Iron–Sulfur Cluster Biogenesis Chaperones: Evidence for Emergence of Mutational Robustness of a Highly Specific Protein–Protein Interaction

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Abstract

Biogenesis of iron–sulfur clusters (FeS) is a highly conserved process involving Hsp70 and J-protein chaperones. However, Hsp70 specialization differs among species. In most eukaryotes, including Schizosaccharomyces pombe, FeS biogenesis involves interaction between the J-protein Jac1 and the multifunctional Hsp70 Ssq1. But, in Saccharomyces cerevisiae and closely related species, Jac1 interacts with the specialized Hsp70 Ssc1, which emerged through duplication of SSC1. As little is known about how gene duplicates affect the robustness of their protein interaction partners, we analyzed the functional and evolutionary consequences of Ssq1 specialization on the ubiquitous J-protein cochaperone Jac1, by comparing S. cerevisiae and S. pombe. Although deletion of JAC1 is lethal in both species, alanine substitutions within the conserved His–Pro–Asp (HPD) motif, which is critical for Jac1:Hsp70 interaction, have species-specific effects. They are lethal in S. pombe, but not in S. cerevisiae. These in vivo differences correlated with in vitro biochemical measurements. Charged residues present in the J-domain of S. cerevisiae Jac1, but absent in S. pombe Jac1, are important for tolerance of S. cerevisiae Jac1 to HPD alterations. Moreover, Jac1 orthologs from species that encode Ssq1 have a higher sequence divergence. The simplest interpretation of our results is that Ssq1’s coevolution with Jac1 resulted in expansion of their binding interface, thus increasing the efficiency of their interaction. Such an expansion could in turn compensate for negative effects of HPD substitutions. Thus, our results support the idea that the robustness of Jac1 emerged as consequence of its highly efficient and specific interaction with Ssq1.

Key words: J-protein cochaperones, Hsp70 chaperones, gene duplication, protein coevolution, mitochondria.

Introduction

Protein mutational robustness, defined as ability to function despite accumulation of mutations (de Visser et al. 2003; Wagner 2005), can have important functional and evolutionary consequences (Masel and Siegal 2009; Masel and Trotter 2010; Rorick and Wagner 2011; Toth-Petroczy and Tawfik 2014). A connection between mutational robustness and gene duplication is well established (Wagner 2008a; Fares 2015), as both sequence divergence analyses (Carretero-Paulet and Fares 2012) and experimental evolution studies (Keane et al. 2014) indicate that duplicates can tolerate more mutational changes than singletons. Little is known, however, about how duplicates’ increased robustness affects the robustness of their partners within their protein interaction network. Here, the results of our analysis of an Hsp70 chaperone duplicate and its singleton J-protein cochaperone partner involved in the biogenesis of iron–sulfur clusters (FeS), which we carried out to approach this question, are presented.

FeS are ancient prosthetic groups critical for function of proteins in many essential life processes, including electron transfer, catalysis, regulation of gene expression, and environmental sensing (for details, see Stehling et al. 2014; Kim et al. 2015). Protein machineries specializing in facilitating cluster assembly on and cluster transfer from a scaffold protein are present in all domains of life. In most proteobacteria and mitochondria of eukaryotes, an Hsp70 molecular chaperone and J-protein cochaperone partner play a critical role in the transfer of clusters to recipient apoproteins (Vickery and Cupp-Vickery 2007; Craig and Marszalek 2011). Although most of the mitochondrial proteins involved in FeS biogenesis were inherited from bacterial ancestors, the evolutionary history of Hsp70 functioning in this process is rather complex (Schilke et al. 2006; Pukszta et al. 2010). The specialized Hsp70
partner involved in FeS biogenesis (called HscA in *Escherichia coli*). Ssc1 not only functions in general protein folding and import of proteins into the mitochondrial matrix but also, unlike DnaK, functions in FeS biogenesis (fig. 1A) (Craig and Marszalek 2011). However, mitochondria of *Saccharomyces cerevisiae* and closely related species contain a second Hsp70, Ssq1 (fig. 1). Ssq1 Hsp70, which is highly specialized for FeS biogenesis, is related to the multifunctional Ssc1 by an ancient gene duplication that took place in a common ancestor of the Saccharomyces and Candida clades (Schilke et al. 2006).

The J-protein Hsp70 cochaperone functioning in FeS biogenesis in mitochondria (called Jac1 in fungi) was inherited from bacteria (Craig and Marszalek 2011). Like all J-proteins, regardless of biological function, Jac1 has the fundamental function of stimulating the ATPase activity of its partner Hsp70, enabling Hsp70 to stably interact with client protein. This ATPase activation requires a transient, but highly specific, interaction between the J-protein’s J-domain and Hsp70. The evolutionary invariant His, Pro, Asp (HPD) sequence motif of the J-domain is critical for ATPase activation in all J-protein cochaperones tested (Mayer and Bukau 2005; Kampinga and Craig 2010). Typically, a single type of Hsp70 functions with multiple J-protein partners. For example, in most eukaryotes the multifunctional mitochondrial Hsp70 functions effectively with three different J-proteins: Jac1 in FeS biogenesis, Mdj1 in protein folding, and Pam18 in protein import (Kampinga and Craig 2010). For FeS biogenesis, Hsp70 binding of only a single client, the scaffold protein for FeS assembly (called Isu1 in *S. cerevisiae*), is required. However, Hsp70 binds to many different client proteins during the processes of protein folding and protein translocation across the mitochondrial inner membrane. Ssq1 exclusively binds Isu1 in *S. cerevisiae*, having lost the ability to bind other client proteins (Dutkiewicz et al. 2003; Schilke et al. 2006). Interestingly, it has also lost the ability to interact with other J-protein cochaperones present in the mitochondrial matrix, Mdj1 and Pam18 (D’Silva et al. 2003; Dutkiewicz et al. 2003).

Thus, Ssq1 and its cochaperone partner Jac1 form a specialized Hsp70 system dedicated solely to FeS biogenesis, providing a clear example of subfunctionalization, where activities present in preduplication parental gene were partitioned between duplicated copies (Hughes 1994; Force et al. 1999). Considered from a protein interaction network perspective (fig. 1B), the emergence of Ssq1 serves as an example of network expansion and rearrangement driven by gene duplication (Wagner 2003; Barabasi and Oltvai 2004; Yamada and Bork 2009). In a preduplication network Ssc1 constitutes a major hub, interacting with three different J-protein cochaperones and a multitude of client proteins (fig. 1B). The organization of the postduplication network differs, with Ssq1 forming an independent interaction module consisting of Jac1 and Isu1, largely functionally isolated from the rest of the network centered on the Ssc1 hub (fig. 1B).

To gain insight into the functional and evolutionary consequences of *S. cerevisiae* Ssq1 specialization on the ubiquitous J-protein cochaperone Jac1, we analyzed this system, taking advantage of the fact that in *S. pombe* Jac1 functions with multifunctional Hsp70 Ssc1. Our in vivo and in vitro
experimental results indicate that in *S. cerevisiae*, the functioning of Jac1 with specialized Sq1 is robust against alanine substitutions within the conserved HPD motif of the J-domain. However, the same HPD substitutions are lethal in *S. pombe*. In biochemical experiments, Jac1 HPD substitution variants stimulated the ATPase activity of *S. cerevisiae* Sq1, but not that of *S. pombe* Sc1. Moreover, analysis of Jac1 phylogeny revealed higher sequence divergence of Jac1 orthologs from species harboring Sq1. We interpret these results as indicating that the emergence of the mutational robustness of Jac1 is a consequence of its highly efficient and very specific interaction with Sq1.

## Results

**Jac1 J-Domain HPD to Alanine Substitution Variants**

Introduction of substitutions at functionally equivalent positions of their sequences is an experimental approach to analyze variation in mutational robustness among proteins (de Visser et al. 2003; Felix and Barkoulas 2015). In the case of Jac1 cochaperones, the HPD sequence of the J-domain, which is critical for functional interaction with partner Hsp70, meets this criterion. Therefore, we decided to compare the effects of individual alanine substitutions in the HPD sequences of Jac1 from *S. cerevisiae* and *S. pombe* in vivo.

First we asked whether deletion of the *S. pombe* gene encoding Jac1Sp is lethal, as is deletion of JAC1Sc for *S. cerevisiae* (Kim et al. 2001), as no experimental work with Jac1Sp has previously been reported. We expressed wild-type Jac1Sp from a plasmid pREP42 JAC1Sp carrying the ura4+ marker and then deleted the copy of the chromosomal gene. Cells were plated on medium containing 5-fluoroorotic acid (5-FOA), which selects for cells having lost the plasmid with the ura4+ marker. No viable colonies were retrieved, indicating that cells that lost the plasmid were inviable (fig. 2A). Therefore, we conclude that the Jac1Sp is essential, as expected.

Next, we constructed JAC1Sp mutants such that the HPD residues were individually replaced with alanine (fig. 2B). To test the in vivo effects of these substitutions, we transformed the *S. pombe* jac1SpΔ strain expressing wild-type Jac1Sp described above, with an additional plasmid expressing the Jac1Sp HPD alanine substitution variants under control of the native promoter. Cells were plated on medium containing 5-FOA to select for cells having lost the plasmid with the ura4+ marker carrying the wild-type Jac1Sp gene. None of the Jac1Sp HPD substitution variants could support growth of *S. pombe* (fig. 2C and E), even though expressed from a multicopy plasmid, and thus present at higher levels than wild-type Jac1Sp expressed from the endogenous chromosomal gene (fig. 2G). Thus in *S. pombe* the HPD motif of Jac1Sp is critical for its function in FeS biogenesis as a J-protein cochaperone of multifunctional Sc1Sp.

Next, we constructed the equivalent HPD to alanine substitutions in Jac1Sc (fig. 2B) and tested them in *S. cerevisiae* jaciScΔ cells, again using the 5-FOA shuffling technique. *S. cerevisiae* jaciScΔ cells expressing plasmid borne copies of JAC1Sc HPD substitution variants were viable, as indicated by growth of individual colonies on 5-FOA plates (fig. 2D and E) at 30°C. Cells retrieved from 5-FOA plates also grew at 30°C on rich media, although the variants grew more slowly (doubling time ~180 min) than the wild-type cells (doubling time ~130 min) and did not form visible colonies at 37°C (fig. 2F and supplementary fig. S1, Supplementary Material online). As in *S. pombe*, the variants in *S. cerevisiae* were expressed at levels higher than the wild-type protein, likely in response to their diminished functionality (fig. 2H). As the substitutions in the HPD were lethal in *S. pombe*, but well tolerated, at least under optimal growth conditions, in *S. cerevisiae*, we conclude that equivalent alterations in the HPD had more severe effects in *S. pombe* than in *S. cerevisiae*.

**HPD Substitutions Do Not Eliminate Jac1Sc-Sq1Sc Functional Interaction**

To test whether the difference in effects of the HPD substitutions in the two Jac1 proteins seen in vivo could be recapitulated in vitro, we purified and analyzed the functional interactions between Jac1Sc and Jac1Sp, and their partner Hsp70s. As the physical interaction between J-domains and Hsp70s is very transient and thus difficult to detect in vitro, we turned to two established assays for testing functional interaction between J-domains and Hsp70s. First, we measured the ability of the different Jac1 proteins to stimulate the ATPase activity of their partner Hsp70, either Sq1Sc or Sc1Sp, in the presence of client protein Isu1. We chose this assay because an increase in the ATPase activity of Hsp70 in the presence of both a J-protein cochaperone and a client protein is a hallmark of a productive chaperone interaction (fig. 3A) (Mayer and Bukau 2005). Two parameters can be determined in such analyses: The maximal stimulation achievable (*Vmax*) and the concentration at which 50% *Vmax* is attained (C0.5). Second, we performed pull-down assays utilizing a fusion between Isu1 and glutathione S-transferase (GST), taking advantage of the ability of GST to bind glutathione. We chose this assay because it measures the efficiency of a final step in the Jac1:Isu1:Hsp70 interaction cycle (fig. 3A), the formation of Hsp70-Isu1 complex, which is dependent on the function of the J-domain of Jac1 (Dutkiewicz et al. 2003; Manicki et al. 2014). Using wild-type proteins we found that in the ATPase assay the *S. cerevisiae* proteins were more efficient than the *S. pombe* proteins, having a C0.5 of 0.04 compared with 3.9 μM (fig. 3B and C). Similarly, several fold higher concentrations of *S. pombe* compared with *S. cerevisiae* proteins were required to pull-down similar amounts of Sq1Sc and Sc1Sp (fig. 3D and E).

Not surprisingly, because of the critical nature of the HPD motif, all six variants had decreased function in both assays compared with the wild-type protein controls. However, the activity of Jac1Sp variants was affected more dramatically than that of the Jac1Sc variants. Jac1Sp variant activity was undetectable in both the ATPase and pull-down assay, even though the activity of wild-type protein was easily measurable (fig. 3C and E). On the other hand, all Jac1Sc HPD
Substitution variants had substantial activity (Fig. 3B and D). For example, the Jac1Sc variants drove complex formation between 18% and 40% as efficiently as native Jac1Sc (Fig. 3D). The D/A and P/A variants were more efficient in Ssq1Sc–Isu1Sc–GST complex formation than the H/A variant, correlating well with the ATPase stimulation measurements.

The biochemical results described above suggested that the robustness against HPD substitutions is higher for the Jac1Sc–Ssq1Sc interaction than for the Jac1Sp–Ssc1Sp interaction. However, our analysis of the caveat that, because we were unable to purify S. pombe Isu1, Isu1Sc was used in all assays. To overcome this caveat, we carried out additional experiments using the Isu1 ortholog from the thermophile fungus Chaetomium thermophilum. C. thermophilum belongs to the Pezizomycotina clade and thus its Isu1 is similarly distant from S. cerevisiae and S. pombe (64% and 63% of amino acid sequence identity, respectively), but like S. pombe, belongs to the preduplication species, and thus harbors a single copy of Ssc1. Moreover, the Jac1 binding site on Isu1 (LVF) is invariant among these three Isu1orthologs (Majewska et al. 2013). When C. thermophilum Isu1 (Isu1Ct) was included in the ATPase assays, both Ssc1Sc and Ssq1Sc were stimulated by their native Jac1 partners (Fig. 3F and G). However, although the P/A variant of Jac1Sc was active in the Ssq1Sc ATPase assay (Fig. 3F), the P/A variant of Jac1Sp did not stimulate the ATPase activity of Ssc1Sp above the background level (Fig. 3G). Therefore, as our results were similar whether Isu1Sc or Isu1Ct was used, we concluded that the differences in robustness against the HPD alanine substitutions observed in our biochemical assays were not affected by use of the Isu1Sc scaffold protein.

Ability of Jac1Sc to Function with Ssc1Sc Does Not Explain Its Robustness against HPD Substitutions

Taken together, our in vivo and in vitro results suggest that substitutions within the HPD motif of Jac1Sp abolish its ability to functionally interact with Ssc1Sp, whereas equivalent substitutions only partially decrease Jac1Sc’s functionality. However, as often the case for yeast duplicates (Kim and Yi 2006; Dean et al. 2008), the subfunctionalization between
Fig. 3. Jac1 HPD substitution variants are active with Ssq1sc, but inactive with Ssc1sp. (A) Jac1 driven Hsp70–Isu1 complex formation. (I) Jac1–Isu1 complex transiently interacts with Hsp70 stimulating its ATPase activity. (II) Upon ATP hydrolysis a stable Hsp70–Isu1 complex forms, whereas Jac1 dissociates. Thus, both the ability of Jac1 to stimulate Hsp70 ATPase activity and the formation of a stable Hsp70–Isu1 complex are measures of the function of the Jac1 J-domain. (B, C) Stimulation of Hsp70 ATPase activity by Jac1 or indicated HPD variants in reactions containing 0.5 μM Hsp70.
Positively Charged Residues on the Surface of the J-Domain of Jac1Sc, but Not Jac1Sp, Are Involved in Its Interaction with Ssq1Sc

That lower concentrations of *S. cerevisiae* proteins were required for both effective ATPase stimulation and binding of Isu1 by Hsp70 suggested to us that the affinity of the interaction between the specialized Jac1Sc-Ssq1Sc pair might be higher than for the Jac1Sp-Ssq1Sc pair. Therefore, we compared the molecular features of the J-domain of Jac1Sc and Jac1Sp first generating structural models (fig. 5A). The Jac1Sc model was based on the available crystal structure (PDB id 3UO3), whereas the model of Jac1Sp was obtained by homology modeling method using L-TASSER structure prediction server (Roy et al. 2010). Both structural models were first relaxed using two-stage energy minimization procedure, as described in Materials and Methods. Subsequently, the homology-based model of Jac1Sp was subject to 20-ns refinement using molecular dynamics (MD) until the root mean square deviation (RMSD) from the initial structure reached a stable value of 0.4 nm. Finally, for both equilibrated models 200-ns-long MD simulations were performed using the CHARMM36 force field (Huang and MacKerell 2013) in GROMACS (Van Der Spoel et al. 2005).

Next, we used the obtained MD trajectories to calculate the mean electrostatic potential around each Jac1 molecule surrounded by an aqueous solution of monovalent ions at physiological concentrations (fig. 5B). Under such conditions Jac1Sc from *S. cerevisiae* generated a patch of uniformly positive electrostatic potential (≥2 kT/e, where k is the Boltzman constant, T is the absolute temperature, and e is the charge of an electron) that was located around the J-domain and stretched up to 1.4 nm from the surface of helix H2 (fig. 5A and 8). In contrast, the electrostatic potential around helix H2 of Jac1Sp from *S. pombe* was markedly weaker and less uniformly distributed with patches of both positively and negatively charged regions. A plausible functional interpretation of these differences is that the positively charged surface of Jac1Sc is important for the highly efficient Jac1Sc-Ssq1Sc interaction, as it could attract negatively charged regions on the surface of Ssq1Sc and pre-orient diffusing proteins for the formation of a productive encounter complex.

To experimentally assess the role of this charged region in functional interaction with Hsp70, we altered four residues of Jac1Sc that are both unique and contribute to this positive electrostatic potential around the J-domain—K20, R35, K38, and R41. With one exception we replaced these residues by ones present at homologous positions of Jac1Sp. Although Jac1Sp has G at the position homologous to Jac1Sc K20, we decided to substitute to an A, reasoning that an A would have fewer structural consequences than a G. Jac1Sc K20A, R35A, K38S, R41L (Jac1Sc-KRKR) had 90% of the ATPase stimulatory capacity of the wt protein, but its *C*₅₀ for Ssq1Sc was reduced 10-fold (fig. 5C). Moreover, combining of the KRKR
substitutions and the H/A substitution within the HPD motif resulted in a variant (Jac\(_{1\text{Sc}}\) ScKRKR + H48A) with no detectable stimulatory ability (fig. 5C). Together, these results are consistent with the idea that the positive electrostatic potential around the J-domain is important for effective Jac1 Sc:Ssq1 Sc interaction, and that the robustness against HPD substitutions depends on the efficiency of this interaction.

**Jac1 Orthologs Functioning with Ssq1 Have Higher Divergence of Amino Acid Sequence than Those Functioning with Multifunctional Hsp70 Ssc1**

Our observation that highly specific Jac1 Sc:Ssq1 Sc interaction was robust against HPD substitutions prompted us to ask whether Jac1 orthologs functioning with specialized Ssq1 have a higher tolerance for mutation accumulation than

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**Fig. 5.** Jac1 Sc has a large patch of positive electrostatic potential around H2 helix of the J-domain that contributes to the efficiency of its interaction with Ssq1 Sc and its robustness against HPD substitutions. (A) Structural models of Jac1 Sc and Jac1 Sp as indicated. Positively charged residues contributing to the patch (Jac1 Sc K20, R35, R37, K38, R41, and Jac1 Sp K87), are indicated with balls and sticks (in blue), HPD motif marked in cyan, helix 1 (H1) and helix 2 (H2) of the J-domain are marked, alignment of the H2 fragment contributing to the charged patch is shown (Top). (B) Using the models in (A), the mean electrostatic potential around Jac1 Sc (left) and Jac1 Sp (right) calculated with APBS software. The visualized surface indicates positive (blue) and negative (red) potential (\(\pm 2 \text{kT/e}\), k is the Boltzmann constant, T is the absolute temperature, and e is the charge on an electron). (C) Stimulation of Ssq1 Sc ATPase activity by Jac1 Sc proteins was measured with 0.5 \(\mu\text{M} \) Ssq1 Sc, 10 \(\mu\text{M} \) Isu1 Sc, and 0.5 \(\mu\text{M} \) Mge1 Sc, and indicated concentrations of Jac1 Sc proteins. Kinetic parameters were calculated as described in figure 3A. Jac1 proteins: WT (WT); quadruple variant containing substitutions K20A, R35A, K38S, and R41L (KRKR); H48A substitutions (H/A) or combination of five substitutions (KRKR + H/A).
those functioning with multifunctional Ssc1. To test this idea, we compared the sequence divergence of Jac1 orthologs that coexist with Ssq1 in the Saccharomyces and Candida clades with that of Jac1 orthologs functioning with Ssc1 in the Yarrowia, Pezizomycotina, and Taphyromycotina clades (fig. 6A). We estimated the evolutionary distances of Jac1 sequences (d) from the Ascomycota Last Common Ancestor (ALCA) to 56 extant Ascomycota species by calculating the expected number of amino acid substitutions per site along branches of the maximum-likelihood (ML) tree (fig. 6A and supplementary fig. S2, Supplementary Material online). We found that the average number of substitutions per site in Jac1 orthologs functioning with Ssq1 (d = 1.59 ± 0.34) was significantly higher (P < 0.01) than the average number of substitutions per site for Jac1 orthologs functioning with Ssc1 (d = 1.38 ± 0.32). Moreover, the d values determined for Jac1 coexisting with Ssq1 were more scattered, ranging from 1.37 to 1.71 within the 25th–75th percentile, while ranging from 1.21 to 1.37 within the 25th–75th percentile for Jac1 coexisting with Ssc1 (fig. 6B). Taken together, these results suggest that the mutational robustness of Jac1 has increased since the emergence of Ssq1.

Using the same methodology we compared the sequence divergence of Ssc1 and Ssq1 orthologs from postduplication (Saccharomyces and Candida) clades with Ssc1 orthologs from preduplication (Yarrowia, Pezizomycotina, and Taphyromycotina) clades (fig. 6B). Although the average number of substitutions per site for Ssc1 orthologs from postduplication clades was higher than those from preduplication clades (d = 0.35 ± 0.05 and d = 0.26 ± 0.06, respectively) and the difference was statistically significant (P < 0.01), their rates of substitutions were markedly lower than those calculated for Jac1 (fig. 6B). In contrast, the substitutions rate for Ssq1 orthologs was dramatically higher (d = 1.40 ± 0.12) and comparable with that of Jac1. The obtained results are consistent with our previously published data that Ssq1 is the fastest evolving Hsp70 in fungi (Kominek et al. 2013). Next, we estimated the variability for each position in the Jac1 amino acid sequence. Using the empirical Bayes method

**Fig. 6.** Jac1 orthologs coexisting with Ssq1 have higher amino acid sequence divergence than Jac1 orthologs coexisting with Ssc1. (A) ML-inferred phylogeny of amino acid sequences of Jac1 from 56 Ascomycota species, rooted using Jac1 from selected bacterial and eukaryotic species. Scale is in substitutions per amino acid site. The complete phylogenetic tree is provided as supplementary figure S2, Supplementary Material online. Clades harboring only Ssc1 are in green: Yarrowia (Y), Pezizomycotina (P), and Taphyromycotina (T); clades harboring Ssq1 and Ssc1 are in magenta: Saccharomyces (S) and Candida (C). (B) Evolutionary distance (d) dispersion, calculated for 28 Jac1 and Ssc1 orthologs from S, C clades (magenta) and 28 Jac1, Ssc1 and Ssq1 orthologs from Y, P, T clades (green), is represented as a box-and-whisker plot (25th and 75th percentiles (box), within 1.5 of the interquartile range (whisker), outliers (gray cross), mean (black dot). All differences between means are statistically significant at P < 0.01, Mann–Whitney U test. (C) Number of changes that each of the Jac1 sequence position underwent during evolution from the ALCA to extant species. For each position the number of changes was plotted separately for S, C clades (magenta) and T, P, Y clades (green), with overlap in gray. Invariant positions in S, C clades, which are variable in T, P, Y clades, are marked by black dots. HPD motif is marked by cyan dots. Jac1 domain structure is indicated below the plot, H1-H8-α-helices.
(Yang et al. 1995) implemented in codeml (Yang 2007), we inferred all Jac1 ancestral amino acid sequences on each of the ancestral nodes on the Jac1 phylogeny. Then, guided by the topology of the Jac1 ML tree, we calculated the number of amino acid changes at each position, from ALCA to 56 extant Ascomycota species, pre- and post-Ssc1 duplication. This analysis revealed an interesting pattern of sequence variability (fig. 6C). Together, we counted 996 inferred changes in the amino acid sequences for Jac1 coexisting with Ssq1 and 712 inferred changes in the amino acid sequence for Jac1 from preduplication clades. Consistently, at the majority of positions (79 of 120 total) sequence variability was higher for Jac1Sp, functioning with Ssq1, suggesting their higher robustness against amino acid substitutions. However, for a limited number of positions, the majority of which localized within helix H2 of the J-domain in close proximity to the HPD motif (fig. 6C, green dots), the opposite was true. This difference in variability suggested that these conserved residues might constitute part of a newly evolved binding interface responsible for highly specific, and more efficient Jac1:Ssq1 interaction.

**Discussion**

A major finding from our work that the mutational robustness of the Jac1 cochaperone has increased as it began partnering with the specialized Hsp70 Ssq1 upon its emergence through duplication of multifunctional Hsp70 Ssc1 is supported by three lines of evidence: 1) Individual alanine substitutions within the HPD motif of the J-domain, which is critical for a functional Jac1:Hsp70 interaction, supported growth in S. cerevisiae where Jac1 functions with Ssq1, but were lethal in S. pombe where Jac1 functions with Ssc1. 2) Purified Jac1Sc variants harboring HPD substitutions were functional in vitro when partnering with Ssq1, as demonstrated by their ability to stimulate the ATPase activity and to promote Ssq1-Lsu1Sc complex formation. Yet, equivalent HPD substitution variants of Jac1Sp were nonfunctional, in both assays with its partner Ssc1Sp. 3) More sequence changes occurred along branches of the phylogenetic tree for Jac1 orthologs from species harboring Ssq1 than for Jac1 orthologs from species harboring only Ssc1, consistent with the idea that higher sequence divergence indicates greater tolerance for amino acid substitutions and, thus, increases of mutational robustness.

Several, mechanisms could explain the higher mutational robustness of Jac1 that functions with specialized Ssq1. One possibility is that both robustness against HPD substitutions and higher sequence divergence of Jac1 are driven by its coevolution with Ssq1. As both Ssq1 and Jac1 are evolving at comparable rates, they could affect each other’s rate of amino acid substitution through mutual induction of compensatory changes. A plausible mechanistic explanation of the coevolution process is an expansion of the Jac1/Ssq1 binding interface, which is supported by our finding of a large, functionally important patch of positive electrostatic potential surrounding the J-domain of Jac1Sc that is missing in Jac1Sp. In addition, measurements of nuclear magnetic resonance (NMR) spectroscopy signal perturbations of the bacterial Jac1 ortholog (HscB) upon its interaction with Hsp70 partner (HscA), specialized in FeS biogenesis, revealed that the binding interface spans the entire J-domain of HscB, as well as residues in the C-terminal substrate binding domain (Kim et al. 2014). Upon expansion of the binding interface, individual residues involved in Jac1:Ssq1 interaction, including the HPD motif, would contribute less to its strength and specificity. Thus, their replacements could be more readily accepted. Moreover, an expanded binding interface might well accumulate compensatory mutations fast enough to offset the effects of slightly deleterious mutations accumulating both within and outside the interacting surfaces (Davis et al. 2009; Tokuriki and Tawfik 2009). Another possible, but not mutually exclusive, explanation of the higher tolerance of Jac1 for mutations is that by functioning with specialized Ssq1 it experienced relaxation of functional constraints. In preduplication species, Jac1 competes with other J-protein chaperones Mdj1 and Pam18 for a common Hsp70 partner (Craig and Marszalek 2011). Such competition among chaperones in a crowded cell environment could constrain amino acid substitutions on their and Hsp70’s surfaces. Thus, lack of such competition could enable the increased rate of the Jac1 and Hsp70 sequence evolution.

The common feature of the mechanisms described in the previous paragraph is their dependence on Ssq1 subfunctionalization following its emergence through gene duplication (Schilke et al. 2006). A connection between gene duplication and mutational robustness is well established in the literature (Wagner 2008a; Carretero-Paulet and Fares 2012; Keane et al. 2014; Fares 2015). Indeed, we observed that in postduplication clades both Ssq1 and Ssc1 evolved faster than their singleton orthologs from pre-duplication species, despite the fact that Ssq1 evolved much faster than paralogous Ssc1. However, a novel finding reported here is that mutational robustness could also increase in a singleton partner interacting with subfunctionalized duplicate. This mutual increase of mutational robustness of interacting partners could affect their evolutionary fate. For example at early stages of Ssq1 evolution, when Jac1 was still able to partner efficiently with both Ssc1 and Ssq1, the increase of mutational robustness of Jac1 driven by its interaction with Ssq1 could have limited the efficiency of purifying selection to purge its mutational variants. This could drive retention and, likely, further coevolution of the Jac1:Ssq1 interaction module. If increase of mutational robustness of interacting partners is a general phenomenon, it could well play a significant role in retention of newly formed protein interaction modules (Wagner 2008b). Such a mechanism, if correct, would also support the importance of neutral evolution for the increase of molecular complexity (Stoltzfus 1999; Lynch 2007; Lukes et al. 2011; Finninger et al. 2012). Moreover, the increase of mutational robustness could confer innovability to interacting proteins allowing them to undergo innovative modifications without loss of functionality (Ferrada and Wagner 2008; Draghi et al. 2010; Toth-Petroczy and Tawfik 2014). Further experimental and computational studies of other highly specialized protein interaction systems are needed to verify the generality of our hypothesis.
Materials and Methods

**Saccharomyces cerevisiae** Strains, Plasmids, and Media

The *S. cerevisiae jAC1Δ* strain, previously described by Andrew et al. (2006), is of the PJ53 background, which is isogenic to W303. *JAC1* mutants were generated in pRS313 and pRS314 (Sikorski and Hieter 1989) using the Stratagene QuikChange protocol, as were all mutants in *E. coli* expression vectors. Yeast were grown on YPD (1% yeast extract, 2% peptone, and 2% glucose) or synthetic media as described by Sherman (1991). All chemicals, unless stated otherwise, were purchased from Sigma.

**Saccharomyces pombe** Strains, Plasmids, and Media

To create the *S. pombe Δjac1-kanMX* strain KGY461 (h+ his3-D1 leu1-32 ura4-D18 ade6-M210) (Burke and Gould 1994) carrying plasmid pREP42, which contains the *ura4*+ marker, with *jac1+* under the *nmt1*+ promoter, was transformed with linear DNA fragment for homologous recombination containing the 535-bp 5′-flanking sequence of *jac1+, kanMX* genetic resistance marker sequence and the 205-bp 3′-flanking sequence of *jac1+. jac1* mutants were generated in pREP41 (Basi et al. 1993) using the Stratagene QuikChange protocol, as were all mutants in *E. coli* expression vectors. All *Jac1* proteins were expressed in *S. pombe* from pREP41 vectors in which the *nmt1*+ promoter was replaced by the 5′-untranslated region of *jac1+* gene (−535 to +1). All *Jac1* proteins expressed in *S. pombe* were tagged with six histidines at the C-terminus. Cells were grown on YES (0.5% yeast extract, 3% glucose, supplements: 0.0075% uracil, 0.025% leucine, 0.0075% adenine, 0.0075% histidine) or Edinburgh minimal medium (EMM) (Moreno et al. 1991). All media were at pH 5.5. Solid media were made by adding 2% agar. Note that because the formal genetic nomenclature is very different for *S. cerevisiae* and *S. pombe*, throughout the text we used unified terminology to avoid confusion: *JAC1* 

**Protein Purification**

Expression of *S. pombe* Jac1 with a polyhistidine tag at the C-terminus was induced in the *E. coli* strain C41(ΔE) carrying pET21d-JAC1HI-S as plasmid by adding isopropyl-1-thio-β-D-galactopyranoside (IPTG) at a final concentration of 1 mM. Cells were harvested, suspended in buffer I (125 mM Tris–HCl, pH 8.0, 10% glycerol, 50 mM NaCl; 5 mM β-mercaptoethanol). After dialysis proteins were loaded on a Q-Sepharose column, washed with buffer J3 and proteins were eluted with a linear 30–300 mM NaCl gradient in J3 buffer. Fractions containing the purest Jac1 were collected, pooled, and dialyzed against buffer J3. Aliquots were stored at −70 °C.

Expression of *C. thermophilum* Isu1 with a polyhistidine tag at the C-terminus, used for the ATPase assays, was induced in the *E. coli* strain C41 carrying pET Duet ISU1C-HIS plasmid by adding IPTG at a final concentration of 1 mM. Cells were harvested, suspended in buffer I (25 mM Tris–HCl, pH 8.0, 10% glycerol, 1 mM PMSF, 0.5 M KCl, 0.05% Triton X-100, 50 mM imidazole, pH 8.0), and lysed using a French press. After a clarifying spin the proteins were subjected to HisBind Resin (Novagen) chromatography. After sequential washing steps with buffers I2 (25 mM Tris–HCl, pH 8.0, 10% glycerol, 1 mM PMSF, 1 M KCl, 0.05% Triton X-100, 50 mM imidazole, pH 8.0) and I3 (25 mM Tris–HCl, pH 8.0, 10% glycerol, 1 mM PMSF, 150 mM KCl, 0.05% Triton X-100, 50 mM imidazole, pH 8.0) proteins were eluted with a linear 50–500 mM imidazole gradient in buffer I1. Fractions containing Isu1C were pooled and concentrated. Next, 100 μl of buffer I4 (1 M KCN in 1 M Tris–HCl, pH 8.0) was added to the protein and incubated for 1 h in room temperature to remove the polysulfane modifications associated with Isu1. KCN was subsequently removed by gel filtration.

**Preparation and Analysis of Mitochondrial and Cell Lysates**

Whole cell lysates of *S. cerevisiae* strains were prepared from 1 ml culture of OD600 = 1.0 by alkaline lysis, as described previously in Yaffe and Schatz (1984). Comparative cell breakage by this efficient method was determined using control antibodies. Lysates of *S. pombe* mitochondria were prepared as described in Moore et al. (1992) and protein concentrations determined using the Bradford (Bio-Rad) assay system with bovine serum albumin as a standard, are expressed as the concentration of monomers.
(SDS-PAGE) gels. The resolved proteins were transferred electrophoretically to nitrocellulose. Specific proteins were detected by antibodies enhanced chemiluminescence using anti-Jac1Sc, anti-Jac1Sp, and control antibodies as indicated in the figure legends.

ATPase Activity of mtHsp70

ATPase activity was measured as described by Dutkiewicz et al. (2003) with 0.5 μM mtHsp70 (Ssq1Sc, Ssc1Sp, Ssc1Sc) 10 μM Isu1 (Isu1Sc, Isu1Ct), 0.5 μM Mge1Sc, and Jac1 (Jac1Sc, Jac1Sp) at the indicated concentrations in buffer A (40 mM Hepes–KOH, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 10 mM MgCl2, and 10% [v/v] glycerol). Reactions (15 μl) were initiated by the addition of ATP (2 μCi, DuPont NEG-003H, 3,000 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. Values were plotted in GraphPad Prism using Michaelis–Menten hyperbolic equation to fit the data. The ATPase activity corresponding to the maximal stimulation (V_m) of the wild-type Jac1 was set to 1. C_0.5 was defined as Jac1 concentration (μM) giving half-maximal stimulation.

Pull-Down Assay

Pull-down experiments were performed as described in Manicki et al. (2014). In short, in a total of 150 μl of reaction volume, 2.5 μM Isu1Sc-GST was incubated with 6 μM Ssq1Sc and 0.1 μM Jac1Sc wt or mutant proteins, or with 30 μM Ssc1Sp and 0.5 μM Jac1Sp wt or mutant proteins in PD buffer (40 mM Hepes–KOH, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 10 mM MgCl2, 5% [v/v] glycerol) for 15 min at 25°C to allow complex formation. Reduced glutathione-immobilized agarose beads were pre-equilibrated with 0.1% bovine serum albumin, 0.1% TritonX-100, and 10% [v/v] glycerol in PD buffer. Forty microliters of beads (~20 μl bead volume) was added to each reaction and incubated at 4°C for 1 h with rotation. The beads were washed three times with 500 μl of PD buffer. Proteins bound to the beads were incubated with 20 μl of 2-fold concentrated Laemmli sample buffer for 10 min at 100°C and aliquots were loaded on SDS-PAGE and visualized by Coomassie blue staining. Every experiment was replicated three times and after quantitation by densitometry average values were plotted as bars in GraphPad Prism with their respective standard deviations.

Jac1 Structural Models, MD Simulations, and Electrostatic Potential Calculations

The initial structure of the S. cerevisiae Jac1Sc protein was taken from the RCSB Protein Data Bank (pdb id 3UO3). The S. pombe Jac1 protein structure was obtained by homology modeling using I-TASSER (Roy et al. 2010). The best model, with the C-score = −1.07 and RMSD = 1.29 to structurally aligned part of the protein, was build based on 1fp0A template (Jac1 ortholog, HscB, from E. coli).

The MD simulation systems consisted of a single molecule of the S. cerevisiae Jac1Sc or S. pombe Jac1Sp protein in a decahedron box solvated with 17,241 water molecules, at physiological concentration of NaCl (150 mM). All simulations were performed with GROMACS (Van Der Spoel et al. 2005) using the CHARMM 36 (Huang and Mackerrell 2013) force field for the protein and ions and TIP3P model for water (Jorgensen et al. 1983). The system was energy-minimized using the steepest descent method in two stages. First, all heavy atoms of the protein were kept fixed; subsequently, all atoms in the system were allowed to relax. The system temperature was maintained at 300 K using v-rescale thermostat (Bussi et al. 2007) with a coupling time of 0.1 ps. The system pressure was held at 1 bar using Berendsen weak coupling algorithm with a coupling time of 2 ps (Berendsen et al. 1984). The van der Waals interactions were evaluated by Lennard–Jones potential with a smooth cut off with switching radius of 0.8 nm and cut off radius of 1.2 nm. Periodic boundary conditions were applied and electrostatic forces were evaluated using the Particle Mesh Ewald algorithm with a short range cut off radius of 1 nm and a Fourier grid spacing of 0.12 nm (Darden et al. 1993). The lengths of all covalent bonds were constrained using P-LINCS (Hess 2008) for the protein and SETTLE (Miyamoto and Kollman 1992) for water. The equations of motion were integrated using the leapfrog Verlet algorithm with a time step of 2 fs. The S. pombe Jac1Sp system was first refined for 20 ns, until the RMSD from the initial structure reached a stable value of 0.4 nm. For each Jac1 protein, 200-ns simulation was performed.

The distribution of the electrostatic potential generated by Jac1 in an aqueous solution at physiological ionic strength was obtained by integrating the linearized Poisson Boltzmann equation using the Adaptive Poisson–Boltzmann Solver (APBS) software (Baker et al. 2001). The concentrations of +1 and −1 ion species were set to 150 mM with an ion exclusion radius of 0.2 nm. Grid size of 161 × 161 × 161 with grid spacing of 0.069 nm was used. We used the Dirichlet boundary conditions with the boundary potential value determined from a Debye–Hückel model for a single sphere with a point charge, dipole, and quadrupole. The electrostatic potential was averaged over the last 100 ns of 200-ns molecular dynamic simulation using frames extracted every 2 ns. All molecular images were created using VMD (Humphrey et al. 1996).

Phylogenetic Analysis and Determination of Sequence Divergence

Genomic data were obtained from the NCBI GenBank, BROAD Institute, Saccharomyces Genome Database, Genolevures, DOE Joint Genome Institute, EBI Integr8, Ensembl Genomes, Yeast Gene Order Browser, Genoscope, Sanger Institute, the Podospora anserina genome project,
Saccharomyces sensu stricto resequencing efforts (Scannell et al. 2011), and the lab of Cecile Neveu-glise (INRA, Thiverval-Grignon, France). Amino acid sequences of Jac1Sc.
S. cerevisiae were used to identify putative homologs within databases of annotated open-reading frames in 56 Ascomycota species, using reciprocal-best-BLAST algorithm locally (Altschul et al. 1990). Sequences were aligned using Clustal Omega v1.2.1 with default parameters (Sievers et al. 2011). Alignment was corrected and trimmed manually. In total, 1,000 ML searches were performed using RAxMLv 8.1.15 (Stamatakis 2006) with 100 rapid bootstrap replicates, under total, 1,000 ML searches were performed using RAxMLv 8.1.15 (Stamatakis 2006) with 100 rapid bootstrap replicates, under the LG model of amino acid substitution and GAMMA model of rate heterogeneity with four discrete rate categories and the estimate of proportion of invariable sites (LG + I + G) (Le and Gascuel 2008), which was determined as the best-fit model of Jac1 protein evolution by ProtTest v3 following Akaike information criterion (Darriba et al. 2011). All analyzed sequences were considered as orthologs based on outgroup criterion and relative position within the genome obtained from OMA synten viewer (Altenhoff et al. 2015).

Evolutionary distances (d) for Jac1 protein were calculated as patristic distances from ALCA to the terminal branches of ML phylogeny (supplementary fig. S2, Supplementary Material online) separately for branches leading to clades harboring only Ssc1: Yarrowia, Pezizymycotina, and Taphyromycotina, or to clades harboring both Ssq1 and Ssc1: Saccharomyces and Candida. Similarly, evolutionary distances (d) were calculated for Ssc1 and Ssq1, based on previously published ML phylogeny (Kominek et al. 2013). The distribution and mean values of d were presented as box-and-whisker plot in Matlab R2015a. The difference between means was compared with the nonparametric Mann–Whitney U test with the significance level \( P \leq 0.01 \).

To determine the Jac1 amino acid variability for each sequence position, ancestral sequences were reconstructed in codeml (Yang 2007) using empirical Bayes method (Yang et al. 1995). Inferred substitutions per site were calculated based on ancestral reconstructions with the highest posterior probability. For every chosen branch of the Jac1 phylogeny, and for every sequence position, the changes between two ancestral states on corresponding nodes have been inferred using python script. For each position, the number of changes was calculated separately for branches leading to clades harboring only Ssc1: Yarrowia, Pezizymycotina and Taphyromycotina, or to clades harboring both Ssq1 and Ssc1: Saccharomyces and Candida, and was visualized as a bar plot in Matlab R2015a.

**Supplementary Material**

Supplementary figures S1 and S2 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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**References**


