doi:10.1016/j.jmb.2012.01.022 J. Mol. Biol. (2012) 417, 1–12

Contents lists available at www.sciencedirect.com

Journal of Molecular Biology

journal homepage: http://ees.elsevier.com.jmb

# Interaction of J-Protein Co-Chaperone Jac1 with Fe–S Scaffold Isu Is Indispensable *In Vivo* and Conserved in Evolution

**Szymon J. Ciesielski [1], Brenda A. Schilke [2], Jerzy Osipiuk [3], Lance Bigelow [3], Rory Mulligan [3], Julia Majewska [1], Andrzej Joachimiak [3,4], Jaroslaw Marszalek [1,2*], Elizabeth A. Craig [2*] and Rafal Dutkiewicz [1]**

[1]*Department of Molecular and Cellular Biology, Faculty of Biotechnology, University of Gdansk, 24 Kladki, Gdansk 80822, Poland*
[2]*Department of Biochemistry, University of Wisconsin–Madison, Madison, 433 Babcock Drive, Madison, WI 53706, USA*
[3]*Midwest Center for Structural Genomics, Department of Biosciences, Argonne National Laboratory, 9700 South Cass Avenue, Building 202, Argonne, IL 60439, USA*
[4]*Department of Biochemistry and Molecular Biology, University of Chicago, 920 East 58th Street, Chicago, IL 60637, USA*

*Received 17 November 2011; received in revised form 18 January 2012; accepted 20 January 2012*
Available online 27 January 2012

***Edited by F. Schmid***

***Keywords:***
Fe–S cluster biogenesis;
mitochondria;
Jac1/HscB;
Isu/IscU;
yeast

The ubiquitous mitochondrial J-protein Jac1, called HscB in *Escherichia coli*, and its partner Hsp70 play a critical role in the transfer of Fe–S clusters from the scaffold protein Isu to recipient proteins. Biochemical results from eukaryotic and prokaryotic systems indicate that formation of the Jac1–Isu complex is important for both targeting of the Isu for Hsp70 binding and stimulation of Hsp70's ATPase activity. However, in apparent contradiction, we previously reported that an 8-fold decrease in Jac1's affinity for Isu1 is well tolerated *in vivo*, raising the question as to whether the Jac1:Isu interaction actually plays an important biological role. Here, we report the determination of the structure of Jac1 from *Saccharomyces cerevisiae*. Taking advantage of this information and recently published data from the homologous bacterial system, we determined that a total of eight surface-exposed residues play a role in Isu binding, as assessed by a set of biochemical assays. A variant having alanines substituted for these eight residues was unable to support growth of a *jac1*-Δ strain. However, replacement of three residues caused partial loss of function, resulting in a significant decrease in the Jac1:Isu1 interaction, a slow growth phenotype, and a reduction in the activity of Fe–S cluster-containing enzymes. Thus, we conclude that the Jac1:Isu1 interaction plays an indispensable role in the essential process of mitochondrial Fe–S cluster biogenesis.



*Corresponding authors. J. Marszalek is to be contacted at Department of Molecular and Cellular Biology, Kladki 24, University of Gdansk, Gdansk 80822, Poland; E. A. Craig, Department of Biochemistry, University of Wisconsin–Madison, Madison, 433 Babcock Drive, Madison, WI 53706, USA. E-mail addresses: marszalek@biotech.ug.gda.pl; ecraig@wisc.edu.

Abbreviations used: PDB, Protein Data Bank; wt, wild type; GST, glutathione *S*-transferase; 5-FOA, 5-fluororotic acid; MDH, malate dehydrogenase.

## Introduction

Iron–sulfur (Fe–S) clusters are prosthetic groups required for the function of proteins involved in numerous, often essential, cellular activities, including redox reactions, enzymatic catalysis, and electron transport, as well as regulatory functions.[1] Although Fe–S clusters can be chemically reconstituted, a set of highly specialized proteins is dedicated to Fe–S cluster biogenesis in living cells. Mitochondria and bacteria have a similarly organized Fe–S cluster biogenesis pathway, carried out by homologous proteins. The central component of the pathway is a scaffold protein on which an Fe–S cluster is assembled, before its transfer to a recipient apoprotein. Mitochondria of the yeast *Saccharomyces cerevisiae*, the organism used in the work presented here, contain two very highly conserved, functionally redundant scaffold proteins, Isu1 and Isu2,[2] which are 83% identical in sequence and hereafter collectively referred to as Isu. The homologous scaffold in prokaryotes is called IscU.

The process of Fe–S cluster biogenesis can be divided into two steps: cluster formation on Isu and cluster transfer from Isu to a recipient apoprotein. Assembly requires the enzymatic function of the cysteine desulfurase Nfs1 to provide the needed sulfur. Additional proteins, Yah1 and Yfh1, also play critical roles. Yah1, an essential ferrodoxin, most likely provides electrons needed for the reduction of sulfur.[1] The role played by the iron-binding protein Yfh1, the yeast frataxin homologue, is not yet well understood. However, it has been implicated as an iron source[3] and/or a regulatory element of Fe–S assembly complex.[4] A specialized Hsp70 molecular chaperone system is central to the transfer process. This system is composed of the Hsp70 Ssq1 and its J-protein co-chaperone Jac1, as well as the nucleotide release factor Mge1. Isu is the only known client protein for the Jac1/Ssq1 pair.[5] Similarly, in *Escherichia coli*, IscU is the only client for the Hsp70 HscA and J-protein HscB pair.[6]

Jac1, like all J-proteins, contains a J-domain of approximately 70 amino acids.[7,8] The universally conserved function of the J-domain, which requires the presence of the invariant histidine:proline:aspartic acid (HPD) motif, is stimulation of the ATPase activity of its partner Hsp70. Such activity is critical for Jac1 function, as a variant in which conserved HPD residues were replaced by three alanines did not efficiently stimulate the ATPase activity of Ssq1 *in vitro*[9] and was unable to rescue the lethality caused by the absence of Jac1 *in vivo*.[10] Stimulation of the ATPase activity of an Hsp70, which is effected by binding of a client protein in Hsp70's peptide binding cleft, as well as the J-domain of its partner J-protein, is critical because when ATP is bound to an Hsp70, client protein binding and release occur very rapidly and thus interaction with a client protein is very transient.[11] Upon hydrolysis of ATP to ADP, Hsp70 undergoes conformational changes that allow stable binding of the client protein by preventing its fast release. Since some J-proteins, including Jac1, are able to bind a client protein on their own, it has been suggested that a critical function of J-proteins is the targeting of a client protein to Hsp70.[12] Such a mechanism is very attractive, as J-protein-dependent client protein targeting would allow orchestration of two events: (i) interaction of a client protein within the binding cleft of Hsp70 and (ii) stimulation of the Hsp70's ATPase activity.

Biochemical results, including the requirement for the simultaneous presence of HscB, HscA, and ATP in a reconstituted *E. coli* system for transfer of Fe–S clusters to an apoprotein, support such a targeting scenario.[13] In addition, the presence of both Jac1/HscB and Isu/IscU is required to observe robust stimulation of Hsp70 ATPase activity *in vitro*, supporting the idea of targeting.[14,15] However, the physiological importance of the targeting mechanism remains to be demonstrated *in vivo* for most Hsp70:J-protein pairs, including the Jac1/Ssq1 and HscA/HscB pairs. The first step of targeting, and very central to the model, is the binding of the J-protein to a client protein.[11] Verification of the importance of such an interaction under physiological conditions requires identification of the residues critical for the Jac1–Isu1 interaction, followed by testing of the importance of such residues for Fe–S cluster biogenesis *in vivo*.

The presence of only a single Fe–S cluster biogenesis system in eukaryotes makes the Ssq1/Jac1/Isu system essential for *S. cerevisiae* and thus a useful model system in which to test these ideas.[16] Previously, we reported[17] that the C-terminal domain (C-domain) of Jac1 is sufficient for Isu1 binding and identified several conserved charged residues on the surface of the C-domain involved in the Isu1 interaction. Replacement of these residues by alanines resulted in an approximately 8-fold decrease in the affinity of Jac1 for Isu1.[17] However, when the variant was expressed as the only Jac1 in the cell, no growth defect was observed. Recently, detailed biochemical and biophysical studies of bacterial HscB identified a set of hydrophobic residues, located on the surface of the C-domain in close proximity to the charged region, as playing a significant role in the HscB:IscU interaction.[18,19] However, the physiological function of these newly identified residues has not been tested, because in bacterial cells, the presence of an alternative redundant pathway of Fe–S cluster biogenesis hampers such studies.[20] Utilizing newly obtained structural information of *S. cerevisiae* Jac1, we now report a combined genetic, biochemical, and physiological analysis of the importance of residues of Jac1 that play a role in the interaction with Isu. Our results support the targeting model, as they indicate that

the interaction between Jac1 and Isu is indispensable *in vivo*.

## Results and Discussion

### X-ray structure of Jac1 from *S. cerevisiae*

To gain Jac1 structural information, we purified recombinant Jac1 protein encompassing residues 10–184, which we call Jac1-C1. As Jac1 is synthesized as a preprotein with a 9-amino-acid pre-sequence at its N-terminus, which is removed upon translocation into mitochondria,[10] Jac1-C1 contains all of the residues found in the mature form of the protein. Jac1-C1 crystallized with two molecules (designated A and B) in the asymmetric unit and diffracted X-rays to a resolution of 2.13 Å. However, the complete structure could not be determined because some segments were not well resolved (see Supplementary Information). Therefore, we prepared a collection of truncation variants of Jac1 to find proteins that yielded improved crystal quality (see Supplementary Information). The best results were obtained for variant Jac1-C2 (residues 5–182). Similar to Jac1-C1, Jac1-C2 crystallized with two molecules (A and B) in the asymmetric unit and diffracted X-rays to 1.85 Å resolution. The structure was determined for residues 8–181 (A) and 12–176 (B), but segments consisting of residues 47–60 and 95–98 (B) were not resolved. The combined models provided good structural information for residues 10–46 and 56–181 of Jac1 (Fig. 1). The overall structure of Jac1 resembles previously reported structures of its orthologs: HscB from *E. coli*[21] [Protein Data Bank (PDB) ID: 1FPO], HscB from *Vibrio cholerae* (PDB ID: 3HHO), and HscB from *Homo sapiens*[22] (PDB ID: 3BVO) with which it shares 29%, 31%, and 28% sequence identity, respectively. The structure of Jac1 is L-shaped and consists of two distinct α-helical domains (Fig. 1): the N-terminal J-domain (residues 11–84) and the C-terminal Isu binding C-domain (residues 101–184). These two domains are connected by a flexible linker (residues 85–100).

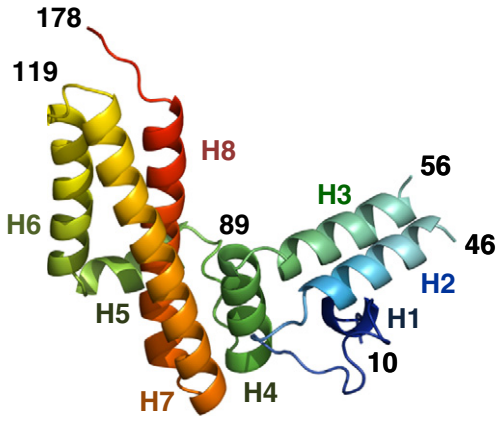


**Fig. 1.** *S. cerevisiae* Jac1 protein structure. Ribbon diagram of Jac1 protein. Rainbow coloring from blue to red indicates the N- to C-terminal positions of the residues in the model. The α-helices are numbered in corresponding colors. Numbers in black correspond to residue positions. The diagram was generated using PyMOL (DeLano Scientific LLC).

As expected, the J-domain contains three α-helices, with helices H2 and H3 comprising an antiparallel coiled coil connected by a loop with the conserved J-domain HPD signature motif (Supplementary Fig. 1a). An unusual feature of the J-domain structure is the disorder present at the ends of the H2 and H3 α-helices including the loop connecting these helices (Supplementary Fig. 1a), as these regions are well defined in the previously solved structures of Jac1 orthologs. The disorder present in both the Jac1-C1 and Jac1-C2 structures may reflect a higher flexibility of the Jac1 J-domain compared to that of orthologs from other species. As noted previously, based on the sequence comparison of Jac1 from *S. cerevisiae* and closely related yeast species with that of other species, the loop region is shorter than the one present in either bacterial (Supplementary Fig. 1b) or human orthologs.[23] The linker between the J-domain and the C-domain also differs significantly between *S. cerevisiae* Jac1 and its orthologs. It contains an additional α-helix (residues 91–100), which is not observed in either the bacterial or the human structure.[21,22] Interestingly, the linker helix is ordered in molecule A of both Jac1 structures and disordered in molecule B, suggesting intrinsic flexibility of this region.

These unique structural features of Jac1's J-domain and linker may have emerged as a result of the complex evolutionary history of mitochondrial Hsp70s and J-proteins involved in the biogenesis of Fe–S clusters in *S. cerevisiae*.[24] In contrast to most eukaryotic species in which Jac1 orthologs partner with the multifunctional mitochondrial Hsp70, which is also involved in facilitating general protein folding and the translocation of proteins into mitochondria, Jac1 in *S. cerevisiae* and closely related yeast species functions with the specialized Ssq1.[24] All evidence indicates that *SSQ1* arose from a duplication of a gene encoding mitochondrial Hsp70, which, in the course of evolution, became highly specialized and now functions exclusively in the biogenesis of Fe–S clusters. We proposed previously, based on computer structure prediction and phylogenetic analysis, that the altered J-domain of Jac1 evolved as consequence of molecular coadaptation between Jac1 and its new Hsp70 partner Ssq1.[23] The unusual structural properties of the J-domain of Jac1 reported here provide strong support for such an evolutionary scenario.

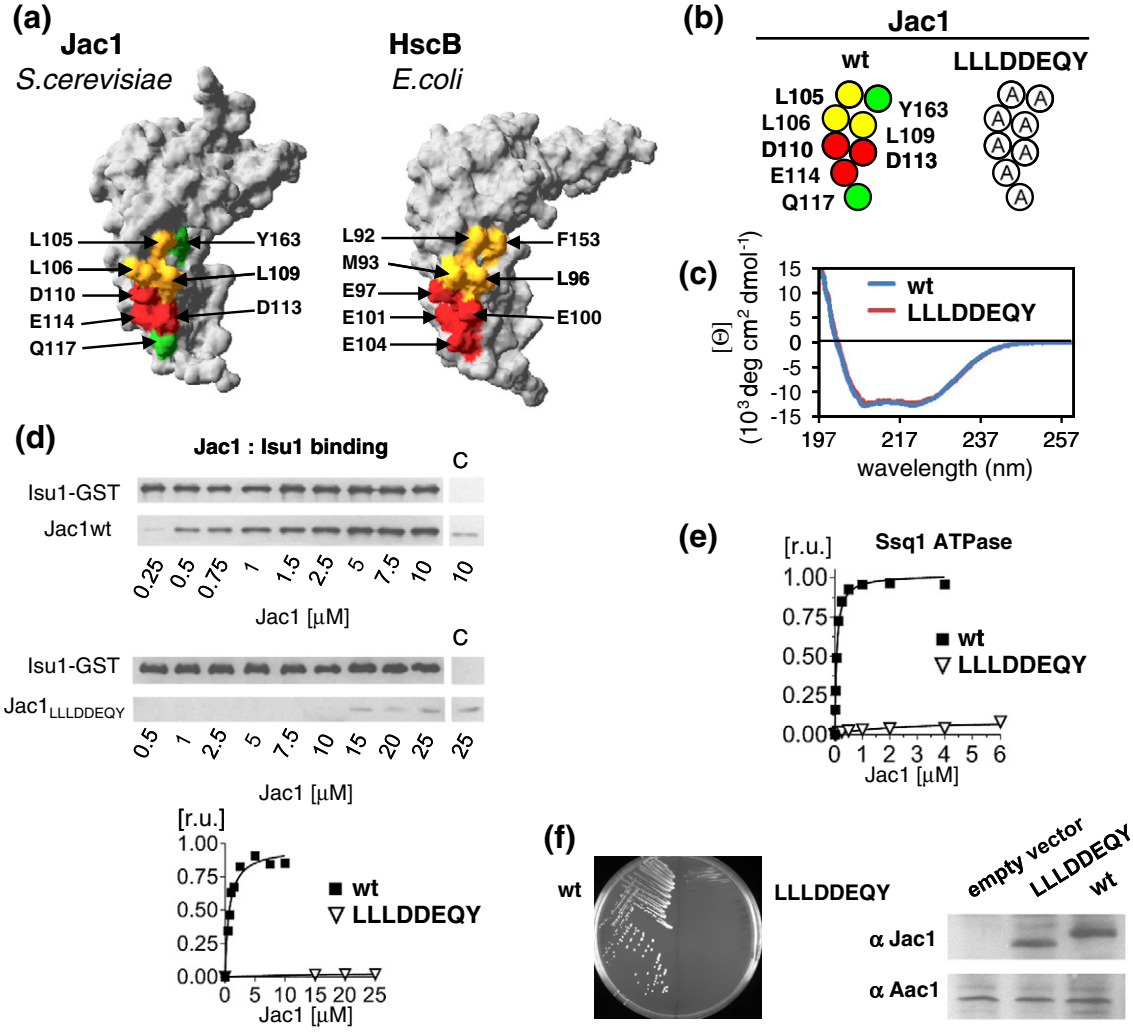


**Fig. 2.** Alanine replacements of conserved charged and hydrophobic residues on the surface of the C-domain of Jac1 result in defective Isu binding and inability to support cell growth. (a) Corresponding residues are highlighted on the surface of protein crystal structures: (left) Jac1 (this report) and (right) HscB (PDB:1FPO) in the region implicated in interaction with Isu/IscU (see the text). Yellow, hydrophobic residues; green, polar residues; red, negatively charged residues. (b) A map of Jac1 surface residues and introduced substitutions to alanine in $Jac1_{LLLDDEQY}$ variant. (c) CD spectra measured for purified wt Jac1 and $Jac1_{LLLDDEQY}$ as described in Materials and Methods. (d) (Top panel) Isu1-GST (2.5 μM) and Jac1 wt or LLLDDEQY variant at the indicated concentrations were mixed to allow complex formation. Glutathione resin was added to pull down the complex. Isu1-GST and Jac1 proteins were separated by SDS-PAGE and visualized by immunoblot analysis using antibodies specific for Isu1 and Jac1. As a control (c), Isu1-GST was omitted for 10 μM Jac1 or $Jac1_{LLLDDEQY}$ as indicated. (Bottom panel) Bound Jac1 was quantitated by densitometry. Values were plotted in Prism using 1:1 binding hyperbola to fit data for wt Jac1 ($K_d = 0.69 \pm 0.12$ μM). $B_{max}$ was set to 1. (e) Stimulation of Ssq1 ATPase activity by wt Jac1 or $Jac1_{LLLDDEQY}$ variant was measured in the presence of 0.5 μM Ssq1, 10 μM Isu1, and 0.5 μM Mge1 and indicated concentrations of Jac1 proteins. Values were plotted in Prism using Michaelis–Menten hyperbolic equation to fit the data. The ATPase activity corresponding to maximal stimulation of wt Jac1 was set to 1. The concentration giving half-maximal stimulation was $C_{0.5} = 0.065 \pm 0.002$ μM. (f) (Top) *jac1*-Δ cells harboring plasmid-borne copies of both wt *JAC1*(*URA3* marked) and wt *JAC1*(*HIS3* marked) or mutant $jac1_{LLLDDEQY}$(*TRP1* marked), as indicated, were plated on glucose-minimal medium containing 5-FOA, which selects for cells having lost the plasmid containing the wt copy of *JAC1*(*URA3* marked). The plate was incubated at 30 °C for 3 days. (Bottom) Immunoblots of 0.1 optical density of cell lysates from indicated strains probed with antibodies specific to Jac1 and Aac1, a loading control. Empty vector—lysate of *GAL-JAC1* strain, harboring a chromosomal copy of *JAC1* under control of glucose-repressible *GAL-10* promoter, prepared following 64 h of growth in glucose-containing media. LLDDEQY—lysate of *GAL-JAC1* strain transformed with plasmid harboring a copy of $jac1_{LLDDEQY}$ mutant under control of the native *JAC1* promoter prepared after 64 h of growth in glucose-containing media. wt—lysate of wt yeast strain.

In contrast to the altered structure of the J-domain, the structure of Jac1's C-domain closely resembles those of other orthologs.[21,22] It is a three-helical bundle with a hydrophobic core formed by the interaction of nonpolar side chains from helices H6, H7, and H8 (Fig. 1). The length and orientation of these helices are almost the same as that found in orthologous structures. In addition, the physicochemical properties of residues exposed on the surface of the C-domain are conserved between Jac1 and HscB (Fig. 2a). Three acidic residues ($D_{110}$, $D_{113}$, and $D_{114}$) on the surface of helix H6 have been

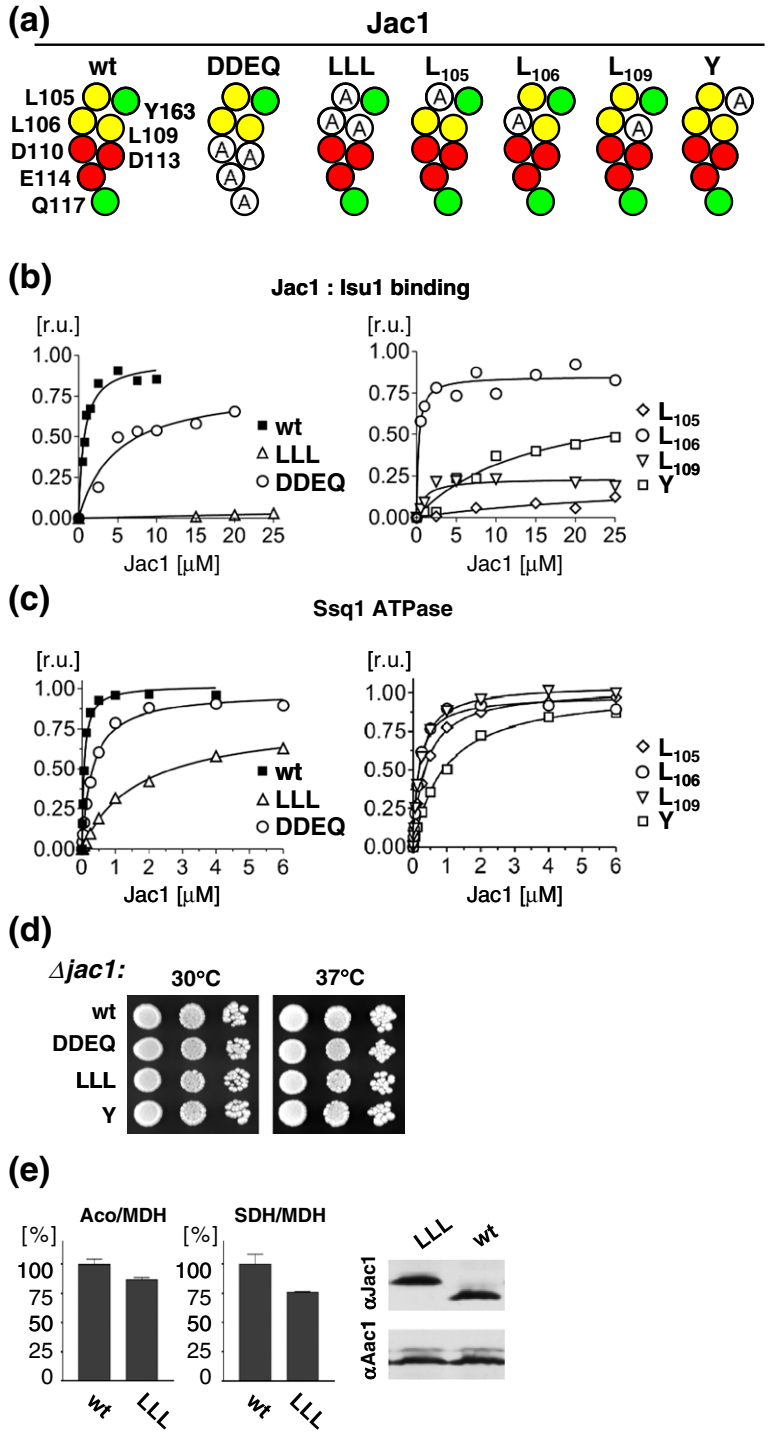


**Fig. 3.** Importance of hydrophobic region for Isu binding *in vitro*. (a) Maps of Jac1 surface residues and introduced substitutions to alanine in Jac1 variants. (b) Isu1-GST (2.5 μM) and Jac1 wt or Jac1 variants as indicated were mixed to allow complex formation. Glutathione resin was added to pull down the complex, and the samples were treated as described in Fig. 1. Bound Jac1 was quantitated by densitometry. Values were plotted in Prism using single binding hyperbola to fit data for wt Jac1 ($B_{max}$ was set to 1; $K_d = 0.69 \pm 0.12$ μM), for $Jac1_{DDEQ}$ ($B_{max} = 0.81 \pm 0.10$ μM; $K_d = 4.73 \pm 1.79$ μM), and for $Jac1_{L106}$ ($B_{max} = 0.85 \pm 0.02$ μM; $K_d = 0.25 \pm 0.07$ μM). (c) Ssq1 ATPase stimulation was measured as described in Fig. 1. Values were plotted in Prism using Michaelis–Menten hyperbolic equation to fit the data. The ATPase activity corresponding to the maximal stimulation of the wt Jac1 was set to 1. Kinetic parameters are listed in Supplementary Table 3. (d) *jac1*-Δ cells harboring plasmid-borne copies of wt JAC1 or mutant *jac1*, as indicated, were plated as 10-fold serial dilutions on glucose-rich medium and incubated at 30 °C and 37 °C for 3 days. (e) (Left) Aconitase activity (Aco) and succinate dehydrogenase activity (SDH) were measured in lysates of mitochondria isolated from *jac1*-Δ cells harboring plasmid-borne copies of wt *JAC1* or $jac1_{LLL}$ grown in glucose-minimal medium. As a standard, the non-Fe–S cluster-containing protein MDH was measured. The ratio of activities of aconitase and MDH or SDH and MDH was calculated and expressed as a percentage of the ratio in wt mitochondrial extracts. Bars represents average values for three repeated measurements with presented error bars as SD. (right) Protein concentrations of Jac1 and Aac1, a loading control, were determined in mitochondrial extracts from cells described above. Mitochondrial extracts were separated by SDS-PAGE, and proteins were visualized by immunoblot analysis using polyclonal antibodies as indicated.

shown previously to be sites for interaction with Isu.[17] Surface-exposed hydrophobic residues ($L_{105}$, $L_{106}$, and $L_{109}$) are situated adjacent to this negatively charged patch on helix H6. A similar hydrophobic patch is present on the equivalent surface of HscB. Detailed biochemical and biophysical studies indicated that three of these residues ($L_{92}$, $L_{96}$, and $F_{153}$) are critical for the HscB:IscU interaction.[18,19] Interestingly, Tyr163 in the Jac1 structure, located on helix H8, occupies a site homologous to Phe153 of HscB, suggesting that it may play a role in the Jac1:Isu interaction (Fig. 2a). Considering the fast rate of Jac1 evolution, as illustrated by only 29% sequence identity between Jac1 from *S. cerevisiae* and *Candida albicans*, a relatively closely related yeast species, the above-described evolutionary conservation of both charged and hydrophobic regions is quite remarkable.

### Alterations in C-domain of Jac1 that abolish Isu1 binding *in vitro* result in a null phenotype *in vivo*

To better understand the interaction between Jac1 and Isu, we constructed a mutant *JAC1* gene encoding a variant having an alanine in place of each of the eight surface-exposed residues (Fig. 2b), encoding $Jac1_{LLLDDEQY}$. We purified $Jac1_{LLLDDEQY}$ and compared its CD spectra to that of wild-type (wt) protein (Fig. 2c). The two CD spectra were indistinguishable, indicating that the extensive alteration of surface-exposed residues did not substantially affect overall structural properties. To test whether $Jac1_{LLLDDEQY}$ was able to bind Isu1, we developed a pull-down assay utilizing a fusion between Isu1 and glutathione *S*-transferase (GST) and thus taking advantage of the ability of GST to bind glutathione. Different concentrations of purified Jac1 were incubated with a fixed concentration of Isu1-GST to allow complex formation. Glutathione resin was then used to pull down Isu1-GST and any Jac1 bound to it. Binding of wt Jac1 was saturable with an apparent $K_d$ of ~0.7 μM (Fig. 2d). $Jac1_{LLLDDEQY}$ did not detectably bind Isu1-GST. Even at 10-fold molar excess, the amount of mutant protein pulled down by Isu1-GST was similar to the background level observed for Jac1 incubated with glutathione resin alone. We also determined the ability of Jac1 to stimulate the ATPase activity of Ssq1 in the presence of saturable amounts of Isu1 (Fig. 2e). In contrast to wt Jac1, which stimulated the ATPase activity of Ssq1 very efficiently with concentration at which half-maximal stimulation was observed ($C_{0.5}$) at ~0.07 μM, $Jac1_{LLLDDEQY}$ was unable to stimulate Ssq1's ATPase above the background level (Fig. 2e).

To determine whether the drastic diminution in the ability of $Jac1_{LLLDDEQY}$ to interact with Isu1 affects the *in vivo* function of Jac1, we transformed a *jac1*-Δ strain harboring a wt copy of the *JAC1* gene and the *URA3* marker on a centromeric plasmid with a second plasmid carrying a different selectable marker and the $jac1_{LLLDDEQY}$ gene. Cells were then plated on media containing 5-fluororotic acid (5-FOA). Only those cells having lost the plasmid containing the *URA3* gene, and therefore the wt copy of *JAC1*, can grow on such media, thus allowing the growth phenotype of cells harboring only a mutant copy of *JAC1* to be scored (Fig. 2f). No 5-FOA-resistant cells carrying $jac1_{LLLDDEQY}$ were recovered. To ensure that the null phenotype was caused by altered protein function rather than by low expression, we measured the level of $Jac1_{LLDDEQY}$. We took advantage of the repression of transcription of the *GAL-10* promoter by glucose. Cells expressing wt *JAC1* under control of the *GAL-10* promoter were transformed with a plasmid carrying $jac1_{LLDDEQY}$ under control of the native *JAC1* promoter or a control vector containing no *JAC1* gene (Fig. 2f). After growth in glucose-containing media, cellular extracts were prepared and Jac1 was detected using immunoblot analysis. Wt Jac1 was depleted below the level of immunodetection whereas the level of $Jac1_{LLDDEQY}$ was similar to that of Jac1 in a wt strain. Therefore, we conclude that $Jac1_{LLDDEQY}$ cannot support cell growth, indicating that the Jac1:Isu1 interaction is essential.

### Three residues contribute most significantly to the Jac1:Isu interaction

To continue our systematic analysis of the contribution of surface-exposed residues to the Jac1:Isu interaction, we created the *JAC1* mutant, $jac1_{DDEQ}$, which encodes alanines in place of the four residues in the conserved charged patch described above: $D_{110}$, $D_{113}$, $D_{114}$, and $Q_{117}$ (Fig. 3a). The ability of $Jac1_{DDEQ}$ to bind Isu1 was impaired in the Isu1-GST pull-down assay. Its apparent $K_d$ value was approximately 6-fold higher than that of wt Jac1, 4.7 μM *versus* 0.7 μM (Fig. 3b). When tested for ability to stimulate the ATPase activity of Ssq1 in the presence of saturable concentrations of Isu1, only a minor decrease in stimulatory activity was detected (Fig. 3c; Supplementary Table 3). No growth defect of cells harboring $jac1_{DDEQ}$, as the only copy of *JAC1*, was observed (Fig. 3d). Thus, we conclude that although the charged region of Jac1 plays a role in Isu1 interaction, its contribution to Isu1 binding is not critical for *in vivo* function and, consistent with previous results,[17] that the affinity of $Jac1_{DDEQ}$ and Isu is higher than that required *in vivo* under typical laboratory conditions.

Next, we turned our attention to the hydrophobic patch. We created a *JAC1* mutant that encoded alanine in place of leucine codons at positions 105, 106, and 109, generating $jac1_{LLL}$ (Fig. 3a), to test how significantly these closely positioned hydrophobic residues contribute to Isu binding. The ability of

$Jac1_{LLL}$ to bind Isu1 was very strongly reduced, as no binding signal above the background level was detected in the pull-down assay (Fig. 3b). $Jac1_{LLL}$'s ability to stimulate Ssq1's ATPase activity was also reduced, but easily detectable. Maximal stimulation was decreased by ~20%, and the concentration ($C_{0.5}$) at which 50% of the maximal stimulation was attained was increased ~24-fold compared to the wt control (Fig. 3c; Supplementary Table 3). Next, we assessed the *in vivo* functionality of the $Jac1_{LLL}$ variant, testing the growth and the activity of two Fe–S cluster-containing enzymes: aconitase and succinate dehydrogenase. Cells expressing $Jac1_{LLL}$ grew normally at both 30 and 37 °C (Fig. 3d). To determine enzyme activity, we prepared extracts from mitochondria of cells expressing wt Jac1 or $Jac1_{LLL}$. The activities were standardized in relation to that of malate dehydrogenase (MDH), which does not contain an Fe–S cluster. Little difference in activity between the strains was observed; aconitase or succinate dehydrogenase activity was 15–25% lower in $jac1_{LLL}$ compared to wt mitochondria (Fig. 3e). That only a slight defect in Fe–S cluster enzyme activity was observed is consistent with robust growth of the mutant cells and is consistent with our previous observation[17] that cells can tolerate significant reduction in the affinity of Jac1 for Isu.

However, because the interaction between Jac1 and Isu1 was significantly reduced upon alteration of leucines 105, 106, and 109 and our overall goal was to identify the most critical residues for Jac1 interaction with Isu, we decided to assess the contribution of individual Leu residues. We created three *JAC1* mutants, each encoding replacement of a single Leu by an Ala (Fig. 3b). $Jac1_{L106}$ bound to Isu1 almost as efficiently as wt Jac1 in the pull-down assay (Fig. 3b). However, both $Jac1_{L105}$ and $Jac1_{L109}$ bound poorly, but detectably (Fig. 3b), while having only a slightly reduced ability to stimulate Ssq1's

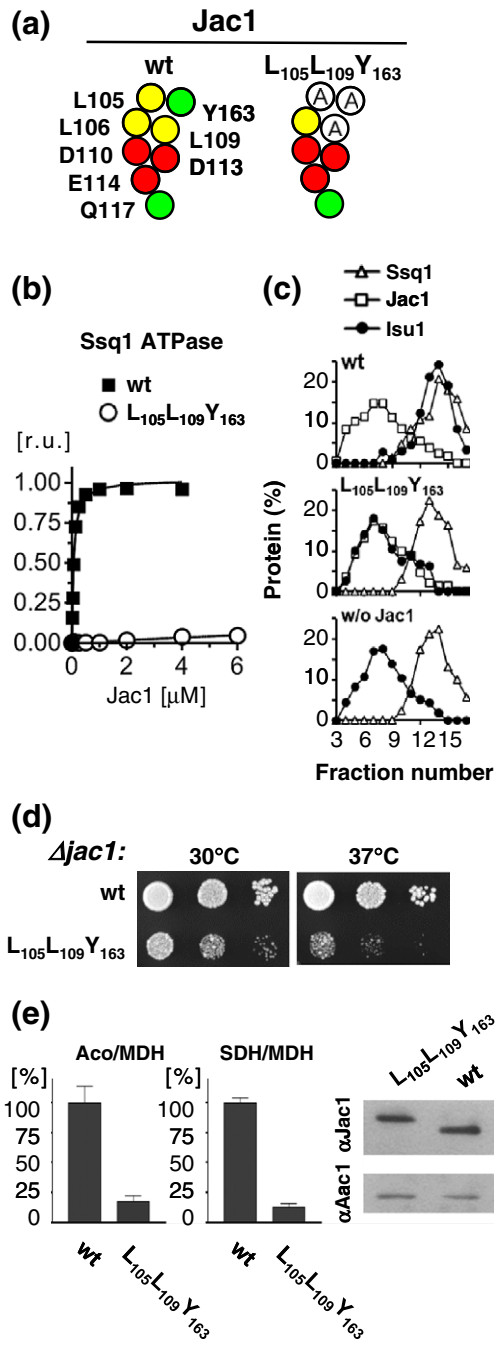


**Fig. 4.** Alanine replacement of L105, L109, and Y163 results in defective Fe–S cluster biogenesis *in vivo*. (a) Map of Jac1 surface residues and introduced substitutions to alanine in $Jac1_{L105L109Y163}$. (b) Ssq1 ATPase stimulation was measured as described in Fig. 1. (c) Isu1 binding to Ssq1 analyzed using glycerol gradient centrifugation. Purified proteins [Isu1 (2.5 μM), Jac1 (5 μM), and Ssq1 (5 μM)] were incubated, as indicated, in the presence of ATP (2 mM) in 70 μl of reaction mixture prior to loading onto a gradient. Plots representing quantification of protein content obtained by densitometry after analysis of protein content of fractions by SDS-PAGE and silver staining. w/o Jac1—a negative control with no Jac1 in the reaction mixture. (d) *jac1*-Δ cells harboring plasmid-borne copies of wt or mutant *jac1*, as indicated, were plated as 10-fold serial dilutions on glucose-rich medium and incubated at 30 °C and 37 °C for 3 days. (e) (Top) Aconitase (left) and succinate dehydrogenase (right) activities were measured in lysates of mitochondria isolated from *jac1*-Δ cells harboring plasmid-borne copies of wt *JAC1* or $jac1_{L105L109Y163}$ grown in glucose-minimal medium. As a standard, the non-Fe–S cluster-containing protein MDH was measured. The ratio of activities of aconitase and MDH or SDH and MDH was calculated and expressed as a percentage of the ratio in wt mitochondrial extracts. Bars represent average values for three repeated measurements with presented error bars as SD. (bottom) Levels of Jac1 and Aac1, a loading control, in the mitochondrial extracts described in the top panel were determined. Extracts were subjected to SDS-PAGE and proteins were detected by immunoblot analysis using polyclonal antibodies as indicated.

ATPase activity (Fig. 3c; Supplementary Table 3). From this analysis, we concluded that two Leu residues, 105 and 109, are candidates for playing an important role in Jac1:Isu interaction *in vivo*.

Finally, we also created a mutant, in which the codon for Tyr163 was replaced by an Ala codon, yielding $Jac1_Y$. $Jac1_Y$ had obviously reduced, but detectable, interaction in both the pull-down and the ATPase assays (Fig. 3a and b). The ATPase defect was the strongest we observed for a single amino acid substitution, with an ~15-fold increase of the $C_{0.5}$ value (Fig. 3c; Supplementary Table 3). Based on these results, we concluded that Tyr163 also plays a significant role in the Isu1 interaction. Next, we assessed the *in vivo* functionality of variants $Jac1_{LLL}$ and $Jac1_Y$. As expected, based on the results described above, cells expressing $Jac1_{LLL}$ or $Jac1_Y$ grew normally (Fig. 3d).

### *jac1*$_{L105L109Y163}$ cells grow slowly and have reduced Fe–S cluster enzyme activity

Alteration of three residues, L105, L109, and Y163, had the largest effects when changed individually. Therefore, we tested the effect of combining the three (Fig. 4a), with a goal of finding a variant that was defective in the *in vitro* assays but, unlike the original $Jac1_{LLLDDEQY}$ mutant, was sufficiently active *in vivo* to support viability. Indeed, $Jac1_{L105L109Y163}$ was strongly defective in ATPase stimulation (Fig. 4b). However, cells expressing this variant as the only copy of Jac1 grew significantly slower than wt cells at 30 °C and extremely poorly at 37 °C (Fig. 4d). To ensure that the phenotypic defect of *jac1*$_{L105L109Y163}$ was not due to low levels of expression, we compared Jac1 levels in mitochondrial extracts prepared from cells expressing wt Jac1 or *jac1*$_{L105L109Y163}$. Wt Jac1 and $Jac1_{L105L109Y163}$ were expressed at comparable levels (Fig. 4e).

As expected, because of the severe interaction defect caused by alteration of individual residues, no interaction of $Jac1_{L105L109Y163}$ was detected in the GST-Isu pull-down assay (data not shown). To further characterize the biochemical properties of $Jac1_{L105L109Y163}$, we also used a previously developed biochemical assay based on glycerol gradient centrifugation of a mixture of Jac1, Isu1, Ssq1, and ATP,[15] which serves as a test of targeting of Isu to Hsp70. In a control experiment, we observed that greater than 80% of Isu1 migrated deep into the gradient, colocalizing with Ssq1, indicating formation of Isu1:Ssq1 complex (Fig. 4c). In contrast, when Jac1 was omitted from the reaction mixture, less than 10% of Isu1 colocalized with Ssq1, indicating that formation of a stable Ssq1:Isu1 complex requires Jac1. However, when we replaced wt Jac1 by $Jac1_{L105L109Y163}$, less then 10% of Isu1 was found in complex with Ssq1. From these results, we concluded that the Jac1 variant defective in Isu1 binding was unable to target Isu1 to its partner Hsp70, Ssq1.

To test whether the *in vitro* and *in vivo* defects of $Jac1_{L105L109Y163}$ are consistent with an effect on Fe–S biogenesis, we compared the activity of two Fe–S cluster-containing enzymes, aconitase and succinate dehydrogenase, in mitochondrial extracts prepared from cells expressing wt Jac1 and $Jac1_{L105L109Y163}$ (Fig. 4e). Activities were ~80% lower in $Jac1_{L105L109Y163}$ extracts than in the wt extracts. As both enzymes require Fe–S cluster for their activity, the slow growth phenotype of a mutant defective in Jac1:Isu1 interaction can be explained by a defective Fe–S biogenesis pathway.

### Phenylalanine can replace tyrosine 163 without functional consequences

Our observation that the three residues, $L_{105}$, $L_{109}$, and $Y_{163}$, play a critical role in the Jac1:Isu interaction is consistent with previously published data[19] showing that the three homologous residues, $L_{92}$, $L_{96}$, and $F_{153}$, are important for the HscB:IscU interaction in *E. coli*. Whereas leucine residues are invariant between Jac1 and HscB, phenylalanine is

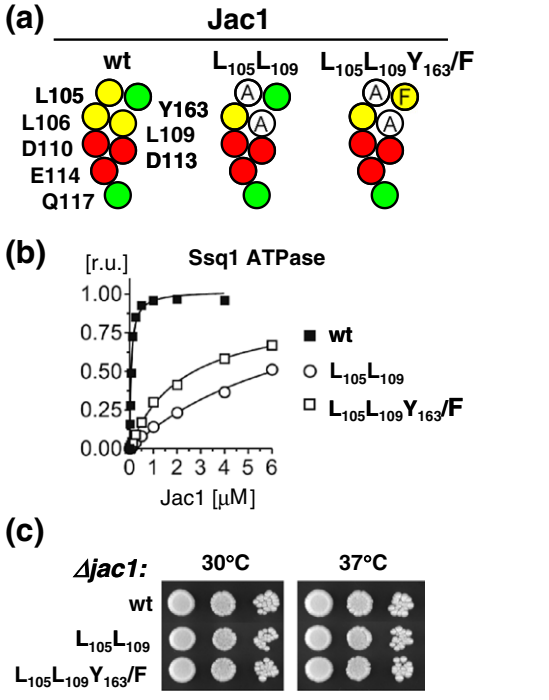


**Fig. 5.** Tyrosine 163 of Jac1 can be functionally replaced by phenylalanine. (a) Maps of Jac1 surface residues and introduced substitutions to alanine in Jac1 variants. (b) Ssq1 ATPase stimulation was measured as described in Fig. 1. (c) *jac1*-Δ cells harboring plasmid-borne copies of wt *JAC1* or *jac1* mutant, as indicated, were plated as 10-fold serial dilutions on glucose-rich medium and incubated at 30 °C and 37 °C for 3 days.

at the position of Tyr163 in HscB. While Phe is predominantly involved in nonpolar interactions, Tyr, with its mixed hydrophobic and polar character, could form hydrogen bonds, as well as participate in nonpolar interactions. To test whether the presence of Tyr at position 163 is critical, or whether Phe would suffice in *S. cerevisiae*, we prepared a Jac1 variant in which Tyr163 was replaced by Phe. To allow scoring of the effects of the Y/F replacement *in vivo*, as well as *in vitro*, we placed it in the context of Jac1 having the $L_{105}A$ and $L_{109}A$ substitutions (Fig. 5a). The ability of $Jac1_{L105A,L109A,Y163F}$ to stimulate the ATPase activity of Ssq1 was comparable to that of the control $Jac1_{L105A,L109A}$ variant (Fig. 5b), indicating that the Y/F replacement had no obvious detrimental effects on biochemical properties of Jac1. Also, no growth defect was visible for cells expressing $jac1_{L105A,L109A,Y163F}$ (Fig. 5c). Thus, these residues can be exchanged for one another without obvious functional consequences, pointing to the conclusion that it is the similar size and hydrophobic character shared by Tyr and Phe that are critical for the Jac1:Isu interaction.

### Perspectives

The results presented here point to two key conclusions: (i) the "adhesive surface" of Jac1's C-domain, consisting of hydrophobic and charged regions and responsible for the Jac1:Isu interaction, is evolutionary conserved and (ii) the Jac1:Isu interaction is indispensable for *in vivo* biological functions.

Hydrophobic residues consisting of leucines 105 and 109 on helix 6 and tyrosine 163 on helix 8 play a critical role in the Isu interaction both *in vitro* and *in vivo*. Yet, $jac1_{L105L109Y163}$ cells are viable. Only when replacements of the hydrophobic residues were combined with replacements of charged residues was a null phenotype observed. Thus, the charged region does play a role, though not a critical one under laboratory conditions. We note that throughout this work, measurements of Jac1 interaction with Isu1 were performed under aerobic conditions; thus, Isu1 was in the apo-form (i.e., no Fe–S cluster was present). However, we reported previously that the biochemical defects measured for apo-Isu1 interaction with both Jac1 and Ssq1 correlated well with *in vivo* Fe–S biogenesis defects determined for cells expressing protein variants defective in apo-Isu1 interactions.[9,17,23,24] Moreover, these findings are consistent with those of a previously published biochemical and biophysical analysis of the HscB:IscU interaction,[18,19] which shows that the three evolutionary conserved hydrophobic residues of HscB have the strongest contribution to IscU binding, with the charged residues contributing to a lesser extent to binding stability. Such a contribution of a number of residues toward the strength of protein:protein interaction across a binding interface has been observed previously for variety of interacting proteins.[25] It is often the case that hydrophobic residues provide stability to the interaction, with the charged region providing specificity and directing the precise orientation of interacting partners.[18] The evolutionary conservation of the spatial orientation of both hydrophobic and charged patches across the binding interface of Jac1 and its bacterial and eukaryotic orthologs is consistent with such a mechanism.

How does our finding that the Jac1:Isu interaction is indispensable *in vivo* fit into the current picture of the role of Jac1 in Fe–S cluster biogenesis? Biochemical data, including those presented here, are consistent with the hypothesis that Jac1, by direct interaction, can target Isu for Ssq1 binding, thus orchestrating the Isu binding and ATPase stimulation events. In the larger picture, a similar mechanism of client protein targeting has been proposed for other J-protein co-chaperones, including bacterial DnaJ and its partner DnaK, as well as their homologues from eukaryotic cells.[8,11] It is also possible that the Jac1:Isu interaction plays roles in addition to the targeting of Isu to Ssq1, as the process of Fe–S cluster assembly on and transfer from Isu to recipient apoproteins is a complex interplay of protein:protein interactions, involving other proteins required for cluster biogenesis.[1] Recent data[26,27] suggest two possibilities: facilitating dissociation of holo-Isu from the desulfurase complex involved in formation of the cluster and stabilization of the ordered conformation of cluster loaded Isu prior to interaction with Ssq1 and cluster transfer. Further experimentation is under way to test these attractive hypotheses.

## Materials and Methods

### Yeast strains, plasmids, media, and chemicals

PJ53 *jac1*-Δ strain of *S. cerevisiae* used in this study is isogenic to *W303* and was described previously by Andrew *et al.*[17] *GAL-JAC1* strain harboring a chromosomal copy of *JAC1* under control of the glucose-repressible *GAL-10* promoter was a kind gift of Dr. Roland Lill (Philipps-Universität Marburg, Germany) and was described by Muhlenhoff *et al.*[28] Full-length mature Jac1 was cloned into pET11a starting at amino acid 10 to give Jac-C1. *JAC1* mutants were constructed by changing selected codons to encode alanine by site-directed mutagenesis (QuikChange protocol, Stratagene) using wt *JAC1* (−350 to +824) cloned into pRS314 or pRS313[29] as a template. An expression vector harboring Isu1-GST was prepared by amplifying the Isu1-GST fusion from p416-Isu1-GST, a kind gift of Dr. Roland Lill described by Gerber *et al.*,[3] using primers that encode an NdeI site at the mature start of Isu1 and a BamHI site downstream of the stop codon for GST. The resulting NdeI-BamHI fragment was cloned

into pET3A (Novagen, Gibbstown, NJ). Yeast were grown on YPD (1% yeast extract, 2% peptone, and 2% glucose) or synthetic media as described by Sherman.[30] All chemicals, unless stated otherwise, were purchased from Sigma.

### Protein purification

Expression of Isu1-GST was induced in the *E. coli* strain C41(DE3) carrying the pET3aISU1-GST plasmid by addition of 1 mM isopropyl-1-thio-D-galactopyranoside at $A_{600} = 0.6$. After 2.5 h, cells were harvested and lysed in a French press in buffer L (25 mM Tris–HCl, pH 8.0, 200 mM NaCl, 1 mM PMSF, 1 mM dithiothreitol, 10% glycerol, and 0.05% Triton X-100). After a clarifying spin, the supernatant was loaded on a 1-ml glutathione agarose column (Fluka) equilibrated with 10 volumes of buffer L. Next, the column was washed with 100 ml of buffer L without PMSF and with 10 volumes of buffer L with 0.5 M NaCl and with 10 volumes of buffer L with 10 mM $MgCl_2$ and 1 mM ATP. After a final wash with 10 volumes of buffer L, proteins were eluted with buffer E (25 mM Tris–HCl, pH 8.0, 200 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 0.05% Triton X-100, and 50 mM reduced glutathione). Fractions containing Isu1-GST were pooled, dialyzed against buffer CM (20 mM Mops, pH 7.0, 25 mM NaCl, 1 mM dithiothreitol, and 0.05% Triton X-100), and loaded onto a 2-ml CM-Sepharose column (Amersham Biosciences) equilibrated with buffer CM containing 25 mM NaCl. The CM-Sepharose column was washed with 10 volumes of buffer CM containing 25 mM NaCl. Isu1-GST was eluted with a 50-ml linear NaCl gradient (25–600 mM) in buffer CM. Fractions containing Isu1-GST were pooled, dialyzed against buffer Q (20 mM Tris, pH 8.0, 10% glycerol, 5 mM β-mercaptoethanol, 0.05% Triton X-100, and 50 mM NaCl), and loaded onto a 1-ml Q-Sepharose column (GE Healthcare). After washing with 10 volumes of buffer Q containing 50 mM NaCl, Isu1-GST was step eluted with buffer Q containing 600 mM NaCl. Fractions containing Isu1-GST were pooled, dialyzed against buffer F (20 mM Tris–HCl, pH 8.0, 10% glycerol, 5 mM β-mercaptoethanol, 0.05% Triton X-100, and 200 mM KCl), and stored at −70 °C.

Recombinant $Mge1_{His}$, $Isu1_{His}$, and $Ssq1_{His}$ were purified as described previously by Dutkiewicz *et al.*[15] $Jac1_{His}$ mutant proteins were purified according to Dutkiewicz *et al.*,[15] but with use of *E. coli* strain C41(DE3) for expression. In all cases, protein concentrations, determined by using the Bradford (Bio-Rad) assay system with bovine serum albumin as a standard, are expressed as the concentration of monomers.

### Pull-down assay

Titration pull-down experiments were performed by incubating indicated concentrations of $Jac1_{His}$ with 2.5 μM Isu1-GST in 150 μl of buffer PD [40 mM Hepes–KOH, pH 7.5, 5% (v/v) glycerol, 100 mM KCl, 1 mM dithiothreitol, 10 mM $MgCl_2$, and 1 mM ATP] for 30 min at 25 °C to allow complex formation. Glutathione-immobilized agarose beads were incubated with 0.1% of bovine serum albumin and 10% (v/v) glycerol in buffer PD. Twenty microliters of beads was added to each reaction and incubated at 4 °C for 1 h with rotation. The beads were washed once with 500 μl and then three times with 200 μl of buffer PD. After the final wash, four times concentrated Laemmli sample buffer (20 μl) was added to the reaction mixtures and samples were incubated for 10 min at 90 °C. Three-microliter aliquots were separated by SDS-PAGE and visualized by immunoblot analysis using polyclonal antibodies specific for Isu1 and Jac1.

### ATPase activity of Ssq1

ATPase activity was measured as described by Dutkiewicz *et al.*,[15] with 0.5 μM Ssq1, 10 μM Isu1, 0.5 μM Mge1, and Jac1 protein at the indicated concentrations in buffer A [40 mM Hepes–KOH, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 10 mM $MgCl_2$, and 10% (v/v) glycerol]. Reactions (15 μl) were initiated by the addition of ATP (2 μCi, DuPont NEG-003H, 3000 Ci/mmol) to a final concentration of 120 μM. Incubation was carried out at 25 °C, and the reaction was terminated after 15 min by the addition of 100 μl of 1 M perchloric acid and 1 mM sodium phosphate. After addition of 400 μl of 20 mM ammonium molybdate and 400 μl of isopropyl acetate, samples were mixed and the phases were separated by a short centrifugation. An aliquot of the organic phase (150 μl), containing the radioactive orthophosphate–molybdate complex, was removed and radioactivity was determined by liquid scintillation counting. Control reactions lacking protein were included in all experiments.

### Glycerol gradient centrifugation

Glycerol gradient centrifugation was carried out as described by Dutkiewicz *et al.*[15] Purified proteins [Isu (2.5 μM), Jac1 (5 μM), and Ssq1 (5 μM)] were incubated in the presence of ATP (2 mM) in buffer G [40 mM Hepes–KOH, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 10 mM MgCl2, and 5% (v/v) glycerol] in a total volume of 80 μl for 10 min at 25 °C. Then, 70 μl of this mixture was loaded onto a 3-ml linear 15–35% (v/v) glycerol gradient prepared in buffer G and centrifuged at 4 °C in a Beckman SW60 rotor for 28 h at 46,000 rpm. Fractions (130 μl each) were collected from the top of the tube, and their protein contents were analyzed by SDS-PAGE followed by silver staining.

### Circular dichroism

Measurements were performed on a Jasco J-815 CD Spectrometer from 197 to 260 nm with 3-s averaging times and 1-nm step size at 25 °C. The protein concentration was 9.8 μM in 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM β-mercaptoethanol, and 10% glycerol in a quartz cuvette with 1-mm path length. Spectra were measured in millidegrees, corrected for buffer effects, and converted to mean residue ellipticity (Θ).

### Mitochondrial enzyme activities

Activities of the respiratory enzymes were measured in mitochondria isolated as described previously by Gambill *et al.*[31] Succinate dehydrogenase activity was measured by

using succinate as a substrate as described by Stehling *et al.*[32] Aconitase activity was measured by monitoring the decrease in absorbance of the substrate isocitrate at 235 nm as described by Stehling *et al.*[32] MDH activity was measured using oxaloacetate as a substrate and monitoring the decrease in absorbance of NADH at 340 nm as described by Stehling *et al.*[32] Data were normalized to the protein content of the mitochondrial samples.

### Accession numbers

Atomic coordinates and structure factors were deposited into the PDB as 3UO2 and 3UO3 for Jac1-C1 and Jac1-C2, respectively.

## Acknowledgements

This work was supported by the Polish Ministry of Science and Higher Education Grant N N301 314137 (R.D.), National Institutes of Health grants GM27870 (E.A.C.) and GM074942, and the U.S. Department of Energy, Office of Biological and Environmental Research, under contract -DEAC02-06CH11357 (A.J.). We thank all members of the Structural Biology Center at Argonne National Laboratory for their help in conducting these experiments. Also, we thank Gyorgy Babnigg and Brian Feldman for statistical analysis and cloning of truncation mutants, respectively.

## Supplementary Data

Supplementary data to this article can be found online at doi:10.1016/j.jmb.2012.01.022

## References

1. Lill, R. & Muhlenhoff, U. (2008). Maturation of iron–sulfur proteins in eukaryotes: mechanisms, connected processes, and diseases. *Annu. Rev. Biochem.* **77**, 669–700.
2. Garland, S. A., Hoff, K., Vickery, L. E. & Culotta, V. C. (1999). *Saccharomyces cerevisiae* ISU1 and ISU2: members of a well-conserved gene family for iron-sulfur cluster assembly. *J. Mol. Biol.* **294**, 897–907.
3. Gerber, J., Muhlenhoff, U. & Lill, R. (2003). An interaction between frataxin and Isu1/Nfs1 that is crucial for Fe/S cluster synthesis on Isu1. *EMBO Rep.* **4**, 906–911.
4. Tsai, C. L. & Barondeau, D. P. (2010). Human frataxin is an allosteric switch that activates the Fe–S cluster biosynthetic complex. *Biochemistry*, **49**, 9132–9139.
5. Craig, E. A. & Marszalek, J. (2002). A specialized mitochondrial molecular chaperone system: a role in formation of Fe/S centers. *Cell. Mol. Life Sci.* **59**, 1658–1665.
6. Vickery, L. E. & Cupp-Vickery, J. R. (2007). Molecular chaperones HscA/Ssq1 and HscB/Jac1 and their roles in iron–sulfur protein maturation. *Crit. Rev. Biochem. Mol. Biol.* **42**, 95–111.
7. Hennessy, F., Nicoll, W. S., Zimmermann, R., Cheetham, M. E. & Blatch, G. L. (2005). Not all J domains are created equal: implications for the specificity of Hsp40–Hsp70 interactions. *Protein Sci.* **14**, 1697–1709.
8. Kampinga, H. H. & Craig, E. A. (2010). The HSP70 chaperone machinery: J proteins as drivers of functional specificity. *Nat. Rev., Mol. Cell Biol.* **11**, 579–592.
9. Knieszner, H., Schilke, B., Dutkiewicz, R., D'Silva, P., Cheng, S., Ohlson, M. *et al.* (2005). Compensation for a defective interaction of the hsp70 ssq1 with the mitochondrial Fe–S cluster scaffold isu. *J. Biol. Chem.* **280**, 28966–28972.
10. Voisine, C., Cheng, Y. C., Ohlson, M., Schilke, B., Hoff, K., Beinert, H. *et al.* (2001). Jac1, a mitochondrial J-type chaperone, is involved in the biogenesis of Fe/S clusters in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **98**, 1483–1488.
11. Mayer, M. P. & Bukau, B. (2005). Hsp70 chaperones: cellular functions and molecular mechanism. *Cell. Mol. Life Sci.* **62**, 670–684.
12. Wawrzynow, A., Banecki, B., Wall, D., Liberek, K., Georgopoulos, C. & Zylicz, M. (1995). ATP hydrolysis is required for the DnaJ-dependent activation of DnaK chaperone for binding to both native and denatured protein substrates. *J. Biol. Chem.* **270**, 19307–19311.
13. Chandramouli, K. & Johnson, M. K. (2006). HscA and HscB stimulate [2Fe–2S] cluster transfer from IscU to apoferredoxin in an ATP-dependent reaction. *Biochemistry*, **45**, 11087–11095.
14. Silberg, J. J., Hoff, K. G. & Vickery, L. E. (1998). The Hsc66–Hsc20 chaperone system in *Escherichia coli*: chaperone activity and interactions with the DnaK–DnaJ–grpE system. *J. Bacteriol.* **180**, 6617–6624.
15. Dutkiewicz, R., Schilke, B., Knieszner, H., Walter, W., Craig, E. A. & 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.
16. 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.
17. Andrew, A. J., Dutkiewicz, R., Knieszner, H., Craig, E. A. & Marszalek, J. (2006). Characterization of the interaction between the J-protein Jac1p and the scaffold for Fe–S cluster biogenesis, Isu1p. *J. Biol. Chem.* **281**, 14580–14587.
18. Fuzery, A. K., Tonelli, M., Ta, D. T., Cornilescu, G., Vickery, L. E. & Markley, J. L. (2008). Solution structure of the iron–sulfur cluster cochaperone HscB and its binding surface for the iron–sulfur assembly scaffold protein IscU. *Biochemistry*, **47**, 9394–9404.
19. Fuzery, A. K., Oh, J. J., Ta, D. T., Vickery, L. E. & Markley, J. L. (2011). Three hydrophobic amino acids in *Escherichia coli* HscB make the greatest contribution to the stability of the HscB–IscU complex. *BMC Biochem.* **12**, 3.
20. Johnson, D. C., Dean, D. R., Smith, A. D. & Johnson, M. K. (2005). Structure, function, and formation of

biological iron-sulfur clusters. *Annu. Rev. Biochem.* **74**, 247–281.
21. Cupp-Vickery, J. R. & Vickery, L. E. (2000). Crystal structure of Hsc20, a J-type co-chaperone from *Escherichia coli*. *J. Mol. Biol.* **304**, 835–845.
22. Bitto, E., Bingman, C. A., Bittova, L., Kondrashov, D. A., Bannen, R. M., Fox, B. G. *et al.* (2008). Structure of human J-type co-chaperone HscB reveals a tetracysteine metal-binding domain. *J. Biol. Chem.* **283**, 30184–30192.
23. Pukszta, S., Schilke, B., Dutkiewicz, R., Kominek, J., Moczulska, K., Stepien, B. *et al.* (2010). Co-evolution-driven switch of J-protein specificity towards an Hsp70 partner. *EMBO Rep.* **11**, 360–365.
24. Schilke, B., Williams, B., Knieszner, H., Pukszta, S., D'Silva, P., Craig, E. A. & Marszalek, J. (2006). Evolution of mitochondrial chaperones utilized in Fe–S cluster biogenesis. *Curr. Biol.* **16**, 1660–1665.
25. Bogan, A. A. & Thorn, K. S. (1998). Anatomy of hot spots in protein interfaces. *J. Mol. Biol.* **280**, 1–9.
26. Kim, J. H., Fuzery, A. K., Tonelli, M., Ta, D. T., Westler, W. M., Vickery, L. E. & Markley, J. L. (2009). Structure and dynamics of the iron–sulfur cluster assembly scaffold protein IscU and its interaction with the cochaperone HscB. *Biochemistry*, **48**, 6062–6071.
27. Shi, R., Proteau, A., Villarroya, M., Moukadiri, I., Zhang, L., Trempe, J. F. *et al.* (2010). Structural basis for Fe–S cluster assembly and tRNA thiolation mediated by IscS protein–protein interactions. *PLoS Biol.* **8**, e1000354.
28. Muhlenhoff, U., Gerber, J., Richhardt, N. & Lill, R. (2003). Components involved in assembly and dislocation of iron–sulfur clusters on the scaffold protein Isu1p. *EMBO J.* **22**, 4815–4825.
29. Sikorski, R. S. & Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*, **122**, 19–27.
30. Sherman, F. (1991). Getting started with yeast. *Methods Enzymol.* **194**, 3–21.
31. Gambill, B. D., Voos, W., Kang, P. J., Miao, B., Langer, T., Craig, E. A. & Pfanner, N. (1993). A dual role for mitochondrial heat shock protein 70 in membrane translocation of preproteins. *J. Cell Biol.* **123**, 109–117.
32. Stehling, O., Smith, P. M., Biederbick, A., Balk, J., Lill, R. & Muhlenhoff, U. (2007). Investigation of iron–sulfur protein maturation in eukaryotes. *Methods Mol. Biol.* **372**, 325–342.