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Authors

Tsang, Felicia

James, Christol

Kato, Michiko

et al.

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Reduced Ssy1-Ptr3-Ssy5 (SPS) Signaling Extends Replicative Life Span by Enhancing NAD^+ Homeostasis in *Saccharomyces cerevisiae*[*]

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Felicia Tsang, Christol James, Michiko Kato, Victoria Myers, Irtqa Ilyas, Matthew Tsang, and Su-Ju Lin[1]

From the Department of Microbiology and Molecular Genetics, College of Biological Sciences, University of California, Davis, California 95616

Background: Signaling pathways regulating NAD^+ homeostasis and their complex interplay with cell longevity remain unclear.
Results: Attenuated SPS (Ssy1-Ptr3-Ssy5) signaling extends replicative life span, which requires increased nicotinamide riboside salvage and functional NADH shuttle.
Conclusion: Enhanced NAD^+ homeostasis contributes to SPS-induced cell longevity.
Significance: Studying SPS signaling as a novel longevity factor helps elucidate the complex regulation of NAD^+ homeostasis.

Attenuated nutrient signaling extends the life span in yeast and higher eukaryotes; however, the mechanisms are not completely understood. Here we identify the Ssy1-Ptr3-Ssy5 (SPS) amino acid sensing pathway as a novel longevity factor. A null mutation of *SSY5* (*ssy5*Δ) increases replicative life span (RLS) by ~50%. Our results demonstrate that several NAD^+ homeostasis factors play key roles in this life span extension. First, expression of the putative malate-pyruvate NADH shuttle increases in *ssy5*Δ cells, and deleting components of this shuttle, *MAE1* and *OAC1*, largely abolishes RLS extension. Next, we show that Stp1, a transcription factor of the SPS pathway, directly binds to the promoter of *MAE1* and *OAC1* to regulate their expression. Additionally, deletion of *SSY5* increases nicotinamide riboside (NR) levels and phosphate-responsive (*PHO*) signaling activity, suggesting that *ssy5*Δ increases NR salvaging. This increase contributes to NAD^+ homeostasis, partially ameliorating the NAD^+ deficiency and rescuing the short life span of the *npt1*Δ mutant. Moreover, we observed that vacuolar phosphatase, Pho8, is partially required for *ssy5*Δ-mediated NR increase and RLS extension. Together, our studies present evidence that supports SPS signaling is a novel NAD^+ homeostasis factor and *ssy5*Δ-mediated life span extension is likely due to concomitantly increased mitochondrial and vacuolar function. Our findings may contribute to understanding the molecular basis of NAD^+ metabolism, cellular life span, and diseases associated with NAD^+ deficiency and aging.

Reduced nutrient signaling activity has been shown to extend life span in a variety of species, but the molecular mechanisms are not completely understood. Because of its short life span and well established molecular genetic techniques, the budding yeast, *Saccharomyces cerevisiae*, is an efficient model, enabling identification and study of novel longevity factors at the molecular level. In budding yeast, several conserved nutrient-sensing pathways such as the cAMP-PKA (cAMP-dependent protein kinase A), TOR (target of rapamycin), and Sch9/AKT (ortholog of mammalian S6 kinase) pathways, regulate longevity, and were shown to also play a role in low glucose-mediated calorie restriction (CR)[2] (1–3). Metabolic factors regulating the downstream processes of these pathways are likely to play pivotal roles in longevity signaling. Due to the complexity of these pathways, determining certain molecular aspects of the underlying mechanisms of longevity is difficult. Unraveling the cross-regulation of metabolic longevity factors, nutrient signaling, and their downstream targets will help elucidate the complex interplay of these factors.

NAD^+ (nicotinamide adenine dinucleotide) metabolism has emerged as a metabolic regulator of longevity. NAD^+ homeostasis is important for many cellular processes; NAD^+, and its reduced form, NADH, mediate essential redox reactions in cellular metabolism. In addition, NAD^+ and its derivatives function as substrates and signaling molecules in key cellular processes such as regulation of Ca^{2+} signaling, chromatin structure, DNA repair, circadian rhythm, metabolic responses, and life span (4, 5). In *S. cerevisiae*, NAD^+ is synthesized from two key intermediates, nicotinic acid mononucleotide and nicotinamide mononucleotide. Nicotinic acid mononucleotide is mainly produced via the *de novo* and nicotinic acid/nicotinamide salvaging pathways. Nicotinic acid mononucleotide is produced by transferring the phosphoribose moiety of phosphoribosyl pyrophosphate to salvaged (or imported) nicotinic acid or to *de novo* tryptophan-derived quinolinic acid, which is catalyzed by phosphoribosyltransferase Npt1 and Bna6, respectively (4). More recently, nicotinamide riboside (NR) was identified as an efficient NAD^+ precursor that is converted to nicotinamide mononucleotide by Nrk1 kinase in a branch of NR salvaging (6, 7). Functional NR and nicotinic acid/nicotinamide

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[1] To whom correspondence should be addressed: Dept. of Microbiology and Molecular Genetics, University of California, One Shields Ave., Davis, CA 95616. Tel.: 530-754-6081; Fax: 530-752-9014; E-mail: slin@ucdavis.edu.

[2] The abbreviations used are: CR, calorie restriction; NR, nicotinamide riboside; SPS, Ssy1-Ptr3-Ssy5; YPD, yeast extract/peptone/dextrose; RLS, replicative life span; qPCR, quantitative PCR.

salvaging pathways are essential for NAD$^+$ homeostasis and life span (1, 8, 9). To date, signaling pathways regulating NAD$^+$ homeostasis remain unclear because of the dynamic nature of these factors. Although the phosphate-responsive signaling (*PHO*) pathway has been connected to regulation of NR salvaging (10), detailed mechanisms underlying the cross-regulation of NAD$^+$ homeostasis, *PHO* and other nutrient sensing pathways are still unclear.

In this study we characterized a long-lived *ssy5*Δ mutant and showed that NR/NAD$^+$ homeostasis plays an important role in *ssy5*Δ-mediated life span extension. *SSY5* is part of the SPS (Ssy1-Ptr3-Ssy5) amino acid nutrient-sensing pathway (11, 12), which is inactive without extracellular amino acids (Fig. 1*A*, *left panel*). SPS pathway is activated when Ssy1 senses extracellular amino acids and sends a signal via conformational changes that trigger subsequent phosphorylations of Ptr3 and Ssy5 by the kinases Yck1 and Yck2 (Fig. 1*A*, *right panel*) (11, 13). Once phosphorylated, the prodomain and catalytic domain of Ssy5 disassociate. The Ssy5 catalytic domain then cleaves the N-terminal cytoplasmic retention domain of both transcription factors, Stp1 and Stp2 (11, 14, 15). Without the cytoplasmic retention domain, Stp1 and Stp2 can bypass the surveillance of inner nuclear membrane ubiquitin ligase Asi complex components, Asi1, Asi2, and Asi3, entering the nucleus to modulate (activate or inhibit) SPS pathway downstream gene expression (15–17). Here we show that SPS signaling is a novel longevity factor and provide evidence linking SPS signaling to NAD$^+$ homeostasis. Our studies may advance the understanding of the interconnection and cross-regulation of NAD$^+$ homeostasis, nutrient-sensing, and longevity pathways.

EXPERIMENTAL PROCEDURES

Yeast Strains, Growth Media, and Plasmids—Yeast strain BY4742 *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* acquired from Open Biosystems (18) was used for this study. Rich media (YPD) and synthetic media were made as described (19). All gene deletions were generated by replacing wild type genes with reusable loxP-*Kan^r*-loxP cassettes as described (20). Multiple deletions were carried out by removing the *Kan^r* marker using a galactose-inducible Cre recombinase (20). The original *ssy5* mutant was identified as a temperature sensitive (*ts*) mutant (*ssy5^{ts}*, Ser-75 to Pro) in a screen for longevity genes carried out as described (21). In brief, we employed the *cdc25-10^{ts}* mutant in an accelerated cell death system to look for genes that (when mutated or overexpressed) can extend the survival of this mutant. *CDC25* encodes a GTP-GDP exchange factor that activates Ras in the cAMP/PKA pathway in response to glucose (22). When shifted to non-permissive temperature at 38 °C, the *cdc25-10^{ts}* mutant exhibits phenotypes similar to G_0 stage cells and survives only ~3 days (21). We have previously identified Bmh1 as a longevity factor using similar screening conditions (21). In a pilot study for optimizing the screening condition, a colony carrying a library plasmid with a *LEU2* marker survived >3 days. However, this extended survival phenotype was not due to the plasmid but instead was due to an unknown *ts* mutation. The identity of this *ts* mutant was revealed by introducing WT *SSY5* using the genomic DNA library, which complemented the *ts* phenotype of *ssy5^{ts}*. Defective SPS signaling impairs leucine uptake; therefore, deleting components of SPS signaling is lethal in leucine auxotrophic backgrounds (23–25) such as BY4742. To make viable SPS mutants, a pPP81 plasmid carrying the *LEU2* gene was introduced before SPS gene deletion. As controls, wild type and other non-SPS mutants used in this study carry pPP81 (26).

Measurement(s) of NAD$^+$, NADH, and NR—Total intracellular levels of NAD$^+$ and NADH were determined using enzymatic cycling reactions as described (26, 27). Relative NR levels were determined by a liquid-based cross-feeding bioassay (8). To prepare cell extracts for intracellular NR determination, ~2.5×10^9 (~250 A_{600} unit) cells (donors of interest) grown to late log-phase (~16 h growth from an A_{600} of 0.1) were lysed by bead-beating (Biospec Products) in 800 μl of ice-cold 50 mM ammonium acetate solution. After filter sterilization, 16 μl of clear extract was used to supplement 8-ml cultures of recipient cells (the NR dependent *npt1*Δ*bna6*Δ*pho5*Δ mutant) (10) with a starting A_{600} of 0.05 in YPD. To determine NR release levels, the supernatant of the donor cell culture was collected and filter-sterilized, and then 1 ml was added to 7 ml of recipient cell culture with a total starting A_{600} of 0.05 in YPD. A control culture of recipient cells in YPD without supplementation was included in all experiments. After incubation at 30 °C for 24 h, growth of the recipient cells (A_{600}) was measured and normalized to the cell number of each donor strain. A_{600} readings were then converted to NR concentrations using the NR standard curve as previously described (8)

Replicative Life Span—All replicative life span (RLS) analyses were carried out on YPD plates with 50 cells per strain for each experiment (27) using a micromanipulator. Statistical analysis was carried out using the JMP statistics software (SAS), and the Wilcoxon rank-sum test *p* values were calculated for each pair of life spans.

Quantitative PCR (qPCR) Analysis of Gene Expression Levels—Cells were grown to log-phase or late log-phase in YPD (~6 or ~16 h of growth from A_{600} of 0.1). Total RNA was isolated using the RNeasy Mini kit, and cDNA was synthesized using Quantitect Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. For each qPCR reaction, 50 ng of cDNA and 500 nM concentrations of each primer were used. qPCR reaction was run on Roche LightCycler 480 using LightCycler 480 SYBR Green I Master Mix (Roche Applied Science) as previously described (28). Average size of the amplicon for each gene was ~150 bp. The target mRNA transcript levels were normalized to *ACT1* transcript levels.

Pho8-dependent Alkaline Phosphatase Activity Assay—The cell extract-based alkaline phosphatase activity assay was carried out as previously described (29) with modifications. ~2–3 A_{600} units of cells grown in YPD for 16 h were collected and washed in 0.85% NaCl with PMSF as described (29). The resultant cell pellet was then resuspended in 600 μl of lysis buffer (20 mM PIPES, 0.5%, Triton X-100, 50 mM KCl, 100 mM potassium acetate, 10 mM $MgSO_4$, 10 μM $ZnSO_4$, 1 mM PMSF) followed by bead-beating. Cell lysates were then centrifuged at 13,200 rpm for 5 min at 4 °C to collect supernatant. 200 μl of supernatant was added to 300 μl of reaction buffer (333 mM Tris-HCl, pH 8.5, 0.53% Triton X-100, 133 mM $MgSO_4$, 13.3 μM $ZnSO_4$, with/without 1.66 mM *para*-nitrophenyl phosphate) and then incu-

bated at 37 °C for 20 min. Reactions were stopped by adding 500 μl of stop buffer (1 M glycine/KOH, pH 11.0). Supernatants were then collected by centrifugation. The alkaline phosphatase activities were determined by normalizing A_{400} readings of colorimetric phosphatase product *para*-nitrophenol to cell number and expressed as 10^{-6} nmol of *para*-nitrophenyl produced/min/cell (29).

Chromatin Immunoprecipitation (ChIP) Assay—Log phase yeast cultures (400 ml) in YPD were induced with phenylalanine at 5 mM for 30 min (30). Induced cells were cross-linked with 1% formaldehyde for 30 min at room temperature and stopped by adding glycine to a final concentration of 125 mM. Cells were pelleted by centrifugation and washed two times with cold Tris-buffered saline. Cells were then suspended in 1 ml of FA-140 lysis buffer (50 mM HEPES, 140 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.1% sodium deoxycholate, 0.1 mM PMSF, 1× protease inhibitor mixture (Pierce)) (31) and lysed by bead-beating. The cell lysate was drawn off the beads and centrifuged at a maximum speed (13,200 rpm) for 30 min at 4 °C. The chromatin pellet was resuspended in 1 ml of FA-140 lysis buffer and sonicated on ice 8 times with 20-s pulses using a Branson 450 Sonifer (output control set at 1.5 and duty cycle held at constant) to shear chromatin to an average length of ~500 bp. Sonicated chromatin solution was centrifuged twice at 10,000 rpm for 10 min at 4 °C. The supernatant was then aliquoted into three tubes (labeled "input", "IP," and "no-Ab"). The IP sample was incubated overnight at 4 °C with anti-HA monoclonal antibody (ab1424, Abcam) at a dilution of 1:150. Both IP and no-Ab samples were incubated with 60 μl of ChIP-grade protein G beads (Cell Signaling Technology) for 2 h at 4 °C and then washed as described (31). DNA was then eluted from the beads 2 times with 125 μl of elution buffer (5× Tris-EDTA plus 1% SDS). The combined DNA solution and input samples were incubated at 65 °C overnight to reverse the cross-linking. The purified DNA samples were analyzed by qPCR, and results were compared with a standard curve prepared from input DNA. The amount of immunoprecipitated DNA from the experimental promoters (*OAC1*, *MAE1*, and *PHO8*), the positive control promoter (*AGP1*) (30), and the negative control (*ACT1*) (32) was determined relative to no-Ab DNA. The oligonucleotide sequences used are provided in Fig. 5*A*. S.D. were calculated from the results for three independent biological replicates.

RESULTS

Yeast Mutants with Reduced SPS Signaling Exhibit Increased Replicative Life Span—A temperature-sensitive *ssy5* loss-of-function mutant was identified in a screen for long-lived mutants (see "Experimental Procedures"). *SSY5* is part of the SPS nutrient-sensing pathway in *S. cerevisiae* (Fig. 1*A*) (11, 12, 33). We first examined whether deleting *SSY5* indeed extended life span. As shown in Fig. 1*B*, cells lacking *SSY5* exhibited an ~50% extension in RLS (cell division potential) compared with wild type (WT). To determine whether the observed life span extension was due to reduced SPS signaling activity, we analyzed mutants lacking specific SPS components. As shown in Fig. 1*B*, all SPS mutants examined, except for *stp2*Δ, showed significantly increased RLS ($p < 0.005$) compared with WT. *STP1* and *STP2* are homologous SPS downstream transcription factors that share overlapping function (30, 34). These results support that the RLS extension observed in the *ssy5*Δ mutant was due to overall reduction in SPS activity. Henceforth *ssy5*Δ was used as a positive control for RLS extension representative of reduced SPS pathway.

Lowered SPS activity may mimic nutrient restriction; thus, we tested whether *ssy5*Δ functions in previously characterized CR pathways to extend life span. As shown in Fig. 1, *C* and *D*, both moderate CR (0.5% glucose *versus* 2% glucose as the normal condition) (1, 27) (Fig. 1*C*) and a genetic mimic of CR, *cdc25-10* (1) (Fig. 1*D*), further extended the long life span of *ssy5*Δ cells. Therefore, reduced SPS activity and CR appeared to modulate life span by different mechanisms.

*Reduced Amino Acid Uptake Does Not Mimic the Long Life Span of ssy5*Δ—As an amino acid sensor, major downstream targets of SPS include several amino acid permeases (35), some of which have been shown to modulate life span (36). Lowered SPS signaling reduced the expression of these permeases (35), raising the possibility that the long life span of *ssy5*Δ was due to reduced amino acid uptake. To address this, we determined the RLS of deletions of amino acid permeases *AGP1*, *BAP2*, and *BAP3*, which are downstream targets of SPS. Agp1 is a low affinity broad range amino acid permease, which transports asparagine, glutamine, and other amino acids (37, 38). The paralogs Bap2 and Bap3 are branched-chain amino acid permeases that transport leucine, isoleucine, and valine (39, 40). Unlike *ssy5*Δ, both the single deletions (*agp1*Δ, *bap2*Δ, or *bap3*Δ) and triple deletion (*agp1*Δ*bap2*Δ*bap3*Δ) of these permeases did not significantly extend RLS (Fig. 1*E*). Therefore, reduced amino acid uptake mediated by these permeases was not responsible for the life span extension observed in *ssy5*Δ cells.

Components of NADH Shuttle System Are Required for SPS Signaling-mediated Life Span Extension—To understand how *ssy5*Δ extends RLS, we examined additional potential downstream targets of SPS. Several components of the putative malate-pyruvate NADH shuttle system (41) (Fig. 2*A*) were suggested to be transcriptionally regulated by SPS (35, 42).

The malate-pyruvate NADH shuttle system consists of mitochondrial enzymes (Mae1; Fig. 2*A*) and cytoplasmic enzymes (Pyc1/Pyc2 and Mdh2; Fig. 2*A*) (41). These enzymes produce permeable or small metabolites that can be transported via carrier proteins (Dic1, Oac1, and Mpc1/Mpc2; Fig. 2*A*) across the mitochondrial inner membrane (41, 43). Because some of these enzymes require NAD$^+$ or NADH for redox reactions, the NAD$^+$/NADH ratio is concomitantly balanced between the mitochondrial and cytoplasmic pools. Thus, this group of enzymes is described as a NADH shuttle although they do not shuttle NADH directly (41).

As shown in Fig. 2*B*, expression of these shuttle components indeed significantly increased in *ssy5*Δ cells. Expression of the negative controls, *MDH1* and *AAT1*, components of another shuttle (malate-aspartate shuttle), was not increased by *ssy5*Δ. Increased expression of *MAE1* and *OAC1* was further validated by Western blot analysis using HA-tagged Mae1 and Oac1 (Fig. 2*C*). Moreover, deleting both *MAE1* and *OAC1* largely abolished the life span extension in *ssy5*Δ cells (*ssy5*Δ *versus*

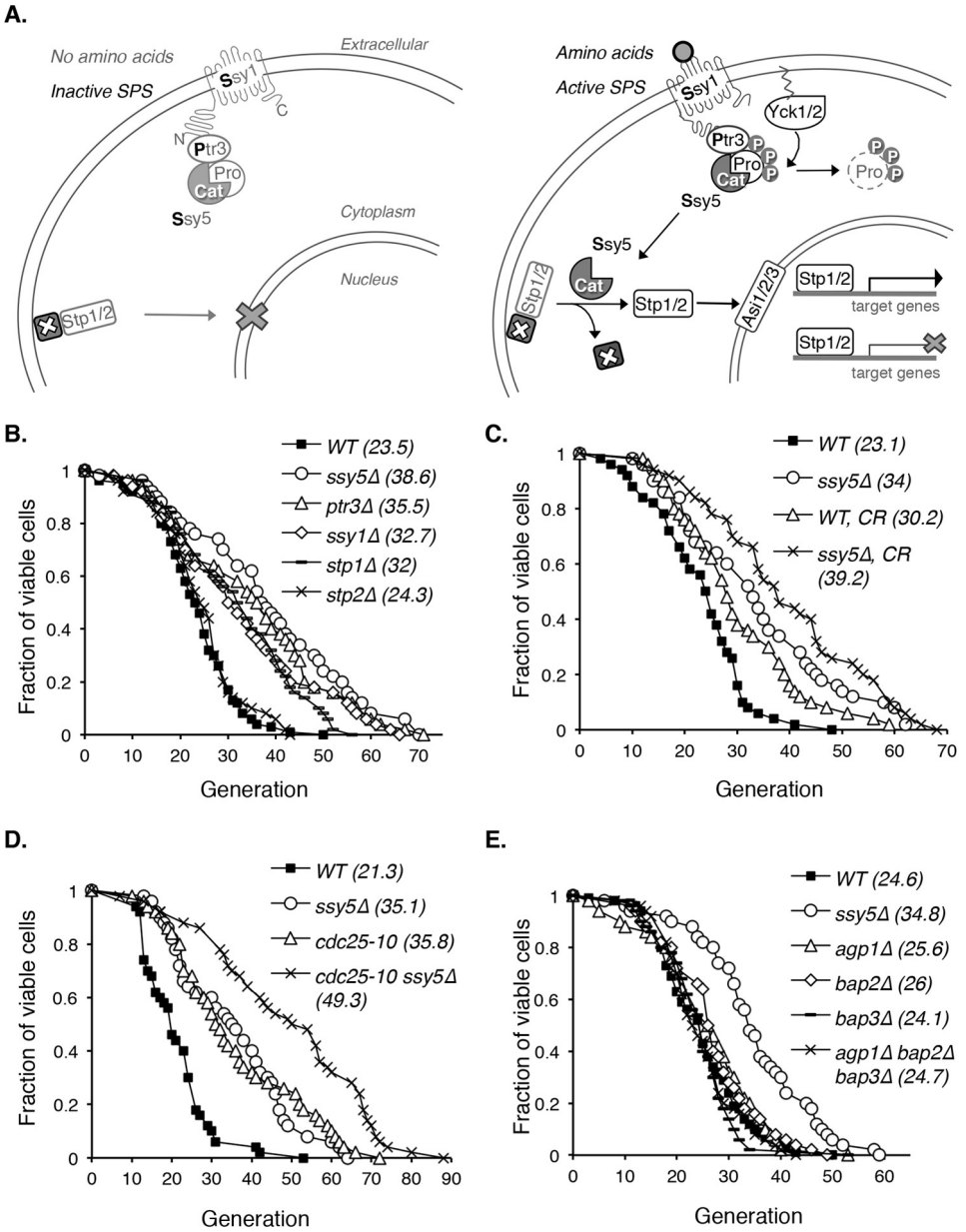

FIGURE 1. **Yeast mutants with reduced SPS signaling show increased replicative life span**. *A*, a simple model of the SPS nutrient-sensing pathway in *S. cerevisiae*. The *left panel* shows inactive SPS without amino acid stimulation. Upon activation by extracellular amino acids, SPS activates transcription factors Stp1 and Stp2 (*right panel*) to modulate (activate or inhibit) downstream gene expression. *Pro*, Ssy5 prodomain; *Cat*, Ssy5 catalytic domain; *P*, phosphorylation. *B*, deletions of various SPS pathway components extend RLS. The RLS of all mutants (except for *stp2*Δ) are significantly increased ($p < 0.005$) when compared with the WT. Δ: gene deletions. *C*, moderate CR further extends RLS in the SPS mutant *ssy5*Δ background (*ssy5*Δ *versus* *ssy5*Δ, CR; $p < 0.05$). *CR*, 0.5% glucose (*versus* 2% glucose in standard growth media). *D*, *cdc25-10*, a CR genetic mimic, further extends life span in the *ssy5*Δ background (*ssy5*Δ *versus* *cdc25-10 ssy5*Δ; $p < 0.005$). *E*, reduced amino acid uptake is not the main cause of RLS extension in *ssy5*Δ cells. Unlike *ssy5*Δ, deletion of SPS downstream amino acid permeases *AGP1*, *BAP2*, and *BAP3* does not extend RLS. Data shown are representative of multiple independent experiments. Statistical analysis of RLS is determined by the Wilcoxon rank sum test.

*ssy5*Δ*mae1*Δ*oac1*Δ; $p < 0.005$) (Fig. 2*D*), suggesting the malate-pyruvate NADH shuttle contributes to *ssy5*Δ-mediated RLS.

To discern the mechanism, we first examined the NAD$^+$/NADH ratio in *ssy5*Δ cells. As shown in Fig. 2*E*, the NAD$^+$/NADH ratio was not significantly changed by *ssy5*Δ, suggesting that the increased malate-pyruvate shuttle expression (Fig. 2*B*) did not alter the ratio. Nevertheless, the malate-pyruvate shuttle is entwined with the TCA cycle, which produces various biosynthetic intermediates including amino acid precursors (44). Due to reduced amino acid uptake (23–25), *ssy5*Δ may increase the malate-pyruvate shuttle expression (Fig. 2*B*) to produce amino acid precursors via the TCA cycle. This predicts increased demand for NAD$^+$ and NADH in *ssy5*Δ cells, prompting us to study whether SPS signaling modulates NAD$^+$ homeostasis.

*NR Production Contributes to NAD$^+$ Homeostasis in ssy5*Δ *Cells*—Because NAD$^+$ and NADH levels did not change in *ssy5*Δ cells (Fig. 2*E*), we determined the level of NR, a NAD$^+$ precursor shown to contribute to NAD$^+$ homeostasis and life span (7, 8). Interestingly, both intracellular and released NR levels were elevated in *ssy5*Δ cells (Fig. 2*F*), suggesting NR salvaging activity was increased, which may contribute to NAD$^+$

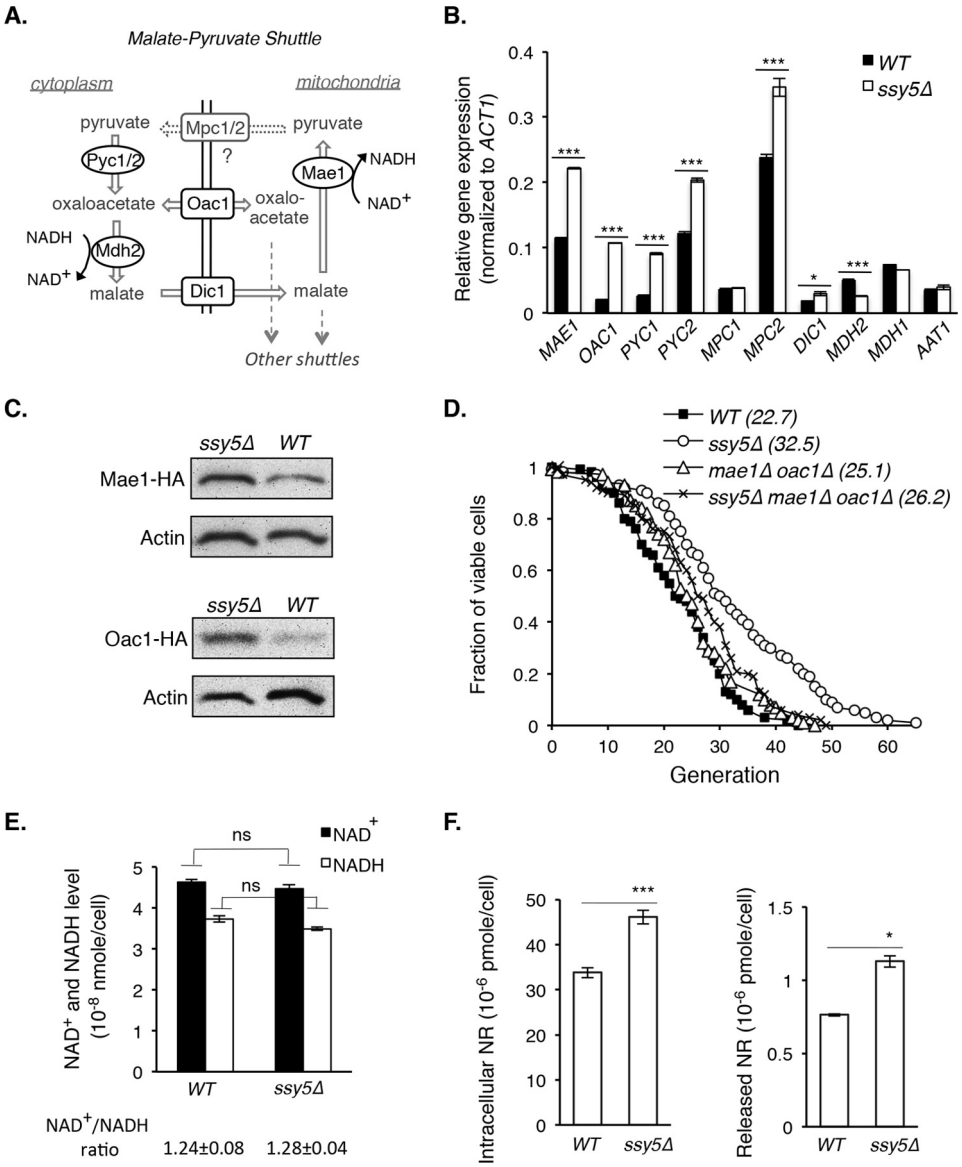

FIGURE 2. **SPS signaling-mediated life span extension requires components of NADH shuttle systems.** *A*, a simplified model of the putative malate-pyruvate NADH shuttle. Cellular NAD^+/NADH ratio is regulated by groups of cytoplasmic (*e.g.* Pyc1/Pyc2 and Mdh2) and mitochondrial (*e.g.* Mae1) enzymes, which produce permeable or small metabolites that can be transported (via carrier proteins Dic1, Oac1, and Mpc1/Mpc2) across the inner membrane. As a result, the NAD^+/NADH ratio and metabolites are balanced between the mitochondrial and cytoplasmic pools. *B*, gene expression of the malate-pyruvate shuttle components increases in the *ssy5*Δ mutant. Results show gene expression comparisons between WT and *ssy5*Δ cells determined by qPCR. *MDH1* and *AAT1* are components of another NADH shuttle, shown as a control. *C*, Mae1 and Oac1 protein levels increase in the *ssy5*Δ mutant. Results show Western blot analysis of HA-tagged Oac1 and Mae1 in both WT and *ssy5*Δ cells. *D*, deletions of both *MAE1* and *OAC1* largely abolish the life span extension by *ssy5*Δ (*ssy5*Δ *versus ssy5*Δ*mae1*Δ*oac1*Δ; $p < 0.005$). *E*, the levels and ratio of NAD^+ and NADH are not changed by *ssy5*Δ. *F*, the level of nicotinamide riboside (*NR*, a NAD^+ precursor) increases in *ssy5*Δ cells. Data shown are representative of multiple independent experiments. *Error bars* denote S.D. derived from triplicate samples. The *p* values were calculated using Student's *t* test (*ns*, not significant; *, $p < 0.05$; ***, $p < 0.005$) except for *D* (Wilcoxon rank sum test).

biosynthesis. To identify the source of elevated NR in *ssy5*Δ cells (Fig. 2*F*), we examined the gene expression of pathways known to produce and salvage NAD^+ and NR (8, 10, 45–47). Expression levels of the genes in NAD^+ synthetic pathways generally remained similar between WT and *ssy5*Δ cells as we expected (Fig. 3*A*), as no change in NAD^+ and NADH levels was observed between these strains (Fig. 2*E*). Therefore, the increased NR in *ssy5*Δ cells (Fig. 2*F*) was not likely from canonical NAD^+ biosynthetic pathways. Instead, elevated NR in *ssy5*Δ cells is more likely due to increased expression of the phosphate-sensing *PHO* pathway (35, 48, 49) components (Fig. 3*B*), some of which were previously shown to produce NR, and therefore contribute to NAD^+ homeostasis (4, 10).

Previously, NR supplementation was shown to rescue the short life span and reduced NAD^+ level of the *npt1*Δ mutant (7). Because we did not observe increases in NAD^+ and NADH levels by deleting *SSY5* in WT background, we examined whether increased NR in *ssy5*Δ cells would improve RLS and NAD^+ homeostasis in the *npt1*Δ mutant. First, we tested if *ssy5*Δ could rescue the short RLS of the *npt1*Δ mutant. As shown in Fig. 3*C*, deletion of *SSY5* rescued the RLS of *npt1*Δ mutant to WT level (*npt1*Δ *versus ssy5*Δ*npt1*Δ; $p < 0.005$). This

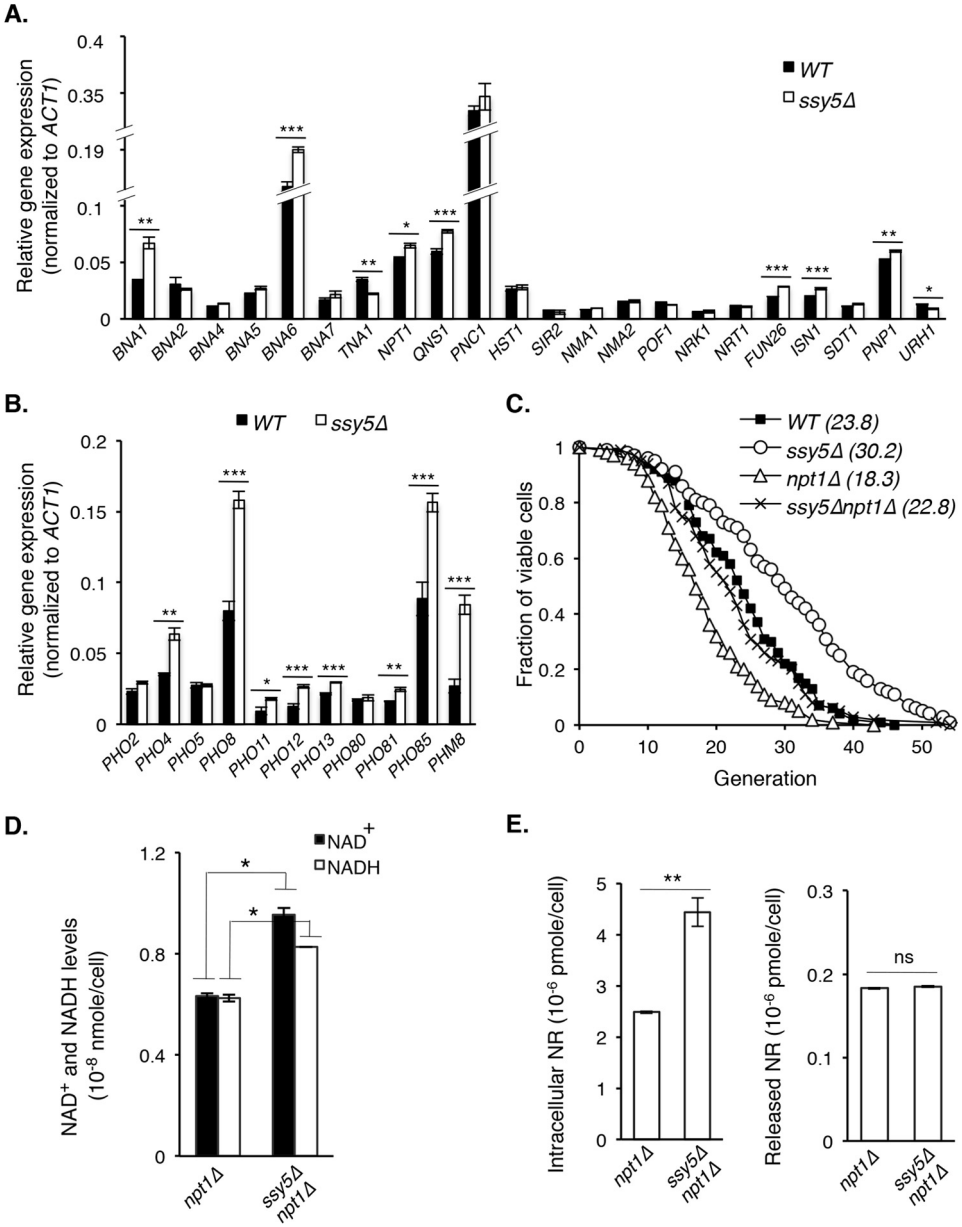

FIGURE 3. **NR production contributes to enhanced NAD$^+$ homeostasis in *ssy5*Δ cells.** *A*, overall gene expression of the NAD$^+$ biosynthetic pathway remains largely similar in *ssy5*Δ cells compared with WT. Results show relative gene expression (normalized to *ACT1*) determined by qPCR. *B*, deletion of *SSY5* increases gene expression of the phosphate sensing (*PHO*) pathway components. Results show gene expression comparisons between WT and *ssy5*Δ cells. *C*, deletion of *SSY5* rescues the short RLS of the NAD$^+$ biosynthesis-deficient *npt1*Δ mutant (*npt1*Δ *versus ssy5*Δ*npt1*Δ; $p < 0.005$). *D*, deletion of *SSY5* increases both NAD$^+$ and NADH levels in *npt1*Δ cells. *E*, deletion of *SSY5* increases intracellular NR levels in *npt1*Δ cells. Data shown are representative of multiple independent experiments. *Error bars* denote S.D. derived from triplicate samples. The *p* values were calculated using Student's *t* test (*ns*, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$) except for *C* (Wilcoxon rank sum test).

was accompanied by increases in NAD$^+$ and NADH levels (Fig. 3*D*) as well as intracellular NR levels (Fig. 3*E*) compared with the *npt1*Δ mutant. These results support that elevated NR in *ssy5*Δ cells contributes to NAD$^+$ homeostasis and plays a role in *ssy5*Δ-mediated RLS extension.

Reduced SPS Signaling Increases the Expression of Specific PHO Signaling and NADH Shuttle Components Independent of PHO2 and PHO4—Next, we sought to identify factors responsible for *ssy5*Δ-induced NR increase. A likely candidate was Pho8, whose expression was increased by *ssy5*Δ (Fig. 3*B*). Pho8 is a vacuolar phosphatase (50) shown to produce NR from nicotinamide mononucleotide (10). We examined Pho8 activity using cell lysates of WT and *ssy5*Δ cells as previously described (29). Indeed, *ssy5*Δ cells showed a slight increase in Pho8 activity (Fig. 4*A*). In addition, deleting *PHO8* abolished the intracellular (Fig. 4*B*, *left panel*) but not the released (Fig. 4*B*, *right panel*), NR increase in *ssy5*Δ cells and correspondingly mitigated the life span extension in *ssy5*Δ cells (Fig. 4*C*). Interestingly, deletion of both *PHO2* and *PHO4*, the transcription factors that regulate *PHO* pathway (49), did not block life span extension in *ssy5*Δ cells (Fig. 4*D*), suggesting that *ssy5*Δ regulates certain *PHO* downstream components independent of *PHO2* and *PHO4*. Indeed, as shown in Fig. 4*E*, *SSY5* deletion still increased *PHO8* expression in the *pho2*Δ*pho4*Δ back-

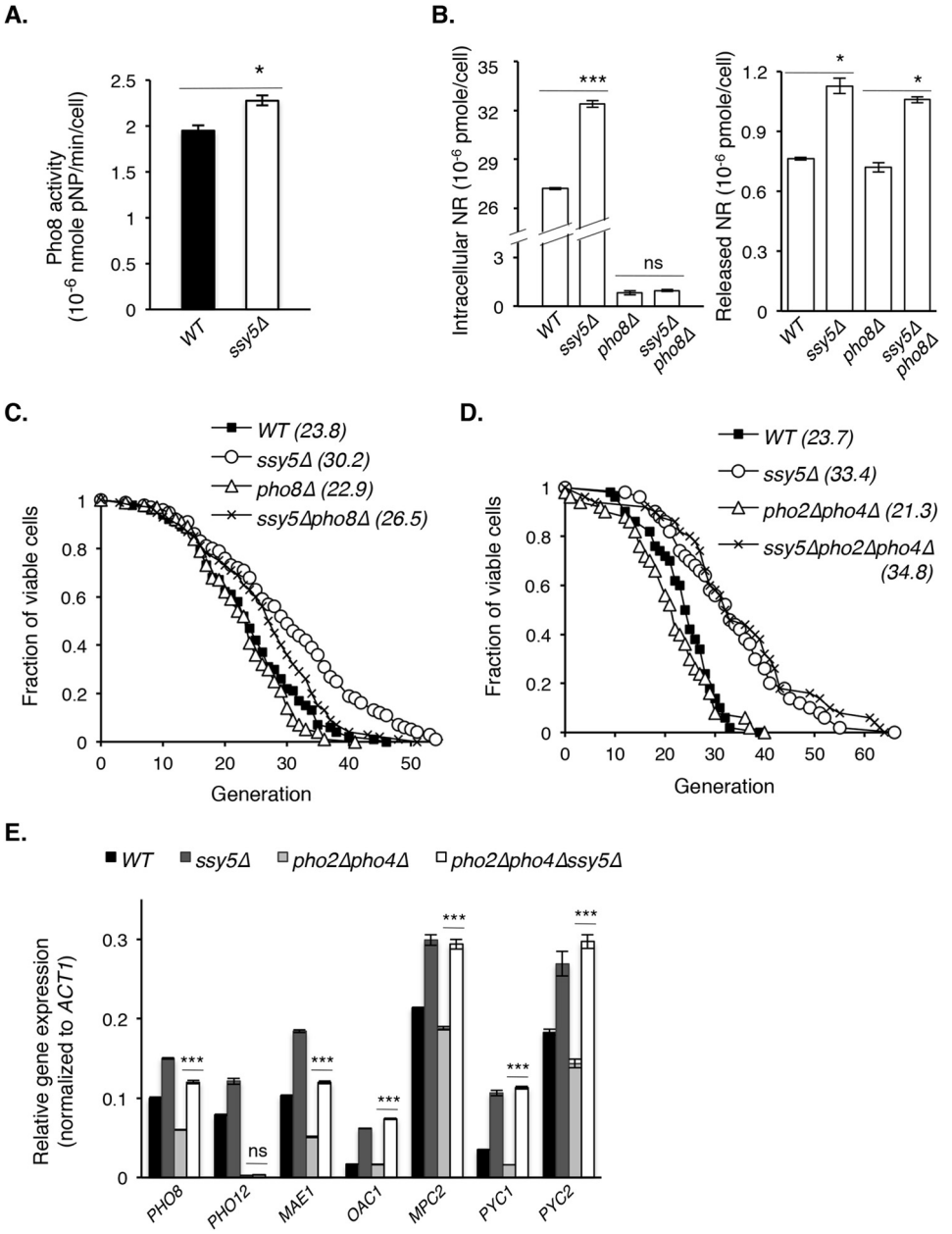

FIGURE 4. **Reduced SPS signaling increases the expression of specific *PHO* signaling and NADH shuttle components independent of *PHO2* and *PHO4*.** *A*, vacuolar Pho8 phosphatase activity is slightly increased in *ssy5*Δ cells. Results show Pho8 phosphatase activity using *para*-nitrophenyl phosphate as a substrate for cell lysate derived from WT and *ssy5*Δ cells. Pho8 activity is reflected by production rate of colorimetric *para*-nitrophenol (*pNP*), determined at A_{400}. *B*, deletion of *PHO8* abolishes intracellular NR increase in *ssy5*Δ cells (*left panel*) but not released NR, which mostly originates from cytosolic phosphatases (*right panel*). *C*, deletion of *PHO8* partially abolishes the life span extension in *ssy5*Δ cells (*ssy5*Δ *versus ssy5*Δ*pho8*Δ; $p < 0.05$). *D*, deletions of both *PHO2* and *PHO4*, transcription factors that regulate *PHO* pathway, do not alter the life span extension in *ssy5*Δ cells. *E*, *ssy5*Δ increases gene expression of *PHO8* and the malate-pyruvate shuttle components independent of *PHO2* and *PHO4*. Results show relative gene expression (normalized to *ACT1*) of WT, *ssy5*Δ, *pho2*Δ*pho4*Δ, and *ssy5*Δ*pho2*Δ*pho4*Δ cells determined by qPCR. Data shown are representative of multiple independent experiments. *Error bars* denote S.D. derived from triplicate samples. The *p* values were calculated using Student's *t* test (*ns*, not significant; *, $p < 0.05$; ***, $p < 0.005$) except for *C* and *D* (Wilcoxon rank sum test).

ground. In contrast, *PHO12* transcript (negative control) is under strict *PHO2/PHO4* regulation (51) and was undetectable in *pho2*Δ*pho4*Δ background (Fig. 4*E*).

In addition to *PHO8*, components of the malate-pyruvate shuttle were required to extend RLS in *ssy5*Δ (Fig. 2*D*). Although potential Pho2/Pho4 binding sequences (CACGTG) (52) were found in the promoter regions of *OAC1* and *PYC1*, deletion of both *PHO2* and *PHO4* did not block *ssy5*Δ-induced increases in gene expression of the malate-pyruvate shuttle components examined (Fig. 4*E*). However, the basal level expression of *MAE1* and *PHO8* decreased in *pho2*Δ*pho4*Δ background (Fig. 4*E*), suggesting some control by Pho2/Pho4. Overall, these observations are in line with the result that *ssy5*Δ extends RLS independent of Pho2 and Pho4 (Fig. 4*D*) and supports a role for NR and NAD^+ homeostasis in *ssy5*Δ-mediated RLS extension.

Stp1, a Transcription Factor of the SPS Signaling Pathway, May Directly Bind to the Promoter Regions of Shuttle System Components—In this study we show *PHO8*, *MAE1*, and *OAC1* are required for *ssy5*Δ-induced RLS (Figs. 2*D* and 4*C*) and that

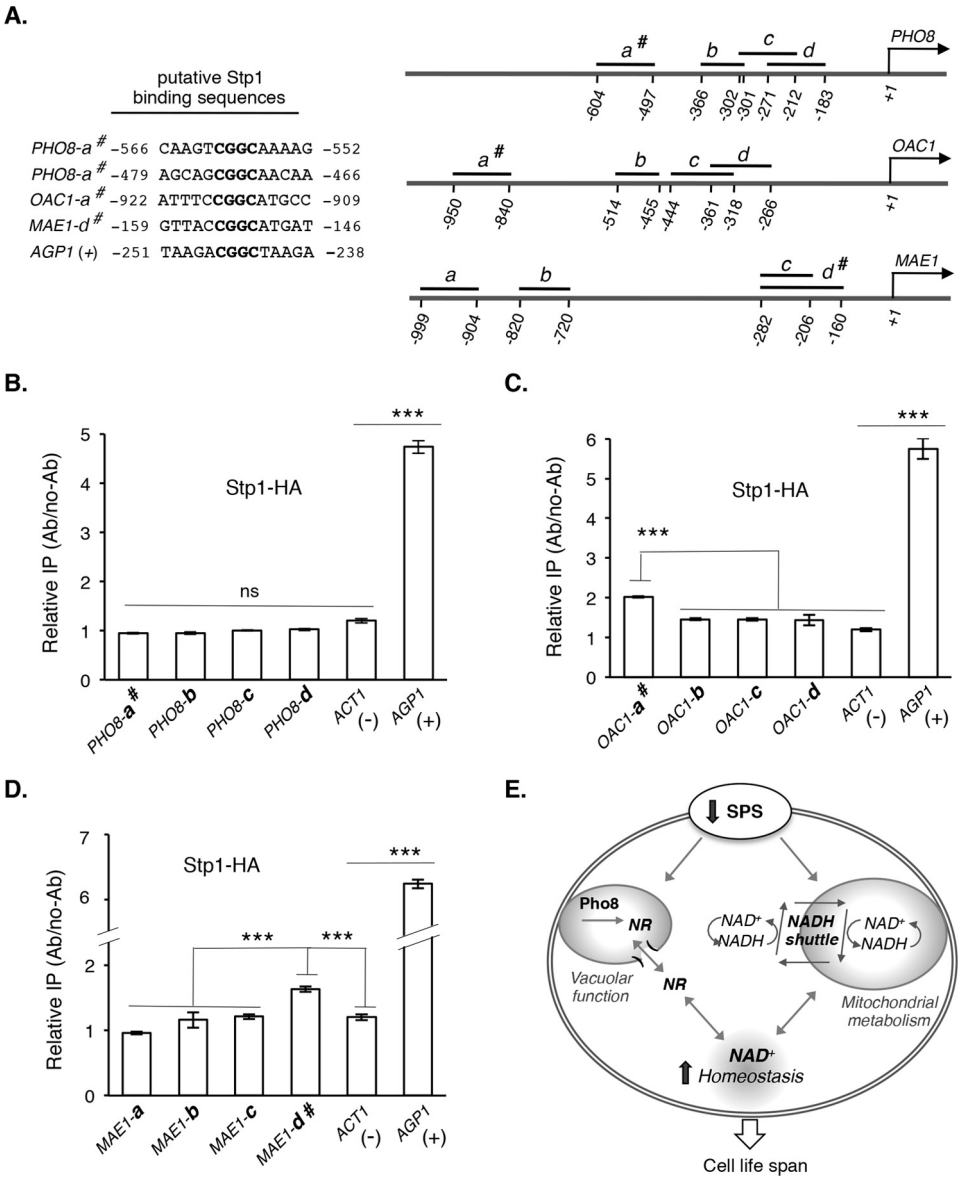

FIGURE 5. **Stp1, a transcription factor of SPS signaling pathway, may directly bind to the promoter regions of NADH shuttle components.** *A*, putative binding sites of Stp1 in the promoter regions of *PHO8*, *OAC1*, and *MAE1* are shown in the *left panel*. DNA fragments used for ChIP analysis are shown in the *right panel*. **#** denotes fragments containing a putative Stp1 binding site. *B*, HA-tagged Stp1 (*Stp1-HA*) does not significantly bind to various promoter regions of *PHO8* compared with *AGP1* as positive control (+) and *ACT1* as negative control (−), determined by qPCR. *C*, Stp1-HA significantly binds to *OAC1-**a**#* but not other promoter regions of *OAC1*. *D*, Stp1-HA significantly binds to *MAE1-**d**#* but not other promoter regions of *MAE1*. *E*, proposed model of how reduced SPS signaling leads to increased NAD$^+$ homeostasis (via NR salvage and NADH shuttle), which works in concert with mitochondrial and vacuolar metabolism to maintain cell function and extend life span. For clarity, additional factors and interactions are not shown. Data shown are representative of multiple independent experiments. *Error bars* denote S.D. derived from triplicate samples. The *p* values were calculated using Student's *t* test (*ns*, not significant; ***, $p < 0.005$).

the expression of these three genes is controlled by SPS signaling (Figs. 2*B* and 3*B*). When SPS-activated Stp1 binds to target promoters, it may activate or inhibit gene expression (Fig. 1*A*). That expression of *PHO8*, *MAE1*, and *OAC1* increased in SPS-defective *ssy5*Δ cells (Figs. 2*B* and 3*B*) suggests that SPS-activated Stp1 binding inhibits expression of these promoters. Thus, in SPS-defective *ssy5*Δ cells, Stp1 is retained in the cytoplasm, resulting in increased expression of these genes. However, it remains unclear whether these genes are direct targets of Stp1. Therefore, we searched for and found putative Stp1 binding sequences (53–55) on the promoter of *PHO8*, *MAE1*, and *OAC1* (Fig. 5*A*, *left panel*). Next, we carried out ChIP analysis of the promoter fragments of these genes using HA-tagged Stp1 (Stp1-HA) to examine whether Stp1 directly binds to these promoters. The *AGP1* promoter fragment *AGP1* (+) was used as a positive control for Stp1 binding as previously described (30). Surprisingly, Stp1 did not appear to bind to the DNA fragment containing two Stp1 binding sites (*PHO8-**a**#*) (Fig. 5*A*) under the condition examined (Fig. 5*B*). Thus, *PHO8* is less likely to be a direct target of Stp1. On the other hand, Stp1-HA showed significantly increased binding to the DNA fragments containing putative Stp1 binding sites (*OAC1-**a**#* and *MAE1-**d**#*) (Fig. 5*A*) for both *OAC1* (Fig. 5*C*) and *MAE1* (Fig. 5*D*). Together, these results suggest that both *MAE1* and *OAC1* are direct targets of Stp1 and that the regulation of *PHO8* expression by SPS signaling likely requires an additional transcription factor(s).

DISCUSSION

In this study we characterized a low SPS activity mutant, *ssy5*Δ and showed that NAD$^+$ homeostasis plays an important role in *ssy5*Δ-mediated life span extension (Fig. 5*E*). Expression of malate-pyruvate NADH shuttle components was increased in *ssy5*Δ cells (Fig. 2*B*). Of these components, deleting *MAE1* and *OAC1* significantly reduced *ssy5*Δ-induced life span extension (Fig. 2*D*). We also showed that Stp1, a transcription factor of the SPS pathway, directly binds to the promoter of *MAE1* and *OAC1* to regulate their expression (Fig. 5, *C* and *D*). In addition to the shuttle components, *ssy5*Δ enhances NAD$^+$ homeostasis by increasing NR salvaging. Deletion of *SSY5* increases NR level (Fig. 2*F*), which partially restores the NAD$^+$ pool (Fig. 3*D*) and life span (Fig. 3*C*) of the short-lived and NAD$^+$-depleted *npt1*Δ mutant. A main source of increased NR in *ssy5*Δ cells is the vacuolar phosphatase Pho8 (Fig. 4*B*), which is also required for full life span extension by *ssy5*Δ (Fig. 4*C*). Interestingly, *ssy5*Δ-induced *PHO8* expression is independent of the canonical *PHO* pathway (Fig. 4*E*). Moreover, SPS-mediated regulation of Pho8 likely involves additional yet-to-be identified transcription factors as Stp1 does not appear to bind to *PHO8* promoter (Fig. 5*B*) under the conditions tested. Together, our studies have unraveled SPS signaling as a regulator of NAD$^+$ homeostasis. Enhanced NAD$^+$ homeostasis due to reduced SPS signaling may concomitantly increase mitochondrial and vacuolar function, all contributing to life span extension (Fig. 5*E*).

Although *ssy5*Δ synergizes with glucose restriction to extend life span (Fig. 1, *C* and *D*), suggesting they function in parallel pathways, they also modulated different components in similar pathways. For example, *ssy5*Δ and CR require different NADH shuttles for life span extension. The malate-pyruvate shuttle plays an important role in the *ssy5*Δ pathway (Fig. 2*D*), whereas the malate-aspartate shuttle is important for CR (26). Interestingly, increased NAD$^+$/NADH ratio was observed in cells under CR (26) but not in *ssy5*Δ cells (Fig. 2*E*). Perhaps increased shuttle activity in *ssy5*Δ cells does not alter the ratio but instead maintains balance between the mitochondrial and cytoplasmic NAD$^+$(H) pools. Another possibility is that increased expression of NR salvaging factors by *ssy5*Δ (Fig. 3, *A* and *B*) leads to NR production (Fig. 2*F* and 3*E*) and replenishes the NAD$^+$ pool (Fig. 3*D*), thereby compensating depletion of NAD$^+$ or NADH. Notably, CR-induced increase in the NAD$^+$/NADH ratio is due to decreased NADH level (56); however, the mechanisms underlying this decrease remain unclear.

One amino acid permease target of SPS signaling, *AGP1*, has been linked to TOR signaling and shown to extend chronological life span (cell survival at a non-dividing G_0 state) when deleted (36). In *ssy5*Δ cells, low SPS activity results in decreased *AGP1* expression, and therefore, longer chronological life span is anticipated. Interestingly, *ssy5*Δ only extends RLS (Fig. 1*B*) but not chronological life span (data not shown). In addition, deleting *AGP1* and additional SPS downstream amino acid permeases, *BAP2* and *BAP3*, is not sufficient to mimic *ssy5*Δ and extend RLS (Fig. 1*E*). These studies support that reduced amino acid uptake mediated by these specific amino acid permeases is not the major cause of *ssy5*Δ-induced RLS extension, although it plays important roles in chronological life span. Instead, our studies suggest that enhanced mitochondrial metabolism plays a key role in *ssy5*Δ-induced life span. The malate-pyruvate NADH shuttle is entwined with the TCA cycle, which supports mitochondrial energy metabolism and produces intermediates for various biosynthetic pathways (44). Because lowered SPS signaling reduces amino acid uptake, *ssy5*Δ cells may compensate by up-regulating expression of malate-pyruvate shuttle components (Fig. 2*B*) to increase amino acid biosynthesis via the TCA cycle. This is in line with *ssy5*Δ cells requiring functional malate-pyruvate shuttle for life span extension (Fig. 2*D*). Overall, this evidence supports a key role for enhanced mitochondrial metabolism in the extended life span of *ssy5*Δ cells. In mammals, impaired mitochondrial metabolism and NADH shuttles have also been implicated in age-associated diseases such as diabetes (57, 58).

Our studies identified Pho8 as an important player in *ssy5*Δ-increased NR salvaging. Pho8 produces NR from nicotinamide mononucleotide as a major source of intracellular NR and contributes to NAD$^+$ homeostasis (10). Because deletion of Pho8 only partially abrogates *ssy5*Δ-induced life span extension (Fig. 4*C*), additional NR homeostasis factors likely play a role. In fact, deleting Pho8 abolishes only intracellular, not released, NR increases in *ssy5*Δ cells (Fig. 4*B*). We previously showed that intracellular NR level largely reflects the NR stored in the vacuole (produced mainly via Pho8) (10). On the other hand, released NR level reflects the NR produced in the cytoplasm (mainly via nucleotidases, Isn1 and Sdt1) (9) because cytoplasmic NR dynamically exchanges with the extracellular environment (4, 10, 28). It would be interesting to determine whether other NR homeostasis factors, such as Isn1 and Sdt1, also play a role in *ssy5*Δ-mediated NR salvaging. Increased Pho8 activity in *ssy5*Δ cells not only increases NR and NAD$^+$ homeostasis but is also likely to contribute to vacuolar function. The vacuolar H$^+$-ATPase (v-ATPase) was previously shown to play essential roles in preserving mitochondrial function in yeast (59). Overexpression of *VPH1*, *VMA1* (v-ATPase subunits), *VPH2* (required for v-ATPase function and assembly), and *AVT1* (a H$^+$-dependent vacuolar amino acid importer) preserved vacuolar acidity and amino acid import and extended RLS (59). These factors are suggested to extend RLS by preserving vacuolar function, allowing proper transport of amino acids and metabolites between the cytoplasm and vacuole. Compromised vacuolar function led to mitochondrial fragmentation and malfunction perhaps due to abnormal accumulation of metabolites and reduced metabolic flexibility (59). Interestingly, *AVT1*, *VMA2*, and *VMA4* have been suggested to be downstream targets of SPS signaling pathway (60–62). Thus, enhanced overall vacuolar function is tightly connected to proper mitochondrial metabolism, and both play important roles in *ssy5*Δ-mediated life span extension.

The vacuole also plays an important role in NR and NAD$^+$ homeostasis. We previously showed the vacuole is a major source of NR production (via Pho8) and storage (10). Our studies suggest NR produced by Pho8 plays an important role in *ssy5*Δ cells, as deleting *PHO8* largely abolished intracellular NR increase (Fig. 4*B*) and mitigated life span extension induced by *ssy5*Δ (Fig. 4*C*). Because NAD$^+$ biosynthesis takes place in the cytoplasm and nucleus, how is vacuolar NR converted to

NAD^+? We have previously identified Fun26 as a putative NR transporter on the vacuolar membrane (10). Fun26 is a homolog of the human ENT (equilibrative nucleoside transporter) protein family that facilitates transport of various purine and pyrimidine nucleosides (63). Deleting *FUN26* causes a significant increase in NR release and accumulation (10). Interestingly, expression of *FUN26* is slightly increased in *ssy5*Δ cells (Fig. 3*A*), suggesting enhanced NR exchange between the cytoplasmic and vacuolar pools. Overall, these studies support a role for vacuolar NR mediating NAD^+ homeostasis in *ssy5*Δ-mediated life span extension.

Maintaining NAD^+ homeostasis is essential for cellular function: aberrant NAD^+ metabolism has been implicated in numerous metabolic and age-associated diseases (5). Due to its complex nature, factors regulating NAD^+ metabolism and homeostasis are not completely understood, although recent studies have identified several regulators of NAD^+ homeostasis. For example, the mitochondria and vacuole were shown to play important roles in the biosynthesis and metabolism of pyridine nucleotides such as NAD^+(H) and $NADP^+$(H) (4, 41, 64, 65). Our studies similarly support that compartmentalization of pyridine metabolites is pivotal for regulating NAD^+ homeostasis. Recently, NAD^+ metabolism was linked to the *PHO* pathway in yeast. Activation of the *PHO* pathway is associated with increased NR production and mobilization (10). Cross-regulation of *PHO* and multiple CR-related nutrient-sensing pathways (cAMP-PKA, TOR, Sch9/AKT) have been reported (4); however, detailed mechanisms remain unclear. Our current studies link SPS signaling to *PHO* signaling in which reduced SPS signaling activates *PHO* gene expression (Fig. 3*B*). However, SPS signaling also regulates certain *PHO* downstream targets (such as Pho8) independent of the canonical *PHO* transcription factors Pho2/4 (Fig. 4*E*), highlighting the complex regulation of these pathways. Most likely, the interplay between longevity signaling pathways is essential for conferring metabolic flexibility in different growth conditions. Future studies to identify and characterize additional signaling factors that regulate cross-talk between NAD^+ homeostasis and longevity regulating signaling pathways are highly anticipated. Overall, our studies contribute to comprehending how nutrient signaling pathways regulate NAD^+ homeostasis and may also provide insight into the underlying mechanisms of diseases related to defects in NAD^+ metabolism.

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