Protection of scaffold protein Isu from degradation by the Lon protease Pim1 as a component of Fe–S cluster biogenesis regulation

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ABSTRACT **Iron–sulfur (Fe–S) clusters, essential protein cofactors, are assembled on the mitochondrial scaffold protein Isu and then transferred to recipient proteins via a multistep process in which Isu interacts sequentially with multiple protein factors. This pathway is in part regulated posttranslationally by modulation of the degradation of Isu, whose abundance increases >10-fold upon perturbation of the biogenesis process. We tested a model in which direct interaction with protein partners protects Isu from degradation by the mitochondrial Lon-type protease. Using purified components, we demonstrated that Isu is indeed a substrate of the Lon-type protease and that it is protected from degradation by Nfs1, the sulfur donor for Fe–S cluster assembly, as well as by Jac1, the J-protein Hsp70 cochaperone that functions in cluster transfer from Isu. Nfs1 and Jac1 variants known to be defective in interaction with Isu were also defective in protecting Isu from degradation. Furthermore, overproduction of Jac1 protected Isu from degradation in vivo, as did Nfs1. Taken together, our results lead to a model of dynamic interplay between a protease and protein factors throughout the Fe–S cluster assembly and transfer process, leading to up-regulation of Isu levels under conditions when Fe–S cluster biogenesis does not meet cellular demands.**

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INTRODUCTION

Iron–sulfur (Fe–S) clusters are essential cofactors of proteins engaged in fundamental cellular processes, such as oxidative phosphorylation, amino acid metabolism, ribosome assembly, and response to changing environmental conditions (Johnson *et al.*, 2005). Maturation of this diverse set of proteins depends on the iron–sulfur cluster (ISC) machinery present in mitochondria, which was inherited from the prokaryotic ancestor of this organelle (Lill *et al.*, 2014). Thus, not surprisingly, most ISC components are essential and evolutionarily conserved from bacteria to human. The fundamental steps in this process have been elucidated (Paul and Lill, 2014). Initially, Fe–S clusters are assembled de novo on a scaffold protein and then transferred to recipient proteins. However, little is understood about how this process is regulated in vivo.

The Fe–S cluster scaffold is a highly conserved, small (14 kDa) protein called Isu. In the yeast *Saccharomyces cerevisiae*, the organism used in this study, Isu is encoded by two genes, *ISU1* and *ISU2*. Isu1 and Isu2 are functionally redundant. However, Isu1 is expressed at higher levels in the cell than Isu2 (Garland *et al.*, 1999; Schilke *et al.*, 1999). Isu interacts with a number of proteins during the biogenesis process (Lill *et al.*, 2014). The assembly step is initiated by Isu forming a complex with Nfs1, a cysteine desulfurase, which functions as a sulfur donor (Webert *et al.*, 2014). Nfs1 binding promotes formation of a stable "assembly complex" composed of Isu and Nfs1 together with one of these factors, Yfh1, the yeast frataxin homologue (Schmucker *et al.*, 2011; Manicki, Majewska, *et al.*, 2014). Yfh1 functions as an iron donor for the cluster and/or regulator of Nfs1 activity (Stemmler *et al.*, 2010; Pastore and Puccio, 2013; Fox *et al.*, 2015). Next the newly synthesized Fe–S cluster is transferred from Isu to a recipient protein, either directly or via specific carrier proteins (Paul and Lill, 2014; Maio and Rouault, 2015). This transfer step is carried out by a J-protein:Hsp70 chaperone system (Dutkiewicz *et al.*, 2003)

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Abbreviations used: ISC, iron–sulfur cluster machinery; WT, wild type.
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and is initiated by binding of J-protein Jac1 to Isu (Ciesielski *et al.*, 2012). Jac1's and Nfs1's binding sites on Isu partially overlap, making their binding mutually exclusive (Majewska, Ciesielski, *et al.*, 2013). Jac1 serves to target Isu to Hsp70, the binding of which facilitates transfer of the Fe–S cluster to a recipient protein (Chandramouli and Johnson, 2006; Bonomi *et al.*, 2011; Uzarska *et al.*, 2013).

The ISC system is located in the matrix of mitochondria. However, it is also required for the biogenesis of cytosolic/nuclear Fe–S clusters and regulation of the cell's response to low iron levels driven by the Aft transcription factors (Yamaguchi-Iwai *et al.*, 1996; Rutherford *et al.*, 2003; Lill *et al.*, 2014). The activity of Aft transcription factors is low when cluster biogenesis is normal but activated when the mitochondrial ISC system is inefficient. Tight regulation of Aft activity is required, as resulting iron accumulation caused by up-regulation of membrane transporters under Aft control can be deleterious if cellular iron levels surpasses the biosynthetic needs (e.g., for Fe–S clusters and heme) and the storage capacity of the cell (Outten and Albetel, 2013; Gomez *et al.*, 2014). Free iron increases the production of reactive oxygen species, damaging biologically relevant macromolecules (Welch *et al.*, 2002). Iron accumulation commonly occurs in human diseases linked to the disruption of Fe–S cluster biogenesis (e.g., Friedreich's ataxia developed due to frataxin dysfunction; Martelli and Puccio, 2014; Stehling *et al.*, 2014; Mena *et al.*, 2015).

An additional feature of the Fe–S cluster biogenesis system is the 10- to 25-fold up-regulation of the levels of Isu, but not other components of the system, upon reduced efficiency of either cluster assembly or transfer steps (Andrew *et al.*, 2008). The majority of this up-regulation is due to an increase in Isu's stability. That is, the up-regulation is posttranslational. Strikingly, this induced stability of Isu appears to depend on the presence of Nfs1, the initial partner of Isu in the assembly step of the process, but not on its catalytic activity (Song *et al.*, 2012). The mitochondrial matrix protease Pim1 is implicated because in its absence, Isu is very stable. Isu accumulates to levels similar to that occurring when Fe-S cluster biogenesis is disrupted (Song *et al.*, 2012). Pim1 belongs to the evolutionarily conserved Lon family of serine proteases (Venkatesh *et al.*, 2012). Like other members of the broader family of ATPases associated with diverse cellular activities (AAA) proteins, Lon-type proteases are oligomeric, and their function is driven by ATP hydrolysis (Koppen and Langer, 2007). Pim1 is a well-established component of the mitochondrial protein quality control system and is responsible for the degradation of damaged and dysfunctional proteins (Baker *et al.*, 2011; Voos, 2013). Less is known about its involvement in the regulation of physiological processes through degradation of specific proteins (Major *et al.*, 2006; Pinti *et al.*, 2015).

The data point to a model in which direct physical interaction of Nfs1, and perhaps other interacting proteins, protects Isu from degradation by Pim1. In this study, we focus on the mechanisms behind the modulation of Isu's degradation and thus abundance. Using purified components, we developed an in vitro assay that allowed us to test this model in the absence of confounding factors that are present in vivo. We also used both in vivo and in vitro analyses to assess Jac1's roles in modulating Isu levels. Taken together, the results extend our understanding of how the stability of Isu is modulated in vivo at different steps of Fe–S cluster biogenesis, providing new insights into how this process is regulated.

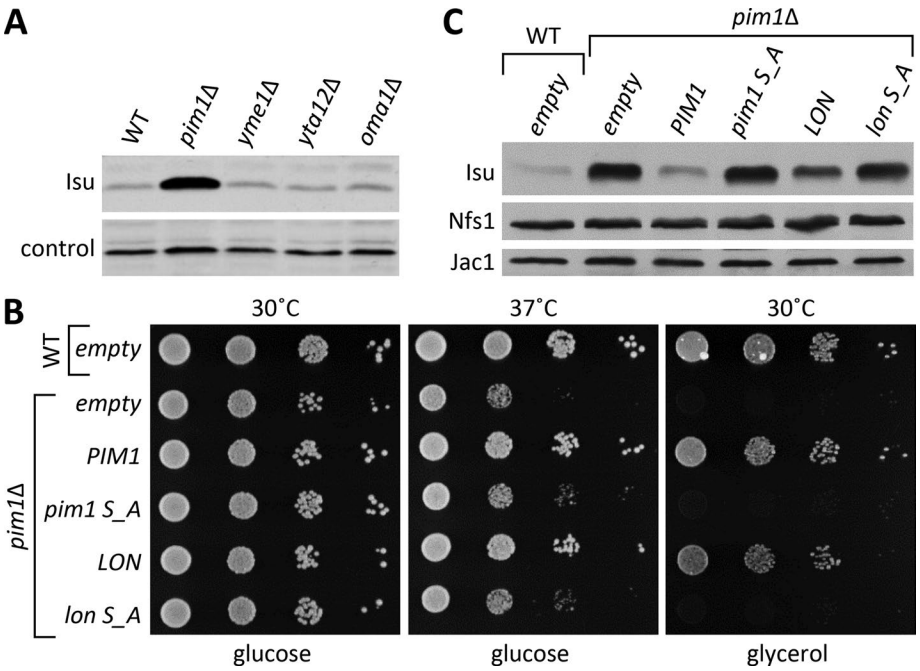

FIGURE 1: Lon-type protease Pim1 regulates Isu levels in vivo and can be substituted for by human LON. (A) Equal amounts of whole-cell lysates, prepared from a WT strain and strains lacking mitochondrial protease Pim1 (*pim1*Δ), *i*-AAA (*yme1*Δ), *m*-AAA (*yta12*Δ), and Oma1 (*oma1*Δ), respectively, were separated by SDS–PAGE and subjected to immunoblot analysis with Isu-specific antibodies and, as a loading control, Mge1-specific antibodies. (B) WT or *pim1*Δ cells, transformed with plasmid vector (*empty*) or vector encoding yeast (*PIM1*) or human (*LON*) Lon-type protease or their proteolytically inactive variants (*pim1 S_A* or *lon S_A*, respectively) were plated as 1:10 serial dilutions on glucose-based synthetic medium (glucose) or glycerol-based rich medium (glycerol) and incubated at the indicated temperatures. (C) Equal amounts of whole-cell extracts prepared from strains described in B were separated by SDS–PAGE and probed with antibodies specific for Isu, Nfs1, and Jac1.

RESULTS

The proteolytic activity of the Lon-type protease Pim1 regulates Isu levels in vivo

Isu levels, in comparison to the wild-type (WT) strain, are markedly higher in yeast cells lacking the mitochondrial Lon-type protease Pim1 (Song *et al.*, 2012), the only soluble protease in the matrix. We used immunoblot analysis to test whether proteases of the inner membrane known to have a range of substrate specificities might also be important for Isu degradation (Koppen and Langer, 2007). We measured Isu levels in *yta12*Δ and *oma1*Δ cells, which lack *m*-AAA and Oma1 proteolytic activity, respectively. As controls, we included a deletion of *YME1*, which encodes the *i*-AAA protease whose active site faces the intermembrane space, as well as a deletion of *PIM1*. Only the *pim1*Δ cells had elevated Isu levels compared with WT cells (Figure 1A), pointing to the Pim1 protease as the key regulator of Isu abundance in vivo.

Because Pim1 has biologically relevant activities other than protein degradation (e.g., DNA binding; Liu *et al.*, 2004), we asked whether its proteolytic activity is critical for regulation of Isu levels in vivo. We

took advantage of a previously described Pim1 variant that is proteolytically inactive due to substitution of the catalytic serine, Ser-1015, by alanine (Pim1 S_A) but maintains its other functions (Rep, van Dijl, *et al.*, 1996; Wagner *et al.*, 1997). We compared the Isu levels in *pim1Δ* cells harboring an empty vector or vectors containing *PIM1* WT or *pim1 S_A* to that in WT cells (Figure 1C). As expected, Pim1 WT expression resulted in reduced levels of Isu, whereas expression of Pim1 S_A did not.

The human mitochondrial LON protease can substitute for Pim1 in vivo

In vivo results clearly link Pim1 to proteolysis of Isu, but they do not directly address what influences Isu's susceptibility to degradation. To begin to address these issues, we decided to develop an in vitro assay, using purified components, to investigate Isu degradation both in isolation and in the presence of factors that act at different steps of Fe–S cluster biogenesis. However, consistent with previous reports, we found the *S. cerevisiae* Pim1 protease to be prone to self-degradation and lose activity in vitro (Stahlberg *et al.*, 1999). On the other hand, the human homologue of Pim1, the mitochondrial LON protease, has been successfully purified and used in in vitro assays (Lu, Lee, Nie, *et al.*, 2013).

Before embarking on in vitro experiments using human LON, we tested its ability to substitute for Pim1 in vivo. To do so, we used a plasmid expressing the mature form of human LON fused with a yeast mitochondrial targeting sequence under the control of the *ADH* promoter. As expected from previous analyses (Suzuki *et al.*, 1994; Van Dyck *et al.*, 1994; Wagner *et al.*, 1997), *pim1Δ* cells grew poorly at 37°C in glucose-based medium and were unable to grow on the nonfermentable carbon source glycerol, as did *pim1Δ* cells expressing either Pim1 S_A or the analogous human LON S_A variant (Figure 1B). However, at 37°C and on glycerol-based medium, cells expressing human LON WT grew nearly as well as those expressing Pim1. We also tested how expression of LON and Pim1, both under the control of the *ADH* promoter, affected the level of Isu in *pim1Δ* cells. Expression of LON WT, but not LON S_A, resulted in reduced levels of Isu, albeit not as low as those effected by expression of yeast Pim1. Whereas cells with LON WT had Isu levels substantially reduced compared with *pim1Δ* cells, Isu levels in cells expressing LON S_A were as high as in cells expressing no Lon-type protease (Figure 1C). Taken together, these results show that human LON is able to partially substitute for Pim1 in vivo and that this ability depends on its proteolytic catalytic site.

LON protease degrades Isu in vitro

Encouraged by the ability of human LON protease to substitute for yeast Pim1 in vivo, we purified it for use in in vitro degradation assays. Having confirmed that Isu1 was stable upon incubation at 30°C (Figure 2), we mixed it with a substoichiometric concentration of LON protease (7.5 μM Isu1; 1.25 μM LON monomer) and removed aliquots over a 30-min time course. The amount of full-length Isu1 present in the reaction decreased with time. Only 4% remained after 30 min. To verify that the decrease was due to LON-dependent proteolysis, we performed additional control experiments (Figure 2). First, since Lon-type proteases require ATP and Mg^{2+} ions for their proteolytic activity (Suzuki *et al.*, 1994; Van Dyck *et al.*, 1994), we performed the experiment as described, except that we left out ATP and Mg^{2+} ions from the reaction buffer. No decrease in the amount of Isu1 occurred over the 30-min time course. Second, we purified and tested the human LON S_A variant. We observed no decrease in full-length Isu1. Taken together,

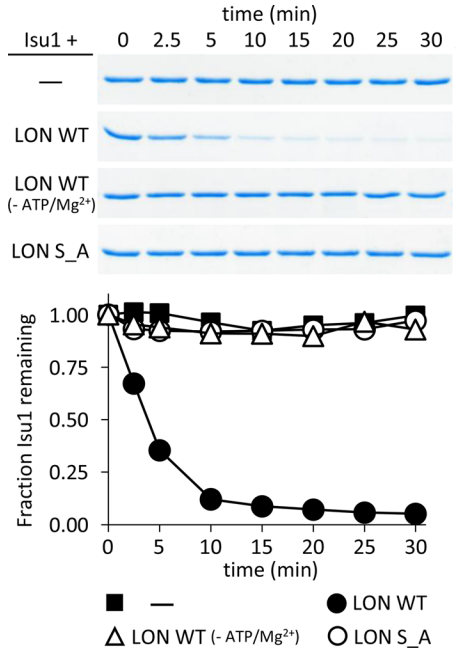

FIGURE 2: LON protease degrades Isu in vitro. Isu1 (7.5 μM) was incubated alone or with human LON WT or LON S_A (1.25 μM) in standard reaction conditions or in the absence of ATP and $MgCl_2$ (–ATP/Mg^{2+}). At indicated times, aliquots were removed, separated by SDS–PAGE, and stained (top). Amounts of full-length Isu1 were quantitated by densitometry and plotted as relative units with the time-zero value set at 1 (bottom).

these in vitro results establish that Isu1 is a substrate of the LON protease.

Binding of cysteine desulfurase Nfs1 prevents Isu degradation in vitro

Because previously published in vivo results suggested that Nfs1 protects Isu from degradation (Andrew *et al.*, 2008; Song *et al.*, 2012), we decided to test the effect of Nfs1 addition on Isu1 degradation in vitro. Nfs1 was purified in complex with Isd11, a protein with which Nfs1 normally forms a heterodimer. Interaction with Isd11 is necessary to maintain Nfs1 in an active conformation (Adam *et al.*, 2006). For simplicity, we refer to the Nfs1:Isd11 complex as Nfs1 throughout. We preincubated Isu1 with a threefold molar excess of Nfs1 before addition of LON to the reaction. Isu1 degradation was inhibited in the presence of Nfs1 (Figure 3A); after 10 min, 92% of Isu1 was intact, compared with 14% in the reaction without Nfs1. To determine whether stabilization of Isu1 depends on direct physical interaction with Nfs1, we took advantage of a previously isolated variant of Nfs1 having a reduced ability to bind Isu1 due to alanine substitutions of residues Leu-479 and Met-482 (Nfs1 LM_AA; Majewska, Ciesielski, *et al.*, 2013). Nfs1 LM_AA did not protect Isu1 from degradation as effectively as Nfs1 WT. After 30 min, only 21% of Isu1 remained in the reaction with Nfs1 LM_AA, in contrast to 67% with Nfs1 WT (Figure 3A).

Next we assessed the concentration dependence of protection of Isu1 degradation by WT and variant Nfs1. We focused on the 20-min time point in a set of reactions having increasing concentrations of Nfs1 WT or Nfs1 LM_AA, ranging from equimolar to a sixfold excess of Nfs1 relative to Isu1. Consistent with the results presented in Figure 3A, protection of full-length Isu1 from degradation was enhanced as concentrations of either WT or variant Nfs1 increased

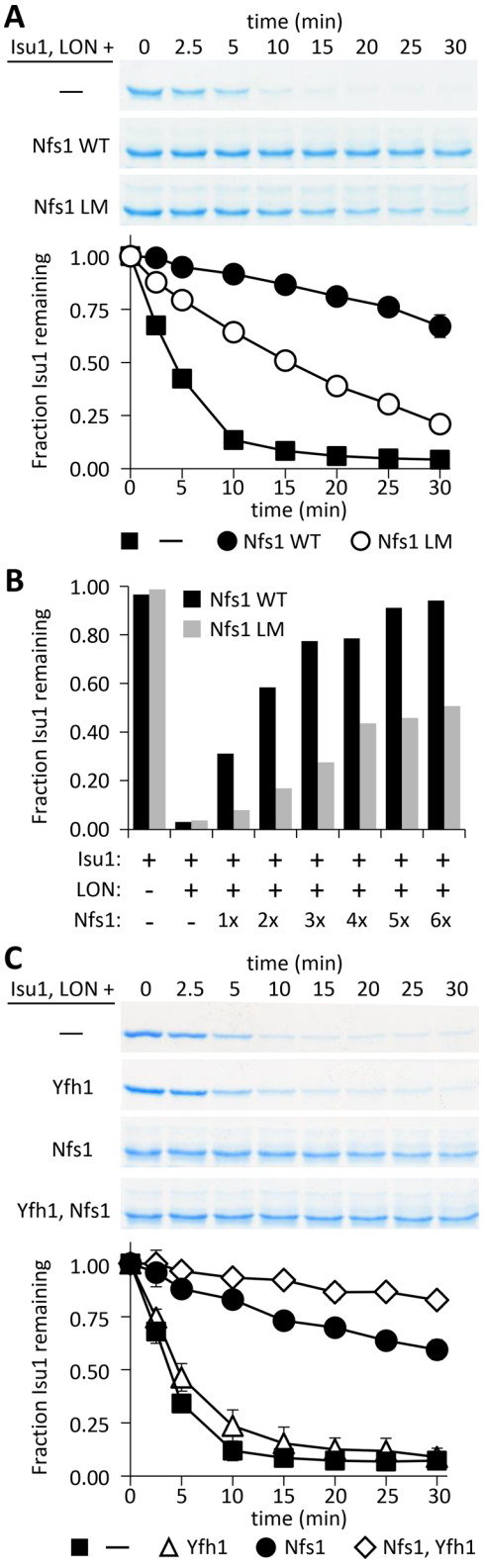

FIGURE 3: Nfs1 binding protects Isu from degradation in vitro. (A) Isu1 (7.5 μM) alone or after preincubation with 22.5 μM Nfs1 WT or Nfs1 LM_AA (Nfs1 LM) was mixed with 1.25 μM human LON protease. After protease addition (i.e., time zero), aliquots were collected at indicated times, separated by SDS–PAGE, and stained (top). Amounts of full-length Isu1 from three independent experiments were quantitated by densitometry and plotted as relative units with the time-zero value set at 1 (bottom). Error bars are shown as ±SD; in most cases, they are obscured by symbols. (B) Reactions were performed as in A with increasing concentrations of Nfs1 WT or Nfs1 LM_AA (Nfs1 LM), with equimolar Nfs1 and Isu1 concentration indicated as 1×. After 20 min, samples were collected, separated by SDS–PAGE, and stained. Isu1 amounts were quantitated by densitometry and presented as a bar graph with time-zero value set at 1. (C) Isu1 (7.5 μM) alone or after preincubation with 22.5 μM Nfs1 WT (Nfs1), Yfh1, or both protein partners (Nfs1, Yfh1) was mixed with LON (1.25 μM). Aliquots were collected at indicated times, separated by SDS–PAGE, and stained (top). Amounts of full-length Isu1 from independent experiments were quantitated by densitometry and plotted as relative units with the time-zero value set at 1 (bottom). Error bars are shown as ±SD.

(Figure 3B). However, at all concentrations tested, WT was more effective than the variant. For example, 58% of Isu1 remained in the reactions with a twofold excess of Nfs1 over Isu1, reaching 94% with a sixfold excess (Figure 3B). In contrast, the amount of Isu1 remaining did not exceed 51% of its initial concentration in the presence of even sixfold excess of the Nfs1 LM_AA variant (Figure 3B). These in vitro results positively correlate Nfs1 binding ability with protection of Isu1, leading to the conclusion that a direct interaction between the two proteins is the mechanism preventing Isu1 degradation.

Because Nfs1 and Yfh1 can bind Isu1 simultaneously, forming a triple complex, we also tested whether Yfh1 affects degradation of Isu1. Isu1 was preincubated with a threefold molar excess of Yfh1 before addition of LON to the reaction (Figure 3C). The presence of Yfh1 did not markedly affect Isu1 degradation. At 15 min after Lon addition, 15% of full-length Isu1 remained, compared with 9% in the absence of Yfh1. However, when Isu1 was preincubated with Nfs1 and Yfh1 simultaneously, we observed a modest increase in Isu1 protection compared with preincubation with only Nfs1. Eighty-three percent of full-length Isu1 remained after 30 min when both Nfs1 and Yfh1 were present, compared with 60% when Yfh1 was excluded. These results indicate that protection of Isu1 from degradation provided by binding of Nfs1 can be enhanced by the additional interaction of Yfh1.

Binding of cochaperone Jac1 prevents Isu degradation in vitro

The binding site of Nfs1 on Isu partially overlaps with that of Jac1 (Figure 4A; Majewska, Ciesielski, *et al.*, 2013), raising the question of whether Jac1 binding can also protect Isu from degradation. To address this question, we preincubated Jac1 WT with Isu1 before adding LON protease. The presence of Jac1 WT resulted in significant inhibition of Isu1 degradation (Figure 4B). Fifty-three percent of full-length Isu1 remained 10 min after LON addition when it was preincubated with a fivefold molar excess of Jac1, compared with 13% remaining in the absence of Jac1 (Figure 4B). To determine whether this protection of Isu1 from degradation depends on Jac1 binding to Isu1, analogous to our Nfs1 analysis, we took advantage of our previously isolated Jac1 variant that is defective in interaction with Isu1. Jac1 LLY_AAA does not form a stable complex with Isu1 due to alanine substitutions of residues Leu-105, Leu-109, and Tyr-163 (Ciesielski *et al.*, 2012). Jac1 LLY_AAA did not significantly protect Isu1 from proteolysis. After 30 min, only 6% of Isu1 remained, comparable to the 4% remaining in the reaction without Jac1 (Figure 4B). Focusing on the 15-min time point, we assessed the concentration dependence of Jac1 protection. Only 8% more Isu1 remained when a sixfold excess of Jac1 LLY_AAA was used compared with that remaining in the reaction lacking Jac1, whereas in the presence of Jac1 WT, 45% of Isu1 remained (Figure 4C).

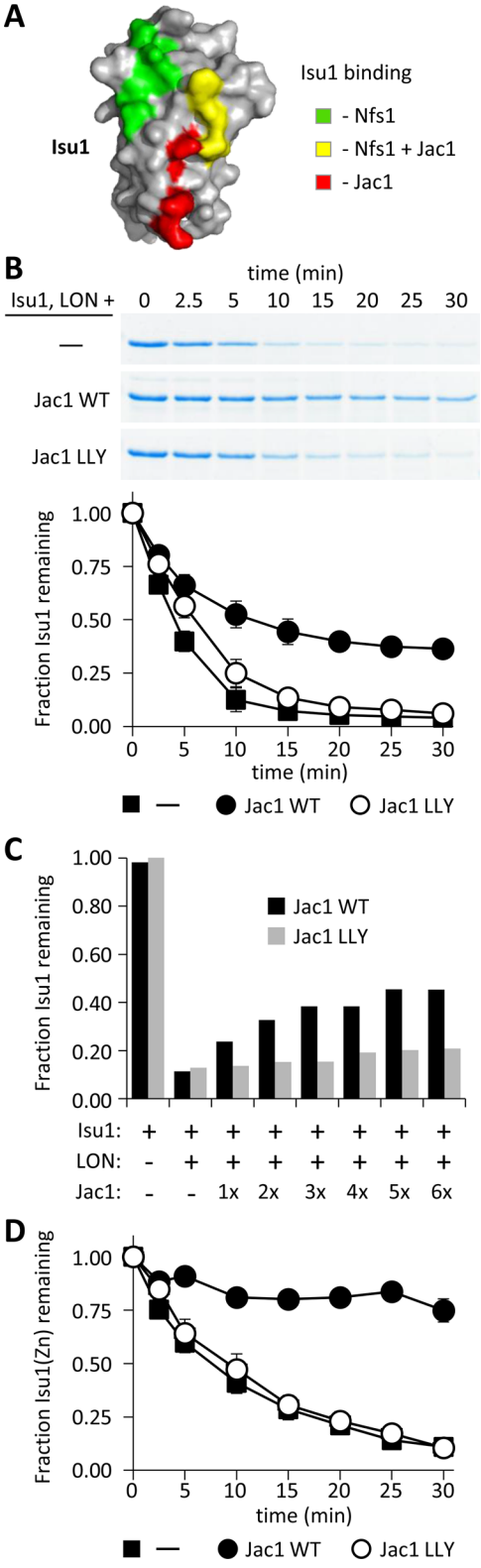

FIGURE 4: Jac1 binding protects Isu from degradation in vitro. (A) Surface representation of Isu1 with residues involved in interaction with Nfs1 and Jac1 highlighted, based on previously published results (Majewska, Ciesielski, *et al.*, 2013); prepared using PyMOL (www.schrodinger.com/pymol/). (B) Isu1 (7.5 μM) alone or after preincubation with 37.5 μM Jac1 WT or Jac1 LLY_AAA (Jac1 LLY) was mixed with LON (1.25 μM). Aliquots were collected at indicated times, separated by SDS–PAGE, and stained (top). Amounts of full-length Isu1 from independent experiments were quantitated by densitometry and plotted as relative units with the time-zero value set at 1 (bottom). Error bars are shown as ±SD. (C) Reactions were performed as in B with increasing concentrations of Jac1 WT or Jac1 LLY_AAA (Jac1 LLY), with equimolar Jac1 to Isu1 concentration indicated as 1×. After 15 min, aliquots were collected, separated by SDS–PAGE, and stained. Amounts of full-length Isu1 were quantitated by densitometry and presented as a bar graph with the time-zero value set at 1. (D) Reactions were performed as in B but with Isu1 preloaded with zinc ions (Isu1(Zn)) instead of apo-Isu1. Amounts of full-length Isu1 at the indicated times were visualized, quantitated, and plotted as in B.

Although addition of Jac1 reduced Isu1 degradation, protection by Jac1 was less effective than that by Nfs1. Given that during the biogenesis process, Jac1 interacts with Isu1 having a coordinated Fe–S cluster, we wanted to test the effectiveness of Jac1 in protecting ligand-bound Isu1. Because it is challenging to stoichiometrically reconstitute Fe–S clusters on Isu in vitro, we took advantage of the fact that Zn^{2+} ions are known to be coordinated by Isu via the same residues that coordinate clusters, thus partially mimicking cluster binding (Iannuzzi *et al.*, 2014). Therefore we incubated purified Isu1 with Zn^{2+} ions and separated the zinc-loaded Isu1 (Isu1(Zn)) from free Zn^{2+} ions. Using the same conditions as described earlier, we examined the degradation of Isu1(Zn) by LON in the absence of Jac1. Isu1(Zn) was degraded somewhat more slowly than ligand-free Isu1. After 10 min, 41% of Isu1(Zn) remained (Figure 4D), compared with 13% of ligand-free Isu1 (Figure 4B). Next we tested the effect of Jac1 WT. Seventy-five percent of the initial amount of Isu1(Zn) remained after 30 min (Figure 4D), compared with the 36% seen with the ligand-free Isu1 reaction (Figure 4B). On the other hand, no protection was observed when Jac1 LLY_AAA was present with Isu1(Zn), similar to the lack of effect on ligand-free Isu1 degradation. Taken together, these results revealed that Jac1 is able to protect Isu1 from proteolysis in vitro via direct binding and that the presence of ligand affects its efficacy.

Jac1 protects Isu from degradation in vivo

Because Jac1 was able to protect Isu1 from degradation in vitro, we next asked whether Jac1 can provide such protection in vivo. If so, we reasoned that increasing cellular concentration of Jac1 would lead to Isu accumulation. On the other hand, a similar excess of a Jac1 variant defective in Isu binding should not, if stabilization requires complex formation with Isu. WT yeast cells were transformed with a plasmid carrying the *JAC1* or *jac1 LLY_AAA* gene under the control of the strong constitutive *GPD* promoter, which resulted in >20-fold increase in Jac1 levels in both cases (Figure 5A). This overexpression of Jac1 WT led to >10-fold increase in Isu levels. Of note, when Jac1 LLY_AAA was overexpressed, no increase in Isu levels was observed; levels were similar to that in control cells having empty vector (Figure 5A).

This result is consistent with the idea that binding to Jac1 protects Isu from degradation in vivo. However, previous in vivo studies showed that maintenance of high levels of Isu, when Fe–S cluster biogenesis is perturbed, depend on the presence of Nfs1 (Song *et al.*, 2012). Therefore we wanted to test whether the stabilization we observed upon overexpression of Jac1 was Nfs1 dependent. We designed an experiment in which Nfs1 was depleted in cells overexpressing Jac1 WT (Figure 5B). Because Nfs1 is an essential protein, we used a yeast strain having chromosomal *NFS1* under the control of the *GAL10* promoter. Growing this strain in galactose-based medium allowed expression of Nfs1, which was repressed upon shift to glucose-based medium. First, as a reference, we determined the

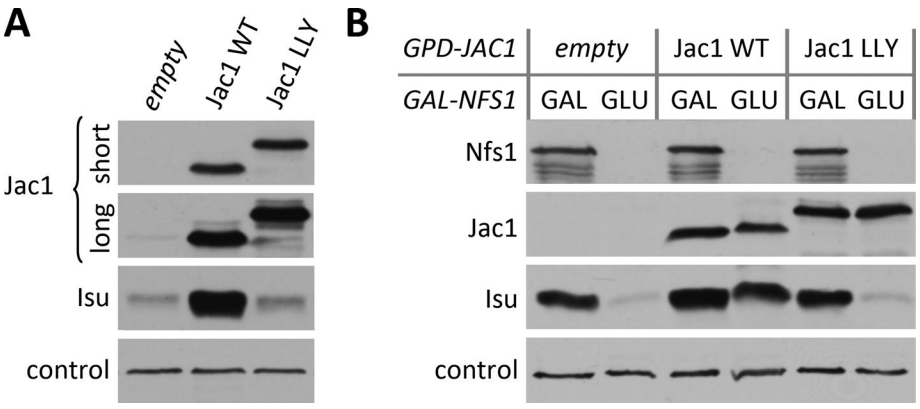

FIGURE 5: Overproduction of Jac1 leads to elevated levels of Isu in vivo. (A) Equal amounts of whole-cell extracts prepared from WT yeast cells transformed with empty vector (*empty*) or plasmids encoding *JAC1* (Jac1 WT) or *jac1 LLY_AAA* (Jac1 LLY) under the *GPD* promoter were separated by SDS–PAGE and subjected to immunoblotting using antibodies specific for Jac1, Isu, and, as a loading control, Ssc1. Both 5-s (short) and 30-s (long) exposures are shown for immunoblots using anti-Jac1 antibodies. (B) To repress Nfs1 expression, a strain with a chromosomal copy of *NFS1* under the control of the *GAL10* promoter (*GAL-NFS1)* was used. *GAL-NFS1* was transformed with the set of plasmids used in A. Cells were grown on galactose (GAL)- or glucose (GLU)-based synthetic medium. Equal amounts of whole-cell extracts were separated by SDS–PAGE and subjected to immunoblot analysis with antibodies specific for Nfs1, Jac1, Isu, and, as a loading control, Ssc1.

levels of Isu in the presence and absence of Nfs1 in a strain with native levels of Jac1 (Figure 5B). As expected, upon Nfs1 depletion, the levels of Isu dropped on the order of 10-fold. Next we asked how Isu levels were affected in the presence of an excess of Jac1 WT or the Isu binding–defective Jac1 LLY_AAA variant. In the case of Jac1 LLY_AAA, the levels of Isu were similar in the presence or absence of Nfs1, comparable to those observed in the reference strain (Figure 5B). However, in cells with excess Jac1 WT but depleted of Nfs1, the Isu level was increased >10-fold relative to that in the reference strain (Figure 5B). Thus Jac1 is able to protect Isu from degradation in vivo, and such protection does not depend on the presence of Nfs1. Taken together, these results indicate that Jac1 binding to Isu protects it from degradation by Lon-type proteases.

DISCUSSION

The results reported here not only validate our earlier hypothesis that Isu is a substrate of the Pim1 protease but also demonstrate that binding to either Nfs1 or Jac1 protects it from degradation. Thus they also provide new insights into how the interplay between Isu's susceptibility to and protection from degradation may contribute to regulation of Fe–S cluster biogenesis in vivo.

Pim1 degradation of Isu and its protection upon partner binding

Nfs1 and Jac1, the two proteins that protect Isu from degradation by Pim1, have overlapping binding sites. Therefore the most straightforward hypothesis is that their interaction physically prevents access of Pim1 to sequences in Isu necessary for its recognition for degradation. Although studies of Pim1, like those on other Lon-type proteases, have generally focused on the degradation of misfolded (Bender *et al.*, 2011) and oxidized (Bayot *et al.*, 2010) proteins as part of a quality control system (Baker *et al.*, 2011; Voos, 2013), a limited number of mitochondrial proteins have been analyzed in vitro as specific substrates under normal physiological conditions (Venkatesh *et al.*, 2012; Pinti *et al.*, 2015). One of these is the α subunit of the matrix-processing protease (MPPα), which, like Isu, is protected from degradation through its interaction with another protein, its partner subunit, MPPβ (Ondrovicova *et al.*, 2005).

The critical Isu residues shared by the Nfs1:Isu and Jac1:Isu binding interfaces form a hydrophobic patch, raising the possibility that these residues are ones recognized by Pim1. Of interest, degradation of MPPα is initiated at hydrophobic residues protected by MPPβ (Ondrovicova *et al.*, 2005). To be a substrate for Lon, it appears that MPPα must be properly folded, as a folding-incompetent variant was not susceptible to degradation by Lon. A second well-characterized Lon substrate is monomeric steroidogenic acute regulatory protein (StAR), which functions at the rate-limiting step in steroid hormone biosynthesis (Granot *et al.*, 2007). Because its degradation serves as a regulatory mechanism (Bahat, Perlberg, *et al.*, 2015), it is not surprising that Lon recognizes the folded protein for degradation (Ondrovicova *et al.*, 2005). Such data are consistent with the idea that Pim1 both recognizes and initiates degradation at sites on the surface of proteins.

Our observation that zinc binding makes Isu less susceptible to Pim1 degradation is also consistent with previous observations that structural compactness determines how fast a substrate is degraded by ATP-dependent proteases, including Lon-type proteases (Koodathingal, Jaffe, *et al.*, 2009). In general, substrate unfolding has been found to be a prerequisite for processive peptide bond hydrolysis of ATP-dependent proteases after recognition (von Janowsky, Knapp, *et al.*, 2005). Although Zn and Fe–S cluster coordination do not have identical effects on the structure, structural data for Isu homologues suggest local rearrangement of the ligand-binding region, resulting in a more compact conformation for both (Kim *et al.*, 2012; Iannuzzi *et al.*, 2014). In addition, we found that zinc coordination also significantly improved Jac1's ability to protect Isu. This additional protection is consistent with the fact that, as a cluster transfer factor, ligand-bound Isu is the physiologically relevant binding partner of Jac1. Indeed, interaction between Isu and Jac1 bacterial homologues was previously found to be more efficient for cluster-bound than for unbound scaffold (Hoff *et al.*, 2000). Data also suggest that bacterial Isu orthologues exist in relatively ordered and disordered forms (Kim *et al.*, 2012; Iannuzzi *et al.*, 2014), raising the possibility that interaction with proteins such as Nfs1 and Jac1 might protect it from digestion by Lon protease by minimizing the amount of time spent in the relatively disordered state rather than protecting a specific protease recognition site per se. More studies will be required to understand the exact mechanism of Isu degradation by Pim1.

Degradation and protection of Isu as part of Fe–S cluster biogenesis regulation

The results reported here are important not only because they establish the principles of Isu protection by components of the cluster biogenesis system, but also because they provide the first evidence that the transfer factor Jac1 plays a role in the stabilization of Isu in vivo. Together they point to the idea that Isu is protected from degradation by Pim1 from the time it enters the Fe–S cluster assembly pathway via its binding to Nfs1 and through the transfer step via its interaction with Jac1. Only on transfer of the Fe–S cluster from it by

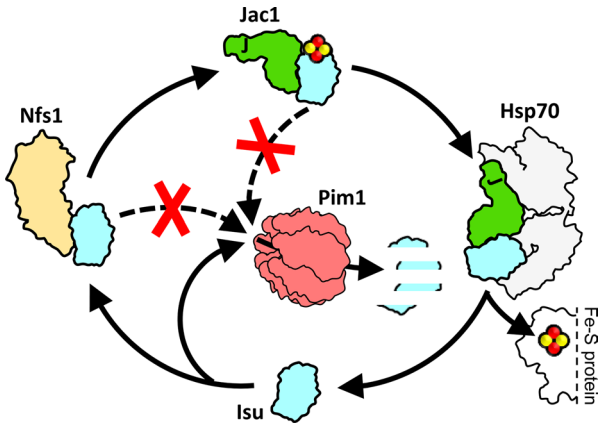

FIGURE 6: Binding to protein partners regulates abundance of Isu scaffold by protecting from degradation by Lon-type protease. Interaction of Isu with Nfs1 and Jac1 during cluster assembly and transfer, respectively, protect Isu from degradation by Pim1 (top). However, upon transfer of the cluster and release from the chaperone system, Isu is susceptible to degradation (bottom). Thus, if the biogenesis process is stalled at either the assembly or transfer step, Isu levels build up, potentially compensating for reduced efficiency of the biogenesis process.

the chaperone system does Isu become susceptible to degradation by Pim1 (Figure 6). Thus, if cluster biogenesis is compromised either during the assembly or the transfer step a build-up of Isu occurs. Such a build-up was shown to be important for maintaining cell growth under such conditions (Andrew *et al.*, 2008).

Isu is the only component of the ISC machinery whose levels are elevated in the cell in the absence of Pim1 or when cluster biogenesis is compromised (Andrew *et al.*, 2008). Of importance, it is also the only component of the assembly process not linked to other mitochondrial processes. For example, Nfs1 also participates in tRNA metabolism (Nakai *et al.*, 2004), and Yfh1 is also involved in the biosynthesis of iron-containing cofactor heme (Pastore and Puccio, 2013). Thus up-regulation of Isu serves as an Fe–S cluster biogenesis–specific regulatory mechanism, distinct from other cellular processes, including those that use iron. When viewed from a broader physiological perspective, such separation may allow balancing of the distribution of iron to distinct cellular processes when it is in limited supply. Indeed, overexpression of Isu under some circumstances can be advantageous for yeast cells, such as prolonged existence in stationary phase, but detrimental when iron is limiting, for example, compromising the activity of heme-requiring enzymes such as ferric reductase (Song *et al.*, 2012).

On the other hand, *S. cerevisiae* has a well-described, comprehensive cellular mechanism, the Aft activation pathway, to overcome disruption of Fe–S cluster biogenesis and other iron-requiring processes when iron is limiting (Lill *et al.*, 2014). Not only does activation of Aft transcription factors increase expression of Fe transporters to bring more iron into cells, it also results in a broad remodeling of metabolic networks by both transcriptional and posttranscriptional mechanisms (Outten and Albetel, 2013). In this way, only the most critical iron-utilizing enzymes are still synthesized, with other metabolic intermediates being produced by non–iron-requiring pathways, even if they are less efficient. Then, what might be the purpose of the Isu-Pim1 regulatory mechanism under iron-limiting conditions? Because Fe–S clusters are important for so many cellular processes, the posttranslational Isu up-regulation caused by reduced degradation, the focus of this report, could be advantageous, serving as a rapidly implemented, stopgap measure. Such a measure would allow cells a window of time to adjust. If such transient up-regulation of Isu were sufficient to overcome a temporary problem, whole-cell metabolic remodeling would not be implemented. In support of this idea, elevation of Isu levels occurs, but not the activation of the Aft pathway, in a strain expressing a Jac1 variant with mildly reduced affinity for Isu (Andrew *et al.*, 2008). Growth of these cells is as robust as that of WT cells, but only if Isu levels remain elevated. Clearly, future experiments are needed to understand the mechanistic relationship between the Aft regulation pathway and the Isu posttranslational degradation pathway in maintaining the overall balance of cellular iron utilization.

MATERIALS AND METHODS

Yeast strains, plasmids, growth conditions, and chemicals

The *S. cerevisiae* haploid strains used for the comparison of different mitochondrial protease deletions (*pim1*Δ, *yme1*Δ, *yta12*Δ. and *oma1*Δ) were derived from BY4742, which is isogenic to S288C (*his3*Δ1 *leu2*Δ0 lys2Δ0 *ura3*Δ0; Brachmann *et al.*, 1998). All other yeast strains used in this study were derived from strain 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-Δ1/met2-Δ1 lys2-Δ2/lys2-Δ2*). The *pim1*Δ strain (*PIM1* deleted with the *KanMX4* cassette) used to assess the compensation for Pim1 by Lon-type proteases and their variants and the *GAL-NFS1* strain harboring a chromosomal copy of *NFS1* under control of the *GAL10* promoter were described previously (Andrew *et al.*, 2008; Song *et al.*, 2012).

To generate p414-Pim1 WT and p414-Pim1 S_A plasmids, the open reading frames (ORFs) of the WT and mutant (Ser-1015 to Ala) *PIM1* gene from *S. cerevisiae* were PCR amplified from plasmids pSDH1 and pSDH4, respectively, with an *Xho*I site engineered at the 3′ end after the stop codon. Plasmids pSDH1 and pSDH4 were a kind gift from Carolyn Suzuki (University of Medicine and Dentistry of New Jersey, Newark, NJ) and described in Rep, van Dijl, *et al.* (1996). The PCR products were digested with *Xho*I and ligated into p414-ADH vector (Mumberg *et al.*, 1995) digested with *Sma*I and *Xho*I. To generate p414-LON WT, the human *LONP1* gene starting at Met-115 codon was PCR amplified from plasmid obtained from the DNASU plasmid repository (Tempe, AZ; https://dnasu.org/DNASU/Home.do; ID HsCD00733037; Cormier *et al.*, 2010) and fused to the mitochondrial targeting sequence of subunit 9 of the *Neurospora crassa* ATPase gene (*Su9*) using PCR sewing. An *Spe*I site was incorporated at the 5′ end of Su9, and an *Xho*I site was incorporated after the stop codon of *LONP1*. The PCR product was digested with *Spe*I and *Xho*I and ligated to a similarly digested p414-ADH vector (Mumberg *et al.*, 1995). The site-directed mutation Ser-855 to Ala was created in this plasmid using the Stratagene (Santa Clara, CA) QuikChange procedure. Plasmids were transformed into a diploid PJ53 strain heterozygous for the *PIM1* deletion, and transformants were sporulated and dissected to obtain *pim1*Δ haploids carrying the desired plasmid.

Plasmids pRS414 harboring *JAC1* or *jac1 LLY_AAA* under the *GPD* promoter were generated by subcloning ORF's PCR amplified from pRS313 plasmids (Ciesielski *et al.*, 2012) to the p414-GPD vector (Mumberg *et al.*, 1995) using *Bam*HI and *Pst*I restriction sites. Protein purification plasmids for Isu1, Jac1 (Dutkiewicz *et al.*, 2003), Nfs1 (Majewska, Ciesielski, *et al.*, 2013), and Yfh1 (Manicki, Majewska, *et al.*, 2014) were described before as indicated. A previously described plasmid for purification of human LON with a polyhistidine tag was a kind gift from Carolyn Suzuki (Liu *et al.*, 2004).

Yeast were grown on glucose-based medium: rich medium (1% yeast extract, 2% peptone, 2% glucose) or synthetic medium, as described (Sherman *et al.*, 1986). Glucose was replaced with 2% galactose in the galactose-based medium or 3% (vol/vol) glycerol in the glycerol-based medium, as indicated. Strains plated as 1:10 serial dilutions on glucose-based medium were incubated for 2 d at 30 and 37°C, respectively, or plated on glycerol-based medium and then incubated for 4 d at 30°C. For the experiments with Nfs1 depletion in vivo, indicated strains initially grown on galactose-based medium were subcultured to glucose-based medium for 64 h to deplete Nfs1 (Song *et al.*, 2012).

All chemicals, unless stated otherwise, were purchased from Sigma-Aldrich (St. Louis, MO). Media components were purchased from Thermo Fisher Scientific (Madison, WI). Restriction enzymes were purchased from New England Biolabs (Ipswich, MA).

Protein purification and immunoblot analysis

Isu1, Nfs1:Isd11 (referred to as Nfs1 in the text), Yfh1, and Jac1 were purified from *Escherichia coli* with C-terminal polyhistidine tags as previously described (Dutkiewicz *et al.*, 2003; Ciesielski *et al.*, 2012; Majewska, Ciesielski, *et al.*, 2013; Manicki, Majewska, *et al.*, 2014). Recombinant human LON protease with a polyhistidine tag on the N-terminus, starting at Met-115, was overexpressed in *E. coli* cells and purified as described previously (Lu, Lee, Nie, *et al.*, 2013).

Immunoblot analysis of protein levels in whole-cell lysates prepared by bead beading was performed using the ECL detection system from GE Healthcare (Pittsburgh, PA) according to the manufacturer's suggestions, using polyclonal antibodies specific for Isu, Jac1, Nfs1, Mge1, and Ssc1 described previously (Andrew *et al.*, 2008).

In vitro degradation assay

Isu1 (7.5 µM) protein was incubated in reaction buffer R (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–NaOH, pH 8.0, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM ATP, 0.15 mg/ml bovine serum albumin) at 30°C. After 10 min, human LON (1.25 µM monomer) was added, and a 10-µl sample of the reaction mixture was collected for each time point, with the time-zero sample taken immediately after adding the protease. The protease activity in collected samples was heat inactivated (5 min at 95°C), and protein content was analyzed using SDS–PAGE and stained using Coomassie Brilliant Blu G. Visualized protein bands for Isu1 were quantitated using ImageJ (Schindelin *et al.*, 2012).

In the time-course experiments with Nfs1, 22.5 µM Nfs1 WT or Nfs1 LM_AA in complex with Isd11 was preincubated with Isu1 for 10 min at 30°C before protease was added. In analogous experiments with Jac1, 37.5 µM Jac1 WT or Jac1 LLY_AAA was preincubated with Isu1. In experiments assessing concentration dependence for Nfs1 and Jac1 variants, a set of reactions, as described, was prepared, each containing the indicated protein content. Briefly, Isu1 (7.5 µM) in buffer R was preincubated with indicated Nfs1 or Jac1 proteins in the concentration range from 1× (7.5 µM) to 6× (45 µM), as indicated. Human LON protease (1.25 µM) was added, and each reaction mixture was incubated at 30°C with sample collection and protein content analysis, as described.

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REFERENCES

Boldface names denote co–first authors.

Adam AC, Bornhovd C, Prokisch H, Neupert W, Hell K (2006). The Nfs1 interacting protein Isd11 has an essential role in Fe/S cluster biogenesis in mitochondria. EMBO J 25, 174–183.

Andrew AJ, Song JY, Schilke B, Craig EA (2008). Posttranslational regulation of the scaffold for Fe-S cluster biogenesis, Isu. Mol Biol Cell 19, 5259–5266.

Bahat A, Perlberg S, Melamed-Book N, Isaac S, Eden A, Lauria I, Langer T, Orly J (2015). Transcriptional activation of LON Gene by a new form of mitochondrial stress: a role for the nuclear respiratory factor 2 in StAR overload response (SOR). Mol Cell Endocrinol 408, 62–72.

Baker MJ, Tatsuta T, Langer T (2011). Quality control of mitochondrial proteostasis. Cold Spring Harb Perspect Biol 3, a007559.

Bayot A, Gareil M, Rogowska-Wrzesinska A, Roepstorff P, Friguet B, Bulteau AL (2010). Identification of novel oxidized protein substrates and physiological partners of the mitochondrial ATP-dependent Lon-like protease Pim1. J Biol Chem 285, 11445–11457.

Bender T, Lewrenz I, Franken S, Baitzel C, Voos W (2011). Mitochondrial enzymes are protected from stress-induced aggregation by mitochondrial chaperones and the Pim1/LON protease. Mol Biol Cell 22, 541–554.

Bonomi F, Iametti S, Morleo A, Ta D, Vickery LE (2011). Facilitated transfer of IscU-[2Fe2S] clusters by chaperone-mediated ligand exchange. Biochemistry 50, 9641–9650.

Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD (1998). Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast 14, 115–132.

Chandramouli K, Johnson MK (2006). HscA and HscB stimulate [2Fe-2S] cluster transfer from IscU to apoferredoxin in an ATP-dependent reaction. Biochemistry 45, 11087–11095.

Ciesielski SJ, Schilke BA, Osipiuk J, Bigelow L, Mulligan R, Majewska J, Joachimiak A, Marszalek J, Craig EA, Dutkiewicz R (2012). Interaction of J-protein co-chaperone Jac1 with Fe-S scaffold Isu is indispensable in vivo and conserved in evolution. J Mol Biol 417, 1–12.

Cormier CY, Mohr SE, Zuo D, Hu Y, Rolfs A, Kramer J, Taycher E, Kelley F, Fiacco M, Turnbull G, *et al.* (2010). Protein Structure Initiative Material Repository: an open shared public resource of structural genomics plasmids for the biological community. Nucleic Acids Res 38, D743–D749.

Dutkiewicz R, Schilke B, Knieszner H, Walter W, Craig EA, Marszalek J (2003). Ssq1, a mitochondrial Hsp70 involved in iron-sulfur (Fe/S) center biogenesis. Similarities to and differences from its bacterial counterpart. J Biol Chem 278, 29719–29727.

Fox NG, Das D, Chakrabarti M, Lindahl PA, Barondeau DP (2015). Frataxin accelerates [2Fe-2S] cluster formation on the human Fe-S assembly complex. Biochemistry 54, 3880–3889.

Garland SA, Hoff K, Vickery LE, Culotta VC (1999). Saccharomyces cerevisiae ISU1 and ISU2: members of a well-conserved gene family for iron-sulfur cluster assembly. J Mol Biol 294, 897–907.

Gomez M, Perez-Gallardo RV, Sanchez LA, Diaz-Perez AL, Cortes-Rojo C, Meza Carmen V, Saavedra-Molina A, Lara-Romero J, Jimenez-Sandoval S, Rodriguez F, *et al.* (2014). Malfunctioning of the iron-sulfur cluster assembly machinery in Saccharomyces cerevisiae produces oxidative stress via an iron-dependent mechanism, causing dysfunction in respiratory complexes. PLoS One 9, e111585.

Granot Z, Kobiler O, Melamed-Book N, Eimerl S, Bahat A, Lu B, Braun S, Maurizi MR, Suzuki CK, Oppenheim AB, *et al.* (2007). Turnover of mitochondrial steroidogenic acute regulatory (StAR) protein by Lon protease: the unexpected effect of proteasome inhibitors. Mol Endocrinol 21, 2164–2177.

Hoff KG, Silberg JJ, Vickery LE (2000). Interaction of the iron-sulfur cluster assembly protein IscU with the Hsc66/Hsc20 molecular chaperone system of Escherichia coli. Proc Natl Acad Sci USA 97, 7790–7795.

Iannuzzi C, Adrover M, Puglisi R, Yan R, Temussi PA, Pastore A (2014). The role of zinc in the stability of the marginally stable IscU scaffold protein. Protein Sci 23, 1208–1219.

Johnson DC, Dean DR, Smith AD, Johnson MK (2005). Structure, function, and formation of biological iron-sulfur clusters. Annu Rev Biochem 74, 247–281.

Kim JH, Tonelli M, Kim T, Markley JL (2012). Three-dimensional structure and determinants of stability of the iron-sulfur cluster scaffold protein IscU from Escherichia coli. Biochemistry 51, 5557–5563.

Koodathingal P, Jaffe NE, Kraut DA, Prakash S, Fishbain S, Herman C, Matouschek A (2009). ATP-dependent proteases differ substantially in their ability to unfold globular proteins. J Biol Chem 284, 18674–18684.

Koppen M, Langer T (2007). Protein degradation within mitochondria: versatile activities of AAA proteases and other peptidases. Crit Rev Biochem Mol Biol 42, 221–242.

Lill R, Srinivasan V, Muhlenhoff U (2014). The role of mitochondria in cytosolic-nuclear iron-sulfur protein biogenesis and in cellular iron regulation. Curr Opin Microbiol 22, 111–119.

Liu T, Lu B, Lee I, Ondrovicova G, Kutejova E, Suzuki CK (2004). DNA and RNA binding by the mitochondrial lon protease is regulated by nucleotide and protein substrate. J Biol Chem 279, 13902–13910.

Lu B, Lee J, Nie X, Li M, Morozov YI, Venkatesh S, Bogenhagen DF, Temiakov D, Suzuki CK (2013). Phosphorylation of human TFAM in mitochondria impairs DNA binding and promotes degradation by the AAA+ Lon protease. Mol Cell 49, 121–132.

Maio N, Rouault TA (2015). Iron-sulfur cluster biogenesis in mammalian cells: New insights into the molecular mechanisms of cluster delivery. Biochim Biophys Acta 1853, 1493–1512.

Majewska J, Ciesielski SJ, Schilke B, Kominek J, Blenska A, Delewski W, Song JY, Marszalek J, Craig EA, Dutkiewicz R (2013). Binding of the chaperone Jac1 protein and cysteine desulfurase Nfs1 to the iron-sulfur cluster scaffold Isu protein is mutually exclusive. J Biol Chem 288, 29134–29142.

Major T, von Janowsky B, Ruppert T, Mogk A, Voos W (2006). Proteomic analysis of mitochondrial protein turnover: identification of novel substrate proteins of the matrix protease pim1. Mol Cell Biol 26, 762–776.

Manicki M, Majewska J, Ciesielski S, Schilke B, Blenska A, Kominek J, Marszalek J, Craig EA, Dutkiewicz R (2014). Overlapping binding sites of the frataxin homologue assembly factor and the heat shock protein 70 transfer factor on the Isu iron-sulfur cluster scaffold protein. J Biol Chem 289, 30268–30278.

Martelli A, Puccio H (2014). Dysregulation of cellular iron metabolism in Friedreich ataxia: from primary iron-sulfur cluster deficit to mitochondrial iron accumulation. Front Pharmacol 5, 130.

Mena NP, Urrutia PJ, Lourido F, Carrasco CM, Nunez MT (2015). Mitochondrial iron homeostasis and its dysfunctions in neurodegenerative disorders. Mitochondrion 21, 92–105.

Mumberg D, Muller R, Funk M (1995). Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156, 119–122.

Nakai Y, Umeda N, Suzuki T, Nakai M, Hayashi H, Watanabe K, Kagamiyama H (2004). Yeast Nfs1p is involved in thio-modification of both mitochondrial and cytoplasmic tRNAs. J Biol Chem 279, 12363–12368.

Ondrovicova G, Liu T, Singh K, Tian B, Li H, Gakh O, Perecko D, Janata J, Granot Z, Orly J, *et al.* (2005). Cleavage site selection within a folded substrate by the ATP-dependent lon protease. J Biol Chem 280, 25103–25110.

Outten CE, Albetel AN (2013). Iron sensing and regulation in Saccharomyces cerevisiae: Ironing out the mechanistic details. Curr Opin Microbiol 16, 662–668.

Pastore A, Puccio H (2013). Frataxin: a protein in search for a function. J Neurochem 126(Suppl 1), 43–52.

Paul VD, Lill R (2014). SnapShot: eukaryotic Fe-S protein biogenesis. Cell Metab 20, 384–384 e381.

Pinti M, Gibellini L, Liu Y, Xu S, Lu B, Cossarizza A (2015). Mitochondrial Lon protease at the crossroads of oxidative stress, ageing and cancer. Cell Mol Life Sci 72, 4807–4824.

Rep M, van Dijl JM, Suda K, Schatz G, Grivell LA, Suzuki CK (1996). Promotion of mitochondrial membrane complex assembly by a proteolytically inactive yeast Lon. Science 274, 103–106.

Rutherford JC, Jaron S, Winge DR (2003). Aft1p and Aft2p mediate iron-responsive gene expression in yeast through related promoter elements. J Biol Chem 278, 27636–27643.

Schilke B, Voisine C, Beinert H, Craig E (1999). Evidence for a conserved system for iron metabolism in the mitochondria of Saccharomyces cerevisiae. Proc Natl Acad Sci USA 96, 10206–10211.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, *et al.* (2012). Fiji: an open-source platform for biological-image analysis. Nat Methods 9, 676–682.

Schmucker S, Martelli A, Colin F, Page A, Wattenhofer-Donze M, Reutenauer L, Puccio H (2011). Mammalian frataxin: an essential function for cellular viability through an interaction with a preformed ISCU/NFS1/ISD11 iron-sulfur assembly complex. PLoS One 6, e16199.

Sherman F, Fink GR, Hicks JB (1986). Laboratory Course Manual for Methods in Yeast Genetics, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Song JY, Marszalek J, Craig EA (2012). Cysteine desulfurase Nfs1 and Pim1 protease control levels of Isu, the Fe-S cluster biogenesis scaffold. Proc Natl Acad Sci USA 109, 10370–10375.

Stahlberg H, Kutejova E, Suda K, Wolpensinger B, Lustig A, Schatz G, Engel A, Suzuki CK (1999). Mitochondrial Lon of Saccharomyces cerevisiae is a ring-shaped protease with seven flexible subunits. Proc Natl Acad Sci USA 96, 6787–6790.

Stehling O, Wilbrecht C, Lill R (2014). Mitochondrial iron-sulfur protein biogenesis and human disease. Biochimie 100, 61–77.

Stemmler TL, Lesuisse E, Pain D, Dancis A (2010). Frataxin and mitochondrial FeS cluster biogenesis. J Biol Chem 285, 26737–26743.

Suzuki CK, Suda K, Wang N, Schatz G (1994). Requirement for the yeast gene LON in intramitochondrial proteolysis and maintenance of respiration. Science 264, 273–276.

Uzarska MA, Dutkiewicz R, Freibert SA, Lill R, Muhlenhoff U (2013). The mitochondrial Hsp70 chaperone Ssq1 facilitates Fe/S cluster transfer from Isu1 to Grx5 by complex formation. Mol Biol Cell 24, 1830–1841.

Van Dyck L, Pearce DA, Sherman F (1994). PIM1 encodes a mitochondrial ATP-dependent protease that is required for mitochondrial function in the yeast Saccharomyces cerevisiae. J Biol Chem 269, 238–242.

Venkatesh S, Lee J, Singh K, Lee I, Suzuki CK (2012). Multitasking in the mitochondrion by the ATP-dependent Lon protease. Biochim Biophys Acta 1823, 56–66.

von Janowsky B, Knapp K, Major T, Krayl M, Guiard B, Voos W (2005). Structural properties of substrate proteins determine their proteolysis by the mitochondrial AAA+ protease Pim1. Biol Chem 386, 1307–1317.

Voos W (2013). Chaperone-protease networks in mitochondrial protein homeostasis. Biochim Biophys Acta 1833, 388–399.

Wagner I, van Dyck L, Savel'ev AS, Neupert W, Langer T (1997). Autocatalytic processing of the ATP-dependent PIM1 protease: crucial function of a pro-region for sorting to mitochondria. EMBO J 16, 7317–7325.

Webert H, Freibert SA, Gallo A, Heidenreich T, Linne U, Amlacher S, Hurt E, Muhlenhoff U, Banci L, Lill R (2014). Functional reconstitution of mitochondrial Fe/S cluster synthesis on Isu1 reveals the involvement of ferredoxin. Nat Commun 5, 5013.

Welch KD, Davis TZ, Van Eden ME, Aust SD (2002). Deleterious iron-mediated oxidation of biomolecules. Free Radic Biol Med 32, 577–583.

Yamaguchi-Iwai Y, Stearman R, Dancis A, Klausner RD (1996). Iron-regulated DNA binding by the AFT1 protein controls the iron regulon in yeast. EMBO J 15, 3377–3384.