Toward the discovery of biological functions associated with the mechanosensor Mtl1p of *Saccharomyces cerevisiae* via integrative multi-OMICs analysis

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Functional analysis of the Mtl1 protein in *Saccharomyces cerevisiae* has revealed that this transmembrane sensor endows yeast cells with resistance to oxidative stress through a signaling mechanism called the cell wall integrity pathway (CWI). We observed upregulation of multiple heat shock proteins (HSPs), proteins associated with the formation of stress granules, and the phosphatase subunit of trehalose 6-phosphate synthase which suggests that mtl1Δ strains undergo intrinsic activation of a non-lethal heat stress response. Furthermore, quantitative global proteomic analysis conducted on TMT-labeled proteins combined with metabolome analysis revealed that mtl1Δ strains exhibit decreased levels of metabolites of carboxylic acid metabolism, decreased expression of anabolic enzymes and increased expression of catabolic enzymes involved in the metabolism of amino acids, with enhanced expression of mitochondrial respirasome proteins. These observations support the idea that Mtl1 protein controls the suppression of a non-lethal heat stress response under normal conditions while it plays an important role in metabolic regulatory mechanisms linked to TORC1 signaling that are required to maintain cellular homeostasis and optimal mitochondrial function.

The fungal cell wall is a physical structure that evolved to shield cells from the changing conditions of the ecological niches in which they thrive, acting as a porous barrier that isolates the delicate plasma membrane (PM) and cytoplasm from the external environment¹,². Fungi have developed a molecular response mechanism named the Cell Wall Integrity Pathway (CWI) to maintain cellular integrity in response to changes in the cellular environment³–⁹. In the CWI, information conveying environmental changes such as chemicals, biomolecules, ions, radiation, osmotic pressure, or thermal motion enters the cell by way of stress mechanosensor proteins embedded within the cell wall-PM composite⁴.

In the budding yeast *Saccharomyces cerevisiae*, the CWI pathway participates in regulation of cellular processes including cell wall biosynthesis, cell wall repair, and maintenance of cell integrity¹,²,⁴,⁸,¹⁰,¹¹, response to oxidative stress¹²,¹³, heat shock¹⁴–¹⁶, hypo osmotic shock¹⁷, glucose starvation and regulation of key cellular metabolic processes such as ribosome assembly and energy metabolism in response to nutrient starvation⁷,¹⁷,¹⁸, impaired

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cell wall synthesis, antifungal drug treatments, and other environmental stresses that can alter the integrity of the cell wall.

The CWI communicates stress signals via five transmembrane mechanosensory proteins consisting of the Wsc-family (Wsc1p, Wsc2p and Wsc3p), and Mid-family (Mid2p and its homologue Mtl1p). Wsc1p has been associated with activation of the CWI pathway in response to Caspofungin, alkaline pH and reorganization of actin during hypo osmotic stress while Mid2p has been associated with activation in response to Calcofluor white, mating pheromone, vanadate, and acidic conditions. The WSC2 and WSC3 genes act as multi-copy suppressors in mutants with glycerol synthesis abnormalities. Mtl1p has been associated with the response to hydrogen peroxide-induced oxidative stress and glucose starvation. These proteins communicate physical and chemical signals to the cell interior via their cytoplasmic domains, by activating a set of downstream effectors that act in a cascade-like fashion. As an initial step, Rom2p is a Rho1p GEF that attaches to the cytoplasmic tail of these mechanosensor proteins in a regulated manner to activate Rho1p GTPase activity which in turn activates Pkc1p. The ensuing sequential signaling cascade includes kinases Bck1p, Mkk1p, Mkk2p and the end-kinase Slt2p. Once a mechanosensor activates the CWI pathway, Slt2p is hyperphosphorylated, it translocates to the nucleus and activates transcription factor Rlm1p and the heterodimeric complex Swi4p/Swi6p by phosphorylation. These transcription factors induce the transcription of specific genes for the synthesis of cell wall components, osmo-protective molecules, and cell cycle regulatory proteins. According to Harrison and co-workers, a linear signal transmission scheme like this cannot be used exclusively to explain every instance of CWI pathway activation. They proposed that in some cases, lateral signals could bypass elements upstream of Pkc1p through non-linear mechanisms that activate Rho1p GTPase which in turn activates Pkc1p. The ensuing sequential signaling cascade includes kinases Bck1p, Mkk1p, Mkk2p and the end-kinase Slt2p. Once a mechanosensor activates the CWI pathway, Slt2p is hyperphosphorylated, it translocates to the nucleus and activates transcription factor Rlm1p and the heterodimeric complex Swi4p/Swi6p by phosphorylation. These transcription factors induce the transcription of specific genes for the synthesis of cell wall components, osmo-protective molecules, and cell cycle regulatory proteins.

Although the functional roles of Wsc- and Mid-family mechanosensors may appear to be redundant, Mtl1p has been designated with unique roles responsible for the response to H2O2-induced stress, also reported for its protective roles is not fully understood although in a study by Petkova et al., they report a "genome-wide microarray analysis to quantify mRNA expression (Affymetrix GeneChip Yeast Genome S98 array) contrasting with more comprehensive analytical methods used in this study. To expand these previous studies, we have therefore designed a strategy for identification of components of the biological network of Mtl1p by analyzing the macromolecular changes occurring at the transcriptome and proteome levels when the MTL1 gene is deleted with further analysis of the systematic effects of these macromolecular changes through metabolite analysis to pinpoint the focus of the mutation. In this manuscript, we present the findings of our studies on the mechanosensor Mtl1p of S. cerevisiae using a multipronged Omics approach. We have conducted quantitative analyses that elucidated the transcriptome, proteome, and metabolome profiles of mtl1Δ strains compared to wild-type controls under normal culture conditions to uncover the biological networks requiring Mtl1p for maintaining metabolic homeostasis and resistance to stress conditions. Because stress–response mechanisms are well conserved, the knowledge derived from experiments using Saccharomyces cerevisiae as a model system provides insights into the mechanisms by which opportunistic fungal pathogens adapt to environmental stresses and can have important translational applications for antifungal drug development.

Materials and methods

Strains. All Saccharomyces cerevisiae strains used in this study.

Confirmation of MTL1 deletion strain. The BY4742 wild-type (WT) and YGR023w (mtl1Δ) strains were obtained from Open Biosystems. The chromosomal deletion of MTL1 was confirmed by PCR with genomic DNA. These strains were used in transcriptome, proteome, and metabolome experiments.

Cell culture conditions. The S. cerevisiae WT and mtl1Δ strains were cultured overnight at 27 °C, with shaking at 210 rpm, in 25 mL of complete synthetic medium (CSM) containing all amino acids and 2% glucose (Sigma), plus 0.67% Nitrogen base supplemented with ammonium sulfate (Fisher Scientific). Two to three cultures prepared with different media batches were used per experiment. The next day, the cultures were replenished with fresh CSM and cultured at 27 °C until they reached an Optical Density (OD600) between 0.6 and 0.7 units. The cells were harvested by centrifugation at 3838xg for 5 min at 4 °C. Cell pellets were washed with 1 mL ice-cold sterile deionized water, and centrifuged as before. Cell pellets were suspended in the appropriate buffer solutions described below for RNAseq, Total Protein, and Total Metabolite extractions, or stored for future use at −80 °C.

RNAseq analysis. Transcriptome library construction and Ion Proton sequencing. Cells were harvested in 2 mL freshly prepared Buffer Y1 (1 M sorbitol, 0.1 M EDTA, pH 7.4, and 0.1% 6-Mercaptoethanol (Sigma M-3148, 98%) with 50 U Zymolase added per 1 × 107 cells (ICN Code 320921 Zymolase Arthrobacter luteus, 20,000U/g) and incubated for 60 min at 30 °C with gentle shaking to generate spheroplasts. These were...
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Sample processing. Five each of WT control and mtl1Δ experimental total protein samples (250 μg per sample) were delivered for quantitative proteomics analysis (Supplementary Table S3). Total protein lysates for WT and mtl1Δ strains were generated from cells pellets disrupted with glass beads in lysis buffer (50mMTris HCl pH7.5, 1% Glycerol, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 5X Protease Inhibitor Cocktail and 5X PMSF) by vortexing for 60 s at high speed alternating with 3 min on ice, repeated for 4 cycles. Total protein concentration was quantified by the DC-Protein Assay (Bio-Rad).

Sample processing began with an acetone precipitation overnight with 100 μg of total protein to concentrate the samples on the next day and wash away unwanted substances. Samples were resuspended in 50 μl of 2X sample buffer (95% Laemli/5% β-mercaptoethanol). SDS-PAGE using Mini-PROTEAN TGX Precast Gel (12% fixed gel) was allowed to run for 15–20 min at 150 V to generate a 1.5 cm band. Gels were stained with Bio-Safe Coomassie G-250 to visualize the quality of the proteome bands present in each lane, and to be able to cut them out. After the proteome gel bands were cut out, they were destained using a 50 mM ammonium/50% Acetonitrile solution at 37 °C. Then, they were reduced with Dithiothreitol (25 mM DTT in 50 mM Ammonium Bicarbonate) at 55 °C, alkylated with iodoacetamide (10 mM IAA in 50 mM Ammonium Bicarbonate) at room temperature in the dark, and digested with trypsin (Promega) overnight at 37 °C. Digested peptides were extracted out of the gel pieces using a mixture of 30% acetonitrile/2.5% formic acid in water. Extracted samples were dried and stored at ~80 °C to wait for TMT labeling procedure and subsequent LC–MS/MS analysis.

TMT labeling and fractionation. As specified by the manufacturer’s protocol, dried extracted samples were reconstituted in 100 mM TEAB and labeled with the TMT 10plex labeling reagents (41 μl, 0.8 mg). The TMT labels were added as described in Table 1. The addition was followed by an hour incubation allowing the labelling reaction to occur, and a quenching step of 15 min. Finally, equal amounts of each sample were mixed to gener-
ate a final pool. We used 75% of the volume per sample for this pool, and kept the remaining 25% of the sample volume in each individual vial which is now stored in our freezer for case the analysis has to be repeated. The final pool was dried to proceed with the fractionation procedure.

Fractionation was performed using the “Pierce High pH Reversed-Phase Peptide Fractionation Kit” and following the manufacturer’s instructions. Briefly, the column was conditioned twice using 300 μl of Acetonitrile, centrifuging at 5000×g for 2 min; and repeating the steps using 0.1% Trifluoroacetic acid (TFA). Sample (final pool) was bound to the column, washed to remove contaminants and any unbound TMT reagent, and eluted 8 times into 8 different vials using a series of elution solutions with different Acetonitrile/TFA percentages. Elution solutions are specified in the manufacturer’s protocol. The entire procedure was performed twice and 16 fractions were generated followed by drying and LC–MS/MS analysis.

Sample preparation for LC MS/MS. The reconstitution of the fractions for mass spectrometry analysis was made using 2.5% acetonitrile/2.5% formic acid in water. A total of 3 μl were transferred to a special sample vial to be able to inject 2 μl of sample into the instrument. The remaining volume of the reconstituted fractions were stored at – 80°C.

LC–MS/MS analysis. For peptide separation on an Easy-nLC1200 instrument (Thermo Fisher Scientific), a PicoChip H354 REPROSIL-Pur C18-AQ 3 μm 120 A (75 μm × 105 mm) chromatographic column (New Objective) was used. The separation was obtained using a gradient of 7–25% of 0.1% of formic acid in acetonitrile (Buffer B) for 102 min, 25–60% of Buffer B for 20 min, and 60–95% Buffer B for 6 min. Making a total gradient time of 128 min at a flow rate of 300 nl/min, with an injection volume of 1 μl per sample.

Q-Exactive Plus (Thermo Fisher Scientific) operates in positive polarity mode and data-dependent mode. The full scan (MS1) was measured over the range of 375 to 1400. The MS2 (MS/MS) analysis was configured to select the top ten most intense ions for HCD fragmentation, configured over the range of 200 to 2000 m/z. A dynamic exclusion parameter was set for 30.0 s.

Database search. Once the Mass Spectrometry analyses were finished, the raw data files were searched with a S. cerevisiae database and downloaded from UniProt (Universal Protein Resource). The raw data was analyzed with Proteome Discoverer software version 2.1 (https://www.thermofisher.com/pr/en/home/industrial/mass-spectrometry/liquid-chromatography-mass-spectrometry-lc-ms/lc-ms-software/multi-omics-data-analysis/proteome-discoverer-software.html) using workflows configured for quantitative proteomics. A dynamic modification for oxidation + 15.995 Da (M) was configured. A static modification of + 57.021 Da (C) generated by the alkylation during processing, and static modifications from the TMT reagents + 229.163 Da (Any N Term, K) were all included in the parameters for the search.

Quantitative proteomics by TMT labeling. The analysis was performed for the datasets related to Mutant (mtl1Δ) and Wild Type (WT) groups to determine the differential protein abundances. The comparisons included five replicates for both mtl1Δ and WT groups as described in Supplementary Table S3, where the Control Group and Experimental Group correspond to MT and WT replicated samples, respectively. Similar to our analysis of differently expressed mRNAs, the datasets were pre-processed with tailored Python scripts for filtering missing values, as well as identification and processing of outliers using the Interquartile (IQR) imputation method. A single channel design for the Experimental Group vs Control Group was implemented and ran using the R Bioconductor software limma version 3.9.0 (https://www.bioconductor.org)43. The results from the statistical analysis of significantly different protein abundances were considered based on a Fold Change FC ≥ 2 and adjusted P value (using the Benjamini–Hochberg adjustment) ≤ 0.05.

Metabolomics analysis. Sample Processing. Cells pellets of WT and mtl1Δ strains were resuspended in 1 ml of pure methanol (Fisher HPLC grade) pre-chilled at – 20 °C, for 5 min (quenching step). The cell suspensions were centrifuged for 5 min at 167×g, 4 °C. The methanol supernatant was removed from each sample with a Pasteur pipet. The samples were resuspended in 1 ml of a 1:1 mixture of methanol and sterile deionized water and homogenized with a Poly Tron PT-2100 homogenizer (set at 15) using two 7-s pulses.

Derivatization of glucose. Metabolic extracts from the WT and mtl1Δ strains (n = 13, each) were collected and dried in a nitrogen gas stream at 50 °C (RapidVap, Labconco). Nitritation was performed as previously described44 by adding 150 μl of 0.2 mM hydroxylammonium chloride in pyridine to the dried sample, and then heating at 90 °C for 40 min. After that, acetylation was performed by adding 250 μl of acetic anhydride, and then heating at 90 °C for 60 min. After that, the sample was dried in a nitrogen gas stream again, and then redissolved in 400 μl of ethyl acetate. Derivatized samples were collected and stored at – 20 °C.

| Strain | Genotype | Source  |
|--------|----------|---------|
| BY4742 | MAT a his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | Open Biosystems |
| YGR023w | MAT a his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mtl1::KanMx4 | Open Biosystems |

Table 1. Saccharomyces cerevisiae strains.
**Derivatization of other metabolic features.** Metabolic extracts from the WT and mtl1Δ strains (n = 7, each) were collected, evaporated to dryness in a nitrogen stream, derivatized with methoxyamination by adding 30 µl of 20 mg/ml solution of methoxyamine hydrochloride in pyridine (Sigma-Aldrich) and incubated at 37 °C for 2 h. Trimethylsilylation was subsequently performed by adding 50 µl of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA + 1% TMCS, Sigma-Aldrich) and incubated for 1 h at 65 °C. Samples were centrifuged at 15,700 × g for 10 min at RT. Supernatants were transferred to glass vials and stored at −20 °C.

**Analysis of glucose content.** Twenty microliters per sample were added to glass vials with inserts to evaluate the glucose content via GC–MS (GC/MS-TQ8050, Shimadzu Inc.) using SIM mode and analytical conditions previously described44. The glucose concentration in the samples was performed by comparing the obtained absorbance values in each sample with the glucose (Sigma) calibration curve.

**Analysis of other metabolites.** Twenty microliters per sample were added to glass vials with inserts followed by the addition of 1 mM 2-fluobiphenyl (Sigma-Aldrich) as an internal standard. Samples were processed via GC/MS-TQ8050 using full scan mode and analytical conditions as previously described45. Peak integration was performed using GCMS Labsolution data analysis version 4.45 software (https://www.ssi.shimadzu.com/products/gas-chromatography-mass-spectrometry/gcmsolution-software.html). Mass spectral library searches of the major chromatographic peaks were conducted using the GCMS Lab solution data analysis software equipped with the NIST14/2014/EPA/NIH database in each data set, which resulted in a final data set consisting of 45 metabolic features selected for the metabolomics analysis. Quantitative analysis of metabolic features in each sample was performed by calculation of a response factor using the internal standard 1 mM 2-fluobiphenyl spiked into each sample before the GC/MS analysis.

**Quality assessment and quality controls in metabolomics analysis.** Reproducibility of metabolite recovery, the performance of sample extraction, derivatization, and instrumentation were validated by the utilization of several blank samples, including a system suitability blanks, and derivatization processing blanks. To evaluate analytical accuracy and precision, an external quality assessment was performed using 1 mM 2-fluobiphenyl spiked into derivatization blank samples before running on the GC/MS (n = 3). The percent of relative standard deviation (%RSD) of 2-fluobiphenyl peak abundances accounted for 3.8%, which demonstrates good reproducibility of the method. For systematic bias mitigation, we performed the randomization of the sample analysis order. Blanks and quality control samples were spaced evenly among the injections to monitor instrument stability.

**Bioinformatic analysis of metabolites.** Metaboanalyst 4.046,47 was used for bioinformatic analysis. Identified concentrations of each metabolite were composed as the data matrix and processed. Data integrity check was performed according to default settings on the Metaboanalyst interface and normalized by OD, and range scaled to improve the pattern recognition for metabolomics data. Differences between WT and mtl1Δ strains were evaluated using Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA). Quality and reliability were assessed by cross-validation using two parameters: the R2(Y) (a measure of how much variation is represented by the model) and Q2(X) (a measure of how accurate the data are classified in the model)48. To evaluate the model performance, class labels were permuted 1000 times to identify whether differences between groups were significant. Mann–Whitney U test was used to identify statistically significant (p < 0.05) metabolic features.

**Functional enrichment analysis of proteome and metabolome data.** To understand the cellular regulatory mechanisms in mtl1Δ strain, we applied an integrative multi-omics analysis of mtl1Δ proteome, and metabolome using Cytoscape and the ClueGO plugin in the Cytoscape which integrates Gene Ontology (GO) terms and creates a functionally organized GO/pathway term network corrected by kappas statistics49 using two-sided (Enrichment/Depletion) tests based on the hypergeometric distribution (Supplementary Table S2). The P value <0.05 was corrected by Bonferroni step down correction method; Min GO Level = 7 and Max GO Level = 16; Kappa Score Threshold = 0.4; Initial Group Size = 2 and sharing Group Percentage = 50.0. The created network represented the pathways as randomly colored nodes, where some of them were partially overlapped depending on their functional relation. The node size represented the term enrichment significance and is highlighted by a large name label. Edges indicate statistically significant associations between GO terms. Since ClueGo mostly evaluates functional enrichment, taking into account the number of analyzed genes/proteins, therefore, to evaluate other functional enrichments where a small number of proteins per term existed, we applied the GeneMANIA plugin in the Cytoscape to model possible interaction networks. GeneMANIA integrates GO terms and creates a functionally organized GO/pathway term network applying Q-values from an FDR corrected hypergeometric test for enrichments and the Benjamini–Hochberg corrections50. Pathways visualizations have been carried out using ClueGo plugin for Cytoscape 3.7.2 and ChemDraw 15 (PerkinElmer).

**Results and discussions**

**Integrative analysis of transcriptome and proteome data.** To assess the effect of Mtl1p in the regulation of stress genes, we measured the mRNA levels in the mtl1Δ strain compared to a WT using Next Generation Sequencing (NGS) and quantitative proteomic analysis by TMT labelling of tryptic peptides generated from total proteins followed by tandem mass spectrometry (MS–MS). After we filtered the results of these experiments according to the selected criteria (FC ≥ 2 or FC ≤ −2 P value ≤ 0.05) we identified a total of 104 differentially regulated mRNA transcripts and proteins (Supplementary Table S1, Fig. 1). Interestingly, 22 down-
regulated ORFs reported in this study were also reported in a previous study. We found 33 upregulated (Group 1) and 12 downregulated proteins (Group 3) that correlated at both the mRNA and protein levels, while there were 42 upregulated (Group 2) and 17 downregulated proteins (Group 4) that were uncorrelated with their corresponding mRNAs (Supplementary Table S1). One protein that was differentially regulated in the mtlΔ strain (Group 2) was the catalytic subunit of the β-1,3-glucan synthase, Fks1p, a protein induced by the CWI pathway in response to cell wall damage (Supplementary Fig. S1). In the mtlΔ strain, this gene exhibits repressed mRNA levels in the RNAseq data yet proteome analysis shows a sevenfold increase in expression this protein (mRNA -2.52 vs Protein +7.07). Remarkably, previous breakthrough studies using transcriptome and proteome data from E. coli and yeast reported that protein and mRNA copy numbers for any given gene may be uncorrelated, highlighting the disconnect between proteome and transcriptome quantitative analysis in single cells,

Although we did not find a mutual relationship in expression between the mRNA and proteins in all cases, genes associated with the stress response such as SSA1, SSA4, HSP26, HSP42, HSP78, HSP82, HSP104, and CTT1 were upregulated at the protein level. Another finding was that the proteins encoded by genes MRPL7, PHB2, ATP17, AIM45, ALD4, HSP78, HSP82, NDE1, and QCR2, all of which encode components of the mitochondria, were upregulated at the protein level in the mtlΔ, while mitochondrial components IDH1, MAE1, NUC1, and SAM50 were downregulated (Fig. 1, Supplementary Table S1). A review that addressed the question regarding the inverted correlation between transcripts and proteins suggests that despite the effectiveness of mRNA expression values shown in a variety of applications, their correlations with protein levels are almost certainly only correlative rather than causative. Therefore, it is most probable that the concentration of proteins and their interactions represent true contributing forces in the cell.

The cAMP/PKA pathway regulates genes containing a Stress Response Element (STRE) consisting of the sequence 5′-CCCT-3′ bound by transcription factors (TFs) Msn2p and Msn4p Activation of PKA by increasing intracellular cAMP levels inhibits the accumulation of carbohydrate stores, respiratory growth, and the transcription of genes bearing an STRE through negative regulation of the Rim15p kinase by PKA and inhibition of nucleocytoplasmic shuttling by TFs Msn2p and Msn4p. To test if the regulated mRNAs in the mtlΔ strain represent genes with STREs, we analyzed the promoter DNA sequences within 500 base pairs of the transcription start site of all 104 differentially regulated genes in the mtlΔ strain (Supplementary Table S1), to identify potential Msn2/Msn4 binding motifs. Only 19 differentially downregulated genes (i.e. HSP42, CAR1, HSP104, ALD4, TDH1, SSA4, CTT1, TSL1, HSP78, GPH1, GLK1, PRB1, YML131W, PG2M, YMR196W, TPS2, HSP150, VPS13, YPL260W) and 6 upregulated genes (i.e. RPS20, IP13, SND3, RPL3, PHB2, YBL036C) contained at least one STRE, indicating that other signaling systems are also involved in regulating the mtlΔ phenotype. In the future, a broader search of all consensus sequences contained in the Yeast Transcription Factor Specificity Compendium database (YeTFaScO) is warranted for these differentially regulated genes. Interestingly, inspection of the promoter regions of WSC1, WSC2, WSC3, MID2, and MTL1 revealed that only the MTL1 promoter contained an STRE, implying that its expression is transcriptionally regulated by TFs Msn2p and Msn4p in response to stress conditions.

Analysis of metabolome data. The biological effect of a differentially expressed gene can ultimately be manifested in the operation of the biological processes(es) in which it participates in the cell. Thus, a global analysis of cellular metabolites can reveal changes in specific metabolic processes that may be attributed to the absence of a given protein. Also, the biochemical steps being affected targeted within these putative metabolic processes may be identified intuitively. Therefore, to better understand the biological processes that require Mtl1p function(s), a global metabolite analysis was conducted in WT and mtlΔ yeast strains.

We identified a total of 46 metabolites in this analysis (Table 2), the identification of glucose was conducted separately, and we did not find any differences between WT and mtlΔ strains (Table 2 Row 46, Fig. 2B). The observed fold-change for glucose in mtlΔA was 1.1 (Table 2, Fig. 2B). For a description of the analysis see “Materials and Methods”. The other 45 metabolites extracted from WT and mtlΔ strains were used for metabolomics analysis.

The OPLS-DA score plot shows a clear separation between WT and mtlΔ strains (Fig. 2A) with an R2Y of 0.95 and Q2 of 0.78. To further evaluate the model, we performed permutation tests (n = 1000). The empirical P values were 0.003 for R2Y and 0.02 for Q2. Thus, a clear distinction between the metabolome of WT and mtlΔ strains was observed (Fig. 2A). Mann–Whitney U test analysis detected 12 features significantly decreased in mtlΔA strain compared to WT. (Table 2, Fig. 2C,D). The most significant metabolic changes were related to amino acid metabolism, purine metabolism, and carboxylic acid metabolism.

Functional enrichment analysis. The functional enrichment analysis was performed exclusively for groups exhibiting upregulated (Groups 1 and 2) and downregulated (Groups 3 and 4) proteins using ClueGo as described in “Materials and Methods” section. We identified 13 top-ranked categories and their associated proteins including cellular amino acid metabolic and biosynthetic process, trehalose metabolism in response to stress, cellular response to heat, cytoplasmic stress granule, flavin adenine dinucleotide binding, glucose metabolic process, stress granule disassembly, purine nucleoside monophosphate biosynthetic process, diphospho- transfersase activity, pyruvate metabolic process, DNA synthesis and protein targeting to mitochondrion (Fig. 3, Supplementary Table S2).

Identified functional enrichments in the cellular response to heat and trehalose metabolism in response to stress in the mtlΔ strain grown at normal conditions and temperature (27 °C) suggest that the deletion of MTL1
Figure 1. Comparison of differential protein and mRNA expression profiles. (Left panel, Proteomic_FC) 104 proteins identified on the right margin, with statistically significant changes in abundance of twofold or greater in the mtl1Δ strain are shown alongside their mRNAs (Right panel, RNAseq_FC) with expression changes of twofold or greater. Note that a significant block of proteins correlated with their mRNA levels, while others did not correlate (see Results and Discussion for details). The color scale indicates relative fold-change (Red = FC ≥ 2, Green = or FC ≤ −2), P value ≤ 0.05.
| # | ID          | Fold change | p value |
|---|-------------|-------------|---------|
| 1 | Alanine     | 1.01        |         |
| 2 | Asparagine  | 0.30        | 0.0006  |
| 3 | Aspartate   | 0.34        | 0.014   |
| 4 | Cysteine    | 0.41        |         |
| 5 | Glutamate   | 0.75        |         |
| 6 | Glutamine   | 0.36        | 0.01    |
| 7 | Glycine     | 0.76        |         |
| 8 | Histidine   | 0.52        | 0.007   |
| 9 | Homocysteine| 0.57        |         |
|10 | Homoserine  | 2.46        |         |
|11 | Isoleucine  | 1.06        |         |
|12 | Leucine     | 0.77        |         |
|13 | Methionine  | 0.21        | 0.04    |
|14 | N-Acetyl-glutamate | 0.87 | |
|15 | Ornithine   | 0.16        | 0.0006  |
|16 | Phenylalanine| 0.28       | 0.007   |
|17 | Proline     | 0.48        |         |
|18 | Serine      | 0.88        |         |
|19 | Thrreonine  | 0.43        | 0.007   |
|20 | Tryptophan  | 0.76        |         |
|21 | Tyrosine    | 0.72        |         |
|22 | α-Aminoadipate| 0.50        | 0.007   |
|23 | Valine      | 1.00        |         |
|24 | 5-Oxoprolinate| 1.45       |         |
|25 | 5-Oxoproline| 0.89        |         |
|26 | Sarcosine   | 1.12        |         |
|27 | Hypoxanthine| 0.32        | 0.0111  |
|28 | Orotate     | 0.34        |         |
|29 | Uracil      | 0.82        |         |
|30 | 3-Ureidopropionate| 0.98| |
|31 | Cystathionine| 0.76       |         |
|32 | Glycolate   | 0.87        |         |
|33 | Myristate   | 0.39        |         |
|34 | Palmitate   | 0.77        |         |
|35 | Stearate    | 1.24        |         |
|36 | Palmitoleate| 0.72        |         |
|37 | Oleate      | 0.82        |         |
|38 | Ricinoleate | 1.47        |         |
|39 | Lactate     | 0.35        |         |
|40 | Citrate     | 0.40        | 0.0012  |
|41 | Succinate   | 1.07        |         |
|42 | Fumarate    | 1.17        |         |
|43 | Malate      | 0.39        | 0.0006  |
|44 | Malonate    | 0.45        |         |
|45 | Niacinamide | 0.69        |         |
|46 | Glucose     | 1.1         |         |

**Table 2.** Difference in metabolite concentrations between WT and mtl1Δ strains.
could be associated with the activation of complex regulatory mechanisms related to non-lethal heat stress. Yeast responds to heat stress via rapid synthesis of heat shock proteins (HSP) and trehalose that serves to protect cellular functions by preventing protein aggregation, unfolding aggregated proteins, or targeting denatured proteins for degradation\(^1\). Thus, we identified significant upregulation of \(\text{HSP104}, \text{HSP150}, \text{HSP26}, \text{HSP42}, \text{HSP78}, \text{and HSP82}\) and enzymes \(\text{NTH1}, \text{TPS2}, \text{TSL1}\) (Supplementary Table S2, Fig. 3). In addition, trehalose is an effective stabilizer of proteins, suppresses protein aggregation upon denaturation\(^1\) and acts as an anti-dehydration agent minimizing the effects of dryness\(^1\). It is recognized that the activity of various trehalose-metabolizing enzymes required the presence of \(\text{HSP104}\) in yeast cells under heat stress\(^1\). Thus, \(\text{HSP104}\) works synergistically with \(\text{trehalose}\) to stabilize the yeast proteome in response to heat stress\(^1\). Moreover, mitochondria have been recognized to play an essential role in heat stress tolerance\(^5\). In agreement with this assumption, we also observed a functional enrichment in protein targeted to the mitochondrion (Fig. 3). It is recognized that most mitochondrial proteins are synthesized in the cytosol and then translocated to mitochondria to perform their functions. An earlier study showed that heat stress could affect mitochondrial protein translocation efficiency, and therefore, the mitochondrial biosynthesis and functioning are repressed\(^6\). Our analysis suggests that the \(\text{MTL1}\) deletion did not repress the synthesis of proteins targeted to the mitochondrion since \(\text{HSP82}\), ubiquinol cytochrome-c reductase, Complex III (QCR2), and \(\text{SSA1}\) were upregulated (Fig. 3).

Functional enrichments in the amino acid metabolic process associated with upregulation in catabolic enzymes \(\text{BAT2}, \text{ARO9}, \text{DUR1,2}, \text{MET13} \text{and CHA1}\) (Supplementary Table S1 Groups 1—2) and amino acid biosynthetic process concomitant with the downregulation of anabolic enzymes \(\text{MET6}, \text{MET17}, \text{LEU1}, \text{GLN1}, \text{GDH1}, \text{MAE1}, \text{ASN1}, \text{SAM2}, \text{SPE3}, \text{and CAR1}\) (Supplementary Table S1 Groups 3–4) suggest that the biosynthesis of amino acids was partially suppressed in the \(\text{mtl1}\Delta\) strain. Pairing proteome and metabolome data produced results consistent with this assumption and explained a significant reduction of several metabolites specifically: \(\text{CHA1}/\text{threonine}, \text{ARO9}/\text{phenylalanine}, \text{ASN1}/\text{asparagine}, \text{MET6-MET17-MET13-methionine}, \text{GLN1}/\text{glutamine}, \text{and CAR1}/\text{ornithine}\) (Supplementary Table S1, Figs. 1, and 2D). Moreover, identified significant decrease in \(\alpha\)-aminoadipate suggests a negative regulation of the anabolic function of the \(\alpha\)-aminoadipic acid pathway in yeast, which is used for lysine biosynthesis\(^6\). A recent study highlights that heat stress in yeast exhibits an anaplerotic character by elevating the expression of a large number of proteins to guarantee constant protein levels for important metabolic processes as a compensative regulatory mechanism against protein aggregation and shutdown of cell growth\(^1\). In contrast, we observed that the \(\text{mtl1}\Delta\) strain displays a cataplerotic character by counterbalancing stress-related detrimental effects by downregulation of anabolic functions.

Previously, it was reported that heat stress could trigger reliance on glycolysis for energy generation and increase the signaling pathway for glucose-regulated gene expression\(^9\). Accordingly, the glucose metabolic process was significantly enriched by the deletion of \(\text{MTL1}\). Glyceraldehyde-3-phosphate dehydrogenase (\(\text{TDH1}\)), glucokinase (\(\text{GLK1}\)), phosphoglucomutase (\(\text{PGM2}\)), the regulatory subunit of the trehalose synthase complex (\(\text{TSL1}\)), aldehyde dehydrogenase (\(\text{ALD4}\)) and malate dehydrogenase (\(\text{MDH2}\)) were associated with the glucose metabolism.

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**Figure 2.** Metabolomics analysis of WT and \(\text{mtl1}\Delta\) strains. (A) OPLS-DA score plot based on WT and \(\text{mtl1}\Delta\) cell metabolomes. (B) Violin plots visualize the non-significant distribution of glucose in WT and \(\text{mtl1}\Delta\) strains (\(n=12\)). (C–E) Violin plots visualize the distribution of significantly altered metabolites in WT and \(\text{mtl1}\Delta\) strains (\(n=7\)).
metabolic process (Supplementary Table S2). However, we have not found any changes in glucose content in the mtl1Δ strain compared to the WT strain (Table 2, Fig. 2B) presumably because of elevated conversion of glucose to trehalose by TPS2 and TSL1 or pyruvate by TDH1 (Supplementary Table S2). The fact that pyruvate metabolic process was also significantly enriched in the mtl1Δ strain supports our assumption.

Figure 3. Functional enrichment analysis of the molecular, biological and cellular regulatory mechanisms in the mtl1Δ strain. (A) Network representations of GO enriched terms in the respective networks in mtl1Δ strain of differentially expressed proteins using ClueGO. Enriched terms are represented as circle nodes based on their kappa score (>0.4) and adjusted P values corrected with the Benjamini–Hochberg method. The enrichment significance of the GO terms is reflected by the size of the nodes. Node color represents the class that they belong. Mixed coloring means that the specific node belongs to multiple classes. Associated proteins for each term are presented as triangles. Color of the font indicates upregulation (red) or downregulation (green). The 13 top-ranked categories of GO biological processes are labeled. Sub-network terms for each category are presented in Supplementary Table S2. Visualization has been carried out using ClueGO plugin for Cytoscape 3.7.2. (https://cytoscape.org). (B) Top-ranked categories visualized on a bar chart, showing adjusted P values (Log10).
the first line of cellular defense mechanisms in S. cerevisiae. Pyruvate efficiently scavenges heat-induced ROS, which results in a reduction of protein carbonylation, stabilization of the mitochondrial membrane potential (ΔΨm) generated by Complexes I, III and IV during electron transport. Consistent with this study, we observed upregulation of glucokinase (GLK1), which converts glucose to glucose-6-phosphate for further glycolytic processing to yield pyruvate as the end product, and downregulation of D-lactate dehydrogenase (LDH3), that is coupled to the reduction of pyruvate to lactate. Consequently, a decrease in lactate was identified that could lead to pyruvate accumulation in mtl1Δ strain (Table 2). However, the downregulation of mitochondrial malic enzyme (MAE1) suggests that oxidative decarboxylation of malate to pyruvate is abolished presumably due to significantly low content of malate (Table 2, Fig. 2C). Elevation of ALD4 suggests that pyruvate is metabolized by decarboxylation to acetaldehyde via the alternate mitochondrial pyruvate dehydrogenase bypass pathway, and converted to acetate by ALD4 which can be further converted to acetyl-CoA. Additionally, it was shown that heat stress depressed the conversion of glucose to glutamate and glutamine, derived from α-ketoglutarate. As a result, both mRNAs and proteins of glutamate dehydrogenase (GHD1) and glutamine synthetase (GLN1) were downregulated, and correspondently the content of glutamate and glutamine were reduced in the mtl1Δ strain (Table 2, Fig. 2D).

An earlier study suggested that in S. cerevisiae, heat stress induces oxidative stress through ROS produced mainly by the heat-damaged mitochondrial respirasome and a set of genes associated with the mitochondrial respiratory chain was downregulated. Functional enrichment analysis of the mtl1Δ strain respirasome showed a positive correlation of proteins in Supplementary Table S1, Group 2 such as NADH dehydrogenase, Complex I (NDE1), Subunit 2 of ubiquinol cytochrome-c reductase (QCR2, Complex III) and F1F0 ATP synthase, Complex V (ATP17) with mitochondria respirasome (Fig. 4A,B). Moreover, enrichment in llinavin adenine dinucleotide binding GO term concomitant with an upregulation of electron transfer flavoprotein AIM45 that serves as a specific electron receptor for several dehydrogenases, including succinate dehydrogenase to convey electrons to ubiquinone in the mtl1Δ strain (Fig. 4B). In addition, we identified the upregulation of fumarate oxidoreductase that reduces fumarate to succinate (OSM1). The previous study suggested that the OSM1/fumarate couple accepts electrons with similar efficiency as cytochrome C. Since we did not find any significant differences in the content of succinate and fumarate (Table 2) in the mtl1Δ and WT strains, it suggests that the mtl1Δ strain has developed an adaptive response to maintain oxidative phosphorylation at a controlled level to support cell growth. Besides its role in oxidative phosphorylation, OSM1 is involved in protein targeting to the mitochondrial pathway which is usually activated to prevent protein aggregation, misfolding, or proteolysis triggered by stress.

Furthermore, we identified functional enrichments in the other specific target pathways related to stress-resistance, such as DNA synthesis involved in DNA repair, purine nucleoside monophosphate biosynthetic process, and diphosphotransferase activity (Fig. 3). It is recognized that enzymatic DNA repair pathways become evolved to cope with the heat stress, which can trigger nuclear mutation frequencies and DNA strand breaks. In the mtl1Δ strain, we found upregulations of two proteins involved in DNA repair such as the largest subunit B220 of RNAPII (RPD21) and DNA polymerase alpha catalytic subunit A (POL1) (Supplementary Table S1, Group 2). DNA repair mechanisms involve either tolerating the damage or protecting of the DNA by removing bases, nucleotides, mismatches, homologous recombinations and non-homologous end-joining to ensure overall survival. A nucleotide excision repair mechanism includes transcription-coupled DNA repair when nucleotides are rapidly removed in the transcribed DNA strand. It is known that RPD21 promotes the intrinsic capacity of RNAPII for transcription bypass of DNA lesions by incorporation bypass of nucleic acids across the lesions. POLI is a component of the replisome, a multi-component enzymatic machine at the replication fork that mediates DNA replication and specifically modulates both imprecise non-homologous end-joining and more complex chromosomal rearrangements.

In Group 2, we also found upregulation of adenylosuccinate synthetase (ADE2) and bifunctional phosphoribosylaminomimidazolecarboxamide formyltransferase/IMP cyclohydrolase (ADE17) which along with GUK1 (Group 1) are associated with purine nucleoside monophosphate biosynthetic process (Fig. 3, and Supplementary Table S2). Purine and pyrimidine nucleotides serve many important functions in cells as precursors to DNA and RNA, energy source, signaling molecules, and cofactor components. De novo purine nucleotide biosynthesis in yeast involves the 10-step production of the purine nucleotide inosine monophosphate (IMP), the common precursor to both AMP and guanosine monophosphate (GMP). ADE2 and ADE17 are involved in the de novo IMP biosynthetic process while GUK1 acts in biosynthetic pathways downstream of GMP and AMP (Supplementary Table S2). Since IMP can also be synthesized from hypoxanthine by the salvage pathway, the possibility of incorporating hypoxanthine into DNA and RNA due to heat stress can cause significant defects in purine nucleotide metabolism. Our metabolomics analysis identified a significant reduction in hypoxanthine in the mtl1Δ strain presumed to be the result of triggering a complex cellular survival adaptive response to the MTL1 deletion (Fig. 2C, Table 2). Moreover, we observed a decrease in uracil (Table 2), which, together with hypoxanthine are miscoding and mutagenic DNA and can interfere with RNA editing and function. Identified enrichments of differentially expressed folic acid synthesis protein (FOLI) and thiamine pyrophosphokinase (THI80) at both protein and RNA levels (Supplementary Table S1, Group 1) associated to the diphosphotransferase activity GO term (Fig. 3) also suggests the triggering of cellular survival adaptive response to the MTL1 deletion. In particular, FOLI is important for the biosynthesis of purines and is required to manage folate deficiency during the heat stress for preventing of massive incorporation of uracil into the DNA. THI80 produces thiamine pyrophosphate from thiamine—an essential coenzyme for several enzymes in carbohydrate metabolic pathways required for yeast’s metabolic adaptation to heat stress and regulation of energy production.

In addition, we observed functional enrichment in cytoplasmic stress granule GO term (Supplementary Table S2, Fig. 3). Stress granules are non-membrane-enclosed RNA granules that dynamically sequester the non-translating messenger ribonucleoproteins, and a large variety of misfolded proteins through protein–protein
interactions which vary under different conditions. In the mtl1Δ strain, we identified upregulations of HSP26, known for its role in sequestration of misfolded proteins to stress-induced foci and RNA helicase (DED1)—a regulator of 48S translation pre-initiation complex assembly, which directly responds to environmental stress. DED1 protein rapidly becomes insoluble by forming gel-like condensates that selectively repress translation of housekeeping mRNAs to promote survival under conditions of severe heat stress.

In addition, our functional enrichment analysis predicted associations of five other proteins that were not yet recognized for their associations with cytoplasmic stress granule assembly, including peptidyl-prolyl cis–trans isomerase (FPR1), RPO21, flavohemoglobin nitric oxide oxidoreductase (YHB1), LEU1 and SAM2 (Figs. 1, 3, Supplementary Table S2). However, proteomics analysis of stress granules coupled with super-resolution microscopy in yeast conducted by another group revealed the occurrence of physical interactions between DED1 and YHB1, RPO21, LEU1, and SAM2, suggesting the possibility that these proteins can be recruited to stress granules. Thus, physical interactions of YHB1 with DED1 could explain its important role in oxidative and nitrosative stress responses. Selective degradation of non-functional or unassembled forms of RPO21 by the 20S proteasome after completion of RNAPII assembly in the cytoplasm and interaction of RPO21 with DED1 could represent its possible recruitment to stress granules as another quality-control mechanism to prevent RPO21 accumulation. Moreover, observed downregulation of SAM2 and LEU1 followed by a decrease in amino acids methionine and leucine correspondently (Table 2, Fig. 2D) could be sensed by the mtl1Δ strain as a stress...
and as such also triggers SAM2 and LEU1 interaction with DED1 and their recruitment to the stress granules. In addition, functional enrichment analysis predicted the association of FPR1 with stress granules assembly. FPR1 is a yeast orthologue of the human FKBP12, which belongs to a family of protein folding chaperones. It was reported that FPR1 is significantly upregulated when yeast cells are exposed to proteotoxicity-induced stress. In silico modeling showed a possibility of polymerization of FKBP12 with its interacting partner FRB into gel-like structures inside living cells. However, the role of FPR1 in the process of stress granules formation in yeast is still not completely elucidated.

Functional enrichment in the cytoplasmic stress granule disassembly GO term was concomitant with upregulations of HSP104 and SSA1 (Supplementary Table S1, Fig. 3). HSP104 is the most crucial thermostolerance-related heat shock protein of Saccharomyces cerevisiae, which has the ability to rescue denatured proteins through disassembly of high-molecular weight aggregates. HSP104 is abundant under normal growth conditions but is substantially induced by heat stress. Interestingly, a recent study showed a novel mitochondrial function in preventing protein aggregation in response to heat stress, which could also trigger assembly of protein aggregates on the mitochondrial surface, where HSP104 facilitates their untangling and transport into the mitochondrial matrix for degradation. SSA1 is a member of HSP70 family which can colocalize with HSP104 in stress granules and might influence stress granules assembly or disassembly enabling efficient protein homeostasis. Several upregulated proteins including PHB2, HSP82, SSA4, HSP78, CCT3 act in de novo posttranslational protein folding, while HSP78 and HSP82, can also induce protein refolding. It was also found that HSP82 is significantly expressed only during heat stress. The accumulation of a significant number of misfolded proteins could have secondary consequences, such as the inhibition of normal protein degradation by the ubiquitin–proteasome system that is controlled in the mtl1Δ strain by upregulation of deubiquitinating enzymes RPT3 and UBP6, which spare ubiquitin from proteasomal degradation.

In addition, stress granules are typically observed when cytoplasmic translation is depressed. Accordingly, we detected a significant downregulation of several cytoplasmic ribosomal proteins (Fig. 1, Supplementary Table S1 Group 4), such as RPL33B, RPL42A, RPS6B and RPL3 (large ribosomal subunits), and RPS1A, and RPS20 (small ribosomal subunits), and RSP5 involved in ribosome stability. A recent study suggested that protein degradation and aggregation induced by heat stress can delay the proteomic response, which could explain the weak correlation of the transcriptome and the proteome (Fig. 1, Supplementary Table S1 Group 4). Importantly, downregulation of RPL33B, RPL42A, RPS6B, RPL3, RPS1A, and RPS20 coupled with upregulation of the component of the mitochondrial ribosome MRPL7 (Fig. 1, Supplementary Table S1 Group 1), that suggests a potential molecular switch from cytoplasmic to mitochondrial translation as part of a complex adaptive response to avoid proteotoxic stress that could impact growth of the mtl1Δ strain (Supplementary Table S2).

Taken together, an analysis of functional enrichment of the mtl1Δ proteome and metabolome suggests that the loss of Mtl1p function can activate complex regulatory mechanisms related to non-lethal heat stress, even under normal culture conditions at 27 °C that disrupts numerous metabolic processes and cellular structures. Based on a preliminary conclusion, mtl1Δ strains develop an adaptive response for survival and growth.

**Modeling of TORC1 contribution to the mtl1Δ interactome.** The MTL1 function is required to maintain ribosomal gene repression, the general stress response through Rho1p, the inhibition of the TORC1, and activation of the cell wall integrity CW1 pathway in response to both glucose starvation and oxidative stress. Since TORC1 functionality was not inhibited in the our mtl1Δ strains, it is able to regulate a variety of anabolic and catabolic processes upon sensing diverse nutrient-derived signals including amino acids such as glutamine, leucine, asparagine, arginine and methionine. These amino acids, excluding arginine, which was not detected in our samples, were lower in the mtl1Δ strains and asparagine and methionine significantly decreased (Table 2, Fig. 2D). Correspondingly, anabolic enzymes encoded by GLX1, ASN1, MET6, and MET17 were significantly decreased at both transcriptional and translation levels (Supplementary Table S1, Group 3). Thus, we hypothesize that certain levels of the aforementioned amino acids required to maintain the mtl1Δ cellular homeostasis is being provided via an amino acid transporter system, allowing TORC1 to sense their levels and trigger appropriate adaptive responses.

Using the GeneMANIA plugin in Cytoscape, we conducted the TORC1 interactome analysis to identify if significantly regulated proteins could interact with TORC1 physically or genetically. Two proteins FPR1 and MDH2 potentially interact physically with TORC1 (Fig. 5). FPR1 is a protein of the FKBP12 family in *S. cerevisiae*, which is a primary target for rapamycin. Addition of rapamycin results in the formation of the FPR1-rapamycin complex required for TORC1 inhibition. Although FPR1 is recognized to play a crucial role in rapamycin’s efficacy, its physiological functions remain unclear. Nevertheless, a previous study suggests that FPR1 interacts with TORC1 and heat shock proteins Hsp90p and Hsf1p during stress. Consequently, the predicted physical interaction of TORC1 and FPR1 in our study might explain the observed functional enrichment in the cytoplasmic stress granules pathway components (Fig. 5, Supplementary Table S2). MDH2 catalyzes the reversible oxidation of malate to oxaloacetate in the citric acid cycle and plays a pivotal role in the malate-aspartate shuttle that operates in the metabolic coordination between cytosol and mitochondria. Since it is known that TORC1 interacts with MDH2 to protect it from degradation during nutrient stress, the predicted physical interaction of TORC1 with MDH2 could be the result of adaptive metabolic changes in the mtl1Δ strain that resulted in functional enrichment of the glucose metabolic process (Fig. 5). Furthermore, GeneMANIA analysis predicted genetic interaction between the TORC1 and QCR2, OSM1, ATP17, MRPL7, RSP5, RPS1A, and RPS20 encoded proteins (Fig. 5). Gene interaction is a broad term used to describe the joint role of multiple genes on biological processes and phenotypic effects. It is recognized that mammalian TORC1 is a positive regulator of key genes encoding electron transport chain proteins and stimulates oxidative phosphorylation. Given that TORC1 is a highly conserved eukaryotic protein, observed upregulation of QCR2, OSM1 and ATP17 and genetic interaction...
with TORC1 supports our hypothesis of a synergistic association of TORC1 with QCR2, OSM1 and ATP17 in shaping the respiratory process in the mtl1Δ strain (Fig. 4B, Fig. 5) and protein targeting to the mitochondrial pathway associated with OSM1. Moreover, this analysis predicted a suppressive genetic interaction of TORC1 with RPS1A, RPS20, RSP5, and a synergistic genetic interaction with MRPL7 (Fig. 5). Since TORC1 regulates translation and ribosome biogenesis in S. cerevisiae104, our data support the contribution of TORC1 to the molecular switch from cytosolic to mitochondrial translation as in the mtl1Δ strains. In human cultured cells, TORC1 activity is inversely correlated with mitochondrial protein synthesis and respiration regulating mitochondrial biogenesis105.

Taken together, we envisage that a deletion of the MTL1 gene in S. cerevisiae can engage TORC1 signaling concomitant with activation of the cytoplasmic stress granules pathway, enhanced glucose metabolism, oxidative phosphorylation, with protein targeting to the mitochondrial pathway and activation of the molecular switch from cytoplasmic to mitochondrial translation.

In summary, a deficiency of the Mtl1 protein in the mtl1Δ strain triggers what we describe as a non-lethal heat stress-like response in yeast cells rendering them more sensitive than the WT to oxidative stress caused by H2O2, as previously described by others12,31. Mtl1p is a key mechanosensory protein for recognizing environmental stress by a mechanism that requires its functional and structural integrity to signal the suitability of temperature, pH, carbon source and nutrient availability for normal growth31. We provide additional evidence supporting that Mtl1p maintains normal cellular homeostasis through a mechanism regulated by TORC131,39. The impairment in Mtl1p function caused by heat denaturation, oxidative stress, or other chemical and environmental threats to cell integrity interrupts this signaling axis and triggers the activation of a general stress response17 that we believe to be more similar to the non-lethal heat stress-like response described here for mtl1Δ strains. We propose that the responses observed in the mtl1Δ mutant mimic a chronic non-lethal heat stress state evidenced by upregulation in the accumulation of heat shock proteins and trehalose synthesis, both of which are factors implicated in the adaptive response to heat stress106. Similarly, with the accumulation of cytoplasmic catalase T (22-fold), the enhanced accumulation of proteins that promote cytoplasmic stress granule formation, the increase in enzymes required for amino acid catabolism with a reduction in ribosome biogenesis. The enhanced

**Figure 5.** Predicted contribution of TORC1 in the mtl1Δ strain interactome. Figure shows the overlay of predicted physical interactions (straight parallel black lines) and genetic interactions (dotted black lines) with GO terms (Fig. 3). Associated upregulated proteins (red) and downregulated proteins (green) are presented as triangles. GO terms concomitant with the predicted TORC1-protein interactions are represented as grey circle nodes. Non-related GO terms and proteins are shown in white. Visualization has been carried out using ClueGO plugin for Cytoscape 3.7.2 (https://cytoscape.org).
susceptibility of the mtl1Δ mutant to H₂O₂ treatment, despite the massive accumulation of catalase T, could be caused by high endogenous levels of ROS. Normally, ROS are mainly produced within the mitochondria. However, proteome and metabolite analysis of the mtl1Δ strain showed upregulation in proteins of the mitochondrial respirasome as indicators that this mitochondrial function was not affected by the MTL1 deletion. This assumption can be tested by direct measurements of mitochondrial physiological activity. Therefore, the enhanced sensitivity to oxidative stress associated with mtl1Δ strains may originate from accumulated ROS generated by other dysregulated metabolic processes. Enhanced catabolism of amino acids and ER stress may represent two potential sources for these ROS.

Conclusions
We have provided new metabolic data showing that an mtl1Δ strain upregulates the accumulation of heat shock proteins and trehalose synthesis, which are known indicators of the adaptive response to non-lethal heat stress that we propose for these strains. Similarly, the accumulation of cytoplasmic catalase T (22-fold), enhanced accumulation of proteins that promote cytoplasmic stress granule formation and enzymes required for amino acid catabolism, combined with a reduction in ribosome biogenesis and increased synthesis of mitochondrial respiratory proteins, indicate a complex cellular stress response characterized by elements of heat stress and nutrient deprivation. Without having found evidence of mitochondrial malfunction, we propose that the down-regulation of ribosomal protein synthesis coupled with the upregulation in synthesis of mitochondrial proteins may represent a shift in translation of cytoplasmic proteins to translation of mitochondrial proteins as an adaptive response to Mtl1p deficiency. The combined data supports the new function of active Mtl1p under normal cellular conditions which is to suppress the non-lethal heat stress response presumably via down-regulation of the MTL1 gene at the transcriptional level followed by triggering an adaptive cellular response to maintain normal metabolic homeostasis.

Data availability
Metabolomics data are available at the NIH Common Fund’s National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench (Project ID PR001034, https://doi.org/10.21228/M81X28). RNAseq data are available at Sequence Read Archive (SRA) repository, with the accession number PRJNA686365 (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA686365). Proteomics data are available at the Proteomics Identification Database (PRIDE). Currently, it is only accessible for authors and reviewers (to access the data please go to https://www.ebi.ac.uk/pride/archive/login, and use these credentials to login: reviewer_pxd023963@ebi.ac.uk, Password: OpR4sZae, then look for the accession number PXD023963). The data will be publicly accessible after the paper is accepted for publication.

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Competing interests

The authors declare no competing interests.

Additional information

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