Reversible solidification of fission yeast cytoplasm after prolonged nutrient starvation

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Summery statement: After prolonged quiescence, fission yeast cell populations switch into a state exhibiting profound immobilisation of sub-cellular components that differs from the cytoplasmic immobilisation states induced by acute energy depletion.
Abstract

Cells depend on a highly ordered organization of their content and they must develop strategies to maintain the anisotropic distribution of organelles during periods of nutrient shortage. One of these strategies is to solidify the cytoplasm, which was observed in bacteria, and in yeast cells with acutely interrupted energy production. Here, we describe a different type of cytoplasm solidification that fission yeast cells switch to, after having run out of nutrients during multiple days of culturing. It provides the most profound reversible cytoplasmic solidification of yeast cells described to date. Our data exclude the previously proposed mechanisms for cytoplasm solidification in yeasts and suggest a mechanism that immobilizes cellular components in a size-dependent manner. We provide experimental evidence that in addition to time, cells use intrinsic nutrients and energy sources to produce this state. Such cytoplasmic solidification may provide a robust means to protect cellular architecture in dormant cells.

Introduction

Cell function and survival require a highly ordered, cell type-specific organization of the cytoplasmic content. How cells generate and maintain a cell type-specific spatial organization is central to understanding the living state. It involves the asymmetric distribution of material, mediated by active transport of key regulatory components, which counteracts the entropic activity of diffusion. This occurs in a highly crowded and dynamic environment, with spatially dispersed constituents ranging in size from small ions and metabolites to macromolecular complexes, and to large, complex structures like the cytoskeletal networks and organelles. Their sub-diffusive motion is influenced by macromolecular crowding, specific and unspecific interactions, and the polymer networks of the cytoskeleton (Luby-Phelps et al., 1986; Tolić-Nørrelykke et al., 2004; Weiss et al., 2004; Wirtz, 2009). Consequently, the cytoplasm was described as a complex viscoelastic fluid, a gel-like material, or a colloidal liquid at the transition to a glass-like state (Fels et al., 2009; Grygorczyk et al., 2015; Luby-Phelps et al., 1986; Miermont et al., 2013; Mitchison et al., 2008; Moeendarbary et al., 2013; Parry et al., 2014; Tolić-Nørrelykke et al., 2004). The maintenance of such a complex, anisotropic cellular architecture and its remodelling in response to environmental changes requires a constant input of energy. Organisms have adopted a variety of strategies to cope with situations in which energy is limited. For example, when nutrients become limiting for growing and dividing cells
they can exit the cell division cycle and enter quiescence, similar to when they start differentiating (Coller et al., 2006). Such nutrient starvation-induced quiescence reverses as soon as nutrients become available again (Lennon and Jones, 2011; Yanagida, 2009). In extreme cases of energy shortage, cells can enter a dormant state with little or no energy consumption (Lennon and Jones, 2011). This mainly involves the formation of specialized cell types such as spores and seeds, some of which can survive for centuries. Some animal species, such as tardigrades or hibernating mammals, manage to adopt dormant states by massively down regulating cellular metabolism (Guppy and Withers, 1999; Reuner et al., 2009; Storey and Storey, 2007). In most cases, it is not known to what extent, if at all, the low energy consumption during such dormant states allows cells to maintain their favoured cytoplasmic organization. One way to preserve cellular architecture during an extended dormancy period is to reduce water content. This increases macromolecular crowding, which can trigger a transition into a glass-like state of the cytoplasm constraining the motion of its cytoplasmic components. This mechanism was shown to operate in bacterial spores, in plant seeds, in metabolically inactive bacteria, and in budding yeast cells acutely depleted of energy energy (Cowan et al., 2003; Dijksterhuis et al., 2007; Joyner et al., 2016; Munder et al., 2016; Parry et al., 2014; Sun and Leopold, 1997). Alternatively, cells replace water with high amounts of carbohydrates, possibly providing cytoplasmic vitrification to prevent harmful fusion events (Elbein et al., 2003; Soto et al., 1999). Furthermore, quiescent cells were shown to introduce structural changes to cytoplasmic components following energy depletion, with many metabolic enzymes and other proteins forming transient assemblies (Laporte et al., 2008; Narayanaswamy et al., 2009; Noree et al., 2010; O’Connell et al., 2012; Petrovska et al., 2014; Sagot et al., 2006). These assemblies were suggested to inactivate enzymatic function and to serve as storage depots. As a collective, they were proposed to form higher-order structures that mediate a solid-like state of the cytoplasm (Munder et al., 2016).

When nutrients become growth limiting for cells of the fission yeast *Schizosaccharomyces pombe*, they will mate and produce dormant spores (Egel, 1989; Tanaka and Hirata, 1982). Cells lacking a mating partner will enter a quiescent state. So far, research on fission yeast starvation has largely focused on the physiology of nitrogen-starved cells or cells acutely depleted of glucose (Costello et al., 1986; Joyner et al., 2016; Oda et al., 2015; Saitoh and Yanagida, 2014; Yanagida et al., 2011). We describe fission yeast cells that have slowly run out of nutrients. After several days of culturing, these cells show a sudden, drastic solidification of their cytoplasm, which we termed “cytoplasmic freezing” (CF). In this state, structures in the size range of 250-350nm show virtually no motion, while molecules the size of GFP can
almost freely diffuse. Cytoplasmic solidification in CF cells is much more profound and robust than in cells with other known liquid- to solid-like transitions and we show that a different molecular mechanism must be involved (Joyner et al., 2016; Munder et al., 2016).

Results

Starved cells have 2 intracellular immobilization states

After 2 days of culture under standard conditions, fission yeast cells have used all the glucose in the medium and arrested growth to enter a quiescent state (Fig. S1A) (Makushok et al., 2016). Imaging cells during the following days, revealed a striking decrease in mobility of virtually all subcellular structures visible by light microscopy, ending with their seemingly complete immobilization (Movie 1). An exception were small particles that erratically moved within vacuoles throughout starvation. We quantified this mobility change by tracking the motion of Lipid droplets (LDs), which are clearly distinguishable on DIC images. We refer to culturing day 2 as starvation day 2 (SD2), culturing day 3 as starvation day 3 (SD3) etc. In exponentially growing cells the dispersed LDs dynamically move throughout the entire intracellular space (Fig. 1A; Movie 1). On SD2, LDs frequently accumulated into 1-2 grape-like structures displaying visible motion, although their overall position within cells remained fairly constant (Fig. S1B). On SD3-SD4 LDs showed similar dynamicity but increasingly, cells contained fewer, larger LDs, presumably due to fusion within the grape-like structures (Fig. S1B). By SD5 and SD6 a larger fraction of cells contained enlarged LDs besides the smaller ones. All these LDs, independent of their size, displayed a complete absence of motion, which continued throughout the following days (Fig. 1A; Movie 1; Fig. S1C). We quantified this drastic mobility change, with a newly developed procedure for automated motion quantification of Bodipy labelled LDs (Listenberger and Brown, 2007; Long et al., 2012). Simultaneous Phloxine B treatment enabled automatic exclusion of dead cells from analysis (Fig. S1D; Methods) (Noda, 2008). Unfortunately, phototoxicity prevented the prolonged imaging required to calculate LD mean square displacement mobility. Instead, we determined a measure commonly used to quantify the co-localization of two fluorescent markers, the Pearson Correlation Coefficient (CC) (Adler and Parmryd, 2010; Dunn et al., 2011). We used the CC to quantify the "co-localization" of a fluorescent marker with itself at two time-points separated by 42 seconds (Fig. S1E-G). The maximal CC of 1 describes a completely static distribution of fluorescence intensity and thus absence of particle displacement. Increased
mobility of fluorescent structures will decrease the CC accordingly. Notably, this method merely provides a measure of whether there is motion but not of its nature. Monitoring the values of all pixels of a cell between two consecutive time points, we obtained a CC of 0.56 in exponentially growing cells, reflecting the strong, seemingly erratic motion of LDs, including occasional jumps (Fig. 1B; Movie 1). After cells had entered a quiescent state at SD2, LD mobility was reduced and LDs mainly wobbled in a given location without major translocations (Movie 1). Accordingly, the CC increased to 0.70 (Fig. 1B). On SD3 there was little change with average median CCs of 0.68. On SD4 we measured a slight increase to 0.75. Only on SD5 did the average median CC start to increase significantly with an average median CC of 0.84. On SD6 it reached 0.966, consistent with complete LD immobilization (Fig. 1B). Thereafter, the average median CC remained high with 0.95 on SD7 and 0.95 on SD8. Remarkably, analysis of individual cell cultures showed that in a given cell population, the final mobility reduction step was abrupt, occurring either on SD5 or SD6 (Fig. 1C). On SD5 the average median CC of a given culture either remained closer to that measured for cultures on SD4, or it jumped to that measured for all SD6 cultures (Fig. 1D). These results suggest that within a population, cells transit to the CF state in a coordinated fashion, within a relatively short period of time. Occasionally, we found a case where an entire cell culture did not transit to CF during SD5-SD8 (Fig. S1H). Since such cell populations could be clearly distinguished, we excluded them from subsequent analysis. Altogether, our measurements suggested the presence of two starvation phases, revealed by two abrupt decreases of LD mobility, which we termed early starvation and deep starvation. We termed the state of cells in deep starvation, where LDs are immobilized, "cytoplasmic freezing" (CF).

To investigate how cells regained LD mobility when exiting starvation, cells in the CF state were supplied with fresh growth medium (Methods). LD mobility became detectable within less than 5 min, showing that the release occurs in a switch-like manner (Fig. 1E; Movie 2). LD mobility increased over the following hour, which led to a broadening of the CC distribution (Fig. 1F,G). Some cells remained in the CF state during this observation period (Fig. 1H, white arrows; Movie 2).

**CF provides robust cell shape preservation**

To further test the extent of cytoplasm solidification in CF cells we generated protoplasts by digesting away the cell wall. When applying the standard protocol for cell wall digestion, exponentially growing cells, as expected, adopted a spherical shape, confirming that it is the
cell wall that provides the cylindrical cell shape (Fig. 2A,B; Methods). In contrast, CF cells treated in the same way, remained cylindrical. Since CF cells may have altered cell wall composition making them resistant to cell wall digestion, we followed cell treatment with live imaging. This confirmed that similar to exponentially growing cells, the majority of CF cells exited their cell wall shells. While exponentially growing cells squeezed through a small opening with massive deformation to immediately adopt a spherical shape, CF cells slipped out of their cell wall shells as solid cylinders (Movie 3). Consistently, this came with a time delay, as it required a larger opening in the cell wall. These data show that CF cells can preserve their shape independent of their cell wall (Fig. 2A). Shape preservation was robust as even after an additional three days of incubation in the continued presence of the digestive enzymes the majority of SD6 protoplasts remained cylindrical (Fig. 2A).

The standard protoplasting protocol uses a hypertonic digestion mix (1.2 M sorbitol), which strongly dehydrates exponentially growing cells to prevent their rupturing (Atilgan et al., 2015). Dehydration strongly increases macromolecular crowding, which could be critical for cell shape preservation of CF cells. To investigate this possibility, we removed the cell walls of CF cells using a hypotonic digestion mix containing 0.5 M sorbitol only. Under these conditions all exponentially growing cells rapidly lysed and no surviving protoplasts formed (Fig. 2C; Movie 4). In contrast, most CF cells still formed cylindrical protoplasts. Again, live imaging confirmed that the majority of CF cells exited their cell wall (Movie 4). Around 70% of the cells remained cylindrical even after an additional incubation day (Fig. 2C). This indicates that dehydration-mediated macromolecular crowding is not responsible for CF protoplast shape preservation. We conclude that CF cells stably fix the entire cellular content in a way that makes them resistant to osmotic pressure.

CF protoplasts produced under hypotonic conditions had a smooth surface while under hypertonic conditions surfaces were shrivelled and indented, suggesting that CF cells can lose some fluid (compare Fig. 2A,C). To quantify the extent to which CF cells dehydrate in a hypertonic environment, we placed them in a buffer solution containing 1.2 M sorbitol and measured the changes in cell width, the best indicator of volume loss, and cell length. While exponentially growing cells on average shrank by 0.83 µm in width and 1.28 µm in length, CF cells only shrank by 0.27 µm and 0.58 µm respectively (Fig. 2E). From these values we estimated cell volumes, assuming a cylinder plus two half spheres representing the cell ends. The predicted volume loss of 19.9% for CF cells was much lower than the 43.5% of exponentially growing cells (Fig. 2F; Methods). It is possible that CF cells are already severely dehydrated and therefore cannot shrink much further. Such dehydration could well be causative
for CF. To test this possibility, we measured the development of cell length and width with daily measurements starting from SD3, which is when most cells had arrested growth and division, and up to SD7. We found no change with cell lengths and widths remaining similar to the values of freshly divided exponentially growing cells (Fig. 2G). Most importantly, no change occurred when cells entered the CF state at SD5 or SD6. This indicates that CF is not linked to fluid loss.

**Subcellular architecture in deep starvation**

To learn more about the cellular state of CF cells, we performed electron microscopy on plastic sections of SD7 cells. Superficially, the cells did not differ much from exponentially growing cells (Fig. 3A). Cell walls were similar in thickness and appearance. Organelles were surrounded by regions without any distinct structure, except for electron dense spots typical of ribosomes. We found neither evidence for increased crowding of sub-cellular structures nor for any kind of filamentous assemblies. However, CF cells lacked the typical elongated mitochondria that span the length of exponentially growing cells (Fig. 3B). Instead, the mitochondria were small and globular with an approximate diameter of 250-350 nm (Fig. 3B). Most of these globular mitochondria were located in polar regions near the cell periphery (Fig. 3A,B). In contrast, organelles such as LDs and vacuoles had the tendency to cluster around the nucleus in the cell centre (Fig. 3A; Movie1; Fig. S1B).

To further explore the sub-cellular architecture of CF cells, we analysed cells expressing GFP- or mCherry-tagged proteins that are known to mark cellular organelles in exponentially growing cells (Table S1). When imaging control cells not expressing any markers with a 488 nm excitation laser and the GFP filter set at SD6, we noticed the gradual appearance of multiple autofluorescent particles (Fig. S2A). By SD6, this autofluorescence had reached such a significant level that it had to be considered when imaging GFP-tagged proteins.

Comparing the known protein localization of exponentially growing cells with that in cells on SD6, we found that the mitochondrial marker cox4p (cox4p-GFP), significantly changed localisation (Fig. 3B) (Yaffe et al., 2003). In exponentially growing cells cox4p-GFP revealed the typical tubular mitochondria organisation. On SD3, mitochondrial tubes were shorter and multiple dynamic globular structures of uniform size became visible, suggesting that the mitochondria were undergoing fission (Fig. 3B; Movie 5). Consistently, on SD6 only globular mitochondria of uniform size were present, mainly near cell poles as in our electron microscopy pictures (Fig. 3B). Similar to LDs, the motion of these globular mitochondria had completely
arrested (Movie 5). Since cox4p-GFP fluorescence was sufficiently bright and stable, we could produce movie sequences that allowed determining the mitochondria mean square displacement. This provided a second, independent measure for CF with a structure comparable in size with LDs but with different subcellular location. On SD3, the mean square displacement of globular mitochondria was consistent with the significant, visible motion. On SD6 the mean square displacement was nearly zero substantiating an almost complete immobilisation of organelle motion also in polar cell regions (Fig. 3C). This suggests that CF is a comprehensive phenomenon of the cytoplasm.

Unlike cox4p-GFP the localisation of the GFP-tagged nucleoporin nup85p did not change much in CF cells as compared to exponentially growing cells (Fig. 3D). Similarly, GFP-tagged AHDL - a luminal marker of the nuclear envelope and the endoplasmic reticulum - still showed the alignment of ER with the entire cell membrane, nuclear envelope localisation and additional, ill defined cytoplasmic membrane structures. Although additional bright dots appeared, this is indicative of an intact ER membrane network, suggesting no major changes in ER and nucleus organisation occurring in deep starvation. Vacuoles also were detectable at SD6, but with clearly reduced size while their number appeared increased (Fig. 3D). In addition, the vacuolar marker confirmed that at SD6, vacuoles lighted up in all cells expressing mCherry-tagged proteins, suggesting vacuolar mCherry accumulation following degradation of the attached protein (Fig. S2B) (Costantini et al., 2015).

The signal of all other organelle markers vanished below the autofluorescence level (Fig. S2C). This suggests that in deep starvation many proteins are either downregulated or loose distinct localisation such that they no longer are detectable by fluorescence imaging.

**Immobilization of cytoplasmic components in CF correlates with their size**

Having shown that fragmented mitochondria and LDs - both ranging in size between approximately 250 nm and 350 nm - were immobilized in CF cells, we wondered whether this also applies to components as small as individual proteins. To test this we used cells expressing Lifeact-GFP (Huang et al., 2012; Riedl et al., 2008). This is a globular, 29 kDa protein of 4.5x2.5 nm, binding to F-actin with a high turnover rate and otherwise seeming biochemically inert, which makes it a good readout compared to endogenous proteins which are more likely to be subject to unknown transient binding interactions (Riedl et al., 2008; Yang et al., 1996). To test the mobility of the free, cytoplasmic Lifeact-GFP pool, we used the "fluorescence loss in photobleaching" (FLIP) method (Bancaud et al., 2010). FLIP involves repetitive bleaching.
of a defined, cellular sub-region. The kinetics of fluorescence loss in the remaining, unbleached cell regions provides a good measure of fluorescent particle motion into the bleached regions, thereby revealing information about the diffusive behaviour of the particle. In CF cells on SD6, Lifeact-GFP depleted fast in the unbleached regions (Fig. 3E). Notably, the strong signal on the stable actin cables also depleted fast. This reveals fast binding/unbinding of the fluorescent protein to actin as well as a rapid, diffusion throughout the cytoplasm that is not significantly reduced compared to the diffusion rate in exponentially growing cells (Fig. 3E). We conclude that in CF cells, larger cellular components are fixed in place, while small molecules can diffuse almost as freely in CF cells as in exponentially growing cells. This is consistent with the presence of a global network structure with a certain mesh size in CF cells.

**CF is not mediated by the cytoskeleton**

Excluding fluid loss, we next investigated the possibility that cytoskeletal filaments form a cell-wide meshwork trapping bigger structures such as the LDs. Microtubules can be virtually excluded as previous work has shown that they completely disappear from cells at around SD4-5, with the exception of a single, very short microtubule stump remaining in some of the cells (Laporte et al., 2015; Makushok et al., 2016). We confirmed this result by imaging cells expressing GFP-tagged alpha2 tubulin (Fig. S3A). In addition, we show that in CF cells at SD6 no microtubules reformed.

To test for a role of F-actin in CF, we used cells expressing the F-actin marker Lifeact-GFP (Huang et al., 2012; Riedl et al., 2008). As previously shown, in exponentially growing cells Lifeact-GFP labelled thin actin filament bundles that align parallel to the long cell axis and dynamic actin particles that concentrated at growing cell poles. On SD2-SD4, the actin particles disappeared except for a few remaining dynamic patches that were distributed throughout the cells. The thin interphase filaments were replaced by thicker, dynamic F-actin bundles (Fig. 4A; Movie 6). These extended along the cell periphery and often curled around the cell ends or curled up inside the cells. On SD6, all actin patches had disappeared and the F-actin bundles had evolved into extremely prominent, very long F-actin bands (Fig. 4A). These bands were completely immobile and extended along the cell circumference while curling around both cell-ends to form a structure often reminiscent of a shoelace (Fig. 4A; Movie 6). The very strong signal of these actin bands suggests that they contain most of the cellular actin pool. Nevertheless, this does not fully exclude that the cells could additionally harbour a global F-actin network. Unlike single microtubules, single actin filaments cannot be detected by
fluorescence imaging or standard electron microscopy. We therefore treated cells with LatrunculinB (LatB), a drug that interferes with F-actin polymerization (Spector et al., 1983). Adding LatB to exponentially growing cells resulted in a fast depletion of all visible F-actin structures (Fig. S3B). In contrast, adding LatB to cells on SD6 did not affect the shoelace-like F-actin structure suggesting that these do not turn over, unless the drug cannot enter CF cells (Fig. S3C). As this result did not allow drawing conclusions on the role of F-actin, we applied LatB prior to the induction of CF on SD3 and incubated cells in the continued presence of the drug until SD6 (Methods). This prevented the formation of the shoelace-like F-actin structures in many cells, which ended up with a dispersed Lifeact-GFP signal (Fig. 4B). In the other cells the marker labelled short stumps and ring-like F-actin structures. Since in this experiment LatB can significantly interfere with the formation of the thick F-actin cables in CF cells, it is unlikely that it would not do so with a network of single actin filaments. Nevertheless, such cells showed CF on SD6 similar to DMSO-treated control cells (Fig. 4C,D; Movie 7). Similarly, cps8-188 cells carrying a temperature-sensitive mutation in the actin encoding act1 gene, switched into the CF state at SD5, when shifted to the restrictive temperature at SD4 (Fig. 4E,F) (Ishiguro and Yamada, 1993). Together these results suggest that F-actin does not critically contribute to CF.

Septins, are another protein family, shown to assemble into filaments that could potentially form a global network in CF cells (Mostowy and Cossart, 2012). The fission yeast genome has seven non-essential septin genes (spn1-spn7) (Longtine et al., 1996; Pan et al., 2007). Of these, spn1p-4p assemble in the cytokinetic ring during cell division, while spn5p-7p are exclusively expressed during meiosis (Fig. S3D) (Abe and Shimoda, 2000; Mata et al., 2002; Onishi et al., 2010; Watanabe et al., 2001). To ensure that the latter are not additionally expressed in deep starvation, we checked the signal of cells expressing GFP-tagged endogenous spn5p-7p on SD6. We found no signal above the autofluorescence (Fig. S3E). We therefore carried on with analysing the non-meiotic septins spn1p-spn4p tagged with GFP or tagRFP. We found that they all formed small clusters in deep starvation (Fig. 4G). Next, we tested cells carrying a deletion of any of the four septins spn1-spn4, which prevent septin filament formation and/or the proper formation of the septin ring in cytokinesis (An et al., 2004; Berlin et al., 2003; Tasto et al., 2003). On SD6 all septin deletion mutants showed CF that was indistinguishable from the wild type (Fig. 4H,I). We conclude that septins are unlikely candidates for mediating CF.
Autophagy accelerates CF establishment

To further investigate the molecular nature of CF, we used our automated quantification of LD motion to screen for gene deletions that prevent CF in cells in deep starvation, using a library of 3400 fission yeast strains each carrying a deletion of a non-essential gene (Methods) (Kim et al., 2010). The strains were screened on SD8 since due to the presence of multiple auxotrophic mutations these strains entered deep starvation with a delay. Of the roughly 500 deletions that did not show CF in deep starvation, we noticed a clear accumulation of mutants affecting autophagy. Autophagy is an evolutionarily conserved mechanism used by cells to remove damaged organelles and to recycle cellular components (Nakatogawa et al., 2009). To investigate the role of autophagy in CF we took a prolonged look at two strains, carrying a deletion of *atg1* (*atg1Δ*) and *atg8* (*atg8Δ*) respectively, which encode essential autophagy pathway components. Both mutants show normal exponential growth and entered quiescence similar to the wild type (Fig. 5A). The cells of both mutant strains entered the CF state but with a two- to three-day delay in comparison with the wild type (Fig. 5B,C; Movie 8). Consistently, cell wall digestion of *atg1Δ* and *atg8Δ* mutant cells in a hypotonic environment produced spherical protoplasts at SD6-8. Only at SD9 cells robustly maintained a cylindrical shape similar to wild type cells at SD6 (Fig. 5D). These results indicate that autophagy is not essential for CF but promotes the cells ability to enter the CF state.

CF differs from other solid-like cytoplasmic states

Two recent studies reported on cytoplasm solidification in budding yeast cells, depleted of energy either by acute glucose depletion (AGD) or by "drug-induced energy depletion" (DED) (Joyner et al., 2016; Munder et al., 2016). These states being reminiscent of CF, we directly compared the respective cells with cells in deep starvation (Methods). For DED cells, 0.5 and 2 h drug treatment was tested as we found incubation times to be critical (Methods). Time-lapse imaging with DIC microscopy showed that unlike in CF cells, LD motion was evident in AGD and DED cells (Fig. 6A; Movie 9). Quantification with the Bodipy-labelling approach revealed an average median CC of 0.878 for AGD cells and 0.900 for 0.5 h DED cells (Fig. 6B). The average median CC of 0.966 for 2 h DED cells was similar to the 0.969 of CF cells, but unlike in CF cells, some LDs showed clearly movement. Notably, the weaker Bodipy fluorescence in CF cells will reduce the precision of automated analysis and result in a lower CC as compared to AGD and DED cells.
Both budding yeast studies contained a fission yeast experiment in which protoplasts were produced from AGD or 2 h-treated DED cells, using the standard protocol. These protoplasts were found to preserve their cylindrical shape unlike untreated cells that became spherical (Fig. 6C; Movie 10; Methods) (Joyner et al., 2016; Munder et al., 2016). In order to investigate the robustness of shape preservation, we extended the observation period of protoplasts in the continued presence of digestive enzymes as done for CF cells (Methods). We found that within 3 hours the vast majority of the initially cylindrical AGD protoplasts had become spherical indicating that these cells can still rearrange their cytoplasm although with slower kinetics as compared to exponentially growing cells (Fig. 6C). DED cells in contrast, mostly remained cylindrical for the first three hours. Only after three days of incubation approximately 20% of the 2 h-treated and 80% of the 0.5 h-treated DED cells had turned spherical (Fig. 6C). This suggests that under hypertonic conditions robust cytoplasmic solidification can only be reached by prolonged drug exposure.

As introduced above the standard hypertonic cell wall digestion mix dehydrates cells to an extent that is critical for protoplast shape preservation. Indeed, cell width and length measurements showed that in a 1.2 M sorbitol solution AGD and DED cells shortened and considerably reduced their width (Figs 6D, S4). Their volume loss was estimated to be 39.4% for AGD cells, 35.1% for 0.5 h-treated DED cells and 38.6% for 2 h-treated DED cells (Fig. 6D). This shows that in hypertonic conditions, AGD and DED cells experience increased macromolecular crowding due to considerable fluid loss within their cytoplasm. To investigate the contribution of volume loss to protoplast shape preservation, we produced protoplasts in hypotonic conditions (Methods). As with exponentially growing cells, this caused rapid lysis of all 0.5 h-treated and the majority of 2 h-treated DED cells (Fig. 6E; Movies 4,10). Time-lapse imaging revealed that the ~ 15% of 2 h-treated cylindrical DED protoplasts did not fully exit the cell wall shell. The parts that did, rounded up, suggesting a fluid content (Fig. 6F; Movie 10). Of the AGD cells, ~ 50% lysed, while the remaining cells adopted a spherical shape, squeezing out of the cell wall shell, in a similar manner to exponentially growing cells in the hypertonic digestion solution (Fig. 6E,F; Movies 4, 10). These results show that unlike for CF cells, experimentally imposed dehydration is critical for protoplast shape preservation of AGD and DED cells.

Taken together, these results indicate that the much more profound cytoplasmic solidification of CF cells is different from that of cells experiencing acute energy depletion.
Starved fission yeast lack extensive protein assemblies

The cytoplasmic solidification of budding yeast DED cells was proposed to be mediated by homotypic assembly of a large number of proteins (Joyner et al., 2016; Munder et al., 2016). To check for a similar behaviour of homologous proteins, we followed several of them, tagged with mGFP and/or mCherry, in 0.5 h-treated and 2 h-treated fission yeast DED cells (Table S2). None of them formed assemblies suggesting that fission- and budding yeast cells react differently to DED (Fig. 7A). Importantly, some of the tested proteins, when tagged with GFP(S65T) instead of monomeric tags, either formed DED-specific assemblies or assemblies in both, untreated and DED-treated cells (Fig. S5A).

While these data do not support protein assembly formation in DED fission yeast cells they cannot exclude this possibility in CF cells. Therefore, we checked all proteins in CF cells on SD6. By SD6, no aggregates were detectable and the signal intensities of most of the GFP-tagged proteins had dropped below the aforementioned autofluorescence (Figs 7B,C, S2A). Western blot analysis showed reduced protein levels for most of these proteins (Fig. S6). Notably, also actin levels were reduced further supporting the above conclusion that F-actin does not mediate CF. Also, pre-existing GFP(S65T)-dependent aggregates disappeared (Fig. S5B). As shown above, cells expressing mCherry tagged proteins, accumulated fluorescence in the vacuolar lumen, in one case accompanied with bright nuclear fluorescence (Fig. 7C). Consistent with the absence of macromolecular assemblies in CF cells, electron microscopy pictures showed no evidence of distinct areas as described for protein aggregates in budding yeast cells (Fig. 3A) (Petrovska et al., 2014). These results suggest that extensive macromolecular protein assemblies are unlikely to be the basic cause of CF.

Discussion

We here introduce cytoplasmic freezing (CF) as the strongest cytoplasmic solidification state of yeast cells found to date. CF occurs in a switch-like manner only after several days of quiescence in almost all cells of a glucose starved population. This phenotype is not modulated by cell cycle stage or cell size as glucose starved cells, with few exceptions, are of similar length and mainly arrested in G2, which is also the cell cycle stage in which fission yeast cells mainly grow (Costello et al., 1986). CF significantly differs in many ways from the two previously described cytoplasmic solidification states that were induced either by acute glucose depletion, AGD, or by DED, drug induced energy depletion of exponentially growing cells (Joyner et al., 2016; Munder et al., 2016). Following acute energy depletion in fission yeast
cells, LD motion is indeed much reduced but in contrast to CF cells, it is still evident by eye. We quantitatively confirmed this difference with our cross-correlation analysis, which faithfully monitors LD displacement between two time points thus enabling direct comparison of LD motion between the different states with high sensitivity. Unfortunately, for technical reasons a more standard comparison using LD mean square displacement was not possible. Such a value we could only obtain for the globular mitochondria in CF cells. The fact that these mitochondria are of very similar diameter as LDs and placed in different locations suggests that generally, the motion of organelles in the 250nm size range is virtually abrogated in CF cells. In addition to the difference in the degree of LD immobilisation, we find several other fundamental differences indicating that the mechanism underlying CF differs from that with which exponentially growing cells respond to acute energy depletion (Joyner et al., 2016; Munder et al., 2016). First of all, CF cells very robustly preserve their cylindrical shape in the absence of a cell wall even in a hypotonic solution where exponentially growing cells as well as AGD and DED cells rapidly lyse and disperse their cellular content. The previously published shape preservation of cell wall depleted fission yeast cells in AGD and DED conditions we find to depend on experimentally contributed cellular dehydration, which critically increased sub-cellular, macromolecular crowding. In addition, under most conditions this shape preservation was not robust and protoplast rounding occurred with a delay. Increased macromolecular crowding due to fluid loss was proposed to underlie cytoplasmic solidification of AGD cells and to contribute in DED cells. Since we find no evidence for dehydration when quiescent cells switch to CF at SD5/SD6 this mechanism cannot be critical. Consistently, we also find no evidence for dehydration during the days preceding CF induction, suggesting that CF is not just a more profound or advanced manifestation of the AGD-induced state. In DED cells, a pH-dependent formation of numerous homotypic protein assemblies was proposed to be the other, more critical mechanism providing cytoplasmic solidification. In CF cells, we find no evidence for the presence of such assemblies again arguing for a different mechanism underlying CF. So far, such homotypic protein assemblies were only shown in budding yeast cells. Interestingly, we do not find any evidence for such assemblies forming in similarly drug-treated fission yeast cells. Notably, this was true only when homologs of the relevant proteins were tagged with monomeric fluorophores. When tagged with the GFP(S65T) variant, which is routinely used by budding- and fission yeast researchers, some of these proteins indeed formed such assemblies (Huh et al., 2003). That certain fluorescent protein tags mediate protein aggregation and thus should be avoided when studying protein assemblies was previously also shown for bacterial cells (Landgraf et al., 2012). However, since we only analysed a limited
number of proteins we cannot exclude that simply by chance, we may have missed the crucial ones.

At this stage we cannot tell what the mechanism causing CF is but it seems that quiescent cells need 3-4 days to become competent for switching into this state. In support of this, we find that blocking autophagy, a conserved mechanism for cell autonomous recycling of macromolecules, delays the onset of CF by 2-3 days. It is plausible that cells use autophagy to provide energy and possibly to produce components that mediate the CF state in the absence of external sources. A possible mechanism is the specific enrichment of small components, such as sugars or other metabolites, in the cytosol. This strategy is used by animals to control the formation of subcellular ice crystals when exposed to freezing temperatures and by plants to increase the longevity of their seeds (Leprince et al., 2017; Storey and Storey, 2017). What speaks against such a mechanism in CF cells is that we would expect it to decrease also the motility of protein-sized components such as Lifeact-GFP, which is not the case. This thus speaks more in favour of another mechanism, where CF cells generate a global polymer network that forms a stable mesh throughout the cytosol. Such a network would preserve cell shape and fix larger cellular components while simultaneously allowing molecules smaller than the mesh size to freely move as observed for Lifeact-GFP. In support of a polymer, is also the fact that in a hypertonic environment the fluid loss of CF cells is little, as compared to exponentially growing cells or AGD and DED cells, given that the ability to retain fluid is typical for hydrogels. Alternatively, fluid retention in CF cells could be due to a complete insulation of quiescent cells from the environment. What speaks against is the fact that they still take up the Bodipy dye and still actively eliminate Phloxine B, our marker for automated detection of dead cells that fill up with the dye (Fullerton et al., 2006; Tamai et al., 1996). Notably, hydrogels can form spontaneously via phase transition once the critical components have reached a certain concentration. This was proposed to occur during various processes, including Balbiani body formation, and it can involve proteins and/or RNAs (Boke et al., 2016; Brangwynne et al., 2009; Han et al., 2012; Jain and Vale, 2017; Kato et al., 2012; Patel et al., 2015). In our case, such a phase transition could account for the tremendous synchrony with which CF occurs in a given cell population, either on SD5 or SD6, assuming that all cells of a population produce the critical components at a similar rate. Prime candidates that could be forming a polymer network are the known filament forming proteins actin, tubulin or the septins. However, protein localisation, electron microscopy imaging and interference experiments do not support a role for any of them.
It seems obvious that CF will preserve overall cell architecture thus relieving cells from using vast amounts of energy in times of prolonged nutrient starvation. Also, CF may provide resistance to various stresses, without the need for metabolic activity. Unfortunately, our starvation conditions, which rely on standard fission yeast liquid culturing, are not optimal for investigating the role of CF in cell survival. The problem is that mortality rates massively increase after SD8. This is likely due to the fact that, under these conditions, cells enter starvation in their own, toxic waste. Thus, in order to perform such studies more favourable starvation conditions will first have to be established.

Nevertheless, CF is very much reminiscent of a recently published process in tardigrades that is essential to provide tolerance to desiccation. For this, the tardigrade cells first need some time to express intrinsically disordered proteins. These eventually undergo a global phase transition converting the cytoplasm into a gel-like state, that fixes the cellular content (Boothby et al., 2017). Key to understanding the mechanism underlying CF will thus be the identification of the responsible components. Corresponding gene deletion strains, will in addition allow testing the CF state for being cytoprotective during a starvation period.

**Methods**

**Yeast cell culturing**

Cells were grown at 25 °C in EMM2, supplemented with thiamine as required, and as described in (Moreno et al., 1991). For CF experiments, cells were cultured to enter starvation as described in (Zaitsevskaya-Carter and Cooper, 1997). Standard precultures in mid-exponential growth phase (EMM2 medium) were diluted to OD 0.02 (spectrophotometer Genesys 10S Vis, Thermo Fisher Scientific, Waltham, Massachusetts) in EMM, with 0.5% glucose (EMMLG) plus supplements as required. Notably, CF occurred also when cells were diluted in 2% glucose EMM (standard starting condition to analyse exponentially growing cells) but the synchrony of induction was reduced and quantitative analysis was hindered by optical inhomogeneity amongst the cells.

Cells were cultured in Erlenmeyer flasks on a shaker (New Brunswick scientific innova 4230; 220 rpm) for up to 8 days. The culture volume did not exceed 1/10 of the total volume of the flask. All strains used are listed in Table S1.

To follow starvation exit, we added glucose by supplementing cells with fresh EMM2 (1:4) and incubating them in an Erlenmeyer flask on the shaker at 25 °C. AGD was done as described
for budding yeast (Joyner et al., 2016). DED was done as described (Munder et al., 2016) (Antimycin A from Streptomyces sp., Merck, Darmstadt, Germany, A8674; 2-Deoxy-D-glucose, Merck, D8375). We performed all DED experiments with 0.5 and 2 h drug incubation, as different incubation times were previously reported for different experiments and for the different drugs tested (Munder et al., 2016). Glucose levels in filtered culturing media (Filtropur S 0.2, Sarstedt 7510401) were measured using a glucose detection kit (Abcam, 102517) and following the manufacturer’s instructions.

Protein tagging and constructs

Protein tagging was performed as described in (Bähler et al., 1998), using the primers listed in Table S3, except that plasmids were equipped with a “happy linker” sequence between gene and fluorophore sequence where indicated as for EB1 in (Jankovics and Brunner, 2006). mGFP(A206K) was generated from the routinely used GFP(S65T) by site-directed-mutagenesis at the dimer interface using forward (AGGTCGACGGATCCTTGGAG) and reverse (GATCTTTCGAAAGTTAGATTGTGGACAGGTAATGGTTGTCTGG) primers, and insertion into the original plasmid using restriction enzymes BamHI / BstBI (Snapp, 2003).

Microscopy

Live imaging was performed at room temperature on a spinning disc microscope (Zeiss Axio Observer Z1, Yokogawa CSU-X) using 63x and 100x NA 1.4 oil plan apo objectives, Andor iQ2.9 software, Andor Neo sCMOS and iXon3 EMMCCD cameras (Andor Technology, Belfast, UK) using 488 nm and 561 nm laser excitation and 525/50 BP and 568 LP emission filter sets. Z stacks with 0.5µm steps were acquired for standard fluorescence unless stated otherwise.

DIC imaging of starved cells was performed on poly-L-lysine (2 mg/mL, Merck, P1399) coated glass bottom dishes (Bioswisstec, Schaffhausen, Switzerland; 5160). In the dishes, we constructed a chamber by adding a cover slip on top of 3 parafilm strips acting as spacers. The dishes were then placed for 2 s on a heating plate at 100 °C, which partially melted the strips such that they glued the coverslip to the dish. The thereby formed chamber was filled with cell culture (~30 µL) by pipetting into the gap between the parafilm strips. To extract LD trajectories from DIC movies, cells were centrifuged at low speed (300rpm, Multifuge 1S-R, Thermo Fisher Scientific) and imaged immediately (5-15 min after mounting) as prolonged
residence in the chamber caused artefacts. For each movie, 100 image frames were acquired at 4 fps.

LD trajectories of cells exiting starvation (Fig. 1E) were extracted from DIC movies using lectin-coated glass bottom dishes (griffonia (bandeiraea) simplicifolia lectin1; Vector laboratories, Burlingame, California; L-1100). A first movie was taken from cells in EMM without glucose (EMM0G) immediately before glucose addition. After 2% glucose addition, movies were taken for up to 60 min.

Live cell imaging for CC quantification was done on lectin coated glass bottom 8-well (ibidi, Martinsried, Germany; 80827) or 10-well slides (Greiner Bio-One, Kremsmünster, Austria; 543079) after centrifugation at 1000 rpm. 1 mg/mL Bodipy (BODIPY 493/503; Thermo Fisher Scientific; D3922) was dissolved in DMSO and used at a final concentration of 4 µg/mL in EMM2 or EMM0G for exponential or starved cells respectively. Phloxine B (Merck, P4030) was dissolved in water to 5 mg/mL, diluted to 100 µg/mL in water, and used at a final concentration of 10 µg/mL. When imaging, we first acquired a single focal plane image of red fluorescent Phloxine B followed by 3 single focal plane images of green fluorescent Bodipy with a time interval of 42 s.

The movies for cox4-GFP particle tracking were made of 300 frames taken at 4 fps in chambers as for DIC imaging.

Protoplasts in high sorbitol were imaged with DIC on lectin coated glass bottom 10-well slides after centrifugation at 1000 rpm. Protoplasts in low sorbitol were transferred to a lectin coated imaging chamber (see above), sealed off with VALAP (Vaseline, Lanoline, Parafilm; 1:1:1) to prevent dehydration, and imaged immediately.

Cell wall digestion

Protoplasts in high sorbitol were generated by enzymatic digestion of cell wall, with slight alterations to a previously published protocol (Kelly and Nurse, 2011). To form protoplasts, cells were incubated at 25 °C with 5 mg/mL Zymolyase 20-T (MP Biomedicals) plus 5 mg/mL lysing enzymes from Trichoderma Harzianum (Merck; L1412) in 500 uL E-buffer + 1.2 M sorbitol in a 2 mL Eppendorf tube for 1 h on a rotor at 25 °C unless stated otherwise.

Protoplasts in low sorbitol were generated by washing cells in E-buffer + 0.5 M sorbitol, centrifuged at minimal speed for 5 min, and resuspended in 50 uL E-buffer + 0.5 M sorbitol plus cell wall digesting enzymes.
For DED, protoplasts were generated in continued presence of 20 mM 2-deoxy glucose and 10 mM antimycin A in E-buffer as described in (Munder et al., 2016).

**FLIP acquisition and analysis**

Fluorescence loss in photobleaching (FLIP) experiments were performed on cells mounted to an imaging chamber sealed with VALAP (see above). Imaging was done at RT on a spinning disc microscope (Nikon Eclipse Ti, VisiScope system, Yokogawa W1) using a 60x water objective, VisiView software, and an Andor EMCCD camera (iXon Ultra 888 back illuminated). A z-stack of 3 planes (1 μm step size) was acquired every second for 100 sec while a small region with 1.12x1.12 μm size near one cell pole was bleached every 5 sec. The mean fluorescence intensity loss of a reference region at the opposite pole was then extracted using Fiji. The analysis was done using Matlab, as described in (Bancaud et al., 2010). The signal was normalized to the last pre-bleach time point. For each condition, 30 cells were analysed - 10 each in three independent experiments.

**Electron microscopy**

Cells were high pressure frozen in solution (reviewed in (McDonald et al., 2010)) using a Wohlwend Compact-2 high-pressure freezer (Martin Wohlwend AG, Sennwald Switzerland). *S. pombe* samples destined for plastic section microtomy were freeze-substituted in 0.1% glutaraldehyde and 1% uranyl acetate in acetone for 48 h and warmed from -90 °C to -50 °C in 8 h (5 °C per hour). Cells were then washed by acetone for 3 times and infiltrated in HM20 solution (25%, 33%, 50%, 67%, 75%, 100% in acetone) (Lowicryl HM20 Embedding Kit, Electron Microscopy Science, Hatfield, PA) over 5 days using Leica EMAFS (Leica, Vienna, Austria). Samples were then polymerized to blocks under Leica EMAFS UV light unit for 72 h.

Plastic blocks were cut into ribbons of 80 (for single projection images) – 250 nm thick plastic sections (for tomographic reconstructions), depending on the questions asked, by Leica Ultracut microtome (Leica Inc., Vienna, Austria) using Diatome Ultra 45° (Diatome AG, Biel, Switzerland). Ribbons were collected on formvar-coated Cu-Rn grids (Electron Microscopy Science, Hatfield, PA) or Carbon Film Finder grids (Electron Microscopy Science, Hatfield, PA), immuno-labelled (optional), stained by uranyl acetate (2% uranyl acetate in 70% methanol) for ~ 4 min and Reynold’s lead citrate for ~ 2 min (the staining time was adjusted based on the thickness of the sections).
Individual pictures of plastic sections, mostly used as a control, were acquired with a FEI Philips CM100 TEM and AMT 2Kx2K bottom-mount digital camera.

**LatB treatment**

LatB (Latrunculin B, Latrunculia magnifica, Merck; 428020) was added from a stock solution of 10 mM in DMSO to a final concentration of 100 µM (1% DMSO). Control cultures were treated with 1% DMSO. For the short-term effect of this LatB concentration, the stock solution was diluted in EMM2 for exp. cells and EMM0G for SD3 and SD6 cells. For 3-day LatB incubations, starved cultures were split in half at SD3. One half was supplemented with LatB, the other with DMSO to serve as control. Both cultures were incubated at 25 °C on the shaker for another 3 days.

**Western Blotting**

After culturing, 0.5x10^8 cells (2 OD595 equivalents) were harvested by centrifugation and resuspended in 200 µL lysis buffer (1% SDS, 8 M urea, 10 mM MOPS, pH 6.8, 10 mM EDTA, 0.01% bromophenol blue) containing 100 µL glass beads and a protease inhibitor cocktail (Promega) (Marguerat et al., 2012). The cells were disrupted by vortexing for 3 x 40 s in a Fastprep FP12 cell disrupter (Thermo Scientific). The protein samples were denatured and reduced with 5 mM DTT for 10 min at 65 °C and clarified by centrifugation before SDS-PAGE and western blot analysis using 1:1000 anti-GFP mouse monoclonal antibody (11814460001, Roche) or anti-actin antibody mouse monoclonal antibody (224-236-1, DSHB).

**Image Analysis**

Routine image processing was done using Fiji/ImageJ. Deconvolution was done using Huygens software (Scientific Volume Imaging) on image stacks acquired using Nyquist criteria. Plots were made using Matlab (MathWorks). Optimal sample sizes were not explicitly calculated.

**LD trajectories from DIC movies**

The DIC movies (100 frames, 4 fps) were stack registered (Fiji plugin “StackReg”). LDs were tracked with the Fiji plugin “Manual Tracking with TrackMate” (settings for semi-automated tracking: Quality threshold: 0.2, Distance tolerance: 0.1, Max nFrames: 0). The LDs were manually seeded in the first time-frame, and the trajectory was considered if the particle
could be tracked for more than 95/100 frames. The manually seeded first trajectory point was excluded from the final trajectory, such that all LD positions were automatically detected. The trajectories were plotted using Matlab.

**Mean square displacement of mitochondria**

Cox4-GFP labelled mitochondria were tracked using the Mosaic particle tracking plugin in Fiji (Mosaic Toolsuite, (Sbalzarini and Koumoutsakos, 2005); Settings: radius: 4, cutoff: 0, per/abs: 2, link: 3, displacement: 2). The subsequent analysis was done in Matlab (Mathworks). Only trajectories longer than 160 frames were considered, and the mean square displacement up to a time lag of 40 frames was computed. The time-averaged mean square displacement for each particle is plotted as a color-coded histogram, and additionally the ensemble-averaged mean is shown.

**CC quantification of LD motion**

Cell segmentation: The Phloxine B signal was log transformed and background subtracted in Fiji (Mosaic ToolSuite). Using pixel classification in ilastik the cell’s inside was separated from the outline and the background. Phloxine B-filled, and therefore dead cells, were marked as a separate class and excluded from subsequent analysis. Cell insides were segmented in Cellprofiler and used as seeds to segment the living cells (Fig. S1D). Subsequently, the 3 Bodipy images, taken at a time interval of 42 s (t1-t3), were stack registered in Fiji (StackReg). We included additional procedures to account for high variability of Bodipy signal intensity amongst individual cells in deep starvation as well as significant differences of entire populations between different starvation days, with particularly low signal at SD2 and SD3 (Fig. S1F). In addition, for some unknown reason, Bodipy signal intensity gradually increased during imaging, with a major increase between t1 and t2 (Fig. S1F). As a result, the software generally detected LDs most efficiently at t2 and t3, which is why we use these timepoints to determine the CC. Because absolute signal intensities are irrelevant when extracting LD dynamics, we equalized fluorescence on our images by performing a log transform and background subtraction (Mosaic ToolSuite). Pixel classification in Ilastik further improved equalization. This generated pseudo images in which pixel values represent the probability to belong to a LD instead of the actual fluorescence intensities (Fig. S1G). From these, we computed the Pearson correlation coefficient (CC) for all pixels of individual cells between t2 and t3, using Cellprofiler. We present individual CC values as dot plots using the function plotSpread from the MathWorks File Exchange
We overlay a boxplot indicating the median and the 25th to 75th percentile of the values. Where several experiments were pooled, the mean of the medians of the single experiments was plotted in blue. As a measure for the variance between individual experiments, blue error bars indicate the 95% confidence interval (95% CI) of the medians. This variance does not describe the variance of the individual medians and might thus underestimate the true variance of the median.

**Cell size measurements**

For comparison of cell size in respective standard culturing and 1.2 M sorbitol containing medium (Figs 2E,F, 6D, S4), cell length and width were measured manually from DIC images using Fiji. Subsequently, we extracted the means of cell length and cell width measured from 3 independent experiments each, for cells culturing in standard EMM medium and in hypertonic buffer containing 1.2 M sorbitol. To estimate the variance \((dL^2, dW^2)\) of the 3 experiments, bootstrapping was performed (999x resampling of each individual experiment), leading to 999 new means from bootstrapped samples. The mean cell volume \(V\) was approximated as a cylinder plus a ball from the mean of the bootstrapped means of measured length and width \((L, W)\) as follows \(V = \pi \times \frac{L}{2} \times (\frac{L}{2} - W) + \frac{4}{3} \times \pi \times \frac{W}{2}^3\) (1). The variance of the cell volume \((dV^2)\) was estimated by Gaussian error propagation with \(dV^2 = \frac{\pi}{2} \times W \times dW^2 + (\frac{L}{2} - W)^2 + (\frac{W}{2} - \frac{L}{4})^2 + \frac{\pi}{4} \times W^2 \times dL^2\) (2). To define the amount of cell shrinkage after transfer to the hypertonic buffer, we divided the mean of the bootstrapped means of cells in hypertonic buffer \((S)\) by the mean of cells in EMM growth medium \((E)\) for length, width, and approximated volume. The variance \((dR^2)\) of the ratio \(R = S/E\) was estimated by Gaussian error propagation with \(dR^2 = (dE/E)^2 + (dS/S)^2 + (dS/E + dE/S)^2\) (3). The mean and 95% CI of these normally distributed fractions are shown in Fig. 2F and Fig. 6D.

Measurements of cell length and width using Phloxine B signal was done in an automated fashion as described above.
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Competing interests

All authors declare no competing interests.
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Figure 1 - Reversible motion arrest of LDs in deep starvation.

A. Upper panels show trajectories of the LDs depicted in the lower DIC image panels with cells taken from 25 sec movies (4 frs) on different culturing days.

B. Dot plots (1 dot/cell) with overlain box plots showing Pearson correlation coefficient-based (CC) quantification of Bodipy-labelled LD dynamics. Boxes represent the 25-75
percentile. The blue line shows the mean of the medians from 4 independent cell populations (n = 820, 1451, 1406, 1380, 1407, 1308, 1260, 1241 cells). Error bars: 95% confidence interval. Note that none of the four cell populations had fully entered CF on SD5. Standard box plots are overlain.

C Dot plot as in (B) showing differing population timing for the transition to CF (SD4-5 and SD5-6 respectively; n = 356, 372, 325, 357 cells).

D Dot plot as in (B) of different SD5 cell populations (n = 323, 375, 387, 323, 453, 377, 424, 327 cells).

E LD trajectories as in (A) of cells on SD5, immediately before and 5 min after glucose addition.

F LD trajectories as in (A) of cells 60 min after glucose addition.

G Dot plots as in (B) showing LD dynamics at consecutive time points during starvation exit (3 independent cell populations, n = 354, 303, 442, 300, 400 cells).

H LD trajectories as in (A) of cells on SD5 immediately before and 60 min after glucose addition, showing LDs remaining immobilized in two of the cells (white arrowheads). Scale bars: 5 µm.
Figure 2 - Robust, cell wall-independent CF cell shape preservation without fluid loss.

A Protoplasts of cells incubated with cell wall digesting enzymes in hypertonic buffer, for 1 h (left panels) and for 3 days (right panel).

B Live imaging of an exponentially growing cell exiting its cell wall (white arrow) when incubated with cell wall digesting enzymes in hypertonic buffer.

C Cell debris (exponentially growing cells) and protoplasts (CF cells) respectively, of cells incubated with cell wall digesting enzymes in hypotonic buffer.
D Live imaging of CF cell exiting its cell wall (arrow) when incubated with cell wall digesting enzymes in hypotonic buffer.

E Cell length and width distribution measured from DIC images of exponentially growing cells and CF cells in standard culturing medium (-S) or incubated for 1h in hypertonic buffer (+S) (3 independent cell populations, n = 547, 391, 643, 404 cells). Boxes represent the 25-75 percentile. The blue line shows the mean of 3 experimental means. Error bars: 95% confidence interval.

F Ratios for cell lengths (yellow), widths (blue), or approximated volumes (red) comparing cells incubated for 1 h in hypertonic buffer to cells taken from standard growth medium (for details see methods). Dots represent means; error bars: 95% confidence interval of bootstrapped mean ratios (Methods).

G Dot plots showing the cell length (left) and cell width (right) distributions from SD3 to SD7. Boxes represent the 25-75 percentile. Blue lines show the mean of 3 means extracted from 3 independent cell populations (n = 1406, 1380, 1407, 1308, 1260 cells). Error bars: 95% confidence interval.

Scale bars: 5 µm.
**Figure 3 - Subcellular architecture in deep starvation.**

A  Electron micrographs of freeze-substituted, plastic-embedded, and sectioned cells during exponential growth (left) and on SD7 (right) showing vacuoles (v), low-density lipid droplets (ld) and tube-like (left panel) or spherical (right panel) mitochondria (m). Note that sections do not show the cell middle. Scale bar: 1 µm.

B  Mitochondria visualized by maximum intensity projections of cox4p-GFP during starvation. The fragmented mitochondria on SD6 are often polarized (white arrows) and mostly cortical, as seen in the single planes of a z stack (lower panel; cell marked by the dotted square in upper panel).

C  Mean square displacement of spherical mitochondria at SD3 and SD6 (300 frames, 4 fps, 6 independent experiments, n = 17439, 15948 particles; 15 particles per cell on average). Plotted are color-coded histograms of each particle’s time-averaged mean square displacement; Dotted lines show the ensemble- and time-averaged mean square displacements. The second plot of SD6 shows a zoomed y-axis. In the first two panels y-axis numbers are x10\(^3\), in the third panel x10\(^4\).

D  Comparison of exponentially growing and SD6 cells expressing a marker of the nucleus (nup85p-GFP), vacuoles (Spac11D3.06-GFP) or the ER (GFP-AHDL). The faint cytoplasmic dots nup85p-GFP cells at SD6 represent autofluorescence rather than nup85p-specific signal (see Fig. S2C). Images show single planes, deconvolved where indicated.

E  Fluorescence loss in photobleaching (FLIP) experiments on Lifeact-GFP in exponential growth (upper panel) and on SD6 (lower panel). Images show maximum intensity projections of 3 planes. Repeated bleaching at the orange square every 5 s. Plot shows bleach corrected and normalized mean signal intensity of white square. Thick lines: mean of pooled cells from 3 independent experiments (n = 27, 32 cells); error bars: 95% CI of the mean; dotted lines: normalized signal of single cells.

Scale bars in B, D, E: 5 µm.
Figure 4 - Interference with cytoskeleton does not affect CF.

A  Lifeact-GFP visualizing F-actin during starvation.

B  Lifeact-GFP from cells on SD6 that were incubated with LatB or DMSO from SD3 onwards.

C  LD trajectories extracted from 25 sec movies (4 frs, droplets depicted in lower DIC images) of wild type cells on SD6 incubated with DMSO or LatB from SD3 onwards.
**D** Dot plots (one dot per cell) showing correlation coefficient-based (CC) quantification of Bodipy-labelled LD dynamics of wild type cells on SD6 from 3 independent cell populations incubated with DMSO or LatB from SD3 onwards. Boxes represent the 25-75 percentile. The blue line represents the mean of the 3 medians extracted from 3 independent cell populations (n = 1539, 1148 cells). Error bars show the 95% confidence interval.

**E** LD trajectories as in (C) at SD5 of wildtype cells and cells carrying the temperature sensitive actin mutation cps8-188, after shifting temperature at SD4 (-20 h) from 25 °C to 36 °C.

**F** Dot plots as in (D) showing quantification of LD dynamics at SD5 of wildtype and cps8-188 cells, after shifting temperature at SD4 (-20 hours) from 25 °C to 36 °C as in (E) and of SD5 and SD6 respectively of the corresponding control cells kept at 25 °C throughout (n = 1056, 1059, 956, 907 cells).

**G** Localization of spn1-4p on SD6. The unspecific signal portion can be estimated from comparison to the autofluorescence from a SD6 wild type cell without fluorescent tag with the same imaging and contrast settings (insets).

**H** LD trajectories as in (C) of cells with spn1-4 deletions on SD6.

**I** Dot plot representation as in (D) showing quantification of LD dynamics in cells with spn1-4 deletions on SD6 (n = 1529, 1311, 1062, 1434, 1044 cells). Fluorescence images represent maximum intensity projections.

Scale bars: 5 µm.
Figure 5 - Autophagy mutants delay transition to CF state.

(A) Optical density (OD) measurement-based growth curves of wild type (grey), atg1Δ (blue), and atg8Δ (rose) cells (four independent cell populations each).

(B) LD trajectories extracted from 25 sec movies (4 frs, droplets depicted in lower DIC images) of atg1Δ and atg8Δ cells on starvation days SD6 and SD9.

(C) Dot plots (one dot per cell) showing correlation coefficient-based (CC) quantification of Bodipy-labelled LD dynamics for wild type, atg1Δ, and atg8Δ cells (3 independent cell populations, n = 657, 733, 481, 422, 521, 361, 598, 447, 296 cells). Boxes indicate the 25-75 percentile.

(D) Protoplasts of wild type, atg1Δ and atg8Δ at SD6 (left), SD8 (middle) and SD9 (right). Cell wall digestion for 4 h in 0.5 M sorbitol conditions.

Scale bars: 5 µm.
Figure 6 - CF differs from other solid like cytoplasmic states.

A  LD trajectories extracted from 25 sec movies (4 frs, droplets depicted in lower DIC images) of CF cells on SD6, of AGD cells and of DED cells with 2 or 0.5 h drug exposure.

B  Dot plots (one dot per cell) showing CC-based quantification of Bodipy-labelled LD dynamics in CF cells at SD6, in AGD cells and in DED cells with 2 or 0.5 h drug exposure.
Boxes represent the 25-75 percentile. The blue line represents the mean of the 3 medians extracted from 3 independent cell populations (n = 795, 562, 350, 447 cells). Error bars show the 95% confidence interval.

C Protoplasts of cells incubated with cell wall digesting enzymes in hypertonic buffer containing 1.2 M sorbitol for 1 h (left panel), 3 h (middle panel) and 3 days (left panel).

D Ratios calculated from measured length (yellow), width (blue), or approximated volume (red) of cells placed in the respective standard culturing medium, or in 1.2 M sorbitol containing buffer for 1 h. To estimate the variance of the 3 experiments, bootstrapping was performed (Methods). Dots represent means, error bars show the 95% confidence interval of the bootstrapped mean ratios estimated with Gaussian error propagation.

E Protoplasts or cell remnants from cells incubated with cell wall digesting enzymes in hypotonic buffer containing 0.5 M sorbitol until complete cell wall exit (20, 15, 10 min respectively).

F Live imaging of protoplast evasion from cell wall (white arrow) under 0.5 M sorbitol conditions. Upper panel: AGD cell, total time 1.5 s; lower panels: DED 2 h cells, total time 2 min and 5min respectively.

Scale bars: 5 µm.
Figure 7 - Fission yeast cells do not form macromolecular protein assemblies.

A Images show fluorescence signal of the indicated fusion proteins in exponentially growing cells (left panels) and DED cells (right panels) incubated for 0.5 or 2 h prior to imaging.
B Images show fluorescence signal of the indicated fusion proteins in exponentially growing cells (left panels) and SD6 cells (right panels). The unspecific signal portion can be estimated from comparison to the autofluorescence from a SD6 wild type cell without fluorescent tag with the same imaging and contrast settings (insets). Images are maximum intensity projections in all panels. All observations were confirmed in 2 independent experiments.

Scale bars: 5 μm.
Figure S1 - LD morphology and mobility analysis in starvation.

A Glucose measurement, comparing filtered medium of an SD2 cell culture (black cross) with culturing medium without added glucose (blue cross) and a standard curve of glucose in water at 0, 2, 4, 6, 8 mM (grey circles) with the calculated curve fit (R²=0.9995).

B LD composition changes from evenly distributed droplets in exponentially growing cells to grape-like LDs (white arrowheads) in cells on SD2. From SD3 to SD5 cells increasingly contain fewer but bigger LDs.

C LD trajectories as in Fig 1A, comparing cells containing grape-like LDs (white arrows; orange trajectories) and cells containing bigger LDs (blue trajectories). The left panel shows cells on SD4, the right panel on SD6.

D Images show Phloxine B-labelled cells (upper panel) as used to segment cells. Segmented cell outlines are shown in green (lower panel). Dead cells internalized Phloxine B (white arrow) and were excluded from segmentation and subsequent analysis.

E The upper panels show the Bodipy signal in the cells of the DIC images in the lower panels, during exponential growth, on SD3 and on SD6. White arrows depict individual cells with an extreme difference in Bodipy signal intensity.

F Bodipy-labelled cells on SD3 imaged at 3 consecutive time points (t1-t3), separated by 42s.

G Same cells as in (E) showing the Bodipy image pixels in the upper panels and in the lower panels the respective pseudoimages resulting from classification with Ilastik. The pseudoimages show the probability of pixels to belong to LDs as was then used to compute the CC between two imaged time points. Note that contrast settings for the same pictures differ in (D) and (F).

H LD trajectories as in Fig 1A, of a cell population in which no motion arrest occurred up to SD8.

Scale bars: 5 µm.
**Figure S2 - Autofluorescence and organelle markers in deep starvation.**

**A**  
Autofluorescence (excitation 488nm, emission 525/50) of wild type cells in exponential growth, on SD3 and SD6. Images show maximum intensity projections.

**B**  
Single focal plane images showing double labelling of gln1p-mCherry and the vacuolar marker Spac11D3.06-GFP.

**C**  
Images show fluorescence of markers for the indicated subcellular structures in exponentially growing cells and on SD6. The unspecific signal portion can be estimated from comparison to the autofluorescence from a SD6 wild type cell without fluorescent tag with the same imaging and contrast settings (to the right of dashed line). All images are maximum intensity projections.

Scale bars: 5 µm.
Figure S3 - Cytoskeleton in deep starvation.

A Microtubules visualized by GFP-atb2p during starvation.
B Effect of 100 µM LatB/DMSO on F-actin in exponential cells. Images represent maximum intensity projections.
C Same as (B) for SD6 cells.
D Spn1-4p localization in exponentially growing cells.
E GFP-tagged spn5-7p in exponentially growing cells (left; corresponding DIC images show cell location) and on SD6 (middle). The unspecific signal portion can be estimated from comparison to the autofluorescence from a SD6 wild type cell without fluorescent tag with the same imaging and contrast settings (to the right of dashed line). Fluorescence images represent maximum intensity projections.

Scale bars: 5 µm.
Figure S4 - Hypertonic environment induces volume loss in acutely energy depleted cells.

Cell length and width of AGD and DED (2 and 0.5 h) cells in standard culturing medium (-S) or in 1.2 M sorbitol containing buffer (+S) from 3 independent cell populations each, measured manually from DIC images (n = 341, 452, 324, 340, 371, 442). The blue line represents the mean of 3 independent experimental means. Error bars show the 95% confidence interval.
Figure S5 - GFP(S65T)-dependent protein assemblies.

A Images show fluorescence signal of the indicated fusion proteins with mGFP and GFP(S65T) respectively in exponentially growing cells (left panels) and DED cells (right panels) incubated for 0.5 or 2 h prior to imaging. Note that corresponding fusion proteins with mGFP and GFP(S65T) show the same imaging and contrast settings.

B Images show fluorescence signal of the indicated fusion proteins in exponentially growing cells (left panels) and SD6 cells (right panels). The unspecific signal portion can be estimated from comparison to the autofluorescence from a SD6 wild type cell without fluorescent tag with the same imaging and contrast settings (insets). Images are maximum intensity projections in all panels. All observations were confirmed in 2 independent experiments.
**Figure S6 - Protein levels in deep starvation**

Western blot of wild type control cells and cells expressing the indicated fusion proteins. anti-GFP (upper panel) and anti-actin antibodies (lower panel) were used for cells in exponential growth (EG) and cells at SD6. For better visualization loading of the different strains was adapted from left to right as follows: 10x, 1x, 5x, 2.5x, 10x, 2x, 2x.

Scale bars: 5 µm.
### Table S1: Strains used in this study

| Strains  | Genotype                                      | Source                                                                 |
|----------|-----------------------------------------------|------------------------------------------------------------------------|
| DB404    | h- sec63-GFP::kanMX6 ura4-D18 leu1-32         | (Yjestica et al., 2008)                                                |
| DB558    | h- wild-type                                  | (Leupold, 1950)                                                       |
| DB559    | h+ wild-type                                  | (Leupold, 1950)                                                       |
| DB933    | h- nup85-GFP::kanMX6 ade6-M216 leu1-32        | This study                                                            |
| DB2003   | h+ cnx1p-HL-GFP::kanMX6 ura4-D18 leu1-32 ade6-M216 ** | This study                                                            |
| DB2057   | h- SV40-GFP-ath2:::leu2 leu1-32              | (Pardo and Nurse, 2005)                                               |
| DB2400   | h+ anpl-GFP::ura4 ade6-216 ura4-D18 leu1-32  | (Yjestica et al., 2008)                                               |
| DB2401   | h+ sec72-GFP::ura4 ade6-216 ura4-D18 leu1-32 | (Yjestica et al., 2008)                                               |
| DB2402   | h+ anpl-mCherry::ura4 ade6-216 leu1-32        | (Yjestica et al., 2008)                                               |
| DB2403   | h+ uch2-mCherry::ura4 ade6-216 leu1-32        | (Yjestica et al., 2008)                                               |
| DB2404   | h- ost1::GFP-ura4 ura4-D18 leu1-32            | (Yjestica et al., 2008)                                               |
| DB2405   | h+ ost1-mCherry::ura4 ade6-210 leu1-32        | (Yjestica et al., 2008)                                               |
| DB3287   | h- spn1::kanMX6                               | (Wu et al., 2010)                                                     |
| DB3293   | h+ spn2::ura4+ ura4D18                       | (An et al., 2004)                                                     |
| DB3297   | h+ spn2-GFP::kanMX6 *                        | (An et al., 2004)                                                     |
| DB3310   | h+ spn3-GFP::kanMX6 *                        | (An et al., 2004)                                                     |
| DB3324   | h+ spn4::kanMX6 *                            | (Wu et al., 2010)                                                     |
| DB3326   | h+ spn5-HL-GFP::kanMX6                       | This study                                                            |
| DB3340   | h+ spn6-HL-GFP::kanMX6                       | This study                                                            |
| DB3410   | h- leu1-32::pAct1-Lifeact-GFP::leu1 *         | (Huang et al., 2012)                                                  |
| DB3422   | h- spn1-GFP::kanMX6 *                        | (Wu et al., 2010)                                                     |
| DB3426   | h+ spn3::ura4 ura4-D18                       | (An et al., 2004)                                                     |
| DB3455   | h+ spn7-GFP::kanMX6 *                        | (Onishi et al., 2010)                                                 |
| DB3587   | h+ spn4-TagRFP::kanMX6                      | This study                                                            |
| DB3623   | h- Pbp1-GFP-AHDL::leu1 ura4-D18 leu1-32 ade6 | (Zhang et al., 2010)                                                  |
| DB3624   | h+ Pbp1-mCherry-AHDL::leu1                   | (Zhang et al., 2010)                                                  |
| DB3726   | h+ Cox4-GFP::leu2 leu1-32 *                  | (Fu et al., 2011)                                                     |
| DB3856   | h- spn104-mCherry::kanMX6 (hsp104***          | (Coelho et al., 2013)                                                 |
| DB4192   | h- gma12-GFP::ura4 ura4-D18 *                | (Wang et al., 2002)                                                   |
| DB4233   | h- cps8-188                                  | (Ishiguro and Yamada, 1993)                                            |
| DB4672   | h+ Pnmt1-TM-mCherry::leu                     | (Zhang et al., 2012)                                                  |
| DB5013   | h- atg8::kanMX6 *                            | Bioneer M-403H-U5 (Kim et al., 2010)                                  |
| DB5018   | h- atg1::kanMX6 *                            | Bioneer M-403H-U5 (Kim et al., 2010)                                  |
| DB5160   | h- cts1-HL-GFP::kanMX6 (ura7****)             | This study                                                            |
| DB5162   | h- pre6-HL-mGFP::kanMX6 (pre6****)            | This study                                                            |
| DB5209   | h+ suc22-GFP (mr4****)                       | (Vejrup-Hansen et al., 2014)                                          |
| DB5310   | h- gln1-mCherry::natR (gln1****)             | (Coelho et al., 2014)                                                 |
| DB5315   | h+ dis2-NEGFP::ura4 ura4-D18 * (glc7****)     | (Alvarez-Tabarés et al., 2007)                                        |
| DB5320   | h+ adh1-GFP::kanMX6 * (adh2****)             | (Sigova et al., 2004)                                                 |
| DB5380   | h+ cts1-mCherry::kanMX6 *(ura7****)           | (Coelho et al., 2014)                                                 |
| DB5381   | h- hsp104-GFP::kanMX6 * (hsp104****          | (Coelho et al., 2013)                                                 |
| DB5470   | h- tif211-HL-GFP::kanMX6 (gcn3****)           | This study                                                            |
| DB5730   | h+ nmt1::GFP-HL-Spac11D3.06::kanMX gln1-mCherry::natR | This study                                                            |
| DB5869   | h- tif211-HL-mGFP::kanMX6 (gcn3****)          | This study                                                            |
| DB5870   | h- tif211-HL-mCherry::kanMX6 (gcn3****        | This study                                                            |
| DB5871   | h- cts1-HL-mGFP::kanMX6 (ura7****)            | This study                                                            |
| DB5872   | h- hsp104-HL-mGFP::kanMX6 (hsp104****        | This study                                                            |

* auxotrophic alleles were eliminated by crossing to wild type (DB559); ** HL = “happy linker” (Methods); *** S. cerevisiae orthologs
Table S2: Proteins tested for assembly formation

| gene name | product | GO cellular component | Fusion protein localisation |
|-----------|---------|-----------------------|-----------------------------|
|           | https://www.pombase.org/ | https://www.pombase.org/ | mCherry | mGFP | GFP(S65T) |
|           |         |                       | EG | DED 0.5/2h | SD6 | EG | DED 0.5/2h | SD6 | EG | DED 0.5/2h | SD6 |
| adh1      | alcohol dehydrogenase         | -                       | na | na | na | na | na | na | C, N | C, N | (C, N) |
| suc22     | ribonucleotide reductase small subunit | cytosol, nucleus, ribonucleoside-diphosphate reductase complex | na | na | na | na | na | na | N | N | N |
| pre6      | 20S proteasome complex subunit alpha 4 | cytosol, nuclear periphery, proteasome core complex | na | na | na | C, N | C, N | (C, N) | na | na | na |
| dis2      | serine/threonine protein phosphatase PP1 | cytosol, nucleus, cell tip, nuclear chromatin, cell division site, DPS complex | na | na | na | na | na | na | C, N | C, N | C, N |
| gln1      | glutamate-ammonia ligase       | cytosol, nucleus        | C, N | C, N | nd | na | na | na | na | na | na |
| tif221    | translation initiation factor eIF2B alpha subunit | cytosol, eukaryotic translation initiation factor 2B complex | C | C | (C) | C | C | (C) | C | A | (C) |
| hsp104    | heat shock protein             | cytosol, nucleus, nuclear envelope | C, N | C, N | nd | C, N | C, N | N | A | A | N |
| cts1      | CTP synthase                   | cytosol                 | C, N | C, N | nd | C, N | C, N | nd | A | A | nd |

C = cytosolic, N = nuclear, A = assemblies present, na = not analysed, nd = not detectable
Table S3: Primers used in this study

| DB   | Primer 1          | Sequence                                                                                   | Primer 2          | Sequence                                                                                   |
|------|-------------------|-------------------------------------------------------------------------------------------|-------------------|-------------------------------------------------------------------------------------------|
| DB3340 | DPE872           | CTGAATATCAGACAAAGGATCCGTCTTTGGAGGACCACCAATTGAAGGTGTTAAAGTTTGAACCTGTCATACAAATGCTAGCTGCTTTGAAGCTCCTCCAGAATCTGGAGCTTC | DPE873           | AATGGCAATAATTGAGGAAGCCATCATAGTACATGTTAAAGTTTGAAGGTGTTAAAGTTCATCCGAGACGTCCCTCTG |
Movie 1 - Motion arrest of LDs in deep starvation

25sec DIC movies (4frames/sec) of cells in exponential growth and starvation days 2-8. Upper panels from left to right show cells in exponential growth and on starvation days 2, 3 and 4. Lower panels show cells at starvation days 5, 6, 7 and 8. Scale bar: 5\(\mu\)m.

Movie 2 - CF exit following glucose addition

25sec DIC movies (4frames/sec) of cells on starvation day 5 before and after glucose addition. Upper panels from left to right show cells before glucose addition and 5min or 10min after glucose addition. Lower panels show cells 15min, 30min and 60min after glucose addition. Scale bar: 5\(\mu\)m.
Movie 3 - Protoplasting of exponentially growing cells in hypertonic conditions

DIC movies (1 frame/10 sec) of protoplast evasion from the cell wall of exponentially growing cells in a dish. Cells are being incubated in 1.2M sorbitol containing cell wall digesting mix. Note that the bottom cell is undergoing cytokinesis. Scale bar: 5µm.
Movie 4 - Protoplasting of cells in hypotonic conditions

DIC movies (1 frame/10 sec) of cells, incubated on a dish with cell wall digesting enzymes in a hypotonic buffer containing 0.5 M sorbitol. Left panel: cells in exponential growth, right panel: CF cells on starvation day 6. Scale bar: 5 µm.

Movie 5 - Mitochondria dynamics during starvation

Movies (2 frames/3 sec) of mitochondria visualized by maximum intensity projections of cox4-GFP expressing cells during starvation. Panels show from left to right cells in exponential growth, cells on starvation day 3 and cells on starvation day 6. Scale bar: 5 µm.
**Movie 6 - F-actin organization and dynamics in starvation**

Movies (1 frame/5 sec) visualizing F-actin dynamics based on maximum intensity projections of Lifeact-GFP expressing cells. Upper panels show from left to right, exponentially growing cells and cells at starvation days 2, 3 and 4 respectively. The lowers panels show cells at starvation days 5, 6, 7 and 8. Scale bar: 5 μm.

**Movie 7 - CF in F-actin depleted cells**

25 sec DIC movies (4 frames/sec) of wild type cells at starvation day 6 incubated from starvation day 3 onwards with DMSO (control, left panel) or LatB. Scale bar: 5 μm.
Movie 8 - Interference with autophagy delays CF
25sec DIC movies (4frames/sec) of, from left to right, wild type, atg1Δ and atg8Δ cells at starvation days 6 (upper panels) and 9 (lower panels). Scale bar: 5µm.

Movie 9 - LD motion in cells suffering of acute energy depletion
Left to right: 25sec DIC movies (4frames/sec) of AGD cells and DED cells with 2h and 0.5h drug treatment respectively. Scale bar: 5µm.
Movie 10 - Protoplasting of cells in hypotonic conditions

DIC movies (1 frame/10 sec) of cells, incubated on a dish with cell wall digesting enzymes in 0.5M sorbitol containing buffer. Panels show from left to right, AGD cells and DED cells with 2h and 0.5h drug treatment respectively. Scale bar: 5µm.