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TITLE:

A Suppressor Screen for the Characterization of Genetic Links Regulating Chronological Lifespan in *Saccharomyces cerevisiae*

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KEYWORDS:

chronological aging, autophagy, *SIR2*, lysine deacetylase, suppressor screen, *Saccharomyces cerevisiae* copy number screen

SUMMARY:

Here is a protocol to identify genetic interactions through an increased copy number suppressor screen in *Saccharomyces cerevisiae*. This method allows researchers to identify, clone, and test suppressors in short-lived yeast mutants. We test the effect of the copy number increase of *SIR2* on lifespan in an autophagy null mutant.

ABSTRACT:

Aging is the time dependent deterioration of an organism's normal biological processes that increased the probability of death. Many genetic factors contribute to alterations in the normal aging process. These factors intersect in complex ways, as evidenced by the wealth of documented links identified and conserved in many organisms. Most of these studies focus on loss-of-function, null mutants that allow for rapid screening of many genes simultaneously. There is much less work that focuses on characterizing the role that overexpression of a gene in this process. In the present work, we present a straightforward methodology to identify and clone genes in the budding yeast, *Saccharomyces cerevisiae*, for study in suppression of the short-lived chronological lifespan phenotype seen in many genetic backgrounds. This protocol is designed to

be accessible to researchers from a wide variety of backgrounds and at various stages of education. The *SIR2* gene, which codes for a histone deacetylase, was selected for cloning in the pRS315 vector, as there have been conflicting reports on its effect on the chronological lifespan. SIR2 also plays a role in autophagy, which results when disrupted via the deletion of several genes, including the transcription factor *ATG1*. As a proof of principle, we clone the *SIR2* gene to perform a suppressor screen on the shortened lifespan phenotype characteristic of the autophagy deficient *atg1Δ* mutant and compare it to an otherwise isogenic, wild type genetic background.

INTRODUCTION:

Aging is the time-dependent loss of integrity in myriad biological processes that ultimately increases the probability of organismal death. Aging is nearly inevitable for all species. On a cellular level there are several well characterized hallmarks that are associated with aging, including: genomic instability, epigenetic alterations, loss-of-proteostasis, mitochondrial dysfunction, deregulated nutrient sensing, cellular senescence, and telomere attrition[1,2]. In single celled organisms, such as yeasts, this leads to a reduction in replicative potential and chronological life span[3,4]. These cellular changes manifest in more complex organisms, like humans, as pathologies that include cancers, heart failure, neurodegeneration, diabetes, and osteoporosis[5-7]. Despite the many complexities that characterize the process of aging, there is conservation of these molecular hallmarks underlying this process across widely divergent organisms[8-10]. Identification of alterations to these pathways during aging led to the realization that they can be manipulated via lifestyle changes – dietary restriction is shown to substantially extend lifespan in many organisms[11]. These pathways converge and intersect with each other and many other pathways, in complex ways. Elucidation and characterization of these interactions offers potential for therapeutic interventions to prolong lifespan and healthspan[12-14].

The conservation of the molecular underpinnings of aging allows for functional dissection of genetic interactions underlying the process through the use of simpler model organisms – including in the budding yeast, *Saccharomyces cerevisiae*[15,16]. There are two established types of aging modeled by budding yeast: chronological aging (the chronological lifespan, CLS) and replicative aging (the replicative lifespan, RLS)[17]. Chronological aging measures the amount of time that a cell can survive in a non-dividing state. This is analogous to the aging that is seen in cells that spend the majority of their life in G_0, such as neurons[4]. Alternatively, replicative lifespan is the number of times that a cell can divide before exhaustion and is a model for mitotically active cell types (e.g., the number of daughter cells that a cell can have)[18].

The overall goal of this method is to present a protocol that allows for the functional dissection of the genetics of aging using *S. cerevisiae*. While there have been many excellent studies performed by many researchers that have led to our current understanding, there remain many opportunities available for budding researchers to contribute to the aging field from early in their academic career. We present a clear methodology that will allow researchers further advance the field of aging. This protocol is designed to be accessible for all researchers regardless of the stage in their academic career by providing the tools necessary to formulate and test their own

novel hypotheses. The advantages of our approach is this is a cost effective method readily accessible to all researchers regardless of institution – and does not require expensive, specialized equipment necessary for some protocols[19]. There are several different ways to design this type of screen, the approach outlined in this work is particularly amenable to screening null mutants of non-essential genes that exhibit a severe reduction in the chronological lifespan compared to an isogenic wild-type strain of yeast.

As our proof of principle, we clone *SIR2*, a lysine deacetylase reported as exhibiting both an extended and a shortened CLS when overexpressed. *SIR2* overexpression was recently found to increase CLS in winemaking yeasts; however, several groups have reported no link between *SIR2* and CLS extension, leaving its role under characterized[20-22]. Due to these conflicting reports in the literature, we selected this gene to add independent research to help clarify the role of *SIR2* in chronological aging, if any. Additionally, increasing the copy number of a *SIR2* homologue extends lifespan in a nematode worm model system[23].

Autophagy is an intracellular degradation system to deliver cytosolic products, such as proteins and organelles, to the lysosome[24]. Autophagy is intimately linked to longevity through its role in degrading damaged proteins and organelles) to maintain cellular homeostasis[25]. Induction of autophagy depends on orchestrating the expression of many genes, and the deletion of the *ATG1* gene results in an abnormally short CLS in budding yeast[26]. *ATG1* codes for a protein serine/threonine kinase that is required for vesicle formation in autophagy and the cytoplasm-to-vacuole (the fungal lysosomal equivalent) pathway[27,28]. Here, we present our method for an increased copy number screen, testing the effect of increased *SIR2* copy on the CLS in a wild type and an *atg1*-null background. This method is particularly amenable to junior researchers and research groups at primarily undergraduate institutions, many of which serve communities underrepresented in the sciences and have limited resources.

PROTOCOL:

1. Identify potential genetic interactions for screening

1.1. Identify the genetic background(s) for characterization, that results in an abnormally shorted chronological life span (CLS) in *Saccharomyces cerevisiae* using the *Saccharomyces* Genome Database (the SGD, https://www.yeastgenome.org[29,30]), which compiles known phenotypic information for this organism.

1.1.1. Select the **Function** tab from the options on the top of the webpage.

1.1.2. Select **Phenotype** followed by selecting **Browse all Phenotypes**.

1.1.3. From the **Yeast Phenotype Ontology** options scroll to the **Development** subheading and select **Chronological Lifespan**, found under the **Lifespan** subheading.

1.1.4. Select the qualifier for **decreased**, which allows for the identification of genes that exhibit

a phenotype that results in a decreased chronological lifespan phenotype when deleted. For this proof-of-method *atg1Δ* was selected, which results in a short-lived CLS phenotype and is disrupted for autophagy[26].

1.2. Identify target gene(s) to screen for genetic interactions that may suppress the phenotype, based on reported or predicted ontology attributes, of the mutant identified in part 1.2. Repeat the phenotype search as found in steps 1.1.1-1.1.4 above, querying for genes that result in a longer CLS when overexpressed in a wild-type background. *SIR2* was selected based on the reported CLS phenotype and reported interactions with autophagy[31,32].

2. Prepare reagents

NOTE: Unless otherwise specified autoclave each solution at 121 ˚C for 20 min to sterilize prior to use.

2.1. YPAD liquid media: Add 1% Yeast Extract, 2% Peptone, 2% Dextrose (glucose), and 40 mg adenine (as adenine sulfate dehydrate) per liter of double distilled water. Mix well with a magnetic stirrer.

2.2. LB liquid media: Add Tryptone (10 g), Yeast extract (5 g), and Sodium Chloride (10 g) per liter of double distilled water. Mix well with a magnetic stirrer.

2.3. Prepare 1000x (100 mg/mL) ampicillin stock, of double distilled water. Mix well and filter sterilized.

2.4. Synthetic complete – Leucine (SC-LEU) liquid media: Add 1.7 g of Yeast nitrogen base w/o amino acids, 2% Glucose, 1.92 g of SC-LEU Dropout mix, 5 g of Ammonium sulfate per liter of double distilled water. Mix well with a magnetic stirrer.

2.5. TE buffer: Mix Tris (10 mM final concentration), EDTA (1 mM final concentration) in the solution with double distilled water. Mix well with a magnetic stirrer.

2.6. Prepare 50% PEG 3350 in solution with double distilled water. Mix well with a magnetic stirrer.

2.7. Prepare 1 M and 100 mM Lithium Acetate solution with double distilled water. Mix well with a magnetic stirrer.

NOTE: To make solid agar plates add 20 g of agar (per liter with double distilled water) to the media prepared in 2.1 and 2.2 above prior to autoclaving. If preparing ampicillin plates add 1mL of the ampicillin to the media in 2.2 above after it has cooled to roughly 60 ˚C. Pour into sterile plates and allow to set for 48–72 h prior to use. Store plates at 4 ˚C for longer storage.

3. Design the cloning strategy to clone *SIR2* into the pRS315 vector

3.1. Design PCR primers to amplify the *SIR2* gene for cloning into the pRS315 vector.

3.1.1. Design primers manually to have 21–22 nucleotide complementarity to the intergenic regions upstream and downstream of the *SIR2*. Ensure that the entire gene, along with the untranslated regions of the mRNA is cloned by mapping those features from the available datasets[33,34].

3.1.2. Ensure that the PCR primer design results in forward and reverse primers that have a melting temperature (Tm) above 53 °C and below 60 °C.

NOTE: Ideally, both primers should have a Tm as close to each other as sequence will allow, with an approximate GC content of between 40–50%, making sure to avoid dinucleotide repeats and balancing GC and AT distribution throughout the sequence.

3.1.3. After the design of the PCR primers that will allow for the generation of the amplicon for cloning, add restriction enzyme digestion (R.E.D.) target sites to the 5' end of each primer that are compatible to the plasmid-cloning vector. In this method, a HindIII restriction enzyme digestion site (5'-AAGCTT-3') is added to the upstream, forward primer and a SacII restriction enzyme digestion site (5'-CCGCGG-3') is added to the downstream, reverse primer.

NOTE: The use of the SacII and HindIII sites requires that the consensus cut site for each endonuclease is not present in the target gene. If either enzyme targets within the target gene, alternative restriction enzymes should be chosen. There are many that are compatible with the polylinker region on the pRS315 vector.

3.1.4. Lastly, add a four nucleotide (5'-NNNN-3') sequence overhang to the 5' end of each primer to allow the restriction enzyme to bind and digest the amplicon. Once the primers have been designed, have the oligonucleotides commercially synthesized for use cloning the *SIR2* gene.

3.1.5. Resuspension of the PCR primers: Centrifuge the PCR primers using a tabletop microfuge at maximum speed for 4 min. Add TE solution to make a stock concentration of 100 µM. Store the stock concentration at -20 °C and dilute 1/10 for use in PCR applications.

NOTE: To make a 100 µM stock, dissolve the primers in a volume of sterile TE buffer that is 10x the amount of nmoles in the primer tube, using microliters of TE. For example, if the tube contains 15.6 nmoles of primer, add 156 µL of TE buffer.

3.2. Isolate wild-type yeast gDNA for PCR amplification of the *SIR2* cloning construct.

NOTE: Several high-quality options are commercially available for isolating yeast gDNA. Utilization of a kit that includes the digestion of the fungal cell wall with zymolyase results in better quality gDNA (higher yield, less impurities). The protocol below consistently returns high concentration and purity. Details of a kit we recommend can be found in the Table of Materials.

3.2.1. Grow 5 mL culture of wild-type yeast for 48-72 h to post-log phase in enriched media, such as YPAD. Pellet the yeast cells at >800 *x g* for 3 min at room temperature, remove the growth media, and resuspend in 120 µL of zymolyase digestion buffer supplemented with 5 µL of zymolyase (2 units enzyme/µL). Mix the sample by vortexing and incubate at 37 °C for 40 min.

3.2.2. Add 120 µL of a chaotropic lysis buffer (e.g., guanidinium chloride), 250 µL of chloroform, and vortex the sample for 60 s.

3.2.3. Centrifuge at >8,000 *x g* for 2 min and transfer the supernatant into a purification column in a sterile collection tube.

3.2.4. Centrifuge at >8,000 *x g* for 60 s and discard the flow through. The gDNA will be bound to the column matrix.

3.2.5. Wash the column twice with 300 µL of an ethanol-based wash buffer, repeating the centrifugation step from above (3.2.4). Discard the flow through after each spin. Transfer the column into a 1.5 mL microfuge tube, add 60 µL of TE buffer, and incubate at room temperature for 60 s. Flash spin the sample for 30 s to elute the DNA.

NOTE: Determine the concentration of the DNA in the sample (Absorbance at 260nm) and the quality (Absorbance 260nm/280nm). A typical yield will be 100–200 ng/ µL of gDNA with absorbance ratio at 260nm/280nm as close to 1.8 as possible.

3.3. Amplify and isolate the pRS315 plasmid vector for cloning.

NOTE: Several high-quality options are commercially available for the purification of plasmid vectors. A silica-based column chemistry is recommended for this step. The changes noted below have led to the highest concentration and purity. Details are found in the **Table of Materials**.

3.3.1. Grow a 5 mL of culture of *E. coli* containing the pRS315 vector overnight in LB+ ampicillin (80 µg/mL) media. Pellet the culture by centrifugation at >8,000 *x g* for 2 min at RT (15–25 °C).

3.3.2. Re-suspend pelleted bacterial cells in 250 µL of TE buffer with RNase A (100 µg/mL) and transfer to a microcentrifuge tube. Ensure that no clumps of cells remain.

3.3.3. Add 250 µL of lysis buffer and mix by inverting the tube 6–8 times. Incubate for 5 min at room temperature. Do not allow lysis to proceed for more than 5 min – a little less is preferable.

3.3.4. Add 350 µL of neutralization buffer and mix immediately and thoroughly by inverting the tube 10 times. Centrifuge for 10 min at >8,000 *x g*.

3.3.5. Carefully transfer the supernatant from above to a silica spin column by pipetting. Centrifuge for 30 s and discard the flow-through.

3.3.6. Add 500 µL of a high salt wash buffer (e.g., a guanidine hydrochloride buffer) and centrifuge as in step 3.3.5. Discard the flow-through. Wash the DNA binding spin column by adding 750 µL of an ethanol-based wash buffer, to remove residual salts, and centrifuge as in step 3.3.5.

3.3.7. Discard the flow through and centrifuge for an additional 2 min at >8,000 *x g* to remove residual wash buffer. Place the spin column in a clean, labeled 1.5 mL microcentrifuge tube. To elute DNA, add 20 µL of TE buffer to the center of the spin column, incubate for 1 min at room temperature, and centrifuge for 1 min at >8,000 *x g*.

3.3.8. Use a spectrophotometer to determine the quantity (Absorbance at 260nm) and the quality (Absorbance 260 nm/280 nm) of DNA. A typical yield will be 1–2 µg/µL.

3.4. PCR amplification of the candidate gene, *SIR2,* from wild-type genomic DNA

3.4.1. To produce an amplicon that is suitable for cloning, utilize a high-fidelity (HF) PCR polymerase to avoid the unintentional generation of mutations into the sequence being amplified.

NOTE: Many different high-fidelity PCR options are commercially available. To facilitate the optimization of the PCR reaction conditions, use two-buffer combination: one that is a standard HF buffer and one optimized for high GC and complex amplicons. Details can be found in the **Table of Materials**.

3.4.2. PCR amplify the *SIR2* construct for cloning as described in **Table 1.**

NOTE: To maximize success in the cloning steps, multiple identical 50 µL can be set up and concentrated by a PCR column clean up step. Be sure to set up one no gDNA template control reaction (negative control).

3.4.3. Set up the PCR cycling conditions as described in **Table 2.**

NOTE: Different primer pairs vary on their annealing temperature and different polymerases function at different speeds. Make sure to optimize the amplification conditions based upon the enzyme selected and the specifications of the primer combination as designed.

3.4.4. Verify the success of the PCR reaction by visualizing the PCR reaction, which would produce an approximately 2.5 kb of DNA fragment, on a 1.0% TAE-agarose gel (with 0.5 µg/mL ethidium bromide for visualization).

3.5. Digestion and ligation of the candidate gene, *SIR2*, into the pRS315 plasmid vector.

3.5.1. Perform restriction digestion of the vector and the insert: 625 ng DNA (either the vector or insert), q.s. water to bring the final reaction volume to 50 µL, 5 µL of buffer, 1 µL SacII, and 1 µL

HindIII. Incubate the restriction digestions at 37 °C for 3 h, followed by 80 °C for 20 min to heat inactivate the enzymes. Digests can be stored at 4 °C prior to proceeding to the next step.

3.5.2. Set up a 15 µL ligation reaction to create the desired plasmid: 6 µL of sterile water, 2 µL digested vector (50 ng DNA), 4 µL digested insert (100 ng DNA), 2 µL T4 reaction buffer, and 1 µL of T4 DNA Ligase. Incubate the ligation reactions overnight at 16 °C, followed by 80 °C for 20 min to heat inactivate the enzyme.

NOTE: Set up a no insert control, substituting an additional 4 µL of sterile water (10 µL total) in lieu of the insert.

3.5.3. Transform the ligation reactions into *E. coli*.

NOTE: There are many options available for competent cells that are available. This protocol uses chemically competent cells that are stored at -80 °C prior to use.

3.5.3.1. Thaw a 50 µL tube of frozen, competent *E. coli* cells on ice until just thawed and immediately add 15 µL of the ligation reaction. Flick the tube several times. Immediately return the tubes to ice and incubate for 30 min.

3.5.3.2. Heat-shock the cells for 20 s in a water bath at exactly 42 °C, and immediately return the tubes to ice for a 2 min incubation. Add 450 µL of room temperature recovery media (e.g., SOC or LB) to each transformation reaction and incubate for 60 min at 37 °C with shaking.

3.5.3.3. For each transformation reaction, make a 1:10 dilution of cells. Using sterile technique, plate 150 µL of the undiluted cells and the 1:10 dilutions onto LB + (80 µg/mL) ampicillin plates[35]. Incubate the plates at 37 °C overnight.

3.6. Screen prospective transformants for the overexpression vector.

3.6.1. Using sterile technique, inoculate the potential transformants that grew into 5mL LB + (80µg/mL) ampicillin and grow overnight. Following the procedure outlined in section 3.3.1–3.3.7 above, isolate the plasmids from every potential transformant and screen for successful integration of the insert by restriction digestion followed by gel electrophoresis on a 1.0% TAE-agarose gel (with 0.5 µg/mL ethidium bromide for visualization).

4. Transform the vector into *atg1Δ* and wild type yeast strains

NOTE: This is performed using a modified lithium acetate transformation protocol[36].

4.1. Pellet 15 mL of wild type and *atg1*-null yeast cells grown overnight in YPAD media to early to mid-log phase (O.D. 600nm = 0.4-0.9) of growth for 3 min at > 800 *x g* at room temperature.

4.2. Decant the supernatant, re-suspend the cell pellet in 1 mL of sterile ddH_2O and transfer the contents to a 1.7 mL microfuge tube. Pellet the cells for 3 min at > 800 *x g* at room temperature.

4.3. Remove the supernatant and re-suspend the cells in 250 µL of 100 mM lithium acetate with gentle pipetting. Split the cells into separate microfuge tubes for each of the transformations that you will perform. Use 50 µL of the cell-lithium acetate mix per transformation.

4.4. Set up a transformation mix. To each of the transformations add: 240 µL of 50% PEG3350, 36 µL of 1.0 M Lithium acetate, and 5 µL of Salmon sperm (or other carrier) DNA, boiled for 5 min and on ice.

NOTE: PEG is very viscous. Pipette and measure carefully. Mix by pipetting after the addition of each component prior to moving on.

4.5. Add 5 µL of the appropriate plasmid for each transformation performed. Vortex each tube to mix thoroughly. Incubate the samples at 30 °C for 45 min. Heat shock samples at 42 °C for 10 min.

4.6. Pellet cells for 3 min at > 800 *x g* at room temperature, carefully remove the transformation mix, and re-suspend samples in 300 µL of sterile ddH_2O. Pellet cells by repeating the spin above, carefully remove water, and re-suspend your samples in 200 µL of sterile ddH_2O.

4.7. Set up 1/10 and 1/100 dilutions for each transformed strain of yeast.

4.8. Using sterile technique plate 150 µL from each sample onto SC-leucine plates to select for the plasmids. Spread the cells uniformly and evenly and allow the plate to dry before inverting and incubating at 30 °C to grow for 48–72 h.

NOTE: Once the appropriate strain is generated, it may be stored long term in 25% glycerol at -80 °C. The quantification of copy number present can be determined by several methodologies, including qPCR, RNA-FISH, or another appropriate measure[37,38].

5. Determine the chronological life span to test for shortened CLS phenotype suppression

5.1. Test the effect of overexpression of the putative suppressor on CLS in the short-lived yeast , *atg1Δ* mutant, by determining the number of colony forming units (CFUs) that remain as a function of time[39].

NOTE: It is necessary to set up this portion of the experiment with the appropriate controls. A typical experiment will compare a wild type (WT) strain of yeast with the empty vector, WT with the suppressor vector, the deletion mutant with the empty vector, and the deletion mutant with the suppressor vector.

5.1.1. Take a single colony of the strain to study and inoculate it into SC-LEU media. Grow the culture at 30 °C for 72 h, with shaking.

5.1.2. Using a hemocytometer, determine the concentration of cells that are present in the culture[40].

5.1.3. Dilute an aliquot of the culture, so that the result is a uniform number of cells in a 150 µL volume of sterile water. Plate the culture using sterile technique onto SC-LEU plates and grow at 30 °C for 72 h. These plates are the day three time-point and the experiment will be normalized to this time-point as 100% viability[39].

NOTE: The number of cells should be 200–500, sufficiently large for the analysis and a manageable number for counting. In this study, we used 200 cells for our plating quantity.

5.1.4. Continue to incubate the yeast cultures at 30 °C, taking regular aliquots and plating as outlined in 5.1.3. Continue this process until the strains are no longer viable, then compile and analyze the results.

NOTE: The complete list of strains used in this study are found in **Table 3**.

REPRESENTATIVE RESULTS

As there are conflicting reports on the role of *SIR2* during aging, we chose this gene for study as a potential suppressor of the *atg1Δ* mutant's shorten CLS phenotype[26]. The role of *SIR2* is somewhat controversial, with conflicting reports on its role in extending CLS, however it has been clearly linked to increased CLS in at least one yeast background, with a role in both autophagy and mitophagy[22,31,32,41].

Our choice of plasmid vector is the pRS315 shuttle vector, which was constructed for ease of genetic manipulation, propagation, and maintenance in both budding yeast and bacteria[42]. This vector contains the *LEU2* nutritional marker, the T7 and T3 promoters, and is a centromeric (*CEN*) vector for stable passage during cell division[42]. The stability of this vector across mitotic cell divisions were more desirable when compared to isogenic vectors that differed by nutritional marker[42]. The pRS315 vector also contains an autonomously replicating sequence, which combined with the *CEN* maintains low, consistent plasmid levels within (and across) a cell population[43].

The *SIR2* gene and the corresponding upstream (5' UTR) and downstream (3' UTR) genomic region's DNA sequence was obtained[33]. To ensure that the transcribed gene contained the necessary components UTRs for stability and translation of the protein product, our initial window was +/- 400 bp. This window expanded to +/- 500bp based on the nucleotide composition within this region, as our initial window did not result in a region conducive for cloning. We designed PCR primers that allow for the amplification of the gene and corresponding regulatory regions, incorporating restriction digestion sites and a four-nucleotide overhang added to the 5' end of each primer (**Figure 1A**). The successful amplification of this region by PCR

results in a DNA fragment 2.469 kb in length (**Figure 1B**).

SIR2 was amplified by PCR, and the products of this reaction were visualized by agarose gel electrophoresis and compared to a no template control reaction (**Figure 2**). *SIR2* amplification was seen in both lanes with the genomic DNA template; the two reactions were pooled and concentrated. Plasmid purification of pRS315 from *E. coli* was performed, which were visualized by agarose gel electrophoresis (**Figure 3**). The samples were quantified by spectrophotometry, and the plasmid prep from transformant #2 was selected for cloning, which had a concentration of 256 ng/µL ($OD_{260/280}$ = 1.87).

The plasmid and the insert were digested with HindIII and SacII, ligated together, and transformed into *E. coli* for amplification and screening. The choice of these restriction digestion sites required the verification that neither site is present in the region to be cloned. The presence of one (or both) sites would require the use of alternative restriction enzymes to clone the SIR2 gene, and there are several to select from in the pRS315 polylinker region[42].

We screened transformants for successful creation of the *pRS315-SIR2* vector by excision of the insert by double digestion with HindIII and SacII followed by the visualization on agarose gel (**Figure 4**). *pRS315-SIR2* vector was transformed into both wild-type and the *atg1Δ* mutant to generate the strains used for CLS characterization. The pRS315 vector is a classical vector that has been widely used and characterized, which is one of the advantages of this particular system. The relative copy number was determined by qPCR (**Figure 5**) as previously described[37]. This resulted in a modest increase in copy number from an average of one copy per cell to an average of 2.5 copies per cell, consistent with previous reports[42].

The chronological lifespan of *atg1Δ+pRS315-SIR2* was compared to an isogenic strain of yeast containing an empty vector instead of an insert (*atg1Δ+pRS315).* The aging cultures were plated at consistent, equivalent dilutions and were grown for 72 h at 30 ˚C prior to imaging and quantification (**Figure 6A**). The number of colonies forming units that grew was determined and normalized to the day 3 time-point (the first one taken) and plotted (**Figure 6B**). We report that there is no statistically significant effect of our *SIR2* construct on the CLS in the *atg1Δ* background. Additionally, we did not see any extension of the CLS in the wild-type background – where our modest *SIR2* overexpression actually produced a decrease in the CLS compared to the empty vector control (**Figure 6B**).

FIGURE LEGENDS:

Figure 1: Design of the construct to clone *SIR2*. The genomic region flanking the *SIR2* gene was utilized to design PCR primers for amplification and cloning of the gene. Primers contain 21nt of complementarity to the region 419 base pairs upstream of the gene (FP) and 351 base pairs downstream of the gene (RP), either the HindIII or SacII restriction digestion site, and a four-nucleotide overhang (**A**). The schematic of the amplicon created by PCR using the primers designed for cloning the *SIR2* genic region, along with the corresponding sizes (**B**).

Figure 2: PCR amplification of the *SIR2* gene visualized by gel electrophoresis. The products of a high-fidelity PCR reaction to amplify the *SIR2* gene were visualized on a 1% agarose gel (with ethidium bromide). Duplicate reactions were performed using yeast gDNA as a template (+) and compared to a no template control (-). The expected amplicon size is 2.469 kb.

Figure 3: Visualization of the pRS315 vector by gel electrophoresis. Duplicated purified plasmid reactions were performed and visualized on a 1% agarose gel (with ethidium bromide). The size of the pRS315 vector is 6.018 kb.

Figure 4: Screening for the *pRS315-SIR2* vector by gel electrophoresis. Potential transformants that contain the *pRS315-SIR2* vector were digested with HindIII and SacII, and then visualized on a 1% agarose gel (with ethidium bromide). Successful creation of the vector will yield a band at 5.963 kb (the pRS315 backbone) and 2.461 kb (the *SIR2* gene). Two potential transformants were compared to an empty vector control. Transformant #1 exhibits the pattern expected by the *pRS315-SIR2* vector.

Figure 5: The *pRS315-SIR2* vector increases *SIR2* copy number in a wild type yeast background. The number of *SIR2* copies present in the wild-type genetic background was determined by quantitative PCR. Values were calculated using the $2^{-\Delta\Delta Ct}$ method with *ACT1* selected as an internal control[44].

Figure 6: The chronological lifespan of *atg1Δ+pRS315-SIR2.* CLS was determined by quantification of the number of viable colony forming units as a function of time. Aging cultures of yeast were diluted to 500 cells/plate and grown for 72 h at 30 ˚C prior to imaging (**A**). Data was normalized to the day three time-point and plotted for visualization (**B**). EV is empty vector (pRS315 with no insert) and SIR2O/E contains the insert (*pRS315-SIR2*).

Table 1: PCR reaction components.

Table 2: PCR cycling conditions.

Table 3: Strains used.

DISCUSSION:

Unravelling the genetics of aging is a difficult challenge, with many opportunities for further study that can potentially yield significant insights into the complex interactions that exist. There are many methods that allow for the rapid generation of loss-of-function mutants for the study of null strains of yeast[45,46]. This method presents a straightforward approach to identify and clone genes onto the pRS315 vector for overexpression suppressor studies. One advantage to this approach is that this allows for a moderate overexpression from a stable vector, which can avoid any unforeseen challenges that could arise from the use of a chromosomal integration[47]. This approach is presented in a manner that will encourage the recruitment of researchers at various levels of their scientific career, with many of the authors on this publication contributing through the identification and cloning of putative suppressors as a component of their education.

In this work, we demonstrate how to use the wealth of data available compiled in the *Saccharomyces* genome database to identify a desired phenotype, in this case genetic links to an altered chronological lifespan. We cloned *SIR2* into the pRS315 vector to test the effect of moderate overexpression on the CLS of the short-lived autophagy deficient mutant, *atg1Δ*. Throughout a 17-day aging time-course there was no effect seen on the CLS in the autophagy mutant and a more accelerated CLS seen in the wild-type background. This can be interpreted as the modest copy number increase in SIR2 does not have an effect on CLS in the *atg1Δ* mutant background. As *ATG1* is a transcription factor necessary to induce autophagy, our conclusions are limited to initiation of the autophagy pathway. Additionally, we do not see an increase on the CLS in our wild type genetic background – perhaps suggesting that CLS extending phenotypes of increasing the copy number of SIR2 may be specific to certain genetic backgrounds and are not ubiquitous.

The critical steps within this protocol include the proper design of the SIR2 construct to clone, and the proper conditions to optimize ligation. Troubleshooting these steps may be necessary to clone a gene for characterization via the CLS assay. One limitation of this approach is that it selects for cells that retain the plasmid and that can re-enter the cell cycle. While this is a marker of fitness, it is essential for follow up study using complementary approaches to dissect the aging phenotype. This can include quantification of cell viability by vital dye staining as well as approaches that do not depend on plasmid retention. There are excellent methods available demonstrating further characterization of the CLS through the quantification of the outgrowth of aged cells or characterization of the replicative lifespan[48-50]. Additionally, our approach is limited to the identification of interactions that are non-lethal, and it would be challenging to differentiate a failed attempt to clone a gene with a successful attempt to clone a gene that results in a lethal phenotype.

Our approach is useful for the identification of gene putative genetic interactions for further study. It is simple and straightforward, and thus far, we used this approach to clone *SIR2*, *AIF1*, *UBI4*, and *MDH1* and are in the process of following up studies with each of these constructs. This technique can be applied to characterize any number of genetic interactions by following the protocol outline in this work.

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DISCLOSURES:

The authors declare that there is no conflict of interest.

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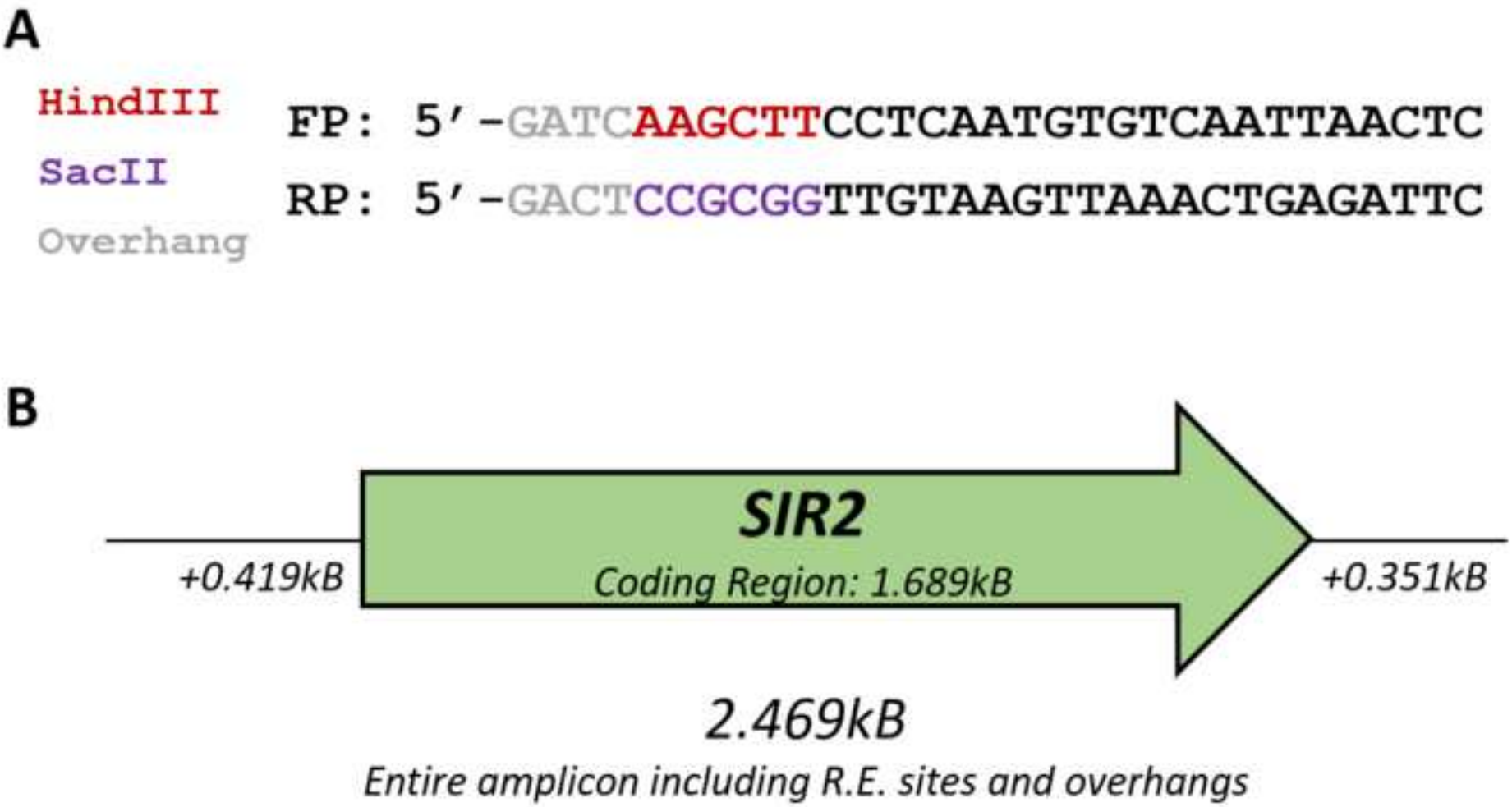

Figure 2

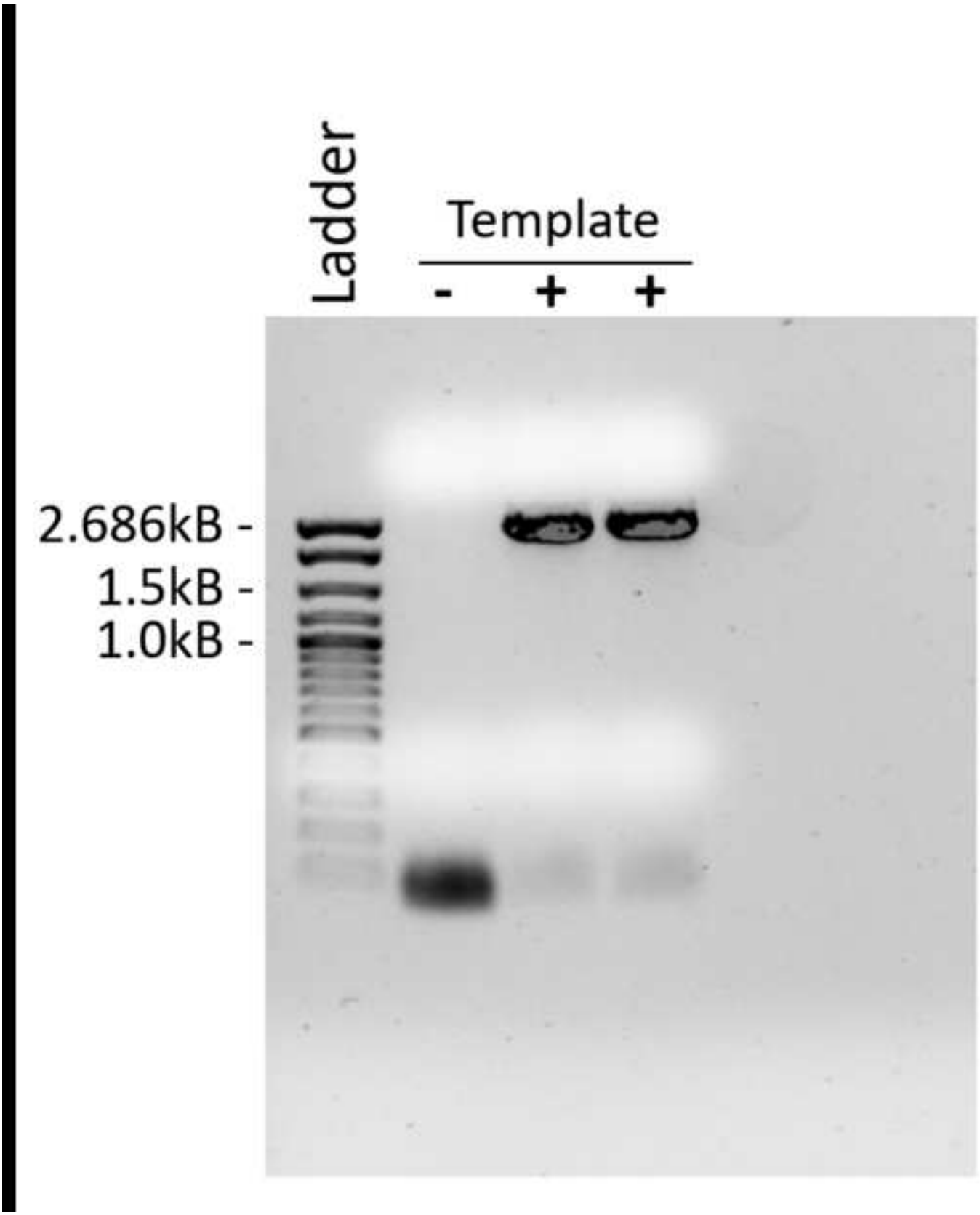

Figure 3

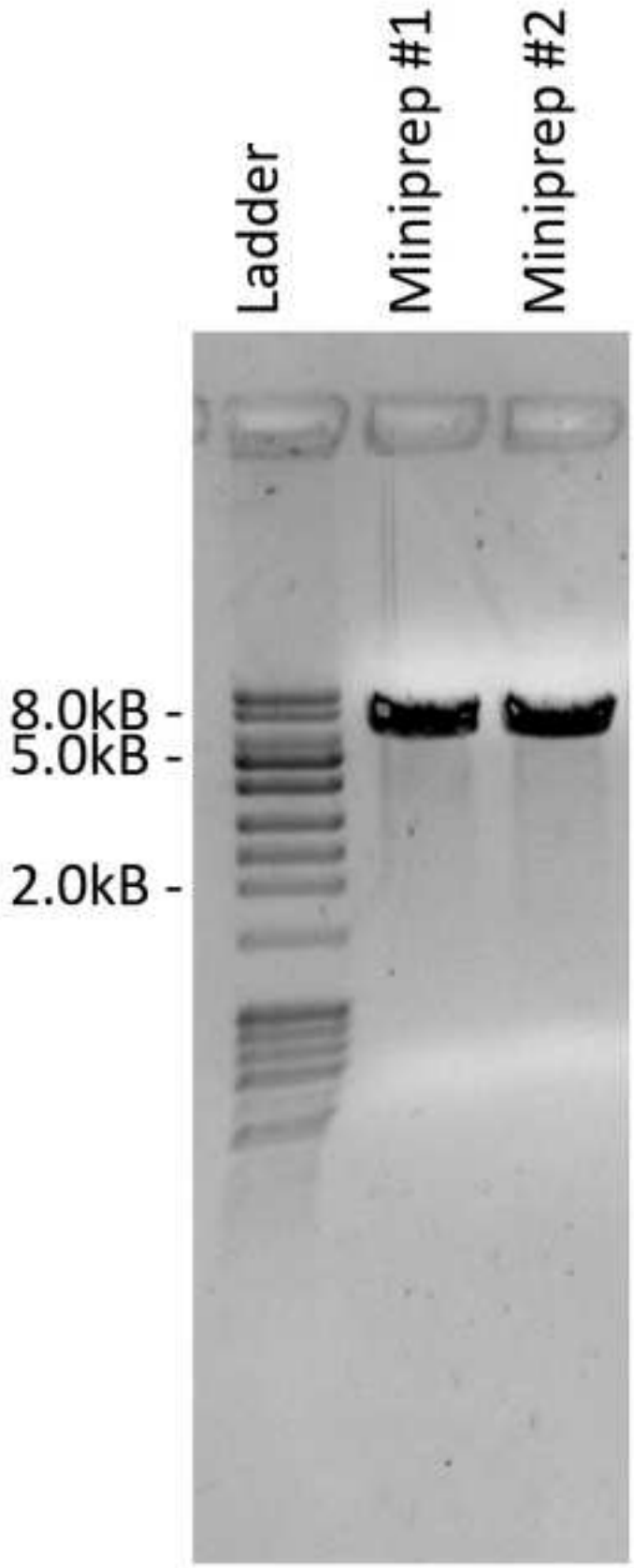

Figure 4

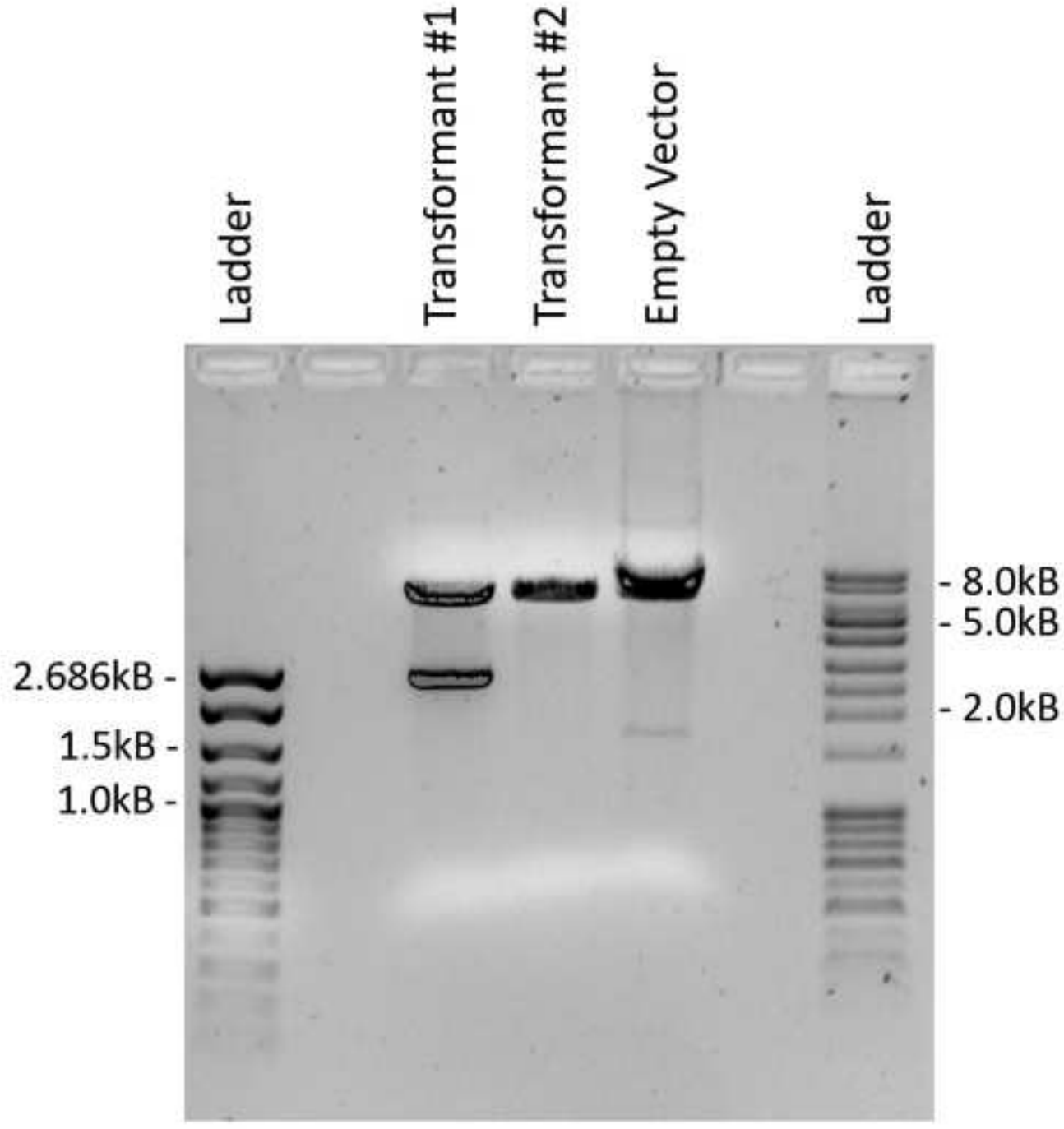

Figure 5

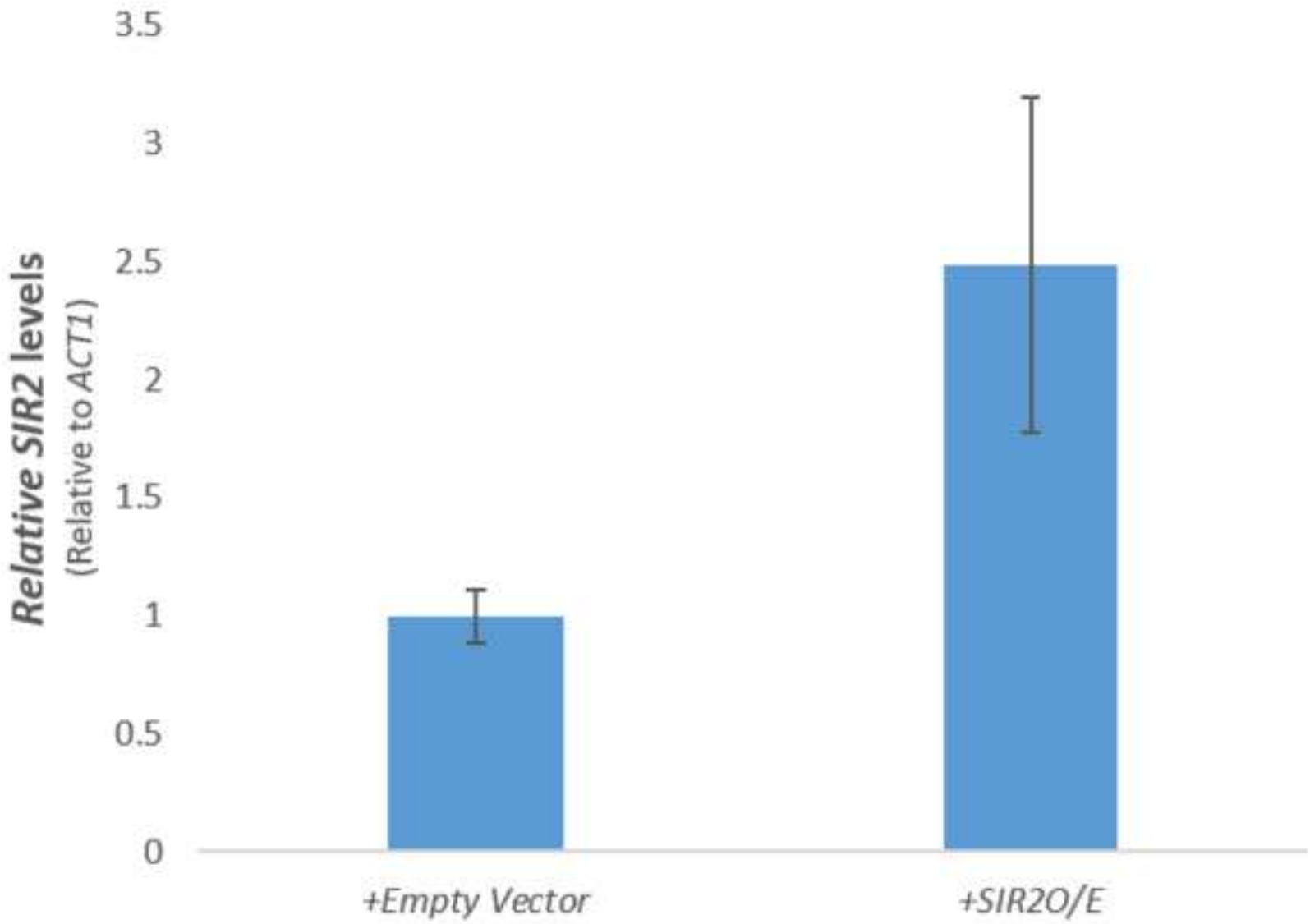

Figure 6

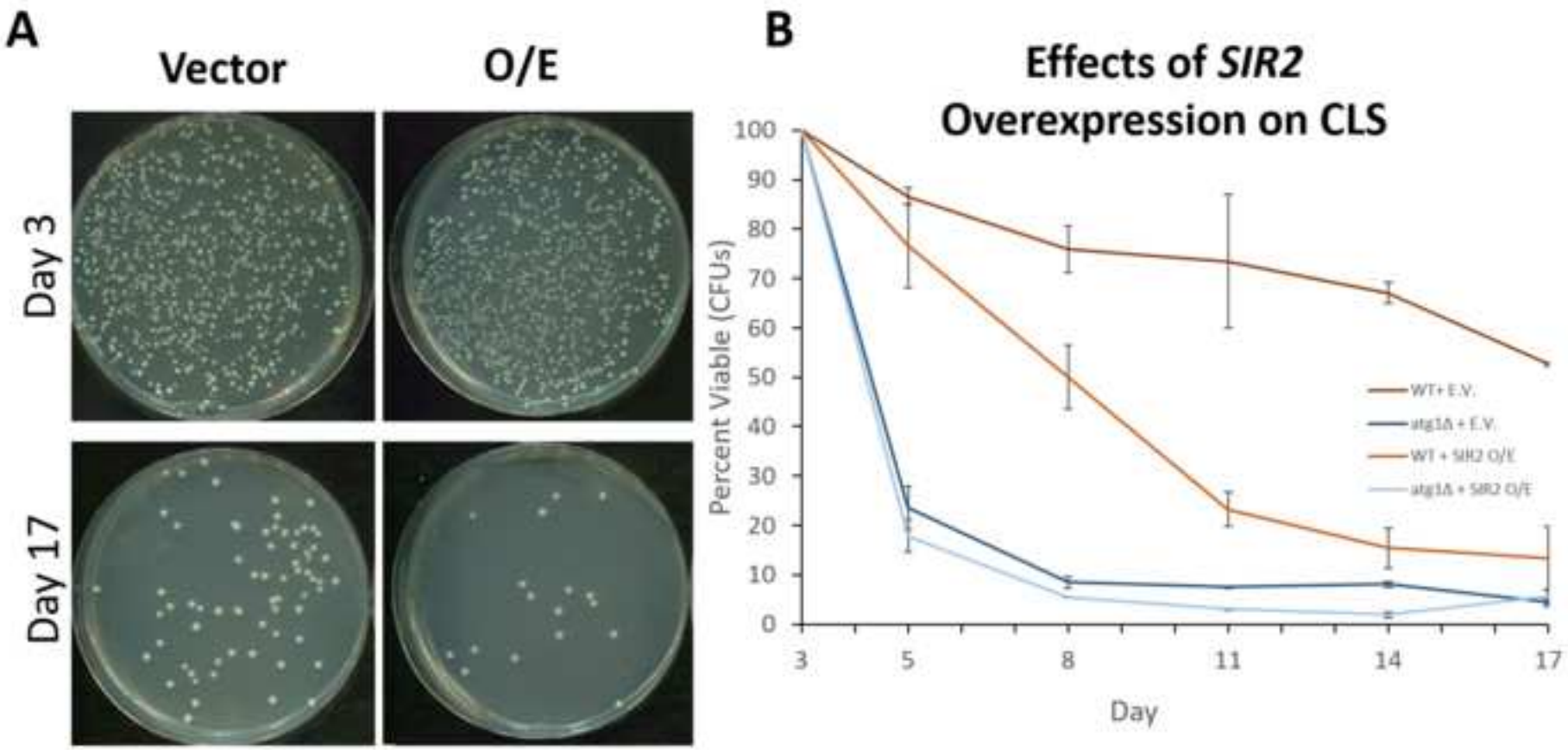

Table 1: PCR reaction components

Component	**Final Concentration**
Nuclease-free water	Q.S. to final volume
Buffer	1X
dNTPs (conc: 10mM)	200uM
Forward Primer (conc: 10µM)	0.5µM
Reverse Primer(conc: 10µM)	0.5µM
Template gDNA	100-200ng
HF polymerase	1 unit/50µL PCR

Table 2: PCR Cycling Conditions

Step	**Temp**	**Time**
Initial Denaturation	98°C	2mins
Cycling (35 cycles)	98°C 53-60°C (primer specific) 72°C	30s 30s 30s per kB
Final Extension	72°C	5-10m
Hold	10°C	indefinitely

Strain:

MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 + pRS315 (LEU vector)

MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 + pRS315-SIR2 O/E (LEU vector)

MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0atg1Δ + pRS315 empty vector (LEU vector)

MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0atg1Δ + pRS315-SIR2 O/E (LEU vector)

Parent:	**Ploidy:**
BY4741	Haploid
BY4741	Haploid
BY4741	Haploid
BY4741	Haploid

Name of Material/ Equipment	Company	Catalog Number
Fungal/Bacterial DNA kit	Zymo Research	D6005
HindIIIHF enzyme	New England Biolabs	R3104S
Phusion High-Fidelity DNA Polymerase	New England Biolabs	M0530S
Plasmid miniprep kit	Qiagen	12123
SacII enzyme	New England Biolabs	R0157S
Salmon sperm DNA	Thermofisher	AM9680
T4 DNA ligase	New England Biolabs	M0202S

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September 10, 2020

Dear Drs. Vineeta Bajaj and Jaydev Upponi and the editors at JoVE,

First and foremost, I hope that this email finds you all safe and well during this most concerning time!

I have read and addressed the concerns and suggestions from all of the reviewers and from the editors. You can find my modifications in the marked up manuscript that I have uploaded and I have responded to each one in a supplement that is attached to this letter. I believe that I have addressed all of the suggestions and that the manuscript is much stronger as a result. I also believe that some of the comments were outside of the scope of the protocol, and I have responded in kind. I would like to thank you all for your help and assistance throughout the preparation and revision of this manuscript, your patience is greatly appreciated. My co-authors and I eagerly await the outcome of this process and hope to continue this process for publication in JoVE.

Warm regards,

James T. Arnone, Ph.D.
Assistant Professor
Department of Biology
William Paterson University of New Jersey

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached file for revision.

Thank you – this has been done.

2. Please address specific comments marked in the manuscript.

Thank you, the authors have read and addressed the comments that are present throughout the manuscript.

3. Once done please highlight 3 pages of the protocol section including headings and spacings to be used for filming purposes. Please ensure that the highlighted section forms a cohesive narration for generating scripts for the video. Some of the shorter steps can be combined to have 2-3 actions per step if needed.

Reviewers' comments:

Reviewer #2:

Manuscript Summary:

Comments to the manuscript Dix et al., JoVE61506R1

All my previous concerns have been answered and I have no further objections. So, I recommend accepting it for publication.

It is certainly discouraging that the presented proof-of-method produces a negative result. Not only does the moderate overexpression of Sir2 fail to rescue the short CLS of the Datg1 mutant, as initially expected, but the same overexpression of Sir2 in a WT background results in a shortening of the CLS. I am aware that these results do not serve as proof-of-concept for the methodology described here, but despite this, as I also said in my first report, this work deserves to be published as it provides a methodological line for the identification of linked genes, which may be of interest to novel yeast researchers or to those who are beginning studies of the same biological process.

Major Concerns:

No

Minor Concerns:

No

The authors agree wholeheartedly with this reviewers' comments. As outlined in the manuscript, the selection of SIR2 was chosen because of the fact that it's links have been somewhat controversial in the published literature. Even though SIR2 plays a role in autophagy and CLS in some reports, it did not have that effect in our experiments… which is still noteworthy and deconvolutes the reported effects (it does not increase longevity is still

noteworthy, and its effects on life-span are outside of the autophagy pathway… verbiage to reflect this more clearly has been added upon revision.

Reviewer #3:
Manuscript Summary:
This revised paper is a great improvement over the original version. I have enjoyed reading the thorough responses to reviewers' comments, as well as the revised manuscript. I am satisfied with most of the responses and the revised version of the manuscript. This revision has addressed most of my concerns, as well as those raised by other reviewers.

Major Concerns:
None

Minor Concerns:
None

Thank you, the authors put a significant amount of work to address the concerns of reviewer #3 and all of the other reviewers as well. We appreciate the constructive feedback and that the manuscript is significantly improved as a result of these efforts.

Reviewer #6:
The manuscript writing has been improved significantly since the original submission, but the paper still suffers from a lack of scientific rigor. Again, there is not much here to advance the field that cannot be found in kit manuals, and the authors did not consider or fully address concerns in the original review. For these reasons, this manuscript is not suitable for JOVE that reaches a wide audience of biologists.

The authors take umbrage with this gross over-simplification. The manuscript does not suffer from scientific rigor, rather the reviewer demonstrates the lack of a thorough reading (or understanding) of the manuscript as a whole. We will address each of the major comments below, however the fact that there are multiple reports that have conflicting results is an important context. This paper clearly demonstrates that SIR2 O/E did not extend lifespan when present at higher copy numbers, and that there was no effect in the autophagy background (which is relevant to other published works). Both of these help to provide a clearer picture while providing a clear, straight-forward approach for researchers (of all ages) to make forays into the field.

Major Comments:
1. I understand now that the authors are attempting to capture the promoter sequence by cloning ~500bp upstream of the start codon. However, this does not guarantee over-expression of the

cloned gene of interest. First, 500bp may not be sufficient to capture all activating regulatory sequences (our PGK1 promoter is 983 bp).

The Nagalakshmi et al. 2008 reference is one that provides a database for an RNA-Seq experiment and the De Boer et al 2012 provides a thorough resource for TF and DNA-sequence interactions throughout the yeast genome on a global level. Both of these works have been cited in the revised submission and it is mentioned to ensure that the region targeted encompasses the relevant features. PGK1 has a larger promoter than most – it is an outlier. However, a thorough reading of this paper would have clearly resulted in this realization if the author wanted to clone this gene per the protocol. In fact, we chose a region bigger because of the sequence composition. This is a concern that was already addressed and no changes were made.

Second, as I am sure the authors know, not all promoters are active at all times, e.g. genes that are repressed during stationary phase and chronological aging. Cloning a repressed gene on a plasmid under its own cis-acting factors will not provide a further increase in expression (by the way, why not use a 2μ plasmid?).

This is true – the authors understand how promoters work. This concern would be more relevant had we taken the reviewers previous comments about chromosomal integration, where there are wide-spread position effects reported by our research group and many others, such as the Sternberg lab and the Gottschling lab (and many more that are too numerous to list here). The advantage of the plasmid vector chosen is that the assemblage of nucleosomes is not the same as in the chromosome – or as found on a 2 micron vector. This plasmid vector would result in a copy number increase – and per this reviewers suggestion we have modified the title accordingly. Additionally, the turnover of SIR2 is slower than, say the RPs and RiBis, which means that there would be higher levels of the protein and these would remain for a period during the stationary phase and CLS. Im sure that the author is aware that this is the case, and the autophagy null background would actually increase this effect. The reviewer is most likely also aware that SIR2 overexpression has been reported to interfere with Rpd3 regulated loci and produces many off target effects at high levels. The value of overwhelmingly high expression is of questionable value.

We believe that this concern is outside of the scope of the protocol and methodology.

A constitutive promoter upstream of the cloning site is needed, such as PGK1, TDH3, TEF1 (maybe ADH1, although this is now questioned) to ensure consistent over-expression under different growth and aging conditions. Here is a reference for the authors to consider (Peng, Microb Cell Fact 2015). I would be happy at some point to share our library of constitutive yeast expression vectors. Nonetheless, RT-PCR data is an absolute requirement to demonstrate over-expression of the cloned gene. Assuming your gene is being overexpressed is insufficient.

The reviewer has made a gracious offer to potentially share resources involving the use of **more "constitutively active" promoters. Per the aforementioned rationale, we are not sold on this being the best approach for this experiment in our hands – we have outlined an approach that should be called a copy number amplification, etc. We have changed the**

title of the manuscript to reflect this. ***The increased copy number has been verified and we have added a new figure (figure 5), with the old figure 5 renamed as figure 6. We believe that this addresses the confusion that we inadvertently caused this reviewer and appreciate the comments and the opportunity to do so. This will make the manuscript stronger.***

2. It is unfortunate that the authors cannot demonstrate a genetic rescue, as they have now made it clear that their SIR2 plasmid in itself shortens CLS (a concern I raised in my original review, if SIR2 levels are even increased at all). So how are we to know this technique works? Again, this is complicated by the lack of data demonstrating over-expression of their cloned construct. Have the authors vetted their technique by simply rescuing the Δatg1 strain with ATG1? At this point, seems to be a necessary internal control.

The number of copies of SIR2 has increased, which can be seen in the new figure #5 that is incorporated into the paper – this was necessary and we appreciate the reviewer's comments here. This would address the original concern and what we do show is consistent with published reports cited throughout the manuscript.

3. Several statements in the "Representative Results" section are inappropriate:

"Our initial, preliminary results indicated that SIR2 overexpression utilizing the pRS315 vector may offer a modest increase in the CLS of the short-lived atg1Δ strain of yeast, however this did not hold up to repetition."

If results are not reproducible, then it should not be published for the scientific audience. Since there are replicates reported in the new Fig. 5B, just eliminate this sentence.

This was meant to illustrate to the target readers the importance of the repetition, which was more appropriate with the first version which simply presented a lower resolution screen. Per this authors suggestion this sentence has been removed.

"Our modest SIR2 overexpression actually produced a decrease in the CLS compared to the empty vector control."

"Modest SIR2 overexpression" is not reported but assumed.

This has been modified to reflect the qPCR analysis of the copy number increase, so this is not an assumption upon revision.

When the authors are confident in their ability to over-express a gene of interest, then there is an obligation to interpret their findings. Instead, they just say "Additionally we did not see any extension of the CLS in the wild-type background." So does this mean that SIR2 does not operate in the same longevity pathway(s) as autophagy? Are there other interpretations?

We have revised the text in the manuscript, reflecting that modest copy number increase of SIR2 does not have an effect on longevity in the atg1-null background. We will not speculate

on every component within this pathway, but simply limiting our conclusions in a manner that is appropriate. These changes clarify the interpretation.

4. The cloning strategy should include a discussion of choosing your restriction sites, as SacII and HinDIII may interfere with cloning any particular gene of interest. Have the authors considered a less outdated cloning strategy, like USER or Gibson assembly?

We have specified this within the manuscript and further clarified this per the reviewer's confusion. This vector has what is called a polylinker region with many restriction sites. We selected these two for cloning, but they must not be present in the final amplified cloning region to work. If they are, it is simple to select other RED sites that do not interfere. That is one of the strengths of the vector. There are certainly other vectors that are possible for use – we chose one that is cost-effective and much better characterized than the other suggestions. It is certainly possible to adapt this to those should the user desire. We believe that our rationale was quite sufficient in the revised manuscript that was submitted.