Cell Morphogenesis Proteins Are Translationally Controlled through UTRs by the Ndr/LATS Target Ssd1

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Abstract

Eukaryotic cells control their growth and morphogenesis to maintain integrity and viability. Free-living cells are further challenged by their direct interaction with the environment and in many cases maintain a resilient cell wall to stay alive under widely varying conditions. For these organisms, stringent and highly localized control of the cell wall's remodeling and expansion is crucial for cell growth and reproduction. In the budding yeast Saccharomyces cerevisiae, the RNA binding protein Ssd1 helps control cell wall remodeling by repressing translation of proteins involved in wall expansion. Ssd1 is itself negatively regulated by the highly conserved Ndr/LATS protein kinase Cbk1. We sought to identify mRNA regions that confer Ssd1-mediated translational control. After validating a GFP reporter system as a readout of Ssd1 activity, we found that 3' untranslated regions of the known Ssd1 targets CTS1, SIM1, and UTH1 are sufficient for Cbk1-regulated translational control. The 5' untranslated region of UTH1 also facilitated Ssd1-mediated translational control in a heterologous context. The CTS1 and SIM1 3' untranslated regions confer Ssd1 binding, and the SIM1 3' untranslated region improves Ssd1 immunoprecipitation of the endogenous SIM1 transcript. However, SIM1's 3' untranslated region is not essential for Ssd1-regulated control of the message's translation. We propose that Ssd1 regulates translation of its target message primarily through UTRs and the SIM1 message through multiple potential points of interaction, permitting fine translational control in various contexts.

Introduction

Many single-celled organisms maintain a cell wall. This barrier is crucial for separating the intercellular space from the environment, but presents a problem: it must be continually remodeled for growth to occur [1–3]. This is a dynamic process that requires both deposition of new wall material and removal or rearrangement of existing linkages. In fungi, the integrity of the cell wall is crucial for survival, and any action to remodel it is tightly controlled such that polarized growth and proliferation is kept in balance with stress resistance and osmotic stability. In the budding yeast S. cerevisiae, cell wall biogenesis and remodeling involves a combination of local wall polymer synthesis and tightly controlled secretion of hydrolases that open up the lattice and allow it to expand. While key components of the budding yeast cell wall organization system have been discovered [1–3], it remains unclear how the opposing extracellular processes of wall synthesis and hydrolysis are kept in balance, properly localized, and coordinated with growth status.

Proper control of wall hydrolases, which could reduce cell integrity if hyperactive, is probably especially crucial for normal cell growth and stress resistance. In budding yeast the mRNA binding protein Ssd1 provides an important part of this control. Ssd1 is highly conserved in fungi; it contains a C-terminal RNaseH–related domain that lacks residues necessary for catalytic function and an N-terminal region with conserved sequence blocks of unknown function and a propensity for prion formation. While Ssd1 can bind bulk RNA [4], large scale analyses of mRNA binding indicate that the protein associates with specific transcripts [5–6]. These include mRNAs that encode cell wall related proteins, notably the cell separation chitinase Cts1 and the “SUN” family wall hydrolases Sun4, Sim1, and Uth1. Ssd1 suppresses translation of these wall remodeling proteins, and this activity is important under commonly occurring stressful conditions, such as ethanol-containing growth medium [7–8]. Consistent with a role in translational control, Ssd1 is recruited to cytoplasmic “P bodies”, which are discrete cytoplasmic foci at which mRNAs removed from active translation accumulate, and also interacts with proteins involved in the control of translation [6,9–10].

Ssd1’s ability to block translation of bound mRNAs is efficiently negatively regulated by the Ndr/LATS family protein kinase Cbk1, which directly phosphorylates Ssd1’s N-terminal region [6]. This phosphorylation does not affect Ssd1’s ability to bind mRNA, but rather appears to reduce its ability to block the translation of associated messages [6]. Cbk1 is an essential component of a highly conserved regulatory system called the “RAM network” that controls final separation of mother and daughter cells and sustained polarized growth that occurs during mating and bud morphogenesis [11–18]. When the function of any component of the RAM network is lost Ssd1 is hyperactivated, causing constitutive translational repression of cell wall remodeling proteins. This loss of hydrolase expression is lethal because it
effectively blocks cell wall expansion, severely restricting bud growth and blocking cell proliferation [6]. Similarly, changing the amino acids in Ssd1 that are phosphorylated by Cbk1 to non-phosphorylatable residues creates a highly toxic ssd1 gain-of-function allele (ssd1-8A) that gives a substantially similar suppression of wall expansion when expressed.

 Intriguingly, while Ssd1 clearly modulates translation of some of the mRNAs it binds, the protein has additional functions. While the effect may be indirect, the decay rates of diverse mRNAs is faster in cells that express functional Ssd1, regardless of whether or not these messages associate with Ssd1 [6], and many genes are differentially expressed depending on the presence of functional Ssd1 [19]. A number of Ssd1-associated transcripts are asymmetrically localized in proliferating cells [20–21], and Ssd1 has been implicated in subcellular localization of one of these (SRL1) [9]. At least one Ssd1-associated message, CLN2, is stabilized by Ssd1 binding to its 5’ untranslated region [22]. It is not known if Cbk1 regulates Ssd1-mediated stabilization of CLN2 or if this 5’UTR-mediated association is related to Ssd1’s function as a translational repressor.

 Ssd1’s apparently diverse functions may reflect the underlying complexity of messenger RNA particle (mRNP) organization, in which different complements of associated regulatory proteins confer distinct mRNA behavior. It is unclear how Ssd1 associates with mRNAs, and understanding this could illuminate what dictates the composition of Ssd1-containing mRNPs. The motif A/G/U/UCAUUCCUU is significantly enriched in 5’ untranslated regions of mRNAs that associate with Ssd1 in affinity purification experiments [5], and a portion of the CLN2 5’ UTR containing a sequence matching this motif mediates Ssd1 association [22]. For brevity, we refer to this motif as the “SEE” (Ssd1 Enriched Element). While it occurs with elevated frequency in Ssd1-associated mRNAs, the SEE is not present in all of them, and the motif has not been directly shown to be sufficient for Ssd1-mRNA binding or Ssd1-regulated translational control. Ssd1 also interacts with the poly-A binding protein Pab1 [23], suggesting that Ssd1-mRNP interactions are complex. We sought to determine if the 5’UTR of an Ssd1-regulated message is sufficient to confer translational regulation on an otherwise unregulated transcript and if Ssd1 acts through other regions of target messages. Given the extensive role of 3’UTR-directed regulation of mRNA translation efficiency as noted.

Materials and Methods
Yeast strains and plasmids
We constructed all yeast in the S288c background strain BY4741 (Open Biosystems). We constructed GFP reporter plasmids the Drag and Drop recombinant cloning method [28], using known annotations of 5’ and 3’ UTR boundaries [29–30] to generate primers for UTR integration into pGREG vectors. We replaced the pGREG GAL1,10 promoter with the TEFL1 or ADH1 promoters and their 5’UTRs from the PCR Toolbox vectors pYM-N18 or pYM-N6 [31], respectively, by subcloning at SaeI-SpeI. We made destabilized GFP Pest reporters by PCR-mediated stitching of the 534 nucleotides encoding the 178 C-terminal residues of Cln2 [32], followed by a stop codon, to the 3’UTR of interest and subsequent recombinant cloning into a pGREG576 N-terminal GFP vector. We sequenced all plasmids and checked fusion protein expression by western blotting against GFP (Roche cat. no. 1181446001). We replaced the endogenous 3’UTR of SIM1 using homologous recombination to integrate a PCR product encoding the CYC1 3’UTR at the 3’ end of SIM1, following the stop codon.

Flow cytometry analysis of GFP reporters
We grew samples for flow cytometry to mid-log (OD600~0.6) in YP (yeast peptone) rich media and washed into PBS, or in YNB (yeast nitrogen base) synthetic media for direct analysis. We grew GFP Pest time course samples to OD600~0.4 and treated cells with 25 μM 1NA-PP1 or an equivalent volume of DMSO vehicle. We removed 1 mL from each sample every hour following treatment, fixed these time points in ice-cold ethanol and stored them in the dark for cumulative analysis. We used a Becton Dickinson LSRII flow cytometer with a 488 nm excitation laser and a 530 nm emission filter (FTC), kept laser power settings the same between experiments and collected a minimum of 10,000 cells per sample. We analyzed flow cytometry data using FACSDiva (Becton Dickinson) or FlowJo (Tree Star) software. Briefly, we gated cells from debris by forward-scatter/side-scatter plots and set baseline GFP fluorescence gates using side-scatter/FTC plots of cells expressing no GFP reporter. We adjusted experiments showing deviations in cell size between samples by taking the ratio of mean FTC fluorescence to mean forward-scatter signal; this is indicated as FSC-normalized. We used mean fluorescence intensity (MFI) statistics of both the population above baseline FTC autofluorescence and MFI of the entire population to make comparisons between reporters. We analyzed mRNA collected from the same flow cytometry samples by real-time qPCR (see below) to evaluate translation efficiency as noted.

Polysome profiling of GFP reporters and Northern blot analysis
We did polysome profiling and subsequent Northern blotting analysis of fractionated RNA as previously described [6]. We amplified probes from genomic DNA using the following primers and labeled with Ready to Go probes (Amersham) at 532P-dCTP. GFP 5’ GTGAAGGGTGATGGAAATAG and 5’ TGTTTGTCGTGCTGCTT~AAGGAC, PGR1 5’ GAATTTGTTCTGTGCTT~GCCA and 5’ TTCTCGGAAGCGCTTACGGA. We performed AUC (Area Under the Curve) analysis of relative polysome and monosome association across three replicate experiments using Prism (GraphPad).

Purification of Ssd1-associated mRNA
We purified Ssd1-associated transcripts in cells expressing Ssd1-TAP from the endogenous SSD1 locus and in cells expressing untagged Ssd1 as previously described [33], but scaled-down 10-fold. We modified the antibody-mediated RNA immunoprecipitation by Ssd1-TAP as follows: we incubated lysates with 2 μg anti-TAP rabbit polyclonal antibody (Thermo-Fisher Pierce cat. no. CAB1001) for 30 minutes at 4°C, followed by 2 hour incubation at 4°C with recombinant Protein G-Sepharose 4B beads (Life Technologies). We recovered mRNA from Protein G-Sepharose 4B beads (washed as described in [33]) using the MasterPure Yeast RNA Purification Kit (Epitect Technologies). We determined relative abundance of recovered mRNA by real-time qPCR. We reverse transcribed (Promega) 2.5 μg RNA
primed only with oligo dT and detected messages by incorporation of SYBR green (Life Technologies) into amplicons generated with the following primers:  

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\begin{align*}
\text{SUN4} & \quad 5'\ ATTGGTTCTTC \quad \text{and} \quad 5'\ TCATCAAGGGCGCAATTTT, \\
\text{GFP} & \quad 5'\ TGGGAAGCTTCACTAGCGA & \quad \text{and} \quad 5'\ AAAGGCGA-GATTGTTGAGC, \\
\text{SIM1} & \quad 5'\ TCTGGTGTGTC & \quad \text{and} \quad 5'\ AAAGTATTGTGACGACAGGGC, \\
\text{ACT1} & \quad 5'\ GGGTATTGATAACGGTTCTGTATG & \quad \text{and} \quad 5'\ ATGATACCTTGGTTGCTGTTAC.
\end{align*}
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We evaluated abundance of Ssd1 target proteins by assaying newly replenished yeast growth media. We treated logarithmically growing cells with 10 μM INAP-PPI or an equivalent volume DMSO vehicle for 1 hour. At OD600 = 0.5, we washed the resulting precipitated protein pellet in acetone. We air-dried the precipitated media at top speed in a 4°C microcentrifuge, aspirated the media following centrifugation, and treated these samples with sodium azide at a final concentration of 20 mM to stop growth. We pelleted the cells and treated 800 μL supernatant media with 89°C ice cold 100% trichloroacetic acid (TCA), incubating on ice for 20 minutes to precipitate secreted proteins. We spun TCA-precipitated media at top speed in a 4°C microcentrifuge, aspirated the media following centrifugation, and washed the resulting precipitated protein pellet in acetone. We air-dried precipitated protein pellets and resuspended them in Tris pH 9.4-buffered SDS-PAGE loading buffer. We lysed cell pellets with 89°C M1NA-PP1 or an equivalent volume DMSO and maintained them on YNB media lacking uracil and leucine. We removed 1 mL aliquots of cells in growth media at 5, 15, and 30 minutes post-media replenishment and treated these samples with sodium azide at a final concentration of 20 mM to stop growth. We pelleted the cells and treated 800 μL supernatant media with 89°C ice cold 100% trichloroacetic acid (TCA), incubating on ice for 20 minutes to precipitate secreted proteins. We spun TCA-precipitated media at top speed in a 4°C microcentrifuge, aspirated the media following centrifugation, and washed the resulting precipitated protein pellet in acetone. We air-dried precipitated protein pellets and resuspended them in Tris pH 9.4-buffered SDS-PAGE loading buffer. We lysed cell pellets from each time point to assay internal protein content by alkaline pH 9.4-buffered SDS-PAGE loading buffer. We lysed cell pellets from each time point to assay internal protein content by alkaline pH 9.4-buffered SDS-PAGE loading buffer. We lysed cell pellets from each time point to assay internal protein content by alkaline pH 9.4-buffered SDS-PAGE loading buffer. We lysed cell pellets from each time point to assay internal protein content by alkaline pH 9.4-buffered SDS-PAGE loading buffer.
When SSD1 is deleted the steady-state levels of many mRNAs increase, whether or not they associate with Ssd1 [6]. To determine if the transcript abundance of both control and hypothetically Ssd1-regulated GFP constructs respond similarly to deletion of SSD1 we measured GFP message levels in cells grown in the cultures used for flow cytometric analysis. We found that abundance of GFP construct mRNAs with either the control CYC1 3' UTR or the Ssd1-regulated CTS1 3' UTR were modestly elevated in ssd1Δ cells compared to SSD1 cells, with no statistically significant difference (p = 0.4764) (Figure S1C). This is consistent with prior demonstration that Ssd1 depresses many transcript levels and does so indiscriminately. Importantly, the small variance in mRNA levels expressed from our GFP reporters indicates that changes in fluorescence from the Ssd1-regulated reporter (Figures S1A and S1B) reflect differences in translation levels. We calculated translation efficiency of our GFP reporters in ssd1Δ and SSD1 cells by dividing the fold change in MFI by the fold change in transcript abundance, and found that after accounting for changes in mRNA abundance due to the absence of Ssd1, the CTS1 3' UTR shows markedly increased translation efficiency when Ssd1 is absent, while the CYC1 3' UTR does not (Figure 1D).

3'UTR-mediated Ssd1 translational control is promoter-independent

To confirm that the CTS1 3' UTR confers context independent Ssd1-regulated translational control we measured expression of GFP from a construct driven by the constitutive ADH1 promoter and containing the ADH1 5' UTR, combined with the CTS1 3' UTR. This \( P_{ADH1} \)-GFP-CTS1\(^{3'UTR} \) reporter showed elevated expression in cells lacking Ssd1 (p = 0.0402), further confirming that the CTS1 3' UTR confers Ssd1-mediated translational regulation (Figure 2A). These findings demonstrate that the 3' UTR from the Ssd1-bound transcript CTS1 can confer Ssd1-dependent changes in expression in two heterologous contexts, and that these changes are likely post-transcriptional, facilitated through Ssd1-mediated translational regulation.

We tested 3'UTRs of several other transcripts that bind Ssd1 and exhibit Ssd1-mediated translational regulation in our GFP reporter system. We found that GFP expressed from an ADH1 promoter with the ADH1 5' UTR and the 3' UTR from the Ssd1-bound transcript STM1 showed consistently lower MFI (p = 0.0444) in cells expressing functional Ssd1 (Figure 2B). We saw a similar statistically significant effect using a GFP reporter with the 3' UTR of the Ssd1-bound transcript UTH1 (p = 0.0126) (Figure 2C). In contrast, GFP reporter constructs with 3'UTRs from the Ssd1-bound transcripts SUN4, TOS1 and SCW10 did not show statistically significant changes in GFP expression dependent on the presence of Ssd1 (Figure S2A). Thus, while 3'UTRs of Ssd1-bound messages do not always confer Ssd1-dependent regulation of GFP translation, 3'UTRs of CTS1, STM1 and UTH1 are sufficient for this. While statistically significant, differences between reporter expression in SSD1 and ssd1Δ cells are generally modest. As discussed further below, this is consistent with strong negative regulation of Ssd1 by the Ndr/LATS kinase

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**Figure 1.** GFP bearing the Ssd1-bound transcript CTS1's 3' UTR is differentially expressed depending on Ssd1 genotype. SSD1 (EL1700) or ssd1Δ (EL1953) cells were transformed with \( P_{TEF1} \)-GFP-CYC1\(^{3'UTR} \) or \( P_{TEF1} \)-GFP-CTS1\(^{3'UTR} \) maintained under G418 selection. (A) Diagram of exogenous reporters used in this figure. (B) Logarithmically-growing cells expressing these reporters in both strain backgrounds were examined by fluorescence microscopy. Representative images of cells expressing \( P_{TEF1} \)-GFP-CYC1\(^{3'UTR} \) showing minimal variance in GFP expression in SSD1 or ssd1Δ, but cells expressed lower levels of \( P_{TEF1} \)-GFP-CTS1\(^{3'UTR} \) in SSD1 than ssd1Δ. All images contrast adjusted in OpenLab software using identical settings. (C) Lysates of the same cells used in (B) were analyzed by Western blot against GFP and the housekeeping gene Pgk1, with equal numbers of cells processed for each strain. Western blots confirm lower GFP levels in SSD1 cells when GFP is expressed with the CTS1 3' UTR, while Pgk1 levels are invariant. (D) Estimation of translation efficiency of GFP-CYC1\(^{3'UTR} \) and GFP-CTS1\(^{3'UTR} \) reporters, determined through division of GFP MFI (mean fluorescence intensity) by relative GFP transcript abundance (shown in Figure S1A), shows significant repression of \( P_{TEF1} \)-GFP-CTS1\(^{3'UTR} \) in SSD1 cells. Data in (D) represent three independent trials. Error bars represent ± SEM, *** indicates P-value <0.001, * indicates P-value >0.05 at 95% confidence intervals as calculated by unpaired two-tailed Student’s t-test.

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Cbk1 in proliferating cells in which the wall is rapidly growing [6]. Additionally, we propose that relatively subtle coordinated translational control has appreciable effects on cell wall organization because Ssd1 regulates a cohort of messages involved in similar processes.

The UTH1 5’UTR confers translational control, while CTS1 and SUN4 5’UTRs do not

We determined if 5’UTR elements from Ssd1-bound transcripts confer Ssd1-dependent expression changes in our GFP reporter system. Of the 5’UTRs tested, only the UTH1 5’UTR showed a significant Ssd1-mediated effect on GFP expression, while the control reporter with the ADH1 5’UTR showed insignificant Ssd1-dependent change in MFI (p = 0.2237) (Figure 2D). Our GFP reporters for testing the 5’UTR of UTTHI contained either the UTTHI 5’UTR alone or a tandem ADH1-UTH1 5’UTR in the presence of a GFP open reading frame and a CYC1 3’UTR. Both test constructs showed highly significant (p<0.0001) elevation of GFP expression in the absence of Ssd1, indicating that the UTH1 5’UTR confers Ssd1-mediated regulation of translation in the presence of additional 5’UTR elements. The 5’UTRs from the Ssd1-bound messages CTS1 and SUN4 did not confer a similar effect (Figure S2B). Both UTTHI and CTS1 5’UTRs contain the SEED present in some Ssd1 targets, while the SUN4 5’UTR lacks this motif [5]. Thus, these data show that presence of the SEED is not sufficient for translational control. However, these UTRs are not strictly comparable: the CTS1 5’UTR contains a single SEED while the UTTHI 5’UTR contains four.

Figure 2. Ssd1 regulates translation of GFP reporters with 3’UTRs from Ssd1-associated transcripts or the UTH1 5’UTR. (A) CTS1 3’UTR reporter GFP MFI difference in SSD1 and ssd1Δ is promoter and 5’UTR independent, as flow cytometry reveals significant Ssd1-dependent differences in expression. (B) SUN4 3’UTR confers significant Ssd1-dependent expression variances on GFP expressed from the ADH1 promoter. (C) GFP expressed from ADH1 promoter in the context of the UTH1 3’UTR is elevated in ssd1Δ compared to SSD1 cells, while the CYC1 3’UTR confers no such effect on GFP expression. (D) UTH1 5’UTR confers decreased expression of GFP in SSD1 cells, either when present as the only 5’UTR element or expressed in tandem with the ADH1 5’UTR, suggesting its effect is context-independent. In (A) and (B), fluorescence data were corrected for variations in cell size apparent in forward scatter (FSC) measurements as described in Materials and Methods. (A) through (D) represent three independent trials. Error bars represent ± SEM, *** indicates P-value<0.001, ** indicates P-value of 0.001 to 0.01, * indicates P-value 0.01 to 0.05, and ‘ns’ indicates P-value>0.05 at 95% confidence intervals as calculated by unpaired two-tailed Student’s t-test.

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3’UTR reporters are sensitive to Ssd1’s phosphorylation state

The Ndr/LATS kinase Cbk1 negatively regulates Ssd1’s translational repression of associated mRNAs; this inhibition is probably very efficient in rapidly growing cells [6]. While proteins encoded by Ssd1-bound transcripts are more abundant in ssd1Δ cells, translational repression is considerably stronger when Cbk1’s phosphorylation of Ssd1 is compromised [6], and total loss of Cbk1 function is lethal in cells that express functional Ssd1 [4,11,38–43]. To study the effect of hyperactive Ssd1 on our GFP reporters we used the cbk1-as allele, which encodes a mutant Cbk1 kinase that is specifically inhibited by the otherwise innocuous cell permeable compound 1NA-PP1 [44–45]. While the kinase encoded by cbk1-as is functional in the presence of the vehicle DMSO it is hypomorphic, resulting in reduced kinase activity compared to that of wild-type CBK1 [14]. Thus, DMSO-treated ( uninhibited) cbk1-as cells exhibit elevated Ssd1 translational repression, and 1NA-PP1-treated cbk1-as exhibit full Ssd1 hyper-activation.

GFP reporters bearing the CTS1 3’UTR or SIM1 3’UTR expressed in uninhibited cbk1-as cells showed a slight decrease in MFI of the population, with no significant additional reduction in MFI with 1 hour cbk1-as inhibition (Figures S3A and S3B). In budding yeast, the half-life of GFP (t½≈7 h) [46] is much longer than the period of a typical cell cycle (~1.5 h), obscuring changes in translation rate in 1 hour inhibition experiments. To address this, we assessed translational repression using a GFP constructs destabilized by appending the PEST domain from Cln2 to the GFP C-terminus (estimated half-life of 30 minutes) [32]. In cells expressing destabilized GFPPEST in the context of the ADH1 5’UTR and the SIM1 3’UTR, the maximal fluorescence of the GFP-positive population, visualized in a flow cytometry histogram, was inversely correlated with Ssd1 activity (Figure 3A). With Cbk1 inhibition, the left edge of the histogram population overlays that of the untransformed GFP negative population, showing that PADH1-GFPPEST-SIM13’UTR expression is repressed in this condition. We did not see a similar change in expression or depletion of signal to background levels when we expressed a control destabilized reporter PADH1-GFPPEST-CYC15’UTR in the hypomorphic cbk1-as background or in cells treated with 1NA-PP1. Destabilized GFPPEST flankled by the ADH1 5’UTR and CTS1 3’UTR and expressed from a constitutive ADH1 promoter showed statistically significant reduction in the fraction of the population exceeding the GFP baseline threshold when Ssd1 is present (p = 0.0186) (Figure 3B)

Destabilized GFPPEST expressed from an ADH1 or TEF1 promoter bearing the Ssd1-regulated CTS1 3’UTR also showed similar reduction in destabilized GFP reporter expression by Western blotting when Ssd1 is present, and that GFP expression is further depleted by expression in the cbk1-as background. We found that the CTS1 3’UTR Ssd1 reporter behaves similarly irrespective of promoter identity (Figure S4A). Several reports have noted the importance of Ssd1 in stressful conditions such as ethanol-rich growth medium, and offer evidence that Ssd1 regulates cell wall remodeling proteins under these circumstances [7–8]. Applying these observations to our GFP reporter system, we grew cells expressing control CTC1 3’UTR or test CTS1 3’UTR GFPPEST reporters in rich media supplemented with 5% (v/v) ethanol to test if these stress conditions would further stimulate Ssd1 activity. While the cells harboring the CTS1 3’UTR reporter in rich media alone showed a modest shift in the population histogram to lower GFP fluorescence in cbk1-as than CBK1 cells, cbk1-as cells grown in 5% ethanol showed a dramatic change in the fluorescence profile that is not observed in CBK1 cells. This effect also depended on the presence of an Ssd1-regulated 3’UTR; in the same strains under the same conditions, the GFPPEST-CYC15’UTR reporter showed no reduction in fluorescence in the presence of the hypomorphic cbk1-as and no response to ethanol treatment in cbk1-as cells (Figure S4B). These results confirm that the 3’UTRs from the Ssd1-associated transcripts SIM1 and CTS1 confer translational control that is sensitive to Ssd1 activity, and that this does not occur when an unassociated 3’UTR is present.

Prolonged Cbk1 inhibition results in complete depletion of Ssd1 reporters

While statistically significant, differences in translation of Ssd1-regulated mRNAs between rapidly proliferating Ssd1Δ and ssd1Δ cells were relatively subtle. In contrast, extended 1NA-PP1 treatment of cbk1-as cells should cause persistent hyperactivation of Ssd1 and substantial translational suppression of mRNAs under its translational control, including our GFP reporters. To test this, we grew cbk1-as or cbk1-as ssd1Δ cells expressing the Ssd1-responsive PTEF1-GFPPEST-CTS13’UTR reporter to mid-log (OD600=0.5) and split the cells into DMSO-treated and 1NA-PP1-treated populations. We allowed treated and untreated cells to grow for 6 hours, fixing samples of each culture at 1 hour intervals for later analysis by flow cytometry. GFP fluorescence was dramatically depleted after 6 hours in cbk1-as cells treated with 1NA-PP1, but persisted at levels similar to the beginning of the time course in cbk1-as cells treated with DMSO and in cbk1-as ssd1Δ cells treated with either 1NA-PP1 or DMSO (Figure 3D). Destabilized GFP reporters were fully depleted only when the CTS1 3’UTR was present, as the fluorescence of cells expressing GFPPEST with the CYC1 3’UTR control reporter in the same conditions were not depleted even under Ssd1 hyperactivation (Figure S4C). Taken together, these results show that translation of a reporter protein ORF from an mRNA with an Ssd1-regulated 3’UTR is shut off when Ssd1 is no longer inhibited by Cbk1.

3’UTR reporters are enriched in monosomes when Ssd1 is hypophosphorylated

Ssd1’s control of cell wall protein expression is evident by altered polypeptide occupancy of its target transcripts when Ssd1 is not phosphorylated by Cbk1 [6]. To assess ribosome occupancy of exogenous GFP reporters we assayed ribosome density along the Ssd1-regulated TEF15’UTR-GFP-CTS15’UTR reporter transcript by polysome profiling, using sucrose gradients to separate bulk mRNP’s of varying levels of ribosome content. We determined the relative abundance of GFP transcript in monosomes and polysomes by northern blotting mRNA recovered from fractionated sucrose gradients. We compared 1NA-PP1-treated cbk1-as cells, where Ssd1 is hyperactive, to 1NA-PP1-treated cbk1-as ssd1Δ cells, where Ssd1 is absent. Hyperactivation of Ssd1 did not alter the bulk mRNA polysome profile as measured by optical density at 254 nm (Figure 4A), consistent with prior findings [6]. In cells containing hyperactivated Ssd1, abundance of GFP mRNA was increased in monosomes fractions, (Figure 4B). The housekeeping gene PGK1, itself not a target of Ssd1, showed invariant polysome and monosome association when Ssd1 was hyperactivated (Figure 4B). Polysome profiling is a qualitative assay subject to inherent variations in density gradient preparation or northern blot analysis; to further quantify our polysome association experiments, we measured the area under the curve (AUC) of the polysome- and monosome-associated regions (determined by bulk mRNA profiles) of both GFP and PGK1 relative mRNA abundance profiles across three replicate experiments (Figure 4B and Figure S5A). Polysome occupancy of GFP-CTS15’UTR was
significantly reduced ($p = 0.04056$) in cells where Ssd1 was hyperactivated, while the PGK1 mRNA was not (Figures S5B and S5C). While statistically significant, monosome association of our GFP reporter in response to Ssd1 hyperactivation was modest, particularly when compared to the shift that occurs with endogenous Ssd1-associated mRNAs [6]. This may be attributable to short length of the GFP transcript, which likely reduces overall maximal polysome density.

3’UTRs of some Ssd1 targets confer Ssd1-mRNA interaction

We sought to determine if UTRs that confer Ssd1-mediated translational repression could physically associate with Ssd1. We performed RNA immunoprecipitation assays to determine if the CTS1 3’UTR could mediate immunoprecipitation of GFP mRNA with TAP-tagged Ssd1. We found that while GFP mRNA produced from either the GFP-CYC1 3’UTR or GFP-CTS1 3’UTR reporters was present in lysates, only the CTS1 3’UTR conferred significant co-precipitation with Ssd1-TAP (Figure 5A). Correcting for the amount of Ssd1-TAP immunoprecipitated across two experiments, we found significant enrichment ($p = 0.0319$) of GFP transcript in SSD1-TAP compared to SSD1 cells only when the CTS1 3’UTR is present (Figure 5B).

We next tested the SIM1 3’UTR’s ability to confer Ssd1 association with mRNA, comparing its pull-down with the CTS1 3’UTR reporter and the endogenous SUN4 transcript. We found

Figure 3. Destabilized GFP reporters show Cbk1-phosphoregulation of Ssd1-dependent changes in expression. (A) Destabilized GFP-Cln2PEST bearing the SIM1 3’UTR shows moderate shifts in population fluorescence depending on the phosphorylation state of Ssd1, but a destabilized GFP bearing the CYC1 3’UTR remains unaffected. Ssd1 phosphorylation state was modulated by the introduction of the hypomorphic cbk1-as allele and treatment of these cells with DMSO or 1NA-PP1. (B) Destabilized GFP-Cln2PEST harboring the Ssd1-regulated CTS1 3’UTR responds to Ssd1 hyperactivation through Cbk1 inhibition. A significant difference in GFP levels was observed by flow cytometry between cbk1-as SSD1 cells treated with DMSO or 1NA-PP1 through the fraction of the population above baseline fluorescence or. The fraction of cells expressing destabilized GFP was significantly dependent on the presence of Ssd1 (compare CBK1 SSD1 to CBK1 ssd1Δ). (C) Prolonged Cbk1 inhibition results in complete depletion of GFP fluorescence in cells expressing an Ssd1-regulated reporter. Flow cytometry was performed on cells fixed at one hour intervals as described in Materials and Methods. We report the relative %GFP positive at each time point $t>1$ h as a fold change relative to the %GFP positive population at $t=0$ h. Additional controls shown in Figure S4C. (A) and (C) are representative trials of replicated experiments. Data in (B) represent three independent trials. Error bars represent ± SEM, ** indicates $P$-value of 0.001 to 0.01 and * indicates $P$-value 0.01 to 0.05 at 95% confidence intervals as calculated by unpaired two-tailed Student’s t-test.

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Figure 4. Ssd1-regulated expression of GFP reporter is due to changes in transcript ribosomal occupancy. Polysome profiling of RNA extracts followed by Northern blot analysis of RNA fractions from across the polysome gradient were used to analyze ribosomal occupancy of the $P_{\text{TFF1-GFP-CTS13^{UTR}}}$ reporter in 1NA-PP1-treated cbk1-as SSD1 and cbk1-as ssd1Δ cells. (A) Bulk polysome $A_{254}$ trace of fractionated sucrose gradients reveals fractions containing polysome-associated mRNA (highlighted by gray chart area) and monosome-associated mRNA (towards top of gradient). No changes in bulk translation are observed when Ssd1 is present (top trace) compared to when Ssd1 is absent (bottom trace). (B) Quantification from Northern blots against GFP of signal intensity across the polysome gradient reveal that the $\text{GFP-CTS13^{UTR}}$ transcript is enriched in monosomes when Ssd1 is present and hyperactivated (compare solid line with square points to dashed line with diamond points). No difference in ribosomal occupancy was observed in Northern blots against the housekeeping gene Pgk1. (A) and (B) are representative plots from three replicate experiments; additional replicates are presented in Figure S5.

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elevated immunoprecipitation efficiency of GFP mRNA when the 
CTS1 3'UTR or SIMI 3'UTR is present compared to the 
CYC1 3'UTR reporter, which was not enriched over a no antibody 
control (Figure 5C). Robust immunoprecipitation of the 
SUN4 endogenous transcript, as previously reported [6], revealed 
variations in overall pull-down efficiency between biological and 
Figure 5. The 3' UTRs of CTS1 and SIM1 confer Ssd1 binding to GFP reporters. (A) Representative samples of Western and Northern blots 
show TAP-tagged Ssd1 immunoprecipitates PTER-GFP-CTS13'UTR reporter. In extracts from strains expressing Ssd1-TAP (see WB: anti-TAP), GFP mRNA 
is immunoprecipitated when the CTS1, but not the CYC1, 3'UTR is present (see Northern: GFP (IP)). In RNA immunoprecipitation input samples, GFP 
mRNA was detected irrespective of its 3'UTR and ACT1 mRNA was present in all samples. (B) The fold enrichment of GFP mRNA immunoprecipitated 
from SSD1-TAP over SSD1 (untagged) cells was quantified over three experiments. The GFP reporter expressed in the context of the CTS1 3'UTR was 
significantly enriched in SSD1-TAP IP samples compared to reporters bearing the CYC1 3'UTR. (C) GFP-CTS13'UTR and GFP-SIM13'UTR reporters, but not 
the control GFP-CYC13'UTR reporter, are immunoprecipitated in SSD1-TAP lysates incubated with anti-TAP antibody. Real-time qPCR detection shows 
variant pull-down efficiency across 3 experiments, as shown by the variance in SUN4 mRNA immunoprecipitation. (D) Three replicate RNA IP 
experiments were normalized using SUN4 mRNA IP as a positive control. GFP-CTS13'UTR and GFP-SIM13'UTR reporters show significant enrichment in 
RNA IP samples compared to GFP-CYC13'UTR reporter. Error bars represent ± SEM and * indicates P-value 0.01 to 0.05 at 95% confidence intervals as 
calculated by unpaired two-tailed Student's t-test. (E) CTS1 3'UTR and CLN2 5'UTR confer 3-hybrid interaction with Ssd1. In either MS2 aptamer 
position, the CYC1 3'UTR does not mediate a 3-hybrid interaction, while the CTS1 3'UTR does mediate a 3-hybrid interaction. Notably, the CLN2 5'UTR 
also mediates a 3-hybrid interaction. AD-IRP co-transformed with IRE-MS2 serves as a positive control, while AD-Ssd1-(1-862)-8E co-transformed with 
IRE-MS2 serves as a negative control.
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technical replicates; we thus normalized GFP mRNA pull-down to the positive control SUN4 for each sample in respective experiments. Both the GFP-CTS1<sup>5′ UTR</sup> and GFP-SIM1<sup>5′ UTR</sup> reporters showed significant enrichment (CTS1: p = 0.0371; SIM1: p = 0.0286) in immunoprecipitated samples compared to the GFP-CYC1<sup>5′ UTR</sup> reporter (Figure 5D). Notably, the enrichment of either the GFP-CTS1<sup>5′ UTR</sup> or GFP-SIM1<sup>5′ UTR</sup> reporters were at most 10-fold less than that of the endogenous transcript SUN4.

This is consistent with a previously observed 10-fold difference between Ssd1’s association with SUN4 and with UTH1, CTS1 and SIM1 [6]. Despite these differences in Ssd1 binding efficiency, there was little correlation with the strength of translational control of GFP reporters (Figure 2). Overall, these results show that at least two 3′UTR elements from known Ssd1-bound transcripts can confer Ssd1 binding to an otherwise unassociated transcript.

We used a yeast 3-hybrid system to test the CTS1 3′UTR’s interaction with Ssd1 in a different way. We found the CTS1 3′UTR, but not the CYC1 3′UTR or IRE (Iron Response Element), mediated 3′-hybrid interaction with a truncated form of Ssd1 (1-862) that includes the RNA binding domain [4]. As a positive control, we also confirmed that the Ssd1-associated CLN2 5′UTR [22] exhibited a similarly robust interaction (Figure 5E). Interestingly, although Ssd1 interaction with the CTD tail of RNA polymerase II [47] suggests that Ssd1 may load onto its target transcripts co-transcriptionally, the MS2 hybrid RNAs in the 3′-hybrid system we employed are transcribed by RNA polymerase III [55].

The SIM1 3′UTR enhances Ssd1 interaction with the endogenous SIM1 transcript

Having found that 3′UTR elements are sufficient for Ssd1-mediated translational regulation of a GFP reporter, we asked if the 3′UTR of an endogenous Ssd1 target transcript is necessary for Ssd1 association with target mRNAs. We replaced the 3′UTR of the Ssd1 target transcript SIM1 at its endogenous locus with the CYC1 3′UTR (Figure 6A) and compared Ssd1 immunoprecipitation of this chimeric SIM1-CYC1<sup>5′ UTR</sup> transcript and the native SIM1 transcript. We found that Ssd1 immunoprecipitation of SIM1-CYC1<sup>5′ UTR</sup> was significantly reduced (p = 0.0422), about two-fold, relative to the endogenous SIM1 transcript (Figure 6B). This difference in precipitation was not a result of variations in the pull-down of Ssd1 between the two test strains (Figure S6), demonstrating that replacing the SIM1 3′UTR with an otherwise unbound 3′UTR reduces Ssd1 association with the SIM1 message.

Ssd1 exerts translational control over SIM1 through redundant means

Since the SIM1 3′UTR promotes Ssd1 association with the SIM1 mRNA and is sufficient for Ssd1-mediated translational control of a GFP-SIM1<sup>5′ UTR</sup> construct, we asked if this 3′UTR is necessary for Ssd1’s translational repression of the SIM1 mRNA. The Sim1 protein, like many encoded by Ssd1 target mRNAs, is a secreted cell wall associated protein [6]; these are generally long-lived, complicating measurement of translational suppression. We therefore analyzed the levels of both cell-associated Sim1 and the fraction of Sim1 secreted from cells into growth medium. For the experiments shown in Figure 6, we used cbk1-as cells with either ssd1A or the wild type SSOL allele, and expressing either the endogenous SIM1 mRNA or SIM1-CYC1<sup>5′ UTR</sup>. We first treated these cells with either DMSO or 1NA-PP1 for about an hour, and then washed them into fresh medium with either DMSO or 1NA-PP1 and took samples of cells and cell-free growth medium at indicated times. When we inhibited cbk1-as in cells containing SSOL and wild-type SIM1 the amount of Sim1 secreted into the media was greatly reduced relative to control DMSO treatment (Figure 6C, left). As expected for a protein with slow degradation, we did not see extensive depletion of cell-associated Sim1 upon Cbk1-as inhibition in SSOL cells. We found SIM1-CYC1<sup>5′ UTR</sup> behaved essentially identically to the endogenous SIM1 mRNA in all assays (Figure 6C, right). These experiments indicate that hyperactivation of Ssd1 represses translation of Sim1, and that the SIM1 3′UTR is not necessary for this effect.

Consistent with absence of translational repression, we saw no significant reduction in the amount of secreted Sim1 in 1NA-PP1-treated cbk1-as ssd1A cells with either the endogenous SIM1 gene or the SIM1-CYC1<sup>5′ UTR</sup> chimeras. Under these conditions, the amount of cell-associated Sim1 was increased. We found that SIM1 mRNA levels were significantly elevated in 1NA-PP1-treated cbk1-as ssd1A cells (Figure S7), which we infer reflects increased SIM1 transcription and results in a corresponding increase in the amount of cell-associated Sim1.

Discussion and Conclusions

Figure 7 presents a graphical summary of our analysis of CTS1 and SIM1 UTRs in Ssd1’s binding and translational control of these mRNAs. While not a general model for Ssd1-mRNA interaction and translational repression, our results indicate that the CTS1 and SIM1 3′UTRs are sufficient for Ssd1-mediated translational control and binding in heterologous contexts. Ssd1-mediated translational repression of these constructs is particularly strong when the Ndr/LATS kinase Cbk1 is inhibited, consistent with this kinase’s direct negative regulation of Ssd1[6]. Intriguingly, the SIM1 3′UTR helps confer robust Ssd1 mRNA binding but is not essential for Ssd1 translational control of the SIM1 mRNA. We therefore suggest that information in both 5′ and 3′ UTRs can play a role in SIM1 repression. Consistent with this, we find that Ssd1 can exert translational control over the UTH1 mRNA through either its 5′ or 3′ UTR (Figure 2D).

As noted, the SEE motif is clearly enriched in the 5′UTRs of some known Ssd1 target transcripts [5,22]. There is no direct evidence that it binds Ssd1 or directs translational control, and not all Ssd1 target transcripts contain the SEE in 5′UTR regions [5,6]. We find that the SEE-containing 5′UTR of CTS1 does not mediate Ssd1-mediated translational regulation of reporter constructs, while other mRNA regions that do not contain this motif can do this. It is notable that the UTH1 5′UTR, which contains four SEE motifs, confers translational control. Thus, it remains possible that the SEE promotes Ssd1-mediated translational control in some contexts. However, the SEE itself appears to be neither necessary nor sufficient for Ssd1 translational control of mRNAs. Overall, our findings suggest that multiple signals can direct Ssd1 to target transcripts, and that the SEE is one of several mechanisms that promote Ssd1 association with a target mRNA.

Our analysis of Ssd1’s association with specific transcripts [6,22] does not discriminate between direct interaction of Ssd1 with mRNA and indirect interaction through another RNA binding protein, several of which are known to associate with Ssd1 [6]. Thus, Ssd1 may influence translation by binding to another protein that interacts with specific mRNAs. Ssd1 association with the CLN2 5′UTR and CTS1 3′UTR by yeast three-hybrid is especially notable, as the hybrid RNAs used in this system are transcribe from an RNA polymerase III promoter. There is strong evidence that Ssd1 associates with the Ser2,5P CTD tail of RNA polymerase III [47], a hallmark of transcriptional elongation, and previous studies have suggested co-transcriptional loading of RNA binding proteins [5]. Our three-hybrid data suggest that co-
transcriptional loading of RNA polymerase II-associated Ssd1 onto an mRNP is not the exclusive mechanism by which Ssd1 binds its target transcripts. Taken together with previously characterized interactions, our findings indicate that Ssd1 may be present in a closed loop mRNP configuration that permits multiple points of Ssd1-mRNP contact. We have shown that CTS1 and SIM1 3' UTRs confer Ssd1 translational control, as can both the 5' and 3' UTRs of the UTH1 mRNA; Ssd1 associates with the CLN2 5' UTR [22] and the polyA-binding protein Pab1 [23]. Thus, cis-elements or proteins bound in both 5' and 3' UTRs may work together, and possibly redundantly, to create a context amenable to Ssd1 association and function. Consistent with redundancy of Ssd1 translational control, the SIM1 3' UTR is sufficient for Ssd1-mediated translational control, but is not essential in the endogenous context of SIM1 (Figure 7B). Intriguingly, analysis of Ssd1 binding to the CLN2 5' UTR indicates that this association stabilizes Chn2 expression [22], distinct from Ssd1 repression of the translation of bound transcripts [6]. Thus, it is possible that Ssd1 exerts variable effects over an mRNA’s behavior depending on context and the complement of RNA binding proteins present on the transcript.

Our results show that Ssd1 can tune expression of proteins through association with UTRs in their mRNAs. In at least some cases, this Ssd1 association allows the Ndr/LATS kinase Cbk1 to control the mRNA’s translation. Ssd1-mediated translational control was not universal for all of the UTRs we tested. This could indicate either a limitation of reporters in studying RBP-mediated translational control, or that Ssd1 regulons present in some UTRs (such as SCW10 or TOS1) may not function in a heterologous context, possibly due to a changed complement of RBPs or mRNA secondary structure. SUN4 presents an intriguing case. We found that neither its 5' nor 3' UTRs are sufficient to confer Ssd1-mediated translational control, and perhaps the SUN4 5' and 3' UTRs must flank the appropriate ORF for Ssd1 translational repression. Interestingly, the SEE motif is present in the SUN4 ORF, but not in the SUN4 UTRs. Other Ssd1-bound transcripts not tested here may also have functionally distinct mechanisms that promote Ssd1 association. These sites may serve complementary roles in shaping the spatiotemporal expression of
proteins encoded by Ssd1 target transcripts, influencing translation, RNA localization and stability in diverse ways. This would allow the Ssd1 system to influence a wide range of processes involved in cell wall maintenance and robustness.

Supporting Information

Figure S1 GFP bearing the Ssd1-bound transcript CTS1’s 3’-UTR is differentially expressed depending on Ssd1 genotype. GFP fused to the CTS1 3’-UTR shows significantly depressed expression in SSD1 cells, determined by: (A), the ratio of GFP MFI of \[P_{TEF1-GFP-CYC13’UTR}\] or \[P_{TEF1-GFP-CTS13’UTR}\] in ssd1Δ over SSD1 cells and (B), the ratio of \[P_{TEF1-GFP-CTS13’UTR}\] to \[P_{TEF1-GFP-CYC13’UTR}\] in ssd1Δ or SSD1 cells, determined by flow cytometry. (C) GFP transcription of reporters is not significantly different as determined by real-time qPCR. Data presented in (A) through (C) represent at least three independent trials. Error bars represent ± SEM. *** indicates P-value<0.001, ‘ns’ indicates P-value>0.05 at 95% confidence intervals as calculated by unpaired two-tailed Student’s t-test.

Figure S2 UTRs from further Ssd1 targets do not confer translational control. We expressed GFP reporters with the indicated 3’ or 5’-UTR in SSD1 and ssd1Δ cells and evaluated their expression by measuring MFI on a flow cytometer as described in Materials in Methods. (A) The 3’-UTRs from the Ssd1-associated messages SUN4, TOS1 and SCW10 do not confer significant Ssd1-dependent variations in expression compared to the control reporter bearing the CYC1 3’-UTR. (B) The 5’-UTRs from the Ssd1-associated messages SUN4 and CTS1 do not confer significant Ssd1-dependent variations in expression compared to control reporters bearing either the TEF1 or ADH1 5’-UTR. The expression of these constructs is nearly identical in SSD1 and ssd1Δ cells. Data represent three independent trials. Error bars represent ± SEM. No P-values calculated between control (gray bars) and test (black bars) constructs were significant (P-value>0.05 at 95% confidence intervals as calculated by unpaired two-tailed Student’s t-test.)

Figure S3 (A) Stable GFP-CTS13’UTR or (B) SIM13’UTR reporters do not respond to 1 hour INA-PP1 treatment (compare cbk1-as +DMSO and cbk1-as +1NA-PP1) as measured by GFP
MFI. Expression in *cbk1-as SSD1* cells is significantly different to expression in *CBK1 ssd1Δ* cells, showing that the *cbk1-as* allele retains kinase activity. GFP perdurance likely masks changes in GFP translation in response to 1NA-PP1 treatment, necessitating the use of destabilized GFP<sup>P<sub>PEST</sub></sup>. In (A) and (B), fluorescence data were corrected for variations in cell size apparent in forward scatter (FSC) measurements as described in Materials and Methods. Error bars represent ± SEM. ** indicates P-value of 0.001 to 0.01, * indicates P-value 0.01 to 0.05, and 'ns' indicates P-value>0.05 at 95% confidence intervals as calculated by unpaired two-tailed Student's t-test.

**Figure S4** Destabilized GFP-CTS1<sup>3'UTR</sup> reporters respond to Cbk1 inhibition and are further reduced under growth in ethanol. (A) Western blotting against GFP confirms destabilized GFP (GFP-Chi<sup>2'PEST</sup>) bearing the CTS1<sup>3'UTR</sup> expression is responsive to Ssd1 phosphorylation state and is depleted on Cbk1 inhibition when expressed from either *ADH1* or *TEF1* promoters, while steady-state levels of the housekeeping gene *Pgk1* are unaffected. (B) Repression of destabilized GFP reporter (uGFP) expression under Cbk1 inhibition depends on the presence of Ssd1 or an Ssd1-regulated 3'UTR. We report the relative %GFP positive at each time point t>1 h as a fold change relative to the %GFP positive population at t=0 h. Flow cytometry was performed on cells fixed at one hour intervals as described in Materials and Methods. (C) Reporter expression under growth in 5% ethanol, a condition where Ssd1 function is critical, was examined by flow cytometry as described in Materials and Methods. Histograms depicting the GFP fluorescence of *CBK1 SSD1* or *cbk1-as SSD1* cells expressing either the Ssd1-bound destabilized reporter (uGFP-CTS1<sup>3'UTR</sup>) or unbound reporter (uGFP-CYC1<sup>3'UTR</sup>) grown in YPD rich media supplemented to 5% or 0% (v/v) final ethanol concentration reveal strong suppression of GFP expression in ethanol-exposed *cbk1-as* cells expressing the bound CTS1<sup>3'UTR</sup> reporter, but not in *CBK1* cells or when an unbound CYC1<sup>3'UTR</sup> is expressed.

**Figure S5** Ssd1-regulated expression of GFP reporter is due to changes in transcript ribosomal occupancy. Polysome profiling of RNA extracts followed by Northern blot analysis of RNA fractions from across the polysome gradient were used to analyze ribosomal occupancy of the *P<sub>TEF1</sub>-GFP-CTS1<sup>3'UTR</sup>* reporter in 1NA-PP1-treated *cbk1-as SSD1* and *cbk1-as ssd1Δ* cells. Experiments were performed as described in Figure 4; here, we show two additional replicates. (A) Relative mRNA abundance traces from Northern blots of two replicate polysome profiling experiments (Trial 2, top and Trial 3, bottom). Highlighted gray regions indicate the mRNA fractions associated with polysomes, determined from total A<sub>254</sub> measurements of fractionated sucrose gradients. GFP mRNA is enriched in monosomes in the absence of Ssd1, while *PGK1* mRNA polysome association changes minimally. (B) Three replicate GFP and *PGK1* ribosomal occupancy maps were analyzed by calculating the total area under the curve (AUC) and determining the fraction of that area encompassed by the polysome-associated region (gray box). We saw a significant difference in *GFP-CTS1<sup>3'UTR</sup>* polysome association, but not for *PGK1*. (C) Data tables for GFP and *PGK1* ribosome AUC calculations show the percent encompassed by the polysome region of each trial, the mean percentage of each transcript in polysome regions, and P-value. Error bars represent ± SEM, * indicates P-value 0.01 to 0.05, and 'ns' indicates P-value>0.05 at 95% confidence intervals as calculated by unpaired two-tailed Student's t-test.

**Figure S6** Immunoprecipitation of Ssd1 is similar is not affected by 3'UTR identify at the *SIM1* locus. Samples representing 0.05% (v/v) of the volume at each indicated experimental stage were removed for SDS-PAGE analysis by Western blotting with anti-TAP. Ssd1 protein is similarly immunoprecipitated in samples expressing the *SIM1* locus with either the *SIM1* or *CYC1* 3'UTR.

**Figure S7** *SIM1* transcription increases in 1NA-PP1-treated *cbk1-as ssd1Δ* cells. We collected mRNA from cells used in the assay for secreted Sim1 (Figure 6C) and measured *SIM1* message abundance by quantitative RT-PCR. As noted in our discussion of Figure 6C, we saw increased cell-associated Sim1 protein in *cbk1-as ssd1Δ* cells treated with 10 µM 1NA-PP1 for 1 hour. *SIM1* message levels were significantly elevated in 1NA-PP1-treated *cbk1-as ssd1Δ* cells compared to *cbk1-as SSD1* cells with the same treatment, and significantly elevated compared to DMSO-treated *cbk1-as ssd1Δ* cells. We saw no significant difference in *SIM1* transcript abundance between *cbk1-as SSD1* and *cbk1-as ssd1Δ* cells treated with DMSO. Data shown are the result of four independent trials, each of which included three technical triplicates. Error bars represent ± SEM, * indicates P-value 0.01 to 0.05, and 'ns' indicates P-value>0.05 at 95% confidence intervals as calculated by unpaired two-tailed Student's t-test.

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**Author Contributions**

Conceived and designed the experiments: AGW YL ELW. Performed the experiments: AGW YL ELW. Analyzed the data: AGW ELW. Contributed reagents/materials/analysis tools: AGW YL. Wrote the paper: AGW ELW.

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