

Title

**Synergistic Roles of the Proteasome and Autophagy for Mitochondrial Maintenance and Chronological Life Span in Fission Yeast**

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**Abbreviations:** ROS (Reactive Oxygen Species), VEG (Vegetative proliferation)

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## Abstract

Regulations of proliferation and quiescence in response to nutritional cue are important for medicine and basic biology. The fission yeast *Schizosaccharomyces pombe* serves as a model, owing to the shift of proliferating cells to the metabolically active quiescence (designate G0-phase hereafter) by responding to low nitrogen source. *S. pombe* G0-phase cells keep alive for months without growth and division. Nitrogen replenishment reinstates vegetative proliferation phase (designate VEG). Some forty genes required for G0 maintenance were identified, but there still remain many to be identified. We here show using mutants that the proteasome is required for maintaining G0 quiescence. Functional outcomes of proteasome in G0 and VEG-phases appear to be quite distinct. Upon proteasome dysfunction, a number of anti-oxidant proteins and compounds responsive to ROS (reactive oxygen species) are produced. In addition, autophagy-mediated destruction of mitochondria occurs, which suppresses the loss of viability by eliminating ROS-generating mitochondria. These defensive responses are found in G0 but not in VEG, suggesting that the main function of proteasome in G0-phase homeostasis is to minimize ROS. Proteasome and autophagy are thus collaborative to support life span of *S. pombe* G0-phase.

## Introduction

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The transition between cell proliferation and G0/quiescent phase is finely controlled in response to extracellular conditions, such as growth factors and nutrient availability(1-3). The regulation of entry and exit to/from G0 phase is strongly relevant to tumorigenesis and tissue regeneration. Failure to maintain terminally differentiated cells in the healthy G0 phase may also lead to diseases such as neurodegeneration. Therefore, regulations of G0 entry, exit and maintenance are medically important topics.

The fission yeast *Schizosaccharomyces pombe* has been adopted as an excellent model organism for cell cycle research and cell biology, owing to its genetic tractability and similarity to higher eukaryotic cells. Like mammalian cultured cells, which are introduced to G0 phase by serum starvation(1), proliferating cells of the fission yeast enter G0 phase in response to low nitrogen(4-6). Following nitrogen withdrawal from the medium, fission yeast cells immediately cease cell growth, perform division twice and arrest in G0 phase (G0 entry). Arrested G0 cells are metabolically active, viable for more than one month (G0 maintenance) and reinstate proliferation by nitrogen replenishment (G0 exit). We take advantage of this feature of fission yeast and adopt this yeast as a model organism to study the regulatory mechanisms of G0 phase. Previously, we identified 7 genes required for G0 entry and 36 genes for maintenance(5, 6), including regulators of transcription, chromatin dynamics, vesicle transport, and the actin cytoskeleton. However, many other factors may remain unidentified.

The ubiquitin/proteasome system, a major proteolytic mechanism in the cell(7), is one possible candidate pathway that may be essential for G0 phase. The ubiquitin/proteasome system is involved in cellular essential pathways, such as cell cycle control and protein quality control. The proteasome is required for M phase progression(8), because the proteasome is responsible for the degradation of the mitotic regulators, Cyclin B and Securin(9-13). In fission yeast, the proteasome is localized to the nucleus, which guarantees the rapid degradation of Cdc13/Cyclin B and Cut2/Securin in the nucleus(14-16). In G0 phase, however, the essential roles of the proteasome have not been understood well so far. In this study, we attempt to elucidate essential proteasomal functions specific to G0 phase. We found the proteasome complex is exported from the nucleus to the cytoplasm upon G0 entry. Inactivation of the proteasome in G0 phase is lethal and leads to the accumulation of cellular reactive oxygen species (ROS) and massive degradation of mitochondria by autophagy (mitophagy), which inhibits further accumulation of ROS. We suggest that the proteasome and selective mitophagy cooperatively contribute to G0 maintenance via reducing lethal accumulation of ROS.

## Results

Striking alteration of the 26S proteasome localization was observed during the shift from VEG to G0 phase of *S. pombe*. Green fluorescent protein (GFP) tagged to  $\alpha 4$  (20S component) or Pad1 /Rpn11 (19S component) was chromosomally integrated under the native promoter and used to locate the 26S proteasome. In VEG, the proteasome is enriched in the nucleus and nuclear envelope(14-16). In G0, nuclear localization signals of  $\alpha 4$ - and Pad1-GFP were greatly diminished with the increase of cytoplasmic GFP-signals, while the signal on the nuclear periphery persisted (Fig. 1a and Fig. S1; G0 and VEG cells are, respectively, round- and rod-shaped). The signals were not intrinsic fluorescence, as cells without GFP-tagging showed no fluorescence (Fig. 1a). The proteasome signals remained in the nucleus for 6h after the removal of nitrogen (–N), when most cells had finished the second division and were competent to mate if cells with the opposite mating type existed. The nuclear proteasome was diminished at 12h at the timing of G0 entry(6) (Fig. 1b). The level of proteasome subunit was unchanged during VEG to G0 transition, while the level of Cdc13 mitotic cyclin greatly reduced(6) (Fig. 1c). The cytoplasmic  $\alpha 4$ -GFP signal returned to the nucleus following treatment with the Crm1/exportin inhibitor leptomycin B (LMB)(17) (Fig. 1d), indicating that the proteasome export to cytoplasm increased at the G0 shift.

To examine whether proteasome-mediated proteolysis existed in G0-phase, we first isolated the 26S proteasome from both VEG and G0 extracts and analyzed the compositions by mass spectrometry (Fig. S2). Reported subunits and accessory proteins of the 26S complex(18) were all present in both VEG and G0, indicating that the regular 26S proteasome existed in G0. Next, proteolysis and ubiquitination in G0 were examined using *mts3-1*(8), a temperature-sensitive mutant of the proteasome subunit Mts3/Rpn12. Cut8 (a proteasomal localization factor whose degradation in VEG depends on proteasomal activity(15, 16)) found to be short lived in G0 like in VEG phase (Fig. 1e). After the addition of cycloheximide, a protein synthesis inhibitor, the level of Cut8 was diminished within 15 min in both VEG and G0 phase in wild type (WT), but not in the *mts3-1*, suggesting that Cut8 was actively degraded in a proteasome-dependent manner at a similar rate in both VEG and G0. Indeed the level of poly-ubiquitinated proteins in G0 was already high in *mts3-1* at a permissive temperature (26°C), and further increased at the restrictive temperature (37°C) (Fig. 1f). These results established that 26S proteasome-mediated proteolysis was active in G0-phase.

The proteasome was essential for maintenance of the viability in G0. The *mts3-1* mutant decreased to 40% viability after 24h at 37°C, which further decreased to 0.1% after 72h at 37°C in G0 (Fig. 1g). WT sustained high viability (>80%) after 72 h at 37°C, and the

G0-arrested *mts3-1* cells did not lose viability at all for at least one week at 26°C (Fig. S1). The other proteasome mutant *pad1-932* also lost the viability in G0 at 37°C (Fig. S1).

Thin-section transmission electron microscopy (TEM) of WT and *mts3-1* (Fig. 1h-j) revealed abnormalities in cytoplasm and the nucleus in *mts3-1* at 37°C. First, mitochondria (indicated by m in WT at 37°C after 24h; Fig. 1h) were greatly diminished in *mts3-1*. Secondly, aberrant electron-dense materials (small arrow) and vesicle-like structures (arrowhead) formed in the mutant nucleus after 24h (Fig. 1i) and the nuclear damages further progressed after 72h (Fig. 1j). Nuclear chromatin structure stained by DAPI (inset) also became deformed. Unexpectedly, the intra-nuclear vesicle-like structures may be lipid-containing, as they were stained by Nile-Red that was used to reveal lipid droplets (storage particles) normally present in cytoplasm(19) (Fig. S3). A notable mutant phenotype at 26°C was that *mts3-1* divided only once and arrested after nitrogen withdrawal (Fig. 1k), while WT cells divided twice before arrest(6). The reason why the second division was omitted in *mts3-1* is unknown.

The decrease of mitochondria in G0 proteasome mutant cells 24h after the shift to 37°C was verified by Mitotracker Green stain(20) (Fig. S4). We then made GFP-tagged Sdh2 (succinate dehydrogenase subunit, a mitochondrial protein) integrated at the C-terminus of the chromosomal *sdh2<sup>+</sup>* gene. Resulting Sdh2-GFP signals expressed under the native promoter in WT and *mts3-1* mutant at 26°C behave like a mitochondria marker, whereas the GFP signal was greatly diminished in *mts3-1* after 24h at 37°C (Fig. 2a and Fig. S4). The protein levels of Sdh2-GFP and Gcv1-FLAG (mitochondrial glycine decarboxylase, SPAC31G5.14) detected by immunoblot showed the time course decrease in *mts3-1* at 37°C after 12h (Fig. 2b). The decrease of mitochondrial protein Sdh2-GFP was observed in other proteasome mutants (*pad1-932*, *pts1-732* mutated in the  $\beta 5$  subunit, *ump1-346*, -620 mutated in the 20S assembly factor(21); Fig. S4). In VEG phase, however, the Sdh2-GFP signal did not decrease at all after a temperature shift to 37°C, when the mutant ceased dividing completely and viability was reduced to 15% (Fig. 2c). Immunoblot (Fig. 2d) showed that Sdh2-GFP did not decrease in VEG even after 24h at 37°C (viability decreased to 0%). These results established that the marked degradation of mitochondria occurred specifically in G0 upon proteasomal inactivation.

To determine whether all of principal mitochondrial proteins disappeared in *mts3-1* mutant in G0, comprehensive proteomic analyses were performed, using liquid chromatography-tandem mass spectrometry(22) (LC-MS/MS; ThermoFisher LTQ). Proteins were extracted from WT and *mts3-1* cells at 37°C for 12h in G0 (control extracts made were from VEG cells cultured for 6h at 37°C). To compare abundance of individual proteins, the

scatter plots were used (Fig 2e-h). Location of each dot (inset) in the logarithmic X- and Y-axis indicates the protein abundance(22, 23) in the two extracts: when the levels of proteins between the two samples are identical or similar, the dots are distributed along the central diagonal line. In WT G0 cells, ~2,000 proteins were detected by LC-MS/MS, in which 256 (light blue dots) were mitochondrial proteins (a total of 696 mitochondria proteins among the 5,000 whole proteins reported in the Sanger Institute GeneDB). In two independently cultured WT G0 cell extracts (37°C, 12 h; Fig 2e), 97% of the 1917 detected proteins were located within the boundary of a 4-fold change in the two samples (indicated by two lines 4.0x and 0.25x). In contrast, 89% of 2043 proteins detected from WT and *mts3-1* G0 cells (37°C, 12h) were within a 4-fold change (Fig. 2f). The abundance of 125 proteins decreased to less than 0.25-fold in *mts3-1* (outside of upper diagonal line), and 60 of them (48%) were mitochondrial proteins (listed in the green columns of Table. S1). Three most greatly reduced (down to 1-5% in comparison with the levels in WT G0) proteins, Sdh2, Cyc1 (cytochrome c) and Ilv5 (acetohydroxyacid reductoisomerase) were mitochondrial proteins. These results showed that many, not all, of mitochondrial proteins reduced their levels in *mts3-1* G0 phase at 37°C. No particular type of mitochondrial proteins was specifically degraded. The experiment was replicated in VEG phase (Fig. 2g to h). As expected from cytological results, the proteomic decrease of mitochondrial proteins was insignificant.

The scatter plot of G0 proteomics data (Fig. 3a) revealed 110 proteins that showed a >4-fold increase in *mts3-1* versus WT at 37°C (Table. S2). Among them, the greatest increase (146-fold) was Hsp16 that belongs to the HSP20/alpha-crystallin heat shock protein family. Hsp16 increased not only by the temperature upshift, but also by cadmium, nutrient starvation, and DNA damage(24). Three others, SPAC11D3.01c (indicated by 1; similar to *N. crassa* conidation protein 6), short chain dehydrogenases SPCC663.06c and SPCC663.08c (2 and 3, respectively) that prominently (>20-fold) increased in *mts3-1* are known to increase in the presence of H<sub>2</sub>O<sub>2</sub>(25). SPAC83.17 (4), Multi protein bridging factor 1 [Mbf1] involved in transcriptional regulation of many process such as lipid metabolism(26), increased 33 fold in *mts3-1* and is responsive to both heat (>5-fold) and cadmium (~2.5-fold) like Hsp16. Indeed many (30%) of 110 proteins that increased >4-fold in *mts3-1* are responsive to H<sub>2</sub>O<sub>2</sub> and/or cadmium stress (colored dots, see captions of Fig 3a). Proteasome dysfunction seems to activate a number of defensive functions directly (H<sub>2</sub>O<sub>2</sub> responsive) and indirectly (cadmium responsive) against oxidative stresses(27).

To examine whether metabolic compounds against oxidative stress were produced in G0 *mts3-1*, metabolomic analysis was undertaken using LC/MS(28). Glutathione (GSH) and ergothioneine, both authentic anti-oxidant metabolites(29), were accumulated in

G0 *mts3-1* (>10-fold; Fig. 3b). Three duplicate experiments produced similar increase. GSH and ergothioneine also accumulated in different proteasome mutant, *pts1* -727 (Fig. S5).

H<sub>2</sub>DCFDA, a chemical compound that produces fluorescence upon the reaction with ROS, was used to stain WT and *mts3-1* cells(30). As seen in Fig 3c, the fluorescent signals were strong in *mts3-1* G0 phase after 24h (viability = 36%), while WT G0 phase cells showed only weak fluorescence. H<sub>2</sub>DCFDA signals began to accumulate in *mts3-1* 12h after the temperature upshift (Fig. S6). At that time point, the viability was high (~80%) so that fluorescence was not generated by dead cells. In VEG, there appeared to be no accumulation of ROS in *mts3-1* after the temperature upshift (viability =15%). The double staining *mts3-1* G0 cells with H<sub>2</sub>DCFDA and Mitotracker showed that the strong signals of H<sub>2</sub>DCFDA were located in the mitochondria and nucleus (Fig. 3d), suggesting that ROS accumulated in the nucleus and mitochondria in *mts3-1* G0.

To explain results described above, we speculated that ROS was generated in G0 *mts3-1* due to the breakdown of mitochondria, but following results suggested that this was not the case. First, it was found that phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, when added to the culture, inhibited a decrease of Sdh2-GFP and Cox2 in G0 *mts3-1* (Fig. 4a). PMSF was reported to inhibit proteolysis in *S. pombe* vacuoles(31), so we examined the involvement of autophagy pathway. The double mutant of *mts3-1* and deletion  $\Delta atg8$  was constructed (Atg8, LC3 homolog, is involved in autophagosome formation(32)) and observed using Sdh2-GFP as the marker of mitochondrial destruction. In the double mutant cells at 24h (37°C), the level of Sdh2-GFP did not decrease (Fig. 4b) and the mitochondrial degradation was virtually absent (Fig. 4c). These findings indicated that mitochondrial degradation in *mts3-1* required Atg8. A surprising outcome was the synthetic lethality of the double mutant in G0 as shown in Fig 4d. The viability of the double mutant sharply decreased after 12h and reached 3%, whereas more than 50% viability was maintained in the single *mts3-1*. Corroborating finding was that the accumulation of H<sub>2</sub>DCFDA fluorescence was observed already in the double mutant at 6h whereas there was no accumulation in the single *mts3-1* at 6h (Fig. 4e). After 24h, fluorescence was much greater in the double mutant *mts3-1*  $\Delta atg8$  than in *mts3-1*. As shown in Fig 4e and Fig. S7, H<sub>2</sub>DCFDA signals were strong along the mitochondria in *mts3-1*  $\Delta atg8$ , suggesting that autophagy actually caused the reduction of ROS (perhaps within mitochondria) by degrading mitochondria that was impaired in G0-phase *mts3-1*. In  $\Delta atg8$  cells, the H<sub>2</sub>DCFDA fluorescence was not significantly different from WT after 24h (Fig. S7). After longer incubation in G0 (two to three weeks) at 26°C, however, the fluorescence was stronger in  $\Delta atg8$  cells, accompanied with the viability decrease after 21 days (Fig. S7). Therefore,

autophagy, though the degree is much less than proteasome function, is also required for long-term survival in G0. This is consistent with the previous report(31).

N-acetyl cysteine (NAC), an anti-oxidant, was added to the G0 culture to examine the effect on H<sub>2</sub>DCFDA fluorescence (Fig. 4e). The fluorescence in *mts3-1Δatg8* was significantly reduced after 6h and 24h. Consistently, the viability of the double mutant with NAC was much higher (83%, 47%, and 39%, respectively, at 20, 24, and 27 h) than the viability of double mutant without NAC (16%, 3%, and 0.3%, respectively, Fig. 4f). The severe lethality of the double mutant might thus be due to the abundance of ROS. Taken together, we concluded that mitochondrial degradation by autophagy is one of many anti-ROS protection events that occurred after proteasome dysfunction in G0 phase.

## Discussion

A principal conclusion in this study is that *S. pombe* G0 cells require proteasome function during the maintenance of G0 quiescence. This conclusion was obtained using several ts mutants in the 26S proteasome subunits. Proteasome genes belong to the group of super housekeeping(6) because of their necessity for both proliferation and quiescence. Note that only 25% of the essential genes in VEG are also needed for G0 maintenance(6): many genes essential for proliferation such as cell division and DNA replication are not necessary for G0 maintenance. The second conclusion is that proteasome functions are quite different between G0 and VEG phase. This is based on striking defective phenotypes of proteasome mutants in G0 phase, regarding the huge increase of oxygen stress responsive compounds and the massive decrease of mitochondria that did not occur in VEG phase. How this difference is explained? The targets of proteasome may change from VEG to G0, more cytoplasmic ones in G0, which may lead to the export of proteasome to cytoplasm in G0 cells. In carbon starved cells of the fission yeast, the proteasome is also exported reversibly to the cytoplasm(33). Necessity of recycling proteins in G0(6) may require a number of cytoplasmic activities for proteasome, whilst in VEG events such as replication, rRNA transcription, mitosis and chromatin dynamics occur in the nucleus. Actual targets of proteasome in G0 remain to be clarified, however. In the quiescent/stationary phase of *Saccharomyces cerevisiae*, Aah1p (adenine deaminase) was reported as a substrate of the proteasome, while its proteolysis is not required for the maintenance of the quiescence(34).

The third, closely related to the second, conclusion of this study is that proteasome dysfunction in G0 elicits defensive responses, mainly the production of anti-oxidant components and the degradation of mitochondria by autophagy. These defensive responses were not found in VEG cells, suggesting that proteasome functions in G0 are directly or

indirectly involved in minimizing ROS. We provide evidence that viability loss in G0 is caused by the accumulation of oxidative stress. In addition, autophagy-mediated mitochondrial degradation as a matter of fact saved proteasome mutant G0 cells. In other word, proteasome collaborates with autophagy in supporting the longevity of G0 cells. Beside the loss of viability, physically aberrant structures were observed in G0 *mts3-1* mutant. Initially observed were the accumulation of electron-dense materials and the internal vesicles in the nucleus. At this timing, viability was high, suggesting that an abnormality in nuclear envelope or nuclear-cytoplasmic shuttling might be initial events that lead to the accumulation of ROS and mitochondrial dysfunction. Note that most of mitochondrial proteins are encoded by chromosomal genes. Aberrant mitochondria possibly producing a bulk of ROS then form, which in turn activate autophagy-dependent mitochondrial degradation. Our results are consistent with this hypothesis. Proteasome and autophagy seem to support life of non-dividing G0 phase cells (chronological life span). Our metabolomic and proteomic analyses identify anti-oxidant metabolites (glutathione, ergothioneine) and many ROS-defensive proteins that increase their levels in *mts3-1* G0 cells. In mammalian neurons, inactivation of the proteasome leads to ROS accumulation, mitochondrial injury and cell death (35-37), which are related to neurodegeneration. Injured mitochondria may be degraded by autophagy (mitophagy). Recent studies suggested that several proteins are involved in mitophagy, such as Atg32 (in budding yeast)(38, 39) and Park2 (E3 ligase responsible for Parkinson disease)(40, 41). Although neither of them has obvious homologues in *S.pombe*, there could be a common mechanism, by which injured mitochondria might be scavenged. Proteasome inhibitors, PS-519 and PS-341, are recently developed for the therapeutic purpose of inflammation and cancer such as multiple myeloma. The present results that proteasome dysfunction produces the autophagy-mediated degradation of mitochondria accompanying a number of defensive cellular functions against the elevated level of ROS, which may cause apoptotic removal of the drug-target mammalian cells, might explain some aspects of these drug effects.

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### **Figure legends**

**Fig. 1**

Proteasome-enriched cytoplasm is required for the maintenance of G0 cells.

**(a)** Upper: proteasome localization in VEG and G0.  $\alpha 4$  subunit and Histone H2A were tagged with green and red fluorescent protein, respectively. G0 cells (lower left, two round cells) and VEG cells (upper right, the rod-shaped cell) were independently prepared and mixed on a glass slide. Scale bar: 2.5 $\mu$ m. Lower: negative controls of no-GFP tagged strain. Scale bar: 5 $\mu$ m. **(b)** Time-course analysis of proteasome localization after –N. **(c)** Protein amounts in (b).  $\alpha$ -TUB represents  $\alpha$ -tubulin as a loading control. **(d)** Incubating G0 culture with 250 nM leptomycin B for 8 h induced a change in the proteasome localization from the cytoplasm to the nucleus. **(e)** The half-life of Cut8, a proteasome substrate, was examined in both G0 and VEG of WT and *mts3-1*. Proteins were extracted at the indicated time points after adding cycloheximide (100  $\mu$ g/ml) and proteins were detected by immunoblotting. **(f)** The amount of poly-ubiquitinated proteins was examined in both WT and *mts3-1* mutants in G0. **(g)** The change of viabilities of WT and *mts3-1* were in G0. **(h-j)** TEM analysis of G0 cells (scale bars: 0.5 $\mu$ m). Arrow: an electron-dense deposit. Arrowhead: the lipid droplet in the nucleus. m: a mitochondrion (no mitochondria were visible in (i, j)). N: the nucleus. Insets show fluorescent images of chromosomal DNA stained by DAPI (scale bars: 2.5 $\mu$ m). **(k)** *mts3-1* mutant cells divided once after –N, whereas WT divided twice.

## Fig. 2

The number of mitochondria was drastically decreased in proteasome mutants in G0.

**(a)** Mitochondria were visualized in G0 using Sdh2-GFP as a marker (green). Cell walls were stained by Calcofluor (white). Scale bar: 2  $\mu$ m. **(b)** Mitochondrial protein amounts were decreased in *mts3-1* at restrictive temperature (37°C) in G0. 24\* is the sample extracted from the culture at 26°C for 24h. **(c)** Mitochondria in VEG. **(d)** Sdh2-GFP levels remained constant at 37°C in VEG. **(e)** Scatter plot of emPAI values (protein abundance, see method) of proteins detected in two different extracts from WT cells, which were independently cultured at 37°C for 12h in G0. A part of the plot is enlarged in the inset to reveal each protein. Mitochondrial proteins are shown in green and others in blue. **(f)** Scatter plot of proteins abundance in both WT and *mts3-1* cells, which were cultured at 37°C for 12h in G0. For both strains, the experiments were repeated twice and the mean emPAI values were analyzed. The names of the proteins with the lowest levels in *mts3-1* are shown. **(g and h)** The same experiment as shown in (e and f) was performed in VEG.

## Fig. 3

ROS accumulated in proteasome mutants in G0.

**(a)** The same experiment as shown in Fig. 2f. Purple, yellow and pale blue dots represent proteins, which are transcriptionally induced over 3-fold by H<sub>2</sub>O<sub>2</sub>, cadmium (Cd) and both of them, respectively. The proteins with the highest levels in *mts3-1* are indicated by numbers (1~4; see text). **(b)** Metabolic analysis revealed glutathione (reduced form; GSH) and ergothioneine in *mts3-1* in G0. Chromatogram patterns of each compound separated by HPLC. Values in red represent the integrated peak area (Y-axis). See text and materials. **(c)** The H<sub>2</sub>DCFDA signal, an indicator of ROS, accumulated in *mts3-1* cells in G0, but not VEG. The time points shown are 0 h and 24h after the temperature shift to 37°C. The viability at each time point is also presented (%). Scale bar: 5µm. **(d)** The H<sub>2</sub>DCFDA (green) signal accumulated in the nucleus (N) and mitochondria (m) in *mts3-1* cells 12h after the temperature shift. Mitochondria were stained by Mitotracker Orange (red). Scale bar: 2 µm.

#### Fig. 4

Autophagy is involved in degrading mitochondria to reduce the lethal accumulation of oxidative stress in G0.

**(a)** PMSF (2 mM) inhibited the decrease of mitochondrial proteins in *mts3-1* in G0. **(b)** Sdh2-GFP signals (green) maintained a tubular structure in the *mts3-1Δatg8* double mutant, whereas the signal was greatly decreased in the *mts3-1* mutant. Both cells were cultured in G0 at 37°C for 24h. Scale bar: 2µm. **(c)** The amount of Sdh2-GFP proteins was kept constant in the *mts3-1Δatg8* double mutant. **(d)** The viabilities of WT (blue), *mts3-1* (red), *mts3-1Δatg8* (orange), and *Δatg8* (green) were examined after the temperature shift to 37°C in G0. **(e)** The accumulation of oxidative stress was examined using H<sub>2</sub>DCFDA. In the *mts3-1Δatg8* double mutant, but not in *mts3-1*, stronger H<sub>2</sub>DCFDA signals appeared 6 h after the temperature shift. At 24 h, the signal in the double mutant was still stronger than that in *mts3-1*. The addition of 30 mM N-acetyl cysteine (NAC) reduced H<sub>2</sub>DCFDA signal intensity in the double mutant at 6 and 24 h. Scale bar: 4 µm. **(f)** NAC treatment rescued the viability of the *mts3-1Δatg8* double mutant (orange solid triangle represents culture without NAC and orange empty triangle represents culture with NAC).

## **Methods**

### ***Strain, medium and culture***

*S. pombe* heterothallic haploids 972 $h^-$  and 975 $h^+$  and their derivatives were used. Complete YE and minimal EMM2 media were used to culture *S. pombe*(42). For G0 induction, exponentially growing cells at 26°C in EMM2 were harvested by vacuum filtration using a nitrocellulose membrane, washed with EMM2-N (EMM2 without nitrogen source), suspended in EMM2-N at a concentration of  $2 \times 10^6$  or  $5 \times 10^6$  cells/ml, and incubated for 24h at 26°C(4). To examine temperature-sensitive mutants, G0 cells of each strain were shifted from 26°C to 37°C.

### ***Immunochemical methods***

For immunoblot analysis, total proteins were extracted using the trichloroacetic acid (TCA) method. Identical amounts of proteins were separated by SDS-PAGE gel and blotted to nitrocellulose membranes. Anti-Cut8, anti-Pad1, anti-Hxk2, anti-Cox2 (a gift from Dr. Bonnefoy), anti- $\alpha$ -tubulin (TAT1; a gift from Dr. Gull), anti-GFP (Roche), and anti-FLAG (Sigma) antibodies were used as primary antibodies. Horseradish peroxidase-conjugated secondary antibodies and an ECL chemiluminescence system (Amersham) were used to amplify signal expression.

### ***Proteomics and metabolite analysis***

To identify immunoprecipitated proteins, we used the procedure reported by Hayashi et al, with some modifications(22). For whole proteome analysis, total proteins were extracted, separated by SDS-PAGE, in-gel-digested and analyzed with LC-MS/MS. All MS/MS spectra were searched against an *S. pombe* non-redundant protein database with the Mascot program (Matrix Science, London, UK). The output data from Mascot was analyzed using in-house software to select reliable peptides and calculate emPAI values(23). For metabolite analysis, we followed methods described previously(28). See Methods. S1 for the detailed methods.

### ***Microscopy***

All images were acquired using an AxioPlan 2 (Zeiss) or DeltaVision microscope setup. For mitochondrial staining, Mitotracker GreenFM and Mitotracker Orange (Invitrogen) were used(20). To obtain mitochondrial images in whole cells, we scanned 21 Z-axis sections at a 0.2- $\mu$ m interval and the obtained images were deconvolved and projected on a 2D plane. For oxidative stress staining via H<sub>2</sub>DCFDA (Invitrogen), we followed the method reported(30). For TEM analysis, see Methods. S1 for the detailed methods.

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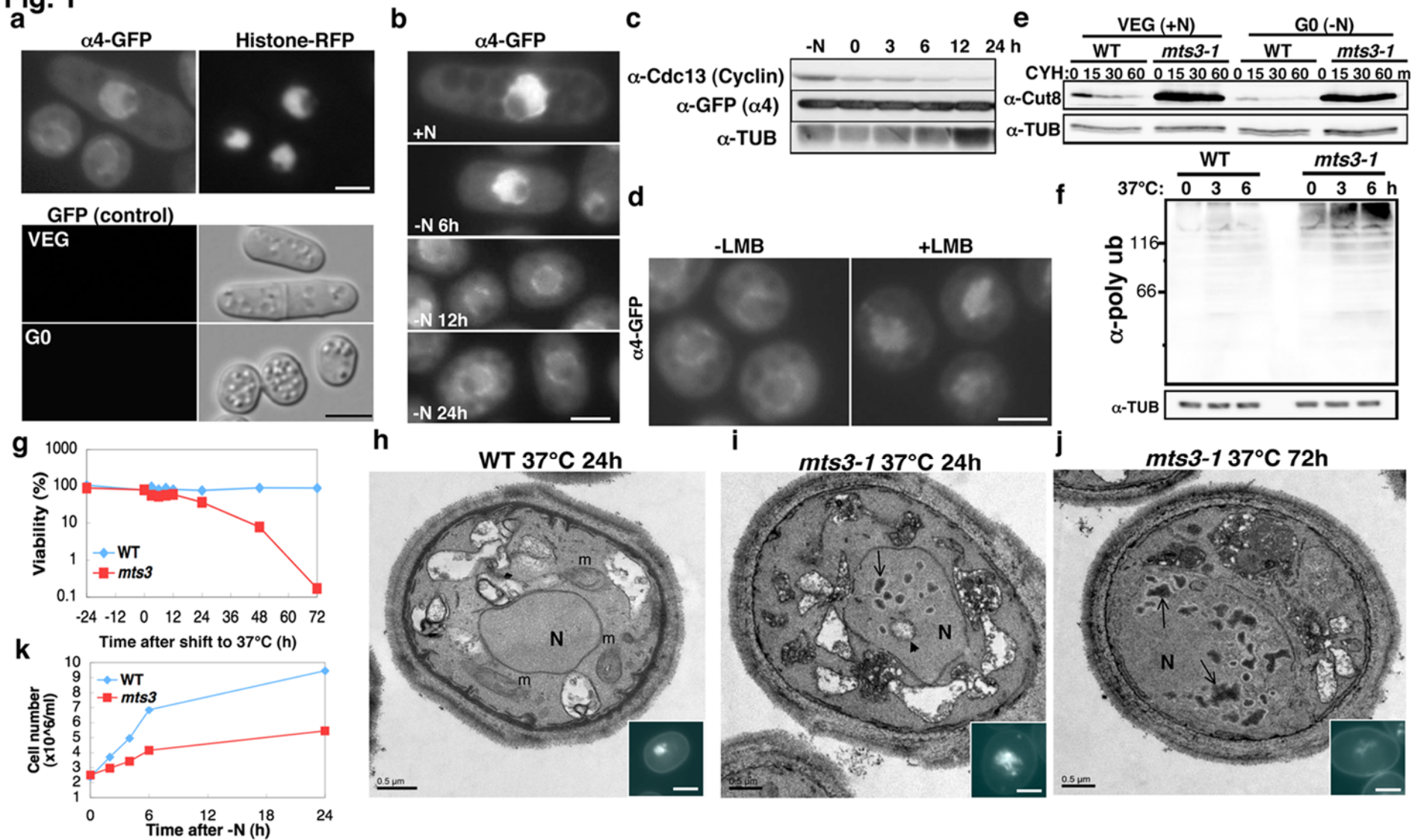
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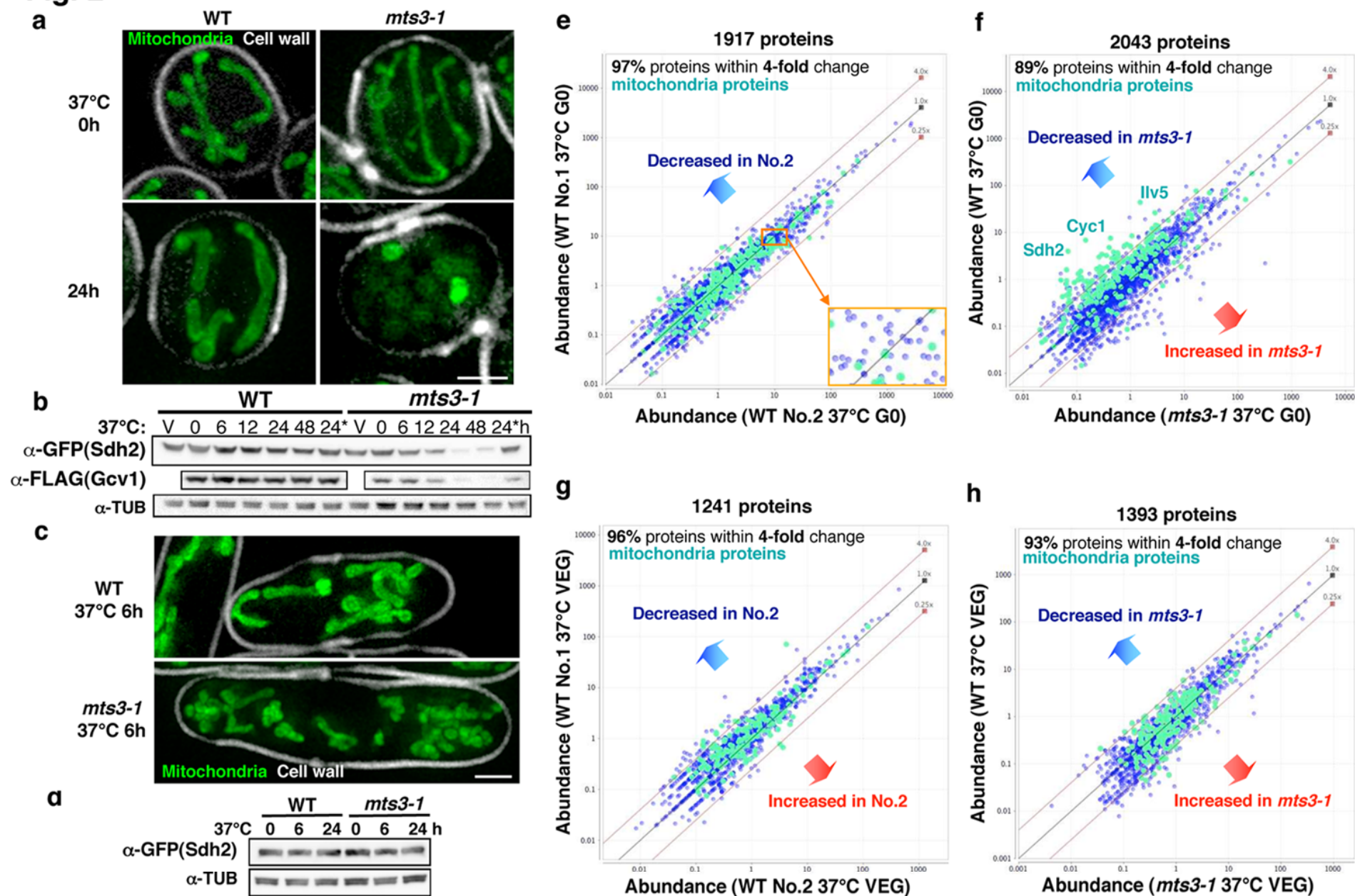
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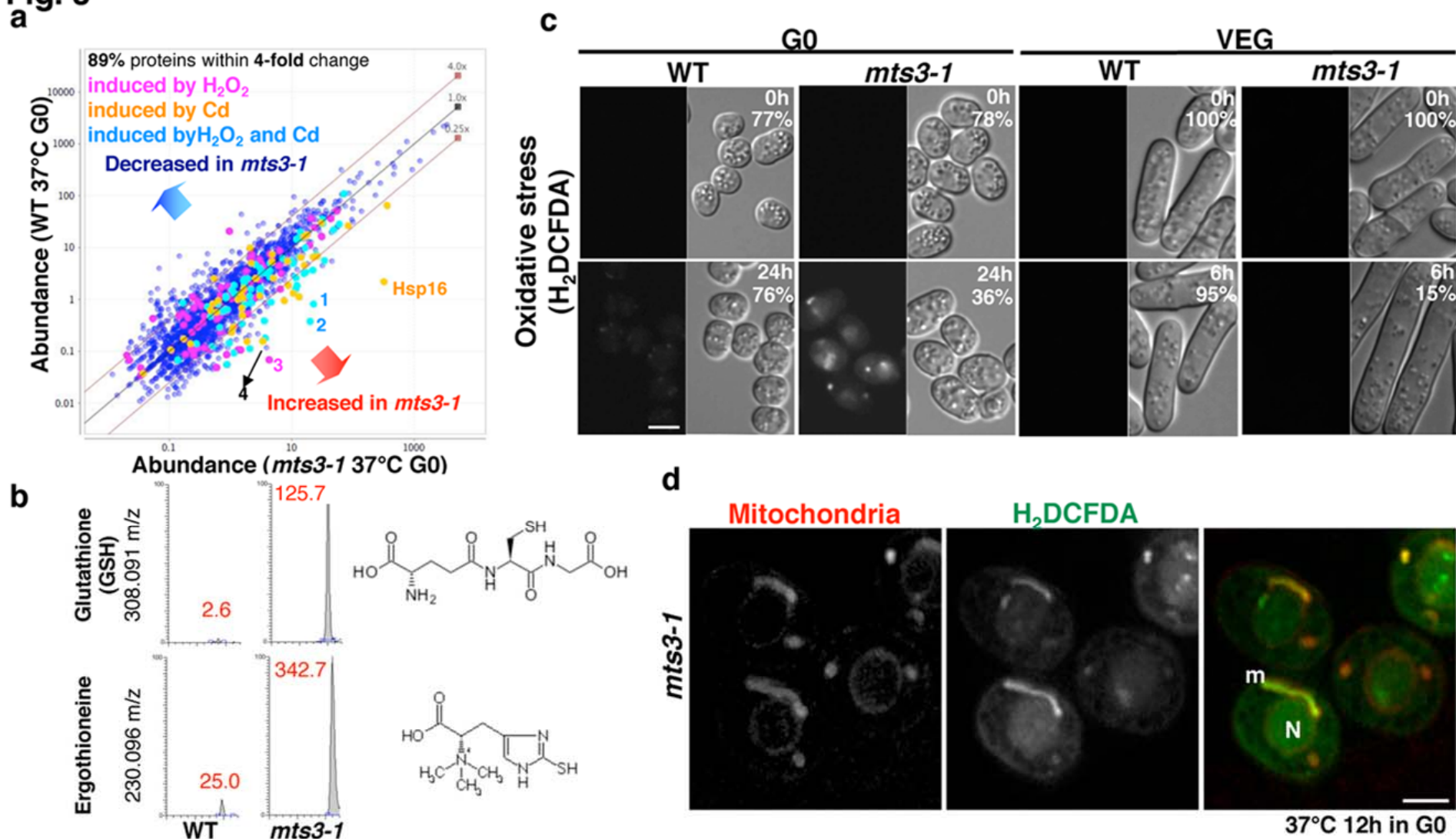
**Fig. 1**

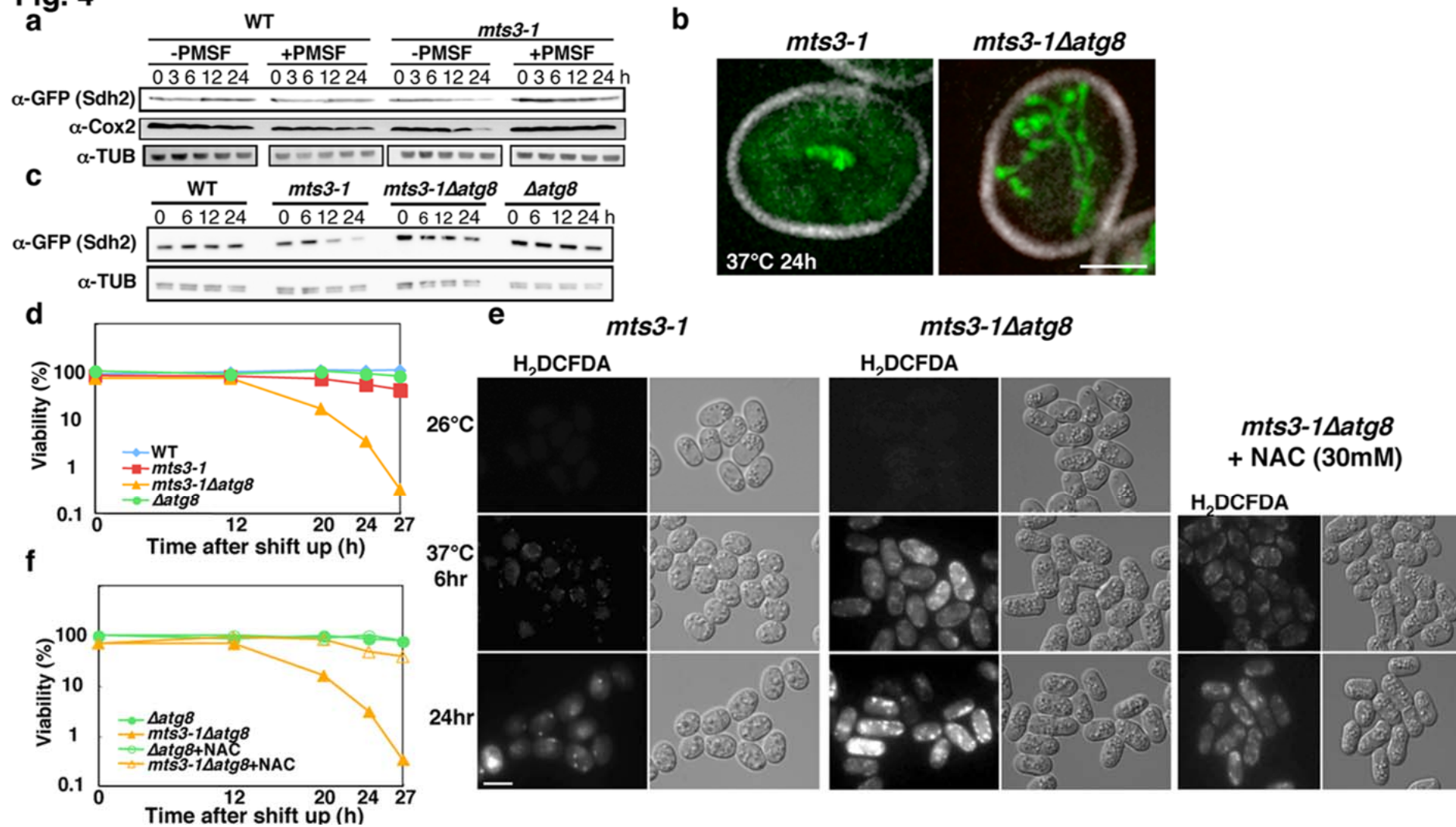


**Fig. 2**



**Fig. 3**



**Fig. 4**

## **Figure legends for Supporting Information**

### **Fig. S1**

(a) The localization of Pad1 (Rpn11)-GFP changed during the transition from VEG to G0. As shown in Fig. 1a, Pad1-GFP was localized to the nucleus and the nuclear periphery, although the nuclear signal was diminished and the signal in the cytoplasm was increased. The signal in the nuclear periphery remained detectable in G0. Scale bar: 2  $\mu$ m. (b) *mts3-1* mutant cells kept the viability high until ten days after –N at 26°C. (c) The temperature sensitive mutant of Pad1/Rpn11 subunit of 19S complex, *pad1-932*, lost the viability at the restrictive temperature (37°C) in G0.

### **Fig. S2**

(a) The  $\alpha$ 4-FLAGx3 protein expressed by the chromosomally integrated gene under the native promoter was immunoprecipitated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The 20 gel slices of G0 and VEG lanes were analyzed by LC/MS/MS after tryptic digestion. The names of the detected subunits or accessory proteins of the proteasome are shown. Blue represents 19S subunits and red represents 20S complex subunits. Black indicates accessory proteins. The experiments were repeated three times and the typical result is shown. (b) List of detected subunits and accessory proteins. Two Rpn13 homologues were observed in *S. pombe*.

### **Fig. S3**

Nile red stained a structure in the nucleus, which could be the vesicle-like structure observed in TEM analysis. *mts3-1* cells were stained by Nile red and Hochst33342 12h after the temperature shift to 37°C in G0. The signals indicated by the arrow were localized in the nucleus, which was stained by Hochst33342. Twenty-one images at 0.2- $\mu$ m intervals on the Z-axis were acquired using the DeltaVision system. One central section is shown after digital image processing. Scale bar: 2  $\mu$ m.

### **Fig. S4**

(a) Mitotracker GreenFM staining showed a decreased number of mitochondria in *mts3-1* at the restrictive temperature (37°C) in G0. Scale bar: 2  $\mu$ m. (b) Mitochondria were visualized by Sdh2-GFP tagging. In *mts3-1* cells, mitochondria were largely decreased at the restrictive temperature (37°C 24h) in G0. (c) In this study, we

isolated four temperature sensitive mutants of proteasome from Hayashi mutant library in our laboratory. All mutants showed a decrease in the number of mitochondria in G0. Sdh2-GFP was used as a mitochondrial marker. In mutants, Sdh2-GFP signals were significantly reduced 24 h after the temperature shift. Weaker signals in the cytoplasm might be GFP signals indicating incorporation of the GFP into vacuoles by autophagy. Scale bar: 2  $\mu$ m. (d) Western blotting showed that Cox2 protein decreased in proteasome mutants.

#### **Fig. S5**

Glutathione and ergothioneine detected by LC/MS were increased not only in *mts3-1*, but also in *pts1-727*, the newly-identified 20S proteasome mutant (see Supplementary figure 5). Trimethyl-histidine, a precursor of ergothioneine, was also increased in *mts3* and *pts1*.

#### **Fig. S6**

H<sub>2</sub>DCFDA fluorescence began to accumulate in *mts3-1* 12h (red line) after the temperature shift to 37°C in G0. Scale bar: 5  $\mu$ m.

#### **Fig. S7**

(a) In the *mts3-1*  $\Delta$ *atg8* double mutant, H<sub>2</sub>DCFDA fluorescent signal was strong in the mitochondria. The double mutant cells were simultaneously stained with H<sub>2</sub>DCFDA and Mitotracker Orange (12h after shift to 37°C in G0). Scale bar: 4  $\mu$ m. (b) In  $\Delta$ *atg8* cells, no significant accumulation of ROS was observed 24h after temperature-shift up in G0. Figures of *mts3-1* and *mts3-1*  $\Delta$ *atg8* are same as Fig. 4. Scale bar: 4  $\mu$ m. (c) ROS was accumulated in  $\Delta$ *atg8* cells fourteen days after G0 entry at 26°C. Scale bar: 5 $\mu$ m. (d)  $\Delta$ *atg8* cells lost the viability after 21 days in G0. The averages of viabilities of three independent experiments were shown. (Error bars: S.D.)

#### **Table. S1**

The list of proteins for which the emPAI ratio (*mts3*/WT) decreased to less than 0.25-fold in *mts3-1* in G0. Mitochondrial residents are shown in green. Sixty of 125 decreased proteins were mitochondrial.

**Table. S2**

The list of proteins for which the emPAI ratio (*mts3*/WT) increased over 4.0-fold in *mts3-1* in G0. Genes, which are transcriptionally induced over 3-fold by H<sub>2</sub>O<sub>2</sub>, Cadmium (Cd) and both of them, are represented by purple, yellow and blue, respectively. SPBC83.17 (Mbf1 indicated by the asterisk)) is induced by heat (>5.0-fold) and Cd (~2.5-fold).

**Supplementary Method. S1*****Proteomics***

To identify immunoprecipitated proteins, we used the procedure reported by Hayashi et al, with some modifications(22). For whole proteome analysis, total *S. pombe* proteins were extracted from G0 or VEG cells by TCA precipitation. TCA solution (100%) was added to cell cultures at a final concentration of 20% and cells were harvested by centrifugation at 4°C. Harvested cells were crushed in 10% TCA solution using glass beads and Multi-beads shocker (Yasui Kikai, Osaka, Japan) and proteins were precipitated by centrifugation at 14,000 x 15min. Precipitants were boiled in 1x LDS buffer (Invitrogen) with 0.16 M Tris and β-mercaptoethanol at 70°C for 10 min. Boiled samples were centrifuged at 14,000 rpm for 5 min to remove cell debris and were quantified using a Bradford assay kit (Bio Rad). An aliquot (33μg) of each sample was separated by NuPAGE Bis-Tris gel (4-12%), stained with Coomassie brilliant blue and sliced into 20 strips from the well to the dye front. After in-gel digestion with modified trypsin (Roche, Nutley, NJ), the resulting peptides were analyzed with online LC-MS/MS on a Finnegan LTQ (Thermo Fischer). All MS/MS spectra were searched against an *S. pombe* non-redundant protein database (including common contaminants such as trypsin and keratin) with the Mascot program (Matrix Science, London, UK). The output data from Mascot was analyzed using in-house software to select reliable peptides and calculate emPAI values(23). The emPAI values are calculated from  $10^{\text{PAI}} - 1$  (PAI = observed peptide number / observable peptide number). The obtained data were applied to in-house software to draw scatter plots.

***Metabolite analysis***

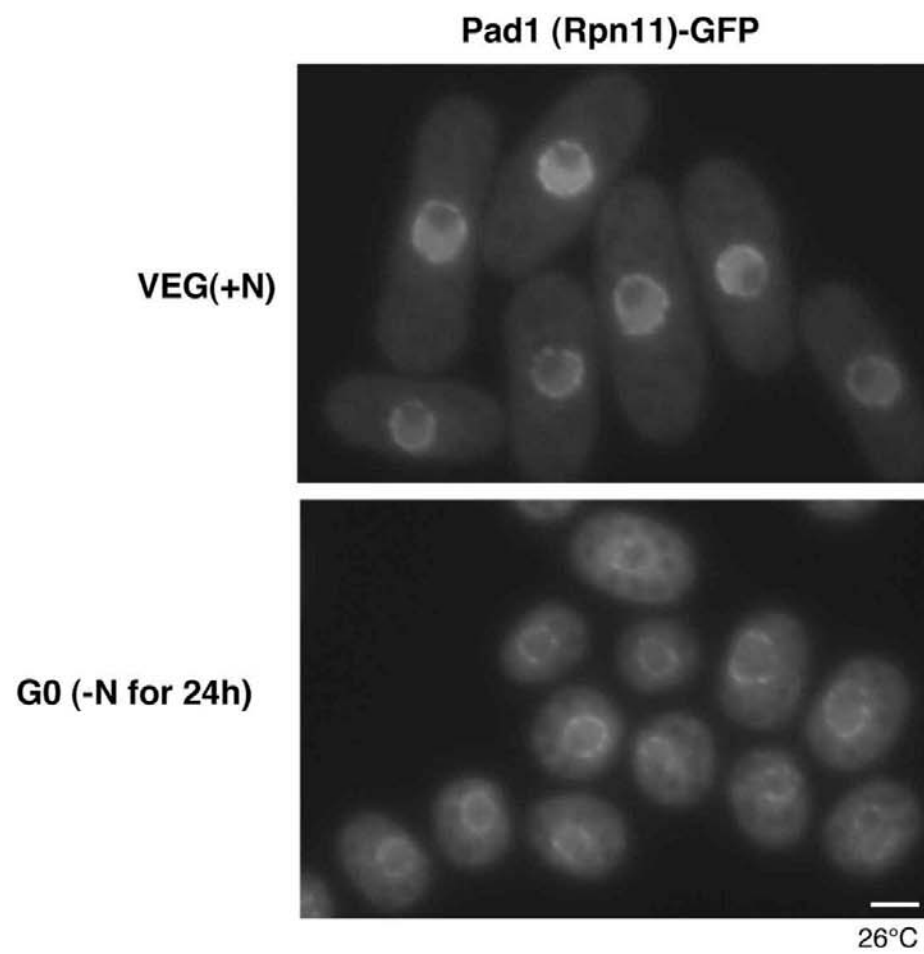
We followed methods described previously(28). Liquid cell culture (50 ml/sample) was filtered using an Omnipore membrane filter (Millipore) and collected cells were

immediately placed into -40°C MeOH. Cell membranes were disrupted using glass beads and a multi-bead shocker in 50% MeOH at 0°C. Proteins were removed from the extracts by filtering on an Amicon Ultra 10-kDa cut-off filter (Millipore) for 60 min at 4°C. Sample solvent was evaporated by centrifugation on a vacuum concentrator (TOMY CC-105) for 60 min and the remaining liquid was re-suspended into 40 µl 50% acetonitrile by gentle pipetting. Samples were then analyzed by LC-MS using a Paradigm MS4 HPLC system (Michrom Bioresources) coupled to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific). LC separation was performed on a ZIC-pHILIC column (Merck SeQuant; 150 x 2.1 mm, 5 µm particle size) using gradient elution with acetonitrile (A) and 10 mM ammonium carbonate + 0.2% ammonium hydroxide (B) as the mobile phase. For MS detection, an electrospray ionization source was used and operated in positive ionization mode with 4 kV spray voltage. Pure ergothioneine and GSH standards were obtained and analyzed for verification of metabolite m/z and retention time.

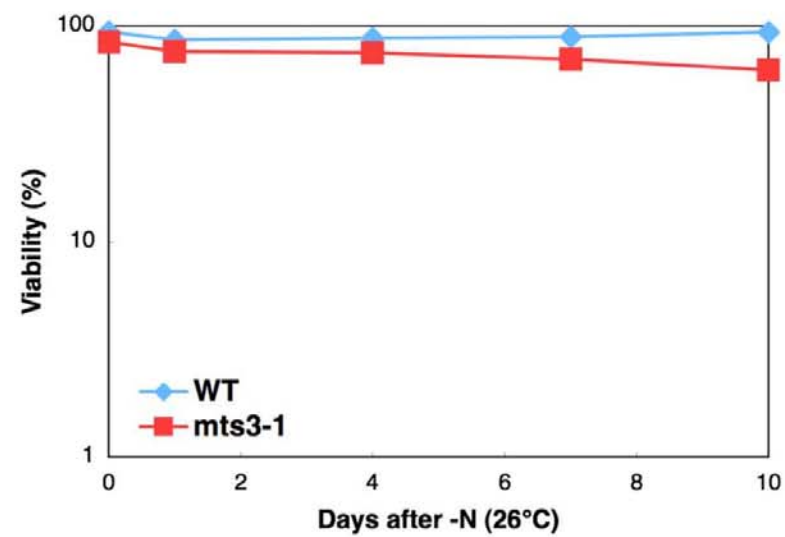
### ***Electron microscopy***

For TEM analysis, cells were fixed with 2% glutaraldehyde in 100 mM phosphate buffer pH 7.2 for 2 h at 26°C, post-fixed with 2% potassium permanganate overnight at 4°C, and embedded in Epon812 (TAAB). Ultra-thin sections were stained in 2% uranyl acetate and Reynold's lead citrate, and viewed with a TEM JEM1230R (JEOL) operating at 100kV.

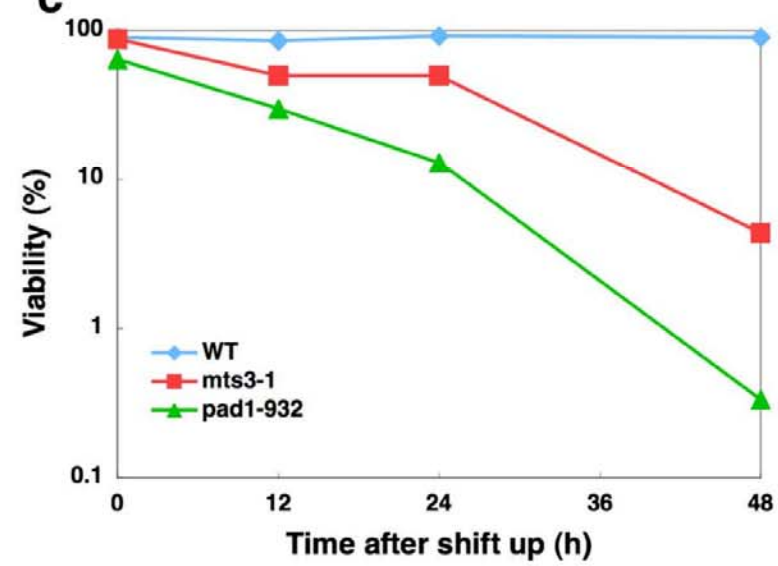
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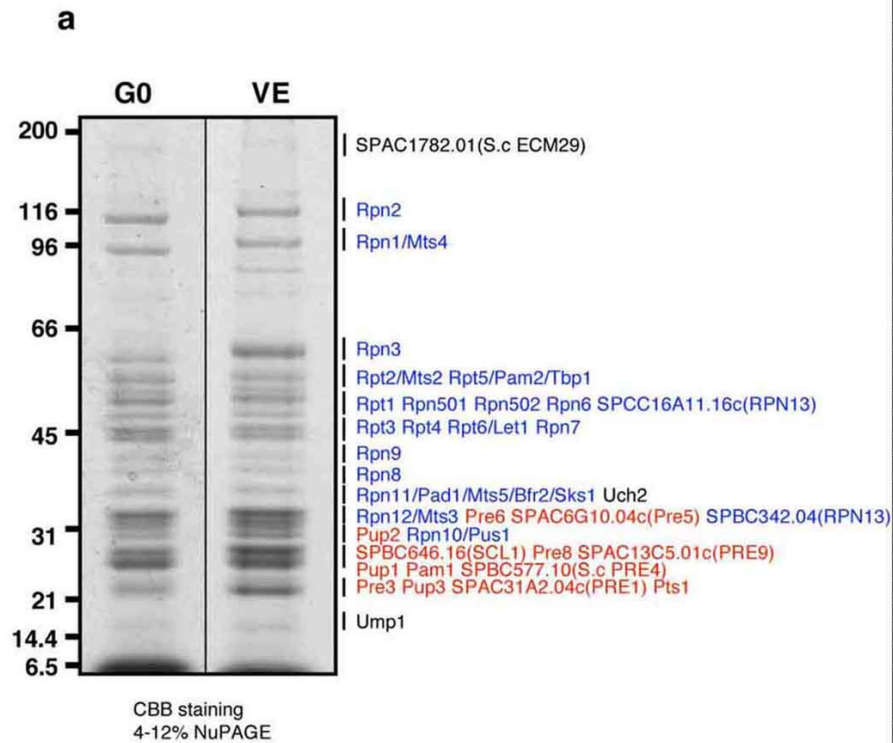


**b**



**c**





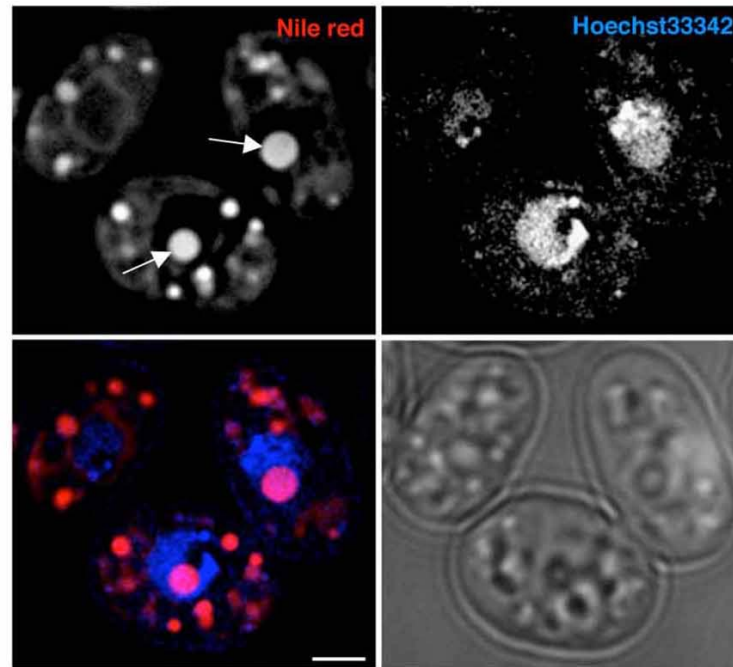
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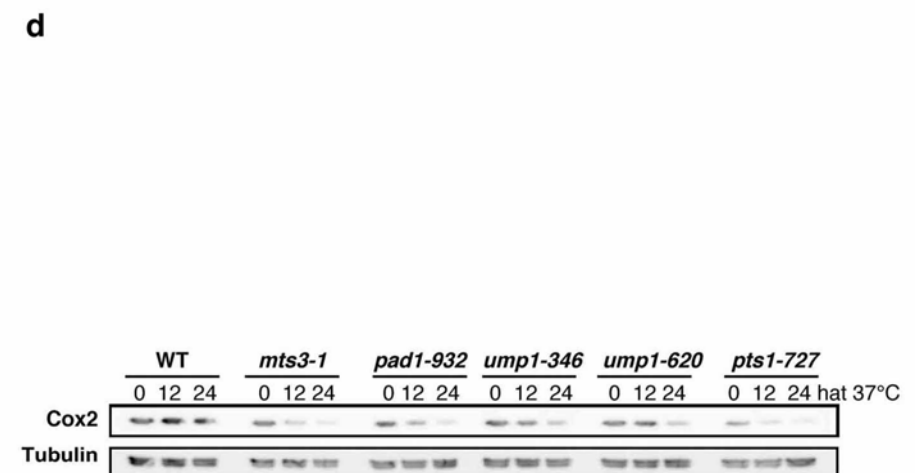
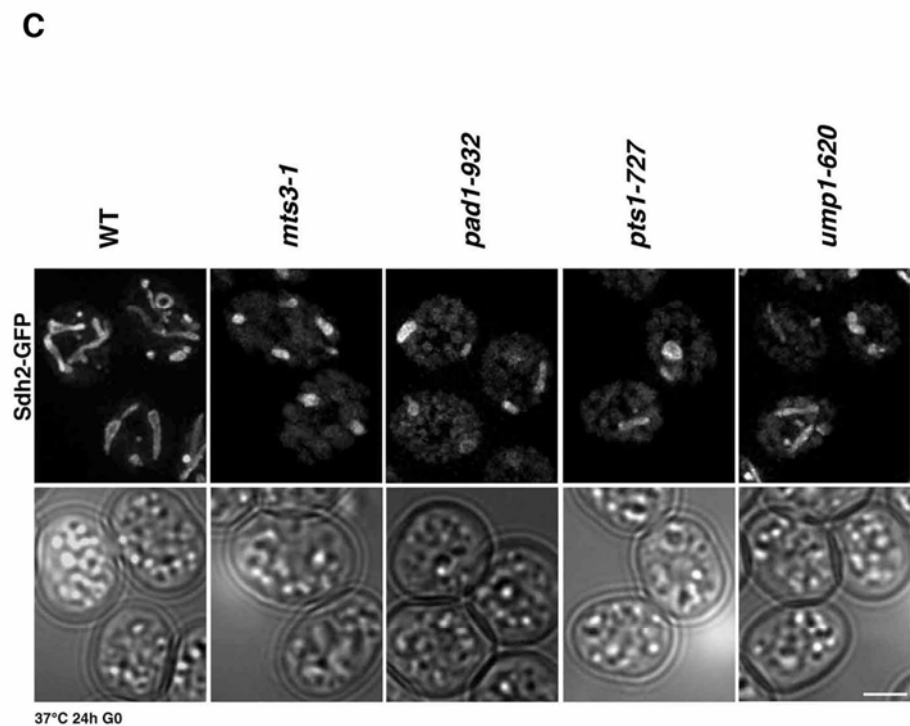
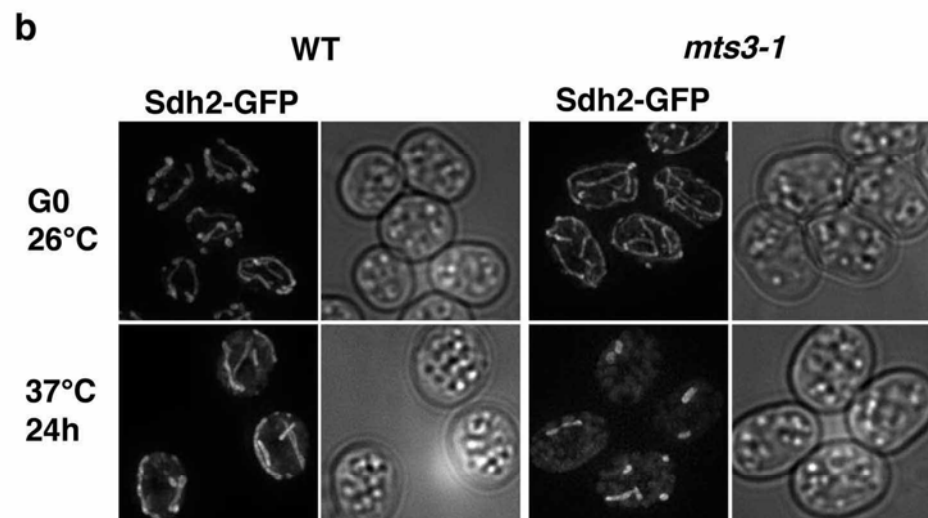
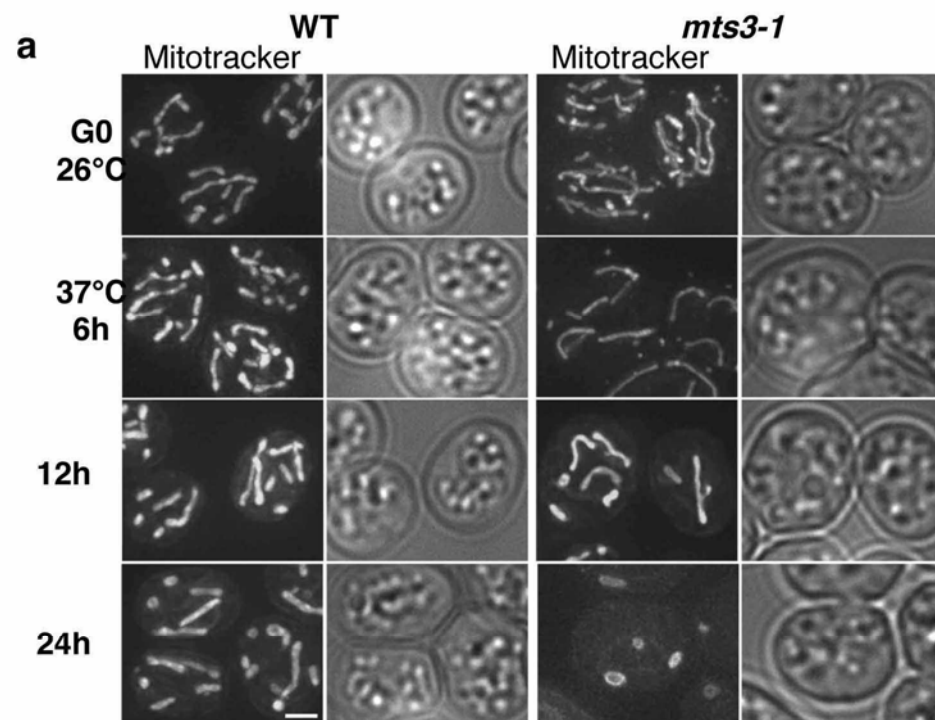
| Systematic name / name                | <i>S. cerevisiae</i> homologue | Identified peptides |     | Coverage (%) |     | MW (kDa) | Function                |
|---------------------------------------|--------------------------------|---------------------|-----|--------------|-----|----------|-------------------------|
|                                       |                                | G0                  | Veg | G0           | Veg |          |                         |
| SPBC646.16                            | SCL1                           | 41                  | 26  | 94           | 71  | 27       | 20S alpha 1             |
| SPCC1442.06/pre8                      | PRE8                           | 26                  | 17  | 98           | 60  | 26       | 20S alpha 2             |
| SPAC13C5.01c                          | PRE9                           | 30                  | 22  | 88           | 65  | 28       | 20S alpha 3             |
| SPBC106.16/pre6                       | PRE6                           | 29                  | 32  | 68           | 68  | 28       | 20S alpha 4             |
| SPAC323.02c/pup2                      | PUP2                           | 33                  | 28  | 79           | 71  | 28       | 20S alpha 5             |
| SPAC6G10.04c                          | PRE5                           | 46                  | 42  | 75           | 74  | 30       | 20S alpha 6             |
| SPCC1795.04c/pre10                    | PRE10                          | 21                  | 19  | 48           | 43  | 28       | 20S alpha 7             |
| SPBC4C3.10c/pre3                      | PRE3                           | 31                  | 29  | 81           | 90  | 25       | 20S beta 1              |
| SPAC23D3.07/pup1                      | PUP1                           | 29                  | 18  | 58           | 42  | 29       | 20S beta 2              |
| SPCC63.12c/pup3                       | PUP3                           | 8                   | 9   | 25           | 34  | 23       | 20S beta 3              |
| SPAC31A2.04c                          | PRE1                           | 10                  | 11  | 49           | 61  | 22       | 20S beta 4              |
| SPAC4A8.13c/pts1                      | PRE2                           | 21                  | 19  | 61           | 61  | 30       | 20S beta 5              |
| SPAC22F8.06/pam1                      | PRE7                           | 28                  | 20  | 72           | 61  | 25       | 20S beta 6              |
| SPBC577.10                            | PRE4                           | 27                  | 16  | 78           | 59  | 29       | 20S beta 7              |
| SPBC16C6.07c                          | RPT1                           | 43                  | 35  | 77           | 68  | 49       | 19S Base                |
| SPBC4.07c/mts2/rpt2                   | RPT2                           | 45                  | 28  | 68           | 54  | 50       | 19S Base                |
| SPCC576.10c/rpt3                      | RPT3                           | 26                  | 24  | 51           | 51  | 44       | 19S Base                |
| SPCC1682.16/rpt4                      | RPT4                           | 37                  | 34  | 69           | 66  | 44       | 19S Base                |
| SPAC3A11.12c/rpt5/pam2/tbp1           | RPT5                           | 38                  | 32  | 54           | 52  | 49       | 19S Base                |
| SPBC23G7.12c/rpt6/let1                | RPT6                           | 34                  | 33  | 69           | 65  | 45       | 19S Base                |
| SPBP19A11.03c/rpn1/mts4               | RPN1                           | 65                  | 51  | 56           | 52  | 98       | 19S Base                |
| SPBC17D11.07/rpn2                     | RPN2                           | 88                  | 63  | 59           | 49  | 107      | 19S Base                |
| SPBC119.01/rpn3                       | RPN3                           | 38                  | 27  | 53           | 44  | 57       | 19S lid                 |
| SPAC1420.03/rpn501/rpn5-a*            |                                |                     |     |              |     |          |                         |
| SPAPB8E5.02c/rpn502/rpn5-b*           | RPN5                           | 39                  | 28  | 59           | 54  | 52       | 19S lid                 |
| SPAC23G3.11/rpn6                      | RPN6                           | 28                  | 21  | 55           | 44  | 47       | 19S lid                 |
| SPBC582.07c/rpn7                      | RPN7                           | 34                  | 28  | 57           | 52  | 47       | 19S lid                 |
| SPCC1682.10/rpn8                      | RPN8                           | 21                  | 11  | 67           | 34  | 36       | 19S lid                 |
| SPAC607.05/rpn9                       | RPN9                           | 35                  | 20  | 71           | 41  | 43       | 19S lid                 |
| SPAC637.10c/rpn10/pus1                | RPN10                          | 13                  | 8   | 43           | 38  | 27       | 19S lid                 |
| SPAC31G5.13/rpn11/pad1/sks1/bfr2/mts5 | RPN11                          | 25                  | 17  | 56           | 55  | 35       | 19S lid                 |
| SPBC16G5.01/rpn12/mts3                | RPN12                          | 24                  | 14  | 64           | 41  | 31       | 19S lid                 |
| SPCC16A11.16c#                        | RPN13                          | 10                  | 10  | 19           | 20  | 44       | 19S lid                 |
| SPBC342.04#                           | RPN13                          | 5                   | 5   | 9.3          | 22  | 32       | 19S lid                 |
| SPCC14G10.03c/ump1                    | UMP1                           | 13                  | 10  | 49           | 48  | 15       | 20S assembly            |
| SPBC409.06/uch2                       | YUH1                           | 21                  | 14  | 52           | 39  | 34       | Ub C-terminal hydrolase |
| SPAC1782.01                           | ECM29                          | 23                  | 18  | 14           | 12  | 191      | stabilizing 26S         |

\*: There are two homologues of Rpn5.

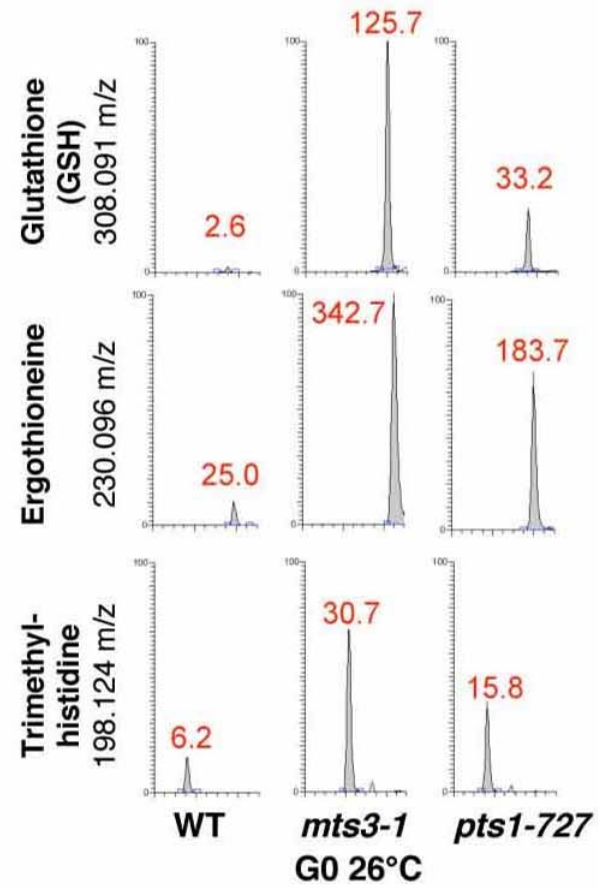
#: There are two homologues of Rpn13.

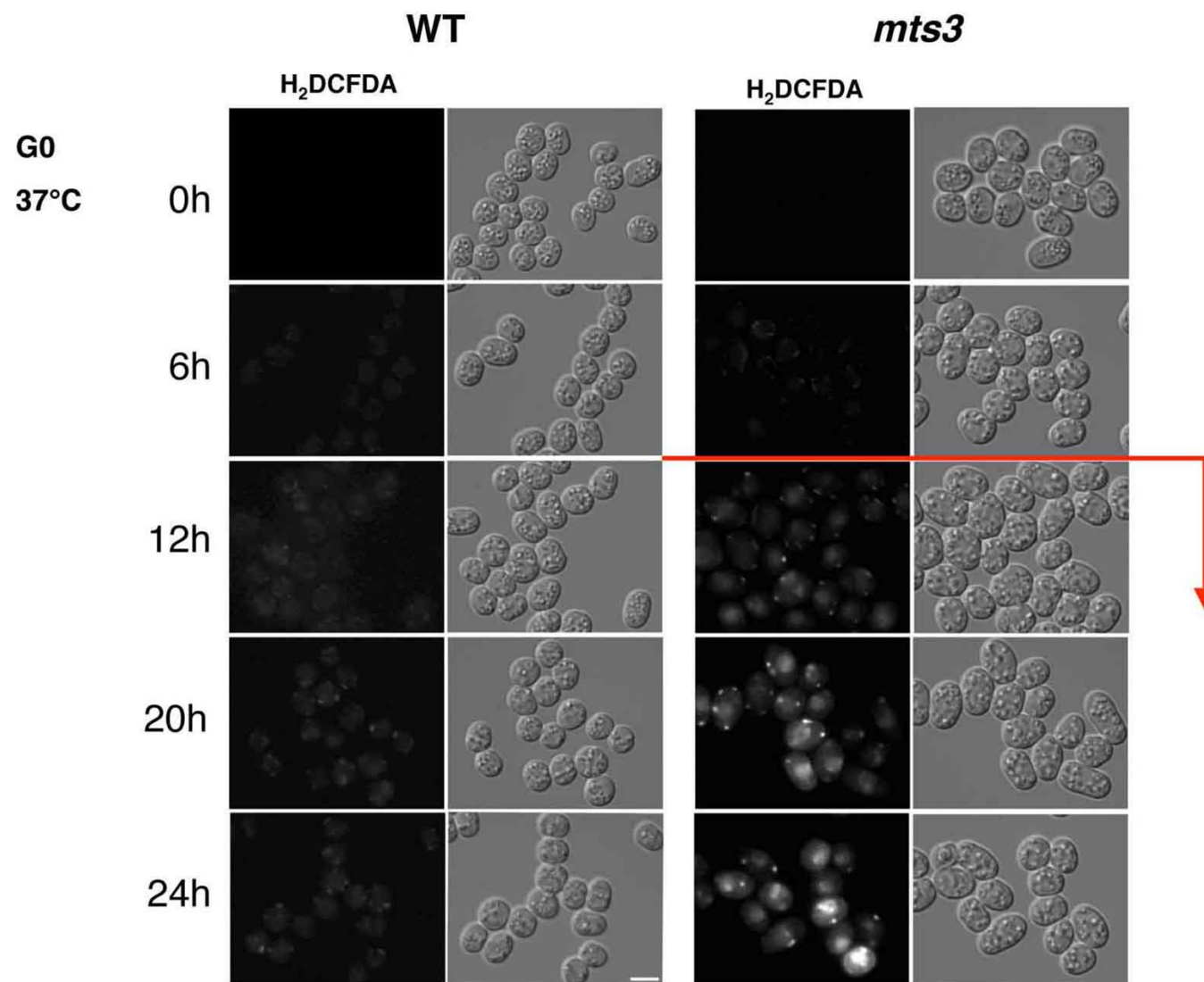
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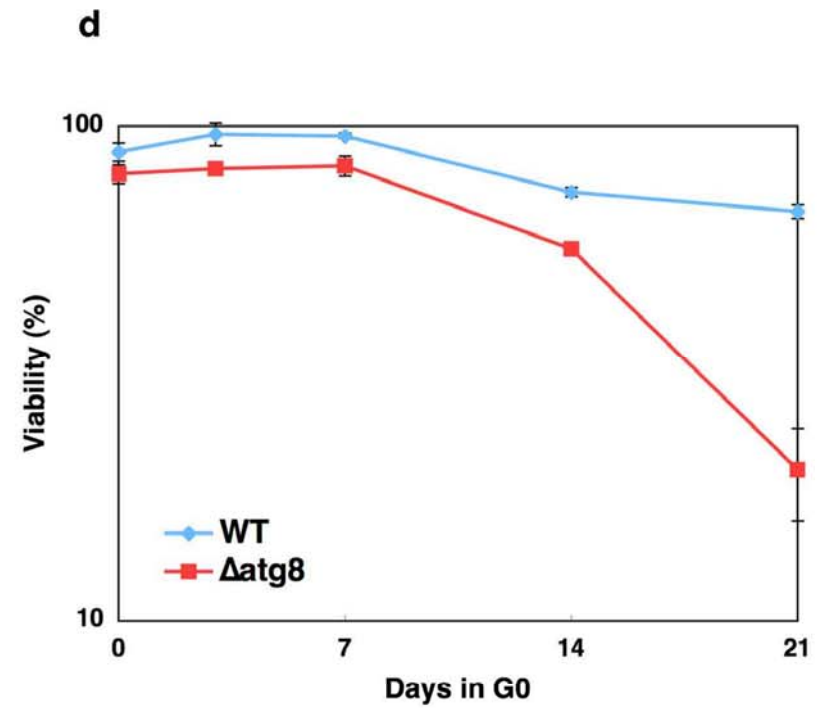
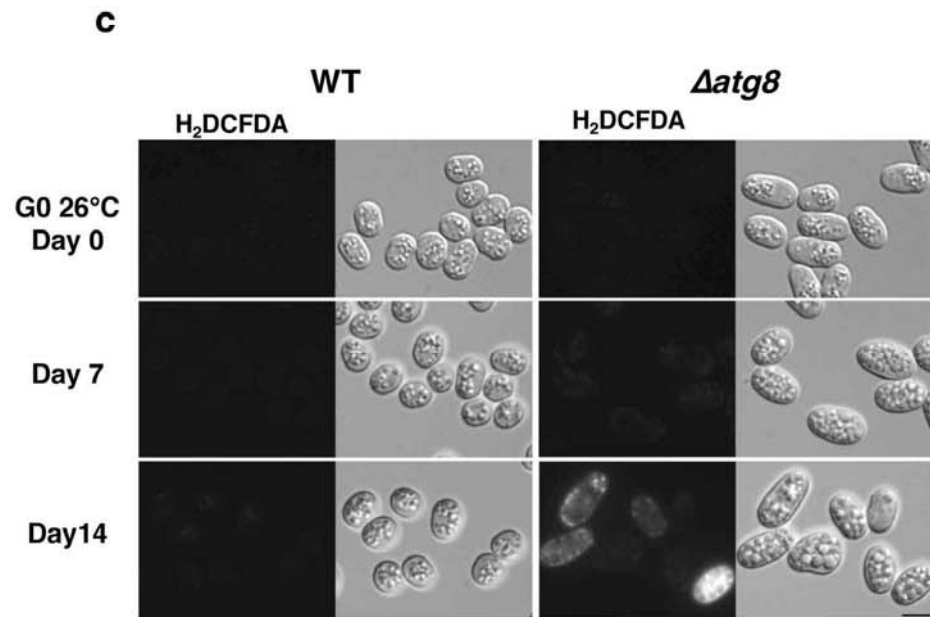
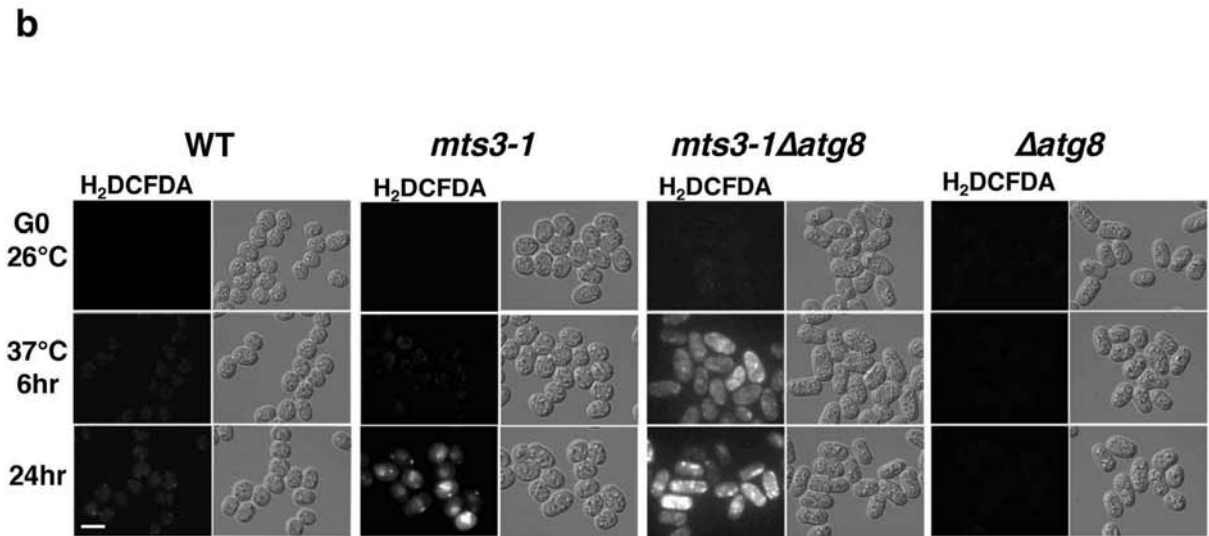
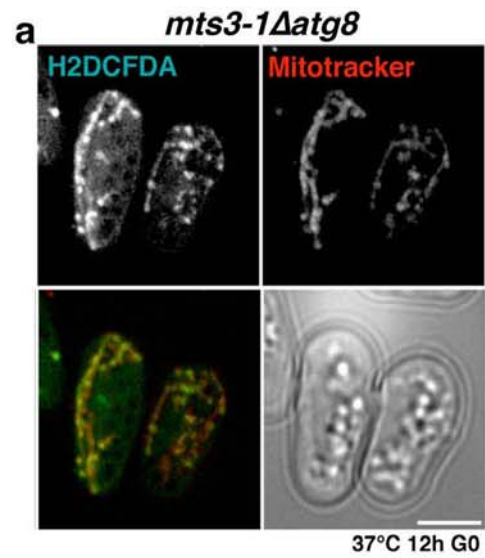




Positive ionization mode (ESI)







| rank | Systematic name | Gene product                                                                                  | ratio (mts3/WT) |
|------|-----------------|-----------------------------------------------------------------------------------------------|-----------------|
| 1    | SPAC140.01      | sdh2, succinate dehydrogenase (ubiquinone) iron-sulfur protein subunit                        | 0.017237734     |
| 2    | SPCC191.07      | cyc1, cytochrome c                                                                            | 0.021365018     |
| 3    | SPBC56F2.12     | ilv5, acetohydroxyacid reductoisomerase                                                       | 0.034667114     |
| 4    | SPBC1861.05     | carbohydrate kinase                                                                           | 0.038058156     |
| 5    | SPCC1682.09c    | guanine nucleotide transporter                                                                | 0.040352681     |
| 6    | SPAC2E1P3.04    | copper amine oxidase                                                                          | 0.046434356     |
| 7    | SPAC31G5.14     | gcv1/n313, glycine decarboxylase T subunit                                                    | 0.052590401     |
| 8    | SPAC17G8.06c    | dihydroxy-acid dehydratase                                                                    | 0.056145702     |
| 9    | SPAC4G9.10      | arg3, ornithine carbamoyltransferase Arg3                                                     | 0.069272546     |
| 10   | SPAC31G5.13     | rpn11/pad1/sks1/bfr2/mts5, 19S proteasome regulatory subunit Rpn11                            | 0.076104234     |
| 11   | SPBC24C6.06     | gpa1, G-protein alpha subunit                                                                 | 0.077219007     |
| 12   | SPAC343.16      | lys2, homoaconitate hydratase Lys2                                                            | 0.079472592     |
| 13   | SPBC776.07      | conserved eukaryotic protein                                                                  | 0.08135805      |
| 14   | SPAC20G8.04c    | electron transfer flavoprotein-ubiquinone oxidoreductase                                      | 0.083210352     |
| 15   | SPBC3H7.03c     | 2-oxoglutarate dehydrogenase (lipoamide) (e1 component of oxoglutarate dehydrogenase complex) | 0.083928498     |
| 16   | SPBP4H10.08     | qcr10, ubiquinol-cytochrome-c reductase complex subunit Qcr10                                 | 0.085743094     |
| 17   | SPBC13E7.04     | atp16, F1-ATPase delta subunit                                                                | 0.086856339     |
| 18   | SPAC23H4.06     | gln1, glutamate-ammonia ligase Gln1                                                           | 0.099162728     |
| 19   | SPBC3B8.04c     | membrane transporter                                                                          | 0.101333065     |
| 20   | SPBC9B6.04c     | tuf1, mitochondrial translation elongation factor EF-Tu Tuf1                                  | 0.104605855     |
| 21   | SPBC16E9.05     | erg6, delta-sterol C-methyltransferase                                                        | 0.106877342     |
| 22   | SPBC215.08c     | arg4, carbamoyl-phosphate synthase Arg4                                                       | 0.109039557     |
| 23   | SPBC25H2.09     | conserved fungal protein                                                                      | 0.109446095     |
| 24   | SPBC428.02c     | eca39, branched chain amino acid aminotransferase Eca49                                       | 0.110259506     |
| 25   | SPBP4H10.15     | aconitate hydratase                                                                           | 0.111028725     |
| 26   | SPAC5D6.04      | auxin family                                                                                  | 0.112204073     |
| 27   | SPCC794.01c     | glucose-6-phosphate 1-dehydrogenase                                                           | 0.113196268     |
| 28   | SPAC17G6.06     | rps2401/rps24-1/rps24, 40S ribosomal protein S24                                              | 0.115663736     |
| 29   | SPAC694.04c     | conserved eukaryotic protein                                                                  | 0.116555091     |
| 30   | SPCC1223.09     | uricase                                                                                       | 0.117571321     |

|    |               |                                                           |             |
|----|---------------|-----------------------------------------------------------|-------------|
| 31 | SPAC589.12    | cell wall organization membrane protein                   | 0.118220669 |
| 32 | SPBPB7E8.02   | conserved protein (fungal bacterial protazoan)            | 0.122633948 |
| 33 | SPBC83.05     | RNA-binding protein                                       | 0.126252093 |
| 34 | SPAC1002.09c  | dld1/dldh, dihydrolipoamide dehydrogenase Dld1            | 0.126321073 |
| 35 | SPAC6F12.07   | tom20, mitochondrial TOM complex subunit Tom20            | 0.126458608 |
| 36 | SPCC576.01c   | sulfonate dioxygenase                                     | 0.130972881 |
| 37 | SPCC1919.12c  | aminopeptidase                                            | 0.138324598 |
| 38 | SPBC21B10.03c | ataxin-2 homolog                                          | 0.14097368  |
| 39 | SPBC27B12.14  | mitochondrial membrane protein complex assembly protein   | 0.141042954 |
| 40 | SPCC1322.16   | prohibitin Phb2                                           | 0.142201696 |
| 41 | SPCC663.03    | pmd1, leptomycin efflux transporter                       | 0.143489968 |
| 42 | SPAC13G6.06c  | glycine cleavage complex subunit P                        | 0.147050306 |
| 43 | SPCC70.03c    | proline dehydrogenase                                     | 0.147890882 |
| 44 | SPAC24C9.05c  | conserved protein (fungal and plant)                      | 0.150779976 |
| 45 | SPCC1620.08   | succinate-CoA ligase (beta subunit)                       | 0.151870563 |
| 46 | SPAC16E8.17c  | succinate-CoA ligase (alpha subunit)                      | 0.152341997 |
| 47 | SPAC19A8.04   | erg5, C-22 sterol desaturase Erg5                         | 0.152812397 |
| 48 | SPBP23A10.10  | ppk32, serine/threonine protein kinase Ppk32              | 0.153989165 |
| 49 | SPAC6G10.08   | idp1, isocitrate dehydrogenase Idp1                       | 0.154369903 |
| 50 | SPAC1296.02   | cox4, cytochrome c oxidase subunit IV                     | 0.155525086 |
| 51 | SPMIT.09      | atp8, F0-ATPase subunit 8                                 | 0.158507042 |
| 52 | SPCC18.18c    | fum1, fumarate hydratase                                  | 0.161325045 |
| 53 | SPAC1B2.05    | mcm5/nda4, MCM complex subunit Mcm5                       | 0.161533452 |
| 54 | SPBC56F2.09c  | arg5, arginine specific carbamoyl-phosphate synthase Arg5 | 0.162527774 |
| 55 | SPAC1782.07   | qcr8, ubiquinol-cytochrome-c reductase complex subunit 7  | 0.165691978 |
| 56 | SPBC27B12.13  | tom40, mitochondrial TOM complex subunit Tom40            | 0.168994063 |
| 57 | SPAPYUK71.03c | C2 domain protein                                         | 0.173651985 |
| 58 | SPAC22E12.14c | sck2, serine/threonine protein kinase Sck2                | 0.175440883 |
| 59 | SPCC1235.15   | dga1, diacylglycerol O-acyltransferase                    | 0.177259585 |
| 60 | SPBC14C8.04   | acetolactate synthase regulatory unit                     | 0.184095184 |
| 61 | SPAC27D7.06   | electron transfer flavoprotein alpha subunit              | 0.184113308 |
| 62 | SPCC1223.08c  | dfr1, dihydrofolate reductase Dfr1                        | 0.186668209 |

|    |               |                                                                                            |             |
|----|---------------|--------------------------------------------------------------------------------------------|-------------|
| 63 | SPCC74.02c    | mRNA cleavage and polyadenylation specificity factor complex associated protein            | 0.189513758 |
| 64 | SPBC146.09c   | lsd1, histone demethylase                                                                  | 0.189788877 |
| 65 | SPAC18B11.11  | GTPase activating protein                                                                  | 0.193179682 |
| 66 | SPCC132.04c   | NAD-dependent glutamate dehydrogenase                                                      | 0.193278887 |
| 67 | SPCC70.02c    | ATPase inhibitor                                                                           | 0.193431581 |
| 68 | SPAC6C3.04    | cit1, citrate synthase                                                                     | 0.194366523 |
| 69 | SPMIT.11      | cox2, cytochrome c oxidase 2                                                               | 0.194616826 |
| 70 | SPBC725.10    | tspO homolog                                                                               | 0.195625537 |
| 71 | SPAC1A6.10    | Moeb/ThiF domain                                                                           | 0.197001786 |
| 72 | SPAC14C4.04   | B22918-2, hypothetical protein                                                             | 0.198955801 |
| 73 | SPAC1039.06   | alanine racemase                                                                           | 0.199519859 |
| 74 | SPBC3E7.16c   | leu3, 2-isopropylmalate synthase                                                           | 0.200632445 |
| 75 | SPBC1289.09   | tim21, mitochondrial inner membrane presequence translocase complex subunit Tim21          | 0.201715544 |
| 76 | SPBC947.15c   | NADH dehydrogenase                                                                         | 0.202609506 |
| 77 | SPACUNK4.15   | 2',3'-cyclic-nucleotide 3'-phosphodiesterase                                               | 0.203525557 |
| 78 | SPAC19B12.04  | SPAC19B12.04/rps3001/rps30-1,SPBC19G7.03c/rps3002/rps30-2/rps30, 40S ribosomal protein S30 | 0.205601776 |
| 79 | SPBP35G2.07   | ilv1, acetolactate synthase catalytic subunit                                              | 0.205731272 |
| 80 | SPAC24C9.06c  | aconitate hydratase                                                                        | 0.206965878 |
| 81 | SPCC320.14    | threo-3-hydroxyaspartate ammonia-lyase                                                     | 0.209106776 |
| 82 | SPBC31F10.15c | atp15, F0-ATPase epsilon subunit                                                           | 0.209248865 |
| 83 | SPAC15E1.03   | rpl36a, 60S ribosomal protein L36/L42                                                      | 0.209248865 |
| 84 | SPAC1556.02c  | sdh1, succinate dehydrogenase Sdh1                                                         | 0.209863604 |
| 85 | SPAC823.05c   | tlg2, SNARE Tlg2                                                                           | 0.210500694 |
| 86 | SPBC29A10.01  | ccr1, NADPH-cytochrome p450 reductase                                                      | 0.210786389 |
| 87 | SPAC869.04    | formamidase-like protein                                                                   | 0.21208836  |
| 88 | SPAC521.03    | short chain dehydrogenase                                                                  | 0.212791872 |
| 89 | SPBP16F5.08c  | flavin dependent monooxygenase                                                             | 0.21302043  |
| 90 | SPCC830.11c   | adenylate kinase                                                                           | 0.213706308 |
| 91 | SPAC17H9.16   | tom22, mitochondrial TOM complex subunit Tom22                                             | 0.214268277 |
| 92 | SPBC29B5.02c  | isp4, OPT oligopeptide transporter family                                                  | 0.215817295 |
| 93 | SPBC17G9.07   | rps2402/rps24-2, 40S ribosomal protein S24                                                 | 0.217652858 |
| 94 | SPBC725.02    | mpr1/spy1, speedy homolog                                                                  | 0.219292207 |

|     |               |                                                                                                                                                      |             |
|-----|---------------|------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|
| 95  | SPBC530.10c   | anc1, adenine nucleotide carrier Anc1                                                                                                                | 0.219592837 |
| 96  | SPAC31G5.04   | homoisocitrate dehydrogenase                                                                                                                         | 0.220421608 |
| 97  | SPAC23C11.13c | hpt1, xanthine phosphoribosyltransferase                                                                                                             | 0.222862044 |
| 98  | SPCC1450.15   | pig-F                                                                                                                                                | 0.224407798 |
| 99  | SPAC23C11.11  | cka1/orb5, serine/threonine protein kinase Cka1                                                                                                      | 0.224995993 |
| 100 | SPBC1215.01   | shy1, SURF-family protein Shy1                                                                                                                       | 0.225835439 |
| 101 | SPBC725.01    | aspartate aminotransferase                                                                                                                           | 0.227935626 |
| 102 | SPBC31F10.07  | cortical component Lsb5                                                                                                                              | 0.229705478 |
| 103 | SPCC18B5.11c  | cds1, replication checkpoint kinase Cds1                                                                                                             | 0.231507884 |
| 104 | SPAC644.09    | alanine racemase                                                                                                                                     | 0.23204236  |
| 105 | SPAC1F12.02c  | p23fy, translationally controlled tumor protein homolog                                                                                              | 0.233152409 |
| 106 | SPAC23A1.03   | apt1, adenine phosphoribosyltransferase (APRT)                                                                                                       | 0.233215492 |
| 107 | SPAC12G12.04  | mcp60/hsp60, heat shock protein Hsp60                                                                                                                | 0.234108739 |
| 108 | SPBC409.10    | ade7, phosphoribosylamidoimidazolesuccinocarboxamide synthase Ade7                                                                                   | 0.234172146 |
| 109 | SPAC823.15    | ppa1, minor serine/threonine protein phosphatase Ppa1                                                                                                | 0.234837347 |
| 110 | SPCC1259.09c  | pyruvate dehydrogenase protein x component                                                                                                           | 0.236374437 |
| 111 | SPBP8B7.05c   | carbonic anhydrase                                                                                                                                   | 0.23815151  |
| 112 | SPAC4G9.04c   | cleavage and polyadenylation specificity factor                                                                                                      | 0.238937026 |
| 113 | SPAC19A8.05c  | vps27, sorting receptor for ubiquitinated membrane proteins                                                                                          | 0.239026252 |
| 114 | SPAC9E9.03    | leu2, 3-isopropylmalate dehydratase Leu2                                                                                                             | 0.239030452 |
| 115 | SPCP1E11.02   | ppk38, Ark1/Prk1 family protein kinase Ppk38                                                                                                         | 0.239428039 |
| 116 | SPAC343.17c   | WD repeat protein, human WDR70 family                                                                                                                | 0.240410695 |
| 117 | SPBC25B2.06c  | btb2, BTB/POZ domain protein Btb2                                                                                                                    | 0.241752986 |
| 118 | SPCC1183.11   | MS ion channel protein 1                                                                                                                             | 0.24253204  |
| 119 | SPAC23C4.09c  | DNA-binding TFAR19-related protein                                                                                                                   | 0.243202073 |
| 120 | SPAC6F12.02   | rst2, transcription factor Rst2                                                                                                                      | 0.243288058 |
| 121 | SPBC119.17    | metallopeptidase                                                                                                                                     | 0.24346154  |
| 122 | SPAC3A11.07   | NADH dehydrogenase                                                                                                                                   | 0.243830431 |
| 123 | SPBC2G2.08    | ade9, C-1-tetrahydrofolatesynthase/methylenetetrahydrof olatedehydrogenase/methylenetetrahydrofolatecyclohydrolase/formyltetrahydrofolate synthetase | 0.244949255 |
| 124 | SPAC1002.03c  | gls2, glucosidase II GlS2                                                                                                                            | 0.246563074 |
| 125 | SPAC1A6.07    | sequence orphan                                                                                                                                      | 0.247279817 |

| rank | Systematic name | Gene product                                                   | ratio (mts3/WT) | Cd+2 | H2O2 |
|------|-----------------|----------------------------------------------------------------|-----------------|------|------|
| 1    | SPBC3E7.02c     | hsp16, heat shock protein Hsp16                                | 146.8581995     | +    | -    |
| 2    | SPCC663.08c     | short chain dehydrogenase                                      | 62.74615283     | -    | +    |
| 3    | SPCC663.06c     | short chain dehydrogenase                                      | 53.17919385     | +    | +    |
| 4(*) | SPBC83.17       | transcriptional coactivator, multiprotein bridging factor Mbf1 | 33.34068723     | -    | -    |
| 5    | SPAC11D3.01c    | conserved fungal protein                                       | 28.0201278      | +    | +    |
| 6    | SPAC32A11.02c   | conserved fungal protein                                       | 21.55927065     | +    | -    |
| 7    | SPCC757.03c     | ThiJ domain protein                                            | 20.57226539     | +    | +    |
| 8    | SPAC139.05      | succinate-semialdehyde dehydrogenase                           | 18.48161055     | +    | +    |
| 9    | SPBC1773.05c    | tms1, hexitol dehydrogenase                                    | 15.83920026     | +    | +    |
| 10   | SPAPJ691.02     | yippee-like protein                                            | 13.9015024      | -    | +    |
| 11   | SPAC13A11.06    | pyruvate decarboxylase                                         | 12.99818471     | -    | -    |
| 12   | SPCC1281.07c    | glutathione S-transferase Gst3                                 | 12.56782373     | -    | +    |
| 13   | SPBC887.01      | acireductone dioxygenase family                                | 12.34299012     | -    | -    |
| 14   | SPACUNK4.17     | NAD binding dehydrogenase family protein                       | 12.32845218     | +    | +    |
| 15   | SPAC513.06c     | dihydrodiol dehydrogenase                                      | 12.26436079     | -    | +    |
| 16   | SPAC977.16c     | dak2, dihydroxyacetone kinase Dak2                             | 12.02308085     | +    | -    |
| 17   | SPCC830.07c     | psi1/psi, DNAJ domain protein Psi1                             | 11.83455111     | +    | -    |
| 18   | SPAC1786.02     | phospholipase                                                  | 11.53960213     | -    | -    |
| 19   | SPAC869.02c     | nitric oxide dioxygenase                                       | 10.07676961     | -    | +    |
| 20   | SPAC4F10.09c    | ribosome biogenesis protein Noc1                               | 10.02147028     | -    | -    |
| 21   | SPBP4H10.07     | ubiquitin-protein ligase E3                                    | 10.01224113     | -    | -    |
| 22   | SPBC12C2.03c    | FAD binding protein                                            | 9.887646211     | -    | -    |
| 23   | SPBC1198.13c    | tfg2, transcription factor TFIIIF complex beta subunit Tfg2    | 9.575179452     | -    | -    |
| 24   | SPCC417.07c     | mto1/mbo1/mod20, MT organizer Mto1                             | 9.566265854     | -    | -    |
| 25   | SPBC725.03      | conserved fungal protein                                       | 9.28405898      | +    | +    |
| 26   | SPAC23G3.09     | taf4, transcription factor TFIID complex subunit Taf4          | 9.108389758     | -    | -    |
| 27   | SPCC757.12      | alpha-amylase homolog                                          | 8.8215832       | -    | -    |
| 28   | SPAC1687.09     | conserved fungal protein                                       | 8.357111058     | -    | -    |
| 29   | SPCC1223.02     | nmt1/thi3, no message in thiamine Nmt1                         | 8.351475911     | -    | -    |
| 30   | SPAC23H4.01c    | sterol binding ankyrin repeat protein                          | 8.295490382     | -    | -    |
| 31   | SPBP4H10.12     | conserved protein (fungal and bacterial)                       | 8.132940624     | +    | -    |

|    |              |                                                                        |             |   |   |
|----|--------------|------------------------------------------------------------------------|-------------|---|---|
| 32 | SPBC3B9.01   | Hsp70 nucleotide exchange factor                                       | 8.036168957 | + | - |
| 33 | SPAC2C4.15c  | ubx2/ucp13, UBX domain protein Ubx2                                    | 7.738747368 | - | - |
| 34 | SPBC115.03   | gfo/idh/mocA family oxidoreductase                                     | 7.729149701 | - | - |
| 35 | SPAC1B3.03c  | wis2/cyp5, cyclophilin family peptidyl-prolyl cis-trans isomerase Wis2 | 7.542579362 | - | - |
| 36 | SPBC2D10.03c | conserved eukaryotic protein                                           | 7.483183967 | - | - |
| 37 | SPAC823.03   | ppk15, serine/threonine protein kinase Ppk15                           | 7.458771177 | - | - |
| 38 | SPCC895.05   | for3, formin For3                                                      | 7.456118631 | - | - |
| 39 | SPBC119.03   | S-adenosylmethionine-dependent methyltransferase                       | 7.3535792   | + | + |
| 40 | SPAC9G1.10c  | inositol polyphosphate phosphatase                                     | 7.342028207 | - | - |
| 41 | SPAC26H5.05  | IPT/TIG ankyrin repeat protein                                         | 7.240172354 | - | - |
| 42 | SPCC14G10.02 | ribosome biogenesis protein Urb1                                       | 7.236706028 | - | - |
| 43 | SPAC17A5.06  | ptr8, transcription factor TFIIH complex ERCC-3 subunit                | 7.20796805  | - | - |
| 44 | SPAC9E9.13   | wos2, p23 homolog                                                      | 7.112611911 | - | - |
| 45 | SPCC18.03    | shuttle craft like transcriptional regulator                           | 7.106318617 | - | - |
| 46 | SPAC5H10.05c | NADHdh_2 domain protein                                                | 6.81479964  | - | - |
| 47 | SPAC23H3.15c | sequence orphan                                                        | 6.654241946 | + | + |
| 48 | SPBC23G7.10c | NADH-dependent flavin oxidoreductase                                   | 6.598656321 | + | + |
| 49 | SPAC56F8.02  | AMP binding enzyme                                                     | 6.528217989 | - | - |
| 50 | SPAC2F7.03c  | pom1, DYRK family protein kinase Pom1                                  | 6.414216795 | - | - |
| 51 | SPBC30B4.02c | R3H and G-patch domain, unknown biological role                        | 6.394966186 | - | - |
| 52 | SPBC19G7.01c | msh2/swi8/mut3, MutS protein homolog 2                                 | 6.285064576 | - | - |
| 53 | SPCC965.07c  | gst2, glutathione S-transferase Gst2                                   | 6.118014916 | + | + |
| 54 | SPAP8A3.04c  | hsp9/scf1, heat shock protein Hsp9                                     | 6.082615309 | + | + |
| 55 | SPBC15C4.05  | ATP-dependent RNA/DNA helicase                                         | 6.058637488 | - | - |
| 56 | SPAC22E12.18 | conserved fungal protein                                               | 5.906127199 | - | - |
| 57 | SPBC1539.10  | ribosome biogenesis protein Nop16                                      | 5.890841874 | - | - |
| 58 | SPAC2H10.02c | 26S proteasome regulator                                               | 5.844724218 | - | - |
| 59 | SPBC11B10.09 | cdc2/swo2, cyclin-dependent protein kinase Cdc2                        | 5.653448517 | - | - |
| 60 | SPBC3H7.07c  | phosphoserine phosphatase                                              | 5.643085113 | - | - |
| 61 | SPAC13G7.02c | ssa1, heat shock protein Ssa1                                          | 5.609986138 | + | + |
| 62 | SPAC2F7.16c  | phospholipase D                                                        | 5.496443501 | - | - |
| 63 | SPBC215.02   | bob1/gim5, prefoldin subunit 5                                         | 5.457598994 | - | - |

|    |               |                                                                                      |             |   |   |
|----|---------------|--------------------------------------------------------------------------------------|-------------|---|---|
| 64 | SPCC18.16c    | fmn1, riboflavin kinase Fmn1                                                         | 5.440689734 | - | - |
| 65 | SPAC20H4.10   | ufd2, ubiquitin-protein ligase E4                                                    | 5.419775645 | - | - |
| 66 | SPAC4G8.05    | ppk14, serine/threonine protein kinase Ppk14                                         | 5.350200091 | - | - |
| 67 | SPBC11C11.08  | srp1, SR family protein Srp1                                                         | 5.309557959 | - | - |
| 68 | SPAC19D5.05c  | U3 snoRNP-associated protein Imp3                                                    | 5.285843471 | - | - |
| 69 | SPBC6B1.02    | ppk30, Ark1/Prk1 family protein kinase Ppk30                                         | 5.225206781 | - | - |
| 70 | SPBC29A3.02c  | his7, phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphohydrolase His7 | 5.223196042 | - | - |
| 71 | SPAC23C11.06c | hydrolase                                                                            | 5.216335057 | + | + |
| 72 | SPCC736.11    | ago1/csp9, argonaute                                                                 | 5.203361813 | - | - |
| 73 | SPBC4F6.07c   | ATP-dependent RNA helicase Mak5                                                      | 5.18123671  | - | - |
| 74 | SPBC31F10.13c | hip1/hir1, hira protein Hip1                                                         | 5.179537419 | - | - |
| 75 | SPCC1682.16   | rpt4, 19S proteasome regulatory subunit Rpt4                                         | 5.153884228 | - | - |
| 76 | SPCC736.07c   | cell polarity protein                                                                | 5.124927963 | - | - |
| 77 | SPBC1105.09   | ubc15, ubiquitin conjugating enzyme Ubc15                                            | 5.122237328 | - | - |
| 78 | SPCC188.08c   | ubp22/ubp5, ubiquitin C-terminal hydrolase Ubp22                                     | 5.081343766 | - | - |
| 79 | SPAC637.03    | conserved fungal protein                                                             | 5.056896177 | + | + |
| 80 | SPBC29A10.16c | cytochrome b5                                                                        | 5.038926778 | - | - |
| 81 | SPBC16E9.16c  | sequence orphan                                                                      | 5.032332597 | + | + |
| 82 | SPAC1F5.03c   | FAD-dependent oxidoreductase                                                         | 5           | - | - |
| 83 | SPCC10H11.01  | prp11, ATP-dependent RNA helicase Prp11                                              | 4.95298747  | - | - |
| 84 | SPAC26H5.04   | vacuolar import and degradation protein Vid28                                        | 4.929505163 | - | - |
| 85 | SPAC637.10c   | rpn10/pus1, 19S proteasome regulatory subunit Rpn10                                  | 4.929139266 | - | - |
| 86 | SPAC16E8.09   | scd1/ral1, RhoGEF Scd1                                                               | 4.782668255 | - | - |
| 87 | SPBC4F6.17c   | mitochondrial matrix chaperone Hsp78                                                 | 4.729265028 | + | - |
| 88 | SPBC887.03c   | noc3, Noc2p-Noc3p complex subunit Noc3                                               | 4.679266344 | - | - |
| 89 | SPAC19G12.08  | fatty acid hydroxylase                                                               | 4.654088269 | - | - |
| 90 | SPAC2F3.05c   | xylose and arabinose reductase                                                       | 4.55290016  | + | + |
| 91 | SPAC1F12.10c  | NADPH-hemoprotein reductase                                                          | 4.468348315 | + | - |
| 92 | SPCC4G3.13c   | CUE domain protein Cue1/4 family                                                     | 4.465687769 | - | - |
| 93 | SPAPB24D3.08c | NADP-dependent oxidoreductase                                                        | 4.461833822 | + | + |
| 94 | SPAC3G6.07    | sequence orphan                                                                      | 4.408353597 | - | + |
| 95 | SPAC664.03    | RNA polymerase II associated Paf1 complex                                            | 4.361076594 | - | - |

|     |              |                                                                   |             |   |   |
|-----|--------------|-------------------------------------------------------------------|-------------|---|---|
| 96  | SPCC63.08c   | ppk36/atg1, serine/threonine protein kinase Ppk36                 | 4.345699119 | - | + |
| 97  | SPCP1E11.04c | pal1, membrane associated protein Pal1                            | 4.32697659  | - | - |
| 98  | SPBC16A3.09c | ufd1, Cdc48-Ufd1-Npl4 complex component Ufd1                      | 4.298752047 | - | - |
| 99  | SPCC1281.06c | acyl-coA desaturase                                               | 4.295212156 | - | - |
| 100 | SPAC26A3.16  | dph1/ucp5, UBA domain protein Dph1                                | 4.295086395 | - | - |
| 101 | SPBC543.04   | conserved eukaryotic protein                                      | 4.264481805 | - | - |
| 102 | SPAC806.02c  | Par A family ATPase iron cluster assembly protein                 | 4.232380317 | - | - |
| 103 | SPBC336.04   | cdc6/pol3/pold/mis10, DNA polymerase delta catalytic subunit Cdc6 | 4.223632666 | - | - |
| 104 | SPBC4B4.01c  | pantothenate kinase                                               | 4.210589297 | - | - |
| 105 | SPAC27F1.05c | 4-aminobutyrate transaminase                                      | 4.208488661 | - | - |
| 106 | SPBC119.13c  | prp31, U4/U6 x U5 tri-snRNP complex subunit Prp31                 | 4.171436572 | - | - |
| 107 | SPCC1281.04  | pyridoxal reductase                                               | 4.122035273 | + | + |
| 108 | SPBC409.13   | 6,7-dimethyl-8-ribityllumazine synthase                           | 4.120889047 | - | + |
| 109 | SPAC23H3.02c | ini1, RING finger-like protein Ini1                               | 4.111807053 | - | - |
| 110 | SPCC1235.14  | ght5, hexose transporter Ght5                                     | 4.017454955 | - | - |