Budding Yeast *SSD1-V* Regulates Transcript Levels of Many Longevity Genes and Extends Chronological Life Span in Purified Quiescent Cells

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Ssd1 is an RNA-binding protein that affects literally hundreds of different processes and is polymorphic in both wild and lab yeast strains. We have used transcript microarrays to compare mRNA levels in an isogenic pair of mutant (*ssd1-d*) and wild-type (*SSD1-V*) cells across the cell cycle. We find that 15% of transcripts are differentially expressed, but there is no correlation with those mRNAs bound by Ssd1. About 20% of cell cycle regulated transcripts are affected, and most show sharper amplitudes of oscillation in *SSD1-V* cells. Many transcripts whose gene products influence longevity are also affected, the largest class of which is involved in translation. Ribosomal protein mRNAs are globally down-regulated by *SSD1-V*. *SSD1-V* has been shown to increase replicative life span¤ and we show that *SSD1-V* also dramatically increases chronological life span (CLS). Using a new assay of CLS in pure populations of quiescent prototrophs, we find that the CLS for *SSD1-V* cells is twice that of *ssd1-d* cells.

INTRODUCTION

Ssd1 is an RNA-binding protein (Uesono *et al.,* 1997; Hogan *et al.,* 2008) that can be distinguished from the hundreds of other RNA-binding proteins by its unusual properties. First of all, *ssd1* is highly pleiotropic. This locus has at least nine different names because of its having been identified in genetic screens for its effect on minichromosome stability, stress tolerance, membrane trafficking, and cell wall integrity, among other things (Uesono *et al.,* 1994; Kosodo *et al.,* 2001; Vannier *et al.,* 2001; Reinke *et al.,* 2004). Systematic global screens have identified ~200 genes that show genetic or physical interactions with Ssd1 (Reguly *et al.,* 2006). These genes show a striking enrichment (Hong *et al.,* 2008) for posttranslational modifiers ($p = 10^{-14}$), including 19 kinases and nine histone deacetylases, and genes involved in the cell cycle and cell morphogenesis ($p = 10^{-8}$). *ssd1* mutants display sensitivity to high osmolarity, caffeine, fungicides¤ and numerous other compounds, which suggests a role for this protein in the maintenance of cell wall integrity (Ibeas *et al.,* 2001; Parsons *et al.,* 2004), but its mechanism of action remains obscure.

Despite, or perhaps because of, its impact on so many different cellular functions, *SSD1* is a common site of varia-

tion in both laboratory strains and natural populations of budding yeast (Wheeler *et al.,* 2003). One possible explanation is that *SSD1-V*, which encodes the full-length "wild-type" protein, reduces the virulence of *Saccharomyces cerevisiae* in one mouse model (Wheeler *et al.,* 2003), but *SSD1-V* is critical in *Candida* (Gank *et al.,* 2008) and several fungal pathogens of plants for evading their hosts' defense systems (Tanaka *et al.,* 2007). In most cases, genetic interactions with *SSD1-V* are positive, whereas the premature termination alleles (*ssd1-d*) cause lethality or a more extreme phenotype. One important exception is the RAM signaling pathway mutants, *tao3* (Du and Novick, 2002), and *cbk1* (Tong *et al.,* 2001; Jorgensen *et al.,* 2002), whose loss of function is lethal if the *SSD1-V* allele is present (Jorgensen *et al.,* 2002). Ssd1-v physically interacts with Cbk1 (Racki *et al.,* 2000)¤ and there are consensus phosphorylation sites for Cbk1 kinase in the Ssd1 protein (Mazanka *et al.,* 2008). These observations suggest that Cbk1 may negatively regulate Ssd1 and that unregulated Ssd1 function is deleterious. Cbk1 is a highly conserved kinase that is localized in the daughter cell, where it influences asymmetric cell fate decisions (Mazanka *et al.,* 2008).

In most cases studied, the *ssd1-d* alleles exhibit properties similar to *ssd1* deletions, which indicates that the *ssd1* truncation results in a loss of function and perhaps a null phenotype (Kaeberlein and Guarente, 2002). However, partial function has been observed with the *ssd1-d2* allele from W303 with respect to cell size, caffeine sensitivity¤ and interaction with cyclin-dependent kinase mutant *cdc28-13* (Sutton *et al.,* 1991). A recent study also showed that *SSD1-V* is required for Hsp104-mediated protein disaggregation and thermotolerance, and in that case, *ssd1-d2* fully complemented the *ssd1* deletion in disaggregation and zymolyase

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype[a]	Source
BY2125	*MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1 ssd1-d2*	Breeden lab W303a
BY2124	*MATα ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1 ssd1-d2*	Breeden lab W303α
BY4260	*MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1 SSD1-V*	Breeden lab
BY6464	*MATα ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1 ssd1Δ::HIS3*	This study
BY6580	*MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1 SSD1-V*	This study
BY6500	*MATa ssd1-d2*	This study
BY6501	*MATα ssd1-d2*	This study
BY6504	*MATa his3-11,15 ssd1-d2*	This study
BY6562	*MATα his3-11,15 ssd1Δ::HIS3*	6504 × 6464
BY6563	*MATa his3-11,15 ssd1Δ::HIS3*	6562 × 6580
BY6564	*MATα SSD1-V*	This study
BY6641	*MATa SSD1-V*	6564 × 6563
W303Va	*MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1 SSD1-V*	Wijnen and Futcher (1999)

[a] All strains are W303 background and include *can1-100 rad5 ho* in addition to the genotype as listed.

sensitivity and partially restored thermotolerance (Mir *et al.*, 2009). These observations make it clear that the common C-terminal truncation of Ssd1 retains some of its functions.

Early studies showed that overexpression of *SSD1-V* could suppress conditional mutations in RNA polymerase I, II, and III (Stettler *et al.*, 1993) and in splicing factors (Luukkonen and Seraphin, 1999). Ssd1 has also been identified through its interaction with the phosphorylated C-terminal repeat of RNA polymerase II (Phatnani and Greenleaf, 2006). These observations are all consistent with Ssd1 having a general effect on RNA levels.

Ssd1 binds RNA in vitro and has some homology to ribonucleases, but it has no discernible ribonuclease activity (Uesono *et al.*, 1997). Recent work has shown that Ssd1-v binds a discrete group of mRNAs in vivo. These mRNAs are enriched for those that encode cell wall components and proteins that localize to the bud (Hogan *et al.*, 2008). Ssd1 does not contain a recognizable RNA-binding domain, and hence its binding to these mRNAs may be indirect.

SSD1-V was initially characterized for its ability to suppress the G1 arrest of a Sit4 phosphatase mutant (Sutton *et al.*, 1991). The role of Sit4 in the cell cycle remains mysterious, but *SSD1-V* has also been shown to restore the viability of *swi4* and *cln1cln2* mutants (Cvrckova and Nasmyth, 1993), all of which are important for the G1-to-S transition. *SSD1-V* also enhances replicative life span (RLS; Kaeberlein *et al.*, 2004), which is defined as the number of cell divisions a mother cell completes in her lifetime (Mortimer and Johnston, 1959). These phenotypes are consistent with a possible role for Ssd1 in the initiation of the cell cycle. We have explored this possibility by characterizing genome-wide transcript levels across the cell cycle in isogenic *SSD1-V* and *ssd1-d* cells. We find that cell cycle regulated transcripts have sharper oscillations in *SSD1-V*, but no specific class is affected. The largest class of affected transcripts are involved in ribosome biogenesis and other pathways that affect longevity. Using a new assay of longevity in purified quiescent cells, we find that *SSD1-V* cells also have a significantly longer chronological life span (CLS) than *ssd1-d* cells.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions

Strains used are all in the W303 background and are listed in Table 1. The *SSD1-V* allele in BY4260 was introduced by four backcrosses to W303Va (Wijnen and Futcher, 1999). The W303 *ssd1-d* prototrophs were made by

homologous gene replacement of auxotrophic markers with wild-type DNA in BY2125 and/or BY2124 (W303 *MATα*). These replacements were verified by sequencing, and then crosses were performed to generate the prototrophic W303 (BY6500 and BY6501) and other auxotrophic combinations, including BY6504 (W303 *MATa his3-11,15*). The entire *SSD1* gene was deleted in BY2125 and replaced by the *HIS3MX6* gene (Longtine *et al.*, 1998) to produce BY6464. The isogenic *ssd1Δ::HIS3* prototroph (BY6562) was generated from crossing BY6464 to BY6504. To introduce the *SSD1-V* allele to BY2125, the *SSD1-V* allele was amplified by PCR from W303Va (Wijnen and Futcher, 1999) and inserted into the *URA3* integrating vector pRS306 (Sikorski and Hieter, 1989) to produce pBD3000. pBD3000 was integrated at the *ssd1-d* locus of BY2125, and the *ssd1-d* and plasmid sequences were evicted by selection against *URA3* (Scherer and Davis, 1979; Boeke *et al.*, 1984) and verified by sequencing to produce BY6580. This W303 *SSD1-V* auxotroph was crossed to BY6562 (*MATα his3-11,15 ssd1::HIS3*) to generate W303 *his3-11,15 SSD1-V,* and then the *his3-11,15* was replaced as above to generate the prototroph BY6564 (*MATα SSD1-V*). The isogenic *MATa* prototrophs were generated by appropriate crosses (Table 1). All studies described in this manuscript were carried out with the *MATa* isolates.

Strains were cultured at 30°C with aeration in yeast rich medium (YPD) with 2% glucose and a 55 mg/L adenine supplement (YSC; Ausubel *et al.*, 2003) buffered to pH 5.8 with succinic acid (10 g/l) and NaOH (6 g/l) and supplemented with 2% glucose. Other nutritional supplements were added as specified (Ausubel *et al.*, 2003) except in the case where fourfold excess supplementation was used for auxotrophic requirements: adenine (final concentration 160 mg/l), uracil (80 mg/l), leucine (240 mg/l), tryptophan (160 mg/l), and histidine (80 mg/l).

Budding and DNA Analysis

Cells were examined by microscopy for the presence of new buds. Two hundred cells were examined every time point. The budding percentage was calculated as the number of budded cells divided by the number of total cells at each time point. To monitor the DNA synthesis through the cell cycle, cells stained with Sytox-Green (Invitrogen, Carlsbad, CA) were analyzed on a FACScan cytometer (BD Biosciences, San Jose, CA) as described in Pramila *et al.* (2002).

Viability Assays of Stationary Phase Cultures

Assays were performed as previously published (Fabrizio and Longo, 2003) with additional supplementation of required compounds as previously described (Fabrizio *et al.*, 2004; Powers *et al.*, 2006), but our YSC media is buffered (see above). Cells were grown to stationary phase (SP) in 25 ml YSC synthetic medium with a fourfold excess of required nutrients with aeration at 30°C in 250-ml flasks. Cultures were sampled at times indicated, diluted, and plated in duplicate on YPD plates. Plates were incubated at 30°C for 3 d before colonies forming units (CFUs) were counted. The percentage of surviving cells was calculated with the plateau CFUs attained by the culture set as 100%.

Fractionation and Purification of Quiescent Cells

Density gradient fractionation of quiescent cells (Q cells; Allen *et al.*, 2006) was scaled down to 2-ml total volume. Cells are grown in 25 ml YPD medium in 250-ml flask for 7 d at 30°C. Separation of Q cells from these SP cultures was done by using RediGrad (GE Healthcare, Waukesha, WI) density gradients. To generate the gradient, RediGrad is diluted 9:1 (vol/vol) with 1.5 M NaCl
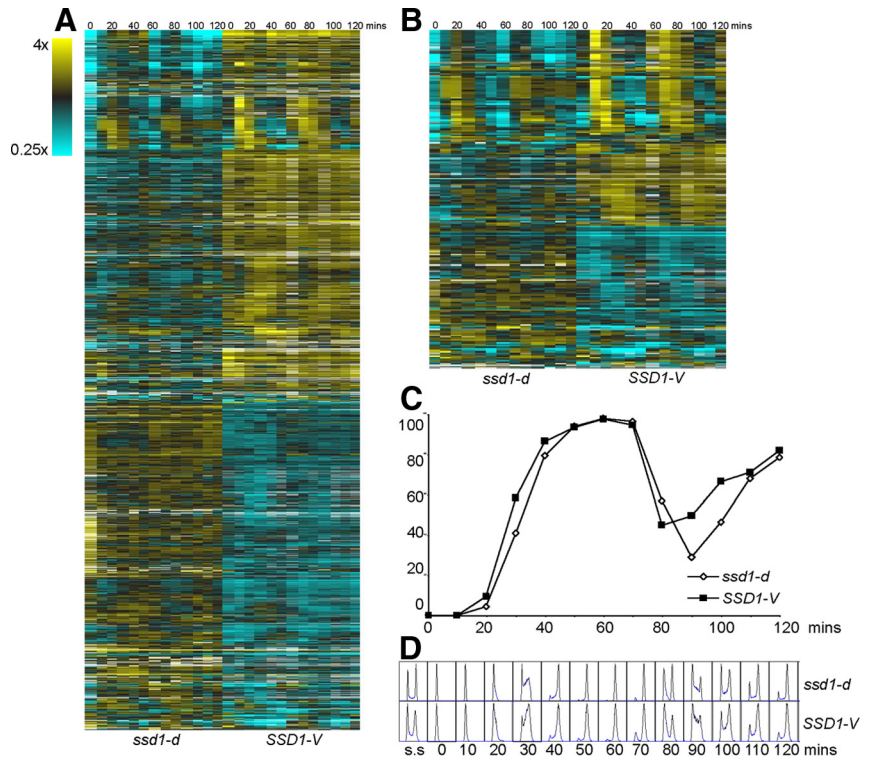
Figure 1. Differentially expressed genes between cells carrying *ssd1-d* versus *SSD1-V*. (A) Heat map of BY2125 (*ssd1-d*) and BY4260 (*SSD1-V*) microarray data through two cell cycles showing the 889 differentially expressed genes identified by DiffExp. Each column represents one time point. Each row represents a gene. (B) Heat map of BY2125 and BY4260 microarray data showing the 227 cell cycle regulated genes that are differentially expressed in cells carrying *ssd1-d* versus *SSD1-V*. (C) Progress through the cell cycle was monitored by budding index and (D) DNA synthesis from 0 min to 120 min after alpha factor release. *ssd1-d*, ◇; *SSD1-V*, ■.

to a final NaCl concentration of 167 mM. Then 1.75 ml gradient solution was put into a 2-ml sterile microfuge tube and centrifuged for 15 min at 13,400 rpm (19,100 × g) at room temperature. About 30 OD$_{600}$ of SP cells in 100 μl Tris buffer was layered onto the preformed gradient¤, and centrifuged at 1370 rpm (400 × g) for 1 h. Q cells were collected from the bottom 0.5 ml of the gradient. Q cell yield was calculated as the percentage of the total loaded OD$_{600}$ units harvested from this bottom fraction.

Chronological Life Span Analysis with Purified Quiescent Cells

Purified Q cells from 1-wk-old SP cultures were inoculated into water at an optical density (A600) of ~1 and incubated at 30°C with aeration. Samples were diluted into distilled water before plating onto YPD plates in duplicate. Plates were incubated at 30°C for 3 d before colony counting. Survival was determined by CFU. CFU at the first week was set to be the initial survival (100%).

Viability Assay

To determine viability, cells were stained using the LIVE/DEAD FungaLight yeast viability kit (Agilent Technologies, Wilmington, DE). The negative viability control was prepared by incubating cells at 75°C for 10 min before the staining. Exponentially growing cells were used for the positive viability control. Cells with no dye added were used as an autofluorescence control. All cell samples were diluted in 1 ml of 50 mM Tris buffer (pH 7.5) with 1 μl of SYTO9 dye and 1 μl of propidium iodide added,¤ and were incubated at 37°C for 15 min before analysis. For flow cytometry, 30,000 cells per sample were analyzed by using 488-nm wavelength excitation and collecting fluorescent emission with filters at 530/30 nm for the FL-1 parameter and 585/42 nm for the FL-2 parameter. To avoid coincidence error, flow rate was maintained at ≤1000 events/s. Cell Quest software (BD Biosciences, San Jose, CA) was used for data collection and analysis.

Microarray Hybridization and Data Processing

Alpha factor synchronizations were carried out as described in Breeden (1997), and the cells were sampled every 10 min for about two cell cycles. Total RNA, 30 μg, was extracted from both *ssd1-d* and *SSD1-V* cells and used for cDNA synthesis. Labeling was performed using the amino-allyl reverse transcription labeling protocol, the labeled cDNAs were hybridized to yeast cDNA arrays (Pramila *et al.*, 2002). Array analysis was performed using the GenePix Prosoftware from Axon Instruments (Foster City, CA). Expression data analysis, normalization, and clustering were done by Genespring GX 7.3.1. The *SSD1-V* dataset is available from the GEO Database (Accession number GSE16911).

Identifying Differentially Expressed Genes

DiffExp (Bar-Joseph *et al.*, 2003) was used to identify genes differentially expressed across the cell cycle between *ssd1-d* and *SSD1-V*. Repeat datasets of *ssd1-d* were used to determine the noise model. The significance threshold for selecting differentially expressed genes was set at 0.005. DiffExp first represents each expression time series as continuous curves so that expression patterns of the same gene are comparable. Then, for each gene, the algorithm computes the global difference between its continuous representation in *ssd1-d* and *SSD1-V* and the corresponding p-value. Finally, it ranks the genes according to their p-values, and outputs those genes with a p-value lower than the given threshold.

Clustering Differentially Expressed Genes

The differentially expressed genes were analyzed by the Clustering of Regression Models (CORM) method (Qin and Self, 2006) to identify clusters of genes whose transcript profiles are similar under two different conditions (*SSD1-V* and *ssd1-d*). The number of clusters was set at 25.

Identifying and Ranking Cycling Genes

To identify the periodic transcripts in *ssd1-d* and *SSD1-V*, we used a method that combines a score for the periodicity of the expression profile with a score for the magnitude of the expression. Previous work (de Lichtenberg *et al.*, 2005; Lu *et al.*, 2007) has shown that such a score performs best among all proposed methods for identifying cycling genes. To compute these scores, we first performed Fast Fourier Transform on the gene expression time series. We then combined the fraction of spectral power around the cell cycle period, and the height of the peak spectrum to obtain the score. Intuitively, the method assigns high scores to genes whose expression level fluctuates strongly with the cell cycle.

Computing the p-value for Differences in Cycling Genes

To evaluate the effects of the *SSD1-V* versus *ssd1-d* on cycling genes, we identified genes that received a high cycling score in one cell type but low in the other. To evaluate the significance of these differences, we used randomization analysis as follows. We assume that the cyclic score assigned to a gene is the sum of its true cyclic score and a random error term that follows the Gaussian distribution $N(0, \sigma^2)$. We estimated σ to be 0.85 based on the zero time point technical replicates. We randomly generated the score for each gene by drawing from a Gaussian distribution $N(0, 1)$. This score was assigned to the gene in both strains. Next, we added a random error term sampled from the error distribution to each gene (independently for each strain). We then counted the number of genes that had a high score (rank
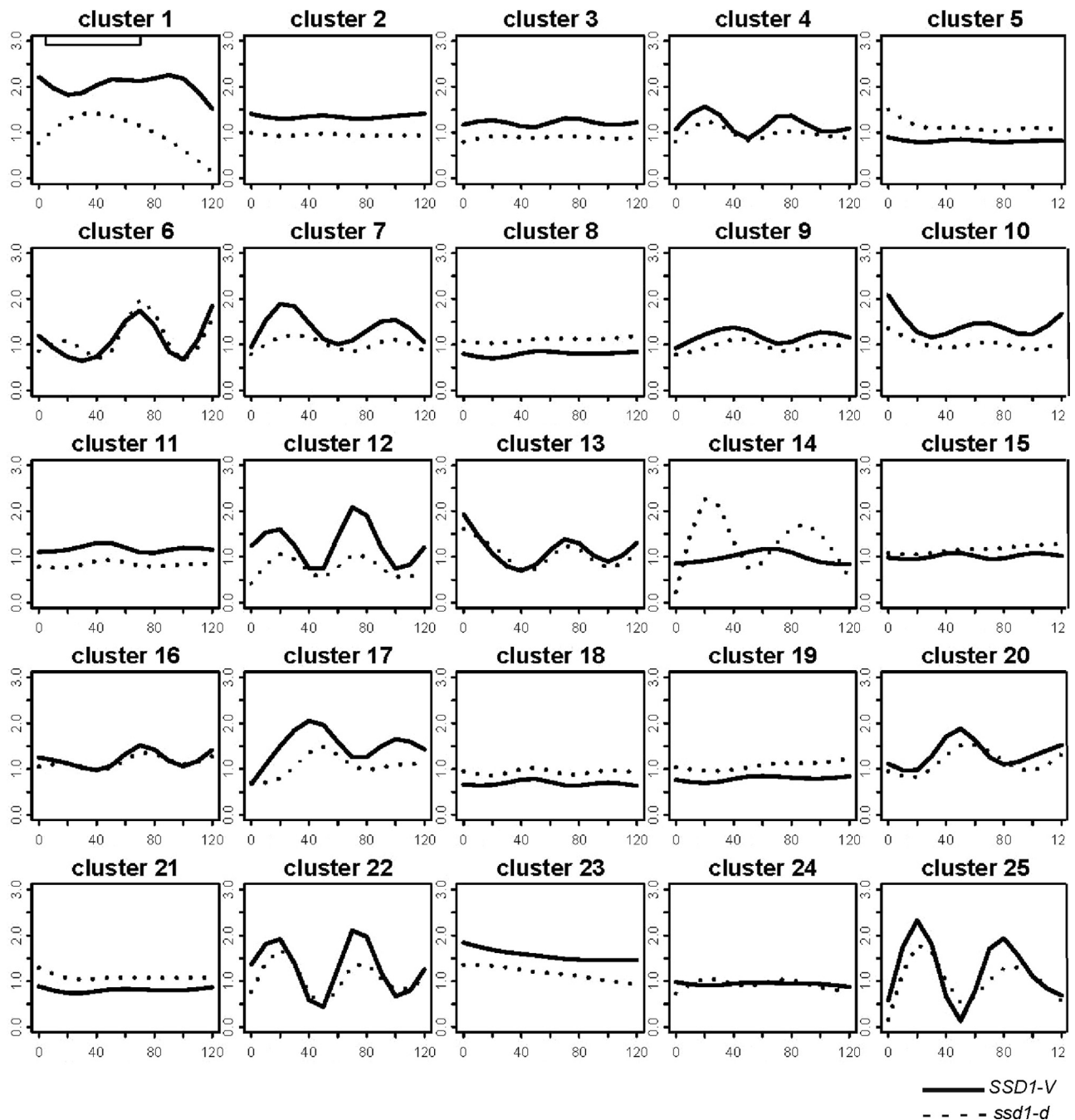
Figure 2. Clustering analysis of the 899 differentially expressed genes in *ssd1-d* versus *SSD1-V*. Twenty-five groups of similarly affected transcripts were found by Clustering of Regression Models (CORM) analysis. *ssd1-d* (BY2125), dashed line; *SSD1-V* (BY4260), solid line.

<1000) in one cell type and a low score (rank >5000) in the other. By repeating this process multiple times, we empirically estimated the probability of observing more than a certain number of genes that are highly periodically expressed in one and not in the other cell type.

RESULTS

SSD1-V Affects Transcript Levels Globally

We have generated three microarray data sets through the cell cycle with W303 (Pramila *et al.*, 2006), which carries the *ssd1-d2* allele,¤ and another data set with an isogenic strain carrying *SSD1-V*. All four data sets were obtained from cells synchronized with alpha factor and followed through two consecutive cell cycles. We have compared the global transcript profiles across the cell cycle using DiffExp (Bar-Joseph *et al.*, 2003) and find that there are 889 genes (15%) that show significant differences between cells carrying *SSD1-V* versus *ssd1-d*. One-half of these transcripts are up-regulated, and the other half are significantly down-regulated by *SSD1-V* (Figure 1A). *SSD1-V* also affects ~20% of the cell cycle–regulated (ccr) transcripts (Figure 1B), but we find no evidence that any specific class of ccr transcripts are enriched among the differentially expressed genes (see Supplemental Figures S1 and S2).

Table 2. Differentially transcribed transcription factors

Gene	Function	p value
A. Transcription factors up-regulated by *SSD1-V*		
YAP5	bZIP transcription factor; interacts with *AFT1*	0
MET4	Transcriptional activator responsible for the regulation of the sulfur amino acid pathway; requires different combinations of the auxiliary factors Cbf1p, Met28p, Met31p and Met32p	1.98E-14
MAL33	MAL-activator protein	1.03E-10
HAL7	Leucine zipper transcription factor; overexpression increases sodium and lithium tolerance	1.52E-09
RDS1	Zinc cluster transcription factor involved in conferring resistance to cycloheximide	3.03E-07
*HCM1**	Transcription factor (fork head domain) regulating genes involved in chromosome dynamics	6.28E-07
*RDR1**	Transcriptional repressor involved in the control of multidrug resistance; negatively regulates expression of the *PDR5* gene	2.35E-06
MSN2	Transcriptional activator related to Msn4p; activated in stress conditions	4.11E-06
AFT1	Transcription factor binds the consensus site PyPuCACCCPu and activates the expression of target genes in response to changes in iron availability	5.22E-06
*HAL9**	Putative transcription factor containing a zinc finger; overexpression increases salt tolerance	5.34E-05
MET18	DNA repair and TFIIH regulator; involved in telomere maintenance	2.33E-04
MET32	Zinc-finger DNA-binding protein, involved in transcriptional regulation of the methionine biosynthetic genes, similar to Met31p	4.38E-04
*MBF1**	Transcriptional coactivator that bridges the DNA-binding region of Gcn4p and TATA-binding protein Spt15p	5.08E-04
INO2	Helix-loop-helix protein	7.75E-04
*NDD1**	Nuclear Division Defective 1	0.002
OPI1	Negative regulator of phospholipid biosynthesis; involved in telomere maintenance	0.002
*YHP1**	One of two homeobox transcriptional repressors (see also Yox1p); restrict ECB-mediated transcription to the M/G1 interval	0.003
RRN3	Protein required for transcription of rDNA by RNA polymerase I	0.003
GAT1	Transcriptional activator of genes involved in nitrogen catabolite repression	0.01
B. Transcription factors down regulated by *SSD1-V*		
ROX1	HMG-domain site-specific DNA binding protein; Heme-dependent repressor of hypoxic genes	4.44E-16
*YER130C**	Transcription factor activity; contains two tandem C2H2-type zinc fingers	1.67E-15
FAP1	FKBP-associated protein that conferring rapamycin resistance	2.04E-08
HAP5	Transcriptional activator of respiratory gene expression	2.80E-05
ARG80	Transcription factor, involved in arginine metabolic process	6.18E-05
*FAP7**	Transcription cofactor activity; Essential NTPase required for small ribosome subunit synthesis	7.18E-05
KRE33	Transcription factor activity; required for 40S ribosomal subunit biogenesis	2.1E-04
RTS2	Similar to mouse KIN7 protein	2.6E-04
*STB1**	Interacts with the putative transcription factor Sin3p	8.4E-04
*FKH2**	Fork Head homolog two	8.6E-04
MSS1	Polypeptide with poly-glutamine and poly-asparagine domains	9.3E-04
MDS3	Negative regulator of early meiotic genes	0.001
*YRR1**	Zn2-Cys6 zinc-finger transcription factor that activates genes involved in multidrug resistance	0.003
*RSF2**	Transcription factor that involved in glycerol utilization, respiration	0.005

*, cell cycle regulated genes

To identify genes whose transcripts might be coordinately regulated by Ssd1, the 889 transcripts that are differentially expressed in *SSD1-V* versus *ssd1-d* were analyzed by CORM (Qin and Self, 2006; Tanaka *et al.*, 2007). This algorithm groups genes that share similar relationships to the covariate, which is *SSD1*. Figure 2 shows 25 clusters of genes whose mean transcript profiles across the cell cycle follow one pattern in *SSD1-V* cells (solid line) and a second, different pattern in *ssd1-d* cells (dotted line). Inspection of these profiles shows that about half the affected clusters are periodic. In nearly every case, the ccr transcripts in the *SSD1-V* profiles have sharper peaks and troughs than those of the *ssd1-d* cells. These effects can also be seen in the heat maps (Figure 1B). We note that budding and DNA synthesis occur slightly earlier in the *SSD1-V* strain than the *ssd1-d* strain (Figure 1, C and D). This slightly accelerated and more synchronous cycle may contribute to the sharper peaks observed in the *SSD1-V* ccr transcripts. It is also possible that *SSD1-V* reinforces these oscillations in a more direct manner. (Cluster membership can be found in Supplemental Table S1).

We have used GO analysis to identify enrichment for functionally related gene products within these clusters. We find that a subset of the Ty1 retro-transposons (in clusters 1 and 11) and subtelomeric helicases (cluster 12) are among the most up-regulated transcripts in *SSD1-V*. However, the sequence homologies within these families make it difficult to know if all or just a fraction of them are up-regulated. We also find that a family of genes involved in iron transport is up-regulated in *SSD1-V* (in clusters 9, 17, and 20). This is likely to be an indirect effect, because *SSD1-V* cells also show a very significant up-regulation of both *AFT1* and *YAP5* mRNA levels (Table 2A). Aft1 and Yap5 are transcription factors that activate transcription of genes involved in iron transport (Yamaguchi-Iwai *et al.*, 1995) and iron storage (Li *et al.*, 2008). Increased expression of these factors may explain why at least 24 of the genes that are bound in vivo by Aft1 (Harbison *et al.*, 2004) are also up-regulated in the *SSD1-V* cells. A subset of these iron transport gene profiles are shown in Supplemental Figure S3.

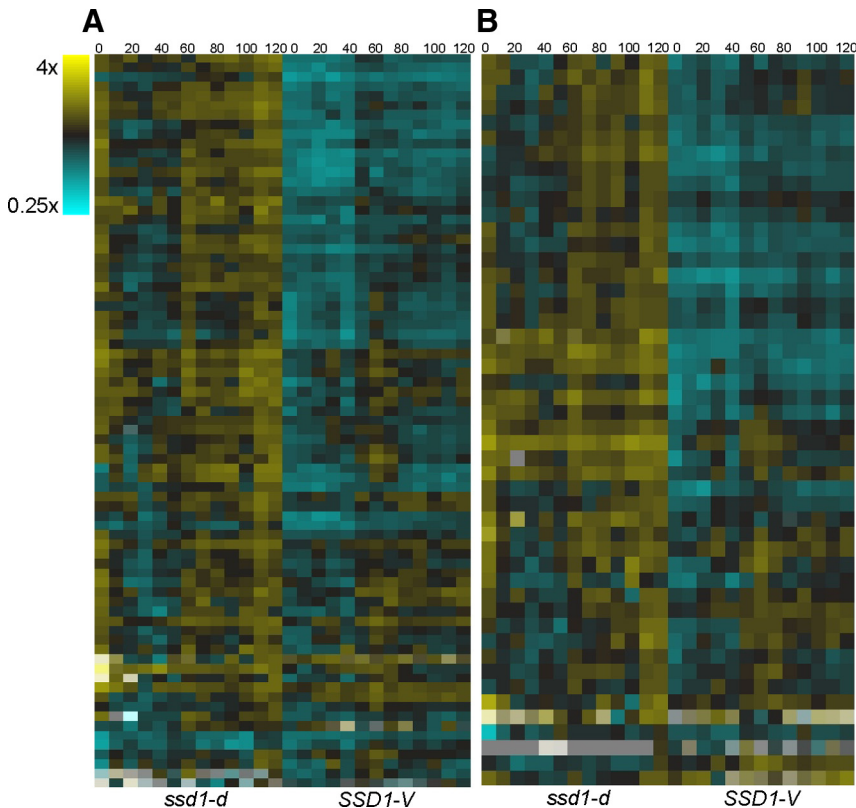In addition of *AFT1* and YAP5, there are 31 other transcription factors that are either up- or down-regulated by

Figure 3. The global impact on the transcript levels of cytoplasmic ribosomal proteins by *SSD1-V*. Data are displayed as in Figure 1. (A) Large subunit protein transcripts. (B) Small subunit protein transcripts.

SSD1-V (Table 2B). We also see increased mRNA levels for transcription factors involved in methionine biosynthesis (*MET4, MET18* and *MET32*), salt tolerance (*HAL7* and *HAL9*) and the stress response (*MSN2*). Efflux pumps involved in multidrug resistance may also be down-regulated indirectly in *SSD1-V* cells, because the Rdr1 repressor of pleiotropic drug response genes (Hellauer *et al.,* 2002) is up-regulated and the Yrr1 activator of drug response genes (Onda *et al.,* 2004) is down-regulated.

Structural Components of Cytoplasmic Ribosomes and Many Ribosome Biogenesis Genes Are Down-Regulated by SSD1-V

The most striking enrichment we observe is for the down-regulation of genes involved in translation in *SSD1-V* cells. Cluster 18 (Figure 2) includes 23 genes involved in ribosome biogenesis (p = 10^{-11}). The promoters of ribosome biogenesis genes contain PAC elements (Dequard-Chablat *et al.,* 1991) and at least one copy of the RRPE element (Hughes *et al.,* 2000). To see if either of these elements could be influenced by *SSD1*'s status, we compared our list of 889 differentially expressed genes with the genes found to contain one or both of these elements in their promoters. Only ~10% of all genes carrying RRPE or PAC elements were differentially expressed, and of those, about half were up- and half were down-regulated (data not shown). We conclude that direct influences on the activities of these promoter elements are unlikely.

In addition, cluster 19 includes 24 genes that encode structural components of cytoplasmic ribosomes (p = 10^{-20}). These genes also display reduced expression in *SSD1-V* cells. Figure 3 shows the transcript levels for all proteins of the large and small subunits of cytoplasmic ribosomes across two cell cycles after release from alpha factor arrest in

ssd1-d and *SSD1-V* cells. These heat maps suggest that the impact of *SSD1-V* on ribosomal (r) protein genes is global. All the r protein transcript levels are lower in the alpha factor arrest (0 time point) and most remain low across two cell cycles in the *SSD1-V* time course. R protein genes are transcriptionally regulated by Rap1, Fhl1, Ifh1¤ and Crf1 (Moehle and Hinnebusch, 1991; Martin *et al.,* 2004; Rudra *et al.,* 2005). None of these factors are affected by *SSD1-V* at the RNA level. Moreover, genome-wide chromatin immunoprecipitation studies have identified 207 promoters that are bound in vivo by Fhl1 (Harbison *et al.,* 2004), but only 38 of those are significantly down-regulated in the presence of *SSD1-V* based on our DiffExp analysis. This, in addition to the fact that the r protein transcripts are affected to differing extents, suggests that another form of regulation is involved.

Differentially Regulated Transcripts of Genes That Interact Physically or Genetically with SSD1

The recent identification of mRNAs that copurify with Ssd1-v (Hogan *et al.,* 2008) led us to first ask if these bound mRNAs are expressed differentially in *SSD1-V* and *ssd1-d* cells. Surprisingly, we found no correlation. Of the 60 mRNAs that *SSD1-V* binds, only eight were differentially expressed and six of these were expressed at a higher level in *SSD1-V* cells. These results do not support the hypothesis that Ssd1 binding to mRNA influences their degradation rate¤, or that Ssd1 is a ribonuclease in vivo.

Table 3 identifies the 20 genes whose mRNA levels are significantly affected by *SSD1* (p < 0.01) and that interact, either genetically or physically with Ssd1 based on BIOGRID (ver. 2.0.49). We find that *SSD1-V* down-regulates mRNA levels for two subunits of the prefoldin complex (Gim3 and Gim1). This may explain why *ssd1* genetically interacts with

Table 3. Differentially transcribed genes reported to interact with *SSD1-V*

Gene	Function	Interaction	Reference	p value
A. Genes up-regulated by *SSD1-V*				
SDS3	(Putative) transcriptional regulator; involved in transcriptional silencing and required for sporulation	Genetic	Vannier *et al.* (2001)	9.50E-11
ATG1 (APG1)	Protein ser/thr kinase required for vesicle formation in autophagy and the cytoplasm-to-vacuole targeting (Cvt) pathway	Physical	Ptacek *et al.* (2005)	1.00E-09
COG8	Required to maintain wild-type vacuolar morphology	Genetic	Collins *et al.* (2007)	3.25E-09
IES1	Subunit of the INO80 chromatin remodeling complex	Genetic	Collins *et al.* (2007)	1.35E-07
RPB8	RNA polymerase subunit(common to polymerases I, II, and III)	Genetic	Briand *et al.* (2001)	3.32E-07
DHH1	(Putative) DEAD box RNA helicase, stimulates mRNA decapping, coordinates distinct steps in mRNA function and decay	Genetic	Moriya and Isono (1999)	8.94E-07
DOT1	Involved in meiosis and transcriptional silencing	Genetic	Collins *et al.* (2007)	2.09E-06
SRO9	Cytoplasmic RNA-binding protein that associates with translating ribosomes; involved in heme regulation of Hap1p as a component of the HMC complex	Physical	Tarassov *et al.* (2008)	3.45E-06
HYP2	Translation initiation factor eIF-5A, possible role in translation elongation	Genetic	Zanelli *et al.* (2005)	3.70E-06
JNM1	Coiled-coil domain protein required for nuclear migration	Genetic	Wilson *et al.* (1991)	1.10E-05
SRB2	Subunit of the RNA polymerase II mediator complex	Genetic	Collins *et al.* (2007)	0.0004
SIT4	Type 2A–related protein phosphatase that functions in the G1/S transition of the mitotic cycle	Genetic	Stettler *et al.* (1993)	0.001
CKB2	Beta' regulatory subunit of casein kinase 2, a Ser/Thr protein kinase with roles in cell growth and proliferation	Genetic	Collins *et al.* (2007)	0.0029
MPT5	Member of the Puf family of RNA-binding proteins; involved in longevity, maintenance of cell wall integrity, and sensitivity to and recovery from pheromone arrest	Genetic	Kikuchi *et al.* (1994)	0.0036
B. Genes down-regulated by *SSD1-V*				
SAP30	Subunit of a histone deacetylase complex that is involved in silencing at telomeres, rDNA, and silent mating-type loci	Genetic	Collins *et al.* (2007)	3.20E-12
GIM3	Subunit of the heterohexameric cochaperone prefoldin complex, which binds specifically to cytosolic chaperonin and transfers target proteins to it	Genetic	Tong *et al.* (2004)	7.50E-06
YKE2 (GIM1)	Subunit of the heterohexameric Gim/prefoldin protein complex involved in the folding of α-tubulin, β-tubulin, and actin	Genetic	Collins *et al.* (2007)	1.11E-05
SDC1	Required for proper transcriptional silencing of rDNA, telomeric silencing	Genetic	Collins *et al.* (2007)	3.25E-05
NBP2	Interacts with Nap1, which is involved in histone assembly	Genetic	Ohkuni *et al.* (2003)	0.00052
NHP10	Protein related to mammalian high mobility group proteins; likely component of the INO80 complex	Genetic	Collins *et al.* (2007)	0.0006
PCL1	G_1 cyclin that associates with PHO85	Physical	Ptacek *et al.* (2005)	0.00094
YPT6	Highly homologous to the human GTPase, Rab6	Genetic	Li and Warner (1996)	0.04226

five of the six prefoldin subunit mutants (Tong *et al.,* 2004; Collins *et al.,* 2007). Among the most affected transcripts are two that encode other RNA-binding proteins (Dhh1 and Mpt5), which are up-regulated by *SSD1-V*. Both of these proteins promote RNA decay via the Ccr4 complex (Garneau *et al.,* 2007; Goldstrohm *et al.,* 2007). *DHH1* and *SSD1* functionally complement each other¤ and double mutants are synthetically lethal (Moriya and Isono, 1999). Mpt5 physically interacts with Dhh1 (Goldstrohm *et al.,* 2006) and genetically interacts with Ssd1 (Kikuchi *et al.,* 1994). Interestingly, *MPT5*, like *SSD1*, is a polymorphic locus in budding yeast (Kennedy *et al.,* 1995). In addition, loss of any one of these four genes (*ssd1, ccr4, mpt5*¤ or *dhh1*) is synthetically lethal with *swi4* (Kaeberlein and Guarente, 2002), which

encodes a transcription factor that is rate-limiting for the G1-to-S transition (McInerny *et al.,* 1997).

Transcripts That Influence Replicative Life Span Are Affected by SSD1-V

One-quarter of the genes listed in Table 3, A and B, are involved in chromatin modification. Topping these lists are two proteins that associate with Rpd3 (*SDS3* and *SAP30*). Rpd3 is a histone deacetylase that influences transcription and silencing across the genome (Rundlett *et al.,* 1996). Both *rpd3* and *ssd1* mutations have been identified as antisilencing factors (Raisner and Madhani, 2008), and both influence replicative aging, but *rpd3* extends (Jiang *et al.,* 2002) and *ssd1* shortens RLS (Kaeberlein *et al.,* 2004). Interestingly,

Table 4. Overlap in gene expression profiles with *SSD1-V/ssd1-d*

Life-span–extending intervention	No. of genes differentially transcribed	Expression pattern	Overlap with *SSD1-V/ssd1-d* and the expression pattern[a]	
SSD1-V/ssd1-d [b]	95	49 up	8	7 up[1]; 1 dn
		46 dn	11	5 dn[2]; 6 up
High osmolarity[c]	117	50 up	11	7 up[3]; 4 dn
		50 dn	11	7 dn[4]; 4 up
Calorie restriction	124	114 up	19	15 up[5]; 4 dn
		10 dn	0	0
HAP4 overexpression	255	255 up	39	15 up[6]; 22 dn

[a] Genes affected: 1. *YBR131W (CCZ1)*, *YDR534C(FIT1)*, *YHL040C(ARN1)*, *YKR075C*, *YLL053C*, *YLR108C*, *YOR383C*; 2. *YDL043C(PRP11)*, *YKL096W(CWP1)*, *YLR200W(YKE2)*, *YMR193C-A*, *YPR078C*; 3. *YLR108C*, *YLR338W(OPI9)*, *YMR169C(ALD3)*, *YMR170C(ALD2)*, *YMR250W(GAD1)*, *YNR076W(PAU6)*, *YPL223C(GRE1)*; 4. *YBL028C*, *YDR152W(GIR2)*, *YDR469W(SDC1)*, *YGR030C(POP6)*, *YHR055C(CUP1–2)*, *YMR193C-A*, *YNL114C*; 5. *YDL105W(QRI2)*, *YDR290W*, *YDR440W(DOT1)*, *YDR534C(FIT1)*, *YEL065W(SIT1)*, *YHL040C(ARN1)*, *YIL084C(SDS3)*, *YIL177C*, *YLL011W(SOF1)*, *YLL062C(MHT1)*, *YNL088W(TOP2)*, *YOR306C(MCH5)*, *YOR374W(ALD4)*, *YOR383C(FIT3)*, *YPL058C(PDR12)*; and 6. *YBL099W(ATP1)*, *YGL149W*, *YGL261C(PAU11)*, *YIL084C(SDS3)*, *YJL102W(MEF2)*, *YJL182C*, *YJR157W*, *YLR338W(OPI9)*, *YLR422W*, *YMR007W*, *YOL131W*, *YOL166C*, *YPL061W(ALD6)*,*YPL172C(COX10)*, *YPL223C(GRE1)*.
[b] See Kaeberlein *et al.* (2004).
[c] This extends the life span of *ssd1-d* cells.

SDS3 is up-regulated ($p = 10^{-11}$), and *SAP30* is down-regulated ($p = 10^{-12}$) by *SSD1-V*. This suggests that the number or nature of Rpd3 complexes formed in *SSD1-V* cells may differ from that of *ssd1-d* cells. BIOGRID indicates that *SSD1* interacts with seven of the 12 subunits of the large Rpd3 complex.

SSD1-V is known to increase mean RLS in at least three genetic backgrounds (Kaeberlein and Guarente, 2002; Longo, 2003; Kaeberlein *et al.,* 2004). Early transcript microarrays identified 97 transcripts that differed by 1.5-fold or greater in exponentially growing *SSD1-V* versus *ssd1-d* cells (Kaeberlein *et al.,* 2004). None of the transcripts identified in that study have been implicated in life span determination. Only a few of those transcripts meet our criteria for differential expression in our *SSD1-V/ssd1-d* comparison (Table 4). These include four of the iron transport genes.

High osmolarity can extend life span (Kaeberlein *et al.,* 2002) and suppress the short RLS observed with *ssd1-d* cells (Kaeberlein *et al.,* 2004). Caloric restriction (CR) and Hap4 overexpression also increase RLS (Lin *et al.,* 2002). Thus we have looked for transcripts whose levels change twofold or greater in microarray data collected after these treatments (Kaeberlein *et al.,* 2002) and are differentially expressed in our study. Table 4 shows that there is about a 10% overlap between our results and these other studies, but none of these genes have been implicated in longevity.

To see if gene products that are known to influence RLS are affected by *SSD1-V* in W303, we looked for differentially expressed genes whose deletion or overexpression has been reported to increase RLS in yeast (Supplemental Table S2). Figure 4A shows the profiles for the four differentially expressed genes from our analysis ($p < 0.01$) that are known to influence RLS. The *SIR2* (Kaeberlein *et al.,* 1999), *MPT5* (Kennedy *et al.,* 1997) and *LAG1* (Jiang *et al.,* 2004) gene products promote longevity, and their mRNA levels are higher in *SSD1-V* cells. Loss of Cyr1 activity promotes longevity (Longo, 2003), and this mRNA is expressed at a lower level in *SSD1-V* cells. Any or all of these transcriptional changes could contribute to increased longevity of *SSD1-V* cells.

Autophagy genes are required for life span extension in worms and flies, and this conserved pathway may play a similar role in yeast (Powers *et al.,* 2006; Alvers *et al.,* 2009).

Autophagy plays an important role in the removal of damaged organelles and proteins, which accumulate in all ageing cells (Vellai, 2009). This process counteracts stresses (e.g., nutrient availability, oxidative damage) by engulfing cytoplasmic organelles and other constituents and transporting them to the vacuole for degradation and recycling (Vellai, 2009). We find that *ATG1* (Table 3) as well as five other autophagy-promoting genes are up-regulated by *SSD1-V* (Figure 4B).

By far the most striking global effect of *SSD1-V* is its down-regulation of transcripts involved in ribosomal structure and biogenesis. Deletion of a single ribosomal protein gene has been shown to extend life span (Kaeberlein *et al.,* 2005b; Chiocchetti *et al.,* 2007), so this global down-regulation would be expected to contribute to the longevity of this strain.

A Specific Assay for Chronological Life Span in Quiescent Budding Yeast

SSD1-V has a significant positive effect on RLS (Kaeberlein and Guarente, 2002; Longo, 2003; Kaeberlein *et al.,* 2004), and we have identified many expression changes that are likely to contribute to this increase in RLS. A possible role for *SSD1-V* in CLS, that is, the length of time that cells can survive in a nongrowing state, has not been tested. Moreover, SP cultures of the W303 *ssd1-d* strain survive longer in this nondividing state than many other strains, including BY4743, which carries the *SSD1-V* allele (Qin and Lu, 2006). This led us to wonder if *SSD1-V* was a significant variable in CLS.

The long-term survival of SP cultures that have exhausted their nutrients and stopped dividing has been used as a means to approximate the CLS of nondividing cells (Fabrizio *et al.,* 2001, 2003). However, a recent study has traced mortality in SP cultures to the accumulation of acetic acid in the media (Burtner *et al.,* 2009), so it is unclear how relevant this assay is as a model of aging in yeast. Moreover, these assays of SP cultures are typically carried out with auxotrophic strains. Starved auxotrophs have short but variable life spans, depending on the limiting nutrient (Henry, 1973; Boer *et al.,* 2008). They also have a nonuniform arrest (Saldanha *et al.,* 2004), accumulate more reactive oxygen species and DNA fragmentation (Gomes *et al.,* 2007)¤ and exhaust their
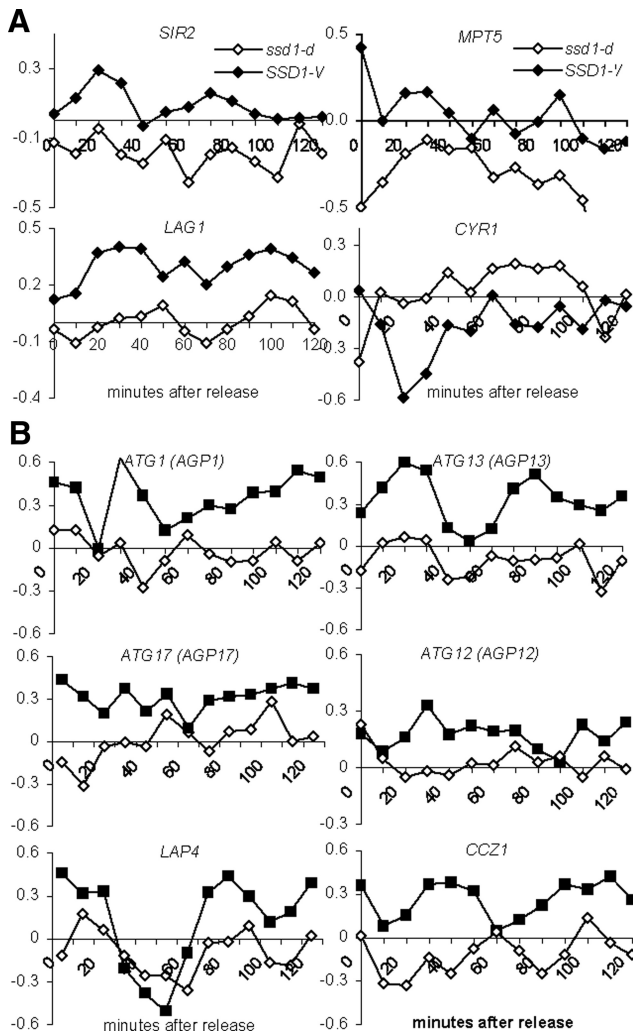
Figure 4. Differentially expressed transcripts that affect the life span extension of yeast cells are affected by *SSD1-V* (closed symbols) versus *ssd1-d* (open symbols). Log$_2$ ratios for each time point plotted using the transcript level in asynchronous populations as the denominator. (A) Microarray expression profiles of four transcripts that influence the RLS are affected in *SSD1-V* versus *ssd1-d*. (B) Microarray expression profiles of the six transcripts that influence autophagy that are up-regulated in *SSD1-V* versus *ssd1-d*.

glucose supply faster than prototrophs (Brauer *et al.*, 2006). In SP cultures, auxotrophs have been shown to be genetically unstable and rely on the DNA damage checkpoint to retain viability (Weinberger *et al.*, 2007). One potential solution to this problem is to add fourfold more of the required compounds than are typically added (Powers *et al.*, 2006; Wei *et al.*, 2008).

For comparison to this recent work, we assayed the long-term survival of SP cultures of *SSD1-V* and *ssd1-d* strains, both as auxotrophs and prototrophs, using fourfold oversupplementation of the compounds required by the auxotroph. Figure 5 shows the survival curves for *SSD1-V* and *ssd1-d* SP cultures in an auxotrophic (left) and prototrophic (right) background. The top panels plot the fraction of the population that can resume cell division and form colonies or CFUs. The bottom plots show the FungaLight viability assay (Molecular Probes, Invitrogen), which quantifies the fraction of cells with sufficient metabolic activity to maintain intact membranes and exclude propidium iodide. By both

assays, in auxotrophs and prototrophs, the *SSD1-V* allele enhances long-term viability compared with *ssd1-d*.

However, these studies also reveal a large and unexpected source of variability in these assays (Table 5). The auxotrophic cultures survive poorly compared with their prototrophic, but otherwise isogenic counterparts. The *ssd1-d* auxotroph retains 50% CFUs for 10 d, but the *ssd1-d* prototroph retains 50% CFUs until day 18. The same is true with the *SSD1-V* pair, but to a lesser extent. The auxotrophic *SSD1-V* culture retains 50% CFUs for 21 d, and the prototroph lasts 30 d. Hence, it is clear that colony-forming ability declines more rapidly in auxotrophs than prototrophs, despite the provision of fourfold excess of required compounds, and this decline is more rapid in auxotrophs carrying *ssd1-d*. In summary, this direct comparison shows that *SSD1-V* promotes long-term survival of SP cells, but prototrophy is also important for survival under these conditions. Auxotrophy has a significant, but variable, negative impact on the reproductive capacity of both strains, and this deleterious effect is not mitigated by oversupplementation of required compounds. We conclude that in order to assess the effect of Ssd1 or any gene product on survival after nutrient exhaustion, the assays must be performed in prototrophs.

Another variable that affects the life span of a SP culture is the fact that these cultures contain cells that are already dead and cells that are viable but cannot resume cell division (Herker *et al.*, 2004; Allen *et al.*, 2006). These cells obscure the properties of the quiescent cells in the population. To specifically assay the longevity of the Q cells, we have used a new purification procedure that separates Q cells from all others by virtue of their increased density (Allen *et al.*, 2006). The cause of this increased density is not understood, but it serves as a convenient property by which to purify a population of cells that retain all the features associated with quiescence. These Q cells arrest with G1 DNA content and maintain high levels of storage carbohydrates. They also show little or no indication of reactive oxygen species or apoptosis (Allen *et al.*, 2006). By assaying the long-term survival of purified, prototrophic Q cells, we can obtain a direct measure of CLS and assess the influence of mutations on the longevity of quiescent cells.

Repeated purifications show that the yield of Q cells is reproducible. However, Q cell yield is dramatically affected by the amount of time the cells are in SP culture before purification and by the genotype of the strain (Table 5). Q cell yield is characteristically higher and drops more slowly in SP cultures of *SSD1-V* cells than for *ssd1-d*. We have also purified Q cells from an *SSD1* deletion (*ssd1Δ*). The Q cell yield over time with the null allele was comparable to that of *ssd1-d*.

SSD1-V Increases the CLS of Q Cells

Q cells were purified from 1-wk-old SP cultures of prototrophic *SSD1-V* and *ssd1-d* cells. These cells were transferred to water and assayed for CFU and membrane integrity over 11 wk (Figure 6). *ssd1-d* Q cells lose reproductive capacity much more rapidly than *SSD1-V* cells, but they retain sufficient metabolic activity to exclude propidium iodide almost as well as *SSD1-V* cells. This indicates that *ssd1-d* is specifically defective in maintaining the long-term replicative capacity of Q cells, but it does not affect their ability to survive in a nondividing, senescent state. We have also assayed the longevity *ssd1Δ* and found little or no difference between the CLS of *ssd1-d* and the null mutant. We conclude that *ssd1-d* displays a phenotype similar to the null mutant in this assay.

Our results are summarized in Table 5. We find that purified Q cells live significantly longer than their respective SP cultures. Instead of dropping to 50% CFUs in 18 d, the *ssd1-d* Q
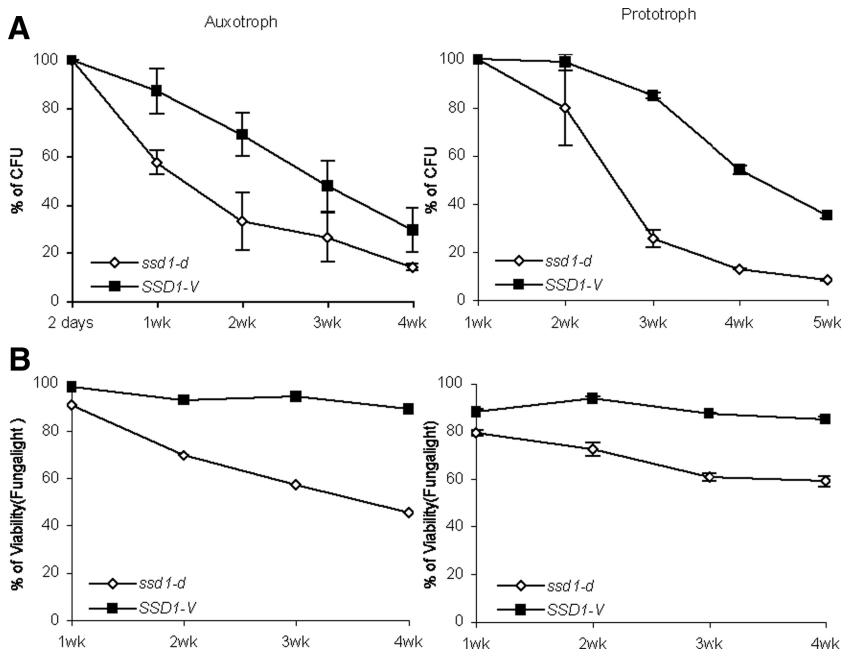
Figure 5. Comparison of the long-term survival of SP cultures of *ssd1-d* and *SSD1-V* in an auxotrophic and a prototrophic background. (A) Survival curves of *ssd1-d* versus *SSD1-V* were measured by colony forming units (CFU). Maximum CFUs for the culture was set as 100% survival. BY6500, prototrophic *ssd1-d* strains; BY6641, prototrophic *SSD1-V* strain; BY2125, auxotrophic *ssd1-d* strains; BY4260, auxotrophic *SSD1-V* strains. (B) The viabilities of both *ssd1-d* and *SSD1-V* in culture were determined by using Fungalight Yeast Viability Kit and plotted from 1 to 4 wk.

cells remain above 50% CFUs for ~33 d. The purified *SSD1-V* Q cells also double the life span measured for the SP culture, and again they surpass the longevity of *ssd1-d* cells by many weeks. We conclude that CLS assays carried out with prototrophic Q cells provide the most direct measure of longevity in the nondividing state. With this assay, we observe a significant enhancement of longevity by *SSD1-V*.

DISCUSSION

Ribosomal Genes Are Globally Down-Regulated by SSD1-V

By far the most striking effect *SSD1-V* has on transcript levels is the global down-regulation of those that are involved in translation. We see significant enrichment of the ribosome biogenesis genes among transcripts expressed at a lower level in *SSD1-V* cells, but only a minority of these transcripts is affected. In addition we find that all of the transcripts that encode cytoplasmic ribosomal (r) proteins are affected to some extent by *SSD1-V*. Interestingly, *SSD1* transcript levels are increased by oxidative and heat stress, and during SP (Gasch *et al.,* 2000).

All these conditions also lead to drops in r protein mRNA levels (Gasch *et al.,* 2000). This is consistent with the possibility that Ssd1 responds to a shift to adverse conditions and globally regulates translational capacity. However, the mechanism of this control is unknown. The promoter elements known to regulate translational machinery are not likely to be the targets, and there is no correlation with the mRNAs bound by Ssd1.

Ribosomal protein mRNAs are highly abundant and account for about one-third of the cell's mRNA (Holstege *et al.,* 1998) and 50% of transcription initiation events (Warner, 1999). In addition, most r protein mRNAs contain introns (Spingola *et al.,* 1999). The abundance of these intron-containing mRNAs has led to estimates that 90% of all splicing events occur on r protein transcripts (Warner, 1999). It follows that a global reduction of r protein mRNAs would significantly reduce the demand for splicing machinery. This probably explains why mutations in Fhl1, a positive regulator of r protein transcription, suppress splicing defects (Maddock *et al.,* 1994). We note that *SSD1-V* shares both the global reduction of r protein mRNAs and the ability to suppress a variety of splicing defects (Luukkonen and Seraphin, 1999).

Table 5. Summary of chronological life span results

	SP survival			Q cell yield (%)[a]		
	Aux[b]	Prot[c]	Q cell CLS Prot[d]	1 wk	2 wk	3 wk
SSD1-V	21 ± 3	30 ± 0.5	57 ± 5	77 ± 3	66 ± 5	48 ± 1
ssd1-d	10 ± 2	18 ± 0.5	33 ± 1	45 ± 9	20 ± 1	7 ± 5
ssd1Δ	n.d.	19 ± 5	41 ± 1	44 ± 2	23 ± 6	9 ± 4

n.d., not determined.

[a] Q cell yield from SP cultures grown for 1, 2, or 3 wk before purification.

[b] Days to 50% CFU in SP culture with auxotrophs.

[c] As in A, but with prototrophs.

[d] Days to 50% CFU with Q cells purified from 1-wk-old SP culture and resuspended in water at 30°C.
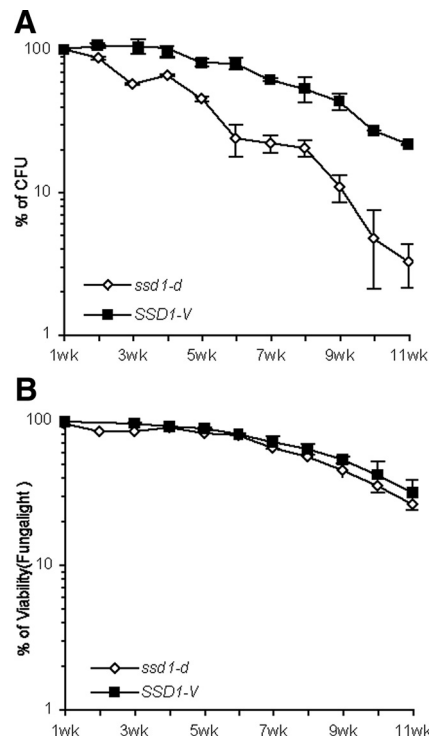
Figure 6. The CLS of Q cells purified from one week old prototrophic cultures of *SSD1-V* and *ssd1-d* cells. Survival of colony forming units plotted as a function of weeks after purification and resuspended in water. (A) Survival curves of Q cells from *ssd1-d* versus *SSD1-V* measured by CFUs. CFUs of Q cells after the first week in water was set as 100% survival. (B) The viabilities of Q cells from *ssd1-d* versus *SSD1-V* in water were determined using Fungalight Yeast Viability Kit and plotted.

Expression Changes in Transcripts That Influence Longevity

We have identified more than 100 transcript changes that could be contributing to the increased RLS that has been seen in *SSD1-V* cells (Kaeberlein *et al.,* 2004). Among the best studied are Sir2 and Mpt5, which are both expressed at higher levels in *SSD1-V* cells. However, both *sir2* and *mpt5* mutants have been tested and found not to be required for the RLS increase observed in *SSD1-V* cells (Kaeberlein and Guarente, 2002; Kaeberlein *et al.,* 2004).

Autophagy is a starvation-induced pathway for degrading and recycling cytoplasmic components that is highly conserved and important for longevity in many systems (Diaz-Troya *et al.,* 2008). We find that six of the 31 autophagy-related (ATG) yeast genes are up-regulated in *SSD1-V* cells. Among these are all three subunits (*ATG1, ATG17,* and *ATG13*) of a highly conserved kinase complex that is essential for the initial induction of autophagy in response to starvation (Diaz-Troya *et al.,* 2008). Atg1/17/13 complexes recruit other Atg proteins to a perivacuolar site where phagosomes are formed (Cheong *et al.,* 2008)¤ and its kinase activity is required for mobilization of these phagosomes (Cheong and Klionsky, 2008). The fact that all three subunits of this kinase are coordinately up-regulated by *SSD1-V* suggests that cells carrying *SSD1-V* may be more efficient at triggering the autophagic response and hence more protected from the stresses that evoke it.

As indicated above, the coordinate reduction in transcripts involved in translation is the most striking signature in the *SSD1-V* microarrays. Loss of even a single ribosomal

protein gene can significantly increase RLS (Kaeberlein *et al.,* 2005a). We have found that nearly all the r protein transcripts and 10% of the ribosome biogenesis transcripts are down-regulated by *SSD1-V* in our strain background. While these effects may not be as dramatic at the protein level, this global reduction would certainly be expected to contribute to the increase in longevity of *SSD1-V* cells.

It has been shown that mutations in several proteins involved in mRNA degradation show all the hallmarks of death by apoptosis in exponentially growing cells (Mazzoni *et al.,* 2003). It is not known whether this apoptosis is elicited by an excess of total mRNA, or by an excess of specific mRNAs that encode apoptosis-triggering gene products. We see no evidence of changes in mRNA levels for specific apoptosis regulators, but it is possible that the total load of mRNA is decreased in *SSD1-V* cells and this contributes to their increased longevity.

SSD1-V Is Important for the Long-Term Survival of Q Cells

Until recently, monitoring the long-term survival of SP cultures has been the only means to estimate the CLS of budding yeast. However, a surprising density shift of the quiescent population within an SP culture enables us to purify these Q cells and study their CLS and other properties in isolation (Allen *et al.,* 2006). A direct comparison of the Q cell CLS assay with the previous assay using SP cultures showed that the SP assay significantly underestimates the CLS of the quiescent population. Moreover, we find that the SP assay is further compromised by the use of auxotrophs. It is not surprising that starvation for amino acids, or other auxotrophic requirements would be deleterious. These compounds are normally produced by wild, prototrophic yeast. As such, wild yeast would not encounter such limitations and would not have evolved a protective response to them. However, the negative effects of auxotrophy that we observe do not appear to do be due to starvation for required compounds. Despite excess supplementation, the prototroph survives SP much longer than its parent auxotroph.

Even with prototrophs, only a fraction of the cells in a SP culture attain the quiescent state (Allen *et al.,* 2006). We also find that the yield of Q cells in these SP cultures diminishes with time in culture and varies with the genetic background. The gradual loss of Q cells from these dense SP cultures is probably not primarily due to the aging process, because there is an extrinsic factor, namely, the acidification of the medium, that appears to drive the loss of viability in SP cultures (Burtner *et al.,* 2009). However, understanding the genetic components that influence Q cell yield could be very important for understanding the mechanism by which cells attain the quiescent state. In addition, our new CLS assay, which follows longevity in a dilute suspension of purified quiescent cells, does not suffer from the same complexities seen with SP cultures and, as such, it offers a new tool for characterizing the chronological aging process.

With this assay, we have shown that *SSD1-V* promotes longevity in quiescent cells. We find that prototrophic *SSD1-V* Q cells have twice the CLS as prototrophic *ssd1-d* Q cells. Because *SSD1-V* also increases RLS, we conclude that *SSD1-V* plays a role in longevity in both dividing and nondividing cells. Consistent with this, we find that loss of *SSD1-V* does not disrupt the metabolic activities required to keep the membranes of nondividing cells intact. Rather, it specifically impairs a Q cell's ability to return to the cell division cycle and form a colony. This inability to resume cell division from quiescence may not be so different from the inability of an old mother cell to divide and give rise to

one more viable daughter cell at the end of her RLS. Hence, it is possible that Ssd1-v is one of a set of core activities that are required for both CLS and RLS. That function of Ssd1 may be difficult to identify because of the large number of transcript differences that are evident in the comparison of *SSD1-V* and *ssd1-d* cells, many of which have known effects on longevity. In this case it may be that the range of processes affected by *SSD1-V* will incrementally or synergistically affect the overall fitness and long-term viability of these cells.

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