Genome-Wide Mechanisms of Lifespan Extension by Dietary Restriction in Yeast

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ABSTRACT

Dietary restriction is arguably the most promising non-pharmacological intervention to extend human life and health span. Yet, only few genetic regulators mediating the cellular response to dietary restriction are known, and the question remains which other transcription factors and regulatory pathways are involved. To gain a comprehensive view of how lifespan extension under dietary restriction is elicited, we compared the chronological lifespan of most gene deletions of *Saccharomyces cerevisiae* between cells aged under restricted and non-restricted conditions. We identified 472 mutants with enhanced or diminished extension of lifespan relative to the WT. Functional analyses of such DR-genes revealed novel processes underlying lifespan extension specifically by dietary restriction. Importantly, our set of DR-genes allowed us to generate a prioritized catalogue of transcription factors, underscoring the relevance of cell-cycle control as a mechanism of chronological-lifespan extension in yeast. In particular, we show that the transcription factor Ste12 is needed for full lifespan extension and cell-cycle arrest in response to nutrient limitation, linking the pheromone-response pathway with cell survivorship. Our global picture of the genetic players of lifespan extension by dietary restriction highlights intricate regulatory cross-talks in aging cells.

INTRODUCTION

Dietary restriction—a reduction in calorie intake without malnutrition, or substitution of the preferred carbon or nitrogen source—extends lifespan in virtually all species studied in the laboratory, from yeast to primates [1–5]. Dietary restriction has been associated with protection against age-associated disease in mice, including neurodegenerative disorders [6] and cancer [7,8], promoting not only a longer lifespan but also healthier aging [9]. Importantly, this intervention reduces the mortality rate in non-human primates [10,11]. Furthermore, dietary restriction delays the onset of aging-related physiological changes in humans [12,13], making dietary restriction the most promising intervention targeted to extend human lifespan. Yet, we are still missing a global picture of the genetic architecture of such lifespan response, which is needed to grant a deeper understanding of the genotype-phenotype relationship of aging and longevity [14].

The budding yeast *Saccharomyces cerevisiae* has been a pivotal model organism in the discovery of the molecular and cellular bases of aging. Two aging models are widely used in this unicellular organism: The replicative lifespan of yeast, which refers to the number of times a single yeast cell can divide, and the chronological lifespan (CLS), which is a measure of the viability of a population during stationary phase throughout time. The latter provides a good model for aging of post-mitotic cells [15,16].

In yeast, there is evidence that dietary restriction results in lifespan extension at least through the modulation of the conserved TOR and Ras/cAMP/PKA pathways,

which regulate cellular growth and maintenance in response to nutrient availability [17,18]. Depletion of Tor1 and Sch9, both components within these pathways, results in CLS extension [4]. Under low nutrient conditions, the serine/threonine kinase Rim15 activates at least transcription factors Msn2, Msn4, or Gis1, activating a maintenance response [19]. However, the *msn2*Δ*msn4*Δ*gis1*Δ triple mutant still shows lifespan extension by DR [20]. Moreover, transcriptomic evidence and database analysis suggest that a larger number of up- and downstream genes are involved in lifespan extension [21,22]; most of these candidates still lack direct phenotypical confirmation. These observations suggest that there is yet to be identified an unknown number of regulators mediating lifespan extension by dietary restriction.

Research on aging has recently taken advantage of genome-wide approaches, enabling a comprehensive description of genes involved in lifespan regulation. For instance, a systematic study of replicative lifespan of most viable deletion strains of yeast revealed biological processes mediating longevity, such as translation, the SAGA complex, and the TCA cycle [23]. In the yeast CLS model, several studies have aimed to estimate in parallel the stationary-phase survival of single-deletion mutants [4,24–27], showing that autophagy, vacuolar protein sorting, regulation of translation, purine metabolism, chromatin remodeling, and the SWR1 complex are major determinants of stationary-phase survival.

The aim of this work was to generate a global picture of the underlying genetics of lifespan extension by dietary restriction. To this end, we compared the CLS of a collection of 3,718 yeast knockout mutants aged under non-restricted and dietary-

restricted media, using a high-resolution parallel phenotyping assay [27]. The sensitivity of our approach to test gene-environment interactions revealed 472 genes that influence the lifespan response to dietary restriction. Subsequent analyses uncovered the major biological processes and a comprehensive catalogue of transcription factors that regulate lifespan extension. In particular, we revealed a link between the pheromone-responsive Ste12 transcription factor and lifespan regulation, suggesting that Ste12-mediated cell-cycle control is a mechanism of chronological lifespan extension in response to nutrient limitation.

RESULTS

Systematic identification of dietary-restriction genes in yeast

In yeast, extended lifespan by dietary restriction is achieved either by limiting the concentration of glucose in the growth medium [28], by amino-acid unbalance [29] or by using a non-preferred source of nitrogen [4]. In this study, the non-restricted medium contained the preferred nitrogen source glutamine, while the restricted medium contained GABA as the sole source of nitrogen [30,31] (see Materials and Methods). Under these conditions, the half-life of the WT strain increased from 20.9 ± 0.4 days to 33.7 ± 1.3 days under non-restricted and restricted conditions, respectively, which represents a 61% extension of the CLS (**Figure 1A**).

To identify the genetic determinants of lifespan extension by dietary restriction at the genome-wide level, we measured the CLS of 3,718 gene-knockout strains

labeled with fluorescent proteins. We used a high-resolution assay based on the measurement of the survival coefficient (*s*) of each knockout strain aged in co-culture with the WT strain [27] under restricted or non-restricted conditions (**Figure 1B**). The quantitative nature of our experimental data allowed us to determine whether the gene deletions had a neutral, deleterious, or beneficial effect on the CLS (**Figure S1**; **Table S1**). We scored 573 significantly short-lived and 254 long-lived single knockout strains in the non-restricted medium (FDR<5%), while dietary restriction resulted in 510 significantly short-lived and 228 long-lived strains.

To validate our large-scale screen, we phenotyped some of the knockout strains among the top hits of (FDR<1%) by using a conventional single-culture CLS assay [32] under both conditions. Twelve out of 16 (75%) strains retested under non-restricted medium recapitulated the CLS effects observed in the genome-wide screen (**Figure S2**; $p<0.05$, *T*-test), while 11 out of 17 (65%) strains tested were consistent with the results of the large-scale screen under dietary restriction (**Figure S3**). This observed rate of false-positive hits is lower than CLS assays that use pooled deletion strains [24,25]. These results show that our profiling approach provides the most accurate CLS score for deletion strains characterized under different dietary conditions.

To gain insight into the genes and cellular processes that mediate the lifespan-extending effects of dietary restriction, we searched for deletion strains that showed differential relative CLS effects when comparing the two dietary conditions (**Figure 1C**). For each gene knockout, we defined a relative lifespan extension, LE, which indicated the degree of lifespan extension by dietary restriction of the mutant

relative to that of the WT, defined as $LE = \ln\left(\frac{s_{DR}}{s_{NR}}\right) + 1$ (see Materials and Methods). The LE of each deletion strain was compared to the LE distribution of 264 independent WT replicates to obtain a *Z*-score for each gene deletion (**Figure 1C**). For higher stringency, we filtered out strains that did not show a CLS effect in either condition (as described in the previous section), and we obtained a list of 472 gene-knockouts with altered dietary-restriction response (FDR<5%; **Table S2**). This comprehensive set, which we termed DR-genes, includes knockouts in which lifespan extension is diminished compared to the WT (LE<1), which suggests that such DR-genes are needed to extend lifespan in response to dietary restriction. This catalogue also includes gene-knockouts that displayed enhanced lifespan extension (LE>1); the majority of these DR-genes had a short lifespan phenotype under non-restricted conditions that was alleviated by dietary restriction. Together, these results show that many gene-environment interactions underlie the lifespan-extension phenotype in yeast.

Functional classification of dietary-restriction genes

To describe which downstream cellular functions influence lifespan extension by dietary restriction, we clustered the 472 DR-genes according to their annotated functional features. We used a kappa statistic approach [33] to group genes by shared GO terms and mutant phenotypes, as reported in the Saccharomyces Genome Database (see Materials and Methods). The analysis was performed separately for genes with diminished (LE<1) or enhanced (LE>1) lifespan. Some of the functional clusters of genes with altered lifespan extension recapitulated most

cellular functions previously related to lifespan regulation (**Figure 2**; **Table S3**), such as autophagy [34–36], respiration and mitochondrial function [37,38], peroxisome biogenesis [39,40], and cytosolic translation [41,42]. This observation shows that our screen was able to independently identify the processes that were previously known to determine CLS and its regulation by the diet regime.

Deletion of genes necessary for cell-cycle arrest resulted in a diminished lifespan extension. Specifically, deletion of genes of the pheromone-responsive FAR complex had short-lived phenotype under DR (**Figure 2;** cluster III). In yeast, the FAR proteins act as inhibitors of the G1-cyclin Cln2, which promotes the G1 to S transition [43,44]. Moreover, mutations in genes for processing and correct localization of different ribonucleoprotein complexes resulted in a strongly diminished lifespan extension (cluster I). While it is well established that ribosomal function is downregulated in response to TOR inhibition or nutrient depletion [41,45], our screen pointed to specific proteins involved in pre-rRNA processing (Slx9), nucleolar rRNA methyltransferase (Rrp8), nuclear export of pre-ribosomal subunits (Arx1), and translational initiation (Bud27). The identification of these DR-genes sheds light on the mechanisms by which dietary restriction regulates ribosomal function. Likewise, deletion of genes involved in nuclear movement along microtubules, such as *DYN1-3*, *JNM1*, *NUM1*, and *PAC1* (cluster X), resulted in diminished lifespan extension, suggesting that microtubule dynamics underlies dietary restriction. In this regard, it is well known that certain cellular processes needed for extended longevity, such as autophagy, require intact function of microtubules [46].

On the other hand, we found clusters of genes in which the knockout strain typically showed enhanced lifespan extension compared to the WT, such as genes with mitochondrial function (**Figure 2**; cluster XV). While dietary restriction shifts the metabolism towards respiration [47], impaired respiration promotes longevity in yeast and nematodes through enhanced retrograde response and activation of anaplerotic pathways needed for lifespan extension by dietary restriction [48,49]. Hence, a higher demand for respiration during dietary restriction could lead to the activation of compensatory pathways; similar feedback mechanisms might account for the alleviation of deleterious effects observed in other deletion strains. For example, the short-lived phenotypes of cell-wall proteins *CWP1, DSE2,* and *TIR3* (cluster XVII) were alleviated under restricted conditions compared to a non-restricted diet. Taken together, these findings suggest that lifespan extension in response to dietary restriction in yeast is a complex phenotype resulting from the interplay of many downstream cellular processes.

A defined set of transcription factors regulate lifespan extension by dietary restriction

Complex phenotypic responses are frequently coordinated by transcriptional regulation, which elicits changes in the expression of a large number functionally-related genes. To investigate the transcriptional regulation of lifespan extension by dietary restriction, we analyzed our set of DR-genes using an algorithm to search for the transcriptional regulators of these genes. In particular, we used TFrank [50], a graph-based approach that takes advantage of available interactions of transcription factors and their targets, to obtain a list of prioritized regulatory

players within the yeast regulatory network (**Table 1**). This approach also allowed us to assess the possible lifespan role of transcription factors that were not included in our genome-wide screen because the deletion strain was unviable or sterile. Transcription factors within the top 5% rank of this analysis included Msn2 and Msn4, as expected; the regulatory role of these two proteins in lifespan and stress response is well established [20,51]. However, many of the transcription factors with high priority had not been previously associated to lifespan extension by dietary restriction in yeast. Noteworthy, the top hits are known to regulate different aspects of cell-cycle progression (e.g. Ace2, Ash1, Tec1, Spf1, and Ste12). We also identified Gcn4, which plays a regulatory role in the replicative lifespan of yeast and is required for full lifespan extension by DR, depletion of ribosomal 60s subunits, or deletion of *TOR1* [52,53]. Bas1—a positive regulator of purine biosythesis, which underlies CLS in yeast [24,27]—was another highly-ranked transcription factor.

Indeed, depletion of some of the highly-ranked transcription factors showed a significant CLS phenotype in our large-scale screen (Ash1, Tec1, Bas1, Snf6, Msn2, and Ixr1; **Table S1**). Nevertheless, we decided to directly interrogate the lifespan role of top-ranked transcription factors, given that many of the genes were not part of our screen and that some strains from the deletion collection carry unlinked mutations [54]. Specifically, we generated *de novo* deletion mutants for seven transcription factors and characterized their CLS under non-restricted and restricted conditions (**Figure 3**). In agreement with the *in silico* inference, deletions of six transcription factors tested had a short-lifespan phenotype under dietary

restriction, while *ash1*Δ was long-lived. Some of the deletion strains were also short-lived under non-restricted conditions, suggesting a general CLS role for those transcription factors (eg. Snf6, Msn2, and Msn4). Most of the observed lifespan effects were moderate, which is in agreement with the fact that transcription factors in yeast typically act on overlapping targets, providing functional compensation to one another [55,56]. These results show that high-TFrank hits are determinants of CLS, confirming that several transcription factors that control cell-cycle progression are required for lifespan extension by dietary restriction in yeast.

Ste12 is needed for full lifespan extension by dietary restriction and cell-cycle arrest in response to nutrient limitation

Among the top hits of our transcription-factor analysis was Ste12, which acts on downstream genes involved in mating or pseudohyphal growth [57–59]. One of our novel clusters of downstream DR-genes included the Ste12-regulated Far complex (**Figure 2**; cluster III), participating in G1 cell-cycle arrest in response to pheromone [60]. To confirm a role for Far7 and Far8 in lifespan extension by dietary restriction, we generated *de novo far7*Δ and *far8*Δ deletion strains and measured their lifespan under both dietary conditions (**Figure S4**). In agreement with our high-throughput screen, we observed that both deletion strains failed to fully extend lifespan under dietary restriction, while there was little to no effect under the non-restricted medium.

To further explore the regulatory role of Ste12 in lifespan extension, we characterized the CLS of the *ste12*Δ strain under a different form of dietary

restriction. In particular, we used synthetic-complete medium with 2% or 0.5% glucose. Lifespan extension was partially abrogated in the *ste12*Δ strain under glucose restriction and, to a lesser extent, under high concentration of glucose (**Figure 4A**). This result confirms a general role of Ste12 in lifespan response to either a non-preferred nitrogen source or the limitation of carbon.

Most methods for measuring post-mitotic survivorship of yeast cells rely on the ability of cells in stationary phase to re-enter the cell cycle upon transfer to fresh medium. Given that Ste12 is a transcription factor involved in cell-cycle arrest, we decided to rule out possible artifacts that could arise from deleting a regulator of the cell cycle. We thus used a more direct method to measure cell survivorship by differential staining of dead and alive cells coupled to flow-cytometry analysis. This experiment showed that, under limited glucose concentration, alive *ste12*Δ populations decayed faster than the WT (**Figure 4B**). The number of alive relative to dead cells confirmed the short-lived phenotype of *ste12*Δ strain under caloric restriction (**Figure S5**). These results indicate that the CLS-effects of the *STE12* deletion are maintained regardless of the methodology used to infer population survivorship in stationary phase.

Phenotypic analysis of the *ste12*Δ deletion strain suggested that this transcription factor participates in lifespan extension by dietary restriction. Still, a bona fide positive regulator of lifespan is expected increase cell survivorship when over-expressed, along its short-lifespan deletion phenotype. We thus investigated whether high *STE12* expression causes lifespan extension under regular non-

restricted conditions. To this end, we generated a copper-inducible *STE12* strain with a GFP fusion to track protein levels. The WT and *pCUP1-STE12* strains were aged under varying concentrations of copper sulfate in 2% glucose SC medium. We found that the CLS of the *STE12*-overexpression strain increased readily as a function of copper concentration (**Figure 4C**), while aging the WT at different copper concentrations had no significant effect on CLS or growth (data not shown). Importantly, the signal of the Ste12-GFP construct in the nucleus also increased as a function copper concentration, confirming that lifespan extension was associated to an increase of Ste12 levels in the nucleus (**Figure 4D**). Together, these results indicate that Ste12 is a novel positive transcriptional regulator of lifespan in yeast.

Finally, we asked whether Ste12-mediated cell-cycle control in response to nutrient limitation is a cellular mechanism of lifespan extension by dietary restriction in yeast. Transcriptional targets of Ste12 are known to mediate arrest of the cell-cycle in G1 phase in response to pheromone signal [57,58], but the role of such Ste12-mediated cell-cycle arrest in aging is unknown. To address this question, we used flow cytometry to monitor the cell-cycle dynamics of yeast populations following nitrogen starvation (**Figure 4E**). Our results show that *ste12*Δ cells failed to arrest the cell cycle at the same rate than the WT strain. This observation suggests that the transcription factor Ste12 integrates nutrient signaling and regulates downstream genes needed for cell-cycle arrest, which may underlie its beneficial effect on cell survivorship.

DISCUSSION

We have screened a collection of 3,718 yeast deletion strains for short and long-lived phenotypes under non-restricted and dietary-restriction conditions. Our quantitative experimental analysis uncovered 472 genes in which dietary restriction modifies the chronological lifespan (CLS) effect of the knockout. To the best of our knowledge, this study yields the most comprehensive phenotypic compendium of genetic players involved in lifespan extension by dietary restriction. Functional classification of such DR-genes pointed to the downstream cellular processes that are involved in this phenomenon, such as autophagy, respiration and mitochondrial function, peroxisome biogenesis, cytosolic translation, the cell-cycle arrest machinery, and genes involved in processing and correct localization of ribonucleoprotein complexes, nuclear movement along microtubules, and cell wall organization.

Our analysis of DR-genes as regulatory targets allowed the establishment of a set of ranked transcription factors mediating the response to dietary restriction (**Table 1**). Deletion of all tested regulators resulted in altered CLS phenotypes (**Figure 3**). Among the top hits were Msn2 and Msn4, two positive regulators of stress-response and lifespan extension which operate downstream of the Tor/Sch9/Ras-PKA pathways that converge on Rim15, the main protein kinase involved in cell survivorship in response to nutrients [3]. Strikingly, high-ranked transcription factors were all regulators of mitotic cell-cycle transitions by either repression of Cln3 specifically in yeast daughter cells (Ace2 and Ash1) [61,62], activation of ribosomal-protein genes and regulation of cell size (Sfp1) [63,64], or cell

differentiation in response to nutrients or pheromone (Tec1 and Ste12) [58]. This observation underscores the role of cell-cycle control as a central mechanism of chronological lifespan in yeast and provides a defined set of transcription factors that could act downstream of Rim15, which plays a key role in controlled cell-cycle arrest upon nutrient limitation [65,66].

We have shown that *STE12* is a positive regulator of lifespan extension by dietary restriction in yeast. Ste12 is a transcription factor downstream of two cell-differentiation programs regulated by MAPK pathways, namely mating and invasive growth [67,68]. We also found that deletion of *STE12* results in a failure to arrest the cell cycle upon nutrient starvation. In the presence of pheromone, the cell cycle is arrested by the action of Ste12, Far1, and the FAR complex (Far3 and Far7-11) [60]; Far1 and Far3 are direct targets of Ste12 [69]. Since efficient G1 arrest protects against replication stress during stationary phase, effectively extending CLS [66,70], we propose that cell-cycle control underlies the lifespan effect of Ste12 through control of the FAR proteins. Concomitantly, deletion of *FAR7*, *FAR8*, and *FAR11* in our screen resulted in diminished lifespan extension. It is thus likely that regulatory elements of the mating pathway are recruited to arrest the cell cycle in dietary restriction independently of the pheromone response, resulting in lifespan extension.

Ste12 is associated to Tec1 during pseudohyphal growth, but not during mating response [71]. Deletion of *TEC1* resulted in diminished lifespan extension by dietary restriction, which suggests that the Ste12 and Tec1 transcription factors could act in concert to promote lifespan in yeast. In addition to controlling cellular

development in response to stimuli, Tec1 is needed for full lifespan extension in response to the Tor1-inhibiting drug rapamycin [72], suggesting that Tec1 acts downstream of the TOR pathway. Also, Ste12 is a regulatory hub in response to rapamycin [73], further strengthening the idea that Ste12 and Tec1 link TOR and MAPK-signaling pathways. However, Tec1 promotes cell-cycle progression by activation of Cln1 [58], in conflict with the fact that the cell-cycle is arrested in response to nutrient limitation. It is thus likely that the players upstream Ste12 and Tec1 transcription factors are involved in an intricate signaling response that results in lifespan extension by dietary restriction.

It remains to be addressed whether the role of the identified transcription factors is conserved. For instance, while *STE12* has no clear homolog in animals, transcriptional networks are rewired through evolution, leading to changes in the regulation exerted by specific regulators, while groups of downstream targets remain associated [74,75]. In addition, the three-kinase module observed in the yeast MAPK pheromone and invasive-growth pathways are conserved in other organisms [76]. In particular, *KSS1* and *FUS3* are key members of the MAPK pathway that regulates cell differentiation programs in yeast [77], while their mammalian counterpart *MAPK1* has been reported to regulate cell-fate determination [78]. Also, the MAPK1/ERK pathway is central to the development of several age-associated diseases in mammals [79]. The study of targets downstream the MAPK pathway in yeast might bring important insights into the regulation of aging in other eukaryotes, including humans.

Our genome-wide screens in provide a comprehensive picture of the mechanisms of lifespan extension by dietary restriction, underscoring a link between the cell-cycle arrest machinery and longevity in yeast. Other cross-talks among DR-genes and their transcriptional regulators may remain to be uncovered, which will shed further light to the gene-environmental wiring of aging cells.

MATERIALS AND METHODS

Strains and media. Fluorescent single-gene deletion strains are prototrophic haploids (*MAT*a *xxx*Δ*::kanMX4 PDC1-mcherry-CaURA3MX4 can1*Δ*:STE2pr-SpHIS5 lyp1*Δ *his3*Δ*1 ura3*Δ*0 LEU2*) derived from crossing the *MAT*α YEG01-RFP SGA-starter with strains from the *MAT*a BY4741 deletion collection, as previously described [27]. All *de novo* single-gene deletions were generated in the YEG01-RFP parental strain (*MAT*α *PDC1-mcherry-CaURA3MX4 can1*Δ*:STE2pr-SpHIS5 lyp1*Δ *his3*Δ*1 ura3*Δ*0 LEU2*) by direct gene replacement with the *natMX4* module conferring resistance to clonNAT; double-knockouts were generated from such *de novo* single-gene deletions using the hygromycin-resistance *hphMX4* cassette. The Ste12 overexpression strain was generated by inserting the *CUP1* promoter and GFP-fusion construct from plasmid pYM-N4 in the 5' region of *STE12* ORF, as described [80].

Non-restricted (NR) aging medium contained 0.17% yeast nitrogen base (YNB) without amino acids and ammonium sulfate, 2% glucose, 0.07% amino acid

supplement mix (Cold Spring Harbor Laboratory Manual 2005), and 25 mM glutamine as nitrogen source. Dietary-restricted (DR) aging medium was prepared substituting glutamine with 25 mM of γ-amino butyric acid (GABA). The choice of a non-preferred nitrogen source for DR instead of limited glucose concentrations overcomes the dramatic metabolic changes due to glucose repression in yeast [81,82], while facilitating the parallel characterization of stationary-phase cultures in low volumes, given that yields are similar under NR and DR conditions. SC medium used for DR based on glucose concentration was 0.17% yeast nitrogen base (YNB) without amino acids, 0.5% or 2% glucose, and 0.2% amino acid supplement mix.

All outgrowth cultures were performed in low-fluorescence medium (YNB-lf), as previously reported [83]. Nitrogen-starvation medium for cell-cycle progression experiments was 2% glucose and 0.17% YNB without amino acids and ammonium sulfate.

Automated CLS screens and data analysis. Fresh cultures of the RFP-tagged gene-deletion collection were replicated in 96-well plates (Corning 3585) with 150 μl of NR or DR aging medium. Saturated cultures were mixed with a CFP-labeled WT reference strain in a 2RFP:1CFP ratio, replicated by pinning into 96-deepwell plates (Nunc 260251) containing 700 μl of NR or DR aging medium, and grown at 30°C and 70% relative humidity, without shaking, in an automated cell-assay system (Tecan Freedom EVO200) integrated to a multi-label plate reader (Tecan M1000). We have previously shown that the CLS effects of mutants aged under low aeration in low-volume cultures change minimally compared to highly-aerated

cultures [27]. Data acquisition and initial processing have been described. In brief, five days after inoculation, 5 μl outgrowth cultures were inoculated every other day into 150 μl of fresh low-fluorescence medium; absorbance at 600nm (OD_{600}) and fluorescence (*RFP* and *CFP*) measurements were taken every 150 min throughout 14 hrs. An apparent survival coefficient, *s*, and its standard error, σ_s, were obtained from the slope of the linear regression (Robustfit, Matlab) of the log of the ratio of *RFP* to *CFP* signal at a fixed interpolation time point in the outgrowth culture (10 hrs), and the number of days in stationary phase, as described [27].

Scoring CLS phenotypes and lifespan extension coefficients. Short- and long-lived knockouts under NR or DR were determined by assigning a *Z*-score to each mutant's *s* coefficient; the distribution's mean and standard deviation were from the measurement of 264 WT_{RFP}/WT_{CFP} independent co-cultures under either condition. Two-tailed *p*-values were obtained from each *Z*-score to compute a false-discovery rate (FDR); we assigned significant phenotypes using a $q<0.05$ cutoff.

The effect of dietary restriction on the gene-deletion strains relative to the WT was evaluated by calculating their relative lifespan extension defined as $LE = \ln\left(\frac{s_{DR}}{s_{NR}}\right) + 1$, where s_{NR} and s_{DR} are the *s* coefficients of a given deletion strain obtained from the screen under NR and DR, respectively. A *Z*-score was assigned to the LE of each mutant compared to the distribution of LE values of 264 independent WT reference experiments, and this was used to obtain an FDR, significant $LE<1$ and $LE>1$ values were assigned using a $q<0.05$ cutoff.

Survival curves based on the outgrowth of individual stationary-phase cultures. Selected strains were grown individually in NR or DR aging medium for 48 hours at 30°C 200 rpm in aerated tubes, then transferred to 96-well plates. This plates were replicated onto 96 deep-well plates containing 700 µl of NR or DR medium and left for the entire experiment at 30°C and 70% relative humidity without shaking. From here on, all experimental steps were performed in an automated robotic station (Tecan Freedom EVO200). After 4 days, 10 µl aliquots were taken with an automated 96-channel pipetting arm to inoculate 96-well plates containing 150 µl of low fluorescence medium. OD_{600} was obtained in a plate reader (Tecan M1000) every 1.5 hours until saturation was reached; this first outgrowth curve was regarded as the first time point (T_0, age = 0 days). Sampling was repeated every 2-3 days for 24-28 days. From these outgrowth curves, we extracted the doubling time and the time shift to reach mid-exponential phase (OD_{600}=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* [32].

Viability data points relative to T_0 were used to plot a survival curve, which was fitted 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. For validation of CLS effects, mutants were taken from the RFP-tagged deletion collection (or generated *de novo*, when indicated) and viability was assayed to calculate death rates in at least 7 experimental replicates, which were compared to replicates of a WT strain; significant CLS effects were considered using a p<0.05 cutoff (T-test).

Functional-cluster visualization. Gene Ontology (GO) associations and phenotype terms were downloaded from the *Saccharomyces* Genome Database (SGD, last updated December 2016) to build two *m* by *n* matrixes, where *m* is the number of DR genes (219 and 253 for LE<1 and LE<1, respectively) and *n* is the number of GO and phenotypic terms (1,748). Each term was used to evaluate the overall agreement between gene-pairs to calculate Cohen's *kappa* ($kappa = \frac{\mathrm{Pra}(a)-\mathrm{Pr}(e)}{1-\mathrm{Pr}\,(e)}$) for each gene pair, where *Pra*(*a*) is the relative observed agreement or the number of terms that a gene-pair shares divided by the total number of terms in the matrix, and *Pr*(*e*) is the probability of agreement by chance, calculated as the sum of probabilities for each member of the gene-pair to be associated or not to each term.

Kappa values were used to build a matrix that represented the agreement between each gene-pair. Gene-pairs that showed $kappa$>0.35 were regarded as likely similar and thus used as cluster seeds to form larger groups of genes; groups sharing more than 50% of their genes were merged in subsequent iterative steps. Clusters with at least four elements were manually named by inspection in the SGD and GO enrichment. Network representation was created using Cytoscape; edges between nodes represent kappa agreement above the established threshold ($kappa$>0.35).

Live/dead staining assay. We used the same scheme of 96 deep-well plates (one plate per replicate) to age cells in SC with 0.5% or 2% glucose. Each day, a single well of each strain was collected. Cells were centrifuged, washed, and dyed with LIVE/DEAD® FungaLigth™ Yeast Viability Kit, following manufacturer's

instructions. Propidium iodide (IP) and Syto®9 fluorescence were measured by cell cytometry (LSRFortessa™, Becton Dickinson) at early stationary phase (4 days after inoculation) and at different time-points until 21 days in stationary phase. IP was excited with a 591-nm laser, fluorescence was collected through a 586/15 band-pass filter; Syto9 was excited with a 488-nm laser, and fluorescence was collected through 525/50 band-pass and 505LP emission filters. Cell-viability percentage was obtained from the total amount of cells measured and subtracting the number of dead-cell events.

Cell-cycle cytometry experiments. WT and mutant strains were grown in flasks containing 50 ml of NR aging medium at 30°C and agitated at 200 rpm until mid-logarithmic phase ($OD_{600}\cong0.5$). Cells were centrifuged and washed twice with sterilized water, and transferred to nitrogen starvation medium. Samples were taken at the moment of transfer (time 0) and 1, 2 and 4 hours after this time point. Fixation and dying with SYTOX™ Green were performed as described by Haase et al [84]. Cells were analyzed by flow cytometry (FACSCalibur™, Becton Dickinson); SYTOX™ Green was excited with a 488-nm laser, and fluorescence was collected through a 525/50 band-pass filter.

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Table 1. A catalogue of transcription factors regulating DR genes in yeast (top 5% rank)

Rank	TF	Weight[a]	% Regulated[b]	Description
1	Ace2	3.15	75.5	Involved in G1/S transition of the mitotic cell cycle; activates cytokinetic cell separation
2	Ash1	2.94	50.4	Negatively regulates G1/S transition of mitotic cell cycle; activates pseudohyphal growth
3	Tec1	2.81	63.0	Transcription factor targeting pseudohyphal-growth genes and Ty1 expression
4	Sfp1	2.55	66.3	Regulates ribosomal-protein genes, response to nutrients and stress, and G2/M transitions of the mitotic cell cycle
5	Ste12	2.45	58.2	Activates genes involved in mating or pseudohyphal-growth pathways
6	Bas1	2.36	44.9	Involved in regulating the expression of genes of purine and histidine biosynthesis
7	Snf6	2.19	37.4	Subunit of the SWI/SNF chromatin remodeling complex
8	Msn2	2.10	54.3	Regulation of transcription in response to a wide variety of stresses
9	Yrm1	1.79	44.5	Transcription factor involved in multidrug resistance
10	Gcn4	1.76	46.7	Activator of amino acid biosynthetic genes; responds to amino acid starvation
11	Ixr1	1.73	26.4	Transcriptional repressor that regulates hypoxic genes during normoxia
12	Abf1	1.723	45.3	DNA binding protein with possible chromatin-reorganizing activity
13	Msn4	1.65	44.0	Regulation of transcription in response to a wide variety of stresses
14	Rap1	1.53	41.4	Essential DNA-binding transcription regulator; role in chromatin silencing and telomere length

a. TFRank weight [50].

b. Percentage of DR-genes regulated by the transcription factor.

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FIGURE LEGENDS

Figure 1. Genome-wide CLS assay revealed short- and long-lived single-knockout strains under non-restricted medium and dietary restriction. (**A**) Survival curves of the WT strain under non-restricted (NR, black) and dietary restriction medium (DR, green). Error bars are the S.E.M of at least 14 experimental replicates. (**B**) Schematic representation of the screening strategy; an RFP-labeled knockout strain ($x\Delta$) with no CLS effect under NR and a short-lived phenotype under DR is aged in co-culture with a CFP-labeled WT strain; a survival coefficient (s) is obtained for each condition. (**C**) Scatter plot of survival coefficients under NR (horizontal axis) and DR (vertical axis) of 3,718 deletion strains; data points above or below the diagonal are colored according to the lifespan extension (LE) significance (q) when compared to the WT replicates (color bar). The right panel shows the cumulative distribution of LE values for mutant strains (circles) and WT replicates (crosses).

Figure 2. DR-genes were clustered using a kappa statistic. Network representation of functional clusters of genes with LE<1 (left) and LE>1 (right). Edges denote agreement between pair of genes ($kappa$>0.35). Node color code is disposed increasingly by mean LE of the cluster. White nodes represent genes that paired only within small clusters (2-3 genes).

Figure 3. Deletion of transcription factors genes that regulate DR-genes has an effect on CLS. Survival curves of gene deletions and WT strains aged under non-restricted medium (NR, solid black line) or dietary restriction (DR, solid green line). Deletion strains (black and green discontinuous lines, for NR and DR, respectively) include genes coding for transcription factors Ace2 **(A)**, Ash1 **(B)**, Tec1 **(C)**, Ste12 **(D)**, Snf6 **(E)**, Msn2 **(F)**, and Msn4 **(G)**. Error bars are the S.E.M. (n=7).

Figure 4. Ste12 is a positive regulator of lifespan extension by DR. (**A**) Survival curves of WT and *ste12*Δ strains aged in 0.5% or 2% glucose. Error bars represent S.E.M. (*n*=7). (**B**) Contour plots showing WT and *ste12*Δ populations of dead and live cells aged under SC 0.5% glucose; fluorescence of Syto9 (alive cells) and propidium iodide (PI, dead cells) is shown in the vertical and horizontal axes, respectively, across time in stationary phase. (**C**) Survival curves of the WT, *ste12*Δ, and *pCUP1::GFP-STE12* overexpression strains aged in SC 2% glucose in non-induced conditions (orange) or induced with 2μM (+), 5μM (++), or 15 μM (+++) copper sulfate. Error bars are the S.E.M. (*n*=7). (**D**) Micrographs of WT and *pCUP1::GFP-STE12* strains with GFP, mCherry and merge filters, showing induction with varying copper sulfate concentrations. **(E)** DNA content labeled with SYTOX Green and measured by flow cytometry; fluorescence of DNA *vs.* normalized cell count is shown for WT and *ste12*Δ strains challenged with a nitrogen depletion shock for 0, 1, 2, or 4 hours. Numbers indicate percentage of cells in the G1 cell-cycle phase.

SUPPORTING INFORMATION

Figure S1. CLS effects from two genome-wide screens. (**A**) Histograms of the normalized counts for 3,719 single-knockout strains under non-restricted medium (NR, black) and dietary restriction (DR, green); WT controls (264) aged under NR (gray line) and DR (light green line) are shown. A distribution of the *s* coefficients of the WT-strain replicates was used to calculate a *Z*-score for each knockout strain and to define short-lived and long-lived strains (see Materials and Methods). Insets show the fraction of short-lived (magenta) and long-lived (purple) deletion strains scored in each screen. (**B**) Venn diagrams show the common number of short- and long-lived knockout strains scored under non-restricted medium (NR, dark gray) and dietary restriction (DR, green).

Figure S2. Validation of hits from genome-wide screen under non-restricted medium. (**A**) The relative death rate of WT and mutant strains, $-1 \bullet ln(r_x/r_{WT})$, aged under non-restricted medium (Glutamine) are shown. Death rates were calculated from the fit of each survival curve to an exponential decay model. A CLS phenotype from the genome-wide screen was considered to be validated when the death rate of the mutant was consistent with the screen (short- or long-lived) and significantly different from that of the WT (p<0.05, two-tailed T-test). **(B)** Survival curves of mutant (blue or green lines) and WT (black lines) strains aged under non-restricted medium; only validated strains are shown along with WT for each experimental batch. Error bars are the S.E.M. (n=7).

Figure S3. Validation of hits from genome-wide screen under dietary restriction. (**A**) The relative death rate of WT and mutant strains, $-1 \bullet ln(r_x/r_{WT})$, aged under dietary restriction (GABA) are shown. Death rates were calculated from the fit of each survival curve to an exponential decay model. A CLS phenotype from the genome-wide screen was considered to be validated when the death rate of the mutant was consistent with the screen (short- or long-lived) and significantly different from that of the WT (p<0.05, two-tailed T-test). **(B)** Survival curves of mutant (blue or green lines) and WT (black lines) strains aged under non-restricted medium; only validated strains are shown along with WT for each experimental batch. The time point of maximum viability (typically time zero) was considered 100% viability. Error bars are the S.E.M. (n=7).

Figure S4. Genes of the FAR complex are required for full lifespan extension by dietary restriction. Survival curves of WT (solid lines) and mutant strains *far7*Δ and *far8*Δ (discontinuous lines) aged under non-restricted medium (NR, black) or dietary restriction (DR, green). Error bars are the S.E.M. (n=7).

Figure S5. *STE12* deletion displays diminished lifespan extension measured by alive/dead characterization. Fraction of alive/dead cells in populations of WT (solid lines) or *ste12*Δ (discontinuous lines) strains aged under 2% (black) or 0.5%

(green) glucose. Alive cell percentage was calculated from dividing the whole cell population in stationary phase cultures by the cells stained with Syto9 only, through time. Error bars are the S.E.M. (*n*=7).

Table S1. Genome-wide CLS screens, complete data set (XLS).

Table S2. Data set of 472 DR genes identified in this study (XLS).

Table S3. Functional clusters of DR genes with diminished or enhanced relative lifespan extension (XLS).

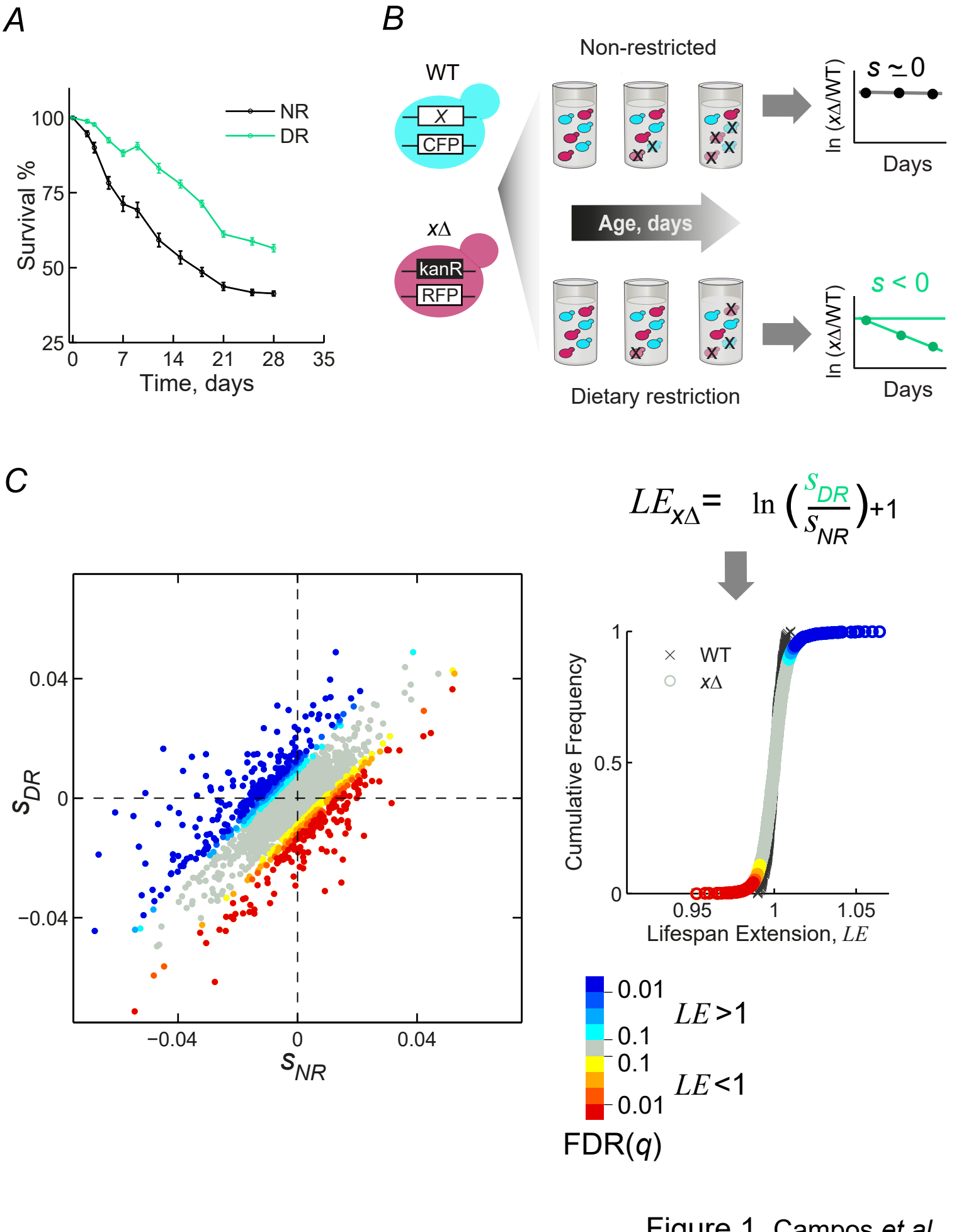


Figure 1_Campos *et al*.

A

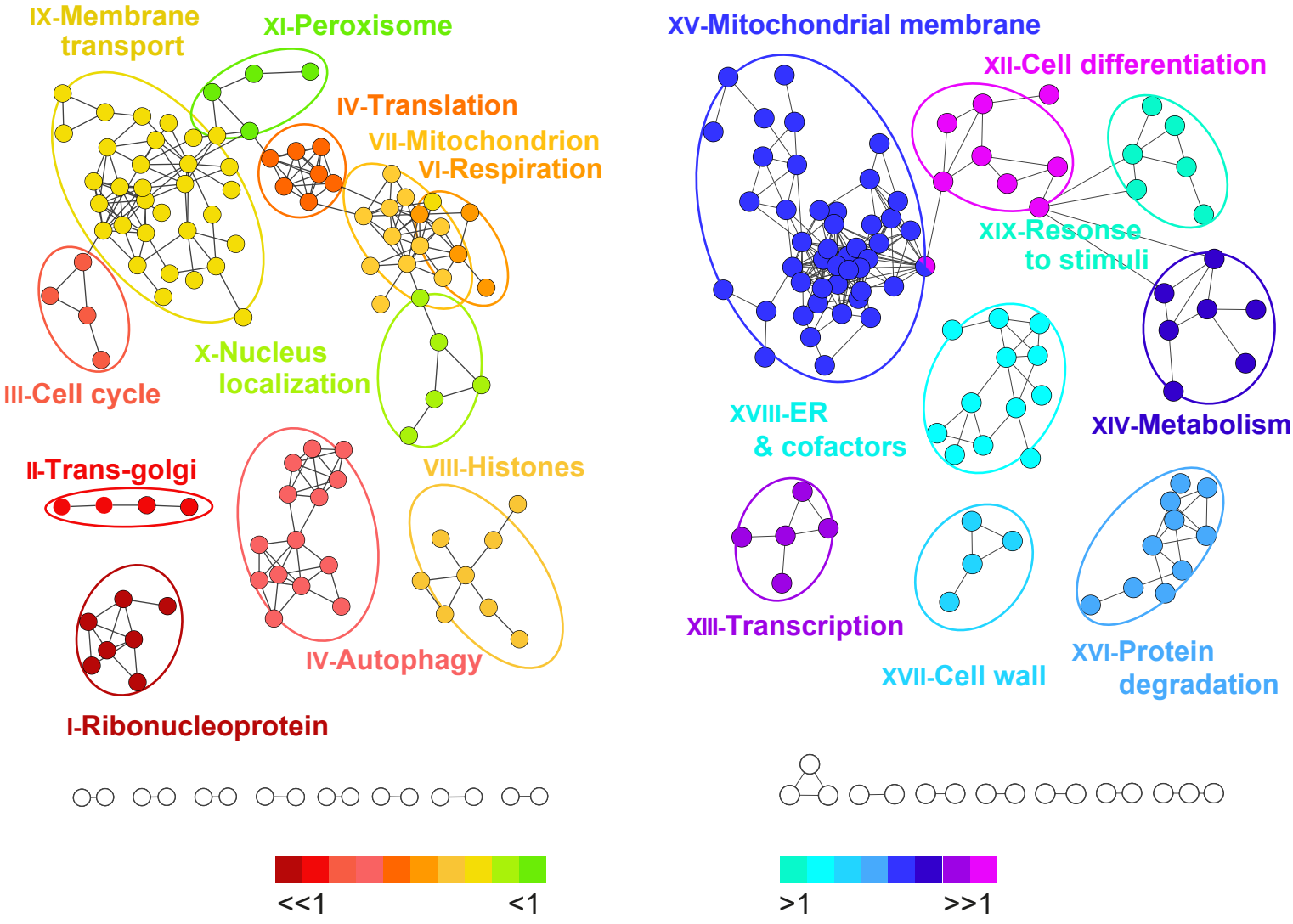


Figure 2_Campos *et al.*

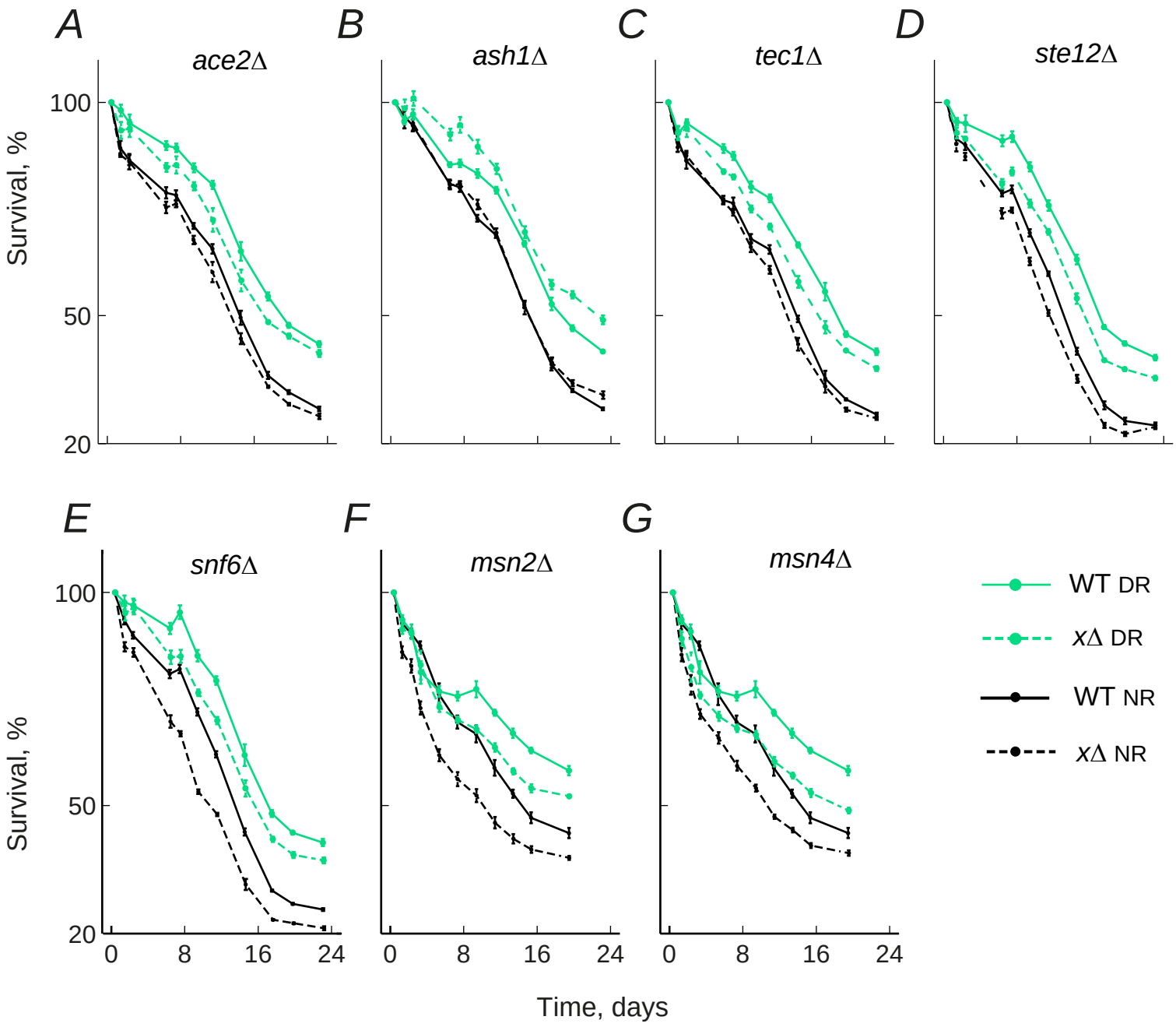
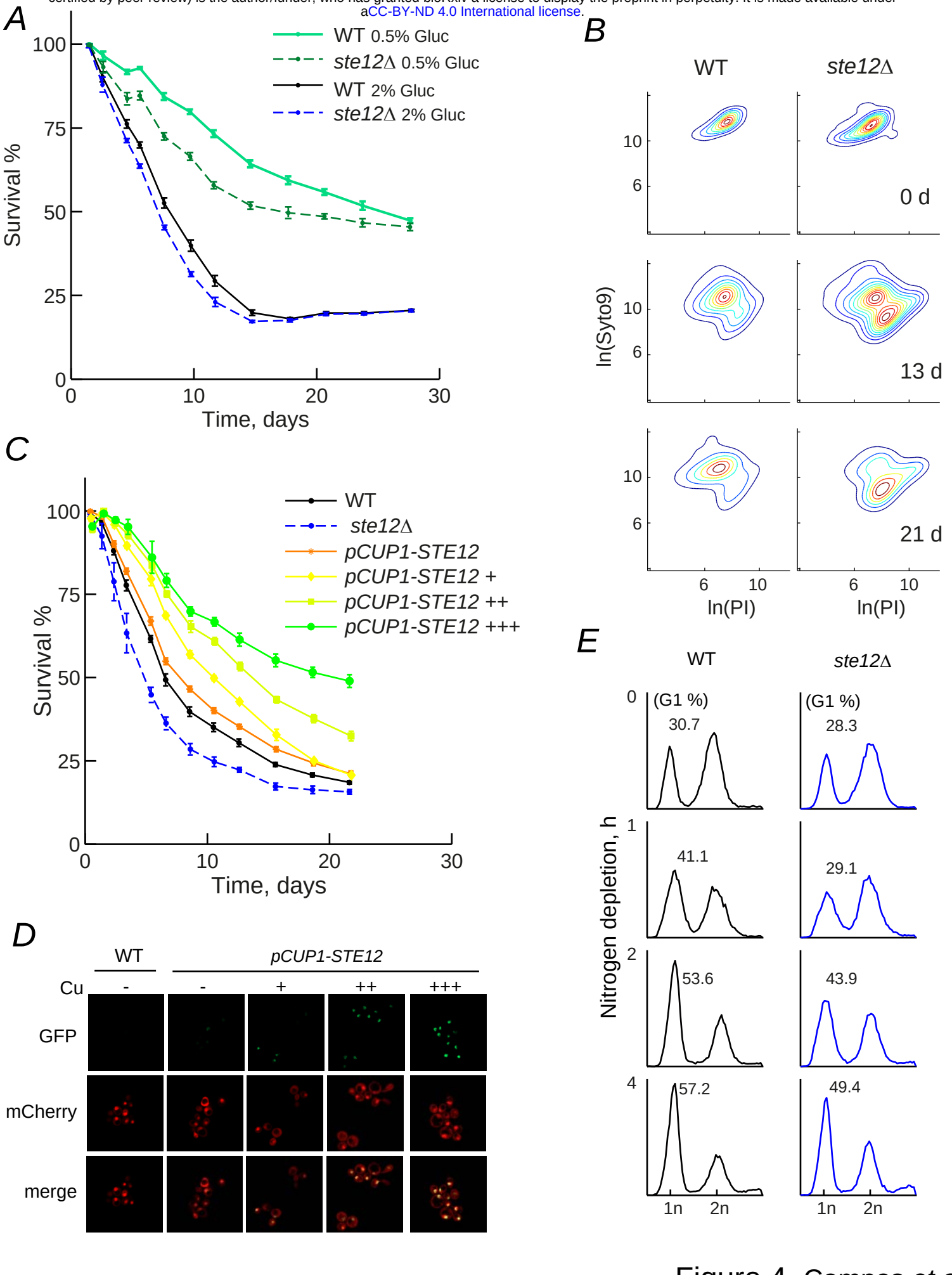


Figure 3_Campos *et al*.



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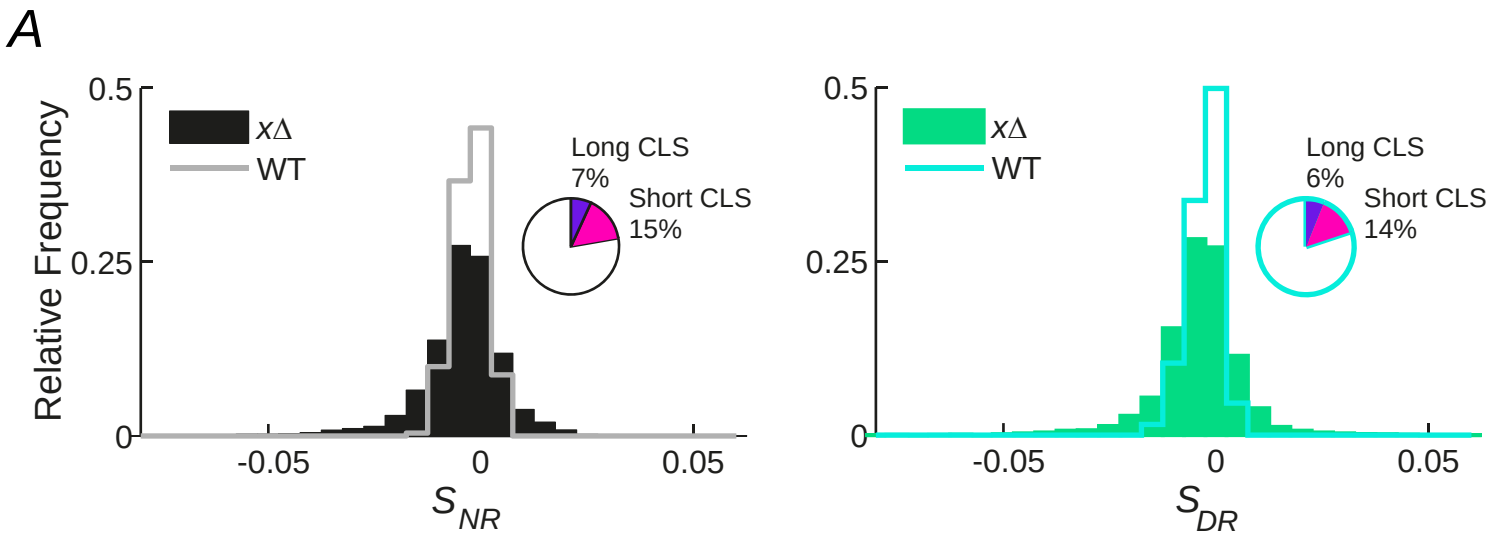


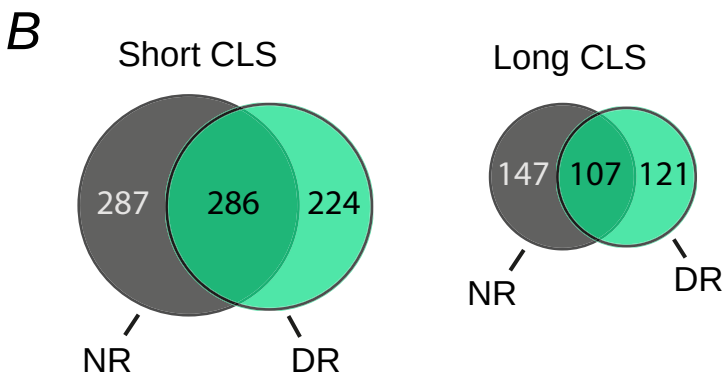
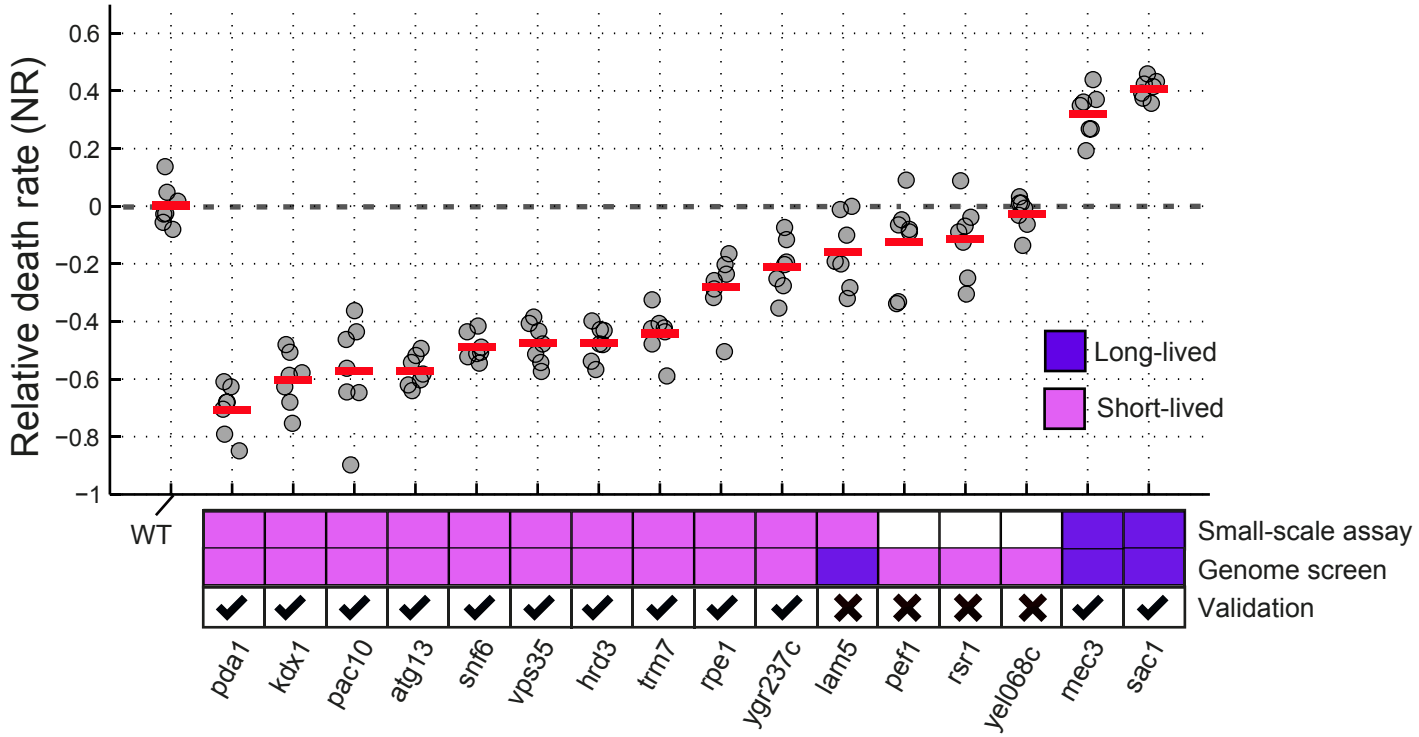
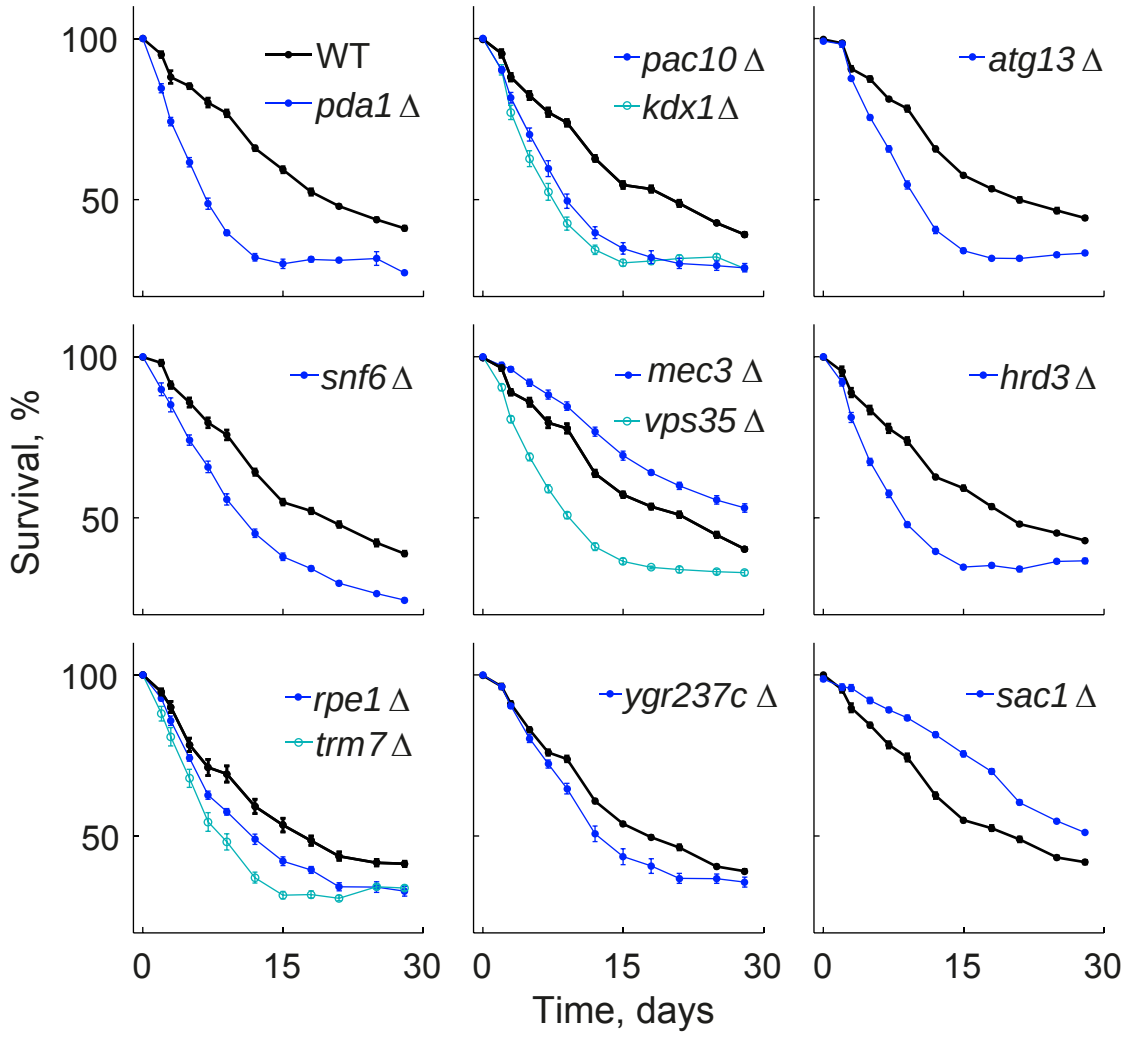


Figure S1_Campos *et al*.

A



B



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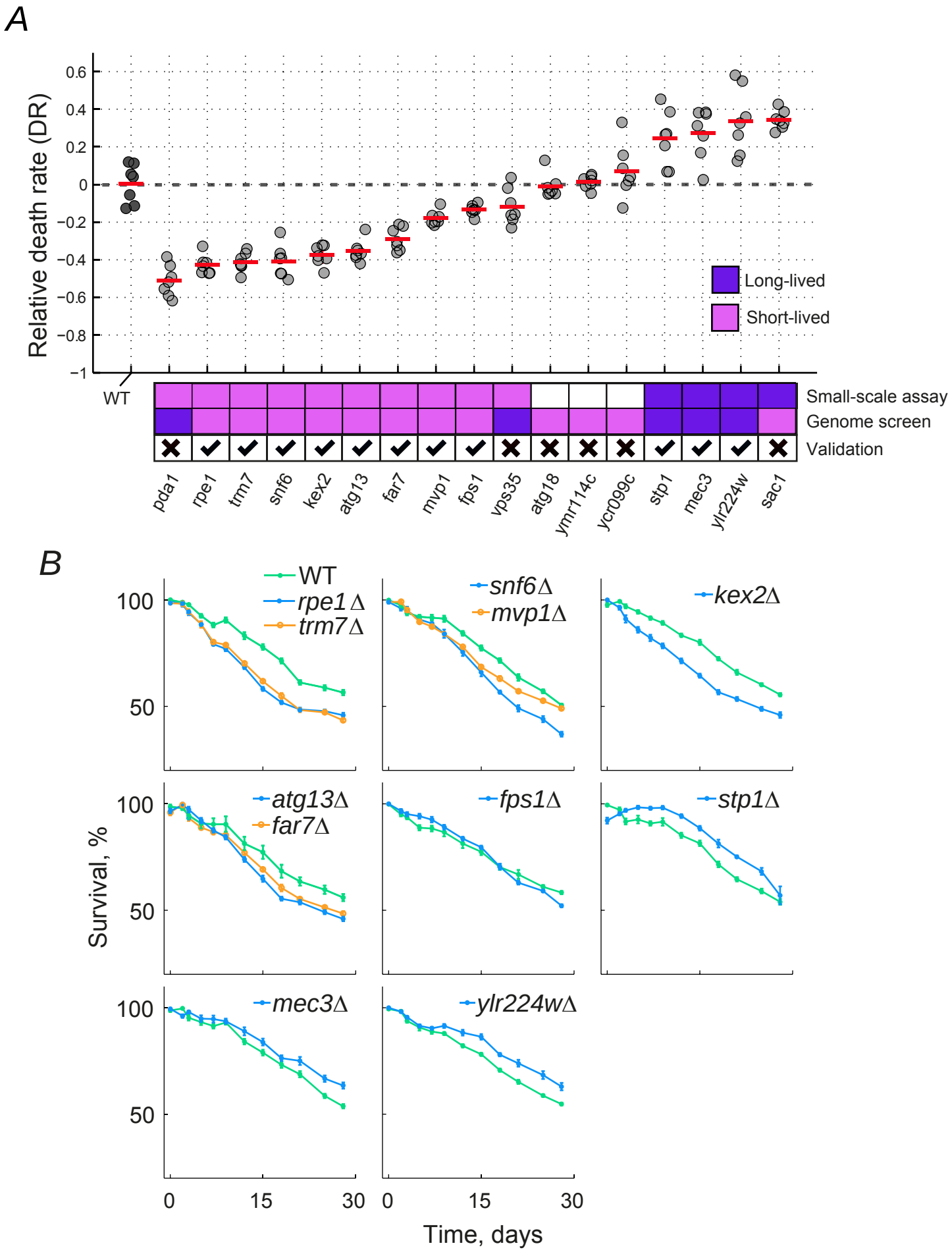

Figure S3_Campos *et al*.

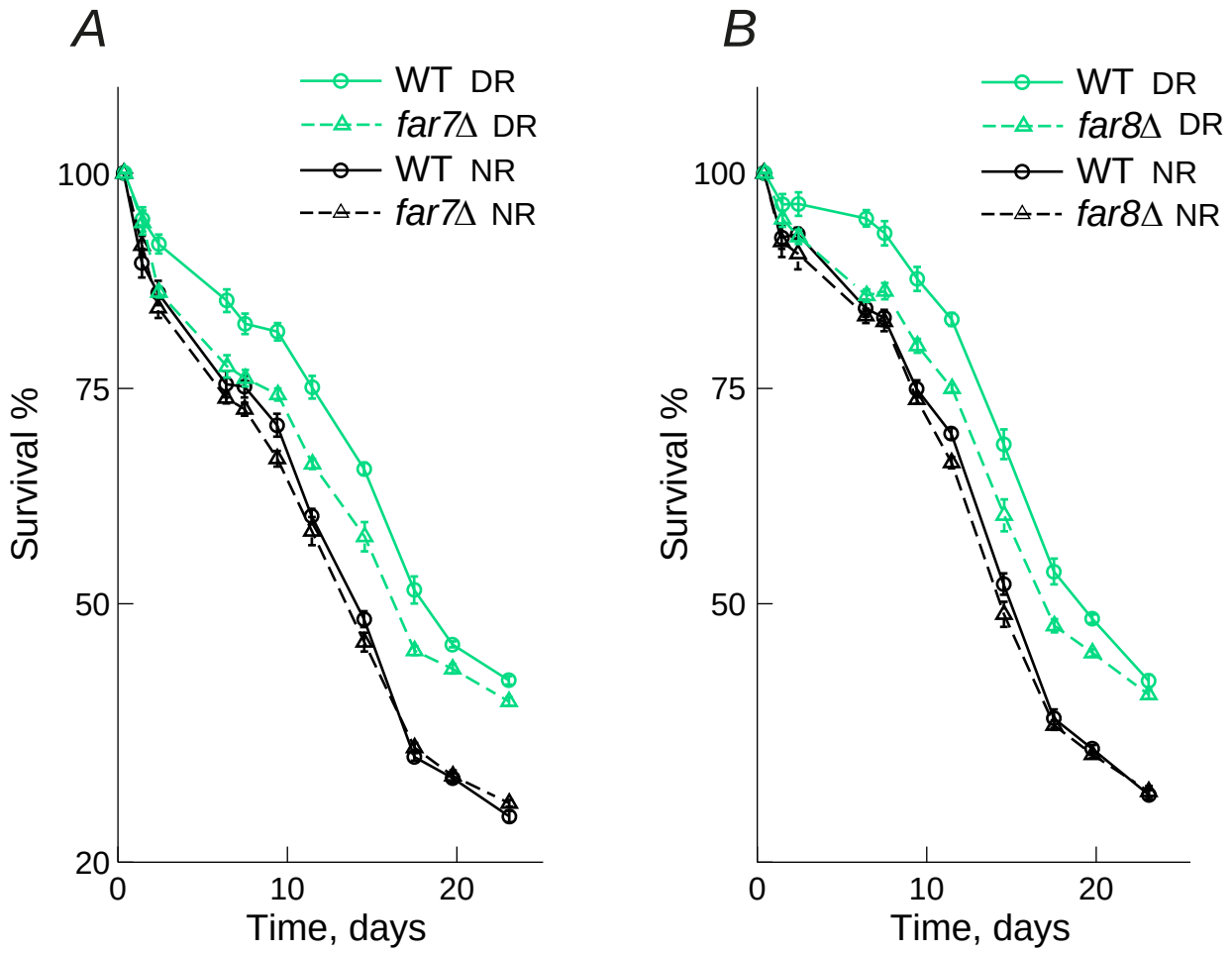

Figure S4_Campos *et al*.

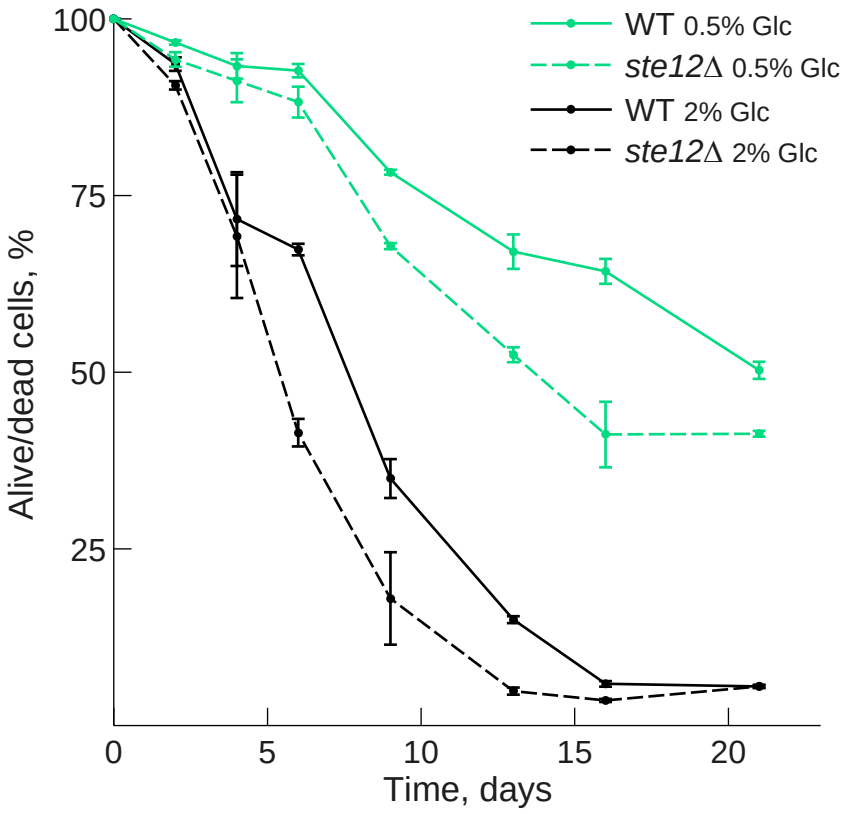

Figure S5_Campos *et al*.