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Co-evolution-driven switch of J-protein specificity towards an Hsp70 partner

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Molecular mechanisms by which protein–protein interactions are preserved or lost after gene duplication are not understood. Taking advantage of the well-studied yeast mtHsp70:J-protein molecular chaperone system, we considered whether changes in partner proteins accompanied specialization of gene duplicates. Here, we report that existence of the Hsp70 Ssq1, which arose by duplication of the gene encoding multifunction mtHsp70 and specializes in iron–sulphur cluster biogenesis, correlates with functional and structural changes in the J domain of its J-protein partner Jac1. All species encoding this shorter alternative version of the J domain share a common ancestry, suggesting that all short *JAC1* proteins arose from a single deletion event. Construction of a variant that extended the length of the J domain of a 'short' Jac1 enhanced its ability to partner with multifunctional Hsp70. Our data provide a causal link between changes in the J protein partner and specialization of duplicate Hsp70.
Keywords: chaperone interaction network; Fe–S biogenesis; Hsp70; J proteins; protein co-evolution

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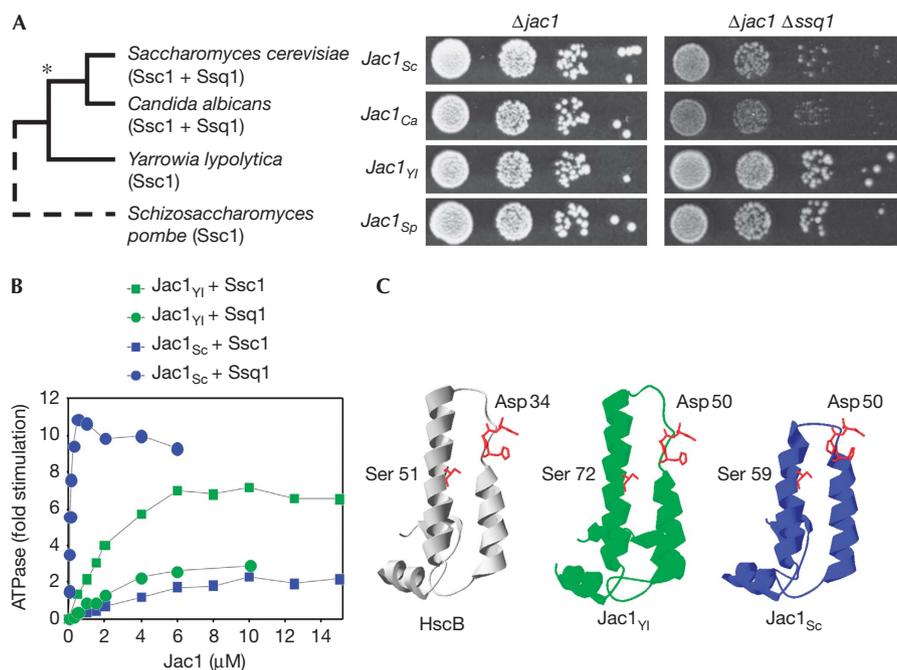
INTRODUCTION

Protein–protein interaction networks can expand by gene duplication and the subsequent divergence of the descendant genes to specialize in one or a subset of the functions performed by the ancestral gene (Lynch & Conery, 2000; Prince & Pickett, 2002; Zhang, 2003). We have taken advantage of the well-studied yeast mitochondrial Hsp70s (mtHsp70s) to investigate the changes that occurred during such specialization, with the goal of identifying changes in partner proteins that accompanied the sub-functionalization of a gene duplicate.

Most eukaryotes, including humans, have a single, multifunctional mtHsp70. However, a subset of fungi, including *Saccharomyces cerevisiae*, also contain a highly specialized mtHsp70 involved in the essential process of iron–sulphur (Fe–S) cluster biogenesis, Ssq1. Ssq1 is encoded by a gene that arose through the duplication of an *mtHSP70* gene in a common ancestor of *Candida albicans* and *S. cerevisiae* (Schilke *et al*, 2006) about 300 million years ago (Taylor & Berbee, 2006). *SSC1*, the paralogue of *SSQ1*, encodes an abundant mtHsp70, which performs the remaining tasks of the ancestral protein, including transport of polypeptides across the mitochondrial inner membrane and protein folding (Craig & Marszalek, 2002). Similarly to other members of the Hsp70 family, both Ssq1 and Ssc1 contain an amino-terminal ATPase and a carboxy-terminal peptide-binding domain, with hydrolysis of ATP inducing conformational changes that stabilize interaction with client proteins. Ssc1, as expected for a multifunctional Hsp70, interacts with various sequences. By contrast, specialized Ssq1 seems to bind to a single protein substrate, Isu1 (Schilke *et al*, 2006), a scaffold on which Fe–S clusters are assembled, facilitating cluster transfer to recipient proteins (Vickery & Cupp-Vickery, 2007).

All Hsp70s, including Ssc1 and Ssq1, require a J-protein co-chaperone, which stimulates the ATPase activity of their partner Hsp70s through the conserved 70-amino-acid (approximately) J domain (Hennessy *et al*, 2005). Two antiparallel helices (Helices II and III), connected by a highly flexible loop, form the core of

Fig 1 | Altered functional properties and structure of the J-domain distinguishes Jac1 orthologues coexisting with Ssq1. (**A**) The ability of *JAC1* orthologues from several yeast species to support growth when Ssc1 functions in iron–sulphur biogenesis. Left panel: species phylogeny. The asterisk indicates *SSC1* duplication. Right panel: Δ*jac1* and Δ*jac1* Δ*ssq1* cells harbouring plasmid-borne copies of wild-type *JAC1* from the indicated yeast species were plated as 10-fold serial dilutions (starting with approximately 5,000 cells) on rich glucose medium and incubated at 30 °C for 3 days. (**B**) Stimulation of the ATPase activity of Ssc1 and Ssq1 from *S. cerevisiae* by Jac1$_{Sc}$ and Jac1$_{Yl}$ in the presence of Isu1$_{Sc}$. ATPase activity in the absence of Jac1 was set to 0. (**C**) The structure of the J domain of HscB (PDB ID: 1fpo), a Jac1 orthologue from *Escherichia coli* and structural models of the J domains of Jac1$_{Yl}$ and Jac1$_{Sc}$. Sc, *Saccharomyces cerevisiae*; Yl, *Yarrowia lipolytica*.

J domains. Ssc1 of *S. cerevisiae* is known to function with two J proteins: Pam18 and Mdj1 (Craig *et al*, 2006). Neither Pam18 nor Mdj1 stimulates the ATPase activity of Ssq1 (D'Silva *et al*, 2003; Dutkiewicz *et al*, 2003), reflecting the fact that Ssq1 has lost the ability to perform the protein translocation and folding activities of Ssc1. Ssq1 functions with a single J protein, Jac1. The ATPase activity of Ssc1 is stimulated by Jac1, but much less effectively than that of Ssq1, suggesting that Jac1 of *S. cerevisiae* has retained some residual ability to function with Ssc1 (Schilke *et al*, 2006).

Taking advantage of the extensive information available on fungal genomes, we determined that the existence of Ssq1 in fungal species correlates with structural and functional changes in the J domain of Jac1. We propose that this correlation uncovers a causal link between changes in the J domain and a shift of J protein specificity towards a new, specialized Hsp70 partner.

RESULTS AND DISCUSSION
Jac1 altered properties upon co-evolution with Ssq1
The ineffective stimulation of the ATPase activity of *S. cerevisiae* Ssc1 by Jac1, along with the severe growth defects of strains lacking Ssq1, suggested that Jac1 might have lost the ability to function efficiently with the multifunctional Hsp70 in organisms that also express an Hsp70 specialized for Fe–S cluster biogenesis. Thus, we assessed the ability of Jac1 orthologues from several fungal species to substitute for *S. cerevisiae* Jac1 (Jac1$_{Sc}$), focusing on three species: *C. albicans*, which like *S. cerevisiae* contains Ssq1, and *Yarrowia lipolytica* and *Schizosaccharomyces pombe*, two species that lack Ssq1 (Schilke

et al, 2006). To determine whether these orthologues are functional in *S. cerevisiae*, we overexpressed each in Δ*jac1 S. cerevisiae* cells. All grew as well as the control strain overexpressing Jac1$_{Sc}$ (Fig 1A).

Similar growth rates indicated that the three *JAC1* orthologues were functional in *S. cerevisiae*, as *JAC1* is an essential gene. However, this test did not provide information as to the identity of their Hsp70 partner, as Ssq1 and Ssc1 were both present. Therefore, by using a Δ*jac1* Δ*ssq1* strain we created a situation in which Jac1 could not cooperate with Ssq1. More robust growth was observed for strains expressing Jac1 from the more distant species, *Y. lipolytica* and *S. pombe*, than from the more closely related species, *C. albicans*, or from *S. cerevisiae* itself (Fig 1A). These results are consistent with the idea that Jac1 orthologues, which function with multifunctional mtHsp70 in their native environment, cooperate efficiently with Ssc1 of *S. cerevisiae*, whereas those that co-evolved with Ssq1 are less effective.

To determine whether the *in vivo* results described above could be correlated with molecular properties of Jac1, we used stimulation of the ATPase activity of Hsp70s as a biochemical measure of Jac1 interaction (Dutkiewicz *et al*, 2003). Two parameters can be determined in such analyses: the maximal stimulation (MS) obtainable and the concentration ($C_{0.5}$) at which 50% MS is attained, a measure of apparent affinity.

Using the Hsp70s Ssc1$_{Sc}$ or Ssq1$_{Sc}$ from *S. cerevisiae* in conjunction with the client protein Isu1$_{Sc}$, we tested two proteins: Jac1$_{Sc}$, a well-characterized Jac1 that coexists with Ssq1, and Jac1 from *Y. lipolytica* (Jac1$_{Yl}$), which works with multifunctional
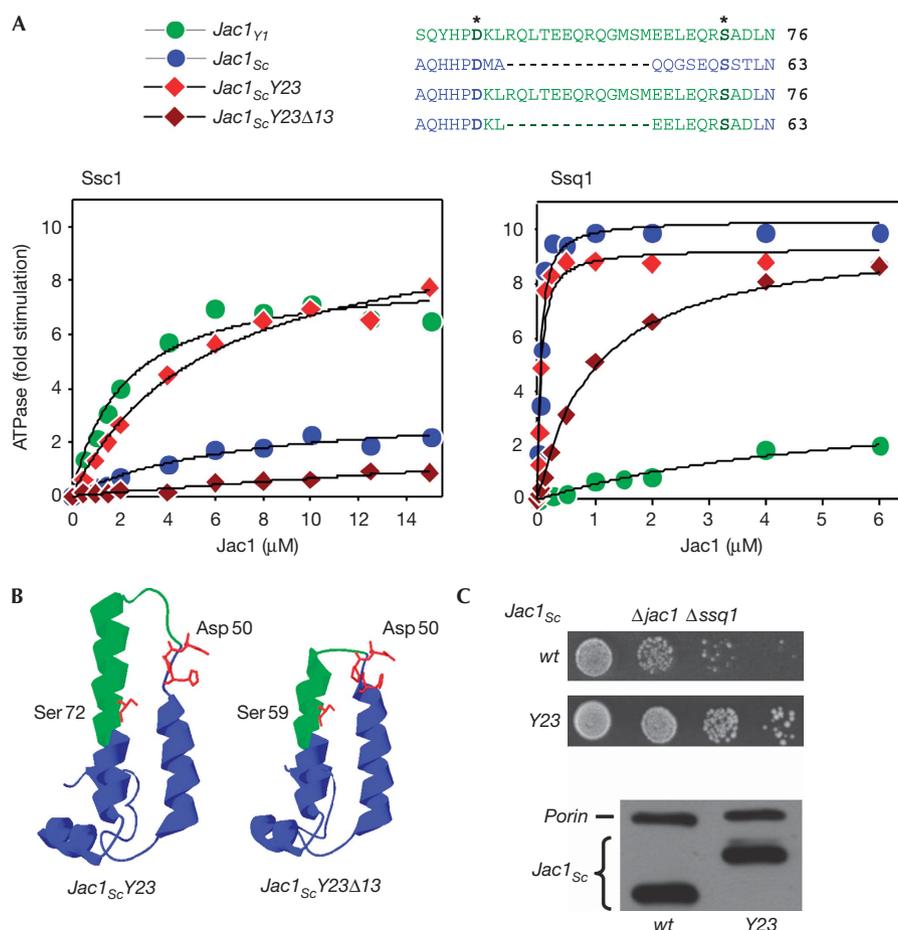
Fig 2 | J-domain structure determines partnership with Ssq1 and Ssc1. (**A**) Stimulation of the ATPase activity of Ssc1 and Ssq1 from *Saccharomyces cerevisiae* by chimaeral Jac1$_{Sc}$Y23 and Jac1$_{Sc}$Y23Δ13 proteins in the presence of Isu1$_{Sc}$. Data from Fig 1A of stimulation by Jac1$_{Sc}$ and Jac1$_{Yl}$ is included for comparison. The curves represent the best fit of the data to a Michaelis–Menten hyperbolic equation (supplementary Table S1 online). (**B**) Amino-acid sequence and structural model of Jac1$_{Sc}$Y23 and Jac1$_{Sc}$Y23Δ13. Green and blue represent sequences derived from *Yarrowia lipolytica* and *S. cerevisiae*, respectively. (**C**) Overexpression of Jac1$_{Sc}$Y23 suppresses the growth defect of Δ*jac1* Δ*ssq1*. Top panels: 10-fold serial dilutions (starting with approximately 10^4 cells) of Δ*jac1* Δ*ssq1* cells expressing plasmid-borne wild-type Jac1$_{Sc}$ (wt) or Jac1$_{Sc}$Y23 (Y23) were plated on a rich glucose medium and incubated at 30 °C for 3 days. Bottom panel: cell lysate from the indicated strains separated by electrophoresis and subjected to immunoblot analysis using antibodies specific for Jac1$_{Sc}$ and, as a loading control, porin. Predicted molecular weight: Jac1$_{Sc}$, 21.6 kDa; Jac1$_{Sc}$Y23, 23.3 kDa. Sc, *Saccharomyces cerevisiae*; Yl, *Yarrowia lipolytica*.

mtHsp70 in its native environment. As expected, Jac1$_{Sc}$ stimulated the ATPase activity of Ssq1 effectively and with high apparent affinity (MS = 10.36-fold; C$_{0.5}$ = 0.050 μM; Fig 1B; supplementary Table S1 online). By contrast, Jac1$_{Sc}$ stimulated the ATPase activity of Ssc1 threefold less efficiently (MS = 3.29-fold) and had more than a 100-fold lower apparent affinity (C$_{0.5}$ = 6.71 μM). By contrast, Jac1$_{Yl}$ stimulated the ATPase activity of Ssq1 poorly (MS = 4.11; C$_{0.5}$ = 6.14 μM), but was significantly better at stimulating the ATPase activity of Ssc1 (MS = 8.28-fold; C$_{0.5}$ = 2.16 μM), even though *Y. lipolytica* is distantly related to *S. cerevisiae*. This effectiveness of Jac1$_{Yl}$ compared with Jac1$_{Sc}$ in stimulating the ATPase activity of Ssc1 is consistent with its ability to outperform Jac1$_{Sc}$ in supporting the growth of Δ*jac1* Δ*ssq1* cells (Fig 1A). Thus, together, the data support the idea that Jac1 and specialized Ssq1 have co-evolved. This co-evolution also resulted in a diminished ability of Jac1 to function with the multifunctional Ssc1.

Conversion of Jac1$_{Sc}$ to function better with Ssc1

Next, we considered whether the functional differences between Jac1 proteins that co-evolved with Ssq1 and those that did not could be explained by alterations in their structure. As no experimentally determined structural information for fungal Jac1 proteins exists, we turned to molecular modelling (details in supplementary information online). The size of the J domains was the most striking difference between the predicted structures (Fig 1C). The Jac1$_{Yl}$ J domain was significantly larger, with 21 residues in Jac1$_{Yl}$ between the aspartic acid of the invariant and critical HPD motif (Asp$_{50}$ in both Jac1$_{Sc}$ and Jac1$_{Yl}$) and the invariant serine in the middle of Helix-III (Ser$_{72}$ and Ser$_{59}$ in Jac1$_{Yl}$ and Jac1$_{Sc}$, respectively; supplementary Fig S1,S2 online). Jac1$_{Sc}$ has only eight residues in the homologous position (Fig 1C). Eleven of these residues in Jac1$_{Yl}$ are predicted to form a loop between the helices, whereas in Jac1$_{Sc}$ two residues form a tight loop (Fig 1C).
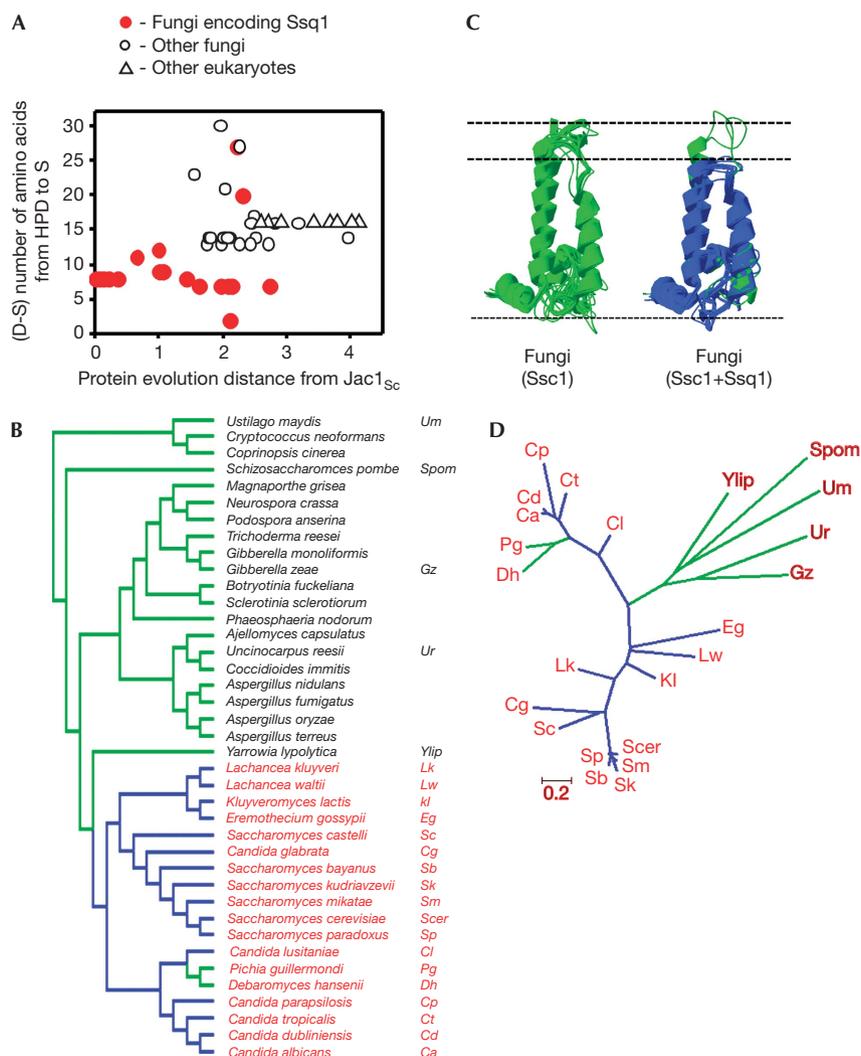
Fig 3 | Across fungal phylogeny, the presence of the 'short' version of the J domain correlates with the presence of *SSQ1*. (A) For each Jac1 orthologue, the number of residues between the Asp of HPD and conserved Ser of Helix-III (D–S distance) was plotted against the evolutionary protein distance calculated for each Jac1 orthologue compared with Jac1$_{Sc}$ (supplementary Table S2 online). **(B)** Fungal phylogeny on the basis of Fitzpatrick *et al* (2006). Blue branches lead to species encoding a 'short' J domain; green branches lead to species encoding J domains with a D–S distance greater than 12 amino acids (supplementary Table S2 online). Species encoding Ssq1 are marked in red. **(C)** The predicted structures of the J domains of Jac1 from the species listed in **(B)**. Blue, 'short' J domains; green, 'regular' J domains. **(D)** Phylogenetic tree inferred from Jac1 structural alignment by maximum likelihood method (details in the supplementary information online). Branches are drawn to scale showing the number of amino-acid substitutions per site.

To test whether these differences near the ends of and between Helices II and III are responsible for the differences in the specificity of the J proteins, we considered whether the J domain of Jac1$_{Sc}$ could be altered to function more efficiently with Ssc1. We constructed a chimeric Jac1$_{Sc}$–Jac1$_{Yl}$ gene, replacing the codons for 10 amino acids of Jac1$_{Sc}$ immediately carboxy-terminal of the conserved HPD motif with 23 amino acids of Jac1$_{Yl}$, yielding Jac1$_{Sc}$Y23 (Fig 2A). Jac1$_{Sc}$Y23 stimulated the ATPase activity of Ssc1 nearly as well as Jac1$_{Yl}$, increasing the MS from 3.3-fold to 10.5-fold (Fig 2A; supplementary Table S1 online). Consistent with the increase in ATPase-stimulatory ability, $\Delta jac1\ \Delta ssq1$ cells expressing Jac1$_{Sc}$Y23 grew better than those expressing Jac1$_{Sc}$ (Fig 2C). Having observed that Jac1$_{Sc}$Y23 functioned more efficiently with Ssc1 than did Jac1$_{Sc}$, we next considered how

these changes affected the ability of Jac1 to stimulate the ATPase activity of Ssq1. Jac1$_{Sc}$Y23 stimulated Ssq1 almost as efficiently as wild-type Jac1$_{Sc}$ (Fig 2A, right panel), suggesting that Ssq1 has not lost the ability to accommodate a longer J domain over time.

Jac1$_{Sc}$Y23 is altered in amino-acid sequence, as well as having an extended structure, compared with Jac1$_{Sc}$. To begin to understand the contribution of each, we deleted the codons for 13 amino acids from Jac1$_{Sc}$Y23, to yield Jac1$_{Sc}$Y23Δ13, which leaves *Y. lipolytica* sequences adjacent to the conserved Ser and HPD, but shortens Helix III and the loop to the size found in the *S. cerevisiae* protein (Fig 2A). Jac1$_{Sc}$Y23Δ13 stimulated the ATPase activity of Ssc1 poorly and that of Ssq1 markedly less well than either Jac1$_{Sc}$ or Jac1$_{Sc}$Y23. This reduced activity with both Hsp70 partners suggests that not only is the structure of the
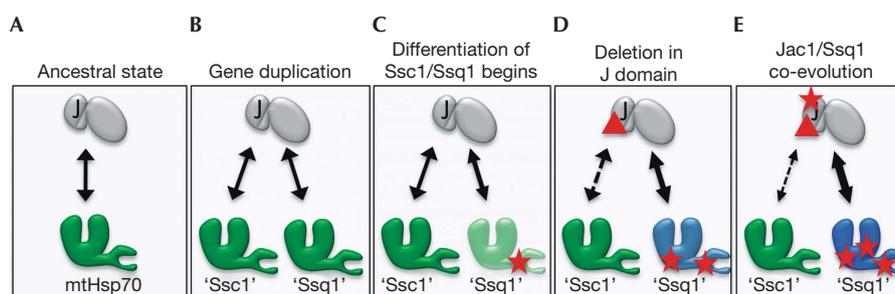
Fig 4 | The hypothetical evolutionary scenario leading to the Ssq1:Jac1 specialized partnership. The lines indicate functional protein–protein interactions, with thick solid lines indicating interactions of greatest strength and dotted lines indicating the weakest interactions. The stars represent amino-acid substitutions that affected biochemical properties of interacting proteins. The triangle indicates deletion in the J domain of Jac1. (**A**) Before gene duplication, Jac1 functioned with multifunctional mtHsp70. (**B**) A duplication of the mtHsp70 gene, resulting in two Ssc1 genes that initially functioned equally well with Jac1. (**C**) Mutations in the Ssc1 gene copy occurred, which became *SSQ1* (**D**) A deletion in *JAC1* occurred, reducing the size of the J domain and resulting in weak interaction with Ssc1. It is noted that this deletion event could have occurred either before—as indicated in (**C**)—or after other alterations in the gene that became *SSQ1*. The hypothesis that the changes in *SSQ1* that facilitated interaction with deleted Jac1 occurred before, rather than after, the *JAC1* deletion is appealing, because it could possibly explain the survival of cells immediately after the occurrence of the *JAC1* deletion. (**E**) Over time, reciprocal changes in *JAC1* and *SSQ1* resulted in highly specific interactions. The colour change of Hsp70 from dark green (**B**) to light green (**C**) to blue (**D,E**) indicates the overall changes in amino acid that occurred after the *SSC1* gene duplication. The Hsp70 encoded by the gene to become *SSQ1* in (**B**) is indicated by 'Ssq1'.

J domain important for specificity, but also that other sequences in the J domain have a role in defining Jac1–Hsp70 interactions.

Co-evolution with Ssq1 altered J-domain structure

The results presented above suggest that the shortened J domain might be a common feature of the Jac1 proteins that have coexisted with Ssq1. To better assess the generality of the structural difference found between the J domains of Jac1$_{Sc}$ and Jac1$_{Yl}$, we extended our analysis to the 39 fungal species for which genomic sequences and species phylogeny are available (Fitzpatrick *et al*, 2006). In particular, we were interested in whether a correlation exists between the presence of the 'short' version of the J domain and the presence of *SSQ1* across fungal phylogeny. Thus, the number of amino-acid residues from the Asp of the HPD motif to the invariant Ser in the middle of Helix III (D–S distance) were counted and plotted against the protein evolutionary distance calculated for each Jac1 orthologue relative to Jac1$_{Sc}$ (Fig 3A; supplementary Table S2 online). Sixteen of 18 Jac1 orthologues from species encoding *SSQ1* (Fig 3B, red lettering) have 'short' J domains, having a D–S distance between 7 and 12 amino acids. By contrast, all J domains of Jac1 orthologues from fungal species lacking *SSQ1* (Fig 3B, black lettering), as well as the eight analysed non-fungal species (Fig 3; supplementary Table S2 online), are longer than 12 amino acids.

Analysis of the fungal phylogeny (Fitzpatrick *et al*, 2006) revealed that all species encoding a 'short' version of the J domain belong to a monophyletic group (Fig 3B, blue branches), suggesting that this version arose in the common ancestor as the result of a single deletion event. Consistent with this idea, analysis of the topology of the phylogenetic tree on the basis of the Jac1 protein sequence indicates that all Jac1 orthologues with 'short' J domains cluster together (Fig 3D). However, there are two outliners within this group, *Pichia guillemondii* and *Debaromyces hansenii*, two closely related species that encode *SSQ1* but have 'long' J domains, with D–S distances of 27 and 20 amino acids, respectively. Their localization on the phylogenetic tree (Fig 3B, green branches), sister

to each other and nested within species encoding a 'short' J domain (Fig 3B), implies that they are descendants of the lineage encoding a 'short' version of the J domain, and that these 'long' J domains are most probably derived features, acquired in the course of evolution long after the emergence of *SSQ1*. Such a secondary re-elongation of the J domain is consistent with our observation that the ATPase activity of Ssq1 can be stimulated efficiently by both 'short' and 'long' J domains (Fig 2A). In summary, the phylogenetic distribution of the 'short' J domain implies that it arose through a single deletion event that corresponds to the timing of the origin of *SSQ1*.

Although this paper focuses on the co-evolution of Jac1 and Ssq1, we also considered the possibility that the third component of the system, the client protein Isu1 (Garland *et al*, 1999; Muhlenhoff *et al*, 2003), might also have co-evolved. However, analysis of an alignment of the sequences of the set of Isu1 orthologues from the species listed in Fig 3B (J.K. and J.M., unpublished results) showed no obvious signs of sequence changes that could be correlated with presence of Ssq1. Therefore, if co-evolution between Isu1 and the Jac1/Ssq1 chaperone system occurred, it is most probably a subtle phenomenon.

Hypothetical evolutionary scenario

Data presented here enable us to develop a scenario regarding the evolution of the Jac1:Ssq1 specialized partnership. Although hypothetical, this scenario, as put forth in Fig 4, purports that the co-evolution of Jac1 and Ssq1 was triggered by deletion in the J domain of Jac1. About 300 million years ago, all fungal genomes encoded a single mtHsp70, Ssc1, which functioned with several J proteins, including Jac1 (Fig 4A). Duplication of the gene encoding mtHsp70 (Fig 4B; Schilke *et al*, 2006) presented the opportunity for division of functions between *SSC1* and the paralogous gene that became *SSQ1* (Fig 4C). Immediately after gene duplication, *SSQ1* and *JAC1* might have accumulated mutations that changed their biochemical properties but did not significantly affect the ability of Jac1 to interact with either Ssc1 or Ssq1. Only after a deletion occurred in *JAC1* (Fig 4D), reducing the

size of the J domain and resulting in weakened interaction with Ssc1, but not affecting interaction with Ssq1, did the Jac1–Ssq1-specific partnership begin to be established. This deletion event might have acted as an evolutionary ratchet, making reversal to the ancestral structure–function relations far more difficult, thus promoting forward co-evolution of Jac1 and Ssq1. Over time, reciprocal changes involving the sequence outside the loop region of Jac1 resulted in a highly specific and efficient interaction between Ssq1 and Jac1, forming a chaperone machinery tuned for functioning exclusively in Fe–S cluster biogenesis (Fig 4E).

Summary

In summary, we propose that the co-evolution-driven reciprocal changes in Ssq1 and Jac1 were triggered by a significant deletion in the J domain of Jac1, allowing selective interaction with Ssq1. Our results have implications for the multiplication and diversification of Hsp70:J-protein interaction network, beyond the specific example of Fe–S cluster assembly. In most cellular compartments, several Hsp70 proteins interact with an even higher number of J proteins (Hennessy *et al*, 2005), functioning in various crucial physiological processes. Thus, co-evolutionary processes in which duplicated chaperones and partner co-chaperones were optimized in a coordinated manner might have had an important role in the formation of specific partnerships. In the broader picture, our results provide experimental evidence supporting the importance of protein co-evolution as a driving force for the expansion and rewiring of the protein interaction networks (Yamada & Bork, 2009).

METHODS

Yeast strains and plasmids. The *S. cerevisiae* strains used are haploid derivatives of PJ53 (W303), described previously (Andrew *et al*, 2006). *JAC1* open reading frames were amplified by PCR: Jac1$_{Yl}$, *Y. lipolytica* (CLIB99); Jac1$_{Ca}$, *C. albicans* (SC5314); Jac1$_{Sp}$, *S. pombe* (1457); and Jac1$_{Sc}$, *S. cerevisiae* described by Andrew *et al* (2006). All were cloned into p414TEF (Mumberg *et al*, 1995). Chimeric *JAC1$_{Sc}$* mutants were obtained by site-directed mutagenesis. All proteins were purified as described previously (Dutkiewicz *et al*, 2003; Schilke *et al*, 2006).

ATPase assays. ATPase activity was measured as described by Dutkiewicz *et al* (2003), with 0.5 μM Ssc1 or Ssq1, 10 μM Isu1, 0.5 μM Mge1 all from *S. cerevisiae* and variable amounts of Jac1. Robust stimulation requires inclusion of the client protein Isu1. Jac1$_{Sc}$ and Jac1$_{Yl}$ bind to Isu1$_{Sc}$ with similar affinity, thus validating the use of Isu1$_{Sc}$ for assays using both *JAC1* proteins (supplementary Fig S3 online). Details are provided in the supplementary information online.

Prediction of protein structure. Homology modelling was performed using *Escherichia coli* HscB (PDB ID: 1fpo) as template. Alignment of the target sequence to the template structure was prepared using the GeneSilico Metaserver (Kurowski & Bujnicki, 2003). Structure models were calculated by using MODELLER (Fiser & Sali, 2003) and their potential accuracy was evaluated by using MetaMQAP (Pawlowski *et al*, 2008). Details are provided in the supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

REFERENCES

Andrew AJ, Dutkiewicz R, Knieszner H, Craig EA, 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

Craig EA, Marszalek J (2002) A specialized mitochondrial molecular chaperone system: a role in formation of Fe/S centers. *Cell Mol Life Sci* **59:** 1658–1665

Craig EA, Huang P, Aron R, Andrew A (2006) The diverse roles of J-proteins, the obligate Hsp70 co-chaperone. *Rev Physiol Biochem Pharmacol* **156:** 1–21

D'Silva PD, Schilke B, Walter W, Andrew A, Craig EA (2003) J protein cochaperone of the mitochondrial inner membrane required for protein import into the mitochondrial matrix. *Proc Natl Acad Sci USA* **100:** 13839–13844

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

Fiser A, Sali A (2003) Modeller: generation and refinement of homology-based protein structure models. *Methods Enzymol* **374:** 461–491

Fitzpatrick DA, Logue ME, Stajich JE, Butler G (2006) A fungal phylogeny based on 42 complete genomes derived from supertree and combined gene analysis. *BMC Evol Biol* **6:** 99

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

Hennessy F, Nicoll WS, Zimmermann R, Cheetham ME, Blatch GL (2005) Not all J domains are created equal: implications for the specificity of Hsp40–Hsp70 interactions. *Protein Sci* **14:** 1697–1709

Kurowski MA, Bujnicki JM (2003) GeneSilico protein structure prediction meta-server. *Nucleic Acids Res* **31:** 3305–3307

Lynch M, Conery JS (2000) The evolutionary fate and consequences of duplicate genes. *Science* **290:** 1151–1155

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

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

Pawlowski M, Gajda MJ, Matlak R, Bujnicki JM (2008) MetaMQAP: a meta-server for the quality assessment of protein models. *BMC Bioinformatics* **9:** 403

Prince VE, Pickett FB (2002) Splitting pairs: the diverging fates of duplicated genes. *Nat Rev Genet* **3:** 827–837

Schilke B, Williams B, Knieszner H, Pukszta S, D'Silva P, Craig EA, Marszalek J (2006) Evolution of mitochondrial chaperones utilized in Fe–S cluster biogenesis. *Curr Biol* **16:** 1660–1665

Taylor JW, Berbee ML (2006) Dating divergences in the fungal tree of life: review and new analyses. *Mycologia* **98:** 838–849

Vickery LE, Cupp-Vickery JR (2007) Molecular chaperones HscA/Ssq1 and HscB/Jac1 and their roles in iron–sulfur protein maturation. *Crit Rev Biochem Mol Biol* **42:** 95–111

Yamada T, Bork P (2009) Evolution of biomolecular networks: lessons from metabolic and protein interactions. *Nat Rev Mol Cell Biol* **10:** 791–803

Zhang J (2003) Evolution by gene duplication: an update. *Trends Ecol Evol* **18:** 292–298