Reactive oxygen species (ROS) production triggers unconventional secretion of antioxidant enzymes

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Abstract

Nutrient deprivation triggers the release of signal-sequence-lacking antioxidant superoxide dismutase 1 (SOD1). We now report that secreted SOD1 is functionally active and accompanied by export of other antioxidant enzymes such as thioredoxins, (Trx1 and Trx2) and peroxiredoxin Ahp1. Our data reveal that starvation increases mitochondrial activity, which generates higher, but non-toxic, levels of reactive oxygen species (ROS). Inhibiting mitochondrial activity or ROS prevents antioxidants secretion. We suggest that in response to ROS production that can permeate the plasma membrane, the cells respond by secreting antioxidants to prevent ROS-mediated damage in the extracellular space. These findings provide an understanding of why cells secrete signal sequence lacking antioxidant enzymes in response to starvation.

Introduction

Unconventional protein secretion (UPS) is defined as the process by which eukaryotic cells export proteins that cannot enter the conventional endoplasmic reticulum (ER) – Golgi complex pathway. This fascinating process was noted upon the cloning of interleukin 1 (IL-1), which lacks a signal sequence for entry into the ER and yet is released by activated macrophages (Auron et al., 1984; Rubartelli et al., 1990). Since then, the repertoire of proteins released unconventionally has expanded considerably. The path taken by this class of proteins to reach the extracellular space varies. Briefly, Type I and II involve direct translocation across the plasma membrane, either via pores or ABC transporters, respectively, while Type III involves incorporation of the cargoes first into an intracellular membrane compartment (Rabouille, 2017; Nickel and Rabouille, 2018). Fibroblast growth factor (FGF)2 and Acyl-CoA binding protein (AcbA/Acb1) are two of the best studied examples of unconventionally secreted proteins thus far. FGF2 follows a Type II route, whereas, Acb1 takes a Type III path via that involves a cellular compartment called CUPS, the cytoplasmic proteins Grh1 and a subset of ESCRTs (Duran et al., 2010; Cruz-Garcia et al., 2014; Steringer et al., 2015; Curwin et al., 2016; Steringer et al., 2017; Cruz-Garcia et al., 2018). More recently, the export of cytoplasmic enzyme superoxide dismutase 1 (SOD1) was reported to follow the same pathway as Acb1, and both depend on a di-acidic motif (Cruz-Garcia et al., 2017). The release of IL-1ß, however, does not appear to have a unifying theme, and depending on the cell type or stimulus different mechanisms have been proposed that include pore formation via gasdermin D, autophagy-mediated, and via an intermediate membrane compartment (Rubartelli et al., 1990; Andrei et al., 2004; Dupont et al., 2011; Liu et al., 2016; Chiritoiu et al., 2019).

The release of Acb1 and SOD1 is triggered by nutrient starvation upon culturing yeast in potassium acetate. The secreted Acb1 in lower eukaryotes such as yeast and slime mold functions in signalling and regulating the starvation induced cell differentiation program of sporulation (Anjard et al., 1998; Kinseth et al., 2007; Manjithaya et al., 2010). In humans, secreted Acb1 also goes by the name of DBI (Diazepam binding inhibitor) and modulates GABA receptor signaling (Costa and Guidotti, 1991; Gandolfo et al., 2001). But what is the physiological significance of secreted SOD1? Here we report that secreted SOD1 is enzymatically active in the extracellular space. Are other antioxidant enzymes secreted along with SOD1? A starvation specific secretome revealed enrichment in cytoplasmic enzymes that function in response to increased reactive oxygen species (ROS) production or oxidative stress. We show that starvation of cells in potassium acetate activates mitochondrial respiration for ATP production, leading to elevated levels of ROS, and both these events are required for unconventional secretion of the antioxidant enzymes, and Acb1. Therefore, during nutrient starvation in potassium acetate, cells sense increased ROS and oxidative stress, leading to secretion of active antioxidant enzymes as a means to protect the extracellular space or perhaps to modulate ROS mediated signalling events.

Results and discussion

Secreted SOD1 is functionally active

Culturing yeast in potassium acetate promotes the unconventional secretion of SOD1, but is secreted SOD1 functionally active (Cruz-Garcia et al., 2017)? Wild-type and mutant SOD1 linked to the neurodegenerative disorder amyotrophic lateral sclerosis (ALS) are reportedly

secreted in a misfolded state when expressed in a motor neuron cell line (Grad et al., 2014), therefore the enzymatic activity of extracellular SOD1 cannot be presumed. We employed a zymography-based assay to directly test whether secreted SOD1 is enzymatically active in yeast cells cultured in potassium acetate. A mild cell wall extraction assay developed to detect starvation specific secretion of Acb1 and SOD1 was performed in non-denaturing conditions (Curwin et al., 2016; Cruz-Garcia et al., 2017). Intracellular and secreted proteins were then separated by native-gel electrophoresis and a standardized in-gel SOD1 activity assay was performed as described previously (Beauchamp and Fridovich, 1971). Our data reveal that both the intracellular and secreted SOD1 are enzymatically active (Fig. 1 A). A corresponding western blot indicated that secreted pool of SOD1 is as active as its intracellular counterpart, suggesting that most, if not all, secreted SOD1 is functionally active (Fig. 1 A).

Identification of a family of secreted antioxidants

Do cells secrete other antioxidant enzymes, like SOD1, upon nutrient starvation? Wild type yeast were cultured in growth or starvation medium, the cell wall proteins were extracted under the conditions described previously (Curwin et al., 2016) and subsequently analysed by mass spectrometry. We also analysed cell wall proteins from cells lacking Vps23, which is required for starvation induced secretion of Acb1 and SOD1, to assist in identification of proteins exported by the same pathway. All analyses were performed in triplicate with statistical evaluation (see Materials and Methods). Using a cut-off of a Log2FoldChange of at least -/+ 1, and a *p* value of less than 0.06, the proteins were classified as starvation versus growth specific, or unchanged (complete tables of the mass spectrometry and statistical analysis are found in Tables S1 and S2, respectively). A total of 136 secreted proteins were identified of which 79 are

growth specific, 35 starvation specific, and 22 exhibited no change upon starvation (Fig. 1 B; Table S2). As expected, the levels of secreted Acb1 and SOD1 were significantly increased upon starvation (log2FC of -3.38 and -2.94 respectively) and their release is Vps23 dependent (Fig. 1 C; Table S2). Of the 35 starvation specific proteins, 26 required Vps23 for their export (Fig. 1, B and C; Table S2). Importantly, none of the starvation specific secreted proteins contained a signal peptide for their entry into the ER, as predicted by SignalP 4.1 (Petersen et al., 2011). In fact, only 27 of the 136 total proteins identified contained a signal peptide, all of which were found to be secreted specifically in growth (Table S2).

We were surprised to find that like SOD1, other enzymes directly involved in redox homeostasis were also secreted in a starvation specific manner. These include the thiol specific peroxiredoxin, Ahp1 that reduces preferentially alkyl hydroperoxides, as well as the cytoplasmic thioredoxins of *Saccharomyces cerevisiae*, Trx1 and Trx2, which function to reduce redox active proteins, including Ahp1 (Holmgren, 1989; Lee et al., 1999). It is also noteworthy that thioredoxin has previously been shown to be secreted unconventionally by mammalian cells (Rubartelli et al., 1992). Closer examination of the starvation specific proteins revealed that 24 are enzymes that function in conserved processes known to affect cellular redox balance (e.g. glycolysis/gluconeogenesis, amino acid/nucleotide biosynthesis, and glycerol metabolism) and 19 are actually annotated to function in response to oxidative stress according to the *Saccharomyces* Genome Database (Fig. 1, B and C; Table 1 and S2). The 15 enzymes involved in response to oxidative stress and dependent on Vps23 for their secretion are highlighted in Figure 1 C and Table 1. Therefore, nutrient starvation upon culture of cells in potassium acetate

leads to unconventional secretion of a number of enzymes that function, either directly or indirectly, in response to oxidative stress.

We focussed on three enzymes mentioned above: Ahp1, Trx1 and Trx2. Like SOD1, they function directly in regulating redox homeostasis and are amongst the more abundant enzymes identified in the secretome (Fig. 1 C; Table S1). Secretion of SOD1 (and Acb1) requires Grh1 (Curwin et al., 2016; Cruz-Garcia et al., 2017), so we first tested the involvement of Grh1 in secretion of these enzymes. A secretion assay of wild-type and *grh1Δ* starved cells revealed that the release of Ahp1, Trx1 and Trx2 upon starvation is also Grh1 dependent (Fig. 1 D). This, along with the finding that they require Vps23 for their release upon nutrient starvation lends us to conclude that these antioxidant enzymes follow the same pathway of unconventional secretion as SOD1 and Acb1.

Starvation leads to enhanced mitochondrial activity and ROS production

Based on the known antioxidant function of these enzymes, we hypothesized that starvation increases ROS production, which signals their unconventional secretion. The major source of ROS in most cell types is the mitochondria, as by-products of aerobic respiration, therefore we decided to investigate mitochondrial organization and function during starvation (Costa and Moradas-Ferreira, 2001; Starkov, 2008; Murphy, 2009). *S. cerevisiae* cells growing in glucose actively repress mitochondrial respiration and rely entirely on glycolysis for energy (known as fermentation) (Entian and Barnett, 1992; Ahmadzadeh et al., 1996). When the carbon source is changed to a non-fermentable one, such as glycerol, ethanol or acetate, cells switch to mitochondrial respiration as the major source of ATP (Boy-Marcotte et al., 1998). Our nutrient

starvation conditions consist of culturing cells in potassium acetate, therefore, we expect an acute decrease in ATP production due to lack of glucose, followed by a switch to mitochondrial respiration for ATP synthesis. To monitor the activity of the mitochondria in starvation conditions, we incubated cells with Mito-Tracker, which preferentially labels actively respiring mitochondria (Kholmukhamedov et al., 2013). We also expressed, exogenously, a mitochondrial targeted DsRed fusion protein that labels mitochondria irrespective of their activity (Meeusen et al., 2004). In cells grown in glucose, Mito-Tracker and mito-DsRed labelled mitochondria are identified as long tubules (Fig. 2, A and B). Upon switch to potassium acetate, an increase in mitochondrial activity was immediately apparent, as indicated by increased intensity of Mito-Tracker labelling (Fig. 2 A). We also noted that mitochondrial morphology was dramatically altered, appearing much larger and rounded, as indicated by both the Mito-Tracker dye and mito-DsRed fusion protein (Fig. 2, A and B). By 2.5 hours of starvation, all cells exhibited a change in mitochondrial morphology and activity.

Based on these observed changes in the mitochondria, we asked whether ATP production was required for unconventional secretion. The chemical 2,4-DNP uncouples electron transport and ATP synthesis by destroying the proton gradient necessary for the mitochondrial ATP synthase function (Loomis and Lipmann, 1948). Therefore, starving cells treated with 2,4-DNP will not generate any ATP, although electron transport and any subsequent ROS production would still occur. As mitochondria represent the sole source of ATP in these culture conditions, it was not a surprise that treatment with 2,4-DNP strongly affected secretion of all cargo proteins tested in a dose dependent manner (Fig. 2 C). Therefore, the process of unconventional secretion is ATP dependent. These data also reveal that uncoupling the events of ATP and ROS production

in the mitochondria is not feasible, as any perturbation to electron transport will inhibit ATP synthesis and therefore secretion.

Direct detection of ROS in living cells by fluorescent dyes or expression of intracellular probes is problematic (Gomes et al., 2005; Belousov et al., 2006; Chen et al., 2010). Therefore, to confirm the production of ROS during starvation we decided to visualize the location of Yap1, a major transcription factor that responds to multiple forms of oxidative stress by its translocation to the nucleus (Schnell et al., 1992; Kuge et al., 1997). As a control, we exposed yeast cells to 1 mM H_2O_2, a strong oxidative stress to the cell that leads to rapid and complete translocation of Yap1-GFP to the nucleus (Fig. 3 A) (Delaunay et al., 2000). Exposure of cells to lower amounts of H_2O_2 caused Yap1-GFP translocation, but to a much lesser extent (Fig. 3 A). In cells starved in potassium acetate, Yap1-GFP could be detected in the nucleus by 2 hours of starvation and in virtually all cells by 3 hours (Fig. 3 B). Clearly then, cells cultured in starvation medium generate and sense oxidative stress, but not to the extent observed upon strong oxidative stress, such as exposure to 1 mM H_2O_2.

Carbonylation of proteins and lipids is an irreversible covalent modification that occurs in response to high levels of ROS and is an indicator of overall cellular damage due to excessive ROS production (Fedorova et al., 2014). Protein carbonylation can be detected by labelling total protein extracts with a fluorescent dye FTC (fluorescein-5-thiosemicarbazide) that specifically reacts with the carbonyl moiety on proteins. Again, we used growing cells exposed to 1 mM H_2O_2 as a control and compared the level of protein carbonylation in growing and starved cells. Our data reveal that starvation of cells in potassium acetate for 2.5 hours did not lead to any

detectable level of protein carbonylation, while cells treated with 1 mM H_2O_2 for 1 hour had clearly increased levels of carbonylated proteins (Fig. 3 C). The combined evidence shows that cells starving in potassium acetate produce and respond to low, non-damaging amounts of ROS.

Elevated intracellular ROS is required for secretion

To test whether ROS production is a pre-requisite for unconventional protein secretion upon starvation we asked if treatment of cells with the antioxidant n-acetylcysteine (NAC) affects secretion. To ascertain that NAC is efficiently taken up by cells and to ensure that it did not affect the ability of cells to sense starvation, growing cells were incubated with 10 mM NAC for 2 hours, washed, and resuspended in potassium acetate without NAC, so the starvation period was unchanged from a typical secretion assay. The cell walls were extracted and analyzed as before, revealing that this pre-treatment with NAC was sufficient to significantly reduce secretion of all the unconventionally secreted cargoes tested (Fig. 3 D). We can thus conclude that starvation of yeast in potassium acetate leads to increased mitochondrial respiration with subsequent ROS production, which is required for unconventional secretion of antioxidant proteins. But is elevated ROS alone sufficient to induce secretion? To test this, we asked whether addition of exogenous H_2O_2 to growing cells would lead to secretion of the antioxidant enzymes. H_2O_2 is only the ROS that can freely cross membranes, while charged ROS, such as the superoxide anion (O_2^-) or hydroxyl groups (OH^-) cannot. We tested addition of 0.1 mM H_2O_2, which was sufficient to induce Yap1-GFP translocation to the nucleus to a similar extent as observed upon starvation (Fig. 3, A and B). Growing and starving cells were exposed to 0.1 mM H_2O_2 for 1 hour or the 2.5-hour duration of starvation, and secreted antioxidant enzymes were analyzed by western blot. The results reveal that exogenous addition of 0.1 mM H_2O_2 does not

induce secretion during growth, nor enhance secretion upon starvation. Therefore, simply creating a pool of cytoplasmic ROS, specifically H_2O_2, is not sufficient to induce unconventional secretion of these antioxidants. This indicates the significance of specific species of reactive oxygen, and/or the source of the ROS, namely the mitochondria, in antioxidant secretion.

Conclusion

We have previously shown that a di-acidic motif contained within SOD1 and Acb1 is required for their starvation specific secretion. In this work, we have focussed on whether the proteins such as SOD1 are functionally active in the extracellular space and the cellular signals that trigger their export. Our findings reveal that SOD1 is secreted in a functionally active form. We have also discovered that cells secrete a number of other enzymes that function together with SOD1 to regulate cellular redox homeostasis. This makes sense because of our findings that culture of yeast in potassium acetate leads to an increased activity of morphologically changed mitochondria, leading to moderate ROS production. The cells therefore respond by exporting antioxidant enzymes. This link is further strengthened by the data that inhibiting mitochondrial activity or ROS blocks unconventional secretion.

Based on our findings, it is reasonable to propose that secretion of SOD1 and other antioxidant enzymes to the extracellular space prevents damage by ROS. The superoxide anion scavenged by SOD1 is damaging to both proteins and lipids. Under starvation conditions, intracellular SOD1 may be unable to contain rapid elevation in superoxide levels, allowing their leakage into the extracellular space via damage to the plasma membrane. The secreted SOD1

activity would then function to scavenge the extracellular superoxide. The activity of secreted Ahp1, whose preferred substrate is an alkyl-peroxide, would repair the damaged lipids of the plasma membrane. Finally, in this scheme, the secreted thioredoxins (Trx1/2) function to reactivate Ahp1 in the extracellular space. Although we did not identify thioredoxin reductase in our secretome analysis, it could be present in low amounts and simply not detected. Trx1 and Trx2 are also secreted in higher relative amounts than Ahp1, as indicated by the western blot analysis, which likely permits continued reactivation of Ahp1 without the need of a secreted thioredoxin reductase activity. This is how we envision a collection of antioxidants are employed in the extracellular space to prevent the damaging effects of superoxide and other ROS species. An alternative, but not exclusive hypothesis, is that the extracellular ROS can act as a secondary messenger and the secreted antioxidants might be used to control the amplitude of ROS mediated signaling (Hurd et al., 2012; Dunnill et al., 2017).

An interesting precedent for the unconventional secretion of functional antioxidant enzymes comes from studies in pathogenic yeast species. Adherence and biofilm formation, that are forms of cellular differentiation, as well as the ability to detoxify ROS, are important virulence factors in *Candida* and *Aspergillus* species. Analyses of cell wall proteins from these species specifically under differentiation conditions, revealed the presence of antioxidant enzymes, including SOD1, as well as many so called “moonlighting” proteins that are highly conserved proteins such as glycolytic and other general metabolic enzymes, much like those identified in our secretome analysis, (e.g. Fba1) (Wartenberg et al., 2011; Ramírez-Quijas et al., 2015; Serrano-Fujarte et al., 2016; Núñez-Beltrán et al., 2017). These studies provide an important connection to how cells respond to ROS by exhibiting in the extracellular space a

number of enzymes that can combat ROS, however, these studies did not systematically address how the respective cytoplasmic antioxidants, and other enzymes, reach the extracellular space. Combined with our new data, we can now state with confidence that, depending on the physiological needs, yeast secrete a number of signal sequence lacking enzymes to function in the extracellular space.

Altogether, our findings reveal a fascinating situation whereby starvation, a physiological relevant condition, triggers the over activation of mitochondria, which then by default results in ROS production and cells respond by secreting a collection of antioxidant enzymes. The challenge now is to elucidate the significance of functionally active antioxidants in the extracellular space.

Materials and Methods

Yeast strains and media

Yeast cells were grown in synthetic complete (SC) media (0.67% yeast nitrogen base without amino acids, 2% glucose supplemented with amino acid drop-out mix from FORMEDIUM). Wild type strain is BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) originally obtained from EUROSCARF. Deletion strains were from the EUROSCARF collection with individual genes replaced by KanMx4. Yap1-GFP was from the Invitrogen collection of C-terminally tagged proteins linked to His3Mx6.

Antibodies

All antibodies were raised in rabbit and have been described previously. Anti-Ahp1, anti-Trx1 and anti-Trx2 were the generous gifts of Shusuke Kuge (Tohoku Medical and Pharmaceutical University), Eric Muller (University of Washington) and Yoshiharu Inoue (Research Institute for Food Science, Kyoto University), respectively. Anti-Cof1 was kindly provided by John Cooper (Washington University in St. Louis) and anti-Bgl2 was a gift from Randy Schekman (UC Berkeley). Anti-Acb1 antibody was generated by inoculating rabbits with recombinant, untagged Acb1, purified from bacteria. Specificity of the serum was confirmed by testing lysates prepared from *acb1Δ* cells.

Cell wall extraction assay

Yeast cells were inoculated at a density of 0.003-0.006 OD_{600}/mL in SC medium at 25°C. The following day, when cells had reached OD_{600} of 0.4-0.7 equal numbers of cells (16 OD_{600} units) were harvested, washed twice in sterile water, resuspended in 1.6 mL of 2% potassium acetate

and incubated for 2.5 h. When growing cells were to be analyzed 16 OD_{600} units were directly harvested. The cell wall extraction buffer (100mM Tris-HCl, pH 9.4, 2% sorbitol) was always prepared fresh before use and kept on ice. To ensure no loss of cells and to avoid cell contamination in the extracted buffer, 2mL tubes were siliconized (Sigmacote) prior to collection. Cells were harvested by centrifugation at 3,000x *g* for 3 min at 4°C, medium or potassium acetate was removed and 1.6 mL of cold extraction buffer was added. Cells were resuspended gently by inversion and incubated on ice for 10 min, after which they were centrifuged as before, 3,000x *g* for 3 min at 4°C, and 1.3 mL of extraction buffer was removed to ensure no cell contamination. The remaining buffer was removed and the cells were resuspended in 0.8 mL of cold TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) with protease inhibitors (aprotinin, pepstatin, leupeptin; Sigma-Aldrich) and 10 µL was boiled directly in 90 µL of 2x sample buffer (lysate). For western blotting analysis, 30 µg of BSA (bovine serum albumin; Sigma-Aldrich) carrier protein and 0.2 mL of 100% Trichloroacetic acid (Sigma-Aldrich) was added to the extracted protein fraction. Proteins were precipitated on ice for 1 h, centrifuged 16,000x *g* for 30 min and boiled in 50 µL 2x sample buffer. For detection, proteins (10 µL each of lysate or wall fractions) were separated in a 12% polyacrylamide gel before transfer to nitrocellulose (GE Healthcare). For preparation of cell wall extracts for mass spectrometry analysis, no BSA carrier protein was added and the proteins were precipitated with acetone and not TCA.

"In-gel" SOD activity assay

After starvation in 2% potassium acetate for 2.5 h, 8 x 16 OD_{600nm} of cells were subjected to the cell wall extraction protocol as described above. Then, cell wall extracts were pooled and

concentrated using Amicon Ultra centrifugal filter 3K devices with buffer exchange into TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) up to a final volume of 75 µl. Also, after incubation in 2% potassium acetate, 16 OD_{600nm} of cells were lysed by bead beating with glass beads in 800 µL of TBS containing protease inhibitors. Lysates were clarified by centrifugation at 16,000x *g* for 15 min. "In-gel" SOD activity assay was carried out on 12% polyacrylamide gels as described earlier (Beauchamp and Fridovich, 1971). Lysates and concentrated cell wall extracts were separated under native conditions for 7 hours at 40mA. Following completion of the run, the gels were incubated in 2.45 mM Nitroblue Tetrazolium (Sigma-Aldrich) in distilled water for 20 min at room temperature in the dark, followed by additional 15 min incubation with 28 µM riboflavin (Sigma-Aldrich) and 28 mM TEMED (Sigma-Aldrich) in 50 mM potassium phosphate buffer, pH 7.8. Then, the gels were rinsed twice in distilled water, illuminated under a bright white light for 20-30 min and photographed using an Amersham Imager 600. Image processing was performed with ImageJ 1.45r software.

Protein sample preparation for mass spectrometry

Samples were reduced with dithiothreitol (27 nmols, 1 h, 37°C) and alkylated in the dark with iodoacetamide (54 nmol, 30 min, 25ºC). The resulting protein extract was first diluted 1/3 with 200 mM NH_4HCO_3 and digested with 0.9 µg LysC (Wako) overnight at 37ºC and then diluted 1/2 and digested with 0.9 µg of trypsin (Promega) for 8 h at 37ºC. Finally, the peptide mix was acidified with formic acid and desalted with a MicroSpin C18 column (The Nest Group, Inc) prior to LC-MS/MS analysis.

Mass spectrometric analysis

The peptide mixes were analyzed using a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) coupled to an EasyLC (Thermo Fisher Scientific). Peptides were loaded directly onto the analytical column at a flow rate of 1.5-2 µl/min using a wash-volume of 4 times the injection volume, and were separated by reversed-phase chromatography using a 25-cm column with an inner diameter of 75 µm, packed with 3 µm C18 particles (Nikkyo Technos, Co., Ltd.). Chromatographic gradients started at 93% buffer A and 7% buffer B with a flow rate of 250 nl/min for 5 minutes and gradually increased 65% buffer A and 35% buffer B in 60 min. After each analysis, the column was washed for 15 min with 10% buffer A and 90% buffer B. Buffer A: 0.1% formic acid in water. Buffer B: 0.1% formic acid in acetonitrile.

The mass spectrometer was operated in data-dependent mode with one full MS scan per cycle followed by the sequential fragmentation of the ten most intense ions with multiple charged (normalized collision energy of 35%). Fragment ion spectra produced via collision-induced dissociation (CID) were acquired in the Ion Trap. All data were acquired with Xcalibur software v2.1.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PXD010849.

Data Analysis

Proteome Discoverer software suite (v1.4, Thermo Fisher Scientific) and the Mascot search engine (v2.5, Matrix Science) (Perkins et al., 1999) were used for peptide identification. Samples were searched against a *S. cerevisiae* database (version February 2014) containing a list of

common contaminants and all the corresponding decoy entries. Trypsin was chosen as enzyme and a maximum of three missed cleavages were allowed. Carbamidomethylation (C) was set as a fixed modification, whereas oxidation (M) and acetylation (N-terminal) were used as variable modifications. Searches were performed using a peptide tolerance of 7 ppm, a product ion tolerance of 0.5 Da and the resulting data files were filtered for FDR < 5 %. Peptide areas were used as input for statistical inference (MSstats v2.6) (Choi et al., 2014).

MitoTracker staining

After incubation in 2% potassium acetate, 10 OD_{600nm} of cells were harvested by centrifugation at 3,000x *g* for 4 min, resuspended at 5 OD_{600nm} /ml in 100 nM MitoTracker Red CMXRos (Molecular Probes) diluted in phosphate-buffered saline, and incubated in the dark for 30 min. Then, ~1.5 OD_{600nm} of cells were harvested by centrifugation at 3,000 *g* for 3 min, resuspended in a small volume, spotted on a microscopy slide, and subsequently live-imaged at 25°C with a DMI6000 B epifluorescence microscope (Leica) equipped with a DFC 360FX camera (Leica) using an HCX Plan Apochromat 100× 1.4 NA objective lens. Images were acquired using LAS AF software (Leica) with 0.08 s exposure times. Image processing was performed with ImageJ 1.45r software.

Detection of total protein carbonylation

For detection of total protein carbonylation wild type cells were grown to mid-logarithmic phase ("growth"), washed twice, and cultured in 2% potassium acetate for 2.5 h ("starvation"). Growing cells were also subjected to 1mM H_2O_2 treatment for 1 h as a positive control. Subsequently cells were lysed by bead beating in carbonylation buffer (20 mM sodium

phosphate, pH 6, 1 mM EDTA, 6 M Urea, plus protease inhibitors) and cell extracts were cleared by centrifugation at 10,000x *g*, for 10 min at 4°C. Supernatants were treated with 1% streptomycin sulphate for 5 min to remove nucleic acids, and cleared again by centrifugation at 10,000x *g* for 5 min at 4°C. Extracts were diluted to 1 µg/µL in carbonylation buffer and 200 µL was labelled with 50 mM fluorescein-5-thiosemicarbazide (FTC) (Fluka) for 2 h in the dark. Proteins were precipitated with TCA, unbound FTC was removed by washing 5 times in ice cold ethanol:ethyl acetate (1:1). Protein pellets were resuspended 50 µL dissolving buffer (20 mM sodium phosphate, pH 8, 1 mM EDTA, 8 M Urea) and samples were prepared with 5x sample buffer without bromophenol blue for separation in a 12% SDS-acrylamide gel by running 5 µg of protein per lane, in 2 separate gels. Total protein was visualized in one gel by silver staining, while fluorescent carbonylated proteins were visualized in the second gel using an Amersham Biosciences Typhoon scanner.

Online supplemental material

Table S1 shows the complete proteomic data of the cell wall extracts analysed in this study.

Table S2 shows the statistical analysis of the mass spectrometry data and the classification of each identified protein as “growth specific”, “starvation specific” or “unchanged”, as “Vps23 dependent” or “Vps23 independent”, or as bearing or not a signal peptide.

Acknowledgements

We thank members of the Malhotra lab for their valuable discussions. V. Malhotra is an Institució Catalana de Recerca i Estudis Avançats professor at the Centre for Genomic Regulation. This work was funded by grants from the Spanish Ministry of Economy and Competitiveness (BFU2013-44188-P and BFU2016_75372-P to VM). We acknowledge support of the Spanish Ministry of Economy and Competitiveness, through the Programmes "Centro de Excelencia Severo Ochoa 2013- 2017" (SEV-2012-0208 & SEV-2013-0347). The mass spectrometry data has been acquired at the CRG/UPF Proteomics Unit, which is part of Proteored, PRB3 and is supported by grant PT17/0019, of the PE I+D+i 2013-2016, funded by ISCIII and ERDF. This work reflects only the authors' views, and the EU Community is not liable for any use that may be made of the information contained therein.

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Figure Legends

Figure 1. Starvation triggers secretion of cytoplasmic antioxidant enzymes

(A) Wild type cells were grown to mid-logarithmic phase, washed twice, and cultured in 2% potassium acetate for 2.5 h. Cell wall proteins ("secreted") were extracted and concentrated with exchange to TBS (Tris-buffered saline). Separately, an aliquot of the same cells was lysed in non-denaturing conditions in TBS ("lysate"). Lysate and secreted proteins were separated in non-denaturing 12% polyacrylamide gels and either transferred to a nitrocellulose membrane for western blot analysis ("anti-SOD1") or subjected to the zymography based in-gel SOD activity assay ("SOD1 activity"). Samples of lysate fractions were loaded in decreasing amount, at a dilution of 1/10 to compare with the concentrated secreted fraction.

(B) Mass spectrometry analysis of cell wall extracted proteins (secreted proteins) from wild type cells growing in nutrient-rich conditions ("growth") versus cultured in potassium acetate for 2.5 h ("starvation"). Cell wall proteins from cells lacking Vps23 were also analyzed under starvation conditions. All three conditions were analyzed in triplicate and statistical analyses were performed to classify the secreted proteins as growth versus starvation specific or unchanged (using a cut-off of a Log2FoldChange of at least -/+ 1, and a *p* value of less than 0.06). Within the starvation specific group, proteins were further classified as dependent on Vps23 or not for their presence in the cell wall, whether they perform an enzymatic activity, and whether they are annotated to affect response to oxidative stress according to the *Saccharomyces* Genome Database (SGD). Complete analyses of proteins identified and the classifications can be found in Tables S1 and S2.

(C) Chart plotting relative protein abundance of the starvation specific/Vps23 dependent enzymes classified as affecting response to oxidative stress. SOD1, Ahp1, Trx1 and Trx2 directly

regulate cellular redox balance, while the remaining are key enzymes in various cellular processes known to affect response to oxidative stress such as glycolysis, gluconeogenesis, amino acid or nucleotide biosynthesis, and glycerol metabolism (Acb1 is included only as a reference). The average protein areas from the mass spectrometry analysis (Table S1) of the three triplicates for each protein was calculated, with the corresponding SEM (error bars), for each condition. A zoom of the low abundance enzymes is shown in the inset (note the scale difference). Complete descriptions of the enzymes can be found in Table 1.

(D) Wild type and *grh1Δ* cells were grown to mid-logarithmic phase, washed twice, and incubated in 2% potassium acetate for 2.5 h. Cell wall proteins were extracted from equal numbers of cells followed by precipitation with TCA ("secreted"). Lysates and secreted proteins were analyzed by western blot and the ratio of the secreted/lysate for the indicated cargo protein was determined and compared to that of wild type in each experiment. Statistical analyses were performed for the indicated unconventional cargo proteins and are represented as fold changes compared to wild type (paired student's t-test). Ahp1=0.07 -/+0.03, p=0.0604; Trx1=0.25 -/+0.04, p=0.0478; Trx2=0.23 -/+0.03, p=0.0065. Error bars indicate SEM, n=3. Cof1 is non-secreted, cytoplasmic protein used to monitor for cell lysis, while Bgl2 is a conventionally secreted cell wall protein.

Figure 2. Nutrient starvation induces morphological changes in mitochondria and increases mitochondrial activity

(A) Wild type cells were grown to mid-logarithmic phase ("growth"), washed twice, and cultured in 2% potassium acetate for 2.5 h ("starvation"). Cells from each condition were

labelled with MitoTracker to examine mitochondrial activity and morphology. Cells were visualized by epifluorescence microscopy. Scale bar, 2 μm.

(B) Wild type cells expressing a mitochondrial targeted DsRed construct were cultured and visualized as in (A). Scale bar, 2 μm.

(C) Wild type cells were grown to mid-logarithmic phase, washed twice, and cultured in 2% potassium acetate for 2.5 h in the presence of 100 nM or 1 mM 2,4-DNP ("low" or "high", respectively) and the corresponding low or high amount of DMSO carrier as control (1:2000 and 1:500, respectively). Cell wall proteins were extracted from equal numbers cells followed by precipitation with TCA. Lysates and cell wall-extracted proteins were analyzed by western blot. 2,4-DNP treatment strongly inhibited secretion of all proteins analyzed, with low dose ranging from 15-30% reduction, while high dose 2,4-DNP virtually blocked secretion of the cargo proteins, ranging from 3-9% reduction (n=4). Cof1 is non-secreted, cytoplasmic protein used to monitor for cell lysis, while Bgl2 is a conventionally secreted cell wall protein.

Figure 3. Starvation leads to a moderate intracellular ROS production, which is a pre-requisite for secretion of antioxidant enzymes

(A) Yap1-GFP expressing cells were grown to mid-logarithmic phase and incubated in the presence or absence of the indicated amount of H_2O_2 for 20 min before visualization by epifluorescence microscopy. Scale bar, 2 μm.

(B) Yap1-GFP expressing cells were grown to mid-logarithmic phase ("growth"), washed twice, and cultured in 2% potassium acetate for 2.5 h ("starvation"). Yap1-GFP was visualized by epifluorescence microscopy. Scale bar, 2 μm.

(C) For detection of total protein carbonylation wild type cells were grown to mid-logarithmic phase ("growth"), washed twice, and cultured in 2% potassium acetate for 2.5 h ("starvation"). Growing cells were also subjected to 1 mM H_2O_2 treatment for 1 h as a positive control. Subsequently, cells were lysed, treated with streptomycin sulphate to remove nucleic acids and protein carbonyl groups were labelled with fluorescein-5-thiosemicarbazide (FTC) for 2 hours in the dark. Proteins were precipitated with TCA, unbound FTC was removed, protein pellets were resuspended and separated by SDS-PAGE. Fluorescence was visualized with an Amersham Biosciences Typhoon scanner, while total protein was visualized separately by silver staining.

(D) Wild type cells were grown to low-logarithmic phase, and either treated with 10 mM n-acetylcysteine (NAC) for 2 h (+) or not (-). Subsequently, equal cell number were washed twice, and incubated in 2% potassium acetate for 2.5 h. Cell wall proteins were extracted and precipitated with TCA ("secreted"). Lysates and secreted proteins were analyzed by western blot. The ratio of secreted/lysate for each cargo was determined and +NAC was normalized to -NAC in each experiment. Statistical analyses were performed for each cargo and are represented as fold change upon NAC treatment (paired student's t-test). Acb1=0.32 -/+0.02, p=0.0335; SOD1=0.27 -/+0.05, p=0.032; Ahp1=0.26 -/+0.03, p=0.0004; Trx1=0.17 -/+0.05, p=0.0597; Trx2=0.13 -/+0.04, p=0.002. Error bars indicate SEM, n=3. Cof1 is non-secreted, cytoplasmic protein used to monitor for cell lysis, while Bgl2 is a conventionally secreted cell wall protein.

(E) For "growth "samples, wild type cells were grown to mid-logarithmic phase and treated (+) or not (-) with 0.1 mM H_2O_2 for 1 hour. For starvation ("starv.") samples, wild type cells were grown to mid-logarithmic phase, equal cell number were washed twice, and incubated in 2% potassium acetate for 2.5 h in the absence (-) or presence (+) of 0.1 mM H_2O_2. Subsequently, cells were processed as in (A) to analyze secreted versus lysate pools of the indicated cargo

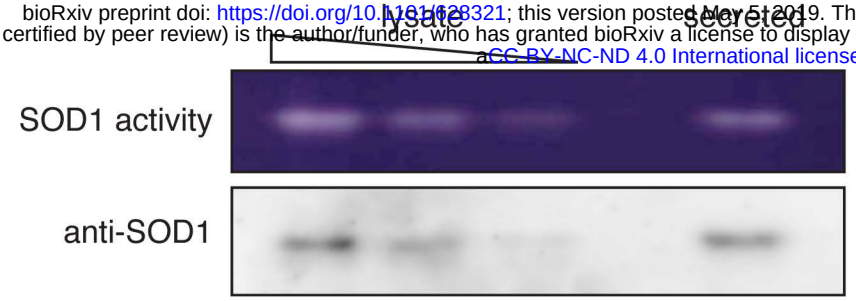
proteins and the same statistical analyses were performed to compare the fold change upon H_2O_2 treatment in the growth or starvation conditions. The observed changes were minor and not found to be statistically significant. Error bars indicate SEM, n=4. Cof1 is non-secreted, cytoplasmic protein used to monitor for cell lysis, while Bgl2 is a conventionally secreted cell wall protein.

Table 1. Starvation specific/Vps23 dependent secreted enzymes with direct or indirect functions in response to oxidative stress

Gene	Protein	Description
YJR104C/ SOD1	Superoxide dismutase [Cu-Zn]	Cytosolic copper-zinc superoxide dismutase; detoxifies superoxide
YLR109W/ AHP1	Peroxiredoxin	Thiol-specific peroxiredoxin; preferentially reduces alkyl hydroperoxides
YLR043C/ TRX1	Thioredoxin-1	Cytoplasmic thioredoxin isoenzyme; part of thioredoxin system which protects cells against oxidative and reductive stress
YGR209C/ TRX2	Thioredoxin-2	Cytoplasmic thioredoxin isoenzyme; part of thioredoxin system which protects cells against oxidative and reductive stress
YIL053W/ GPP1	Glycerol-3-phosphate phosphatase	Hydrolyzes glycerol-3-P; involved in glycerol biosynthesis
YKL060C/ FBA1	Fructose-bisphosphate aldolase	Catalyzes conversion of fructose 1,6 bisphosphate to glyceraldehyde-3-P and dihydroxyacetone-P; required for glycolysis and gluconeogenesis
YCR053W/ THR4	Threonine synthase	Catalyzes formation of threonine from O-phosphohomoserine
YJR105W/ ADO1	Adenosine kinase	Required for the utilization of S-adenosylmethionine
YGL253W/ HXK2	Hexokinase-2	Phosphorylates glucose in cytosol; predominant hexokinase during growth on glucose
YCL050C/ APA1	AP4A phosphorylase	Catalyzes phosphorolysis of dinucleoside oligophosphates
YGR234W/ YHB1	Nitric oxide oxidoreductase	Flavohemoglobin that plays role in oxidative and nitrosative stress responses
YHR163W/ SOL3	6-phospho-gluconolactonase	Catalyzes the second step of the pentose phosphate pathway
YAR015W/ ADE1	Adenylosuccinate synthetase	Required for 'de novo' purine nucleotide biosynthesis
YJR070C/ LIA1	Deoxyhypusine hydroxylase	Metalloenzyme that catalyzes hypusine formation
YDR071C/ PAA1	Polyamine acetyltransferase	Acetylates polyamines

Figure 1

A



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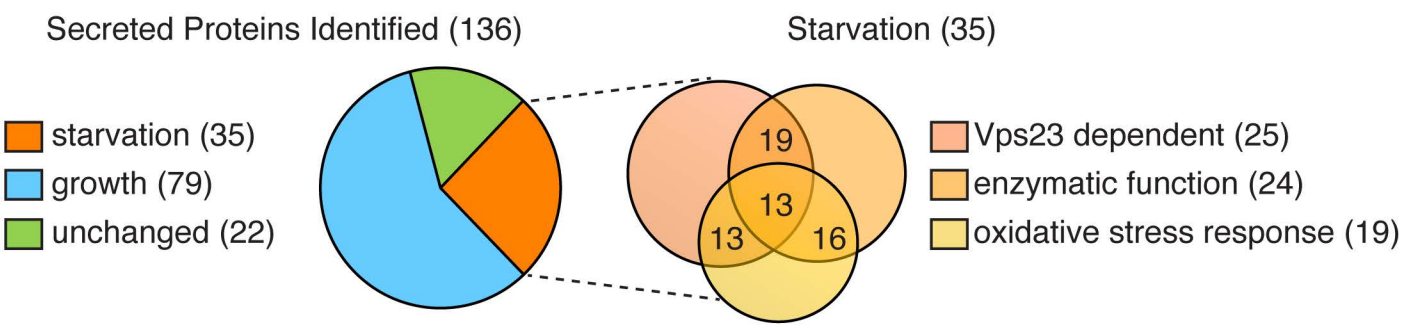

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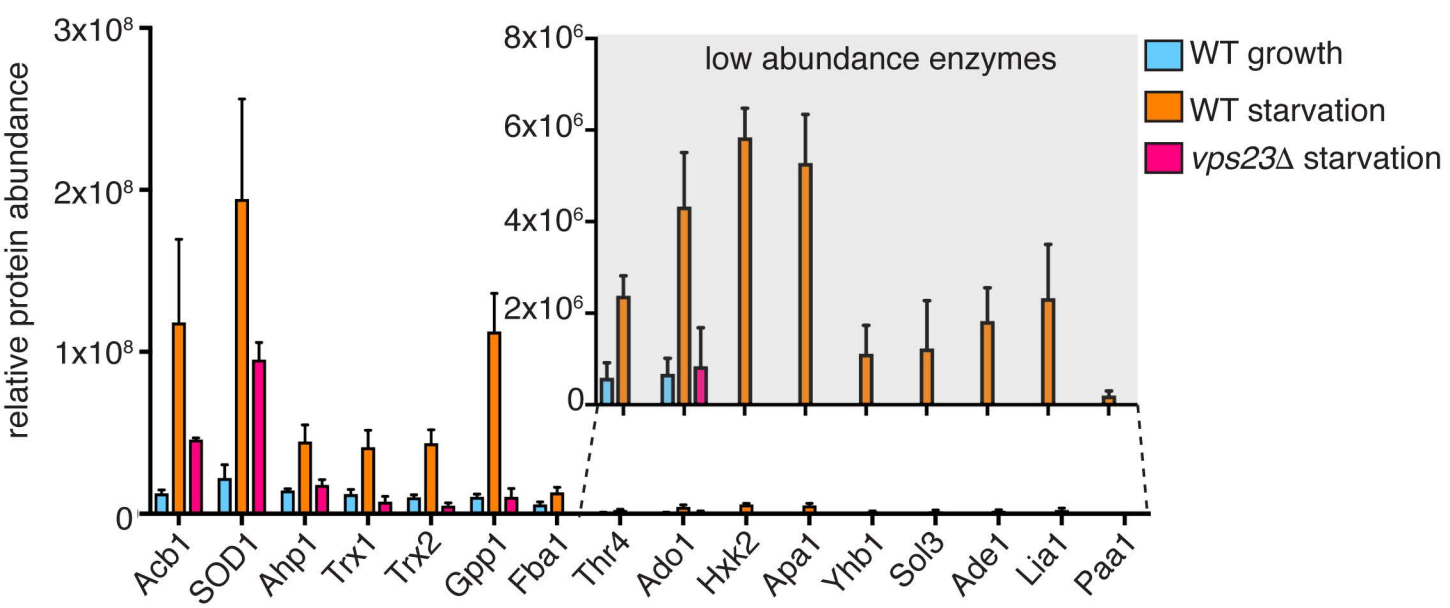

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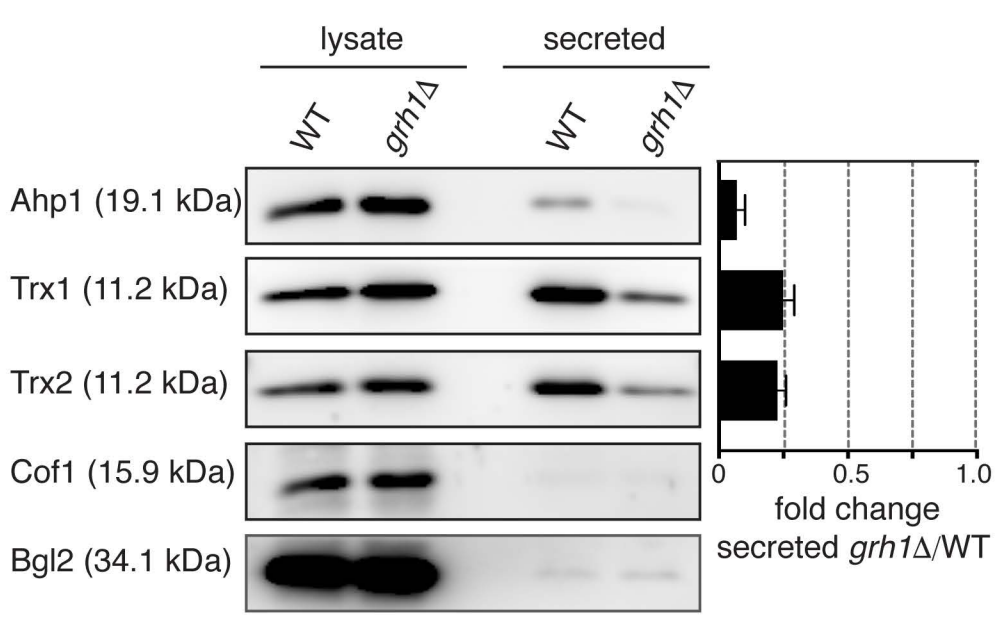
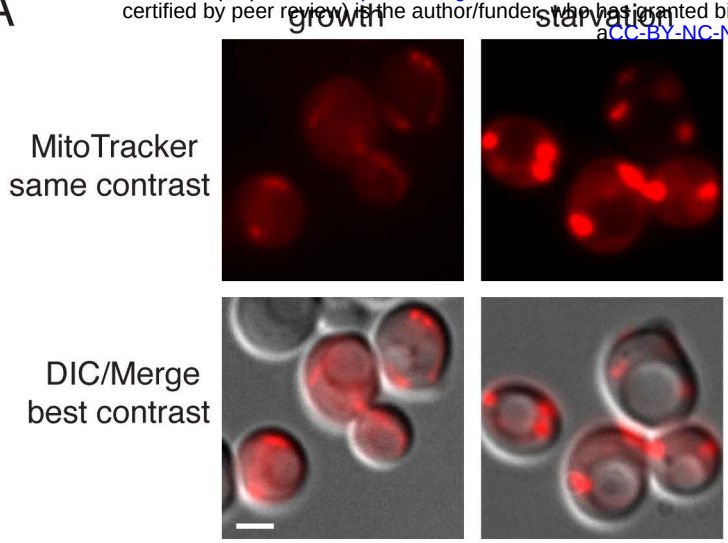


Figure 2

A



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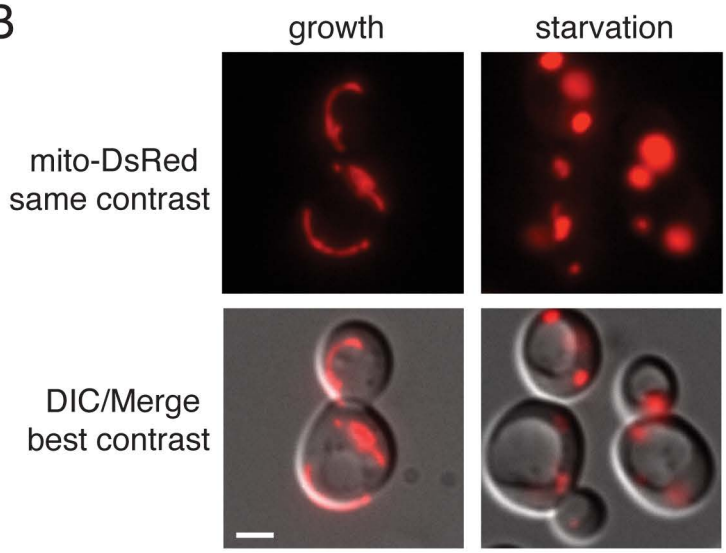

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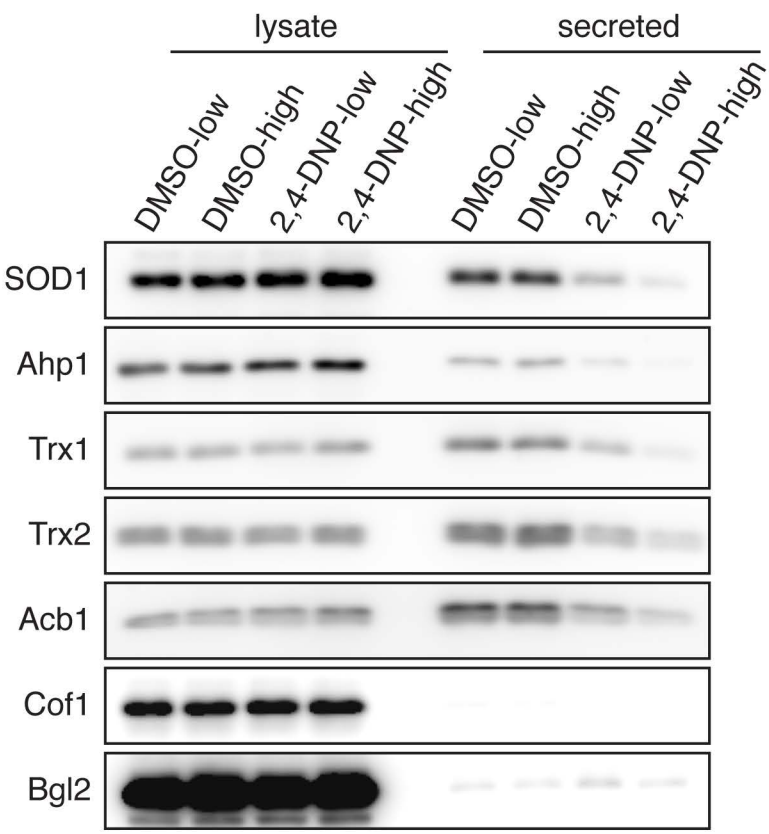

Figure 3