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Stratification of Yeast Cells during Chronological Aging by Size Points to the Role of Trehalose in Cell Vitality

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Abstract

Cells of the budding yeast *Saccharomyces cerevisiae* undergo a process akin to differentiation during prolonged culture without medium replenishment. Various methods have been used to separate and determine the potential role and fate of the different cell species. We have stratified chronologically-aged yeast cultures into cells of different sizes, using centrifugal elutriation, and characterized these subpopulations physiologically. We distinguish two extreme cell types, very small (XS) and very large (L) cells. L cells display higher viability based on two separate criteria. They respire much more actively, but produce lower levels of reactive oxygen species (ROS). L cells are capable of dividing, albeit slowly, giving rise to XS cells which do not divide. L cells are more resistant to osmotic stress and they have higher trehalose content, a storage carbohydrate often connected to stress resistance. Depletion of trehalose by deletion of *TPS2* does not affect the vital characteristics of L cells, but it improves some of these characteristics in XS cells. Therefore, we propose that the response of L and XS cells to the trehalose produced in the former differs in a way that lowers the vitality of the latter. We compare our XS- and L-fraction cell characteristics with those of cells isolated from stationary cultures by others based on density. This comparison suggests that the cells have some similarities but also differences that may prove useful in addressing whether it is the segregation or the response to trehalose that may play the predominant role in cell division from stationary culture.

Keywords

Chronological aging; yeast; trehalose; cell size; centrifugal elutriation

Introduction

The yeast *Saccharomyces cerevisiae* is a frequently used model organism in aging studies and has already helped to unravel a number of important phenomena, for instance the importance of nutrient sensing signaling pathways in regulation of aging (Longo et al. 2012). Yeast have been used to model various aspects of aging, one of which is chronological aging. In yeast, this is measured as the time of cell survival in stationary-phase liquid cultures (Fabrizio and Longo 2003). Stationary phase occurs after exponential and post-diauxic phase and requires metabolic reprogramming of cells. It was generally assumed that cells reaching this phase were in a uniform, non-dividing quiescent state until they eventually died (Burhans and Weinberger 2012).

More recently, a growing body of evidence shows that, on reaching stationary phase, yeast cells differentiate into several subpopulations with distinct physiology, whether grown in liquid culture or as colonies on solid medium (Allen et al. 2006; Palková et al. 2014). This finding has opened up a new field of study of unicellular yeast differentiation, which may help us to understand the development of higher-eukaryotic tissues. For instance, Cap et al. (2012) described metabolic similarities which subpopulations of multicellular yeast colonies share with tumor-affected metazoan tissues. Studies in liquid cultures resulted in isolation and characterization of two stationary-phase cell fractions, termed quiescent (Q) and non-quiescent (NQ) cells (Allen et al. 2006; Aragon et al. 2008; Davidson et al. 2011). Understanding of the entry of cells into and exit from quiescent state is relevant for stem cell and cancer biology.

In yeast liquid cultures, differentiation into Q and NQ cells was observed after glucose exhaustion from the media, and it correlated with accumulation of storage carbohydrates, glycogen and trehalose (Allen et al. 2006; Shi et al. 2010; Li et al. 2013). Trehalose is a carbohydrate that has various beneficial properties to yeasts. It is a stress-protectant. It also acts as a chemical chaperone and an osmolyte to minimize water loss under osmotic stress (Jain and Roy 2010), and it consequently has a role in aging (Kyryakov et al. 2012). Q cells have been reported to contain high levels of trehalose, which could explain their higher vitality comparing to NQ cells. However, Li et al. (2013) claim that trehalose is not solely responsible for attaining the quiescent characteristics of these cells.

In several studies, formation of a population of very small cells was observed after glucose depletion (Aragon et al. 2008; Murakami et al. 2012; Volejnikova et al. 2012; Li et al. 2013). Li et al. (2013) hypothesized that this population consisted of daughter cells which would eventually give rise to a Q-cell lineage. In our study, we aimed to examine the size differentiation observed in chronologically-aged yeast liquid cultures. We were able to divide the cultures according to cell size by means of centrifugal elutriation. We characterized the size-fractions obtained and found that differently sized cells show remarkably distinct physiology. The isolated subpopulation of small cells was less vital and more vulnerable to stress, while bigger cells unexpectedly showed properties similar to Q cells. We also were interested in addressing the question of what triggers yeast cells to differentiate. We found that trehalose, and more importantly its proper level, might indeed play a role in cells differentiation and aging.

Materials and methods

Strains, media, and growth conditions

Yeast strains were derived from *Saccharomyces cerevisiae* JC482 (*MAT a*, *ura3-52*, *leu2*, *his4-539*; from J. Cannon, University of Missouri) and BY4741 (*MATa*, *his3 1*, *leu2 0*, *met15 0*, *ura3 0*). The strains *hog1* and *tps2* in the BY4741 genetic background were obtained from EUROSCARF. The knock-out strains in JC482 genetic background were generated by PCR-based deletion using the *KanMX* module cassette (template plasmid pUG6) and appropriate primers according to the *Saccharomyces* Genome Deletion Project web page (http://www.sequence.stanford.edu/group/yeast_deletion_project/deletions3.html). The strains were grown in YPD medium containing 1% yeast extract, 2% peptone and 2% D-glucose (all w/v). 250 mL flasks containing 50 mL of medium were inoculated from an overnight culture to the final density of 7.5×10^5 cells mL⁻¹ fresh medium, and the cells were grown at 30°C on a rotary shaker at 160 rpm.

Fractionation of cell cultures

Cell fractions were isolated by centrifugal elutriation as described previously (Woldringh et al. 1995; Laun et al. 2001) with some modifications. Cells were harvested after 1 (18 hours), 4, 7, 10, 14 and 21 days of cultivation and were separated according to their diameter using the Beckman elutriation system and rotor JE-5.0 with a standard elutriation chamber. The cells were vortexed for 1 min, harvested at 3000 rpm at 4°C, and re-suspended in PBS buffer (phosphate buffer saline; pH 7.4, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄). The elutriation chamber was loaded with 20 mL of cell suspension corresponding to approximately 2×10^9 cells. To separate cell fractions with different diameters, the chamber was loaded at a flow rate of 11 mL min⁻¹ and a rotor speed of 3200 rpm. Cells of diameter under 2 µm were elutriated and not collected. Then, the flow rate was set to 19 mL min⁻¹ and rotor speed to 2700 rpm (fraction XS, diameter 2–4 µm), 2400 rpm (fraction S, diameter 4–5 µm), 2000 rpm (fraction M, diameter 5–6 µm), and 1350 rpm (fraction L, diameter 6 µm). To mechanically separate the small daughter cells adhering to mother cells in the L fraction, the L fraction was sonicated twice for 10 seconds with the lowest intensity on ice. They were loaded into an elutriator chamber spinning at 3200 rpm. The flow rate was 19 mL min⁻¹ and the rotor speed to 2700 rpm (fraction XS' – daughter cells), and 1350 rpm (fraction L' – mother cells).

Determination of cell concentration and cell-size distribution

The cell concentration and the cell-size distribution were measured (~20 000 cells, interval steps = 0.0375 µm) using a CASY TT[®] cell counter (Roche Diagnostics, Penzberg, Germany), which operates on the principle of a pulsed, low-voltage electric field. Additionally, the cell-size distribution profiles of aging cultures was assessed from the forward light-scatter data obtained by flow cytometry (BD LSR II, BD Biosciences, California, USA) and by direct microscopic measurement (Olympus IX81).

Cell viability assay

Two different assays were performed to evaluate cell viability, a clonogenic assay and propidium iodide (PI) staining. For the clonogenic assay, 100 μ L diluted samples of known total cell number and containing 100–200 viable cells were spread onto YPD (2% agar) plates and incubated at 30°C for 48 h; afterwards, the number of colony forming units (CFU) was determined. For PI staining, 10^6 cells were incubated with 0.3 mg mL⁻¹ PI for 10 min in the dark. The percentage of positive cells was assessed by flow cytometry (BD LSRII, emission filter 585/42 nm, excitation wavelength 488 nm). A total of 20000 cells per sample were evaluated using BD FACSDiva software (BD Biosciences, California, USA).

High-resolution respirometry

The cell respiration rate was measured using the Oroboros Oxygraph-2k respirometer, and the data were processed using DATLAB4 software (Oroboros Instrument, Innsbruck, Austria). Measurements were carried out on whole cells in two 2-mL chambers under physiological conditions (stirring speed 750 rpm, temperature 30°C) in YPD medium with a reduced concentration of glucose (0.1%). After baseline respiration was measured, doses of 2 μ L 1 M KCN were added to assess non-mitochondrial O₂ consumption. The respiration rate is expressed in pmol s⁻¹ (10^6 cells)⁻¹.

ROS measurement

The assessment of ROS content in cells was performed by a method described previously (Madeo et al. 1999) with minor modifications. Briefly, 5×10^6 cells were harvested by centrifugation. The pellets were washed in 1 mL PBS, re-suspended in 250 μ L of 2.5 μ g mL⁻¹ DHE (dihydroethidium, Sigma-Aldrich, Missouri, USA) in PBS, and incubated in the dark for 10 min. The percentage of positive cells was assessed by flow cytometry (BD LSRII, emission filter 585/42 nm, excitation wavelength 488 nm). A total of 20000 cells per sample were evaluated using BD FACS Diva software (BD Biosciences, California, USA).

Trehalose assay

The trehalose assay was performed according to Parrou and François (1997) with minor modifications. Briefly, cell samples (2×10^8 cells) were quickly harvested for 2 min at 3000 rpm in capped Eppendorf tubes. The pellets were washed in 1 mL ice-cold water and re-suspended in 0.25 mL of 0.25 M Na₂CO₃. Cell samples were incubated at 95–98°C for 4 hours and then 0.6 mL of 0.2 M sodium acetate and 0.15 mL of 1 M acetic acid were added to each sample. Half of each sample was incubated overnight with 0.025 U mL⁻¹ of trehalase (T-8778, Sigma-Aldrich, Missouri, USA) at 37°C, and the second aliquot of the suspension was used as a control. The next day, both suspensions were centrifuged for 3 min at full speed. 1 mL of glucose oxidase reagent (GLU GOD 250, PLIVA-Lachema, Brno, Czech Republic) was added to 20 μ L of the supernatant from each sample and incubated for 30 min at 37°C. Absorbance at 498 nm was determined to assess the quantity of glucose liberated from trehalose.

Heat stress resistance

One millilitre of the cell suspension was maintained at 50°C for 15 min, diluted and plated on YPD for assessment of CFU. The relative growth was determined as the ratio of CFU of heat-shocked and untreated cells.

Osmotic stress resistance

Cells from fraction XS, L, and the entire culture were inoculated to a final concentration of 7.5×10^5 cells mL⁻¹ into fresh YPD medium with 0.5 M NaCl. After 24 h at 30°C, the cell count was measured in each culture. The relative growth of cells was expressed as the ratio of the cell count in YPD containing NaCl to the cell count in YPD alone (Navarre and Goffeau 2000).

Statistical analysis

All experiments reported in this work have been performed at least three times (n = number of repetitions). The results are expressed as the mean ± standard deviation. Statistical analyses of the differences between groups were performed using one-way ANOVA or two-way ANOVA for multiple comparisons with Turkey's post hoc test and Student's t test or paired test when appropriate (GraphPad Prism version 6, GraphPad Software, California, USA). Values of p<0.05 were considered significant (*), p<0.01 very significant (**) and p<0.001 extremely significant (***).

Results

Chronologically-aged yeast cultures contain different-sized cells

Yeast chronological lifespan is a measure of culture viability in the stationary phase of cultivation. We observed that the size distribution profile of cultures changes with the increasing time the cells spend in commonly used YPD (2% glucose, 2% peptone, 1% yeast extract) medium. Post-diauxic and early stationary phase cultures have broader size distribution profiles compared to the profiles of actively dividing exponential cultures (Fig. 1a). This shows that smaller and larger cells are formed later during cultivation. Interestingly, the peak of size distribution shifts to smaller diameters by the end of the culture's chronological lifespan (day 21 in Fig. 1a), suggesting that the ratio of smaller cells prevails over that of larger ones.

This observation raised a question whether cell size is a parameter important for yeast in aging cultures. To answer the question, we divided chronologically aged yeast cultures (strain JC482) into different-size fractions by means of centrifugal elutriation. Centrifugal elutriation is a method which efficiently fractionates suspensions on the basis of particle sizes. We set the experimental conditions so as to obtain four cell-size fractions designated XS (diameter 2–4 µm), S (4–5 µm), M (5–6 µm), and L (6–8 µm). The cell sizes in the individual fractions were verified by microscopic observation and by flow cytometric analysis. Fig. 1b shows the actual cell-size distribution of the fractions (a representative experiment at day 7).

Size fractions contain both Q and NQ cells

Previously, Allen et al. (2006) reported the isolation of two stationary-phase cell fractions with distinct physiology, termed quiescent (Q) and non-quiescent cells (NQ). The subpopulations were obtained by density gradient centrifugation where the upper fraction (lower density) consisted of NQ cells and the lower fraction contained Q cells. To test whether the size-fractions isolated by our method of choice might correspond to either Q or NQ cells, we performed the density gradient centrifugation as described by Allen et al. (2006) with the XS and L fraction. Independently of cell size, every fraction formed upper and lower bands, containing Q and NQ subpopulations, and we, therefore, concluded that neither of the size fractions is comparable to Q and NQ. Consequently, these two methods of separation provide different subpopulations.

The fate of size fractions during cultivation

During cultivation the ratio between fractions, notably the XS and L fractions, changes. Fig. 1c shows the percentage of cells in individual size-fractions isolated during cultivation. It is clear that, except on day 1, the percentage of the XS fraction (cells with smallest diameter) increases with time, while the percentage of the L fraction (cells with largest diameter) gradually diminishes.

Moreover, cells in the largest size fraction were found to be budding ($77\pm5\%$, data not shown), while small cells were typically not ($6\pm1\%$, data not shown) (Fig. 1d, 1e). We hypothesized that cells in the L fraction were actively dividing even in stationary phase (under nutrition stress) and produced damaged daughters that eventually became prevalent in the culture. Therefore, the culture's demise might be caused by a decrease in the number of larger cells. To test this, we compared the characteristics of larger mother cells and their small daughters. Buds of L mother cells were removed by gentle sonication and isolated as the XS' fraction by an additional round of centrifugal elutriation. It is worth noting that sonication in our conditions did not harm the cells, as we did not observe any significant increase in propidium iodine (PI) stained cells. (Only damaged cells are permeable to PI). We found that cells in the XS' fraction were of lower quality comparing to the L mother cells, and showed characteristics similar to the cells commonly isolated in the XS fraction (see below). These results suggest that XS cells are indeed produced as lower quality daughters of the L cells.

Characterization of size fractions – small and large cells show remarkable differences in their physiology

In order to characterize the isolated size fractions, we assessed their viability, respiratory metabolism and stress resistance. As recommended in the review by Mirisola et al. (2014), the viability of the cultures was determined by two methods – clonogenic assay and staining with PI. Already on day 4, the CFU value of the XS fraction dropped to $49\pm17\%$ (Fig. 2a). Interestingly, the L fraction exhibited CFU values over 100% (up to 135% CFUs on day 4). This could be explained by the fact that, unlike XS cells, L fraction cells contain buds. The buds probably separated from these cells when they were plated, giving rise to individual colonies and adding to the final cell count. This fact might underestimate XS cell viability in the clonogenic assay. However, PI staining confirms the difference in the viability of these

two fractions, as the XS fraction showed approximately 30% less PI negative cells on days 4–10 compared to the L fraction (Fig. 2b). Consequently, both methods lead to the conclusion that cells in the XS fraction are significantly less viable than cells in the L fraction (Fig. 2a, 2b).

The cells in the XS fraction were not only less viable, but those in this fraction that were still alive took longer to properly adapt to a new environment. This was shown in a post-cultivation experiment, in which the XS and L fractions of a 7 day-old culture were transferred to fresh YPD medium (2% glucose). The XS cells were delayed by two hours in cell cycle re-initiation compared to the L fraction cells (Supplementary, A1).

To determine the condition of mitochondria, we monitored the respiration rate and the ROS production for the entire culture and for the isolated fractions. When the culture entered stationary phase, the respiration rate declined to almost non-measurable values by day 10 (data not shown). The L fraction contributed greatly to the overall respiration rate of the culture, since it reached the highest values on all specified days. On the contrary, the respiration in the XS fraction was negligible. Already on day 1, this fraction showed a four-fold decrease compared to the L cells (Fig. 2c)

Production of ROS was assessed by staining with dihydroethidium (DHE). With extended cultivation time, the amount of DHE positive cells gradually increased (Supplementary, A2). The XS fraction showed the highest percentage of DHE positive cells in post-diauxic and early stationary phase. For example, on day 10 the XS fraction exhibited $54 \pm 13\%$ DHE positive cells, while the percentage of DHE positive cells in the other fractions was about 30% (Fig. 2d). It is possible that the higher amounts of ROS in XS cells cause impaired mitochondrial function which results in a very low respiration rate. In contrast, the L fraction cells seem to have the most coupled mitochondria. On day 4, they showed a very high respiration rate, yet the lowest percentage of DHE positive cells. Interestingly, on day 14 there is a shift in the trend. The difference between the L and XS cells is reduced in the ROS production and also in the viability assessed by PI staining (Fig. 2b, 2d), which may be a sign of the imminent demise of the entire culture.

To examine whether the less vital XS fraction is also more vulnerable to stress conditions than the “fitter” L fraction, we applied heat shock and osmotic stress (see Experimental procedures). Both fractions could withstand the heat shock stress (Supplementary, A3), but there was a significant difference in their ability to resist osmotic stress. The XS cells showed a four-fold decrease in growth under osmotic stress conditions compared to the L cells (Fig. 3a).

The osmotic stress resistance experiments lead to the idea that XS and L cells might differ in the activity of stress response pathways. The HOG pathway controls the osmotic regulation in cells (Schüller et al. 1994). To test its involvement in the XS and L cells stress response, we constructed a knock-out strain *hog1⁻*, missing the MAP kinase Hog1. However, the deletion did not influence our strain, (nor strain BY4741), in any of the studied parameters (data not shown). Based on all the results, we conclude that the cells of XS and L fractions

are two distinct subpopulations. They differ not only in their morphology (size), but especially in their physiology. Therefore, they may play different roles in the aging cultures.

The role of trehalose in cell fitness

An important stress response pathway involved in aging is the trehalose pathway. Shi et al. (2010) showed that upon exit from the quiescent state, trehalose serves as a preferential energy source; and cells lacking trehalose start growing more slowly and show poor survival. Indeed, we found that the content of trehalose in the XS fraction, which showed a growth delay in the post-cultivation experiment and poor viability, was six-times lower than in the L fraction (Fig. 3b, days 4–10).

To confirm the role of trehalose, we interrupted the trehalose biosynthetic pathway by deletion of the *TPS2* gene coding for trehalose-6-phosphate phosphatase. Tps2p is a subunit of the trehalose-6-phosphate synthase/phosphatase complex and is involved in the last reaction of trehalose synthesis (for review Eleutherio et al. 2014). The deletion of *TPS2* resulted in decreased trehalose levels, but the trehalose was not completely depleted (Fig. 4a). Similar to the observation of Shi et al. (2010), we believe that cells acquire the trehalose from the yeast extract component of YPD medium.

We hypothesized that L cells lacking trehalose would gain the characteristics of XS cells, including impaired mitochondrial function, higher ROS production and lower viability. The deletion of *TPS2* resulted in little or no significant differences between the XS and L size fractions in cell viability and ROS production (Fig. 4b, 4c, 4d). Interestingly, the L fraction cells did not lose their vitality, instead the XS fraction cells improve their vitality. The L-fraction *tps2* cells showed unchanged values in the ROS amounts and viability compared to the wild-type L-fraction cells (Fig. 5b, 5d, 5f, 5h). Quite unexpectedly, the XS-fraction *tps2* cells improved in these characteristics (Fig. 5a, 5c, 5e, 5g).

Discussion

Over the past years, evidence has been accumulating in support of the idea that unicellular yeast are able to differentiate into specialized cell types and could therefore serve as a primitive model of developing metazoan tissues (for review Palková et al. 2014). In the present study, we approached the differentiation of chronologically aged yeast liquid cultures from the perspective of cell size, as we observed that the size distribution profiles of cultures changed during cultivation. After leaving exponential phase after day 3 of cultivation, cells became morphologically heterogeneous; populations of smaller and larger cells formed. Presence of very small cells in older cultures was also documented in several other studies (Murakami et al. 2012; Aragon et al. 2008; Volejnikova et al. 2013; Li et al. 2013), but their provenance and importance are still not fully understood. We separated chronologically-aged liquid cultures according to cell size by centrifugal elutriation and identified and characterized the two most distinct cell subpopulations – small cells (2–4 µm in diameter), isolated in the XS fraction, and significantly larger cells (6 µm in diameter) found in the L fraction.

The differentiation of yeast into specialized cell types was previously described in giant colonies grown on solid media (Cap et al. 2012) and in stationary liquid cultures (Allen et al. 2006). Cap et al. (2012) identified two subpopulations occupying specific positions within a colony, U cells located in the upper layer and L cells in the lower layer. The subpopulations were characterized morphologically and physiologically, and their representative characteristics can be found in the Tab. 1. In this model, U cells have properties that predetermine them for long-term survival, while L cells are less viable and are thought to provide a source of nutrients for cells in the upper layer. The first study that shed light on stationary phase liquid cultures was done by Allen et al. (2006). They isolated subpopulations formed after glucose exhaustion termed quiescent Q and non-quiescent NQ, whose characteristics are also listed in Tab. 1. Q cells are more vital, and they are arrested in G_0 . Therefore, they are suitable for studies of the quiescent state. On the other hand, NQ cells are a less viable and a less healthy heterogeneous subpopulation.

The subpopulations of cells we identified, XS and L cells, resemble stationary phase NQ and Q cells, respectively, in most studied characteristics. Less similarity is found with populations of U and L cells on solid media, although the main idea of differentiation into more vital and less vital subpopulations is conserved. This is consistent with the findings of Cap et al. (2009) that the physiology of yeast grown on solid media and liquid cultures is different. Consequently, we tested whether our fractions obtained by centrifugal elutriation are indeed Q and NQ subpopulations isolated by density gradient centrifugation and found that our method of separation leads to different fractions. Centrifugal separation XS and L cells is primarily based on size, while density is the most important factor for the separation of Q and NQ cells.

When one compares isolated subpopulations in our study and NQ and Q cells in the study by Allen et al. (2006), the most striking difference is seen in the ability of cells to divide. While their vital fraction, Q cells, is quiescent and consists of 91 % unbudded daughter cells, our vital L fraction is heterogeneous in terms of cell cycle stage. This differentiates the model proposed by Davidson et al. (2011) and our model. From their proteomics studies, Davidson et al. (2011) suggest that the differentiation into cell types must occur before exhaustion of glucose, during exponential phase. After that, differentiated cells produce daughters of their own type; the Q mother cells give rise to Q daughter cells, NQ cells to NQ daughters. However, they propose that most Q mothers eventually become NQ. They hypothesized that due to their properties, NQ cells serve as long-term nutrient storage reserves for Q cells.

From our results, we conclude that the vital L fraction consists of slowly dividing cells even in the absence of nutrients, therefore primarily consisting of mother cells. Our results do not provide evidence that a given cell type gives rise to the same cell type. The L-fraction mother cells under starvation conditions give rise to less healthy daughters (isolated in the XS fraction), which lose their capacity to reproduce and ability to respond to the environment by proper activation of stress responses. In the XS fraction, there are approximately only 30% of cells which can proliferate in nutrient rich conditions (Fig. 2a). We assume that in nutrient-deprived media this percentage will be even lower. Our results contrast with those of Li et al. (2013), which suggest that small cells generated after diauxic shift contribute to the generation of the Q-cell population.

It is puzzling, that cells isolated in the XS fraction gradually predominate in the older cultures, while the percentage of cells isolated in the L fraction decrease (Fig. 1c). The possible explanation is that the L cells are dividing, producing and raising the percentage of daughter XS cells, and at the same time they themselves die out. The L cells are the most vital subpopulation, but this is valid only until day 14 of cultivation. After that, there is a decrease in their quality, and the differences between the fractions become less evident. Increase in the XS subpopulation and decrease in the quantity and quality of the L subpopulation eventually lead to culture extinction.

The interesting question is how cells decide their fate. In chronological aging, the important prerequisite for cell survival is to respond properly to nutrient deprivation through nutrient-sensing pathways like TOR and RAS/PKA pathways (Fabrizio and Longo 2003, Wei et al. 2013, Georgieva et al. 2015). For example, cells unable to down-regulate RAS/PKA pathway due to *RAS2^{val19}* mutation fail to activate stress responses and to gain the properties of stationary phase cells (Hlavata et al. 2003). Cells which properly sense the diminishing concentration of available nutrients start to store reserve carbohydrates, glycogen and trehalose. Trehalose was described not only as an energy reserve molecule, but also as a stress-protectant and anti-aging molecule (Jain and Roy 2010; Kyryakov et al. 2012). Moreover, trehalose enables cells to exit quiescence more readily (Shi et al. 2010).

We observed a dramatic difference in the ability to store trehalose between XS and L cells. Therefore, we assume that XS cells, which are unable to store trehalose, might be defective in the adaptation to stress conditions, which explains their poor vitality. Trehalose is formed while cells are undergoing the shift from fermentative to respiratory metabolism (Futcher 2006), and it was shown that the trehalose pathway regulates mitochondrial respiratory chain content (Noubhani et al. 2009, López-Lluch et al. 2015). Since XS cells have very low respiration capacity, it points to defects in trehalose metabolism.

We hypothesized that higher levels of trehalose are the reason why L cells are so vital, which correlates with the fact that L cells start to lose their quality once the trehalose content decreases to a certain level (Fig 3b, day 14). However, deletion of the trehalose-6-phosphatase gene, *TPS2*, which results in a significant decrease in cellular trehalose, did not affect the L cells in measured parameters. Unexpectedly, we observed that XS *tps2* cells, which were not supposed to be affected by *tps2* deletion since they already did not depend on trehalose reserve, improved in their characteristics. They showed higher viability and lower ROS production, compared to the wild type XS cells. Recently, Kyryakov et al. (2012) reported that during aging under caloric restriction conditions an appropriate concentration of trehalose must be present in cells to ensure the beneficial effects, because trehalose can also operate as a pro-aging compound. In chronologically-aged cells, chaperones promote refolding of aberrantly folded proteins. Trehalose is believed to interfere with this process by shielding hydrophobic amino acid residues commonly present in misfolded proteins.

Taking the pro-aging effect of trehalose into account, we propose that the daughter XS cells are indirectly influenced by the trehalose content of their mothers (Fig. 6). L mother cells benefit from trehalose stores at least for the first 14 days of cultivation. They are viable,

respiratory competent, able to readily reproduce when fresh media is provided, and have low levels of ROS. However, the daughters they produce are quite damaged. When the levels of trehalose are significantly decreased by *tps2*⁻, the L cells give rise to healthier daughters. If trehalose interferes with aberrant protein refolding, it might in fact lead mother cells to segregate the damaged proteins into daughters. There is a well-documented concept of asymmetrical inheritance in replicative aging, where an aged mother during cell division selectively keeps damaged molecules to itself while generating a rejuvenated daughter cell (Lai et al. 2002; Jazwinski 2005; Henderson and Gottschling 2008; Zdrag-Tecza et al. 2009; Klinger et al. 2010, Nystrom and Liu 2014, Lewinska et al. 2014). In contrast, in chronological aging, this asymmetry is apparently reversed, as our results show.

Our results add to the body of literature indicating the remarkable heterogeneity of slowly dividing yeast cell cultures. Various methods have been applied to fractionate cells into different subpopulations in these cultures. The subpopulations thus obtained often share certain characteristics; however, they also differ. An example of this is the similarity of our XS- and L-fraction to the NQ and Q cells obtained by others, which stops at the level of continued budding. Our L-cells bud continuously, albeit slowly, unlike Q cells which remain dormant but primed to resume growth. We do not know the source of this difference. However, we provide evidence that XS and L cells are distinguished by the larger trehalose content of the latter. The trehalose makes its way into the buds produced by L cells, resulting in a deleterious effect on the XS cells to which they give rise, a conclusion based on the effects of the deletion of *TPS2*. Curiously, it is the Q cells that have the higher trehalose content, compared to NQ cells. This is consistent with their higher vitality, and it is comparable to the higher vitality of L cells compared to XS cells. Here the similarity stops. Somehow, L cells tolerate the trehalose, but their XS progeny do not. We suggest that the trehalose inherited by the latter cells is detrimental. This leads to the question of why this is the case. The answer to this question may come from further comparison of XS/L and NQ/Q cell pairs. It could be due to differences in segregation of trehalose in these cell pairs or to differences in the response to this carbohydrate. Our further research will be directed towards the elucidation of these differences.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Cell-size heterogeneity of yeast cells during a 21-day cultivation. **a** Representative cell-size distribution profiles on days 1, 10, and 21. **b** Four subpopulations isolated from the culture by centrifugal elutriation based on cell size. Histograms show subpopulations of different diameters which were designated XS fraction (smallest cells), S fraction, M fraction, and L fraction (largest cells) from day 7 of culture in a representative experiment. **c** Percentage of cells in the individual fractions obtained by centrifugal elutriation on selected days (n = 6–10). Nomarski microscopic image of **d** XS and **e** L fraction on day 7. Scale bar, 20 μm . The data are mean $\pm$ SD.

Fig. 2.

Reproductive capacity, viability, and mitochondrial function of wild type strain. **a** CFU and **b** PI staining of the individual fractions on selected days. **c** Basal respiration rate as an assay of mitochondrial activity was measured in the fractions on selected days by high-resolution respirometry (n = 3–6). **d** ROS production was determined in the individual fractions on selected days (n = 4–7) by flow cytometry using staining with DHE. The data are mean ± SD.

Fig. 3.

Resistance to osmotic stress and trehalose content of isolated fractions of wild type strain. **a** Analysis of osmotic stress resistance of cells in the XS and L fractions on selected days was performed by comparison of final cell concentration after 24 hours growth in YPD medium with 0.5 M NaCl and in YPD alone as control ($n = 3$). **b** Trehalose content of the individual fractions was determined as the conversion of trehalose to glucose on selected days ($n = 3-5$). The data are mean $\pm$ SD.

Fig. 4. Reproductive capacity, viability, ROS production, and trehalose content of *tps2Δ*. **a** Comparison of trehalose content of wild type and *tps2Δ* of the entire culture on selected days determined as glucose release **b** CFU of *tps2Δ* deletion, **c** PI staining, and **d** ROS production of the individual fractions of *tps2Δ* on selected days. The data (n = 3–6) are mean ± SD.

Fig. 5.

Comparison of wild type and *tps2* of XS (a, c, e, g) and L (b, d, f, h) fraction; a, b PI staining, c, d CFU, e, f ROS production, and g, h trehalose content determined as glucose release. The data (n = 3–8) are mean ± SD.

Chronologically aged cells

Fig. 6.

Involvement of trehalose in yeast chronological aging. Hypothesis: Pro-aging effect of trehalose influences daughter cells in later days of cultivation. Mother cells containing higher level of trehalose give rise to damaged daughter cells while *tps2*-deleted mother cells produce healthier daughter cells (after day 4 to day 21). Trehalose might influence proteostasis and protein segregation to daughter cells. Trehalose content is depicted as blue circles.

Tab. 1

Comparison of differentiated yeast fractions during chronological aging.

Giant colonies Cap et al. (2012)		Stationary liquid culture cells Allen et al. (2006), Aragon et al. (2008), Davidson et al. (2011), Shi et al. (2010)		Stationary liquid culture cells This study	
Cell type/Characteristics	U cells	L cells	Q cells	NQ cells	XS cells
Vitality	Vital	Less vital	Vital	Less vital	Less vital
Osmotic stress resistance	N/A	N/A	N/A	N/A	Lower
Trehalose content	N/A	N/A	Higher*	Lower*	Lower
Oxygen consumption	Lower	Higher	Higher	Lower	Lower
ROS production	Lower	Higher	Lower	Higher	Higher
Budding	N/A	N/A	Unbudded	Heterogeneous	Heterogeneous
					Unbudded

N/A means not analysed.

* Measured during continuous cultivations/yeast metabolic cycles (Shi et al. 2010)