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Los mecanismos celulares de la extensión de tiempo de vida por metformina en levadura

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Abstract

The antidiabetic drug metformin extends the lifespan of mice, nematodes and yeast. While the mechanisms by which it exerts its effects (at a cellular and organismal level) are just starting to be characterized, it is still unclear which are the relevant genes for its lifespan-extension effect. Moreover, since metformin modulates the cell in a pleiotropic manner, a systems-level study to uncover its lifespan-extension mechanisms is indispensable. Here, we perform a large-scale functional genomics assay to identify genes, processes, and pathways that participate in metformin lifespan-extension in the budding yeast *Saccharomyces cerevisiae*. Specifically, we measured the stationary-phase survival of single gene-deletion strains grown with or without metformin and compared the lifespan phenotypes of the deletion strains with that of the wild type strain. We focused our study in single gene-deletion strains for 1,478 genes with an orthologue in humans. Genes whose deletion results in a change in the lifespan phenotype from the expected due to metformin treatment are our candidates for mediators of the drug's lifespan extension. Based on our results, we found that genes involved in intracellular transport and protein localization, the tricarboxylic acid cycle, and protein glycosylation—particularly N-linked glycosylation—are relevant for the cellular-level lifespan extension effect of metformin in yeast. Our study provides one of the first large-scale views of how metformin modulates cellular mechanisms leading to longevity.

Resumen

El fármaco antidiabético metformina extiende el tiempo de vida de ratones, nemátodos y levaduras. Mientras que los mecanismos por los cuales ejerce sus efectos (a nivel celular y de organismo completo) se han empezado a caracterizar, aún no se sabe cuáles son los genes relevantes para su efecto de extensión de tiempo de vida. Además, ya que la metformina modula la célula de manera pleiotrópica, es indispensable un estudio a nivel de sistemas para describir sus mecanismos de longevidad. En este estudio, realizamos un ensayo de genómica funcional a gran escala para identificar genes, procesos y vías que participan en la extensión de tiempo de vida por metformina en la levadura *Saccharomyces cerevisiae*. Específicamente, medimos la supervivencia en fase estacionaria de cepas de deleción génica crecidas con o sin metformina y comparamos sus fenotipos de longevidad con el de la cepa silvestre. Enfocamos nuestro estudio en cepas con deleciones sencillas para 1,478 genes con ortólogo en humanos. Los genes cuya deleción resulta en un cambio en el fenotipo de longevidad respecto a lo esperado debido al tratamiento con metformina son nuestros candidatos de mediadores del efecto de extensión de tiempo de vida. Basado en los resultados, encontramos que genes involucrados en transporte intracelular y localización de proteínas, el ciclo del ácido tricarboxílico y la glicosilación de proteínas—particularmente N-glicosilación—son relevantes para la extensión de tiempo de vida por metformina a nivel celular en levadura. Nuestro estudio provee uno de los primeros panoramas a gran escala de los mecanismos celulares que modula la metformina y que resultan en longevidad.

1. Introduction

1.1 The origins of metformin as an antidiabetic drug

Metformin is an antidiabetic drug used as first-line treatment of type 2 diabetes (T2D). It is a derivative of guanidine, a compound found in extracts of *Galega officinalis*, a perennial herb used in medieval Europe to treat conditions prevalent in that period, like plague, snake bites and St. Vitus dance (also known as dancing mania) (Bailey & Day, 2004). Extracts of *G. officinalis* were also used in traditional medicine to relieve symptoms now ascribed to diabetes mellitus, such as thirst and frequent urination (Bailey, 2017). Nevertheless, its use was discouraged later due to its toxic secondary effects; today, *G. officinalis* appears in the database of FDA poisonous plants (FDA, 1978). In the 1920's, compounds extracted from the herb, like guanidine, as well as natural and synthetic guanidine derivatives, were reported to lower blood glucose (Bailey, 2017; Watanabe, 1922) and some were introduced for the treatment of diabetes. But just as *G. officinalis*, most of them were found to be toxic. Toxicity, and the increase in availability of insulin, overshadowed their use, which decreased in 1930's (Bailey, 2017). Hence, regarding diabetes treatment, guanidine-based compounds were forgotten for two decades. However, in the mid-1940s, during the search for antimalarial and anti-influenza agents, these compounds were rediscovered. This time, other type of guanidine-derivatives was tested, namely biguanides, compounds resulting from the fusion of two guanidines. Among them, in 1949, metformin (named flumamine at that time) emerged as a compound with anti-influenza properties and a blood glucose lowering secondary effect (Bailey, 2017). Intrigued by metformin's blood-glucose lowering potential, Jean Sterne began to research the pharmacodynamics of several guanidine-based compounds, including metformin. His study, which confirmed metformin's efficacy and minimal secondary effects (Bailey, 2017; Sterne Jean, 1957), played a pivotal role in the drug's introduction into clinical practice. Although metformin was first synthetized in 1922, it was introduced for diabetes therapy in late 1950's in Europe, and in 1995 in the USA (Bailey, 2017).

At the suggestion of Sterne, it was marketed under the name of "Glucophage", which means glucose eater (Thomas & Gregg, 2017).

1.2 More than an antidiabetic drug

Metformin decreases hepatic glucose production through the simultaneous induction of glycolysis, suppression of gluconeogenesis and improvement of insulin sensitivity in the liver (Salpeter et al., 2008). Today it is the most commonly prescribed drug to treat T2D (Bailey, 2017) for its effectiveness, safety, and low cost. Moreover, it has been shown to promote many other therapeutic effects which has expanded its approved uses and suggests potential future applications (Thomas & Gregg, 2017). It reduces body weight when used alone or in combination with lifestyle changes as shown in meta-analyses of randomized controlled trials with obese adolescents (12 to 19 years) (Bouza et al., 2012) and adults (22 to 88 years) (Domecq et al., 2015). The drug also reduces effectively the levels of androgens, whose excessive production in women triggers the development of polycystic ovary syndrome (PCOS). Therefore, besides T2D, it is prescribed both for the treatment of obesity and PCOS. Metformin could also protect against several cardiovascular diseases (CVD) linked to T2D and various subtypes of cancer (Eurich et al., 2013; Heckman-Stoddard et al., 2017; Maruthur et al., 2016). However, regarding the potential antineoplastic effect of metformin, the results from animals and epidemiological studies have been inconsistent, hence conclusive evidence is still missing (Heckman-Stoddard et al., 2017).

Metformin effects have motivated a substantial amount of research, but amongst them the one that has arguably generated most excitement is its effect on lifespan. Longevity by metformin has been demonstrated repeatedly by studies with model organisms: mice, worms and yeast that are treated with metformin have a greater mean survival that their control counterparts (Anisimov et al., 2011; Borklu-Yucel et al., 2015; Martin-Montalvo et al., 2013; Onken & Driscoll, 2010). Moreover, aging populations of mice and worms show a slower decline in physical performance when treated with the drug, which suggests that it also promotes a youthful physiology (Martin-Montalvo et al., 2013; Onken & Driscoll, 2010). In

humans, a correlation was reported between its use for treating diabetes and a decrease in all-cause mortality (Bannister et al., 2014; Turner, 1998). In the most recent study, Currie's group used retrospective observational data from 78 241 diabetic subjects followed during a 5½ year period and compared their all-cause mortality outcomes to that of a matched control group of individuals that were not diabetics and were not taking the drug. By using parametric survival models, they observed that T2D patients treated with metformin had a longer survival than matched, non-diabetic individuals, despite the later had less morbidity. This result created high expectations in the research field and, currently, there is a keen interest to investigate how metformin affects aging as well as aging-related diseases in humans. However, to date no study has been carried out in order to determine whether a lifespan extending effect by metformin is also induced in heathy humans. In that regard, new promising clinical studies have been set up and some of them have already started (Alfaras et al., 2017).

1.3 Longevity-relevant metformin mechanisms

Because of its importance in health, many studies have been carried out to decipher the cellular mechanisms involved in the therapeutic effects of metformin observed at an organismal and cellular level. Most of the known effects elicited by metformin can be largely explained by its effect on pathways with multiple downstream targets: activation of AMP-activated kinase (AMPK) (Zhou et al., 2001), and inhibition of the mechanistic target of rapamycin (TOR) (Howell et al., 2017). These are conserved major sensors and regulators that integrate signals of the cellular nutrient and energetic status and influence a large amount of physiological responses. Upon activation of the AMPK pathway by the drug, glucose production by hepatocytes is inhibited, glucose uptake by skeletal muscle cells is stimulated, fatty acid oxidation is induced, and expression of lipogenic enzymes is suppressed (Zhou et al., 2001). All these cellular effects of metformin are essential for its effectiveness in the treatment of T2D and obesity. Moreover, activation of AMPK pathway also results in the inhibition of TOR complex 1 (TORC1), one of the two functional complexes in which TOR is found (Howell et al., 2017). This inhibition, in turn, influences a series of responses that altogether

have been proposed to have an antineoplastic effect (Amin et al., 2019) and induce lifespan extension. Longevity by TOR signaling inhibition has been reported in different model organisms; in mice (mTORC1) (Harrison et al., 2009), *D. melanogaster* (dTOR) (Kapahi et al., 2004), *C. elegans* (CeTOR) (Jia et al., 2004), and *S. cerevisiae* (TOR1) (Bonawitz et al., 2007). This highlights the importance of the activation of AMPK by metformin, and the subsequent inhibition of TOR, for the aging-delay effect of the drug. Nevertheless, TORC1 is also inhibited by metformin through mechanisms independent of AMPK activation. The best described of these mechanisms involves the liberation of TORC1 from the endosome/lysosome, in a Rag GTPase-dependent manner (Kalender et al., 2010), by the action of the scaffolding protein AXIN (Zhang et al., 2014). This prevents TORC1 from binding its positive regulator, Rheb, which causes its inhibition. The relevance of TOR inhibition by metformin in lifespan extension is clear and this is in fact a mechanism in common induced by different anti-aging interventions and compounds, like caloric restriction, rapamycin and resveratrol (Anisimov, 2013). On the other hand, the relevance of many other proteins and processes modulated by metformin has not been explored. This is important since the drug induces pleiotropic effects at a cellular level, which has been observed in high-throughput studies performed with *S. cerevisiae* aimed at describing the systemic perturbation of the drug (Borklu-Yucel et al., 2015; Stynen et al., 2018). In one of these studies, Michnick's group used a large-scale biochemical approach to measure the changes in homo-oligomerization of proteins due to metformin treatment (Stynen et al., 2018). With this approach, which can capture the integrated passive and active effects of cellular perturbations, they observed an enrichment in several processes related to metabolism, transport, signaling and others. In a second study, aimed at describing the transcriptional reprogramming induced by metformin, Ulgen's group found that metformin mainly regulates sulfur-containing amino acid biosynthesis, DNA repair, growth, transcription and mitochondrial processes, such as oxidative phosphorylation (Borklu-Yucel et al., 2015). Moreover, they performed and compared the individual transcriptional profiles induced by caloric restriction and metformin supplementation and observed that

they don’t significantly overlap. This result was remarkable because for a long time metformin was considered a caloric restriction mimetic since they are both anti-aging drugs that operate through common mechanisms: AMPK and TORC1 (Dhahbi et al., 2005). Furthermore, these results emphasize the need for a deeper understanding of the cellular mechanisms of metformin and for determining which of the implicated proteins are relevant for its lifespan extension effect.

In this study, we performed a pharmacogenomics assay to measure the longevity phenotypes under metformin of a collection of single-gene deletion strains, for genes with an ortholog in human, of the budding yeast *Saccharomyces cerevisie*. We looked for genes whose deletion affects the longevity phenotype elicited by metformin, to find mediators of its lifespan extension effect.

2. Objectives

2.1 General objective

To determine genes and processes relevant for the lifespan-extension effect of metformin in *Saccharomyces cerevisiae*

2.2 Particular objectives

1. To describe the dosage-effect curve of metformin to extend *S. cerevisiae* longevity.

2. To identify genes that participate in the longevity effect, using single-gene deletion strains.

3. Methods

3.1 Strains and media

Fluorescent single-gene deletion strains collection are prototrophic haploids (*MATa xxxΔ::kanMX4* PDC1-mcherry-CaURA3MX4 *can1*Δ:STE2prSpHIS5 *lyp1*Δ *his3*Δ1 *ura3*Δ0 LEU2). The collection was derived from crossing 1,482 viable deletion strains (*MATa xxxΔ::kanMX4 his3Δ1* ura3Δ0 leu2Δ0 met15Δ0) for genes with a human ortholog (from here on referred to as humort collection) from the BY4741 Saccharomyces Genome Deletion Project (Chu & Davis, 2007) with the MATα Y8205-RFP SGA-starter (MATα Pdc1-RFP-CaURA3 *his3*Δ1 *ura3*Δ0 *can1*Δ:STE2pr-SpHIS5 *his3*Δ1 *ura3*Δ0 *lyp1*Δ::Direct repeat). Genes with a human ortholog were selected with the BioMart tool from Ensembl (Kinsella et al., 2011). Fluorescent control strains were derived from crossing MATα Y8205-RFP SGA-starter or MATα Y8205-CFP SGA-starter with MATa *his3*Δ::kanMX4 *his3*Δ1 *ura3*Δ0 *leu2*Δ0 *met15*Δ0.

Synthetic Complete (SC) Aging medium contained 0.67% yeast nitrogen base (YNB) without amino acids, 0.2% amino acid supplement mix and 2% glucose. For the metformin dose-response assay, the aging medium was supplemented with metformin at 5, 10, 25, 40, 50, 65, 80, 100 and 200 mM. Metformin aging medium (SC-Met) was prepared by supplementing the SC aging medium with metformin at 40 mM.

3.2 Generation of yeast collection and selection of reference

The humort-RFP collection of 1,482 fluorescent single-gene deletion strains and control WT strains (control-RFP and control-CFP) for the CLS screen were generated by the mating methodology known as synthetic genetic array (SGA) (Tong & Boone, 2006). The parental MATa BY4741 humort collection of single-gene deletion strains was arrayed in nineteen 96-well microplates (Corning 3585). In each plate, 85 wells contained one of the deletion strains, 10 dispersed wells

contained the control strain of the humort collection and 1 was empty (**Figure 1A**). The empty well was left to monitor and control for inter-well contamination and systemic positional errors. The humort collection array was inoculated with a manual replicator on a solid layer of MATα Y8205-RFP SGA-starter strain culture for mating. Mating was followed by diploid selection, sporulation, and three rounds of haploid selection, to generate the humort-RFP collection and control-RFP strains. Simultaneously, the same procedure was followed for mating between the control strain of the humort collection and the Y8205-CFP SGA-starter strain to generate control-CFP. Selectable markers for *HIS*$^+$, *URA*$^+$ and *G418 (geneticin)*$^+$ traits were used in order to select *MATa* mating type, fluorescence and the gene disruption, respectively, in the genotype of the humort collection, control-RFP and control-CFP strains (**Figure 1B**). For a greater certainty on the absence of systemic inter-well contamination or flipped order of the strains, a pair of confirmation PCR reactions were carried out for 19 humort-RFP deletion strains. The first PCR reaction (primers A and B) was performed to confirm deletion of the ORF of interest in each case and the second (primers A and kanB) to confirm the presence of the kanMX4 module replacing the ORF (**Figure 1C**). From the 19 tested humort-RFP deletion strains, 18 were randomly chosen strains of each odd microplate of the collection, for which a primers set was available in the laboratory. Deletion strain 19 was the only one from humort-RFP microplate 19 for which a primers set was available. Therefore, only one strain was tested from that microplate. Seventeen validated as positive for the deletion, while two as negative (**Figure 1D**).

The confirmation PCR program was designed with the following guidelines:

Step	Temperature (°C)	Time (min)
1 – Initial denaturalization	98	3:00
2 – Denaturalization	98	0:30
3 – Primer annealing	62	0:40
4 – Final extension	72	0:30

5 – Cycle repeat (Go to step 2, 36x)		
6 – Final extension	72	5:00

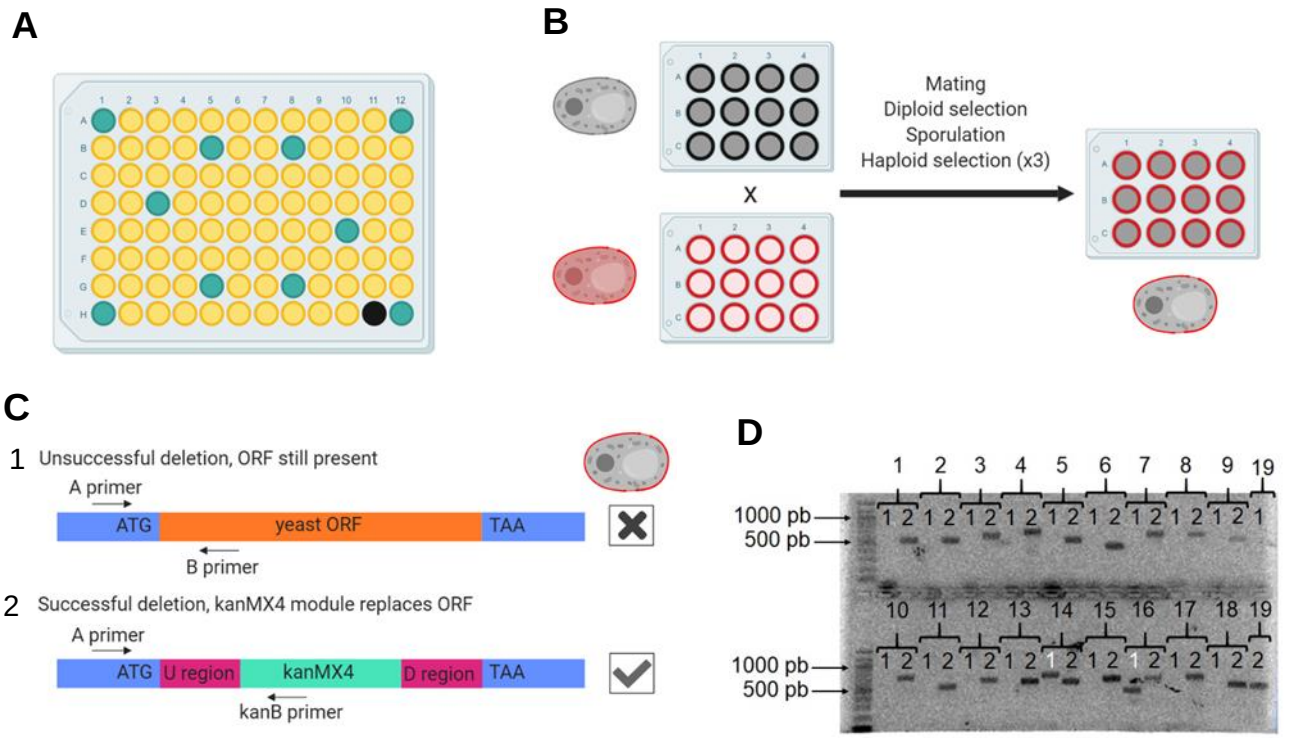

Figure 1. Generation of deletion strains and validation. A) MATa BY4741 humort collection and humort-RFP array. B) SGA outline. C) Strain confirmation outline. One and two indicate the different reactions performed. D) Confirmation electrophoresis gel. Numbers 1-19 indicate the strain tested. Numbers 1 and 2 indicate the reaction.

To select the reference strains (ref-RFP and ref-CFP) among the control strains (control-RFP and control-CFP) generated from the SGA, growth doubling time and death rate of the control strains in SC and SC-met medium was estimated; the control strains with the most central phenotypes, were selected. A detailed description on doubling time and death rate estimation is written in section 4.6 of Methods. A total of 190 control strains were generated from the SGA for each fluorescent label, from which 20% (38 from each) were randomly chosen to be tested; five replicates from each control strain were considered. To select the control strains with the most central phenotypes, the median of the doubling time and death rate values from all replicates was subtracted from the mean values of the replicates of each control strain. For each trait (doubling time or death rate) in each treatment (SC or SC-met) for each fluorescent label (CFP or RFP), a ranking was generated from the absolute value of the subtraction; the absolute value closer to zero was assigned the number one rank. The control strains that scored better in all tests of rankings were selected as reference strains (ref-RFP and ref-CFP).

3.3 Automated co-culture-based CLS screen and data analysis

RFP-tagged deletion strains were replicated in 96-well plates with 180 µl of SC medium. These strains were arrayed in 19 plates. Ten independently obtained RFP-tagged WT-strain controls were replicated in each plate along with the deletion strains. A CFP-tagged WT strain culture was replicated in independent 96-well plates with 180 µl of SC medium. The cultures were grown to saturation at 30 °C. The RFP-tagged strains were mixed with the CFP-tagged strains in a 2:1 ratio to a final volume of 150 µl. Five µl of the mixed cultures were inoculated with a manual replicator in deep-well plates (Nunc 260251) with 750 µl of SC with or without 40 mM metformin. The co-cultures (aging cultures) were grown to saturation in a chamber with controlled temperature of 30 °C and relative humidity of ~ 70%. From then on, all experimental steps were performed in an automated robotic station (TecanFreedom EVO200). Five days from the inoculation day, 10 µl

of the aging cultures were inoculated in 96-well plates containing 150 µl of fresh low-fluorescence medium for outgrowth, with an automated 96-channel pipetting arm. The outgrowth cultures were kept at 30 °C and monitored as follows: Every 2 h for 16 h (8 data points in total) outgrowth cultures were vigorously shaken and transferred to a Tecan multilabel plate reader (M1000) to collect data for red and blue fluorescence channels and optical density at 600 m (OD_{600}). This procedure was performed for four consecutive days and every two days from then on.

To obtain the CLS of each deletion strain population we modeled the rate of death by using an equation that relates the change in the logarithm of the ratio of RFP and CFP fluorescence throughout the experiment to parameters obtained from outgrowth kinetics of the co-cultures (Avelar et al., In prep):

$$1)\quad \ln\left(\frac{x_{RFP}}{w_{CFP}}\right)_{T_0 t1,t2 \cdots T_7 t1,t2} = \ln\left(\frac{N_x}{N_w}\right)_{T_0} + (r_w - r_x)T_i + (g_x - g_w)(t_j) + C_{Tt}$$

Which can also be expressed as

$$2)\quad ln\,(\frac{x_{RFP}}{w_{CFP}})_{\,T_i,\,t_j} = A_x + S \cdot T_i + G \cdot t_j + C_{T_i,\,t_j}$$

where A represents the ratio of viable cells at the start of the experiment, S is the difference in exponential death rates between the wild-type and the mutant, G Is the difference between the growth rates of the mutant and wild-type and C accounts for the variation between different plate measurements for every day of experiment (T_i) and data points in the outgrowth curve (t_j). As C is a parameter that accounts for plate noise-variation, it is the same for all the co-cultures in the same plate for each $T_i t_j$.

Raw measurements went through several filters before analysis: autofluorescence background was eliminated, fluorescence signal from RFP and CFP single cultures was subtracted from the co-cultures signal, and outgrowth curves data was filtered, only stationary phase time points were selected for the analysis. Wells from which only a few measurements (<15) were obtained throughout the entire experiment, were eliminated. After filtering, data from 1414 deletion strains

remained in the analysis. At each data point of the outgrowth curve of a co-culture an equation (2) was defined, using the measured data. Therefore, for each plate, a system of linear equations was constructed. By solving the system, a value of S (equation 2) was approximated for each co-culture, which is referred as the relative survival coefficient of the deletion strain. At the end, we obtained an S coefficient for each one of our 1414 deletion strains.

3.4 Identification of lifespan-extension metformin mediators

A linear regression model was used to characterize the relationship among the relative survival coefficients obtained without metformin and the relative survival coefficients obtained with the metformin treatment. To assess the effect of metformin treatment on the gene deletion strains relative to the WT, we estimated the residual distance from the data points to the fitted line. A 95% confidence interval for new observations was computed by using the curve fitting tool from MATLAB. The data points outside the boundaries were selected to obtain the list of genes with the largest residual distance. These genes were termed as relevant for the lifespan extension effect of metformin and were classified into two main categories: the ones above the confidence interval, as inhibitors, and the ones below, as inductors of the metformin effect.

The validity of the regression model was evaluated by a diagnosis of the linear relationship assumption among the SC S variable and the SC-met S variable. To that end, we observed the behavior of the residuals and asked if they followed a linear trend along the fitted values of the model. In the plot of residuals vs fitted, the observations lined up approximately horizontal at zero, indicating the linearity assumption is appropriate (**Figure 2**).

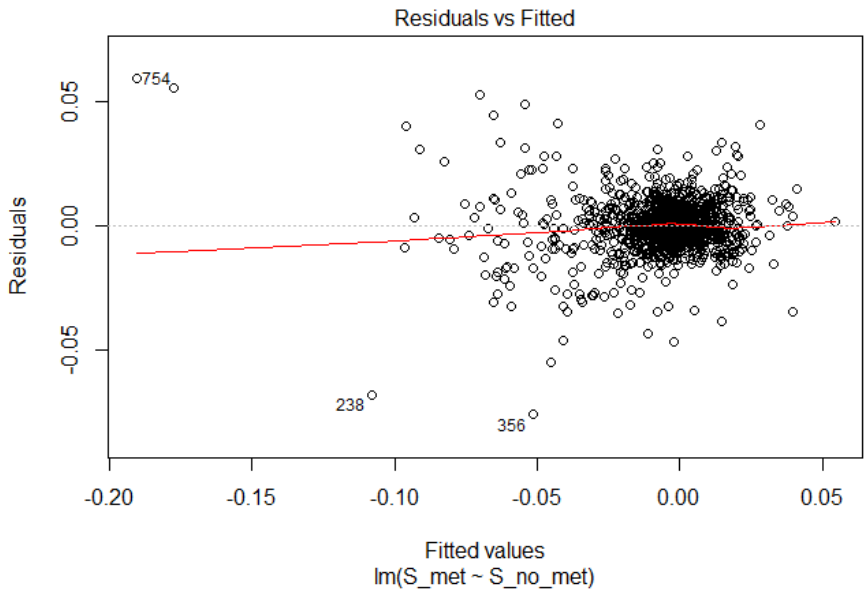

Figure 2. Evaluation of linear regression assumption. The red line shows the average value of the residuals at each value of fitted values. Linearity degree can be interpreted by the level of transposition of the red line to the gray dashed line. Each point corresponds to a deletion-strain from the study.

3.5 Gene Ontology (GO) term enrichment analysis

The GO enrichment analysis for the list of metformin-mediator genes was performed using the GOrilla online tool (Eden et al., 2009) with two unranked lists. The target was the list of metformin mediator-genes (79), obtained from the analysis of lifespan phenotypes from single-gene deletion strains, and the background list was made up of the 1414 genes considered in the study. A significance threshold of p-value < 0.01 was selected. A representative, higher level GO term was selected out of the overlapping terms to reduce redundancy.

3.6 Small-scale CLS assay measured by outgrowth kinetics

A WT strain of *Saccharomyces cerevisiae* was grown in SC medium for 48 hours at 30°C and 200 rpm in aerated tubes, then transferred to 96-well plates. These plates were replicated onto 96 deep-well plates containing 750 µl of SC or SC-

metformin, at the concentration indicated and left for the entire experiment at 30°C and 70% relative humidity without shaking. From then on, all experimental steps were performed in an automated robotic station (TecanFreedom EVO200). Three days after the inoculation day of deep-well plates, 10 µl of the aging cultures were inoculated in 96-well plates containing 150 µl of fresh low-fluorescence medium for outgrowth, with an automated 96-channel pipetting arm. The outgrowth cultures were kept at 30 °C and monitored as follows: Every hour outgrowth cultures were vigorously shaken and transferred to a Tecan multilabel plate reader (M1000) to collect data for optical density at 600 nm (OD_{600}), until stationary growth phase was reached; this first outgrowth curve was regarded as the first time point (T_0 age = 0 days). Three-four consecutive days and every two days from then on, the aging cultures were outgrown as previously described and optical density was collected. From these OD data, we extracted the doubling time and the time shift to reach mid-exponential phase (OD600=0.3) that occurred between the first day of measurements (T_0) and each day in stationary phase (T_n). Relative cell viability was calculated from these data, as reported by Murakami et al. (Murakami et al., 2008).

Viability data points relative to T_0 were used to plot a survival curve, which was fitted (all data points) to an exponential decay model ($N(T) = N_0 e^{-rT}$) where N_0 is the percentage of viability at T_0, T is time in days, and r is the rate of death.

3.7 CLS assay in standard aeration conditions

Three colonies of each strain were inoculated into tubes with 2 ml of SC medium and incubated for 24 hr. To prepare the aging cultures, the pre-inoculates were diluted 1:100 v/v in 50 ml tubes with 10 mL SC with or without metformin. The aging cultures were incubated for 48 hours with agitation (200 rpms) at 30°C. Viability of the aging cultures at each measuring point was obtained by monitoring the change in the growth-kinetics parameters with respect to time of the outgrowths, as described above for the small-scale outgrowth-kinetics based assay and reported by Murakami *et al*. 2008.

4. Results

4.1 Dose-response relationship between metformin and lifespan extension

We characterized the dose-response effect of metformin dosage on chronological lifespan (CLS) extension in wildtype (WT) laboratory strain of *Saccharomyces cerevisiae*, in order to find the optimum concentration of the drug to increase lifespan. The survival of the WT strain, as a function of time, was our proxy for their CLS. For this, we made cultures of yeast in SC medium with different concentrations of metformin and compared the CLS obtained in each treatment (**Methods 4.6**). We used concentrations of metformin previously reported to elicit an effect in *S. cerevisiae* and added other concentrations in between and at the opposing borders of the gradient. We tested nine concentrations (5, 10, 25, 40, 50, 65, 80, 100 and 200 mM) and aged the yeast cultures for three weeks. We measured the lifespan of the aging cultures of the WT strain using an established method for quantitative analysis of yeast CLS based on outgrowth kinetics (Murakami *et al*. 2008) adapted to a high-throughput robotic platform (see Methods). Most of the metformin treatments increased survival rate of the WT yeast populations, compared to the non-metformin condition, and this effect was dose-dependent until 40 mM. From then on, the survival rate decreased with each successive increase in concentration of metformin (**Figure 3A, B**). Hence, we determined that 40 mM was the optimum concentration of metformin to increase lifespan in *S. cerevisie* under the conditions of the experiment. To validate these results, we repeated the experiment using a reduced number of concentrations under standard conditions of full aeration. The methods and analysis were carried out as described previously (Murakami et al., 2008). We tested four concentrations (2, 5, 40 and 100 mM). The concentration of 40 mM induced a higher survival rate of the WT yeast population than the control, 2 and 5 mM. Nevertheless, the yeast

population had a lower survival rate under this treatment than under 100 mM of metformin (**Figure 3C, D**). The difference in phenotypic response in the full-aeration condition vs the low-aeration condition to metformin concentrations may be due to the differences in the metabolisms elicited by each condition (Bisschops et al., 2015), which can impact in the responsiveness to metformin. Even though 40 mM was not the optimum concentration under full-aeration, we decided to use it in the following large-scale experiments for two reasons: 1) it had a large beneficial effect in both conditions, and 2) the successive large-scale CLS screen to identify the mediators of metformin lifespan-extension effect was going to be performed under low-aeration.

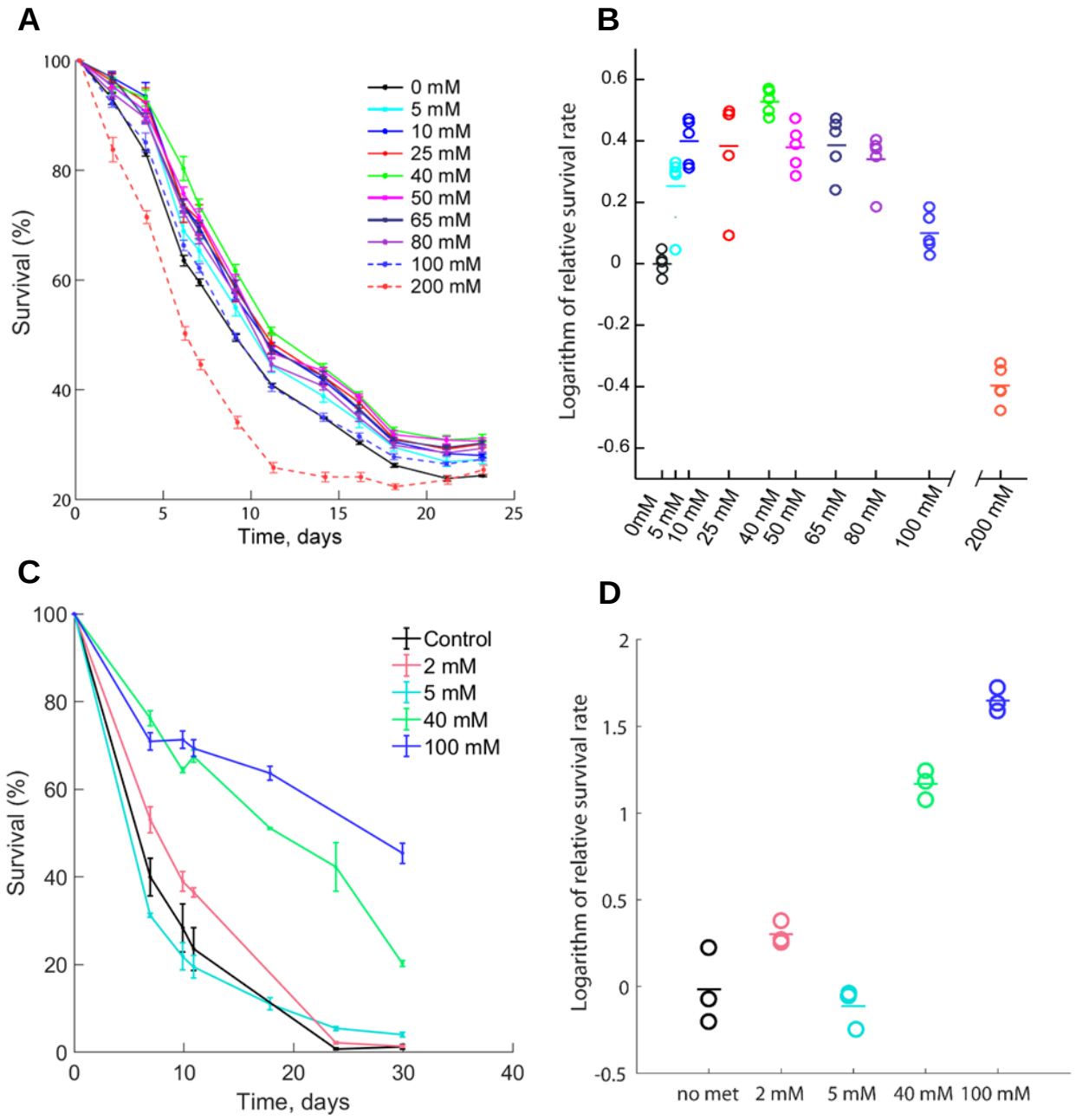

Figure 3. CLS of yeast is affected by metformin concentration.
Survival curves of WT cultures treated with different concentrations of metformin, and their corresponding relative death rates. The survival curves were obtained with a CLS high-throughput outgrowth-kinetics method (A) and a small-scale outgrowth-kinetics CLS method, (C) based on Murakami *et al*. 2008. B and D) The relative survival rates are determined from the slope of the exponential fit to the survival curves showed in A) and C), as a function of time, shown in log-scale.

4.2 Systematic identification of mediators of metformin lifespan-extension

To identify the genes that are potentially participating in the lifespan extension effect of metformin in *Saccharomyces cerevisiae*, we measured the CLS of a collection of 1478 viable single-gene KO strains under bare SC medium (SC) and 40 mM metformin in SC (SC-met). For tracking purposes, all strains were systematically mCherry-labelled by mating the KO-strains array with a fluorescent strain of the opposite mating type (Tong et al., 2001). To characterize the survival of the KO strains, we used an assay developed in our laboratory, based on the measurement of a relative survival coefficient (S) of each knockout strain aged in co-culture with the WT strain (Garay et al., 2014) (**Figure 4A**). The S coefficient of the KO strains, sometimes herein expressed just as S, was used as a proxy measure for the CLS and it is an indicator of the difference between the death rates of the KO strain and the WT strain in co-culture. The sign and magnitude of the S coefficient reflects how fast the KO strain population is dying relative to the WT strain population from the same co-culture. Thus, there are three possible general outcomes:

1) Positive *S*; indicates that the death rate of the WT is higher than the death rate of the KO

2) Negative *S*; indicates that the death rate of the KO is higher than the death rate of the WT

3) *S* coefficient around zero; indicates that the strains die at the same rate.

We compared the S coefficients obtained in SC and the S coefficients obtained in SC-met for each KO strain, in order to find deviations from the predicted relationship modelled from the entire dataset. Specifically, we looked for KO strains whose S coefficient in metformin was smaller (negative deviation) or larger (positive deviation) than the predicted (**Figure 4B**). To model the relationship between the S of the KO strains in SC and SC-met we made a linear regression by a least-squares method (**Figure 4C**).

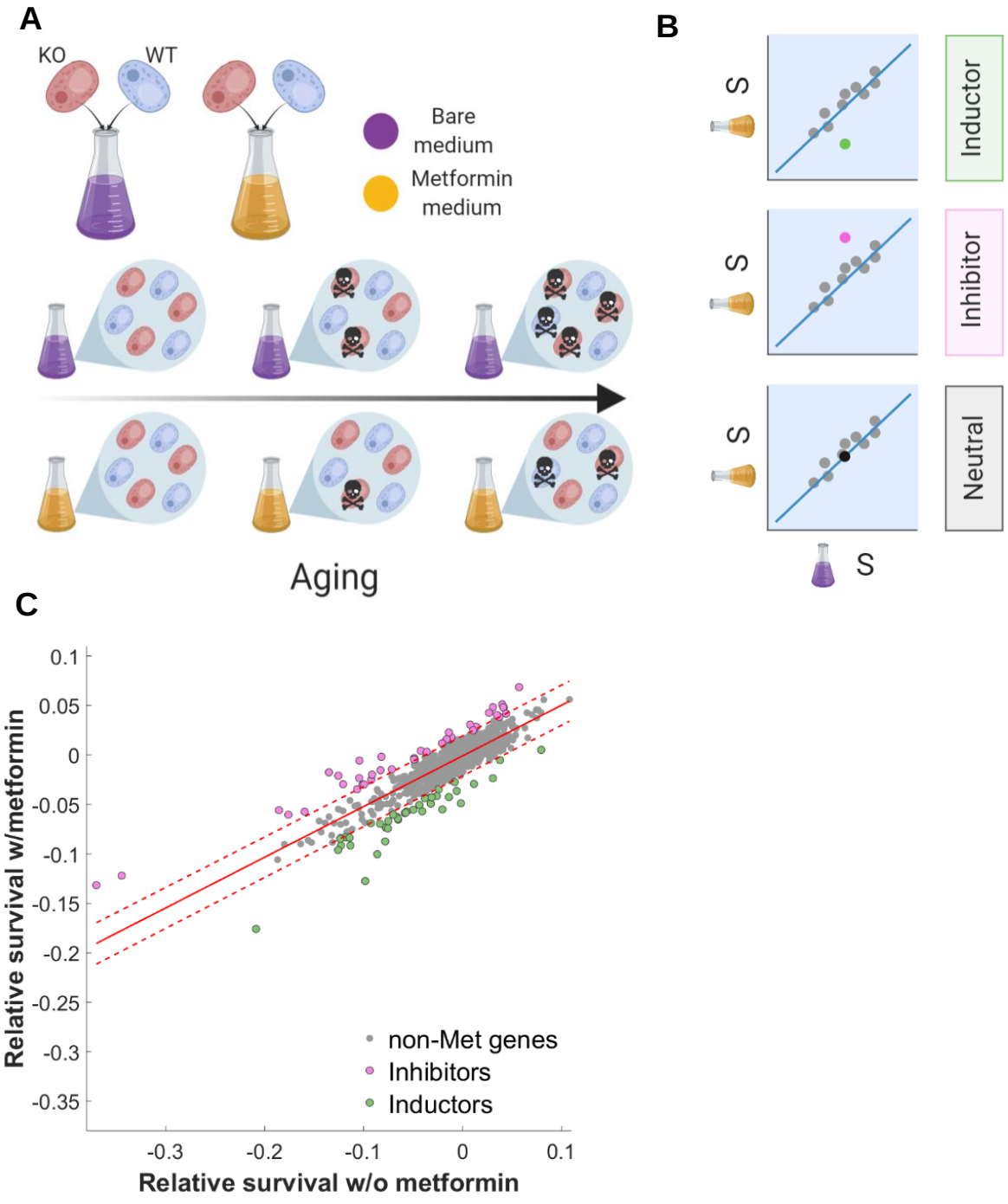
A
KO
WT
Bare medium
Metformin medium
Aging
B
S
Inductor
Inhibitor
Neutral
S
S
C
Relative survival w/metformin
Relative survival w/o metformin
0.1
0.05
0
-0.05
-0.1
-0.15
-0.2
-0.25
-0.3
-0.35
-0.3
-0.2
-0.1
0
0.1
non-Met genes
Inhibitors
Inductors

Figure 4. Large-scale competence-based CLS assay to find mediators of metformin lifespan-extension effect. A) Outline of the experiment. Co-cultures of a WT yeast strain and each one of the deletion strains (KO) in bare medium and metformin medium were grown to exponential phase. The deletion strains were labelled with RFP and the WT strain with CFP, to independently track them. The survival of the strains in aging was followed by the measurement of the change in relative fluorescence as a function of time. A relative survival coefficient (*S*) was obtained for each deletion strain in each treatment. B) Diagram of the comparison of the S of all deletion strains obtained in each treatment and selection of hits. Deletion strain with the greatest deviations from regression model to all data were selected and the corresponding deleted genes were labelled inhibitors or inductors. Genes whose deletion enhances survival by metformin correspond to the inhibitors (green circles). Genes whose deletion results in a decrease of survival than predicted are the inductors (pink circles). C) Relative survival comparison of each deletion-strain (1414 in total) in SC (X-axis) and SC-met (Y-axis). S stands for relative survival. Red line is the linear regression to all data. Dashed red lines are the 95% level prediction interval.

To find the KOs with the greatest positive or negative deviation from the relationship modelled from the entire data, we looked for the observations that fell outside a 95% prediction interval. We classified the observations in two types depending of the directionality with respect to the regression model. On the one hand, the observations above the prediction interval were labelled as “inhibitors” because the gene deletion causes an increase in the S coefficient of the KO strain in SC-met from the regression model (**Supplementary 1**). On the other hand, the observations bellow the prediction interval were labelled as “inductors” because the gene deletion causes a decrease in the S coefficient of the KO strain in SC-met from the regression model (**Supplementary 2**).

This large-scale functional approach allowed us to obtain valuable pieces of information regarding longevity-relevant proteins, cellular mechanisms and processes of an anti-aging intervention. While previous large-scale studies have made extensive and significant contributions to the understanding of the systemic cellular perturbations elicited by metformin (Borklu-Yucel et al., 2015; Stynen et al., 2018), with our approach we can identify longevity-relevant cellular participants and roughly describe their corresponding function. We can find genes whose presence is required to induce an efficient lifespan extension. These genes are the ones belonging to the inductors group. Among the 39 genes that crossed the 95%

threshold and classified into the inductors group, we found Sak1, the Snf1 complex activator kinase, Also, we found the genes Tpk1 and Tpk2, which are involved in the PKA pathway and are regulated by Snf1 (Nicastro et al., 2015). Snf1 is the yeast ortholog of AMPK and its activation has been associated with the beneficial effects of the drug (Zhou et al., 2001), as described in the introduction. On the other hand, we found Ste20, which is involved in the MAPK signaling that regulates the filamentous growth and the mating pathway (Dan et al., 2001). Both of these pathways are regulated by the TOR pathway (Vinod et al., 2008), which is suppressed by metformin. This gene is positively associated with aging progression and its suppression by metformin is believed to have a relevant participation for its lifespan extension effect (Barzilai et al., 2016; Howell et al., 2017). The presence of these genes in the inductors group supports that in *S. cerevisiae* the modulation of AMPK and the TOR pathway by metformin has a role in the effects of the drug that lead to longevity. With our approach we can also find genes whose deletion results in a larger lifespan extension effect of metformin than in the WT. The genes belonging to this group are labelled as inhibitors and with our analysis we found 40 of them. Among them we found VPH1, a subunit of the vacuolar ATPase, whose deletion induces a large survival in SC and a greater than predicted survival in metformin medium (SC-met). Interestingly, the V-ATPase, was proposed as one of the main molecular targets of metformin, through targeting VPH1 (Kim & You, 2017).

4.3 Identification of processes involved in the lifespan extension effect of metformin

To get a broader picture of the biological processes that are relevant for the lifespan extension effect of metformin we performed a Gene Ontology (GO) terms enrichment analysis of the inductors and inhibitors groups. The enriched GO terms (p-value < 0.01) in the inductors and the inhibitors gene groups are indicated in **Table 1** and **Table 2**, respectively. In the inductors group, the TCA cycle, peptidyl-amino acid modification and regulation of localization were enriched (**Table 1**). While in the inhibitors group protein glycosylation, phosphatidylinositol biosynthetic

process, carbohydrate derivative biosynthetic process, protein retention in Golgi apparatus and the endosomal transport were enriched (**Table 2**). We observed some degree of clustering of the genes specifying some of the processes, which reflects their similar phenotype in each of the two conditions (SC and SC-met) (**Figure 5**). Protein glycosylation genes in the inductors' category have a neutral (close to zero) or slightly negative relative survival in SC whereas in metformin they have a neutral or slightly positive relative survival, respectively (**Figure 5A and B**). Likewise, endosomal transport genes, particularly protein retention in Golgi apparatus genes, mostly have a negative phenotype in SC, whereas in metformin their deleterious phenotype was alleviated and reached neutrality (**Figure 5C and D**). The genes of the rest of the enriched processes, however, didn't show a process-specific phenotypic pattern. Inductor genes showed an enrichment in tricarboxylic acid (TCA) cycle, nevertheless they were present in both categories (inductors and inhibitors) (**Figure 5E**). Idh1 and Idh2, which catalyze the oxidation of isocitrate to alpha-ketoglutarate, were present in the inhibitors' group. On the other hand, Fum1, Lsc1, Mdh1, Lsc2, which catalyze most of the final steps of the TCA cycle, from the nucleotide-dependent conversion of succinyl-CoA to succinate, to the interconversion of malate and oxaloacetate, were present in the inductors' group.

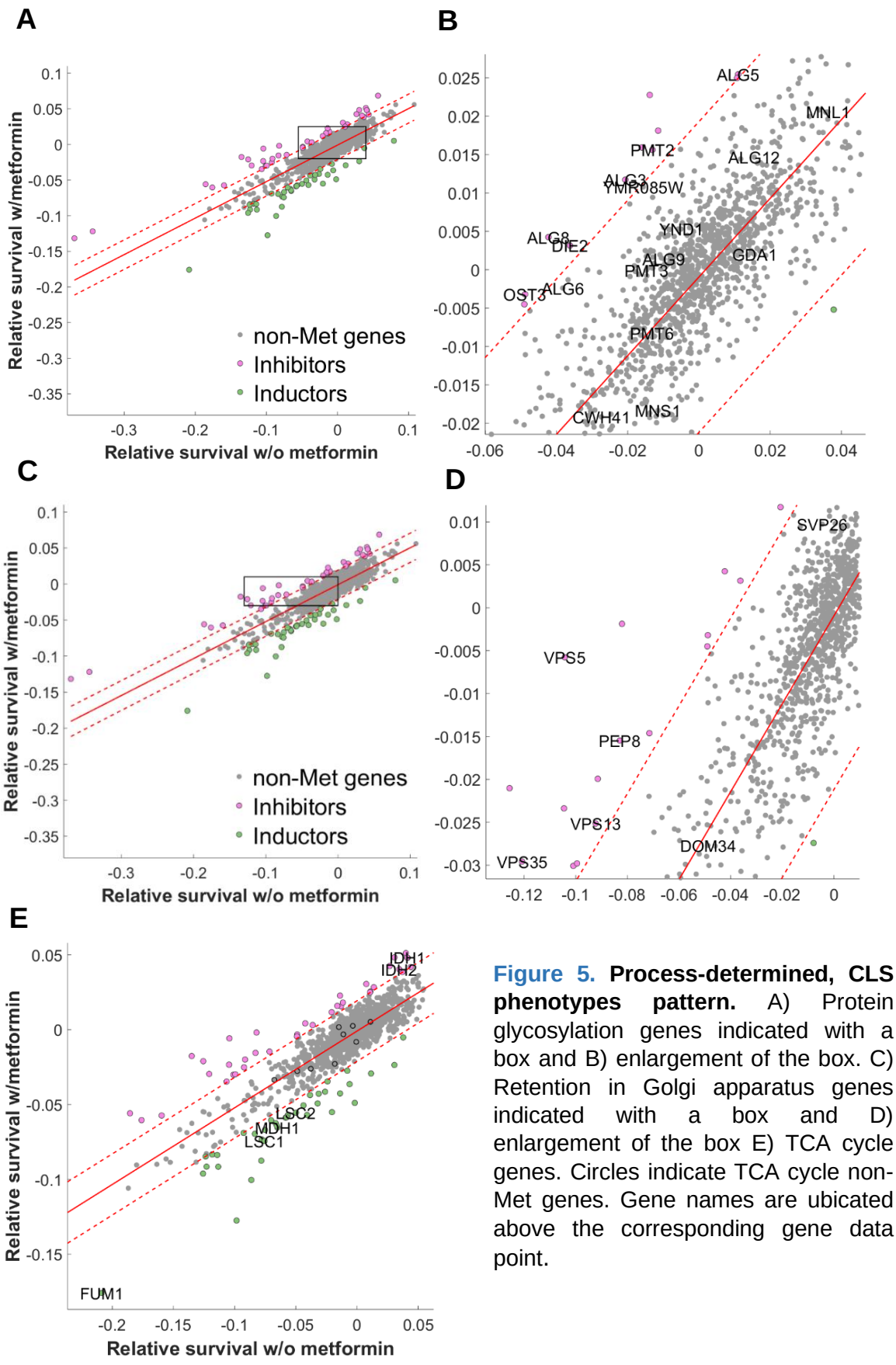

Figure 5. Process-determined, CLS phenotypes pattern. A) Protein glycosylation genes indicated with a box and B) enlargement of the box. C) Retention in Golgi apparatus genes indicated with a box and D) enlargement of the box E) TCA cycle genes. Circles indicate TCA cycle non-Met genes. Gene names are ubicated above the corresponding gene data point.

Table 1. Enriched GO terms (*p*-value < 0.01) for the inhibitor genes. N is the total number of genes; B is the total number of genes associated with a specific GO term; n is the number of genes in the target set; b is the number of genes in the intersection. Enrichment = (b/n) / (B/N).

Description	P-value	Enrichment	N	B	n	b
Protein glycosylation	$5.09x10^{-06}$	11.77	1412	18	40	6
Phosphatidylinositol biosynthetic process	$9.69x10^{-06}$	14.71	1412	12	40	5
Protein retention in Golgi apparatus	$6.30x10^{-05}$	15.69	1412	9	40	4
Endosomal transport	$9.43x10^{-05}$	6.03	1412	41	40	7
Late endosome to vacuole transport	$6.00x10^{-04}$	6.79	1412	26	40	5
Carbohydrate derivative biosynthetic process	$8.82x10^{-04}$	4.26	1412	58	40	7

Table 2. Enriched GO terms (*p*-value < 0.01) for the inductor genes. N is the total number of genes; B is the total number of genes associated with a specific GO term; n is the number of genes in the top of the user's input list or in the target set when appropriate; b is the number of genes in the intersection. Enrichment = (b/n) / (B/N).

Description	P-value	Enrichment	N	B	n	b
tricarboxylic acid cycle	$4.08x10^{-04}$	10.34	1412	14	40	4
peptidyl-amino acid modification	$5.84x10^{-04}$	3.97	1412	73	40	8
regulation of localization	$7.54x10^{-04}$	5.17	1412	42	40	6

4.4 Metformin dose-dependent lifespan effect in deletion strains

The inductors are those genes required to induce an effective, or “wild type”, lifespan extension by metformin. Nevertheless, the participation of the inhibitors in the lifespan extension effect of metformin is quite complex to describe. In order to construct a description of the plausible roles of the inhibitors regarding the longevity effect of the drug, we performed a dose-response CLS assay with a subgroup of deletion strains for those genes. For this part, we selected 14 inhibitors out of the total 39. We mainly chose genes from the enriched GO terms (11 out of 14); the rest were chosen based on their functional association with the former. Specifically, we chose seven genes from the protein glycosylation GO term, three from the endosomal transport GO term, one from carbohydrate derivative biosynthesis GO term, and three with different roles in intracellular trafficking (**Table 3**). We decided to perform a further exploration of the CLS phenotype, under a metformin gradient, of these genes because we were interested in getting an insight of the role of endosomal transport and protein glycosylation on the longevity outcome of metformin cellular modulation. The endosomal transport is a process required for the intracellular targeted movement of substances mediated by endosomes. This process acts as a hub of metabolism, and an aberrant regulation of it, is implicated in the development of neurodegenerative disorders, like Parkinson’s disease and Alzheimer’s disease (Vagnozzi & Praticò, 2019), which are aging-related. Also, recently, endosomes have been proposed as being a main target organelle of metformin, and their implication in the lifespan extension effect is being studied (Kim & You, 2017). Interestingly, the endosomal transport and the protein glycosylation process cooperate in the regulation of protein targeting to different cellular destinations. The appropriate glycosylation of proteins through the N- linked glycosylation pathway in the endoplasmic reticulum (ER) is essential for a correct sorting of proteins through endosomes or for their retention in Golgi and ER (Y. S. Liu et al., 2018; Nagai et al., 1997). Moreover, the protein glycosylation profile is an aging biomarker (Vanhooren et al., 2010) and its disruption has been associated with the development of aging-related diseases, like cancer and Parkinson (Videira &

Castro-Caldas, 2018). Nevertheless, while the endosomal transport has been related to the anti-aging effect of metformin (Kim & You, 2017), the participation of protein glycosylation has not been mentioned before.

For this assay, we prepared co-cultures of each of the chosen deletion-strains and the WT strain, as described in Methods section 4.3, in bare medium (no metformin) and medium supplemented with seven different concentrations of metformin (160, 80, 40, 20, 10, 5, and 2.5 mM). The optimal concentration of metformin to increase lifespan (40 mM) was included, for comparative purposes, as well as two high toxic concentrations (80 mM and 160 mM), as observed in the WT (**Figure 3**). When the co-cultures reached stationary phase and stopped dividing, we followed their survival. Unfortunately, we were forced to stop the experiment at day five due to technical complications. Because of this, we obtained more subtle relative survival coefficients (S), reflecting smaller differences in the survival between the deletion strain and the WT in its co-culture, than those obtained with the data of a complete experiment. However, we still could observe remarkable dose-response patterns with some of the gene-deletions. First, deletion strains for protein glycosylation genes show a similar linear increase in S with increasing concentrations of the drug, even at 80 and 160 mM (**Figure 6**). Thus, deletion strains show less or similar survival as the WT at low concentrations, but they have higher survival than the WT at high toxic concentrations. This suggests that protein glycosylation gene-deletions induce a cellular effect that confers protection against high concentrations of metformin that are normally toxic. On the other hand, deletion strains for genes related to endosomal transport show different graded dose-response relationships (**Figure 7**). Deletion of VPS24, PEP8 or PEP1 results in negative S coefficients. As metformin concentration increases, the S coefficient becomes less negative (the deletion strain's survival and that of the WT strain in its co-culture become increasingly similar), until a high concentration is reached and no further increase in S is observed. Also, they show a maximum S at a concentration of around 40 mM.

Table 3. Selected genes for the metformin dose-response CLS assay. The cellular function in which each gene participates was borrowed from the SGD database.

Cellular process	Gene name	Cellular function
Protein glycosylation	ALG3	N-linked glycosylation
	ALG5	N-linked glycosylation
	ALG6	N-linked glycosylation
	ALG8	N-linked glycosylation
	DIE2	N-linked glycosylation
	OST3	N-linked glycosylation
	PMT2	O-linked glycosylation
Endosomal transport	VPS35	Endosome-Golgi retrograde protein transport
	PEP8	Endosome-Golgi retrograde protein transport
	VPS24	Late endosome to vacuole transport
Carbohydrate derivative biosynthesis	SPE2	Spermidine biosynthesis
Related to intracellular trafficking	PEP1	Endocytic transport: Protein targeting to vacuole
	VPH1	Endocytic transport: Vacuolar acidification
	SSK2	MAPK pathway

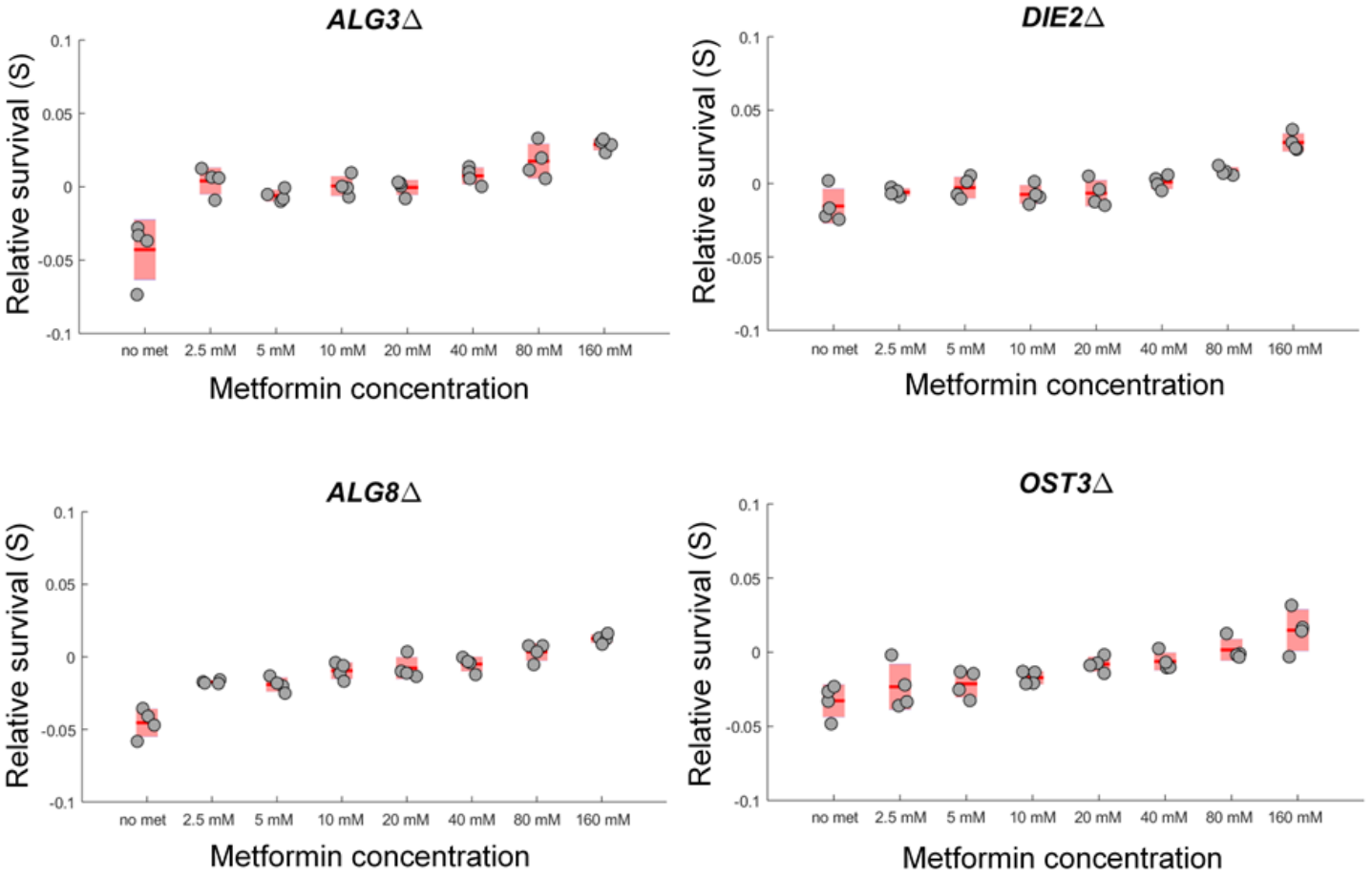

Figure 6. Dose-response effect of metformin on relative survival of deletion-strains for protein glycosylation genes. Relative survival (S) data of co-cultures of the deletion strain indicated in each case and a single WT strain under different concentrations of metformin. Grey dots are the result of each replicate (n=5). The mean of the S of all replicates in each condition is indicated with a red line. Zero is the neutral relative survival (WT survival == deletion-strain survival).

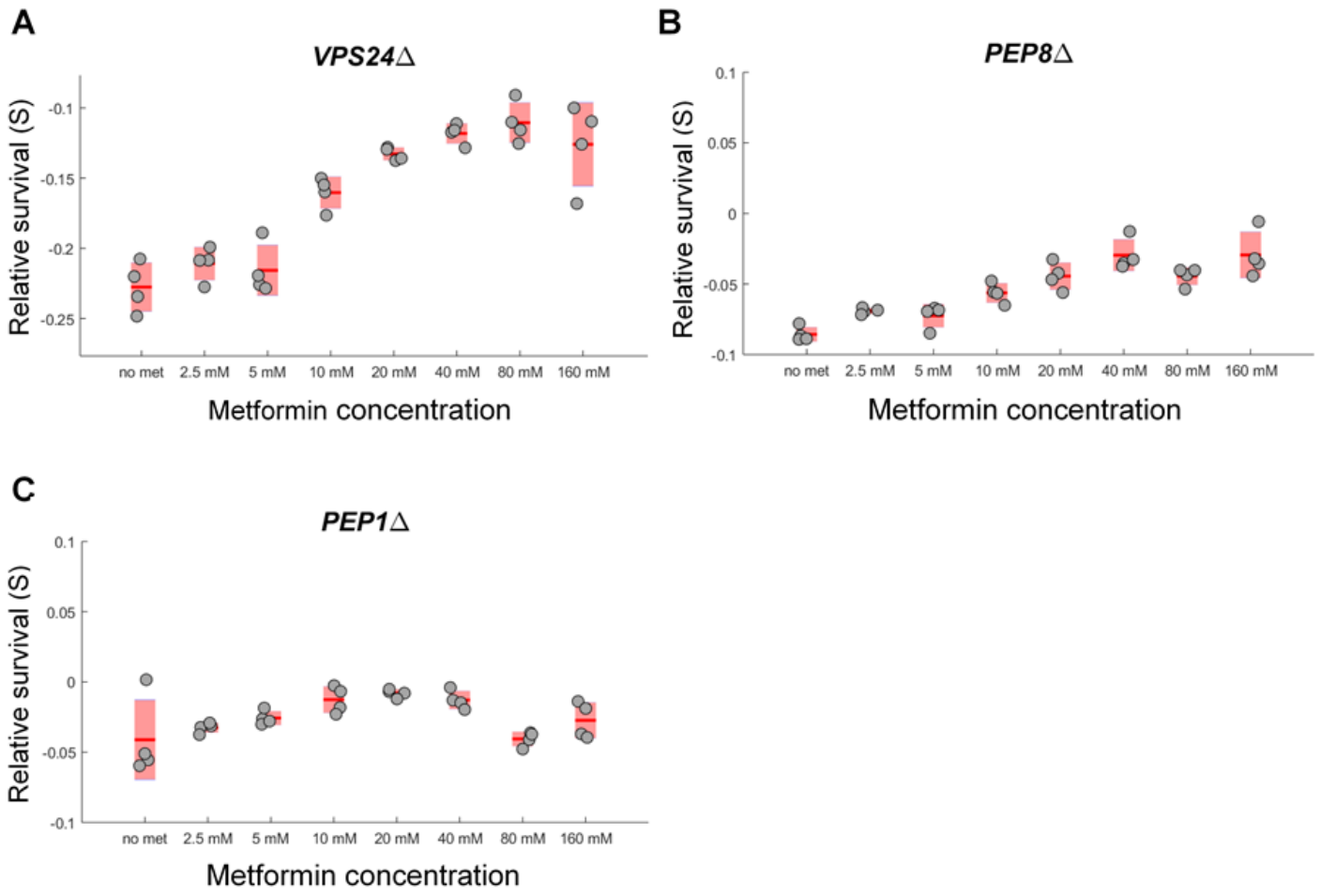

Figure 7. Dose-response effect of metformin on relative survival of deletion-strains for endosomal transport genes. A-D) Relative survival (S) data of co-cultures of the deletion strain indicated in each case and a single WT strain under different concentrations of metformin. A-B) Deletion strains for genes belonging to the endosomal transport GO term. C) Deletion strain for a gene that is included in the enriched GO terms but have a cellular function that is associated with the endosomal transport. Grey dots are the result of each replicate (n=5). The mean of the S of all replicates in each condition is indicated with a red line. Zero is the neutral relative survival (WT survival == deletion strain survival).

5. Discussion

Many independent valuable studies have been building an intricate yet incomplete picture of the global effect of metformin. From such studies we've discovered the wide extent of effects that the drug has in the organism. We now know that the drug impinges on the microbiota, different cell tissues and many diverse processes inside cells. Moreover, its broad-spectrum modulation within the organism results in several therapeutic effects. One of the most outstanding effects, as reported from model organism studies, is lifespan extension. This has brought about an imperative need for a systemic cellular description of the longevity-relevant effects of metformin. In this study, we employed the budding yeast *Saccharomyces cerevisiae* as a tool for a large-scale identification of genes and processes involved in the lifespan-extension effect of metformin.

5.2 Optimal metformin concentration to increase lifespan under low-aeration condition is 40 mM

Herein, we reported that the optimal concentration of metformin to increase lifespan under high-throughput, low-oxygen conditions is 40 mM. This concentration is very close to the commonly employed concentration (50 mM) in studies carried on *S. cerevisiae* (Borklu-Yucel et al., 2015; Li et al., 2019; Stynen et al., 2018). However, we observed that maximal survival under standard conditions of full aeration was obtained with a different concentration of metformin (100 mM). This difference in optimal concentration to increase lifespan between conditions (low vs standard full aeration) could result from different responses to metformin elicited under the cellular metabolic landscapes specific to each oxygen availability condition (Bisschops et al., 2015). Particularly, under hypoxia, and other stressors, TORC1 is inactivated by the recruitment of TSC2 to the lysosome, as reported in *Drosophila* (Lee et al., 2019) and immortalized human cells (Demetriades et al., 2016). Thus, we expect that, under our high-throughput low-oxygen condition in yeast, TOR pathway was partially inhibited. These hypothetic differences in TOR activity between conditions could explain the differences in concentrations required to induce a maximal survival in each oxygen availability condition. Furthermore, we are aware that the optimum metformin

concentration for lifespan-extension in yeast determined here is higher than that found in diabetic patients' plasma, which remains in the micromolar range after typical doses of 1.5–2.5 g per day (Madiraju et al., 2018). Nevertheless, it is not uncommon to use high doses of drugs in yeast due to the presence of a semi-permeable cell wall and a drug efflux system (Sá-Correia et al., 2009; Stynen et al., 2018), which might alter the effective dose of a bioactive compound (Zimmermann et al., 2018). Moreover, metformin mostly exists in a positively charged protonated form under physiological conditions (Foretz et al., 2014), which hinders passive diffusion through cell membranes. However, this difficulty is offset in human cells, as well as other multicellular organisms', by the activity of organic cation transporters (OCT1, OCT2, OCT3, MATE1, MATE2, PMAT, and OCTN1) that facilitate the uptake of metformin despite its physicochemical properties (X. Liang & Giacomini, 2017). On the other hand, orthologs of some of these cation transporters exist in yeast but none has proven to influence metformin responsiveness. We therefore expect that different extracellular concentrations of metformin can induce the same cellular effects in different organisms. Consistent with the above, Jingqi Fang *et al.* reported that a low, dose of metformin (100 µM), comparable to the plasma concentrations in metformin-treated diabetic patients, delays senescence of human diploid fibroblasts and mesenchymal stem cells. On the other hand, higher concentrations (>1 mM) abrogated this effect (Fang et al., 2018). Also, they observed that metformin's geroprotective effects were induced, partially, through the upregulation of the endoplasmic reticulum-localized glutathione peroxidase 7 (GPx7). Interestingly, they observed as well that in *Caenorhabditis elegans* metformin upregulated GPX-6 (GPx7 *C.* ortholog) and that this was required for an efficient lifespan extension of worms. Human cell line cultures and worms were treated with considerably different concentrations of metformin (100 µM vs. 50 mM, respectively) yet the same phenotypic effect (lifespan extension) was induced through the modulation of orthologous pathways. Thus, we expect that a concentration of 40 mM metformin, despite being high, induces an intracellular modulation in yeast that resembles that elicited by a micromolar clinically relevant dosage in human cells.

5.3 Pharmacogenomics assay revealed the genes and processes required by metformin to extend lifespan

By means of a large-scale co-culture-based survival assay with *S. cerevisiae,* we were able to identify genes whose presence is required for metformin lifespan extension at a cellular level. The enriched biological processes in this gene set, that we called inductors, were peptidyl-amino acid modification, regulation of localization and the TCA cycle. We observed that genes from the peptidyl-amino acid modification GO term code for proteins that have a role in histone modifications (methylation or acetylation), protein phosphorylation, transcription regulation and DNA repair. Among them, we found genes associated with the SNF1/AMPK pathway (Sak1, Tpk1 and Tpk2) and the TOR pathway (Ste20). The serine/threonine kinase Sak1p is one of the three constitutively active upstream kinases that phosphorylate Snf1p, the catalytic subunit of the SNF1 heterotrimeric complex, which activates the SNF1/AMPK pathway. SNF1 complex shares functional conserved functions with the AMP-activated Kinase (AMPK) in higher eukaryotes and, AMPK as well as Sak1 orthologs of *C. elegans*, are required for the lifespan and healthspan benefit of metformin (Onken & Driscoll, 2010). TPK1 and TPK2 encode isoforms of the catalytic subunit of cAMP-dependent protein kinase (PKA) which is the effector kinase of a pathway that has been implicated in metabolism, growth, differentiation and lifespan (Santangelo, 2006). PKA and SNF1/AMPK pathway are mutually regulated in an antagonistic manner (Nicastro et al., 2015). In the presence of glucose, cAMP is synthesized by adenylate cyclase Cyr1, this in turn activates PKA, which negatively regulates AMPK in a direct (Djouder et al., 2010) or indirect manner, perhaps though phosphorylation of Sak1 (Barrett et al., 2012). Conversely, in response to nutrient limitation signaling, Snf1 is activated, which reduces the intracellular content of AMP and impairs PKA function (Nicastro et al., 2015). The outcome of this crosstalk induces the transcription of genes that allow the cell to adapt to different nutrient availability conditions and this, in turn, modulates aging (Salminen et al., 2016). Thus, here we observed that proteins that regulate and are regulated by SNF1/AMPK are required for longevity by metformin in *S. cerevisiae*. On the other hand, Ste20 is a member of the MAPK pathway, which is activated in response to poor nutritional conditions and osmotic stress, and controls invasive/filamentous growth and mating

(Bardwell, 2006). Transcriptionally activated by the TOR pathway, G1 cyclin Cln1/2 regulates the MAPK pathway, precisely through the modulation of Ste20 (Vinod et al., 2008). Filamentous growth in *S. cerevisiae* results in pseudohyphae formation, a differentiation program that has been associated to lifespan extension by caloric restriction (Campos et al., 2018). Interestingly, the genetic background of the yeast strain used in this study prevents it to form pseudohyphae due to a mutation in the gene FLO8, required for filamentous/invasive growth (H. Liu et al., 1996). This suggests that, even though the yeast strain used here is unable to filamentous growth, this program could have a role in longevity by metformin. Noteworthy, among the inhibitors (genes whose deletion enhances longevity by metformin, discussed in the following section) we found Kss1, a MAPK pathway kinase that acts downstream of Ste20. Interestingly, a mammalian ortholog of this protein, MAPK1, is involved in development of cardiac hypertrophy, which is a hallmark of cardiac aging that has been reported to be reduced by metformin treatment (Mohan et al., 2019).

Regarding the regulation of localization enrichment, we observed that most of the unique genes from this GO term have a role in the regulation of different stages of the endocytic pathway. Akl1, involved in endocytosis, Mak3, involved in targeting to Golgi, and Gos1, involved in Golgi transport. The endocytic cycle is an endosome-mediated process by which cells internalize soluble and membrane components, which are either recycled to the plasma membrane or degraded in vacuoles/lysosomes (Huotari & Helenius, 2011). Moreover, each stage of the pathway is connected to the Golgi apparatus via transport vesicles, which mediate transport of newly synthesized lysosomal proteins (Huotari & Helenius, 2011). The implication of the endosomal trafficking in the longevity effect of metformin has been suggested previously from studies in *D. melanogaster* and *C. elegans* (Kim & You, 2017). Also, it was proposed that the late endosome/lysosome could be a main site of action of metformin through a modulation of the Vacuolar-type-ATPase (V-ATPase) (Chen et al., 2017; Kim et al., 2016). Interestingly, in mammals, the endosome/lysosomal membrane is a subcellular site where the activities of AMPK and TOR are regulated in a nutrient-dependent, mutually exclusive manner. In the presence of amino acids or under nutrient-rich conditions, TOR localizes to the endosome/lysosome in an active form. Conversely,

under glucose starvation, AMPK localizes to the endosome/lysosome and binds a complex composed of the V-ATPase, Ragulator, consisting of LAMTOR1 through LAMTOR5 (late endosomal/lysosomal adaptor, MAPK and mTOR activator 1–5), the scaffold protein AXIN and the AMPK-regulator LKB1. As a result of this interaction AMPK is activated and TOR is released from the membrane and becomes inactive (Zhang et al., 2014). Thus, a modulation of the endosomes from the endocytic transport provides a direct link between the two principal effects of metformin: TOR inhibition and AMPK activation. Similarly, in *S. cerevisiae*, TOR complex 1 (TORC1) is regulated by a mechanism that depends on the vacuole membrane-localized EGO complex, which is functionally analogous to the Ragulator complex in mammals (Lahiri & Klionsky, 2019). Also, under glucose limitation, SNF1 (AMPK ortholog) localizes around the vacuole, which might regulate its function. This suggests that in *S. cerevisiae*, the endosome/vacuole could play an important role for SNF1 and TOR regulation. Nevertheless, the relevance of the endocytic transport for longevity by metformin needs a further exploration.

Finally, we observed that the TCA cycle is required for the longevity effect of metformin. Noteworthy, we also observed members of this cellular process among the inhibitors' list of genes. The deletion-strains phenotypes for these genes, however, were consistent with the previously reported modulation of this process by metformin. In a study aimed on characterizing the modulation of biochemical properties (*i.e.* binding affinity, post-translational modifications, localization, and abundance) by metformin, Stynen *et al.* observed that the drug modulates the TCA cycle genes in different directions (Stynen et al., 2018). On the one hand, metformin increases at least one of those properties for Fum1, as well as for Cit2, Mdh3, Icl1 and Mls1. On the other hand, it decreases at least one of them for Idh1 and Idh2 as well as Aco1 and Kgd1. Herein, we observed that Fum1 and Idh1-2 were part of different hit categories; Fum1 was a member of the inductors, while Idh1 and Idh2 were part of the inhibitors, which could be explained by the differential modulation of TCA cycle genes by metformin, as previously reported. This modulation suggests that the proteins that are positively regulated by the drug, which correspond to the final steps of the TCA cycle (from succinate to citrate production), are required for longevity induction. Also, that the negative regulation of

proteins from the TCA cycle, which participate in the first steps of the cycle (from isocitrate to succinyl-CoA production) by metformin is partial, since their deletion enhances the longevity effect of the drug. The integrated effect of the drug on the proteins that comprise the TCA cycle, as evidenced from Stynen's and our study, might result in change in the abundance of different metabolites and cofactors. One of the most outstanding effects is an increase in citrate abundance, which may leak into the cytoplasm and be used for lipid biosynthesis. Citrate in the cytoplasm is cleaved by ATP citrate lyase into acetyl-CoA and oxaloacetate. The acetyl-CoA produced is carboxylated into Acetyl manolyl-Coa, which activates lipid biosynthesis (Ferré & Foufelle, 2007). This outcome seems contradictory since it has been shown that metformin can cause mild weight loss. Nevertheless, it's been reported that a supplementation of citrate in the diet induces a protective effect against diabetes-induced heart damage in mice (Q. Liang et al., 2016). Also, citrate synthase levels have been shown to be lower in insulin resistant compared with insulin sensitive individuals obese (Heilbronn et al., 2007), as well as to decay with aging (Crane et al., 2010). Thus, citrate abundance increase by metformin could explain in part cardiovascular protection and, perhaps, improvements in insulin sensitivity in diabetes patients and longevity by the drug.

5.4 Metformin alleviates detrimental effects associated with deletion of endocytic pathway genes

We also found genes whose deletion induced a relative survival increase in metformin than predicted, which we called inhibitors. These genes showed an enrichment in different processes related to endocytic transport, biosynthesis and protein glycosylation. We observed that the deleterious effect in relative survival due to the deletion of genes associated with endocytic trafficking is alleviated efficiently by metformin. Golgi apparatus is a central organelle in endocytic trafficking that serves as a major protein sorting hub (Banfield, 2011). As a result, it is subjected to a constant efflux and influx of proteins, from which it should be able to discriminate the ones that must be retained from the ones for delivery. Thus, deletion in genes associated with this GO term, results in a deficient localization of transmembrane proteins in the trans-Golgi network (TGN) (Redding et al., 1996), protein sorting (Rothman et al., 1989) and late

endosome-to-Golgi retrieval (Reddy & Seaman, 2001), which compromises survival. Metformin induces a large-spectrum modulation of biochemical states of proteins important for localization and associated with different stages of endocytic transport (Stynen et al., 2018). Also, the modulation of V-ATPase activity by metformin (Chen et al., 2017; Kim et al., 2016), which was mentioned in the previous paragraph, could result in a pH change within endosomal compartments, which may influence endosomal maturation and trafficking (Kim & You, 2017). This could explain how metformin can compensate deleterious phenotypes of gene-deletions associated with endocytic transport, like protein retention in Golgi apparatus (as reported here); perhaps through the improvement of cargo recognition and progression of endosomal trafficking. This idea is supported by the observation that some genes that regulate the endocytic pathway are required for the longevity effect of metformin, as discussed in the previous section. The above suggests that the endosomal trafficking might be a core part of the cellular effects of metformin regarding longevity. Some genes related to its regulation are required for longevity by metformin and the detrimental effects of impaired trafficking are alleviated with the drug. This is a striking idea, since the regulation of endosomal trafficking by metformin could explain other cellular effects associated with treatment with the drug, some of which have shown a connection with longevity. As discussed earlier, the endosomes are considered a hub for AMPK and TOR signaling regulation. Moreover, modulation of endosomal trafficking impinges in processes like autophagy (Tooze et al., 2014) and affects recycling of plasma membrane transporters and receptors (Grant & Donaldson, 2009). Autophagy is a process conserved in eukaryotes by which cytoplasmic components like macromolecular complexes and organelles are delivered to the vacuole or lysosome for its degradation and recycling of molecules (Reggiori & Klionsky, 2013). Induction of this process by metformin in mice has been related to protection against cardiomyopathy (Kanamori et al., 2019) and alleviation of hepatic steatosis (Song et al., 2015). Moreover, an increased autophagic capacity has been shown to induce longevity in yeast (Alvers et al., 2009), worms (Chang et al., 2017) and mice (Pyo et al., 2013). Regarding plasma membrane proteins cycling, it has been proposed that metformin induces a redistribution of plasmatic membrane glucose transporters in rodent enterocytes, which is relevant for blood glucose regulation by the

drug (Sakar et al., 2010). These processes depend on the endocytic transport, thus, the suggested endosome modulation by metformin treatment might function as a core for a simultaneous regulation of their activities.

Members of the endocytic pathway also regulate the activities of other organelles, such as mitochondria. Mitochondria are metabolic organelles that are crucial for production of ATP and metabolic intermediates. Also, they have a role in several cellular processes, including iron and calcium homeostasis, regulation of apoptosis, and synthesis of steroid hormones and iron-sulfur clusters (Braymer & Lill, 2017; Friedman & Nunnari, 2014; Miller, 2013). Moreover, mitochondria function decay is considered a hallmark of aging, which is evolutionary conserved, from yeast to humans (Baccolo et al., 2018). In yeast, mitochondria physiology and integrity is regulated to a large extent by their interaction with vacuoles, endosomes and the endoplasmic reticulum (ER) (Soto-Heredero et al., 2017). Noteworthy, among our hits (inductors and inhibitors) we found several key genes that code for proteins that are related to mitochondrial dynamics, morphology and functions. Some of them, moreover, are required for or regulated by interorganelle communication between mitochondria and organelles from the endocytic pathway. This was interesting since mitochondria has been proposed to be one of the main targets of metformin through a regulation of its respiratory activity (El-Mir et al., 2000), which has been suggested as fundamental for the drug's longevity effect (Barzilai et al., 2016). Vps13, a gene here identified as inhibitor, is a member of a tethering complex by which direct physical contacts are stablished between mitochondria and vacuoles. This interaction provides a route for transport of lipids from the ER to the mitochondria, and its critical when ER-mitochondria contact sites are lost. Also, among the inductors we found genes (Crd1 and Mdm35) that are crucial for the synthesis of lipids (cardiolipin and that have a role in the organization of mitochondrial inner membrane domains (Osman et al., 2011). Cardiolipine and phosphoethanolamine are mitochondrial lipids that are proposed to participate in mitochondrial fission by setting spatial marks that determine the placement of division sites (Friedman & Nunnari, 2014). Interestingly, interactions between mitochondria and ER, endosomes and lysosomes are associated with mitochondrial division and division site placement via different mechanisms (Todkar et al., 2019). Also, interorganellar communication

have reported to have an effect in the opposite direction. In yeast, altered cardiolipin synthesis in mitochondria causes vacuolar damage, which can affect cell survival. Thus, this network of organelle communication supports the hypothesis of the endocytic pathway as being at the core of metformin effects that regulate longevity.

5.4 N-glycosylation deletions protect against metformin high-dosage toxicity

Also, we show here that protein glycosylation gene-deletions cause an enhanced response to metformin. Protein glycosylation, specifically N-glycosylation, is a protein modification process that takes place in the endoplasmic reticulum (ER) and consists in the attachment of an oligosaccharide, referred as glycan, to the amide nitrogen of an asparagine (Asn) residue of a protein. N-glycans play an important role in folding and quality control of newly synthetized glycoproteins (Molinari, 2007). They are also required for the distribution of many proteins to the apical membrane of polarized cells (Vagin et al., 2009) and to the lysosome (Moharir et al., 2013). Furthermore, accurate and fine-tuned N-glycosylation is crucial for the activity and correct sorting and retention of proteins in Golgi and ER (Y. S. Liu et al., 2018; Nagai et al., 1997). Metformin modulation of protein-glycosylation proteins in *S. cerevisiae* has been reported (Stynen et al., 2018). Nevertheless, we observed that single-gene deletion of N-glycosylation pathway genes (Alg3 and Alg8) decreases its sensitivity against cytotoxic effects of high metformin doses (>80mM). This suggests that deletion of N-glycosylation genes may offset a secondary toxic effect of the drug. Also, this pathway represents a potential therapeutic target, to induce its inhibition in combination with metformin and, as a result, decrease cytotoxic side effects that are evident at high doses. Sensitivity due to a high metformin-dosage was shown to be partially mediated by the activity of an endosomal-resident Na^+/H^+ exchanger, a protein that mediates the efflux of H^+ from the lumen, as reported in *C. elegans* (CeNHX-5) and *D. melanogaster* (DmNHE3) (Kim et al., 2016). Deletion of this Na^+/H^+ exchanger (NHE) partially alleviated detrimental effects elicited by a 100 mM metformin-dosage, which is toxic in the WT of both organisms (Kim et al., 2016). This protein, as well as its function and localization in membranes from the endocytic pathway, is conserved from yeast to mammals. Furthermore, from studies with *S. cerevisiae* (Nhx1) and mammalian (NHE6) orthologs, it was shown that it contributes to the fine-tuning of the luminal pH (Brett et al., 2005; Prasad & Rao, 2015)

This, as a result, regulates the rate of the endocytic cycle (Brett et al., 2005). Interestingly, the mature form of Nhx1 protein, as well as NHE6, is highly N-glycosylated (Miyazaki et al., 2001; Wells & Rao, 2001) and perhaps this post-translational modification might be important for its function. Thus, deletion of N-glycosylation genes could result in a functional and localization impairment of Nhx1, which might explain in part why deletion of genes from this pathway protects yeast against metformin's high-dosage cytotoxic effects. However, the physiological role for the glycosylation of this NHE protein is not known. Also, even though a deletion-strain for Nhx1 gene was considered in the study, it didn't display an inductor's nor inhibitor's phenotype, under our selection criteria. Validation of this result with a *de novo*-generated deletion-strain for this gene is essential to reduce the probability of a false-negative result.

6. Perspectives

The pharmacogenomics study here exposed provided us with a description of the conserved genes between human and yeast, and its associated cellular mechanisms, that are relevant for the longevity effect of metformin. It also paved the way to formulate hypotheses related to testing the relevance of the endocytic transport in the lifespan-extension effect of the drug, and the protective capacity of glycosylation genes' inhibition. To this end, it is important to validate our hits considering biological replicates, lower metformin concentrations and physiologically relevant oxygenation regimes. In this regard, an approach based on differential staining of dead and alive cells coupled to flow-cytometry analysis is adequate, since it allows aeration of yeast cultures in a medium-throughput manner.

Here, we demonstrated that the endosomes-mediated intracellular transport is required for the effect of metformin, since deletion of some genes that regulate this process abolishes lifespan-extension. Also, we observed that metformin alleviates detrimental phenotypes that result from an endosomal trafficking impairment. Moreover, deletion of genes involved in protein glycosylation result in an improvement in the longevity effect elicited by metformin. Since protein glycosylation represents a crucial step for an accurate protein sorting through endosomal traffic and both processes are relevant for the lifespan-extension effect of metformin, it is interesting to explore how they interact in combination with the drug. To this end, we could generate yeast double-deletion strains for different combinations of genes involved in these two processes and study their phenotype under a metformin treatment. This, on the one hand, would give us a better understanding of the cellular mechanism underlying the longevity effect of metformin in *S. cerevisiae*. On the other hand, this information would be valuable for the design of metformin-derivates that elicit a better cellular response.

Finally, we could study the effect of metformin in KO mice with for genes that participate in the endosomal trafficking and protein glycosylation, to validate the involvement of these processes in the longevity effect of the drug. This will allow us to confirm the participation of these processes in the longevity mechanisms of metformin and to know

whether these targets and processes are relevant for drug design and clinical applications.

7. Main findings

- The optimum concentration of metformin to extend lifespan in *S. cerevisiae* is 40 mM, under a low-aeration, large-scale condition; while at a full aeration condition, the optimum concentration is higher (100 mM). These concentrations are proposed to induce similar intracellular effects than a clinically relevant micromolar-range metformin concentration.
- The required human orthologous genes to induce an efficient lifespan extension by metformin in yeast are related to protein modification, localization and the TCA cycle. On the other hand, metformin induces an intracellular effect that compensates for deficiencies in endocytic transport-related processes. This modulation could improve endosomal maturation and trafficking.
- Metformin more efficiently increases relative survival in protein glycosylation gene-deletion strains. These deletions could perhaps be compensating for the toxic secondary effect of the drug that is evident at high concentrations.

8. Appendices

Appendix 1. Inhibitors. Genes whose deletion results in an increase in survival by metformin, above a 95% prediction interval

Gene	Met S	No Met S	Deviation	Biological process
VPS28	-0.13166	-0.37015	0.058881	Ubiquitin-dependent protein sorting to vacuole
VPS24	-0.12199	-0.34447	0.055393	Late endosome to vacuole transport
GUP1	-0.01761	-0.13507	0.05252	Misfolded protein quality control
VPS5	-0.00578	-0.10425	0.048569	Endosome-Golgi retrograde protein transport
AFG3	-0.02102	-0.12563	0.044268	Assembly of mitochondrial enzyme complexes; degradation of misfolded proteins
PEP1	-0.00188	-0.08208	0.041111	Protein sorting to vacuole
VPH1	0.068499	0.057106	0.040196	Complex assembly; vacuolar acidification
UBI4	-0.05585	-0.18553	0.040121	Protein degradation via the ubiquitin-26S proteasome system
ZRC1	0.048304	0.030676	0.033538	zinc transport from cytosol to vacuole
VPS35	-0.02958	-0.1208	0.033246	Endosome-Golgi retrograde protein transport
CYS3	0.05118	0.040009	0.031634	Cysteine biosynthesis
MGM1	-0.02337	-0.10454	0.031119	Mitochondrial morphology and fusion
LCB5	0.022766	-0.01383	0.030796	Synthesis of long-chain base phosphates
VPS30	-0.06042	-0.17605	0.0307	Vacuolar protein sorting; autophagy
RPL2b	0.042598	0.026764	0.029836	Ribosomal 60S subunit; translation
IDH1	0.048193	0.041037	0.028121	Subunit of isocitrate dehydrogenase; TCA
PEP8	-0.01548	-0.08293	0.027942	Endosome-Golgi retrograde protein transport
FIS1	-0.01993	-0.09146	0.027862	Mitochondria fission; peroxisome abundanse
SPE2	0.048038	0.041488	0.027735	Biosynthesis of spermidine and spermine
SSK2	0.030545	0.007623	0.027587	MAPK pathways
ALG8	0.004245	-0.04228	0.026848	Protein N-glycosilation
VPS41	-0.05731	-0.15966	0.025414	Late endosome to vacuole transport
CWH43	0.015923	-0.01608	0.025107	GPI biosynthetic process
TMS1	0.018115	-0.01146	0.024932	Unknown
IDH2	0.040379	0.034598	0.023604	Subunit isocitrate dehydrogenase; TCA
PMT2	0.015624	-0.01308	0.023268	ER quality control
ALG3	0.011721	-0.02069	0.023264	N-linked glycosylation
VPS13	-0.02514	-0.0923	0.023086	Late endosome to vacuole transport
SAC1	-0.01461	-0.07148	0.02295	Sphingolipid biosynthesis; protein trafficking and processing
OST3	-0.0032	-0.04883	0.022761	N-linked glycosylation
DIE2	0.003146	-0.03623	0.02265	N-linked glycosylation
SWI3	-0.03007	-0.10093	0.022571	Chromatin remodelling; transcription
RPL34a	0.028424	0.013784	0.022311	Ribosomal 60S subunit; translation
DID2	-0.02979	-0.0995	0.022121	Late endosome to vacuole transport
YPK9	-0.0045	-0.04903	0.021558	Transmembrane transport

LAS21	-0.03463	-0.10654	0.020884	Glycosylphosphatidylinositol biosynthesis
ALG5	0.025451	0.011009	0.020759	N-linked glycosylation
GLN3	0.038573	0.037141	0.020496	Transcriptional regulation
KSS1	0.024975	0.010697	0.020443	MAPK pathways
LSM7	0.041869	0.044036	0.02026	mRNA decay, non-coding RNA processing

Appendix 2. Inductors. Genes whose deletion results in a decrease in survival by metformin, bellow a 95% prediction interval

Gene	Met S	No Met S	Deviation	Biological processes
SPT3	-0.12748	-0.0984	-0.07613	Histone acetylation
FUM1	-0.1759	-0.20869	-0.06806	Fumarase; tricarboxylic acid cycle (TCA)
MSD1	-0.10039	-0.08632	-0.05522	Mitochondrial aspartyl-tRNA aminoacylation
GOS1	-0.0489	-0.00178	-0.04704	V-SNARE protein, Golgi transport
MSF1	-0.08749	-0.0782	-0.04649	Mitochondrial phenylalanyl-tRNA synthetase
PRE9	-0.05506	-0.02042	-0.04365	Alpha 3 subunit of the 20S proteasome
TPK1	-0.02359	0.030641	-0.03833	PKA pathway
MDM35	-0.05697	-0.0408	-0.03512	Mitochondrial respiratory chain complex assembly
LSC1	-0.0743	-0.07551	-0.03468	Alpha subunit of succinyl-CoA ligase; TCA
STE20	0.005112	0.079445	-0.03463	MAPK pathways
MON1	-0.04258	-0.0138	-0.03457	Protein targeting to vacuole, autophagy
TPK2	-0.02926	0.011524	-0.03421	PKA pathway
SIN3	-0.07332	-0.07778	-0.03254	Histone deacetylation; transcription regulation; DNA repair
HAP5	-0.09155	-0.1134	-0.03252	Respiration
RIM11	-0.03636	-0.00601	-0.03233	Signal transduction during entry into meiosis
TRM10	-0.04926	-0.03217	-0.03184	tRNA methylation; translation regulation
MDH1	-0.06534	-0.06506	-0.03107	Mitochondrial malate dehydrogenase; TCA.
SAK1	-0.09613	-0.12578	-0.03075	AMPK pathway
RAD6	-0.06423	-0.06546	-0.02975	Postreplication repair; proteosomal degradation; response to unfolded protein
LSC2	-0.05543	-0.04984	-0.02896	Beta subunit of succinyl-CoA ligase; TCA
EAF3	-0.05848	-0.05752	-0.02807	Histone acetylation; transcription regulation; DNA repair
MAK3	-0.05878	-0.0583	-0.02797	Targeting of substrates to the Golgi apparatus
NIP100	-0.09149	-0.12295	-0.02757	Cell division
PMS1	-0.06693	-0.07514	-0.02749	Mismatch repair; mitosis and meiosis
SSO2	-0.06244	-0.06642	-0.02747	Fusion of vesicles at the plasma membrane
HNT2	-0.0572	-0.05661	-0.02726	Nucleoside catabolism
AKL1	-0.05056	-0.04373	-0.02721	Endocytosis regulation
SUB1	-0.04142	-0.02605	-0.02714	Transcription regulation
MTG1	-0.06956	-0.08348	-0.02585	Essential for respiratory competence
CTK1	-0.04277	-0.0313	-0.02579	Transcription and translation regulation
OCT1	-0.08369	-0.11422	-0.02424	Mitochondrial protein processing

OTU2	-0.0052	0.037838	-0.02363	DNA damage response
TIF4631	-0.06051	-0.07016	-0.02363	Translation initiation; ribosomal large subunit biogenesis
MRS2	-0.04365	-0.03833	-0.02307	Mitochondrial Mg(2+) maintenance
MXR2	-0.02741	-0.00787	-0.02243	Cellular response to oxidative stress
BCS1	-0.08335	-0.11838	-0.02177	Complex III assembly
AVL9	-0.03455	-0.02405	-0.02129	Post-Golgi vesicle-mediated transport
COQ5	-0.06896	-0.09265	-0.02056	Ubiquinone (Coenzyme Q) biosynthesis
CRD1	-0.08471	-0.1237	-0.0204	Mitochondrial morphology and function

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