising number of strains in which punctate foci were formed. Manual retesting of 256 punctate strains was carried out, and 180 strains showed a clearly discernible punctate phenotype (Fig. 1*A*) in 1 or more of the media conditions tested, in contrast to the proteins' normal soluble form in exponentially growing cells (14). Although proteins in multiple functional classes were identified, proteins involved in intermediary metabolism and stress response predominated (Table S1).

We hypothesized that one of the reasons these foci had not been observed in previous localization screens (14, 15) was that they could transiently form and disperse under particular cellular conditions. To test this hypothesis, several strains that showed a particularly robust punctate phenotype (Ade4, Gln1, Ura7, and Shm2) were assayed for the reversibility of the punctate phenotype upon the addition of fresh media. All four were found to revert to the cytosolic, diffuse state. The time scales for recovery were on the order of 1–2 h. A representative time lapse experiment is shown in Fig. 1*B* for the Gln1-GFP strain. Upon regrowth to stationary phase, the punctate phenotypes were reestablished. Cycling between punctate and diffuse phenotypes could be readily achieved by cycles of media addition and growth.

To identify the signals responsible for the formation of protein assemblies, strains showing punctate phenotypes were grown in a variety of drop-out media. The Ade4-GFP appears to transition to a punctate state in adenine dropout medium, whereas glucose removal or total nutrient depletion led to the formation of robust Gln1-GFP foci (Fig. 2). Punctate body formation was quite specific, with adenine depletion leaving Gln1-GFP diffuse and glucose depletion leaving Ade4-GFP diffuse. Reversal was also highly specific. Adenine and the related nucleotide hypoxanthine dissolved the foci formed by adenine depletion in Ade4-GFP cells, whereas guanosine and histidine did not. Similarly, glucose was sufficient to dissolve the Gln1-GFP foci formed by glucose depletion. As with foci cycled by quiescence and media addition, cycling between punctate and diffuse phenotypes could be readily achieved by alternating addition of complete and nutrient dropout media (Fig. 2). These results are similar to those observed in the dynamic formation and dispersal of the purinosome (21) but seem to occur more extensively, including even an enzyme involved in basal nitrogen metabolism.

To determine whether protein assembly formation might be downstream of a larger signaling or regulatory pathway, we assayed whether new protein synthesis was required for punctate formation. Ade4-GFP punctate body formation was inhibited by the addition of the protein translation inhibitor cycloheximide, whereas Gln1-GFP punctate body formation was not, again indicating differences between the pathways or mechanisms involved in metabolite-specific protein assembly formation. Cycloheximide also differentially inhibited the reversal of the metabolite-specific assemblies. Dissolution of the Gln1-GFP foci was partially inhibited by inhibiting protein synthesis, whereas Ade4-GFP reversal was not affected.

The metabolite-specific, reversible formation of protein assemblies is a unique phenomenon that might be associated with a conventional protein aggregation pathway. Autophagy is the predominant protein recycling mechanism that operates under nutrient-limiting conditions such as nitrogen starvation. The protein Atg2 is known to assist in the assembly of punctate, autophagosome vesicles (16) and is required for both autophagy and cytoplasm to vacuole targeting (17), and we therefore assayed Gln1-GFP and Ade4-GFP localization in autophagy-defective strains lacking *ATG2* (Fig. 3). The autophagy defect did not affect formation of foci in either strain. Punctate proteins might also have been targeted for vacuolar degradation by other routes, and we therefore assayed foci formation in a *vps33*Δ strain, characterized by fragmented vacuoles, albeit acidified and with active V-ATPase. This vacuolar defect severely impairs Gln1-GFP punctate formation and restricts Ade4-GFP punctates to the bud neck. The fates of the protein assemblies were assayed in the presence of PMSF, a serine protease inhibitor that is known to inhibit vacuolar proteases. Both Ade4-GFP and Gln1-GFP punctates were insensitive to the addition of PMSF. Thus, although factors involved in vacuole formation influence punctate formation, the proteins do not appear to be targeted for degradation. The differences in outcome between the Ade4-GFP and Gln1-GFP punctates also once again emphasize that different protein assemblies seem to be formed and regulated independently.

If the protein assemblies were not being degraded, it was possible that they were being stored during nutrient starvation and become

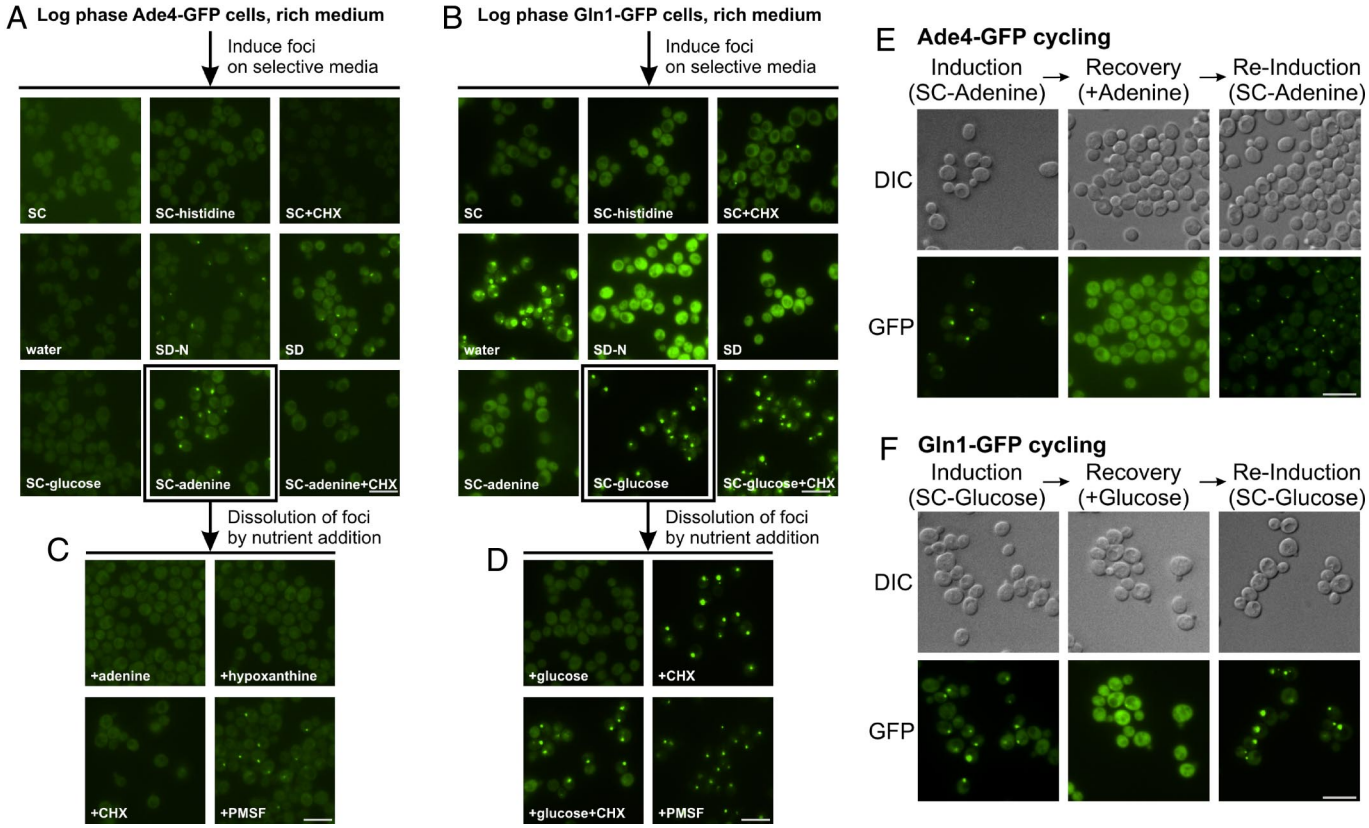

Fig. 2. Ade4-GFP and Gln1-GFP strains demonstrate metabolite-specific induction and dissolution of punctate foci. Specificity of induction (or dissolution) was measured by transferring log-phase cells from rich (or dropout) medium into different test media and imaging after 2 h. (*A*) Removal of adenine is necessary and sufficient to induce formation of Ade4-GFP foci, as seen after growth in synthetic complete adenine dropout medium (SC-adenine). However, adding cycloheximide (CHX) to the adenine dropout medium (SC-adenine+CHX) blocks punctate body formation, indicating a requirement for protein translation. Induction was also observed in SD medium or medium lacking a nitrogen source (SD-N). (*B*) In contrast, removal of glucose is necessary and sufficient for induction of Gln1-GFP foci, as shown after growth in synthetic complete glucose dropout medium (SC-glucose); addition of cycloheximide (SC-glucose+CHX) has no effect. (*C* and *D*) Similar metabolite-specificity is seen for dissolution of foci. Ade4-GFP foci can be dispersed after readdition of adenine or related hypoxanthine; Gln1-GFP can be reversed by readdition of glucose. Dissolution of Gln1-GFP foci requires protein synthesis, whereas Ade4-GFP does not, instead requiring protein synthesis for maintenance of the foci. Addition of the vacuolar protease inhibitor PMSF shows no effect. (*E* and *F*) Formation and dissolution of punctate foci can be cycled by alternating nutrient depletion and addition. (*E*) Ade4-GFP expressing cells cycling in response to depletion and addition of 20 μg/mL adenine. (*F*) Gln1-GFP expressing cells cycling with successive depletion/addition of 2% glucose. To reduce image saturation and clarify absence of foci, GFP fluorescence images of strains showing foci are scaled to different intensity ranges than those of nonpunctate strains. (Scale bar: 10 μm.)

available when the cell exits its quiescent state. A number of other cellular mechanisms for entering a poised, quiescent state are already known, such as mRNA storage by P-body formation (11), actin bodies (12) and genomic positioning of transcription factors (10), although none of these mechanisms are known to involve either the specific enzymes or the sheer number of proteins observed in this study. Interestingly, we saw no colocalization of Ade4 or Gln1 punctates with P-body marker proteins (Figs. S1 and S2) and only limited colocalization with actin bodies (Fig. S3).

We attempted to directly examine a protein assembly by carrying out high-speed centrifugation on Gln1-GFP cell lysates. Although the GFP signal was minimal in the clarified supernatant, much of the Gln1-GFP assemblies were associated with the pellet fraction (Fig. 4) and sucrose gradient fractionation (7–47%) similarly indicated that the punctates were denser than polysomes. We also attempted to purify punctates on a discontinuous Ficoll gradient (4%, 8%, and 16%). Again, most of the Gln1-GFP pelleted. We also attempted to quantitate the GFP (by Western blot analysis) and to quantitate the presence of foci that were similar in size to cell punctates ($\approx$1 μm, by visual inspection of fluorescence micrographs). Most of the Gln1-GFP was in the pellet fraction, with only a small, floating peak (maximal at Fraction 10) (Fig. 4) detected within the gradient, accounting for $<$3% of total GFP. As a basis for comparison, given that vacuoles are known to float at much lower densities, these results potentially indicate that the punctates are larger or heavier than vacuoles (18). The pelleted protein assemblies could be dissolved by detergent or protease treatment, but not by ribonucleases. However, rich medium could not reverse the formation of the isolated assemblies, again indicating that punctate formation and dissolution occurred in the context of some more extensive cellular pathway or mechanism.

The finding that the metabolite-specific, reversible protein assemblies could be readily isolated suggested that we could further determine what factors were involved in assembly formation and maintenance. We identified proteins in the soluble and insoluble fractions of stationary phase wild-type cells, using mass spectrometry. Protein fractions collected from soluble and insoluble fractions of the cells were treated with Rapigest, an acid labile detergent, digested with trypsin, and analyzed by 2-dimensional liquid chromatography/tandem mass spectrometry, using a LTQ-Orbitrap mass spectrometer (Fig. 5). In all, 1,605 proteins were identified at a 5% false discovery rate. The quantities of proteins in the soluble and insoluble fractions were further determined by APEX analysis (19). The pellet fraction of the stationary phase was indeed significantly enriched for cytoplasmic proteins (Fig. 5 and Dataset S1). Of the 68 proteins observed both by our microscopy screen and

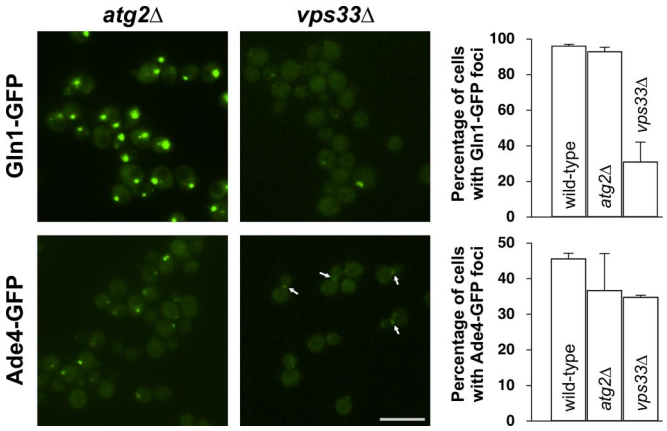

Fig. 3. Punctate foci do not appear to be targeted for degradation by autophagosomes or vacuoles. Ade4-GFP and Gln1-GFP foci form effectively in an autophagy defective strain (*atg2Δ*). Fragmentation of vacuoles in a *vps33Δ* strain reduces expression levels of Ade4-GFP and Gln1-GFP (as measured by total fluorescent intensity), severely impairs Gln1-GFP foci, and appears to produce Ade4-GFP foci that localize to the bud neck (arrows). Nonetheless, foci form in >20% of the vacuole defective cells, as quantified at right. (Scale bar: 10 μm.)

mass spectrometry, 61 (90%) were detectable in the pellet. Because the same protein assemblies can be isolated and characterized using a completely different technique, this precludes the possibility that the original microscopy observations might result from GFP-tag-based aggregation. We further verified Gln1, Ade4, Glt1, Rio2, and Pbs2 aggregation by immunofluorescence, assaying for non-GFP tagged proteins via an alternate epitope tag (Fig. S4) and verified, using doubly epitope tagged strains (Fig. S5), that Ade4 and Gln1 do not colocalize. In addition, the proteins from strains producing punctate foci are computationally predicted to have evolved to self-assemble, separate from any perturbation by GFP [$P < 0.001$, Mann–Whitney U test of self-assembly propensities predicted by the Tango algorithm (20)].

As a test of the hypothesis that the observed widespread reorganization into assemblies was in general dynamic and reversible, we attempted to determine whether cytoplasmic proteins found enriched in the stationary phase pellet could reversibly move to the soluble phase upon growth recovery with new medium. The partitioning of proteins between the insoluble and soluble phases in stationary (S) versus recovered (R) cells was determined using a statistical scoring parameter (difference Z score; see *Materials and Methods*). A positive value for a given protein indicated that this protein had largely partitioned back into the soluble phase in the recovered cells compared with the stationary cells. We generated a list of 114 proteins that satisfied this criterion above a ≈90% confidence cut-off. Not only were many (33 proteins) (Table 1) of the proteins the same as those found to display a punctate phenotype from the cell microarray screen, as had already been shown, but the phenomenon could be now extended to a number of additional cytoplasmic proteins.

Conclusions

Overall, the evidence strongly suggests that metabolite-specific, reversible protein assemblies are perhaps a widespread phenomenon in cell biology. The assemblies can be observed by 2 orthogonal techniques (microscopy and mass spectrometry), are not associated with typical aggregation phenomena, and appear to be controlled by distinct pathways. Although the Ade4-GFP foci show cyclohexamide-sensitive reversible induction and cycloheximide-insensitive dissociation to adenine or the related hypoxanthine, Gln1-GFP foci display such reversible behavior in response to a carbon source like glucose, but with cycloheximide insensitive induction and sensitive dissolution. These assemblies may be independent macromolecular

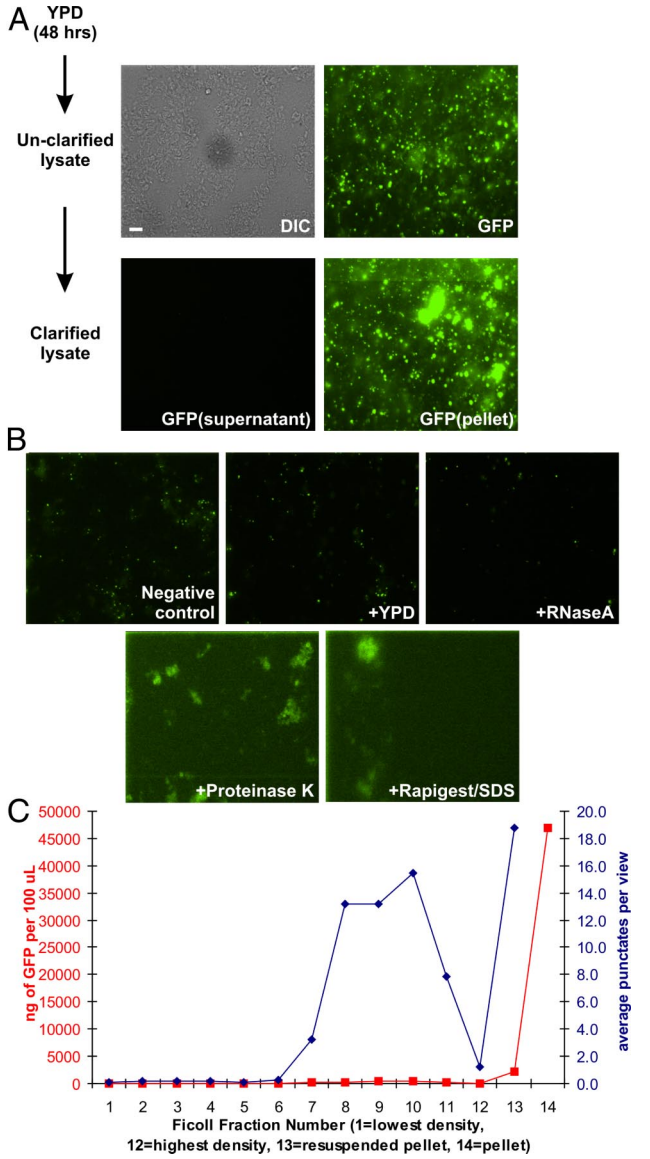

Fig. 4. Physical characterization of Gln1-GFP foci indicates stable proteinaceous structures sensitive to proteases and detergents, but not ribonuclease. (*A*) Enrichment of Gln1-GFP punctate bodies from cells grown in rich medium (YPD) to stationary phase and lysed by glass beads. Lysate was clarified by centrifugation at 13,000 × *g*. GFP-tagged foci are highly enriched in the insoluble pellet relative to the supernatant. (*B*) Preparations of Gln1-GFP foci prepared as in *A* could be dissolved by addition of proteinase K and protein detergents (Rapigest, SDS), but not by addition of rich medium (YPD) or ribonuclease A, indicating no structural requirement for RNA and that additional factors are required for in vivo reversibility. (*C*) Biochemical fractionation of Gln1-GFP punctate bodies. The distribution of Gln1-GFP punctate bodies separated on a Ficoll gradient, expressed as GFP (ng/100 μL; red curve and left-hand axis) and average number of visible punctate foci per micrograph (blue curve and right-hand axis; not measurable in pellet). (Scale bar: 10 μm.)

depots for the storage of enzymes of intermediary metabolism during cellular stasis. In favor of this hypothesis (and again arguing against an entry into degradative pathways), we see that punctate foci persist for up to 1 week (up to 1 week tested) in stationary phase, but can still be reversed at the end of this time. These observations are consistent with those previously made by Brengues et al. (11) that polysomes also reappear in cells recovering from nutrient starvation, lending further credence to the suggestion that exit from and reentry into the cell cycle is accompanied by

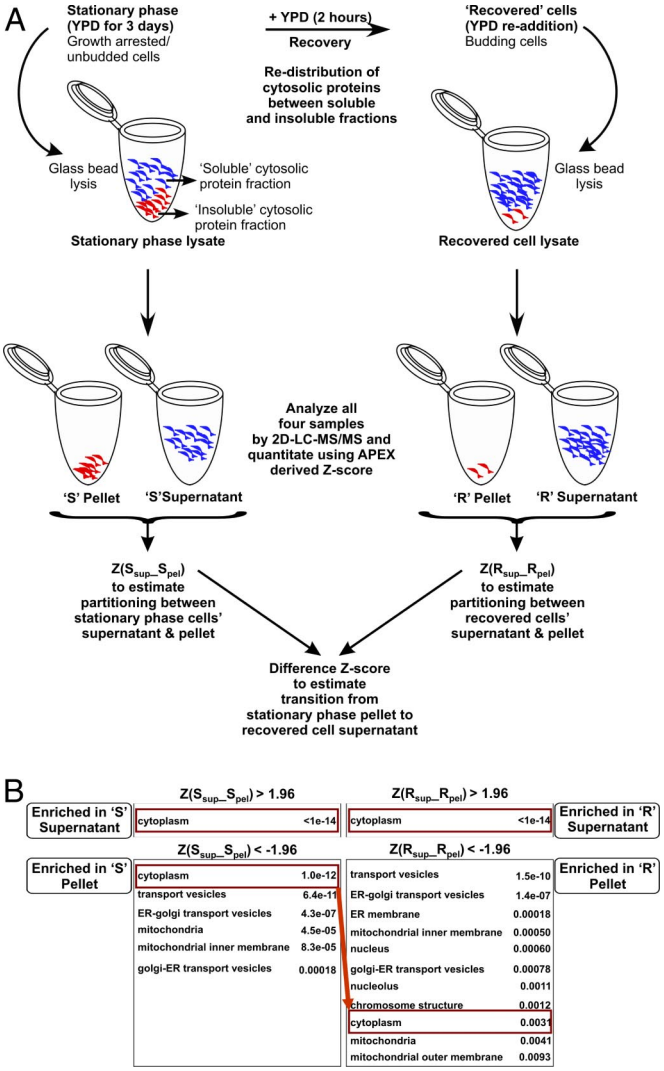

Fig. 5. Overview of mass spectrometry shotgun proteomics analysis of stationary phase and recovered yeast cell protein solubility. (*A*) Schematic of shotgun proteomics analysis of insoluble foci. Two parallel BY4741 cultures were grown to stationary phase; one was recovered by addition of fresh YPD medium. After lysis of each sample and fractionation into their respective soluble and insoluble protein fractions, proteins were identified and quantified by 2-dimensional HPLC/tandem mass spectrometry of their constituent tryptic peptides. (*B*) Statistical enrichment of proteins from known subcellular compartments among those identified by mass spectrometry to be preferentially partitioned (95% confidence level; $|Z| \geq 1.96$) to either the soluble or insoluble protein fraction in stationary phase ($S_{supernatant}$, S_{pellet}, respectively) or recovered ($R_{supernatant}$, R_{pellet}, respectively) cells. Enrichment is calculated as the hypergeometric probability of the overlap of each set of proteins with those classified in each Munich Information Center for Protein Sequences (MIPS) subcellular localization (28), as measured using FunSpec (29). Proteins from the stationary phase insoluble fraction are strongly enriched for cytoplasmic proteins ($P < 10^{-11}$); upon recovery with fresh YPD medium, this enrichment drops dramatically ($P < 10^{-2}$), consistent with the transition of numerous cytoplasmic proteins from insoluble to soluble fraction upon recovery. As controls, the soluble protein fraction of the stationary phase and recovered cells both show similarly strong enrichment for cytoplasmic proteins ($P < 10^{-14}$); similarly, other insoluble protein components such as transport vesicles are similarly enriched in both stationary phase and recovered samples.

large-scale reorganization of protein complexes. The recent studies of the purinosome (21) also seem to indicate that multienzyme complexes can become microscopically discernible and colocalize. As with our own work, nutrient depletion appeared to favor complex formation. It is possible that functional foci formation enhances substrate channeling and metabolite flux control during cellular nutrient stress. If so, it would appear that this is a general phenomenon, extending well beyond purine biosynthesis, and that a large fraction of the cytoplasmic metabolic proteins organize into extensive physical structures after nutrient depletion. The general nature of the depot phenomenon is further emphasized because multiple mechanisms of formation and dissolution appear to be used.

Table 1. Thirty-three proteins observed both by microscopy to form punctate cytoplasmic foci in stationary phase cells and by mass spectrometry to reversibly transition from the insoluble fraction of stationary phase cells to the soluble fraction upon re-addition of nutrients

Common gene name	Systematic gene name	Biological process
ADE12	YNL220W	Purine nucleotide biosynthesis
ADE17	YMR120C	Purine nucleotide biosynthesis
ADE5,7	YGL234W	Purine nucleotide biosynthesis
ADH2	YMR303C	Fermentation
ALA1	YOR335C	Ala-tRNA aminoacylation
ARC1	YGL105W	tRNA-nucleus export
CDC19	YAL038W	Glycolysis
CDC60	YPL160W	Leu-tRNA aminoacylation
CPR6	YLR216C	Protein folding
CYS4	YGR155W	Cysteine biosynthesis
GLN1	YPR035W	Nitrogen metabolism
GLN4	YOR168W	Gln-tRNA aminoacylation
HSC82	YMR186W	Response to stress
HSP42	YDR171W	Response to stress
HSP82	YPL240C	Response to stress
HTS1	YPR033C	His-tRNA aminoacylation
ILS1	YBL076C	Protein biosynthesis
IRA1	YBR140C	Sporulation
KIC1	YHR102W	Cell wall organization
PAB1	YER165W	Translation initiation regulation
RPL4B	YDR012W	Protein biosynthesis
RPS11B	YBR048W	Protein biosynthesis
SBP1	YHL034C	RNA metabolism
SSB1	YDL229W	Protein biosynthesis
SSB2	YNL209W	Protein biosynthesis
STI1	YOR027W	Protein folding
THS1	YIL078W	Protein biosynthesis
TPS2	YDR074W	Response to stress
UGA1	YGR019W	Nitrogen utilization
UGP1	YKL035W	UDP-glucose metabolism
UGT51	YLR189C	Sterol metabolism
URA2	YJL130C	Pyrimidine base biosynthesis
VAS1	YGR094W	Val-tRNA aminoacylation

Materials and Methods

Media and Yeast Strains. Detailed information for materials used in this study, including yeast strains, growth media and conditions, and construction of epitope-tagged yeast strains, can be found in *SI Materials and Methods*.

Media Transfer Experiments. Ade4-GFP and Gln1-GFP punctate foci were induced by growing Ade4-GFP and Gln1-GFP cultures in YPD to $OD_{600} \approx 0.5$, centrifuging at 2,000 × *g* for 5 min and resuspending in assay media. The resuspended cultures were shaken at 30 °C for 2 h, and imaged. Reversal of Ade4-GFP punctate foci was induced by adding back test additives [histidine, 20 μg/mL (22); hypoxanthine, 20 μg/mL (22); cycloheximide, 100 μg/mL (23); PMSF, 1 mM in 1% ethanol (24); or glucose, 2% (22)] at concentrations equal to those in synthetic complete (SC) medium (22) to the SC-adenine culture medium. For Gln1-GFP, reversal was induced in a similar manner, except that additives were added to SC-glucose culture media. Recovery was allowed to progress for 2 h at 30 °C in a shaker before imaging. For cycling back to the punctate state, recovered Gln1-GFP or Ade4-GFP cells were again induced by depleting glucose or adenine, respectively.

High-Throughput Imaging by Cell Microarrays and Manual Confirmation. Spotted cell microarrays (13) were manufactured from the *S. cerevisiae* GFP-tagged yeast strain collection (14) (Invitrogen) and applied to identify foci-forming strains as described in *SI Materials and Methods*.

Confirmation of 256 strains exhibiting punctate localization was performed by growing strains in SC medium for 48 h, manually transferring onto poly(L-lysine)-coated slides, and imaging without fixation. A total of 180 strains were confirmed to consistently form punctate foci (Table S1). Of the 256 strains, 27 strains failed to form punctate foci (Table S2). The remaining strains showed intermittent/rare foci and were not considered further, with the exception of Ade4-GFP, which showed reproducible and robust foci formation in stationary phase in YPD medium, but intermittent formation in SC medium (both 48 h).

Confirmation of Foci Formation by Immunofluorescence. TAP-tagged yeast strains (Open Biosystems) were grown at 30 °C in YPD or appropriate dropout medium, to mid-log or stationary phase, as appropriate, then fixed, spheroplasted, incubated with FITC-conjugated rabbit anti-goat IgG (Zymed), and imaged using standard immunofluorescence protocols as described in *SI Materials and Methods*.

Biochemical Fractionation of Gln1-GFP Foci. Biochemical fractionation of Gln1-GFP foci was done by a modification of a protocol used by Teixeira et al. (23), described in full in *SI Materials and Methods*. Discontinuous Ficoll gradient fractionation of Gln1-GFP foci was performed using a modified version of the vacuole purification protocol from Reider and Emr (18), as described in *SI Materials and Methods*.

Mass Spectrometry-Based Validation and Quantitation of Punctate Formation. Foci formation and dissolution to the diffuse state were verified with an epitope-tag independent, tandem mass spectrometry (MS/MS)-based approach (Fig. 5). We analyzed protein abundances from supernatant and pellet fractions from stationary and recovered cells (referred to as S_{sup}, S_{pel}, R_{sup}, and R_{pel}, respectively) as described in *SI Materials and Methods*. After proteolysis of the 4 protein fractions, mass spectrometry was performed on the resultant tryptic peptides as described in Lu et al. (19) and detailed in *SI Materials and Methods*.

To measure the relative enrichment of each protein between the soluble and insoluble cell fractions, we used the differential Absolute Protein Expression measurement (APEX) technique (19, 27), as described in *SI Materials and Methods*. Based on the numbers of observed MS/MS spectra associated with each identified protein from each cell fraction, we calculated the differential protein enrichment (i.e., bias toward either the soluble or insoluble protein fraction) as a *Z* score.

Two kinds of analyses were performed on the *Z* score data. First, the *Z* scores were correlated with Munich Information Center for Protein Sequences (MIPS) subcellular localization (28) categories. Proteins were selected above a 95% confidence threshold for their statistically significant partitioning toward the stationary phase soluble fraction (244 proteins) or insoluble fraction (315 proteins), and similarly for significant bias toward the recovered cells' soluble (211 proteins) or insoluble fractions (476 proteins). These 4 sets of proteins were analyzed using the FUNSPEC program (29) for known MIPS subcellular localizations.

As expected, for proteins from the recovered cells, proteins of known cytoplasmic location were significantly enriched in the supernatant whereas the respective pellet showed enrichment for transport vesicles and various organelle-associated proteins, validating the mass spectrometry-based approach for analyzing protein partitioning between supernatant and pellet fractions. In contrast, both the supernatant and the pellet fractions of the stationary phase were significantly enriched for cytoplasmic proteins (Fig. 5), thus corroborating the microscopy observations that cytoplasmic proteins tended to accumulate in the pellet of stationary phase cell lysates.

Second, we generated a list of proteins that transitioned significantly from the stationary phase pellet to the recovered phase supernatant. If $Z(R_{sup}\text{–}R_{pel})$ was greater than $Z(S_{sup}\text{–}S_{pel})$ or $Z(R_{sup}\text{–}R_{pel}) - Z(S_{sup}\text{–}S_{pel}) > 0$, then the partitioning from supernatant to pellet would be greater in the stationary phase compared with the recovered phase. This was used to identify the list of proteins preferentially enriched in the stationary phase pellet compared with that of the recovered phase. Using the difference in the 2 *Z* scores as a metric (divided by $\sqrt{2}$ to correct for normality), a list of 114 proteins satisfying the above criterion was generated with a ≈90% confidence cut-off ($|\text{Difference } Z| > 1.64$) (Table S3). We found the proteins in this list to be enriched for proteins found to display a punctate phenotype from the microscopy screen, with 33 proteins in both sets (Table 1), confirming the overall trend observed in the microscopy. All identified proteins and associated *Z* scores are listed in Dataset S1.

Calculation of Potential to Aggregate or Self-Assemble. To examine whether the groups of proteins observed/not observed by microscopy to form punctate foci can be distinguished by their propensity to aggregate, we calculated a TANGO score (20) for each of the 207 proteins. A higher TANGO score indicates a larger predicted propensity to aggregate or self-assemble. The 180 proteins that formed foci have a mean TANGO score of 917 ± 533, whereas the 27 proteins that did not form foci have a mean TANGO score of 489 ± 386. The latter 27 proteins have significantly lower average TANGO scores than the 180 proteins that formed foci ($P < 0.001$ for a Mann–Whitney *U* test).

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