Glutathione and Gts1p drive beneficial variability in the cadmium resistances of individual yeast cells

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Summary
Phenotypic heterogeneity among individual cells within isogenic populations is widely documented, but its consequences are not well understood. Here, cell-to-cell variation in the stress resistance of Saccharomyces cerevisiae, particularly to cadmium, was revealed to depend on the antioxidant glutathione. Heterogeneity was decreased strikingly in gsh1 mutants. Furthermore, cells sorted according to differing reduced-glutathione (GSH) contents exhibited differing stress resistances. The vacuolar GSH-conjugate pathway of detoxification was implicated in heterogeneous Cd resistance. Metabolic oscillations (ultradian rhythms) in yeast are known to modulate single-cell redox and GSH status. Gts1p stabilizes these oscillations and was found to be required for heterogeneous Cd and hydrogen-peroxide resistance, through the same pathway as Gsh1p. Expression of GTS1 from a constitutive tet-regulated promoter suppressed oscillations and heterogeneity in GSH content, and resulted in decreased variation in stress resistance. This enabled manipulation of the degree of gene expression noise in cultures. It was shown that cells expressing Gts1p heterogeneously had a competitive advantage over more-homogeneous cell populations (with the same mean Gts1p expression), under continuous and fluctuating stress conditions. The results establish a novel molecular mechanism for single-cell heterogeneity, and demonstrate experimentally fitness advantages that depend on deterministic variation in gene expression within cell populations.

Introduction
Individual genetically uniform cells within cell cultures exhibit marked phenotypic differences (i.e. heterogeneity), despite being of the same genotype. Such heterogeneity may be manifest in a wide range of phenotypes, many of which are fundamental to organism fitness and/or development. For example, individual cells of the same genotype may exhibit differing tendencies to differentiate and to express motility determinants, pathogenic cells may display variable degrees of virulence, and cells may have differing degrees of resistance to antimicrobial treatments and environmental stressors (reviewed in Avery, 2006). In addition, the principal control processes that regulate cell function (e.g. gene transcription, translation) at any moment in time may be differentially activated in different cells within genetically uniform populations. Recent studies have highlighted the contribution of stochasticity (noise) to such molecular-level variation (Kaern et al., 2005; Newman et al., 2006; Volfson et al., 2006; Struhl, 2007). Other potential drivers of heterogeneity involve epigenetic transitions in the state of the DNA molecule, and deterministic oscillatory changes in the physiological state of the cell (e.g. during the cell cycle) (Lloyd, 1993; Avery, 2006). There have now been a number of studies on gene expression noise in individual cells, and also on heterogeneity at the whole-cell phenotype level. However, very few studies have attempted to relate these aspects experimentally.

Exploiting the yeast model of heterogeneity, it was shown that cell cycle- and age-dependent regulation of the Cu,Zn-superoxide dismutase (Sod1) were key factors driving the variable Cu resistances of individual cells (Howlett and Avery, 1999; Sumner et al., 2003). It has now been established that Sod1p and other gene products can also act to suppress heterogeneity (in other phenotypes) among cells (Bishop et al., 2007). The latter study showed that enhanced phenotypic heterogeneity in certain mutants gave rise to increased rare-cell survival under severe stress. A specific advantage like this arising from heterogeneity touches on the other central question pertaining to heterogeneity: it has been hypothesized that cell populations benefit from phenotypic heterogeneity by the creation of subpopulations that could be better equipped to persist during perturbation and/or to exploit new niches (Tolker-Nielsen et al., 1998; Booth, 2002; Sumner and

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Avery, 2002). Consistent with such benefits, there is evidence of evolutionary selection for mechanisms generating phenotypic heterogeneity (True and Lindquist, 2000; Fraser et al., 2004; Raser and O’Shea, 2004). Furthermore, modelled simulations of competitions between heterogeneous and non-heterogeneous populations indicate that the heterogeneous populations could be more competitive, at least under certain conditions (Thattai and van Oudenaarden, 2004; Kussell and Leibler, 2005; Blake et al., 2006). Recently, Blake et al. (2006) also showed experimentally that phenotypic heterogeneity arising from the stochastic process of transcriptional bursting confers benefits. However, there remains a lack of such experimental evidence on benefits arising from heterogeneity, and this is particularly so for heterogeneity with a deterministic (i.e. non-stochastic) basis. An effective approach to addressing the latter central question would require an incisive means of manipulating the degree of deterministic heterogeneity expressed by cell populations. To date, this has been an elusive goal.

This study aimed to tackle both the questions of underlying molecular mechanism and of fitness consequences pertaining to heterogeneity. This required elucidation of a novel means to manipulate heterogeneity. Stress resistance provides a convenient binary output for measuring heterogeneity (Davey and Kell, 1996; Booth, 2002; Sumner and Avery, 2002). Heterogeneous resistance to oxidative stress is of particular interest, because of the well-documented role of reactive oxygen species (ROS) in disease aetiology (Halliwell and Gutteridge, 1999). Furthermore, ROS may be central to the modes of action of many stressors (Jamieson, 1998). Organisms have evolved enzymatic and non-enzymatic mechanisms for protection against oxidative stress. A major example of the latter is the essential metabolite glutathione, which provides reducing power for ROS scavenging (Wheeler and Grant, 2004). Glutathione also forms conjugates with xenobiotics such as cadmium for subsequent vacuolar detoxification (Li et al., 1997). Virtually all work to date on glutathione-dependent stress resistance phenotypes has relied on observations averaged across large numbers of cells, which mask effects at the single-cell level. However, it has been reported that the redox state of glutathione cycles in individual Saccharomyces cerevisiae cells during short-period, 40 min metabolic oscillations (ultradian rhythms) (Murray et al., 1999). Such oscillations become synchronized (and so recordable) during continuous yeast culture (Murray et al., 2003; Lloyd, 2006). Recent work has revealed that yeast metabolic oscillations are coupled to a periodicity in expression of different classes of genes and metabolites, extending across most of the genome and metabolome (Klevecz et al., 2004; Tu et al., 2005; Li and Klevecz, 2006; Murray et al., 2007). DNA synthesis in cells appears to be restricted to the reductive stage of the rhythms, which would limit the possibility of DNA damage from respiration-derived ROS (Chen et al., 2007). Furthermore, it has been reported that sensitivity to pro-oxidants such as menadione, H$_2$O$_2$ and cadmium fluctuates in continuous yeast cultures, with a similar periodicity as the ultradian oscillations (Wang et al., 2000; Tonozuka et al., 2001). In the present study, we observed marked cell-to-cell heterogeneity in glutathione-mediated stress resistance that was dependent on the GTS1 gene product. GTS1 transcription is known to modulate normal ultradian rhythmicity (Tonozuka et al., 2001; Adams et al., 2003). Therefore, manipulation of GTS1 transcription provided a means to manipulate the degree of heterogeneity in yeast cultures, and so test the hypothesis that deterministic heterogeneity confers a fitness advantage. Such an advantage was demonstrated under both continuous and fluctuating stress conditions.

**Results**

*Variation in GSH content determines phenotypic heterogeneity in cadmium resistance*

It was hypothesized that any variation in the cellular GSH contents of individual yeast cells could cause heterogeneity in GSH-dependent phenotypes. To test this, heterogeneity in a gsh1Δ-deletion strain (defective for the rate-limiting step of glutathione synthesis) was compared with that of wild-type cells. Heterogeneity was compared according to the relative gradients of dose–response curves, as described previously (Sumner et al., 2003; Bishop et al., 2007). Dose–response plots for the gsh1Δ mutant were shifted left relative to the wild type (Fig. 1). This indicated a culture-averaged sensitisation of gsh1Δ cells to Cd and H$_2$O$_2$, consistent with previous results (Wu and Moye-Rowley, 1994; Grant et al., 1996). Moreover, loss of Gsh1p was also associated with decreased cell-to-cell variability. Thus, loss of viability of mutant cells occurred over a narrower range of stressor doses (producing a steeper dose–response curve) than wild-type cells. An approximate 15-fold decline in viability of gsh1Δ cells (from 35% to 2.4%) resulted from only an ~11% increase in Cd concentration (from 90 to 100 μM), whereas a similar loss of viability in wild-type cells required a ~70% increase in Cd concentration (Fig. 1A). No viable gsh1Δ cells could be detected at > 100 μM Cd. This degree of homogeneity resulting from a single gene knockout [equating to a heterogeneity ratio (HR) value ~0.41; see Experimental procedures] was unprecedented in our experience, and was highly reproducible (further plots for wild-type and gsh1Δ cells are shown in Fig. 4B and D). GSH also contributed slightly to heterogeneous H$_2$O$_2$ resistance (Fig. 1B), but this effect (i.e. the difference in kill gradient for gsh1Δ versus wild-type cells) was considerably less marked than for Cd.
Glutathione-dependent variation

GSH-dependent Cd detoxification in yeast involves the formation of bis(glutathionato)cadmium (Cd-GS₂) adducts (Li et al., 1997). These adducts are transported to the vacuole by Ycf1p. To see whether vacuolar uptake of Cd-GS₂ (rather than adduct formation alone) was required to establish GSH-dependent heterogeneity, heterogeneous Cd resistance was also tested in a ycf1Δ mutant. Dose–response plots for the gsh1Δ and ycf1Δ mutants were superimposed (Fig. 1A). This indicated that the vacuolar detoxification pathway is the route through which heterogeneity is perpetrated. This was substantiated with a vac1Δ mutant which is defective for vacuole formation and maintenance. The vac1Δ mutant exhibited similarly decreased heterogeneity in Cd resistance (data not shown). As well as the Ycf1-mediated Cd detoxification mechanism, GSH also supplies reducing power for general antioxidant defence. This role is independent of Ycf1p. Accordingly, deletion of YCF1 influenced neither culture-averaged nor heterogeneous H₂O₂ resistance (Fig. 1B). Because GSH could affect heterogeneity independently of Ycf1p (in the case of H₂O₂), the data suggested that the relationship between Ycf1p and heterogeneity seen with Cd (Fig. 1A) was more likely to be a consequence of heterogeneous GSH rather than heterogeneous Ycf1p levels.

To confirm that the single-cell phenotypes were not inheritable (i.e. not genotypic), a number of ‘resistant’ colonies isolated after an initial exposure to the stressor on agar were subcultured to YPD broth in the absence of stressor. These were grown for 24 h in the broth before resistance was retested (non-heritable stress resistance is normally lost within 24 h of out-growth) (Bishop et al., 2007). Cadmium was selected for these tests because it yielded the strongest phenotype in gsh1Δ cells and because of its genotoxicity (Jin et al., 2003). This should enhance detection of any genotypic variation. The initial isolation of ‘resistant’ wild-type and gsh1Δ cells (via colony formation on Cd) was at Cd concentrations (see Fig. 2 legend) that gave either 100% viability (minus-Cd control), ~50% viability or ~10% viability. Resistant isolates subcultured from these plates (see above) were retested for resistance at each Cd concentration. Wild-type isolates that had not previously been exposed to Cd (‘control’ isolates) exhibited the anticipated ~50% and ~10% colony formation when tested at the relevant Cd concentrations (Fig. 2). With one exception out of six colonies tested, the same was true of cultures derived from Cd-resistant wild-type cells originally plated onto YPD supplemented with either Cd(NO₃)₂ (A) or H₂O₂ (B). Viability (colony formation) was determined after incubation for up to 8 days at 30 °C, and converted to percentages by reference to growth on unsupplemented control medium. The points represent means from three replicate determinations ± SEM. Typical results from one of at least three independent experiments are shown.

Fig. 1. Influence of Gsh1p and Ycf1p on heterogeneous resistance to Cd and H₂O₂. Exponential-phase cultures of S. cerevisiae BY4743 (○) or isogenic deletion strains gsh1Δ (□) or ycf1Δ (■) were plated onto YPD supplemented with either Cd(NO₃)₂ (A) or H₂O₂ (B). Viability (colony formation) was determined after incubation for up to 8 days at 30 °C, and converted to percentages by reference to growth on unsupplemented control medium. The points represent means from three replicate determinations ± SEM. Asterisks denote isolates exhibiting Cd resistance that is significantly higher (P < 0.05, according to Student’s t-test) at both tested Cd concentrations than for control cultures not previously exposed to Cd. Typical results from one of at least three independent experiments are shown.

Fig. 2. Single-cell Cd resistance in wild-type cells is non-heritable. Exponential-phase wild-type (BY4743) or gsh1Δ cells were originally plated onto YPD supplemented with Cd(NO₃)₂ concentrations that gave either 100% viability (minus-Cd control), ~50% viability (wild type, 150 μM Cd; gsh1Δ, 80 μM) or ~10% viability (wild type, 200 μM Cd; gsh1Δ, 100 μM); viability being defined as colony-forming ability. Single-colony isolates from these plates (which in the case of the Cd-supplemented plates were Cd-resistant colonies) were subsequently inoculated directly in to liquid YPD medium in the absence of Cd(NO₃)₂ and, after 24 h exponential growth, % resistance to Cd was retested by plating aliquots of each isolate onto YPD agar supplemented or not with Cd(NO₃)₂. Data are shown for eight wild-type and eight gsh1Δ isolates, each obtained from plates that originally yielded the % viabilities indicated, and each retested for % resistance at the Cd concentrations which normally give ~50% (□) or ~10% viability (■) of the relevant strains (see above for relevant concentrations). Percentage viabilities were calculated with reference to growth on minus-Cd control plates, and data are averaged from three replicate determinations ± SEM. Asterisks denote isolates exhibiting Cd resistance that is significantly higher (P < 0.05, according to Student’s t-test) at both tested Cd concentrations than for control cultures not previously exposed to Cd.
type colonies (‘50’ and ‘10’ isolates in Fig. 2). This indicated that these cells did not retain their resistances during the intervening growth period in the absence of Cd. Therefore, the variable Cd resistances of wild-type cells was primarily due to phenotypic rather than genotypic (inheritable) heterogeneity. In contrast, occasional Cd resistance in gsh1Δ cultures appeared to be primarily genotypic rather than phenotypic: five out of six resistant isolates exhibited inheritable Cd resistance (Fig. 2) (note that the higher Cd concentration gave closer to 0% than the normal 10% viability in some gsh1Δ retests, reflecting the difficulty of reproducing equivalent dosages in successive experiments where the kill gradient is very steep; Fig. 1A). The results indicated that much of the residual detected variability in gsh1Δ cells was probably of genotypic origin. Therefore, the relative gradients shown in Fig. 1A actually underestimate the true impact of GSH on phenotypic (non-genotypic) heterogeneity. It is concluded that elimination of GSH synthesis eliminates most of the non-genotypic heterogeneity in Cd resistance, i.e. there is no GSH-independent mechanism that makes a substantial contribution to this heterogeneity phenotype.

The GSH-specific fluorescent dye monochlorobimane (mBCI) (Stevenson et al., 2002) was used to substantiate that the above effects on heterogeneity were due to single-cell GSH content. The specificity of mBCI for GSH was confirmed by the severe decrease in fluorescence in gsh1Δ cells versus wild-type cells (Fig. 3A). The intensity of mBCI fluorescence in individual wild-type cells varied markedly, and some cells exhibited negligible discernible fluorescence (Fig. 3A). This heterogeneity in GSH content was reflected by the dispersion of mBCI fluorescences among wild-type cells when analysed by flow cytometry (Fig. 3B). Note that in contrast to certain other forms of heterogeneity (Smits et al., 2006), we found no evidence for bistability (distinct subpopulations) in mBCI fluorescences among cells. Our flow cytometry data indicated a graded phenotype, and this correlated with the continuous gradients seen in dose–response curves. To verify that single-cell resistance was related to single-cell GSH content, wild-type cells were sorted into subpopulations comprising cells with the lowest ~20% or highest ~80% GSH contents (mBCI fluorescences). The sorted subpopulations were then tested for Cd resistance at a range of Cd concentrations (Fig. 3C). Percentage resistance to Cd was up to 10-fold greater in high-GSH cells than in low-GSH cells. The results show that single-cell GSH content is a key determinant of single-cell Cd resistance. Furthermore, the gradients of the plots (Fig. 3C) indicated that the degree of heterogeneity in the low- and high-GSH subpopulations was decreased markedly compared with that in a total-cell population (HR ~0.57 and ~0.49 respectively). This substantiated that the extent of heterogeneity in GSH content of cell populations determines the extent of heterogeneity in Cd resistance.

It should be emphasized that the results shown in Fig. 3C are with cells sorted according to their ‘basal’ GSH contents, i.e. before any exposure to Cd. Therefore, the results demonstrate that it is this basal GSH content that determines single-cell Cd resistance. This supports the hypothesis (Sumner and Avery, 2002; Bishop et al., 2007) that it is the initial (pre-induction) state of cells on first contact with stressor that is the major determinant of heterogeneous resistance in this type of experiment. Indi-
GSH1 response (e.g. individual survivors may subsequently mount an adaptive response (e.g. GSH1 induction in response to Cd) which should confer longer-term resistance and continued growth in the presence of stressor (Sumner and Avery, 2002), but this response does not determine the initial heterogeneity.

Gts1p, required for normal ultradian oscillations, is important for GSH1-dependent heterogeneity

Levels of GSH are known to cycle during, and to help regulate, short-period metabolic oscillations in continuous cultures of S. cerevisiae (Murray et al., 1999). This could account for the cell-to-cell heterogeneity in GSH content. We confirmed such an oscillation among cells from the asynchronous batch populations used here. This involved sorting the high-GSH cells from cultures, to give a GSH-synchronized (homogeneous) subpopulation. Subsequently, changes in GSH content of these cells were monitored over time according to mBCl fluorescence. We confirmed such an oscillation among cells from the asynchronous batch populations used here. This involved sorting the high-GSH cells from cultures, to give a GSH-synchronized (homogeneous) subpopulation. Subsequently, changes in GSH content of these cells were monitored over time according to mBCl fluorescence. We observed a ~40 min oscillation in GSH content, which was sustained for at least two cycles before the waveform became dampened as the subpopulation became less synchronous (Fig. 4A). This ~40 min oscillation matched that observed elsewhere in oscillation-synchronized continuous cultures (e.g. Adams et al., 2003; Klevecz et al., 2004; Murray et al., 2007). The product of the GTS1 gene is required for generating normal oscillations (Wang et al., 2001; Adams et al., 2003; Xu and Tsurugi, 2007), and Gts1p also binds to Ycf1p (Kawabata et al., 1999). Therefore, it was hypothesized that the GSH/Ycf1p-dependent heterogeneity in Cd and H2O2 resistance described above could be established by the Gts1p-dependent oscillatory behaviour of individual cells. Consistent with this, cells of a gts1Δ mutant were less heterogeneous than wild-type cells in their resistances to Cd and H2O2 (Fig. 4B and C). This effect was strongest for Cd, particularly when it is taken into account that the wild-type strain starts to decrease in viability at a slightly lower Cd dose than the mutant, which accentuates the heterogeneity difference between the strains (Fig. 4B). In the case of Cd, the effect of GTS1 deletion on heterogeneity (HR ~0.69) was less marked than that of GSH1 (or YCF1) deletion (HR ~0.41; Fig. 1A). In contrast, these genes had equivalent effects on heterogeneity in H2O2 resistance (HR ~0.78 in both cases). Examination of heterogeneity in a gsh1Δ, gts1Δ double mutant revealed that the effects of GSH1 and GTS1 deletion on heterogeneous Cd resistance were not additive (Fig. 4D). This suggests that the gene products modulate heterogeneity via the same pathway.

\[ P_{GTS1} \text{-regulated GTS1 expression suppresses oscillations and heterogeneity in GSH content} \]

The influence of GTS1 on metabolic oscillations in S. cerevisiae is known to be regulated at the level of GTS1 transcription. Oscillations are markedly dampened when GTS1 is expressed under the control of a constitu-
tive promoter (Tonozuka et al., 2001; Wang et al., 2001). Consequently, we postulated that expressing GTS1 behind an alternative promoter could provide a means to manipulate the degree of heterogeneity in stress resistance within cultures. This approach would avoid GTS1 deletion, an important prerequisite for assigning a fitness effect specifically to heterogeneity (versus a culture-averaged change). For the same reason, we would need to express GTS1 at a culture-averaged level equivalent to that in the wild type (albeit with altered heterogeneity). Therefore, we replaced the genomic GTS1 promoter with the tet-regulated promoter system. This system gives constitutive gene expression at an averaged level that is scaleable with the concentration of doxycycline supplied (Belli et al., 1998a,b). To verify that P_{TE}GTS1 expression had the anticipated effects on heterogeneity, the constructed strain (P_{TE}GTS1) was stained with mBCl and analysed for cell-to-cell variation. The GSH contents of individual cells were more homogeneous in P_{TE}GTS1 cultures (and in gts1Δ cultures; not shown) than in the wild type, as reflected by the narrower range of cellular mBCl fluorescences (Fig. 5A). The derived coefficients of variation (CVs) for mBCl fluorescence were 114.5 and 70.2 for wild-type and P_{TE}GTS1 cultures, respectively. A comparable dampening of heterogeneity in the calculations of Drakulic et al. (2005), mean intracellular GSH was observed also in gts1Δ cells with a P_{TE}GTS1 construct integrated at the HO site (data not shown). This suggested that the observed effects on heterogeneity were not dependent on genomic locus.

Heterogeneous cell populations out-compete homogeneous cells during stress

To equalize mean Gts1p expression in wild-type and P_{TE}GTS1 cultures, GTS1 transcript levels were measured with quantitative real-time polymerase chain reaction (qRT-PCR) over a range of doxycycline concentrations (Fig. 6A). As expected, the levels of GTS1 mRNA in the P_{TE}GTS1-regulated strain decreased relative to those in wild-type cells with increasing doxycycline concentration. GTS1 expression was not affected by doxycycline in wild-type cells, consistent with observations elsewhere (Wishart et al., 2005). Doxycycline at 1.0 μg ml⁻¹ gave a GTS1 expression level that was just lower than that of wild-type cells (Fig. 6A), and no further repression of the P_{TE}GTS1 construct was observed at higher doxycycline concentrations. A concentration of 0.8 μg ml⁻¹ doxycycline was selected for further experiments. This gives equivalent averaged-expression of GTS1 in the two strains (Fig. 6A), but with altered consequences for heterogeneity (Fig. 5). We also employed Western blotting to confirm that the averaged level of the Gts1p protein was the same between the two strains at 0.8 μg ml⁻¹ doxycycline, in either the absence or presence of Cd(NO₃)₂ (Fig. 6B). Moreover,
GSH levels were also equalized between the strains, as described above (Fig. 5A).

Dose–response assays were used to test whether the effects of P\textsubscript{TET}\textsuperscript{GTS1} regulated GTS1 expression on variability in cellular GSH content (Fig. 5) resulted in effects on heterogeneous stress resistance. This prediction was borne out, as the P\textsubscript{TET}\textsuperscript{GTS1} strain exhibited decreased cell-to-cell heterogeneity (steeper dose–response plots) compared with the wild-type when tested over a range of Cd or H\textsubscript{2}O\textsubscript{2} concentrations (Fig. 6C). This result was reflected by comparison of the Cd resistances of sorted high/low-GSH subpopulations (see Fig. 3) with the P\textsubscript{TET}\textsuperscript{GTS1} strain. Whereas the wild-type subpopulations exhibited a >50% difference in survival with Cd (Fig. 3C), equivalent tests with the corresponding P\textsubscript{TET}\textsuperscript{GTS1} subpopulations yielded only a ~30% survival difference (data not shown). Another key observation with the dose–response curves (Fig. 6C) was that the P\textsubscript{TET}\textsuperscript{GTS1} plots were shifted left relative to the wild-type plots. This indicated culture-averaged sensitization to Cd and H\textsubscript{2}O\textsubscript{2} in the P\textsubscript{TET}\textsuperscript{GTS1} strain. Because culture-averaged GTS1 expression and Gts1p levels (Fig. 6A and B), as well as mean GSH content (Fig. 5), were similar in the two cultures, it could be inferred that the advantage in wild-type cells was attributable specifically to heterogeneity.

To test this inference more rigorously, the cell division rates of the P\textsubscript{TET}\textsuperscript{GTS1} and wild-type strains during exponential-phase growth in broth were determined over a range of Cd concentrations. The two strains exhibited very similar growth in the absence of Cd and at non-inhibitory Cd concentrations (<12.5 \mu M Cd). However, the inhibitory effect of higher Cd concentrations on relative division rate was more marked in P\textsubscript{TET}\textsuperscript{GTS1} than in wild-type cultures (Fig. 7A). Thus, at 75 \mu M Cd(NO\textsubscript{3})\textsubscript{2} the division rate of the wild type was still ~30% of that measured in the absence of Cd, whereas the corresponding determination for the P\textsubscript{TET}\textsuperscript{GTS1} strain was only ~11%.

Fig. 6. Homogeneous GTS1 expression causes homogeneous stress resistance.
A. GTS1 expression (culture-averaged) in exponential-phase P\textsubscript{TET}\textsuperscript{GTS1} cells was determined at varying doxycycline concentrations and plotted relative to GTS1 expression measured in wild-type (BY4743) cells. GTS1 mRNA in extracts was determined quantitatively by quantitative PCR in triplicate, with reference to ACT1 mRNA.
B. Western blotting was used to determine Gts1p levels in exponential-phase wild-type and P\textsubscript{TET}\textsuperscript{GTS1} cells, incubated at 0.8 \mu g ml\textsuperscript{-1} doxycycline in either the absence or presence of 12.5 or 37.5 \mu M Cd(NO\textsubscript{3})\textsubscript{2}. Each lane was loaded with protein extracted from 2 \times 10\textsuperscript{6} cells [the decrease in Gts1p levels in Cd-treated cells is consistent with Cd-dependent inhibition of protein synthesis, as reported elsewhere (Lafaye et al., 2005); Cd has no effect on Gts1p as a proportion of total cellular protein (Vido et al., 2001)]. The lower panel shows densitometry analyses of Gts1p in each lane. The first lane contains protein markers.
C. Exponential-phase cultures of wild-type (○) or P\textsubscript{TET}\textsuperscript{GTS1} strains (●) were plated onto YPD supplemented with Cd(NO\textsubscript{3})\textsubscript{2} or H\textsubscript{2}O\textsubscript{2} and 0.8 \mu g ml\textsuperscript{-1} doxycycline (to give equivalent culture-averaged GTS1 expression; see A). Viability (colony formation) was determined after up to 8 days incubation at 30°C, and converted to percentages by reference to growth on unsupplemented control medium. The points represent means from three replicate determinations ± SEM. Typical results from one of at least three independent experiments are shown.
Colony-forming units (cfus) were enumerated after 3 days incubation at 30°C; culture-averaged Gts1p expression did not differ significantly between the two strains (these analyses were with the strains growing separately under the same conditions as used in the main experiment, either in the absence or in the presence of Cd). Thus, out-competition of P_TETGTS1 cells by wild-type cells during stress can be linked to the enhanced phenotypic heterogeneity of the wild-type cells.

Discussion

The results presented here reveal a novel deterministic mechanism by which cells generate heterogeneity. In addition, that insight is exploited in demonstrating experimentally that such heterogeneity confers a fitness advantage in cell populations. It is of particular interest that heterogeneity was attributable to cell-to-cell variation in GSH content. Glutathione, an essential metabolite, affects a broad range of cellular phenotypes (Wheeler and Grant, 2004). The influence of GSH seems likely to extend also to heterogeneity in many of those phenotypes. However, interestingly, deletion of GSH1 was found previously to have no significant influence on heterogeneous resistance to copper (Sumner et al., 2003). That former study used a shorter-term assay of heterogeneity than the colony-growth assay used here, the latter offering higher reproducibility. Nonetheless, we have shown how the nature of the heterogeneity assay has little influence qualitatively on outcome, relative to the gene–phenotype combination being studied (Bishop et al., 2007). Therefore, this difference between the studies appears to relate...
to the different types of metals examined. Consequently, the heterogeneity effects described here with Cd and, to a lesser extent, H2O2 should not necessarily be presumed to extend to any of the stressors that are known to be potentially influenced by GSH (Wheeler and Grant, 2004).

Deletion of GSH1 almost eliminated heterogeneity in Cd resistance here. As a consequence, relatively small increments of increasing Cd concentration were required to be able to observe intermediate levels of killing (i.e. other than 0% or 100%) in gsh1Δ cells. The residual heterogeneity in gsh1Δ cells appeared to be of genotypic rather than non-genotypic origin, according to inheritability experiments. It cannot be discounted that there was also some epigenetic component to the inheritability (Xu et al., 2006), although the fact that the influence of GTS1 on heterogeneity was not affected by expression from an alternative genomic locus (HO) provides one argument against locus-dependent epigenetic regulation. Moreover, the degree of homogeneity accomplished here is unique in our experience. This underscores not only the importance of GSH in driving heterogeneous Cd resistance, but also the potential value of this system as a broader model of population homogeneity.

Although not redox active, like several other metals Cd is thought to have an oxidative mode of toxicity. Thus, Cd-induced lipid peroxidation causes membrane permeabilization and loss of viability, cellular thios become depleted during Cd exposure, and the cellular response to Cd stress has marked overlaps with that to oxidative (especially peroxide) stresses (Howlett and Avery, 1997; Lee et al., 1999; Avery, 2001; Avery et al., 2004). Nevertheless, GSH-dependent detoxification of Cd differs from that of H2O2, in that the latter involves vacuolar sequestration via Ycf1p (although Ycf1-mediated peroxide detoxification has been reported in glutaredoxin-overexpressing cells; Collinson et al., 2002). This difference in the action of GSH against Cd and H2O2 could explain why GSH1 deletion had a greater effect on heterogeneity in Cd resistance than in H2O2 resistance in this study, i.e. the GSH-Ycf1p pathway might make a more important contribution to phenotypic ‘noise’ than the GSH-dependent mechanisms involved in H2O2 detoxification (e.g. supply of reducing equivalents to peroxidases). In keeping with this suggestion, it is known that the Gts1 protein – which was shown here to help establish GSH-dependent heterogeneity, according to epistasis tests (Fig. 4D) and flow cytometry-based assays (Fig. 5) – additionally binds Ycf1p, with potential consequences for Ycf1 activity (Kawabata et al., 1999). If Ycf1p function as well as GSH content fluctuates according to single-cell Gts1p activity, this could serve to amplify ‘noise’ in the GSH-Ycf1p pathway of Cd resistance. Noise amplification mechanisms have been described in other biochemical systems (Samoilov et al., 2005). On the other hand, GSH1 (or YCF1) deletion had a greater effect on heterogeneity than GTS1 deletion in the case of Cd resistance, but not in the case of H2O2 resistance (see Figs 1 and 4).

One interpretation of this result is that the specific Gts1p–Ycf1p interaction, which is relevant only to Cd, could in fact help to stabilize heterogeneity. This alternative scenario would partly counterbalance the redox oscillation-associated heterogeneity within the GSH-Ycf1p pathway (also affected by Gts1p) (Murray et al., 1999), potentially offering a mechanism for fine-tuning the degree of culture heterogeneity. Further investigation of such possibilities will require a better understanding of the functional significance of the Gts1p–Ycf1p interaction (Kawabata et al., 1999), which has not yet been elucidated.

The role of Gts1p in the heterogeneity described here adds further insight to this protein’s function. Gts1p was originally considered a putative clock protein and essential for yeast metabolic oscillations (Mitsui et al., 1994; Wang et al., 2001). However, more recent work has indicated that oscillations can persist in gts1Δ mutants, albeit with shortened phase and decreased stability (Adams et al., 2003). Similarly, oscillations were dampened by Ptet-regulated constitutive expression of the GST1 open reading frame (ORF) in the present study. The collective evidence indicates that Gts1p function interfaces with the central oscillating loop and is a key regulator of ultradian rhythms in yeast (Jules et al., 2005; Xu and Tsurugi, 2007). These oscillations drive genome-wide fluctuations in gene expression (Klevecz et al., 2004; Tu et al., 2005; Li and Klevecz, 2006). However, their study to date has been restricted to continuous cultures or cell cycle-synchronized batch cultures, in which the oscillations become synchronized across cell populations. The single-cell and sorting assays exploited here have demonstrated the importance of Gts1p-dependent variation also within asynchronous batch cultures, and so offer the opportunity to extend study of these oscillations to such heterogeneous populations.

The influence of GTS1 on yeast metabolic oscillations is regulated at the level of GTS1 transcription (Tonozuka et al., 2001; Wang et al., 2001). This was exploited here to manipulate heterogeneity. Through the use of Ptet, we achieved our aim of maintaining culture-averaged GTS1 expression and GSH content while suppressing cell-to-cell heterogeneity (by approximately 40% according to relative CV values for population mBCl fluorescence). Thus, comparison of wild-type (heterogeneous) cells with Ptet:GTS1 (homogeneous) cells provided a novel model system for dissecting the impact specifically of heterogeneity on culture fitness. Such an impact became evident during stress, as the heterogeneous population out-competed the homogeneous population in three different experimental assays, despite identical growth rates under non-stress conditions. The latter observation indicates
that generation of heterogeneity has no metabolic cost at the population level, at least in the example studied here. The results provide direct experimental evidence to support the increasingly suggested hypothesis that phenotypic heterogeneity confers fitness advantages in cell populations during stress (Conrad, 1977; Tolker-Nielsen et al., 1998; Sumner and Avery, 2002; Thattai and van Oudenaarden, 2004; Kaern et al., 2005; Avery, 2006). It can be inferred that Gts1p-regulated redox oscillations in wild-type cultures generate cells with varying GSH contents, as suggested previously (Murray et al., 1999). Moreover, we show that the cells with higher than average GSH contents have an advantage in the face of \( \text{H}_2\text{O}_2 \) or Cd stress. Such cells could be critical for the persistence of the organism during stresses that kill large numbers of cells in a population.

The advantage of heterogeneity was evident here even during non-lethal (but growth-slowing) Cd stress (see Fig. 7A). These growth data indicated that the relationship between single-cell GSH content and Cd resistance is not linear: such a relationship would be expected to yield similar culture-averaged growth rates between populations under Cd stress when, as was the case here, culture-averaged GSH content is similar. Rather, the results are consistent with a threshold Gts1p or GSH level (achieved only in a fraction of wild-type cells, and fewer, if any, \( P_{\text{TET}} \text{GTS1} \) cells) above which Cd resistance is elevated markedly, leading to faster net population growth. Similar threshold effects on the outcomes from heterogeneity have been described elsewhere (Blake et al., 2006). The stress-specific advantage of wild-type cells was also marked in competitions under alternating Cd-stress and non-stress conditions. This is the type of situation in which phenotypic heterogeneity has been considered likely to be particularly advantageous (Thattai and van Oudenaarden, 2004; Kaern et al., 2005; Avery, 2006). However, our long-term agar growth assays reveal that intervening growth periods without stress are not a prerequisite for such advantages.

It should be emphasized that disadvantages of homogeneity may not be apparent when a less-heterogeneous subpopulation has an alternative advantage, such as enhanced mean GSH. This was illustrated in the sorting experiments where sorted high-GSH cells had higher Cd resistance than the total population, despite lower heterogeneity (Fig. 3C). Such effects can be controlled against experimentally by equalizing mean expression between test and control cultures, as we did here with the \( P_{\text{TET}} \text{GTS1} \) and wild-type strain comparisons.

In conclusion, recent microarray studies have led to the proposal that ultradian rhythmicity in yeast serves to separate temporally cellular processes that may be incompatible, such as respiration (with associated generation of ROS) and the restructuring of chromatin to enable DNA replication. The present study reveals another outcome of Gts1p-dependent rhythmicity: the generation of heterogeneity which is beneficial to cell populations. This demonstration of a benefit from deterministic variation among cells completes the picture initiated by a recent study of phenotypic variation that is of stochastic origin (Blake et al., 2006). Therefore, this work has elucidated a connection between short-period biological rhythmicity and cell individuality. It also provides new experimental evidence to help explain why these processes may have evolved in cell populations.

### Experimental procedures

#### General culture conditions

Yeast strains were maintained and grown in YPD broth [2% (w/v) bacteriological peptone (Oxoid), 1% yeast extract (Oxoid), 2% D-glucose] or in YNB medium [0.69% yeast nitrogen base without amino acids (Formedium), 2% (w/v) D-glucose] supplemented as required with amino acids or uracil (Ausubel et al., 2007). When selection was needed, either hygromycin B (Invitrogen) or G418 (Sigma) was added to media to a final concentration of 250 \( \mu \text{g} \text{ ml}^{-1} \). Where necessary, media were solidified with 2% (w/v) agar (Sigma). Experimental *S. cerevisiae* cultures were inoculated from overnight starter cultures grown from single colonies, and cultured to exponential phase (OD600 ~2.0) in liquid medium at 30°C, 120 r.p.m.

#### Yeast strains and DNA manipulations

*Saccharomyces cerevisiae* strains BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0), BY4742 (MATa; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0), BY4743 (MATaα; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0/lys2Δ0; LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0) and derivative single deletion mutants were obtained from Euroscarf (Frankfurt, Germany). A gts1Δ1, gsh1Δ1 double deletion strain in the BY4743 background was created after disruption of *GSH1* in gts1Δ1 single mutants from each of the haploid BY4741 and BY4742 backgrounds, using the *HphNT1* cassette (Janke et al., 2004) to replace the entire *GSH1* ORF by short flanking homology (SFH) PCR (Wach et al., 1997). Transformation was by the lithium acetate method (Gietz and Woods, 2002), and appropriate integration of the cassette was confirmed by diagnostic PCR (Wach et al., 1997). The haploid double mutants were mated by co-incubation overnight on YPD agar without selection, followed by two washes in sterile distilled water and incubation for 5 days on YNB agar supplemented with appropriate amino acids for selection.

To construct a cassette giving tet-regulated *GTS1* expression, plasmid pCM225 containing \( P_{\text{TET}} \) (consisting of KanMX4 as the selectable marker, the tetracycline-responsive tTA activator gene and the *tetO* promoter) (Belli et al., 1998a) was cut with PvuII and BglII, recessed DNA ends were filled with Klenow and ligated to produce plasmid pMS01. A 1.2 kb PCR fragment containing the *GTS1* ORF was amplified from yeast genomic DNA with addition of terminal BclI and Hpal sites,
and cloned between the BamHI and Hpal sites of pMS01 to yield plasmid pMS02 containing PTETGTS1. A 1 kb region upstream of the GTS1 ORF (and preceding the GTS1 start codon) was amplified using primers which incorporated terminal NotI and BstEII restriction sites and ligated between the corresponding sites in pMS02. The resulting plasmid, pMS06, was digested with NotI and SfiI to release a 6.2 kb cassette containing PTETGTS1 and targeted for integration into the genome at the native GTS1 locus via the cloned 1 kb upstream fragment and the GTS1 ORF. This PTETGTS1 cassette was transformed into BY4741 and BY4742 cells, G418-resistant transformants were screened for appropriate integration and haploid strains mated as described above to create the diploid PTETGTS1 strain. To produce an equivalent cassette targeted to the HO locus, pMS02 was cut with NotI and SapI and recessed ends filled with Klenow to generate a blunt KanMX4, PTETGTS1 fragment. This fragment was ligated into pBSTHO at the Klenow-filled EcoRI site, which lies in between ~500 bp regions cloned from the 5’ and 3’ ends of the S. cerevisiae HO ORF (Payne, 2006). The product, pMS04, was digested with NotI and AarI to generate a 6.4 kb PTETGTS1.HO cassette, which was transformed into BY4741 and BY4742 cells, in which the GTS1 ORF had previously been deleted by SFH PCR using the HphNT1 cassette, as described above. The resultant strains were mated to create a diploid PTETGTS1.HO strain. All DNA cloning and genetic manipulations were performed in Escherichia coli strain DH5α (Invitrogen). Restriction digests, DNA ligations, sequencing and PCR were carried using standard protocols (Ausubel et al., 2007). All primer sequences are available upon request.

**Dose–response curves**

Exponential-phase experimental cultures (see above) were diluted in fresh YPD, and cells (~200 per plate) were spread plated on YPD agar supplemented or not with the specified concentrations of Cd(NO₃)₂ or H₂O₂. All media for experiments involving PTETGTS1-regulated GTS1 expression additionally were supplemented with 0.8 µg ml⁻¹ doxychline. Colonies were enumerated after incubation at 30°C for up to 8 days, to allow for any slow growth under the stress conditions. Percentage resistances were calculated with reference to control incubations in the absence of stressor. HRs were calculated according to Schmitt et al. (1990). Residual DNA was removed using a NanoDrop spectrophotometer (NanoDrop Technologies) and integrity confirmed by formaldehyde agarose gel electrophoresis. Samples were snap-frozen in liquid nitrogen and stored at −20°C until use. Reverse transcription reactions were performed using Superscript III reverse transcriptase (Invitrogen) with 2 µg of RNA template per sample. Residual RNA was removed by treatment with E. coli RNase H (Invitrogen).

The relative abundance method was used to determine resultant cDNA levels by quantitative RT-PCR. Reactions (in triplicate) comprised 15 pmol each of gene-specific primers (HPLC purified, Sigma–Genosys), 2 µl cDNA template (10⁻¹ dilution), 12.5 µl of 2× QuantiTect SYBR Green PCR Master Mix, made up to 25 µl with RNase-free water. PCRs [95°C for 15 min (95°C for 30 s, 52°C for 30 s, 72°C for 30 s) for 40 cycles] were monitored using a MX4000 RT-PCR thermocycler (Stratagene). Initial template concentrations were calculated using the MX4000 software, and results were normalized using ACT1 as reference mRNA.

**Determination of GTS1 transcript levels**

RNA was extracted from cells using the hot acid phenol method (Schmitt et al., 1990). Residual DNA was removed by treatment with RNase-free DNase (Promega), and treated RNA was recovered using an RNeasy Mini kit (Qiagen). The absence of protein or DNA contamination was confirmed according to A₂₆₀/A₂₈₀ ratios and standard PCR tests respectively. RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies) and integrity confirmed by formaldehyde agarose gel electrophoresis. Samples were snap-frozen in liquid nitrogen and stored at −20°C until use. Reverse transcription reactions were performed using Superscript III reverse transcriptase (Invitrogen) with 2 µg of RNA template per sample. Residual RNA was removed by treatment with E. coli RNase H (Invitrogen).

The relative abundance method was used to determine resultant cDNA levels by quantitative RT-PCR. Reactions (in triplicate) comprised 15 pmol each of gene-specific primers (HPLC purified, Sigma–Genosys), 2 µl cDNA template (10⁻¹ dilution), 12.5 µl of 2× QuantiTect SYBR Green PCR Master Mix, made up to 25 µl with RNase-free water. PCRs [95°C for 15 min (95°C for 30 s, 52°C for 30 s, 72°C for 30 s) for 40 cycles] were monitored using a MX4000 RT-PCR thermocycler (Stratagene). Initial template concentrations were calculated using the MX4000 software, and results were normalized using ACT1 as reference mRNA.

For semiquantitative RT-PCR, 1 µl of 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴ dilutions of cDNA were used as templates in 50 µl PCRs [95°C for 5 min (95°C for 30 s, 52°C for 30 s, 72°C for 30 s) for 30 cycles; 72°C for 5 min] containing 50 pmol of each gene-specific primer. Red Hot Taq polymerase (AB Gene)
was used for amplifications, and products were examined by agarose gel electrophoresis. The intensities of products at template dilutions in which the reaction had not progressed to saturation were estimated with ImageJ software (National Institutes of Mental Health, Maryland, USA) using ACT1 as reference mRNA. All primers were designed using Primer 3 software (Rozen and Skaltsky, 2000).

Western blotting

Preparation of S. cerevisiae lysates and Western blotting were performed using standard procedures (Ausubel et al., 2007). Proteins isolated from 2 × 10^6 cells were separated by SDS-PAGE (10%) using an Invitrogen Nu-PAGE electrophoresis system, and blotted to PVDF membrane (Westram). Blots were probed with rabbit polyclonal anti-Gts1p primary antibody (a gift from Dr Kunio Tsurugi, Yamanashi Medical University) (1:2000 dilution), and alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody (Sigma) (1:3000 dilution). Gts1p was detected with BCIP-NBT (Sigma) and quantified by densitometry with a Quantity One system (Bio-Rad).

Flow cytometry, cell sorting and fluorescence microscopy

Samples of exponential-phase cells (2 × 10^7 cells) in YPD were stained with the reduced-glutathione (GSH)-specific dye monochlorobimane (mBCl; Molecular Probes) (Stevenson et al., 2002) at 100 μg ml⁻¹ for 10 or 15 min at 30°C. Cells were washed and resuspended in 2 ml PBS, briefly sonicated (Sanyo Soniprep 10 s, 2 μm), and analysed using a Coulter Epics Altra flow cytometer (Beckman) equipped with a UV laser. Data for fluorescence from mBCl via a 450 DF65 filter and samples were removed at intervals for staining with 

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