Formation of intracellular glutamine synthetase bodies depends strongly upon cellular age and glucose availability

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ABSTRACT

The enzyme glutamine synthetase serves key roles in central nitrogen metabolism, catalyzing the biosynthesis of glutamine, as well as regulating ammonia assimilation and integrating metabolic signals to balance nitrogen use. The budding yeast enzyme was recently found to form intracellular bodies (GS bodies) composed of glutamine synthetase and Hsp90 chaperones following various types of nutrient depletion or chemical stress. In order to better quantify and characterize the *in vivo* formation of GS bodies, we developed an assay for their formation in single yeast cells using imaging flow cytometry, which enables the quantitative measurement of rates of GS body formation and their population penetrance. Either reduction of supplied glucose, or addition of the competitive inhibitor of glycolysis, 2- deoxyglucose, markedly enhanced the formation of GS bodies. The occurrence of GS bodies increased with increasing cell size, a proxy for cell age, while treatment with rapamycin antagonized their formation. Direct measurement of GS body formation as a function of replicative age showed that mother cells exhibited a significantly higher incidence of GS bodies than daughter cells, and the frequency of GS body formation increased with increasing replicative cell age. Thus, we find that yeast glutamine synthetase bodies form in a manner strongly dependent on available glucose and increase markedly with cell age.

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INTRODUCTION

Evidence is building that protein aggregation *in vivo* may be far more common than expected, with widespread protein aggregation observed in fission yeast cells (Matsuyama et al. 2006; Hayashi et al. 2009), in nutritionally or chemically stressed budding yeast cells (Narayanaswamy et al. 2009; Jacobson et al. 2012; Tkach et al. 2012; O'Connell et al. 2014), and in aged nematodes (David et al. 2010), among many other conditions and organisms. Many of these proteins are observed to assemble *in vivo* into large intracellular bodies (O'Connell et al. 2012), forming a wide array of intracellular structures, from cytoplasmic dots to domains to oil droplet-like structures. As such, intracellular protein aggregates join a growing array of cellular bodies with differing properties and associated proteins, including nuclear stress bodies (Biamonti 2004), stress granules (Buchan and Parker 2009), actin bodies (Sagot et al. 2006), proteasome bodies (Laporte et al. 2008), aggresomes (Kopito 2000), inclusion bodies (Shively 1974), and P-bodies (Ingelfinger et al. 2002), among others (O'Connell et al. 2012). Many of these intracellular bodies, especially those found from high-throughput cell imaging screens, await detailed characterization, and key questions include defining molecular constituents and possible cellular roles, if any, for these various intracellular bodies (Cioce and Lamond 2005; Buckingham and Liu 2011; Biamonti 2004; Cotto, Fox, and Morimoto 1997; Biamonti and Vourc'h 2010; Campbell et al. 2007; Zhao et al. 2013; Xia et al. 2008; Yeates et al. 2008).

Among many metabolic enzymes now known to assemble into intracellular bodies (O'Connell et al. 2012), the enzyme glutamine synthetase is notable for serving a key role in central nitrogen metabolism, catalyzing the condensation of glutamate with ammonia to form glutamine. The endogenous *S. cerevisiae* protein (Gln1p) is a 42 kDa enzyme that assembles into a homodecamer (He et al. 2009). The homologous *E. coli* glutamine synthetase forms a dodecamer, and the purified enzyme shows the remarkable property of assembling *in vitro* into fibers through dodecamer-dodecamer stacking interactions (Miller 1974; Dabrowski et al. 1994). Moreover, intracellular *E. coli* glutamine synthetase aggregation is known to be induced *in vivo* by oxidation, preceding its degradation (Smith 1991; Berlett 1997; Bosshard et al. 2010; Levine 1981).

Recently, it has been shown that a green fluorescent protein fusion to glutamine synthetase expressed in *S. cerevisiae* from the native genomic locus (Gln1-GFP) forms intracellular protein bodies (GS bodies) following various types of nutrient deprivation, chemical stress, or DNA damage (Narayanaswamy et al. 2009; (O'Connell et al. 2014); Tkach et al. 2012). The bodies have been confirmed to form independently of the GFP fusion partner, both through assaying with alternative epitope tags (HA or TAP tags) and through the use of mass spectrometry to confirm that the endogenous untagged protein also forms insoluble protein bodies (Narayanaswamy et al. 2009; O'Connell et al. 2014) . GS bodies are not observed to co-localize with major cellular organelles (e.g., nucleus, endoplasmic reticulum, or vacuoles) nor with other canonical intracellular protein bodies, e.g. actin bodies and P-bodies (Narayanaswamy et al. 2009). The bodies survive cell lysis, and mass spectrometry analysis indicates that they are composed almost exclusively of glutamine synthetase and the Hsp90 chaperones Hsp82p and Hsc82p (O'Connell et al. 2014). Gln1p is normally expressed in exponentially growing yeast cells in rich medium at very high levels, approximately 350,000 molecules per cell (Ghaemmaghami et al. 2003), and models such as the "life-on-the-edge" theory would suggest that the protein might be expressed natively close to its limit of solubility during exponential cellular growth (Tartaglia et al. 2007; Ciryam et al. 2013).

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In order to better understand the *in vivo* conditions that might drive a highly expressed, soluble protein into compact, insoluble protein bodies, we developed a high-throughput single cell assay for GS body formation based on imaging flow cytometry. Using this assay, we quantified the formation of GS bodies in response to available glucose and cell age. In particular, we hypothesized that depleting cellular energy levels would lead to less effective protein homeostasis and a concomitant aggregation of Gln1p. Indeed, GS bodies have been previously observed to form in stationary phase cells as well as in cells transferred into glucose dropout medium (Narayanaswamy et al. 2009)(e.g., as shown in **Figure 1A**). We therefore quantified the kinetic dependence of GS body formation on available glucose and in response to addition of an inhibitor of hexokinase. We find that GS body formation is strongly dependent on glucose, as is the number and size of bodies formed. Treatment with the compound rapamycin, which globally reduces protein synthesis rates and increases levels of autophagy, strongly reduced the incidence of GS bodies. Finally, the tendency to form GS bodies was strongly correlated with replicative aging of the yeast, significantly greater in mother cells than daughter cells, and generally increased with cell size, raising the possibility that the bodies were preferentially formed or retained in mother cells.

MATERIALS AND METHODS

Growth and microscopy of yeast

Yeast strains had a genetic background of BY4741 [genotype, MATa his3_1 leu2_0 met15_0 ura3_0]. Strains expressing GFP-tagged proteins were obtained from the OpenBiosystems GFP collection. Rich (YPD) medium containing Yeast Extract (1%), Peptone (2%) and glucose (2%) was purchased from Sunrise Sciences. Synthetic complete medium (SC) contained 1x Yeast Nitrogen Base (BD Biosciences/Difco) without amino acids, synthetic drop-out medium supplement mix or was purchased from Sigma. Cultures were started from by picking from freezer stocks into YPD and growing overnight, before transferring to new media for regrowth. Log phase cells were imaged and prepared for mass spec at an O.D. of approximately 2. Stationary phase cells were grown a minimum of 48 hours before imaging or lysing for mass spec analysis. All cultures were maintained shaking at 30°C.

For assays in the presence of 2-deoxy glucose, cells in late log phase, defined as an optical density of 2/ml, were spun down, washed with SC-glucose, re-suspended in SC-glucose with 2% 2-deoxyglucose (Sigma) for 2h shaking at 30C.

Cells were imaged on a Nikon E800 fluorescence microscope with Photometrix Coolsnap CCD camera under oil immersion at 100x magnification. DIC images and fluorescent images in the GFP channels were collected using standard filter sets. Cell lysate was imaged on a Nikon TE2000-E with a Photometrics Cascade II camera at 60x magnification. Images were processed using Nikon Elements AR.

For assessing effects of glucose depletion, cells in early log phase (~2 O.D./ml) were centrifuged and resuspended in SC with glucose concentrations ranging from 0 to 2% (at ~1 O.D./ml). At 30 minute or 1 hour intervals, 300µl of culture was removed and fixed by incubation with 4% formaldehyde at room temperature for 60 min, and washed with PBS before storing at 4°C. Cell aliquots were then analyzed using the imaging flow cytometry assay. The change in percent penetrance of GS bodies across each cell population over time was fitted with an allosteric sigmoidal equation to calculate the onset of GS body formation. The time at fifty

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percent maximum penetrance was plotted against glucose concentration to determine glucose dependence of GS body formation.

Imaging flow cytometry for high-throughput quantification of GS body formation

Cells were imaged on an Amnis ImageStream imaging flow cytometer at 60x, collecting both brightfield and GFP fluorescent channels. For each replicate, 50,000 cell images were collected at a flow rate of 40mm/s and a flow width of 7mm. Images were analyzed using Amnis IDEAS 4.0 software and custom templates to filter images based on proper focus and cell viability. Cell images were computationally sorted into populations based on foci phenotype and properties of the fluorescence signal. The percentage of cells containing GS bodies was calculated as the proportion of cells with greater than 30% of total cellular GFP fluorescence signal in an area less than approximately 1.25 μm^2. We observed foci size to scale with integrated fluorescence intensity (F) according to the equation (eqn 1):

$$A = \frac{6 \times 10^{-5}\,\mu\text{m}^2}{\text{relative flourescence units}} \times F + 1.2\,\mu\text{m}^2 \qquad (1)$$

where (F) is the integrated fluorescence intensity of the smallest number of contiguous pixels that account for at least 30% of total cellular fluorescence and (A) is the sum of the area of those pixels.

Fluorescence-activated cell sorting and cell imaging

In order to determine the effects of increasing cell age on the formation of GS bodies, we grew cultures of Gln1-GFP yeast to stationary phase in YPD (5 days) and fixed cells as described above. Bud scars on cells were fluorescently marked by incubation for 20 minutes with calcofluor-white (100 µl/ml PBS cell suspension). The brightest 1% of calcofluor-white fluorescent cells were isolated using FACS, resulting in a population enriched for older mother yeast cells. Cells were imaged as described above, additionally monitoring calcofluor-white fluorescence using a standard DAPI filter set to detect the bud scars. To improve bud scar and GS body imaging, 15 µm deep Z-stacks of 3 µm thick slices were captured for each imaged field. Maximum intensity projections were calculated for each Z-stack and, together with the raw data, used to measure cell size (as estimated by cross sectional area at the focal plane), number of bud scars, and count of GS bodies per cell.

Quantifying rapamycin effects by imaging flow cytometry

Gln1-GFP yeast cultures were inoculated from frozen cultures and grown to 0.2 OD/ml in SC in triplicate, then subcultured and grown to approximately 1 OD/ml in SC before the addition of rapamycin (Sigma) in DMSO to reach final concentrations of 200, 100, 20, 1, and 0 nM rapamycin (DMSO only; no differences were observed relative to a control sample without DMSO). Cells were grown an additional 30 h until cultures had passed the diauxic shift before fixing with formaldehyde, as described above, and analyzed using the imaging flow cytometry assay.

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Results and Discussion

Formation of GS bodies depends strongly upon available glucose

In order to quantify the formation of GS bodies at the single cell level and across many individual cells, we first developed a high-throughput assay based upon imaging flow cytometry: For a given aliquot of Gln1-GFP expressing yeast cells, the cells are fixed with 4% formaldehyde to prevent potential changes in protein localization prior to analysis, then approximately 50,000 cells are individually photographed using imaging flow cytometry and the formation of GS bodies measured on a per cell basis using an automated image analysis pipeline **(Figure 1B)**. The assay is thus significantly higher in throughput than manual analysis of standard fluorescence microscopy images, and the correspondingly higher cell counts allow for greater precision and statistical power in quantifying the occurrence of GS body formation across populations of cells.

Using this assay, we quantified the extent of GS body formation as a function of available glucose in the growth medium, measuring the frequency of cells exhibiting detectable GS bodies as a function of time and for differing concentrations of glucose in the medium **(Figure 1C).** Gln1-GFP tagged yeast cells growing exponentially in 2% glucose were transferred to medium either lacking glucose altogether, or supplemented with varying glucose concentrations, and cell aliquots were assayed using imaging flow cytometry. We found that decreased glucose concentrations led to a corresponding decrease in the onset time of GS body formation **(Figure 1C,D)**, confirming glucose, or possibly cell energy level, dependence to GS body formation.

The count of GS bodies per cell also depends upon available glucose levels

In the above assays, the frequency of cells forming GS bodies clearly depended upon glucose levels. We further examined the count of GS bodies per cell to determine if the count varied on a cell-to-cell basis. Notably, the majority of cells transferred into glucose dropout medium formed one or two large GS bodies **(Figure 2)**, as, for example, can be seen in the cell images of **Figure 1A**.

However, when Gln1-GFP tagged yeast cells were subcultured into growth medium both lacking glucose and containing 2-deoxyglucose (2DG), a competitive inhibitor of hexokinase, many small foci formed per cell rather than one or two large foci, as commonly seen in glucose depletion alone **(Figures 1A, 2).** Thus, GS body population penetrance and count per cell show dependencies on available glucose and hexokinase activity, respectively. One possibility may be that GS bodies form initially as numerous small foci that must coalesce into one or two large foci; for example, one mechanism involving active transport is required to establish aggresomes (Johnston, Ward, and Kopito 1998; Muchowski et al. 2002). Alternatively, 2DG treatment may simply affect other steps required for large GS body formation.

Rapamycin treatment markedly reduces GS body formation

Because GS body formation increases with stress and decreases with metabolic activity, we speculated that rapamycin treatment might reduce GS body formation. Within cells, rapamycin binds to Frp1p, which in turn inhibits the TOR Complex 1 (TORC1)(Stan et al. 1994; Choi et al. 1996). Following TORC1 inhibition, cells behave as if starved, arresting division in G_0 (Cardenas et al. 1999) and releasing the repression of autophagy and the Msn2/Msn4-mediated stress response pathways (Kamada et al. 2000; Monteiro and Netto 2004). Rapamycin also decreases translation initiation and ribosome biogenesis, collectively decreasing the steady-state protein concentration inside cells (Martin, Soulard, and Hall 2004). These mechanisms thus

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directly affect cellular processes related to stress, aging, and aggregation in a manner that we might expect to reduce GS body formation.

In order to test this hypothesis, we grew Gln1-GFP tagged yeast in a range concentrations of the drug spanning 2 orders of magnitude for 24 hours. Using the imaging flow cytometry assay, we found a clear dose-dependent reduction in GS body formation in response to rapamycin treatment, with half maximal population penetrance of GS body formation occurring in response to treatment with approximately 1 nM rapamycin (**Figure 3B**). Only a small proportion of cells exhibited GS bodies upon treatment with 20 nM or higher rapamycin doses (e.g., **Figure 3A**). Notably, many specific cellular mechanisms might contribute to this reduction, including both the decreased cellular protein levels increased autophagy known to follow rapamycin treatment.

GS bodies form at higher rates in replicatively older cells

Many of the mechanisms that cells use to contain and clear protein aggregates are known to decline in efficacy with age across a variety of organisms. Chaperone expression decreases (Lund et al. 2002), proteasome activity diminishes (Tonoki et al. 2009), and chaperone-mediated autophagy slows with increasing cellular and organismal age (Cuervo and Dice 2000), among other trends. This phenomenon potentially contributes to the accumulation of aggregation in age-related diseases, such as plaques in Alzheimer's disease (Morimoto and Cuervo 2009). Recent work in nematodes has shown that widespread protein aggregation occurs normally in the course of aging (David et al. 2010). Given such associations between aging and protein aggregation, and given our observed reduction of GS body formation by rapamycin treatment, we therefore next asked if GS body formation correlated with cell age. Two easily assayable proxies of replicative age in yeast are an expansion of cell volume (Pichová, Vondráková, and Breitenbach 1997) and the accompanying accumulation of bud scars with each replicative cycle (Mortimer and Johnston 1959; Müller et al. 1980).

We observed a clear correlation between the frequency of GS body formation and measured cell diameter, as assayed using fluorescence microscopy (Pearson $r^2 = 0.78$; **Figure 4A**). To obtain an independent assay of cell size, we isolated the largest and smallest 5 percent of yeast cells using low-angle forward scattering fluorescence activated cell sorting (FACS), then imaged the cells by fluorescence microscopy, manually measuring cell diameter and scoring the occurrence of GS bodies. This assay confirmed the same general trend ($p \leq 10^{-4}$; **Figure 4B**).

Due to exponential growth, any mixed population of yeast cells is dominated by young cells, with old cells found only rarely. Thus, in order to directly estimate replicative age by measuring the accumulation of bud scars, we first enriched for yeast cells that had undergone more replicative budding cycles *via* the use of FACS on the basis of the fluorescent bud scar marker dye calcofluor-white. We then imaged the sorted cells using fluorescence microscopy and directly counted both bud scars and GS bodies per cell (e.g., as in **Figure 5A**).

The frequency of cells with GS bodies increased steadily with cell age, as measured by bud scar count, from a base rate of ~76% in unbudded cells and saturating at approx. 95% of the population among cells with 10 or more bud scars (**Figure 5B**). Moreover, cells with more bud scars were found to be more likely to contain a higher count of GS bodies per cell (though the correlation was not linear) with the largest increase in the mean number of GS bodies per cell occurring between cells with no bud scars and cells with one bud scar (**Figure 5C**). The difference was more marked when we narrowed the analysis strictly to pairs of mother and daughter cells that had yet to undergo cell separation. Mother cells were significantly more likely

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to contain GS bodies than their connected daughter cells, and also to exhibit higher counts of GS bodies per cell ($p \leq 10^{-10}$; **Figure 5D**), suggesting the possibility that mother cells might preferentially retain GS bodies, a hallmark of replicative aging in yeast cells for senescence factors (Aguilaniu et al. 2003)(Kaeberlein 2010).

CONCLUSIONS

In summary, we describe a high-throughput single cell assay for the formation of intracellular protein bodies by the metabolic enzyme glutamine synthetase. Using this assay, we demonstrate that reduced glucose availability or the addition of a hexokinase inhibitor both strongly induce GS body formation; the latter also increases the count and decreases the size of the GS bodies formed. The formation of GS bodies increased both with increasing cell age and cell size; in contrast, treatment with the drug rapamycin suppressed GS body formation. Thus, GS body formation depends strongly both upon available glucose levels and replicative cell age.

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FIGURE LEGENDS

Figure 1. Intracellular Gln1-GFP protein bodies form in a manner dependent on glucose availability.

(A) As assayed by fluorescence microscopy, Gln1-GFP tagged yeast strains in log phase growth exhibit visible foci (GS bodies) when starved for glucose either by removal of glucose from the growth medium or by addition of a non-metabolizable competitive inhibitor, 2-deoxyglucose. (B) Schematic view of GS body counting workflow. Approximately 50,000 individual yeast cells are imaged per sample on an Amnis ImageStream; cell images are then processed automatically to select in-focus, individual cells with measurable GFP fluorescence. Cells that pass these filters are analyzed for the intensity and size of the 30% intensity mask, which classifies cells into GS body and non-GS body containing populations. (C) Quantifying GS body formation over time across a range of range of glucose concentrations indicates that reducing glucose available in the growth medium significantly enhances the onset of GS body formation. (D) Plotting the concentration of glucose for a yeast culture against the time to 50% population penetrance reveals a marked correlation between available glucose and the rate of GS body formation.

Figure 2. Inhibition of glycolysis induces higher numbers of GS bodies per cell.

Removal of glucose from the growth medium slows and eventually halts glycolysis as residual glucose is depleted, and leads to strong induction of one or two visible foci per cell after 2h. Direct inhibition of glycolysis by the addition of a competitive inhibitor of glucose hexokinase, 2-deoxyglucose (2DG; added at 2%), induces formation of many small foci rather than one or two large foci, suggesting that the assembly of one or two large foci per cell may be an energy dependent process.

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Figure 3. Rapamycin treatment strongly reduces the population penetrance of GS bodies.

A) Widefield fluorescent microscopy images illustrating the marked decrease in GS body formation following rapamycin treatment. B) The proportions of cells exhibiting visible GS bodies were measured in an unbiased fashion by automatically imaging cells at high-throughput using imaging flow cytometry, identifying and quantifying the relevant cell subpopulations as shown. Rapamycin treatment induced a dose-dependent reduction in the proportions of cells with GS bodies across a concentration range of 1 to 200 nM.

Figure 4. GS body formation correlates with cell size, a proxy for cell age.

A) Increasing cell size is a proxy marker for age, and positively correlates with the likelihood of having GS bodies, as measured by fluorescence microscopy (Pearson r^2=0.78; n = 100 cells). B) GS bodies form at higher rates in larger cells, as assayed by fluorescence activated cell sorting (FACS) yeast cells by size and manually scoring GS bodies ($p \leq 10^{-4}$; n = 100 cells).

Figure 5. GS body formation increases with replicative age and occurs more frequently in mother cells than daughter cells.

A) Maximum intensity projection of a z-stack of widefield fluorescence microscopy images of calcofluor white stained Gln1-GFP expressing cells B) Cells with more bud scars show a higher incidence of GS bodies and C) have higher counts of GS bodies per cell (ANOVA, $p \leq 10^{-4}$), as measured for yeast cells FACS sorted on the basis of the fluorescent bud scar dye calcofluor white, manually counting the number of budscars and GS bodies per cell. Notably, the population penetrance of GS body incidence rises significantly with age. In contrast, the count of GS bodies per cell, which rises between unbudded cells and cells with 1 or more bud scars, shows no significant dependence on replicative age after the first budding. D) In order to directly quantify the formation of GS bodies in new buds relative to their mothers, we reanalyzed the

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549 data in (B) focusing only on the subset of cell images with mother and daughter cells still
550 connected, as diagrammed in the figure. Mother cells were more likely both to have GS bodies
551 and to exhibit multiple GS bodies than their own reproductively *naïve* daughters (Mann-Whitney
552 U test, $p \leq 10^{-10}$).

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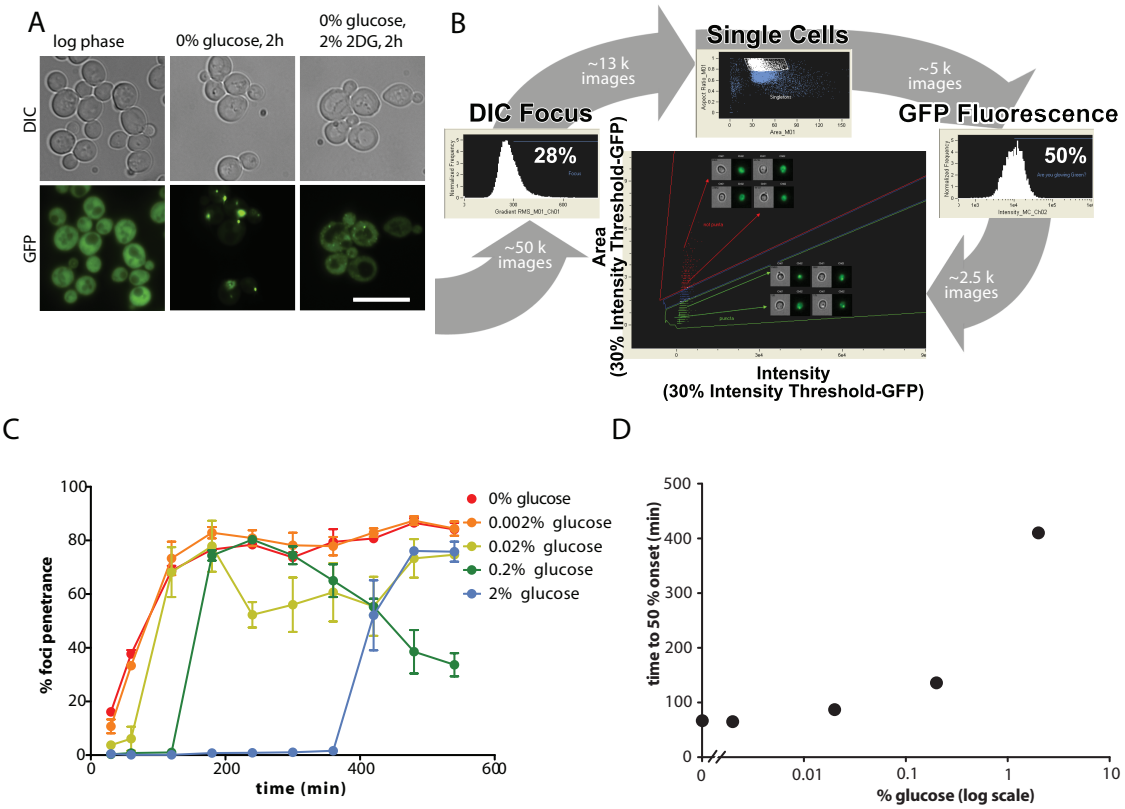

Figure 1

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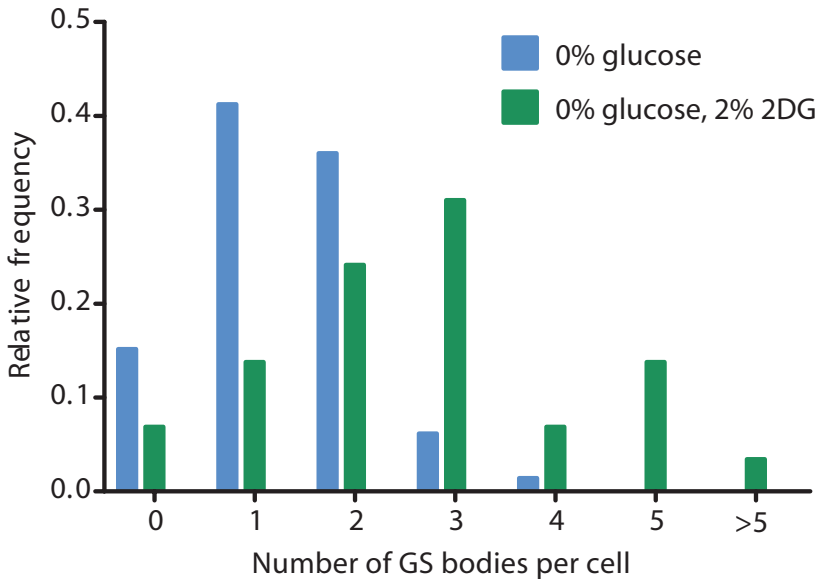

Figure 2

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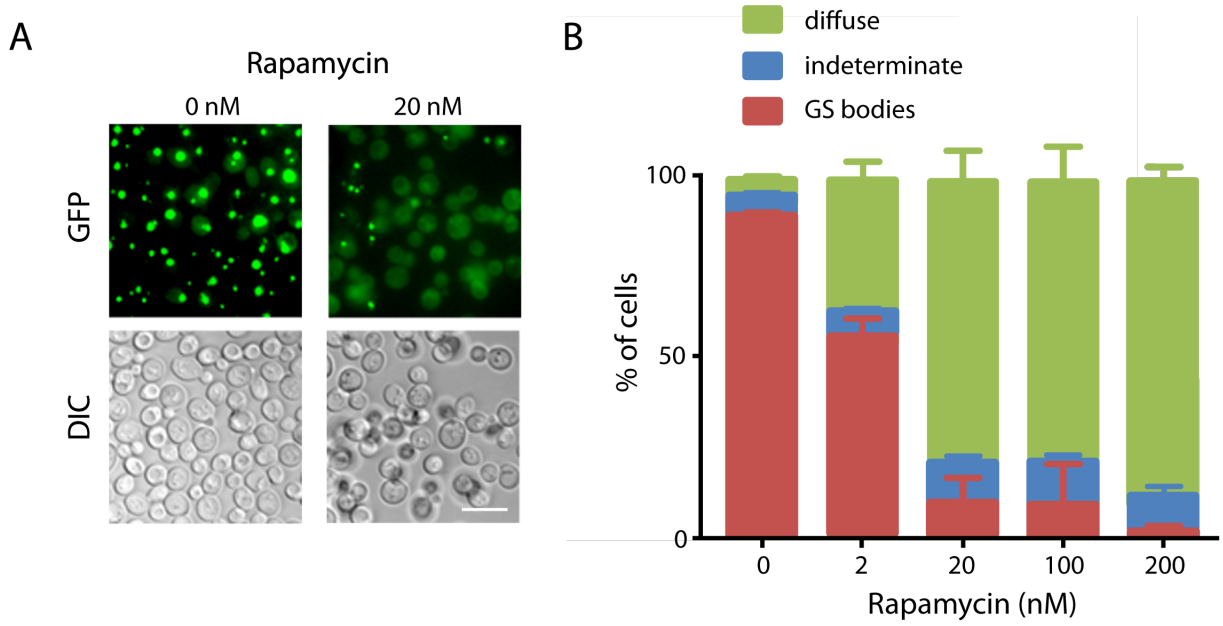

Figure 3.

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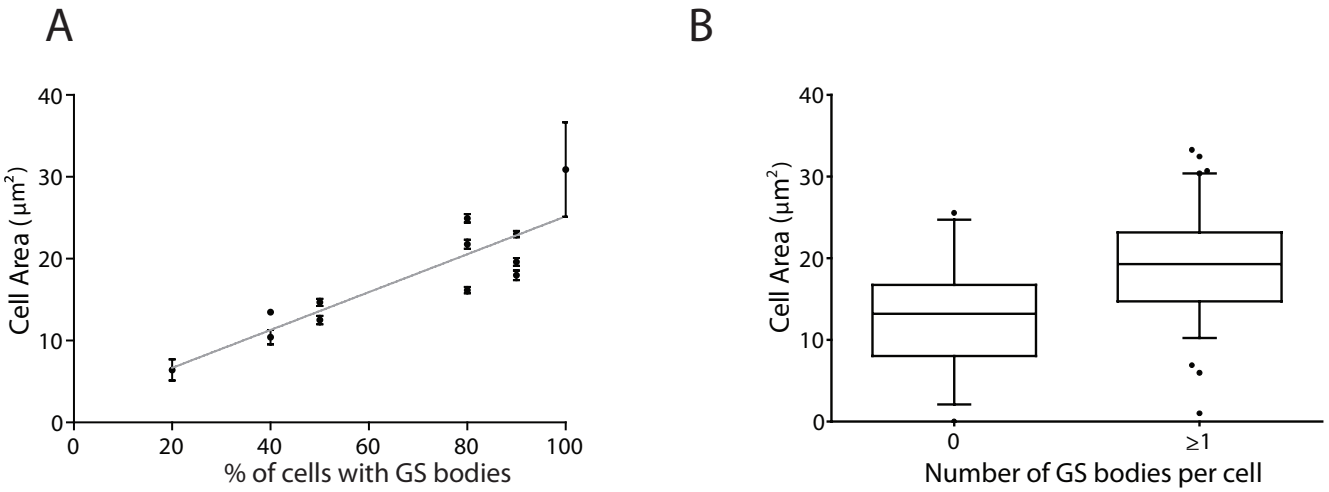

Figure 4.

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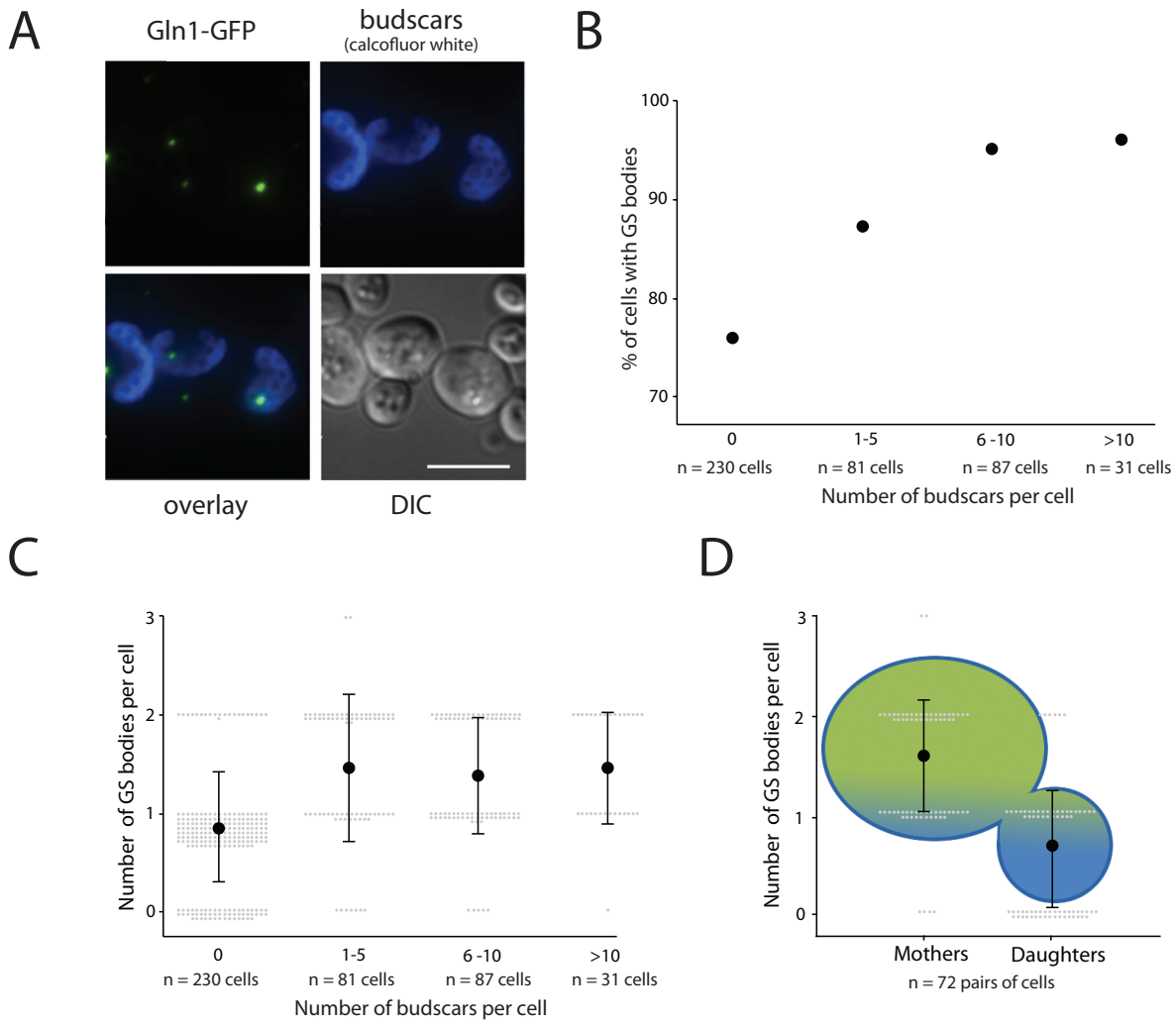

Figure 5.

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