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Posttranslational Regulation of the Scaffold for Fe-S Cluster Biogenesis, Isu

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Isu, the scaffold protein on which Fe-S clusters are built in the mitochondrial matrix, plays a central role in the biogenesis of Fe-S cluster proteins. We report that the reduction in the activity of several components of the cluster biogenesis system, including the specialized Hsp70 Ssq1, causes a 15–20-fold up-regulation of Isu. This up-regulation results from changes at both the transcriptional and posttranslational level: an increase in ISU mRNA levels and in stability of ISU protein. Its biological importance is demonstrated by the fact that cells lacking Ssq1 grow poorly when Isu levels are prevented from rising above those found in wild-type cells. Of the biogenesis factors tested, Nfs1, the sulfur donor, was unique. Little increase in Isu levels occurred when Nfs1 was depleted. However, its presence was required for the up-regulation caused by reduction in activity of other components. Our results are consistent with the existence of a mechanism to increase the stability of Isu, and thus its level, that is dependent on the presence of the cysteine desulfurase Nfs1.

INTRODUCTION

Iron-sulfur (Fe-S) clusters are prosthetic groups required for the function of proteins in many cellular processes that involve redox reactions, enzymatic catalysis, and electron transport, as well as in some regulatory roles. Even though Fe-S clusters can be chemically reconstituted, virtually all organisms contain a highly conserved, complex assembly and transfer system that facilitates the biogenesis of Fe-S cluster–containing proteins (Barras *et al.*, 2005; Johnson *et al.*, 2005; Lill *et al.*, 2006; Lill and Muhlenhoff, 2006). The central component of this system in many prokaryotes and all eukaryotes is a scaffold protein, called IscU or Isu, respectively, on which a Fe-S cluster is assembled, before transfer to a recipient apo-protein. Mitochondria of the yeast *Saccharomyces cerevisiae* contain two very highly conserved, functionally redundant scaffold proteins, Isu1 and Isu2, which are 83% identical and hereafter collectively referred to as Isu (Garland *et al.*, 1999; Schilke *et al.*, 1999). Fe-S cluster biogenesis is an essential process; either Isu1 or Isu2 must be expressed for cells to maintain viability.

Although Isu, in its role as a scaffold, is central to Fe-S cluster biogenesis, in vitro and in vivo evidence link several other proteins to the process. These include Nfs1, Yfh1, and Yah1, which are implicated in the assembly of the cluster on Isu. Nfs1, the cysteine desulfurase, acts as a sulfur donor, catalyzing the release of sulfur from cysteine, is the best established (Li *et al.*, 1999; Muhlenhoff *et al.*, 2004; Dutkiewicz *et al.*, 2006). The iron-binding protein Yfh1, the yeast frataxin homolog, has been proposed to serve as the iron donor. In support of this idea, frataxin is capable of donating iron for Fe-S cluster reconstitution on Isu in vitro (Yoon and Cowan, 2003), and cells lacking Yfh1 have low Fe-S enzyme activities (Rötig *et al.*, 1997). Yah1, an essential ferredoxin, also appears to play a role in cluster assembly, perhaps providing the electrons needed for the reduction of sulfur in cysteine to sulfide (Lange *et al.*, 2000). Reduction in levels of Nfs1, Yfh1, or Yah1 leads to a decrease in the amount of Fe-S clusters associated with Isu, as expected for factors involved in cluster assembly (Muhlenhoff *et al.*, 2003).

Proteins important in transfer of the cluster from Isu to an apo-protein include a pair of specialized molecular chaperones, the J-protein, Jac1, and its Hsp70 partner, Ssq1, which also requires the nucleotide release factor Mge1. Jac1 binds directly to Isu and targets it to Ssq1, stabilizing the Ssq1–Isu interaction by stimulating the ATPase activity of Ssq1 (Dutkiewicz *et al.*, 2003, 2006; Vickery and Cupp-Vickery, 2007). The chaperone pair is thought to remodel the structure of holo-Isu, facilitating transfer of the cluster to an apo-protein (Muhlenhoff *et al.*, 2003; Dutkiewicz *et al.*, 2006). *JAC1* is an essential gene; *SSQ1* is not, although cells lacking Ssq1 grow extremely poorly (Schilke *et al.*, 1996). The viability of *ssq1*Δ cells is attributed to the ability of Ssc1, the abundant Hsp70 involved in protein translocation and folding in the mitochondrial matrix, to function with Jac1 in the Fe-S cluster biogenesis process, albeit significantly less efficiently than Ssq1 (Schilke *et al.*, 2006). In addition to the chaperone system, Grx5, a nonessential monothiol glutaredoxin, plays a role in transfer of cluster from holo-Isu to an apo-protein, although its mechanism of action is not understood (Muhlenhoff *et al.*, 2003). Other proteins implicated in Fe-S cluster biogenesis include the nonessential proteins Isa1, Isa2, and Nfu1, though their functions may be more specialized (Schilke *et al.*, 1999; Jensen and Culotta, 2000; Muhlenhoff *et al.*, 2007).

When yeast cells are compromised in Fe-S cluster biogenesis, not only are the activities of Fe-S proteins decreased, the so-called "iron regulon," a set of genes regulated by the paralogous transcription factors Aft1 and Aft2, is induced (Yamaguchi-Iwai *et al.*, 1995; Rutherford *et al.*, 2001, 2005;

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Chen *et al.*, 2004; Gerber *et al.*, 2004). Aft1 and Aft2 bind to a promoter sequence element designated the iron regulatory element, activating genes involved in iron uptake from the extracellular environment, as well as iron transport across organellar membranes (Garland *et al.*, 1999; Rutherford *et al.*, 2003). Although these two transcription factors clearly have overlapping DNA binding site specificities, they are not functionally identical. A number of genes having an iron-regulatory element in their promoters are activated by Aft1 and Aft2, but others require either Aft1 or Aft2 for activation (Rutherford *et al.*, 2003; Philpott and Protchenko, 2008). The signal that initiates the activation of Aft1 and Aft2, and thus the iron regulon, is not known. However, several cytosolic components important in the activation of Aft1 and Aft2 have recently been identified (Ojeda *et al.*, 2006; Kumanovics *et al.*, 2008). One physiological outcome of the activation of the iron regulon in response to a decrease in Fe-S cluster biogenesis is an increase in mitochondrial iron levels at the expense of cytosolic iron (Lill and Muhlenhoff, 2008). This effect is dependent on Aft1, because mitochondrial iron levels are not increased in *aft1*Δ cells (Rodriguez-Manzaneque *et al.*, 2002; Yang *et al.*, 2006).

We previously observed that the levels of Isu are substantially higher in cells lacking the specialized Hsp70 Ssq1 than in wild-type cells (Knieszner *et al.*, 2005). We have investigated the basis of this up-regulation and report that both transcriptional and posttranslational mechanisms contribute to the rise in Isu levels, with an increase in the stability of Isu protein being predominant. This increase, which is biologically important, as robust cell growth requires this higher level of Isu when chaperone activity is diminished, requires the presence of Nfs1 and is not dependent upon the increase in iron levels that occurs when cluster biogenesis is compromised.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Cell Growth Conditions

Strains of *S. cerevisiae* used in this study were derived from PJ53, which is isogenic to W303: *trp1-1/trp1-1 ura3-1/ura3-1 leu2-3112/leu2-3112 his3-11,15/his3-11,15 ade2-1/ade2-1 can1-100/can1-100 GAL2*$^{+}$*/GAL2*$^{+}$ *met2-*Δ*1/met2-*Δ*1 lys2-*Δ*2/lys2-*Δ*2*. Deletions of *ISU1*, *ISU2* (Schilke *et al.*, 1999), *YFH1* (Voisine *et al.*, 2000), and *SSQ1* (Aloria *et al.*, 2004), replaced by *LEU2, HIS3, HIS3,* and *HPH*, respectively, have been described previously. Null alleles of other genes were obtained by replacing the entire coding region with the indicated marker using standard techniques: *AFT2::KanMX4, GRX5::MET2, NFS1::TRP1, AFT1::LEU2,* and *AFT1::KanMX4.* The *KanMX4* cassette was obtained from the homozygous diploid yeast genomic knockout collection (Open Biosystems, Huntsville, AL; Winzeler *et al.*, 1999). *ISU1** were generated by site-directed mutagenesis (QuikChange protocol, Stratagene, La Jolla, CA) to change the Aft-binding site at position −245 to −239 of *ISU1* from TGCACCC to TGCAGGG. A *URA3*-marked expression vector having the *ISU1** mutation was used for a two-step disruption in *isu1*Δ*::LEU2.* The presence of *ISU1** was determined by sequencing *ISU1* PCR products generated from chromosomal DNA. *FET3-lacZ*, using published start and end points with the promoter from −506 to +3 (Li and Kaplan, 2001), was first constructed in *pBS1-lacZ* (Schilke *et al.*, 1996) and then subcloned into pRS316 (Sikorski and Hieter, 1989) to change the marker.

ISU1 from −4 to 502 was cloned into *pCM184*(Gari *et al.*, 1997). Strains having Isu1 under control of the tetracycline repressible promoter were created by transforming *ssq1*Δ *aft1*Δ *isu1*Δ *isu2*Δ or *aft1*Δ *isu1*Δ *isu2*Δ strains harboring a wild-type *ISU1 URA3*-marked expression vector (pRS316-Isu1; Schilke *et al.*, 1999) with *TRP1*-marked *tet*^{R}*-ISU1* and counterselecting for pRS316-Isu1 on medium containing 5-flourorotic acid (5-FOA). *NFS1* from 489 to 1918 was cloned into the pRS313 vector. QuikChange was used to change amino acid isoleucine 191 to serine generating $nfs1_{I191S}$ (Li *et al.*, 1999). The construct used for generating precursor protein utilized as a control in the pulse experiments contains *ISU1* from −42 to 725 in *pGEM-7Zf+* (Promega, Madison, WI).

To select for suppressors of slow growth phenotype of cells lacking Ssq1, an *ssq1*Δ strain was mutagenized by transforming with the yeast::mTn3*lac*ZLEU2 DNA library digested with Not I and selecting for Leu+ cells (Burns *et al.*, 1994). The Leu+ transformants from each plate were resuspended in water, and 2 OD_{600} equivalents were spread on plates containing 5-FOA. Cells able to grow on 5-FOA were crossed to *ssq1*Δ carrying pRS316-SSQ1. The resulting diploids were sporulated and dissected, and the segregation of the *LEU2* marker with resistance to 5-FOA was determined. Candidates that suppressed the growth defect of *ssq1*Δ on 5-FOA were transformed with pRSQ to rescue the DNA sequence adjacent to the transposon insertion as described (Burns *et al.*, 1996). The DNA flanking the Tn3 element was sequenced and found to be an insertion after nucleotide 80 of *AFT1*. To verify that inactivation of *AFT1* was the reason for suppression of the 5-FOA growth defect, the *AFT1* open reading frame (ORF) was replaced with *LEU2* by homologous recombination.

Because of slow growth and suppressor accumulation all *ssq1*Δ and *ssq1*Δ *aft2*Δ strains were obtained fresh from dissection of a heterozygous diploid and used immediately. Strains were crossed, sporulated and dissected to yield the various combinations described in the text. Haploid progeny were recovered and their phenotype confirmed. For analysis of the effects of Isu levels on cell growth, *ssq1*Δ *aft1*Δ *isu1*Δ *isu2*Δ *tet*^{R}*-ISU1* was grown overnight in rich glucose-based medium (YPD, 1% yeast extract, 2% peptone, and 2% glucose). The following day, dilutions were made into YPD with either no addition or addition of 2 μg/ml doxycycline. The doubling time was calculated by taking OD_{600} measurements over time, and lysates were prepared after cells had been exposed to the drug for at least 24 h. Yeast were grown on YPD or on synthetic media as described (Sherman *et al.*, 1986). When indicated, the iron chelator, bathophenanthroline disulfonic acid (BPS), was added to YPD plates at 120 μM.

Gal-Nfs1 and *Gal-Jac1* (Muhlenhoff *et al.*, 2003) were introduced into wild type derived from PJ53 or $jac1_{LKDDEQ}$ strain (Andrew *et al.*, 2006) by three rounds of crossing and tetrad dissection. The cells were grown overnight in galactose-based medium (1% yeast extract, 2% peptone, and 2% galactose) and then shifted to YPD for repression of *GAL1* promoter.

Northern Blots

RNA was isolated from 10- to 15-ml cultures of indicated strains by the hot phenol method (Ausubel *et al.*, 1997). Ten micrograms of total RNA was separated on a 1% MOPS-formaldehyde gel, transferred to a nylon membrane, and hybridized in ULTRAhyb (Ambion, Austin, TX) overnight with radiolabeled double-stranded DNA probes. Probes were generated by random priming with [α-^{32}P]dCTP (6000 Ci/mmol; Perkin Elmer-Cetus, Boston, MA), using the Prime-A-Gene kit (Promega), and PCR products containing the coding region of each gene. PCR products were generated using additional sequence adjacent to the gene in the plasmid. The plasmid used to generate *PGK1* probe was a gift from Marvin Wickens (University of Wisconsin, Madison, WI). After stringent washes, membranes were exposed using a PhosphorImager detection screen (GE Healthcare, Piscataway, NJ), visualized on a Typhoon 941 (GE Healthcare), and the signal was quantified with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). For reprobing, blots were boiled in 1% SDS and rehybridized with a new probe.

Pulse and Pulse-Chase Analysis

For pulse analysis four independent *ssq1*Δ *aft1*Δ *isu2*Δ *ISU1** and 2 *aft1*Δ *isu2*Δ *ISU1** transformants were treated in duplicate. 5 OD_{600} units of cells were grown to midlog phase, pelleted, and washed in media lacking methionine. Cells were resuspended in media lacking methionine, and 10 μl of [^{35}S] Express Protein Labeling Mix (>1000 Ci/mmol; Perkin Elmer-Cetus) was added and incubated at 30°C for 2 min to incorporate label. These conditions were predetermined to be in the linear range of incorporated radiolabel. Cells were lysed by adding SDS lysis buffer (2% SDS, 90 mM HEPES, pH 7.5, 30 mM dithiothreitol), boiling and freezing on liquid nitrogen. Twenty microliters of *ssq1*Δ *aft1*Δ *isu2*Δ *ISU1** and 100 μl of *aft1*Δ *isu2*Δ *ISU1** were brought up to a volume of 1 ml in lysis buffer (150 mM NaCl, 50 mM HEPES, pH 7.5, 5 mM EDTA, and 1.0% Triton X-100) and centrifuged to pellet cells. The supernatant was then moved to a new tube, and an aliquot was removed and precipitated with trichloroacetic acid, for total incorporated counts. Isu1, 0.5 μl, precursor synthesized in vitro using a rabbit reticulocyte lysate system (Promega) with [^{35}S]methionine (>800 Ci/mmol; MP Biomedicals, Solon, OH) was added to control for immunoprecipitation efficiency. Because processing of synthesized transcripts is so rapid, little or no in vivo precursor was detectable even in the case of the 2-min pulse.

Isu antibody, 1 μl, was added to the cell lysates and incubated for 2 h at 4°C with rotation. Preliminary experiments were performed with increasing concentrations of cell extracts to ensure that the antibody was sufficient for quantitative immunoprecipitations because extracts from different strains had varying concentrations of Isu. A 50% slurry of protein A beads, 20 μl, was added and incubated for 1 h at 4°C. The protein bound to the beads was washed with wash buffer (Lysis buffer + 0.1% SDS), resuspended in sample buffer, and separated by SDS-PAGE. The gel was dried and exposed to a PhophorImager detection screen, and bands were quantified with ImageQuant software. Counts were normalized for total incorporation as well as immunoprecipitation efficiency as indicated by the amount of precursor pulled down.

For pulse-chase analysis, 10 OD_{600} units of cells were used with 20 μl of protein labeling mix as described for pulse analysis except the radiolabeled

precursor was omitted in some cases. After the pulse, cells were resuspended in 10 ml of minimal media plus 0.2 mg/ml methionine and cysteine and placed back in flasks at 30°C for the time course. At each time point cells were collected, lysed, and analyzed as described above. The zero time point was set as 100% protein, and the other time points are presented as a percentage of the zero time point. The supernatants were then reprecipitated with an Ssc1-specific antibody as a control. Data were fit to a single two-parameter exponential decay ($y = ae^{-bx}$) using SigmaPlot (Systat Software, San Jose, CA).

Other Procedures

For β-galactosidase assays, multiple independent transformants were grown in minimal media to an OD_{600} of 0.5–1.2, and β-galactosidase activity in Miller units was measured as described previously (Miller, 1972). All chemicals, unless stated otherwise, were purchased from Sigma (St. Louis, MO).

For expression of $Isu2p_{strep}$ having a strep tag at the C-terminus, DNA encoding mature Isu2p (amino acid 27–156) was cloned into pET3a-Strep-tag vector (Andrew *et al.*, 2006) to create pET3a-Isu2-Strep-tag. $Isu2p_{strep}$ and $Isu1p_{strep}$ were purified from *Escherichia coli* according to the protocol previously described for $Isu1p_{strep}$ (Andrew *et al.*, 2006).

Immunoblot analysis was carried out using the ECL detection system (GE Healthcare) according to the manufacturer's suggestion and polyclonal antibodies specific for Isa1, Isa2, Isu, and Jac1 (Voisine *et al.*, 2001), Mge1 (Laloraya *et al.*, 1994), and Nfs1, Nfu1, Ssc1 (Kang *et al.*, 1990), Yah1 and Yfh1. The anti-Isu was raised in rabbits against mature His-tagged Isu1 protein, whereas the anti-Isa1, anti-Isa2, anti-Nfu1, and anti-Yfh1 antibodies were raised in rabbits against glutathione *S*-transferase (GST)-tagged protein. Anti-Nfs1 and anti-Yah1 were gifts from Andrew Dancis (University of Pennsylvania, Philadelphia, PA).

Immunoblots were conducted on 0.1 OD_{600} units of whole cell lysates prepared by bead beading or 10 μg of mitochondrial preparations as indicated. Mitochondria were prepared by differential centrifugation. Large quantities were prepared as previously described (Gambill *et al.*, 1993). For time-course experiments, ~0.2 g of cells was harvested and lysed by bead beating in sorbitol/Tris buffer (0.6 M sorbitol, 0.1 M Tris-HCl, pH 7.5). Lysates were centrifuged at 3000 × *g* for 5 min to remove cell debris; the resulting supernatants were centrifuged at 16,000 × *g* for 10 min. The pellets were resuspended in sorbitol/Tris buffer and washed by repeated centrifugation. Final pellets were resuspended in sorbitol/Tris buffer.

RESULTS

Expression of Both Isu1 and Isu2 Are Increased in ssq1Δ

As an initial step toward our goal of understanding the basis of the up-regulation of levels of Isu in the absence of Ssq1, we sought to determine the relative contributions made by Isu1 and Isu2 to the total Isu levels. First, we assessed the reactivity of antibodies raised against purified Isu1 to the 83% identical Isu2. When equivalent amounts of purified Isu1 and Isu2 were subjected to immunoblot analysis, the signals obtained were indistinguishable (Figure 1A, two left lanes). We conclude that these antibodies recognize the two proteins equally well. The ratio of the signals obtained when these antibodies were used to analyze extracts from cells expressing only Isu1 or Isu2 (*isu2*Δ and *isu1*Δ, respectively) was ~7:1. Supporting the idea that Isu1 is normally more abundant than Isu2, the signal obtained using extracts from wild-type cells was very similar to that from *isu2*Δ cells. Because our interest was in the up-regulation that occurs in cells lacking Ssq1, we also compared Isu levels in extracts from *ssq1*Δ and *isu2*Δ *ssq1*Δ. In both cases the signals were more than 15-fold higher than in extracts from cells expressing Ssq1. We conclude that Isu1 is the predominant *ISU* protein in wild-type cells, and its levels dramatically increase when Ssq1 is absent.

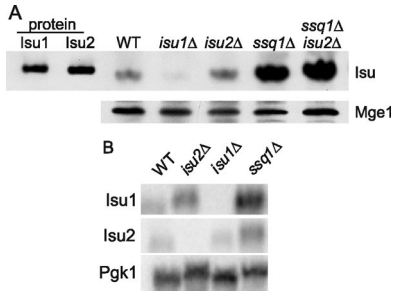

Figure 1. Comparison of *ISU1* and *ISU2* protein and mRNA levels. (A) Expression of Isu1 and Isu2. Purified $Isu1p_{strep}$ or $Isu2p_{strep}$, 5 ng of both, (left lanes) or 0.1 OD_{600} units of whole cell lysates from the indicated strains (right lanes) were subjected to electrophoresis and immunoblot analysis with Isu-specific antibodies or as a control Mge1-specific antibodies. WT, wild type. (B) Equivalent amounts of total cellular RNA isolated from the indicated yeast strains were separated by electrophoresis, blotted to a membrane, and subjected to hybridization with probes encompassing the ORFs of *ISU1*, *ISU2* and, as a control, *PGK1*.

Next we asked whether *ISU1* and *ISU2* mRNA levels are increased in *ssq1*Δ cells. Such an increase would be consistent with previous observations that *ISU1* and *ISU2* mRNA levels increase when Aft1 is activated (Garland *et al.*, 1999) and that the iron regulon is up-regulated in cells lacking Ssq1 (Knight *et al.*, 1998). To determine directly whether *ISU* mRNA levels increase when Ssq1 is absent, we assessed *ISU* mRNA levels in four strains: wild type, *isu1*Δ, *isu2*Δ, and *ssq1*Δ. Unlike *ISU1* and *ISU2* proteins, the mRNAs can be distinguished, because of differences in size and divergence of nucleotide sequence (Figure 1B). *ISU1* mRNA levels were very similar in wild-type and *isu2*Δ cells, as were the levels of *ISU2* mRNA in wild-type and *isu1*Δ cells. However, in an *SSQ1* deletion strain, both Isu1 and Isu2 mRNAs levels were increased on the order of 2–3-fold, relative to the wild-type strain. This 2–3-fold increase in *ISU* mRNA levels is not sufficient to explain the 15–20-fold higher level of Isu in cells lacking Ssq1 than wild-type cells.

Deletion of AFT1 Suppresses the Severe Growth Defect of ssq1Δ

Because the modest increase in *ISU* mRNA levels is insufficient to account for the high level of Isu in *ssq1*Δ, we wanted to further explore the basis of this regulation. However, a more comprehensive examination was hampered both by the severely compromised growth of *ssq1*Δ cells (Schilke *et al.*, 1996) and by the propensity of the *ssq1*Δ strain to accumulate suppressor mutations. Therefore, we sought situations in which *ssq1*Δ cells grew more vigorously, whereas Isu up-regulation was maintained. We isolated insertion mutations in *AFT1* as suppressors of the growth defect of *ssq1*Δ cells (see *Materials and Methods*) and subsequently determined that a complete deletion of *AFT1* resulted in substantial rescue of the growth defect caused by deletion of *SSQ1* (Figure 2A). As *AFT1* is specifically needed to up-regulate iron transporters of the plasma and mitochondrial membranes (Yamaguchi-Iwai *et al.*, 1995; Rutherford *et al.*, 2003), such suppression is consistent with the idea that slow growth of *ssq1*Δ could partially be rescued by the depletion of iron from growth medium (Knight *et al.*, 1998). To test the idea in our strain background that iron overload is in part responsible for the poor growth of Δ*ssq1* cells, we added the iron chelator BPS to growth medium. *ssq1*Δ cells were able to form colonies in the presence of BPS, although growth was compromised compared with wild-type cells (Figure 2B). Also consistent with the idea that iron overload is deleterious, deletion of *AFT2*, which does not result in reduction of iron levels, did not suppress the growth defect of *ssq1*Δ cells (data not shown).

We then asked if *ISU* mRNA and protein was up-regulated in *aft1*Δ *ssq1*Δ cells. Similar levels of both mRNA and protein were found whether or not Aft1 was present (Figure 2, C and D). Because Aft2, the paralog of Aft1, also binds iron regulatory elements, we tested expression levels of *ISU*

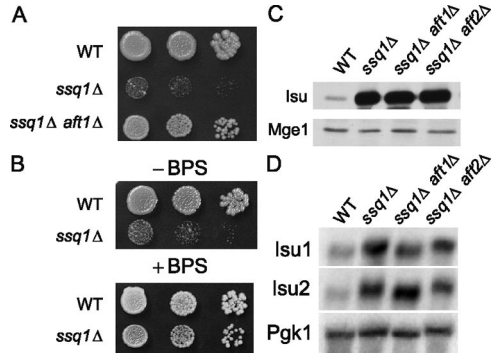

Figure 2. Effect of absence of Aft1 and Aft2 on *ssq1*Δ cells. (A) Serial dilutions at 1:10 of the indicated strains were spotted onto glucose-rich medium, and the plate was incubated for 3 d at 30°C. WT, wild type. (B) Dilutions at 1:10 of the indicated strains were spotted onto glucose-rich medium containing 120 μM BPS, and the plate was incubated for 3 d at 30°C. (C) Cell extracts from indicated strains were separated by SDS-PAGE and subjected to immunoblot analysis with Isu-specific antibodies, or, as a loading control, Mge1-spcific antibodies. (D) Total RNA extracted from the indicated yeast strains were analyzed by Northern blots using probes for the ORFs of *ISU1*, *ISU2,* and as a control, *PGK1*.

mRNA in an *ssq1*Δ *aft2*Δ strain. Isu1 and Isu2 mRNA levels were both up-regulated, consistent with the idea that either Aft1 or Aft2 is capable of activating *ISU1* and *ISU2.* However, most relevant for this report, Isu up-regulation was maintained in *aft1*Δ *ssq1*Δ cells, making the *AFT1* deletion background appropriate for our studies.

Contributions of Transcriptional and Posttranscriptional Regulation of Isu

To more thoroughly assess the contribution of posttranscriptional regulation to Isu levels, we worked toward establishing conditions under which the amounts of *ISU* mRNA levels were the same in cells lacking or expressing Ssq1. First, we addressed the issue of transcriptional regulation of *ISU1*, the more highly expressed of the paralogs (Figure 3).

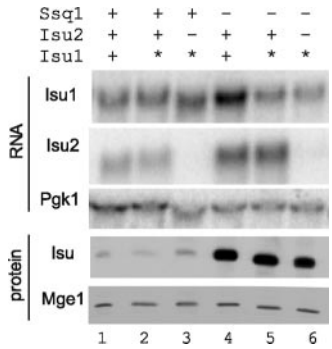

Figure 3. Isu is regulated at the posttranscriptional level. Equivalent amounts of total mRNA (top panels) or cell extracts (bottom panels) from the indicated yeast strains lacking *AFT1* were subjected to separation by electrophoresis, blotted to membranes, and analyzed by hybridization using probes for the ORFs of *ISU1, ISU2* and, as a control, *PGK1* in the case of RNA or using Isu-specific antibody and, as a loading control, Mge1-specific antibody for immunoblot analysis. The presence of the wild-type gene is indicated by (+), absence of the gene by (−); The asterisk refers to *ISU1** in which the Aft consensus binding site has been altered as described in the text.

The *ISU1* promoter has a site that fits the consensus for an Aft1/2-binding site (Garland *et al.*, 1999; Rutherford *et al.*, 2003). We introduced a mutation changing the core consensus sequence from CACCC to CAGGG, generating the promoter mutant we designate *ISU1**. Analogous mutations in other Aft1/2 target gene promoters destroy the functionality of the iron regulatory response element (Yamaguchi-Iwai *et al.*, 1996). Indeed, the increase in levels of *ISU1* mRNAs seen in *ssq1*Δ cells was obviated when the promoter mutations were present (Figure 3, compare lanes 4 and 5, top panel), indicating that Aft1/2 transcription factors are responsible for the up-regulation of Isu1 mRNA.

ISU protein levels were also compared. The levels of *ISU* protein were 10–15-fold higher in the absence, compared with the presence, of *SSQ1* (Figure 3, lanes 1–3 compared with 4–6). Most importantly, Isu1 levels were on the order of 10-fold higher even when ISU mRNA levels were the same, that is with *ISU1* under the control of the *ISU1** promoter and in the absence of *ISU2* (Figure 3, compare lanes 3 and 6). We conclude that substantial up-regulation of Isu1 occurs at the posttranscriptional level when Ssq1 is absent.

Difference in the Rate of Isu1 Degradation in the Absence of Ssq1

To begin to understand the basis of the posttranscriptional up-regulation of *ISU* protein, we next determined the rate of synthesis and degradation of *ISU1* protein. Four independently obtained strains *ssq1*Δ *aft1*Δ *isu2*Δ *ISU1** and two isogenic *aft1*Δ *isu2*Δ *ISU1** control strains were analyzed. Cells were pulse-labeled for 2 min with [^{35}S]methionine and [^{35}S]cysteine. The total amount of radioactivity incorporated by the two strains was comparable, as expected, because of their similar growth rates. Extracts prepared from the strains were subjected to immunoprecipitation with Isu-specific antibodies. The rate of synthesis of Isu1 was statistically indistinguishable in cells containing or lacking Ssq1 (Figure 4A, inset).

Because there was no significant difference in the rate of synthesis of Isu1 between the two strains, we next examined the rate of Isu1 degradation. Cells were pulse-labeled and then "chased" with an excess of nonradiolabeled amino acids to prevent further incorporation of radioactivity. Extracts prepared from samples collected over a period of 4 h were subjected to immunoprecipitation, and the radioactivity was quantified. The half-life of Isu1 from the two strains differed by about fourfold, with Isu1 in cells expressing Ssq1 having a half-life of 29 min and in cells lacking Ssq1 a half-life of 111 min (Figure 4A). As a control, the half-life of the other Hsp70 of the mitochondrial matrix, Ssc1, was also determined. The half-life of Ssc1 was similar in *aft1*Δ *isu2* Δ *ISU1** and *ssq1*Δ *aft1*Δ *isu2* Δ *ISU1**, 112 min and 105 min, respectively (Figure 4B). We conclude that there is a significant difference in the degradation rate of Isu1 in the two strains, leading to higher levels of Isu1 in cells lacking Ssq1.

These results are consistent with the idea that the increase in stability of Isu1 is a response to an alteration in the process of Fe-S cluster biogenesis. However, it is also possible that the increase in stability of Isu is simply a consequence of its increased abundance. To test this idea, we utilized an *ISU1* gene placed under the control of the *tet*^{R} promoter. Using this construct, Isu1 could be expressed at the higher level typically found in *ssq1*Δ cells, even when Ssq1 was present (Figure 4C, inset). The rate of degradation of overexpressed Isu1 was only slightly slower in such cells compared with the rate in the cells having normal levels of Isu1, 28 min compared with 21 min (Figure 4C). We con-

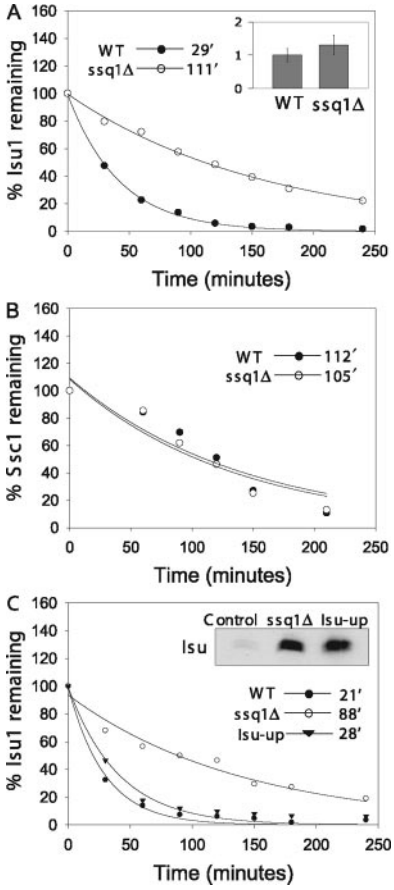

Figure 4. Synthesis and degradation of Isu1 in the presence and absence of *SSQ1*. Cells were pulse-labeled for 2 min at 30°C by addition of ^{35}S-labeled amino acids, and samples were removed and subjected to immunoprecipitation and analysis as described in *Material and Methods*. When indicated, a chase was initiated by addition of unlabeled amino acids (0 time). (A) Inset, analysis of rate of protein synthesis of two independent control *aft1*Δ *isu2*Δ *ISU1** WT and four experimental *ssq1*Δ*aft1*Δ *isu2*Δ *ISU1** (*ssq1*Δ), carried out in duplicate. Incorporation by control cells into Isu1 was arbitrarily set at 1, with the resulting values being 1.0 ± 0.2 and for *ssq1*Δ, 1.3 ± 0.3. (A–C) Pulse-chase analysis of rates of degradation. Calculated $t_{1/2}$ is indicated in minutes (e.g., 21′). (A and C) Isu1 or (B) Ssc1, as a control. Level at 0 time was set as 100%. Data were fit using SigmaPlot to a single two-parameter exponential decay ($y = ae^{-bx}$). Strains analyzed. WT: *aft1*Δ *isu2*Δ *ISU1** (●); *ssq1*Δ: *ssq1*Δ *aft1*Δ *isu2*Δ *ISU1** (○); Isu-up: *aft1*Δ *isu1*Δ *isu2*Δ *tet^R-ISU1* (▼). (C) Inset, extracts from indicated strains were subjected to immunoblot analysis using Isu-specific antibodies.

clude that the rate of Isu1 degradation is not dependent upon its concentration in the cell.

Biological Importance of Isu Up-Regulation

The experiments described above establish that the levels of Isu are up-regulated when Ssq1 is absent. However, they do not answer the question of whether these higher levels are biologically important. To address this issue, we again utilized the *tet^R-ISU1* construct. As discussed above, Isu1 is present at levels near that found in *ssq1*Δ cells when expression is driven from the *tet^R* promoter (Figure 5, right lanes). Cells grew with similar doubling times (2 h) whether Isu1 expression was driven by the endogenous promoter or by the *tet^R* promoter.

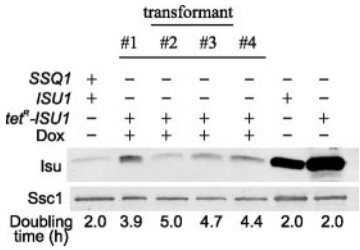

Figure 5. Expression of Isu1 in the absence of Ssq1. Cell extracts were prepared from strains having the *aft1*Δ *isu2*Δ mutation, in addition to the indicated genetic alterations. As indicated by the line, four independent transformants were analyzed. Each had the *isu1*Δ mutation on the chromosome and harbored a plasmid carrying the *ISU1* gene under the control of the tet^R promoter (*tet^R-ISU1*). When indicated, doxycycline (Dox) was added (+) to 2 μg/ml. Extracts were separated by electrophoresis and subjected to immunoblot analysis with Isu-specific antibody, and as a loading control Ssc1-specific antibody. The doubling times for the strains in hours (h) are indicated at the bottom.

To test the effect of lower levels of Isu1 on growth, we took advantage of the fact that addition of doxycycline represses transcription from the *tet^R* promoter. Four independently derived transformants carrying the *ssq1*Δ mutation and expressing *ISU1* from the *tet^R* promoter were analyzed. In the presence of drug, expression of Isu1 was dramatically lowered in all four (Figure 5, lanes 2–5), with levels ranging from very similar to that found in the control cells containing *SSQ1* to about threefold higher (lanes 3 and 2, respectively). All four grew more slowly in the presence of drug when Isu1 expression is reduced (compare lanes 2–5, and 7). However, their growth rates differed. The higher the level of Isu, the more rapid the growth rate. For example, cells having levels similar to that of the control strains expressing Isu1 from its own promoter had a doubling time of ~5 h compared with 2 h (compare lanes 1 and 3); cells having threefold higher levels grew more rapidly, with a doubling time of ~3.9 h (compare lanes 1 and 2), but still almost twice as slowly as control cells. Thus, we conclude that in the absence of Ssq1 higher levels of Isu1 protein are necessary for robust growth.

Specificity of Isu Regulation

Having determined that up-regulation of Isu in the absence of the Hsp70 Ssq1 occurs at both the transcriptional and posttranslational levels, we tested the specificity of the response. First, we asked whether other components of the Fe-S cluster biogenesis pathway were also up-regulated in the absence of Ssq1. The levels of eight additional, known or putative, components of the pathway (Nfs1, Yfh1, Yah1, Mge1, Jac1, Isa1, Isa2, and Nfu1) were compared in extracts from wild-type and *ssq1*Δ mitochondria. None of the proteins tested differed in levels by more than twofold (Figure 6). Thus, Isu is atypical, if not unique, among components of

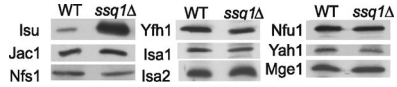

Figure 6. Level of components of Fe-S cluster biogenesis system in the absence of Ssq1. Equivalent amounts of mitochondrial extracts prepared from wild-type and *ssq1*Δ cells were separated by SDS-PAGE and probed with polyclonal antibodies specific for the indicated proteins.

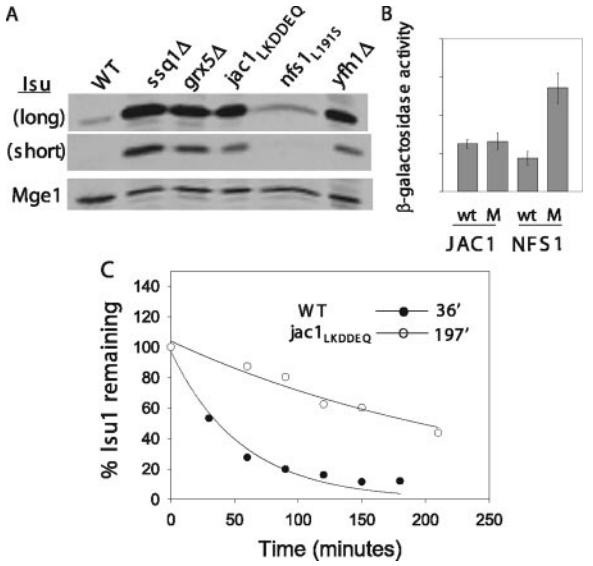

Figure 7. Effect of reduced activity of components of the Fe-S biogenesis system on Isu levels. (A) Cell lysates, prepared from indicated strains, were separated by SDS-PAGE and subjected to immunoblot analysis with Isu-specific and, as a loading control, Mge1-specific antibody. The Isu blot was exposed for 2 min (long) to visualize Isu in the wild-type strain and for 15 s (short) to allow comparison of levels of Isu in the high expressing strains. (B) *FET3-lacZ* reporter activation was measured in *jac1*Δ harboring wild-type *JAC1* (WT) or $jac1_{LKDDEQ}$ (M) on a plasmid; *nfs1*Δ harboring *NFS1* (WT) or $nfs1_{I191S}$ (M) on a plasmid. β-Galactosidase activities of samples from multiple transformants of indicated strains are shown. (C) Pulse-chase analysis of Isu1 degradation in *isu2*Δ $jac1_{LKDDEQ}$ compared with the control *isu2*Δ expressing wild-type *JAC1*. Calculated $t_{1/2}$ is indicated in minutes (e.g., 36′). The level at 0 time was set as 100%. Data were fit using SigmaPlot to a single two-parameter exponential decay ($y = ae^{-bx}$).

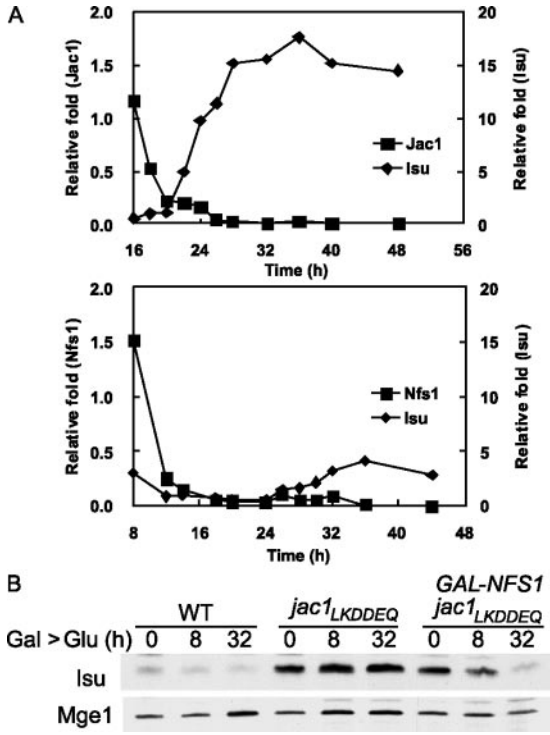

Figure 8. Effect of Nfs1 depletion on Isu expression. (A) *GAL-JAC1* (top) and *GAL-NFS1* (bottom) were grown in galactose-based medium and then shifted to glucose-based medium at zero time. Because expression of both Jac1 and Nfs1 were expressed above normal levels from the *GAL1* promoter, samples were taken as the level of these proteins approached normal (at 16 h in the case of Jac1 repression; 8 h in the case of Nfs1 repression). Crude mitochondrial extracts were prepared and analyzed as in A using antibodies specific for Isu, Jac1, or Nfs1. The signal was quantified after immunoblot analysis using antibodies specific for Isu, Jac1, or Nfs1. (B) Wild type, $jac1_{LKDDEQ}$, and *GAL-NFS1* $jac1_{LKDDEQ}$ cells were shifted from galactose- to glucose-based medium at zero time. Cells were harvested at the indicated times, and mitochondrial extracts were prepared and analyzed by immunoblots using antibodies specific for Isu and as a control, Mge1.

the Fe-S cluster biogenesis pathway in regards to up-regulation in response to the absence of Ssq1.

Next we addressed the effect of reduced activity of components of the Fe-S cluster biogenesis pathway other than Ssq1 on levels of Isu. We utilized deletion mutants for testing the nonessential components Yfh1 and Grx5. To test the essential components, Jac1 and Nfs1, we used the partial loss of function mutants, $nfs1_{L191S}$, a temperature-sensitive (ts) mutant (Li *et al.*, 1999) and $jac1_{LKDDEQ}$, which although having no obvious growth defect, encodes a protein partially defective in binding to Isu (Andrew *et al.*, 2006). Substantially higher levels of Isu were observed in *grx5*Δ and *yfh1*Δ, as well as $jac1_{LKDDEQ}$, but not in $nfs1_{L191S}$ (Figure 7A). Because the difference between the $jac1_{LKDDEQ}$ and $nfs1_{L191S}$ was striking, we considered the possibility that the *NFS1* mutant had less of an effect on Fe-S cluster biogenesis than the *JAC1* mutant.

The degree of up-regulation of the "iron regulon" is an indication of the severity of the defect in Fe-S cluster biogenesis. We therefore monitored the response by testing expression from the promoter of *FET3*, which encodes the high-affinity plasma membrane iron transporter whose expression is driven by Aft1 and Aft2, utilizing a *FET3:lacZ* fusion. β-Galactosidase activity was threefold higher in $nfs1_{L191S}$ mutant compared with the control strain, but the $jac1_{LKDDEQ}$ mutant and its control strain had very similar activities (Figure 7B). Thus, all available data indicate that the *NFS1* mutant has greater effects on Fe-S cluster biogenesis than the *JAC1* mutant.

To determine whether the increase in Isu levels in $jac1_{LKDDEQ}$ cells is due to an increase in stability compared with cells expressing wild-type Jac1, we carried out a pulse-chase analysis of protein degradation. The half-life of Isu1 from the two strains differed by ~5.4-fold, with Isu1 in cells expressing wild-type Jac1 having a half-life of 36 min and in cells expressing $Jac1_{LKDDEQ}$, a half-life of 197 min (Figure 7C). As expected, the level of Isu1 mRNA was similar in both strains (data not shown). Thus, Isu1 is regulated at the level of protein degradation in cells having reduced Ssq1 or Jac1 activity.

Dependence of Isu Up-Regulation on Nfs1

To extend the comparison between Jac1 and Nfs1, we placed the expression of these proteins under the control of the *GAL1* promoter, allowing us to monitor Isu levels after repression of either Jac1 or Nfs1 upon transfer from galactose- to glucose-based medium. In the case of Jac1, the levels of Isu1 began increasing when Jac1 levels reached 25% of normal, rising to ~15-fold higher 6 h later (Figure 8A). Nfs1 depletion showed a very different pattern. About 12 h after Nfs1 dropped to below 25% of normal levels, Isu levels began to rise, but only about fourfold, and dropped to about twofold by the end of the experiment (Figure 8A). In sum, a decrease in Nsf1 activity did not result in a substantial increase in Isu levels.

Of the five components tested for the effect of their activity on the expression of Isu, only Nfs1 depletion failed to show a 10-fold or greater increase in levels. Therefore, we decided to ask whether Nfs1 was required for the increase in Isu levels to occur. To this end we combined the *jac1*$_{LKDDEQ}$ mutation with the Gal:Nfs1 repression system. In the galactose-based medium, conditions under which expression of Nfs1 was not repressed, Isu levels were high, as expected. However, after inhibition of Nfs1 synthesis the level of Isu dropped (Figure 8B). Our results are consistent with Nfs1 being necessary for the increase in Isu levels.

DISCUSSION

We report that the cellular level of the Fe-S cluster scaffold Isu is regulated both at the transcriptional and the posttranslational levels, that is via changes in the rates of mRNA synthesis and protein degradation. The rise in protein stability accounts for the majority of the increase in Isu levels. Upregulation occurs in most, but not all circumstances in which mitochondrial Fe-S cluster biogenesis is compromised. This up-regulation of Isu is biologically important when Fe-S cluster biogenesis is compromised, because its prohibition compromises cell growth.

Both the transcriptional and posttranslational regulation is apparently unique to Isu among the known biogenesis factors, as no significant increase in the levels of the eight other components of the Fe-S biogenesis system tested was observed. In the case of the transcriptional response, this conclusion is supported by additional information. The promoters of both *ISU1* and *ISU2* contain a consensus binding site for the Aft1 and Aft2 transcription factors, whereas none of the other genes encoding the components we tested have such a site. Nor have they been shown to be up-regulated in genome-wide microarray studies when Aft1/2 are activated, for example, in the case of the deletion of *YFH1* (Foury and Talibi, 2001) or depletion of *YAH1* (Hausmann *et al.*, 2008). Perhaps Isu's uniqueness is not surprising, because it is the scaffold on which the clusters are assembled and thus is the central player in the process of Fe-S cluster biogenesis.

How the increased stability of Isu we observed relates to this pathway of Aft1/2 activation remains open. However, two pieces of evidence indicate that the increased stability of Isu does not depend on increased mitochondrial iron levels, one of the outcomes of Aft activation. First, the *jac1*$_{LKDDEQ}$ mutant mounts a robust response, but iron levels are not increased in its mitochondria. Second, the increase in mitochondrial iron levels does not occur in cells lacking Aft1 (Rodriguez-Manzaneque *et al.*, 2002; Yang *et al.*, 2006 and data not shown). However, large increases in Isu levels can occur in the absence of Aft1. Conversely, the increase in mitochondrial iron levels does not depend on Nfs1, as it was previously shown that a decrease in Nfs1 activity leads to an increase in mitochondrial iron levels (Li *et al.*, 1999).

What is the mechanism behind the regulation of Isu stability? The idea that the increase in protein stability is simply an indirect result of the 3- to 4-fold increase of protein concentration due to the mRNA up-regulation is ruled out: The stability of Isu was the same in a wild-type strain with Isu at normal levels or when overexpressed 15-fold. Nor do our results suggest that the increase correlates with either the efficiency of cluster formation or cluster transfer from Isu, as absence of components involved in both results in increased Isu protein levels. High levels of Isu were first observed in cells lacking Ssq1, which is important for cluster transfer (Dutkiewicz *et al.*, 2006; Knieszner *et al.*, 2005). However, an increase in Isu levels was also observed in the absence of Yfh1, the putative iron donor for cluster formation. Indeed, Lill and coworkers have shown that upon depletion of Yfh1, cluster formation on Isu is greatly diminished (Gerber *et al.*, 2003).

Perhaps the best clue as to mechanism, is our finding that of the five components of the Fe-S biogenesis system tested, decrease in activity of four resulted in a robust increase in Isu levels. The outlier was Nfs1, whose depletion did not result in a substantial increase in Isu levels. More importantly however, Nfs1 was required for a *JAC1* mutant to sustain high Isu levels. Nfs1, a cysteine desulfurase, serves as the sulfur donor for cluster synthesis, which is thought to be the first step in cluster synthesis (Smith *et al.*, 2001). Whether Nfs1 binding to Isu fosters a conformation of Isu that is required for stabilization or whether its activity or presence is required to transmit a signal will require further exploration.

Whatever the exact mechanism behind the increase in stability of Isu, it is important to note that the increase is important for cells to maintain robust growth in the absence of the specialized Hsp70 Ssq1. Although we found this response because of our analysis of the effect of mutations that compromised the Fe-S biogenesis system, it likely occurs normally when yeast are exposed to environmental conditions that either limit Fe-S cluster formation or affect cluster stability, such as oxidative stress. As a regulatory mechanism, increase in protein stability has an advantage of potentially being rapid, taking less time and less expenditure of energy than an increase in transcription that requires not only mRNA transcription but translation and protein translocation as well. Interestingly cells have evolved not one, but at least two, mechanisms to up-regulate Isu, underscoring the importance of maintaining the functionality of the essential and multifunctional proteins having Fe-S clusters.

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