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ORIGINAL PAPER

# Plasticity of the Hsp90 chaperone machine in divergent eukaryotic organisms

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**Abstract** Hsp90 is critical for the regulation and activation of numerous client proteins critical for diverse functions such as cell growth, differentiation, and reproduction. Cytosolic Hsp90 function is dependent on a battery of co-chaperone proteins that regulate the ATPase activity of Hsp90 function or direct Hsp90 to interact with specific client proteins. Little is known about how Hsp90 complexes vary between different organisms and how this affects the scope of clients that are activated by Hsp90. This study determined whether ten distinct Hsp90 co-chaperones were encoded by genes in 19 disparate eukaryotic organisms. Surprisingly, none of the co-chaperones were present in all organisms. The co-chaperone Hop/Sti1 was most widely dispersed (18 out of 19 species), while orthologs of Cdc37, which is critical for the stability and activation of diverse protein kinases in yeast and mammals, were identified in only nine out of 19 species examined. The organism with the smallest proteome, *Encephalitozoon cuniculi*, contained only three of these co-chaperones, suggesting a correlation between client diversity and the complexity of the Hsp90 co-chaperone machine. Our results suggest co-chaperones are critical for cytosolic Hsp90 function in vivo, but that the composition of Hsp90 complexes varies depending on the specialized protein folding requirements of divergent species.

**Keywords** Aha1 · Co-chaperone · Tetratricopeptide repeat · Immunophilin · Hop · p23

**Abbreviations**

| | |
|---|---|
| Hsp | heat shock protein |
| TPR | tetratricopeptide repeat |
| BLAST | basic local alignment search tool |

## Introduction

Hsp90 (heat shock protein, 90 kDa) is a highly abundant molecular chaperone that accounts for 1–2% of cellular proteins under normal growth conditions. Two isoforms of Hsp90 are expressed in the cytosol of yeast and mammalian cells, and expression of at least one isoform is essential for viability in all eukaryotes examined. Cytosolic Hsp90 interacts with client proteins in an ordered ATP-dependent pathway that is dependent on additional chaperones, such as Hsp70 and Hsp40, as well as a number of co-chaperones that regulate Hsp90 function in vivo and in vitro. Over the last two decades, Hsp90 has been shown to be required for the maturation of hundreds of diverse client proteins, including steroid receptors and other classes of transcription factors, a variety of serine/threonine and tyrosine kinases, and additional proteins such as telomerase and the hepatitis B virus reverse transcriptase. The number of identified Hsp90 co-chaperones has also grown. Twelve distinct co-chaperones are expressed in *Saccharomyces cerevisiae*, and mammalian cells express homologs of all of those plus additional co-chaperones not present in *S.*

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*cerevisiae* (Pearl and Prodromou 2006; Wegele et al. 2004; Zhao et al. 2005). Although the specific functions of the co-chaperones are still under investigation, there is increasing evidence that some Hsp90 clients preferentially require the function of only a subset of co-chaperones and that closely related co-chaperones may have differential effects on client function (Felts et al. 2007; Riggs et al. 2004; Smith and Toft 2008).

The interaction of clients and co-chaperones with Hsp90 is regulated by ATP and ATP-induced conformational changes in Hsp90. Hsp90 may be divided into three domains: an amino-terminal ATP-binding domain, a middle domain, and a carboxy-terminal domain. Co-chaperone binding sites have been identified in each domain of Hsp90, and co-chaperones have diverse mechanisms by which to regulate function (Pearl and Prodromou 2006; Wegele et al. 2004). Some co-chaperones regulate the ATPase activity of Hsp90, while others likely have specialized in vivo functions, such as direct contact with client proteins, localization, or trafficking (Pratt et al. 2004; Riggs et al. 2004).

The increasing number of known Hsp90 co-chaperones raises important questions of which co-chaperones and which biological functions of these co-chaperones are most critical for Hsp90 function in vivo. A greater understanding of these questions may help explain why cytosolic Hsp90 requires multiple co-chaperones, while bacterial Hsp90 (HtpG) and organellar forms of Hsp90 (TRAP1 of mitochondria and Grp94 of the endoplasmic reticulum) appear to lack co-chaperones even though they have similar functions in binding and hydrolyzing ATP (Felts et al. 2000; Richter et al. 2007). The goal of this study was to gain a greater understanding of how the complexity of the Hsp90 molecular machine varies from organism to organism by analyzing the conservation of ten distinct co-chaperones across 19 disparate organisms. Surprisingly, no individual co-chaperone was present in all 19 species, although five (Hop, PP5, Aha1, p23 and Sgt1) were present at least in 16 out of 19 organisms examined. In contrast, some co-chaperones, such as Cdc37, were limited to fewer species. These results indicate that regulation of cytosolic Hsp90 activity by co-chaperones is likely essential for viability in eukaryotes, but that the overall composition of the Hsp90 molecular chaperone machine varies in a species-specific manner.

## Materials and methods

### Identification of co-chaperone orthologs in divergent species

To identify orthologs of known co-chaperones, the protein sequence of the human co-chaperone was used in a directed Psi-BLAST (Basic Local Alignment Search Tool) search against the genome of indicated species (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to identify the best match. If the alignment was poor (below 0.005 threshold using Psi-Blast), a reciprocal best-hit search was used to determine if the identified ortholog in a species had the closest match to the desired human co-chaperone. If the target co-chaperone was not the closest match in the reverse search, it was not counted in the analysis. In those cases in which no clear ortholog was identified using the human sequence, an additional search using the protein sequence of the closest relative was used. The closest relative was determined from the tree of related Hsp90 sequences (Fig. 3).

Two species did not have complete protein databases deposited in Genbank and were identified by different methods. To identify homologs in *Cyanidioschyzon merolae*, the protein sequence of the human co-chaperone was used in a Blast search against the protein database or a tBLASTn search against the *C. merolae* nucleotide database (http://merolae.biol.s.u-tokyo.ac.jp/). If the protein had not been annotated, the Editseq and/or Seqbuilder programs (Lasergene, Madison, WI, USA) were subsequently used to identify an open reading frame corresponding to the co-chaperone of interest. The sequence tag listed for *C. merolae* co-chaperones refers to the larger DNA clone harboring the open reading frame. To identify homologs in *Thalassiosira pseudonana*, BLAST searches were conducted using the database of the DOE Joint Genome Institute (http://genome.jgi-psf.org/Thaps3/Thaps3.home.html). The name of the protein coding sequence assigned by the Joint Genome Institute was listed in Table 1.

MegAlign (Lasergene) was used to build a consensus of the best hit from each species (ClustalV or ClustalW) and confirm that selected orthologs exhibit sequence similarity in the most highly conserved regions of each protein. Sequence alignments showing known domains are included in the Supplemental Figures. Table 1 shows the Genbank accession numbers of co-chaperone orthologs identified in the different species. For those without a convincing homolog, the 'na', for not apparent, appears. In almost all cases, the Genbank accession number is unique to one particular co-chaperone. The exception is that in three species, *Theileria annulata*, *Tetrahymena thermophila*, and *Thalassiosira pseudonana*, a single protein is counted as representing both Cyp40 and FKBP52 homologs. These proteins belong to a family of recently described proteins called 'dual immunophilins', since they contain separate FK506-binding domains and cyclophilin domains separated by TPR repeats (Barik 2006). For the purpose of this study, these were counted as having functions similar to both Cyp40 and FKBP52, although this has not been confirmed experimentally.

*Identification of Hsp90 orthologs* A prior study examined a wide range of Hsp90 sequences and established a system for naming Hsp90 orthologs (Chen et al. 2006). The Genbank

**Table 1** Orthologs of Hsp90 co-chaperones

| Species | Hop/Sti1 | PP5 | Cns1 | FKB52 | Cyp40 |
|---|---|---|---|---|---|
| *Homo sapiens* | AAH39299 | NP_006238.1 | CAI18995.1 | NP_002005.1 | NP_005029.1 |
| *Drosophila melanogaster* | AAF51511 | NP_524946.1 | NP_525106.2 | NP_524895.2 | NP_648338.1 |
| *Caenorhabditis elegans* | NP_503322.1 | NP_741697.2 | NP_495087.1 | NP_508026.1 | na |
| *Saccharomyces cerevisiae* | NP_014670 | NP_011639.1 | NP_009713.1 | na | NP_013317.1 |
| *Schizosaccharomyces pombe* | NP_588123.1 | NP_596740.1 | NP_594238.1 | na | NP_594787.1 |
| *Leishmania major* | AAB37318.1 | XP_001682421.1 | XP_001685813.1 | XP_001682742.1 | XP_843581.1 |
| *Encephalitozoon cuniculi* | na | na | na | na | na |
| *Chlamydomonas reinhardtii* | XP_001691869.1 | XP_001690473.1 | na | XP_001694809.1 | XP_001690215.1 |
| *Arabidopsis thaliana* | AAU95460 | NP_565985.1 | NP_563702.1 | NP_189160.3 | NP_565381.1 |
| *Dictyostelium discoideum* | XP_629588.1 | XP_639169.1 | XP_637811.1 | XP_638885.1 | XP_638951.1 |
| *Trypanosoma brucei* | XP_8449661.1 | AAG40278.1 | XP_823346.1 | XP_828079.1 | XP_827280.1 |
| *Giardia lamblia* | XP_767235 | XP_001706296 | na | XP_001708385.1 | na |
| *Tetrahymena thermophila* | XP_001008334.1 | XP_001030142.1 | XP_001019203.1 | XP_976669 | XP_976669 |
| *Plasmodium falciparum* | XP_001348498.1 | XP_001350266.1 | XP_001347776.1 | XP_001350859.1 | na |
| *Cryptosporidium parvum* | XP_001388209.1 | XP_001388227.1 | XP_628247.1 | na | na |
| *Entamoeba histolytica* | XP_655642.1 | XP_653228.1 | XP_652329.1 | XP_656239.1 | na |
| *Theileria annulata* | XP_955292.1 | XP_951772.1 | na | XP_951815 | XP_951815 |
| *Cyanidioschyzon merolae* | AP006500.2 | AP006498.2 | AP006502.2 | AP006490.2 | na |
| *Thalassiosira pseudonana* | Thaps3:3416 | na | Thaps3:23210 | Thaps3:31535 | Thaps3:31535 |
| | p23/Sba1 | Aha1 | Sgt1 | Pih1 | Cdc37 |
| *Homo sapiens* | NP_006592.3 | AAH00321 | NP_006695.1 | NP_060386.1 | NP_008996.1 |
| *Drosophila melanogaster* | NP_649925.1 | NP_610121.2 | AAL49336.1 | AAL68384.1 | AAO45184.1 |
| *Caenorhabditis elegans* | NP_498126.1 | NP_506715.1 | NP_505751.1 | NP_505775.3 | AAC71172.1 |
| *Saccharomyces cerevisiae* | NP_012805.1 | Q12449 | NP_014700.1 | NP_011899.1 | NP_010452.1 |
| *Schizosaccharomyces pombe* | NP_594586.1 | NP_595881.1 | NP_595340.1 | na | NP_59752.1 |
| *Leishmania major* | XP_001686111.1 | XP_001682427.1 | XP_001682910.1 | XP_843540.1 | na |
| *Encephalitozoon cuniculi* | na | NP_586059.1 | NP_597459.1 | na | NP_585992.1 |
| *Chlamydomonas reinhardtii* | XP_001692527.1 | XP_001693239.1 | XP_001702976.1 | XP_001698460.1 | na |
| *Arabidopsis thaliana* | CAC16575.1 | NP_566410.1 | NP_001031704.1 | na | na |
| *Dictyostelium discoideum* | Q55FM3 | XP_646215.1 | XP_645851.1 | XP_640978.1 | na |
| *Trypanosoma brucei* | XP_827315.1 | XP_827854.1 | XP_001219018.1 | XP_827333.1 | na |
| *Giardia lamblia* | XP_001706046.1 | XP_001706051.1 | XP_001709713.1 | na | na |
| *Tetrahymena thermophila* | XP_001014692.2 | Q22R57 | XP_001032996.1 | XP_001016640.1 | na |
| *Plasmodium falciparum* | XP_001348684.1 | XP_001351138.1 | XP_001352198.1 | XP_001350744.1 | na |
| *Cryptosporidium parvum* | CAD98707 | XP_628382.1 | na | na | XP627758.1 |
| *Entamoeba histolytica* | na | na | XP_656074.1 | na | na |
| *Theileria annulata* | XP_952229.1 | na | na | na | XP954244.1 |
| *Cyanidioschyzon merolae* | AP006499.2 | AP006498.2 | na | na | na |
| *Thalassiosira pseudonana* | Thaps3:2160 | na | Thaps3:269341 | na | Thaps3:34996 |

accession numbers for Hsp90 orthologs used in this analysis are: *Dictyostelium discoideum* EAL73152; *Entamoeba histolytica* EAL47778.1, EAL47746.1, XP_649616; *Encephalitozoon cuniculi* NP_584635.1; *Schizosaccharomyces pombe* NP_594365.1, AAC41646.1; *S. cerevisiae* AAA02743.1, AAA02813.1; *Caenorhabditis elegans* NP_506626.1; *Drosophila melanogaster* NP_523899.1; *Homo sapiens* NP_005339.3, NP_031381.2; *Leishmania major* XP_001685759.1; *Trypanosoma brucei* XP_823307.1; *T. thermophila* EAR89535.1; *Plasmodium falciparum* NP_704028.1; *Cryptosporidium parvum* XP_626924.1; *T. annulata* XP_952473.1; *Cyanidioschyzon merolae* Q84KP7; *Chlamydomonas reinhardtii* XP_001695264.1, *Arabidopsis thaliana* NP_200076.1, NP_200414.1, NP_200412.1, NP_200411.1. The *Giardia lamblia* sequence is a combination of two separate records, XP_001707991.1 and XP_01705478.1. The Hsp90 sequence of *Thalassiosira pseudonana* is referred to as Thaps3:6285 (http://genome.jgi-psf.org/Thaps3/Thaps3.home.html).

Phylogenetic trees were inferred for these HSP90 proteins using UPGMA (as implemented in Megalign), and neighbor joining and protein parsimony algorithms (as implemented in PHYLIP) (Felsenstein 2005). A bootstrap analysis was performed for each of these three methods, and although the trees inferred by each method were slightly different, the differences occurred at nodes with very weak statistical

support. None of the HSP90 trees reflected the species tree of myosin paralogs from Wickstead and Gull (Wickstead and Gull 2006).

*Co-chaperones included in this study* The co-chaperones included in this study are shown in Fig. 1. Most of these Hsp90 co-chaperones were first identified by virtue of their co-purification with Hsp90 complexes isolated out of yeast or mammalian cell extracts (Hop, PP5, p23, Sgt1, FKBP52, Cyp40 and Cdc37). Additional co-chaperones were identified by virtue of physical or genetic interactions with Hsp90 of *S. cerevisiae* (Pih1, Aha1 and Cns1). Many of the co-chaperones (PP5, Cyp40, Hop, FKBP52, Sgt1, and Cns1) contain tetratricopeptide repeat domains and likely compete for binding to the carboxy-terminal MEEVD sequence of Hsp90. The remaining co-chaperones Cdc37, p23, Aha1 and Pih1 do not contain TPR domains, but with the exception of Pih1, their binding sites to Hsp90 have been identified (Pearl and Prodromou 2006; Riggs et al. 2004; Wegele et al. 2004; Zhao et al. 2005).

*Hop* Hop (Hsp70–Hsp90 organizing protein in vertebrates, Sti1 in yeast) is an abundant, highly conserved protein that is able to interact simultaneously with the carboxy-termini of Hsp70 and Hsp90 through separate tetratricopeptide repeat (TPR) domains. TPR1 contains the primary binding site for Hsp70, and TPR2A contains the primary binding site for Hsp90 (Scheufler et al. 2000). Hop was able to stimulate the ATPase activity of the Hsp70 Ssa1 and inhibit the ATPase activity of Hsp90 and plays a critical role in mediating transfer of clients from Hsp70 to Hsp90 (Pearl and Prodromou 2006; Riggs et al. 2004; Wegele et al. 2006). Hop is one of the most well-studied Hsp90 co-chaperones and is one of the five proteins required for in vitro activation of steroid hormone receptors (Felts et al. 2007).

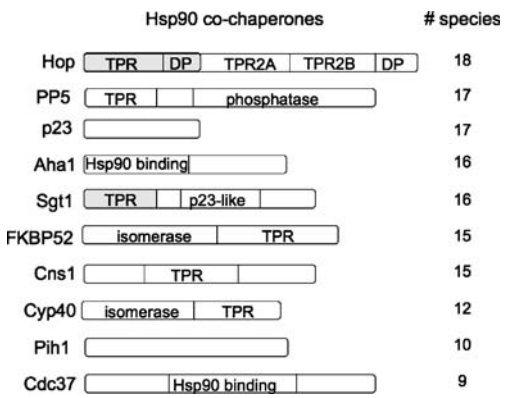


**Fig. 1** Hsp90 co-chaperones included in this study. Co-chaperones are listed in order of their prevalence in the 19 species examined. Many co-chaperones interact with the carboxy-terminus of Hsp90 through tetratricopeptide repeat domains (TPR domains). As discussed in the text, in some species, the orthologs of Hop or Sgt1 appear to lack specific TPR domains, and these domains are *shaded* in the figure. In addition, some species encode 'dual immunophilins' that contain a TPR domain plus distinct FK506 or cyclosporin domains, which may substitute for FKBP52 and Cyp40

*PP5* PP5 (Ppt1 in *S. cerevisiae*) is a serine–threonine phosphatase that binds Hsp90 through its TPR domain. As described in two recent reviews, PP5 interacts with a wide range of proteins involved in signaling pathways, including Raf, ATM/ATR, and the Rac GTPase. PP5 may also regulate the functions of Hsp90 and other co-chaperones by dephosphorylating the heat-shock transcription factor, HSF, as well as Hsp90 itself (Golden et al. 2008; Hinds and Sanchez 2007; Wandinger et al. 2006).

*p23* p23 (Sba1 in *S. cerevisiae*) was first identified as a component of steroid hormone receptor complexes, but is found in a wide range of client protein complexes. p23 binds the amino-terminus of Hsp90 in an ATP-dependent manner, stabilizing the ATP-bound form of Hsp90, which is characterized by dimerized amino-termini. p23 has an inhibitory effect on the ATPase activity of Hsp90 and may be involved in regulating client protein release (Pearl and Prodromou 2006).

*Aha1* The amino-terminal half of Aha1 (activator of Hsp90 ATPase) binds the middle domain of Hsp90. Aha1 stimulates the ATPase activity of Hsp90 by causing a conformational change that allows a catalytic loop of the middle domain to interact with ATP bound to the amino-terminal domain (Panaretou et al. 2002).

*Sgt1* Sgt1, which cooperates with Hsp90 in kinetochore assembly in yeast and humans, contains domains with homology to both TPR domains and p23, but does not appear to modulate the ATPase activity of Hsp90 (Catlett and Kaplan 2006).

*FKBP52* FKBP52 was first identified in complexes with steroid receptors, but has since been identified in complex with multiple types of client proteins (Riggs et al. 2004). FKBP52 and Cyp40 are large members of the FK506 and cyclosporin-binding families of immunophilins, FKBP and CyP, respectively. FKBP52 contains both a TPR domain and a peptidylprolyl isomerase domain. Vertebrate cells also contain a similar protein, FKBP51. While FKBP51 and FKBP52 share a similar domain structure, the two proteins have antagonistic actions toward some steroid receptors, which may be a result of direct contact between the receptors and the co-chaperones (Riggs et al. 2004).

*Cns1* Cns1 is a TPR-containing protein that interacts with both Hsp70 and Hsp90. Cns1 was able to activate the ATPase activity of the Hsp70 Ssa1 ATPase, but did not affect the

ATPase activity of Hsp90 (Hainzl et al. 2004). However, Cns1 binds Hsp90 in an MEEVD-dependent manner, and overexpression of *CNS1* rescues the temperature-sensitive defects caused by a mutation in Hsp90 (Nathan et al. 1999; Tesic et al. 2003).

*Cyp40* Cyp40 was first identified in steroid receptor complexes. Cyp40 contains both a TPR domain and a cyclosporin-binding, peptidylprolyl isomerase domain. *S. cerevisiae* contains two homologs of mammalian Cyp40, Cpr6, and Cpr7, that have different functional properties, reinforcing the concept that even closely related co-chaperones may have different effects on Hsp90 and client protein activity (Mayr et al. 2000; Riggs et al. 2004).

*Pih1* Pih1, along with Tah1, was identified as an Hsp90 co-chaperone during a comprehensive screen of yeast proteins that have genetic and physical interactions with Hsp90. Pih1 does not contain any recognizable motifs, but bound full-length Hsp90 in pulldown assays and bound the carboxy-terminus of Hsp90 in a yeast two-hybrid assay (Zhao et al. 2005).

*Cdc37* The middle domain of Cdc37 binds directly to the amino-terminal domain of Hsp90 and inhibits Hsp90 activity by contacting a residue required for ATP hydrolysis. The amino-terminal domain of Cdc37 is able to bind protein kinases directly (Pearl and Prodromou 2006).

This study was limited to co-chaperones that have specific physical or genetic interactions with Hsp90. Molecular chaperones that cooperate with Hsp90 in some cellular functions, such as Hsp70, the Hsp70-related protein Sse1/Hsp110, as well as Hsp70-binding co-chaperones, including Hsp40s, Hip, and CHIP (Wegele et al. 2004) were excluded. Most of the other co-chaperones excluded also contain TPR domains, which may exhibit specific binding to Hsp70 or Hsp90 (Scheufler et al. 2000): Tah1 (Zhao et al. 2005); Tpr2, which also contains a J domain and interacts with Hsp70; Tom70 a mitochondrial import receptor; Xap2, a protein found in aryl hydrocarbon (AhR) complexes (Riggs et al. 2004); and GCUNC-45, a co-chaperone that interacts with the progesterone receptor (Chadli et al. 2006). The list of co-chaperones continues to grow, and a current listing of Hsp90 co-chaperone and client proteins is maintained by Didier Picard: (http://www.picard.ch/downloads/downloads.htm)

## Results

*Identification of orthologs of Hsp90 co-chaperone proteins* The sequence of each human co-chaperone was used in directed BLAST searches to identify the best match in 18 other species with complete genomes. The species were chosen to provide a good breadth across the whole eukaryotic kingdom (Wickstead and Gull 2006). These include the Metazoa *H. sapiens*, *D. melanogaster*, and *C. elegans*, and the yeasts, *S. cerevisiae* and *S. pombe*. Parasitic organisms include obligate intracellular parasites *L. major, T. annulata, C. parvum*, and *P. falciparum*; amitochondriate (lacking mitochondria) parasites such as *E. cuniculi*, *E. histolytica*, and *G. lamblia*; and the extracellular protozoan *Trypanosoma brucei*. The Plantae were represented by *A. thaliana*, the red algae *C. merolae*, and the green algae *C. reinhardtii*. The remaining species were the diatom *T. pseudonana*, the free-living amoebae *D. discoideum*, and the ciliated protozoan, *T. thermophila*. Multiple sequence alignments were used to confirm that identified orthologs contained highly conserved residues or domains required for function and/or Hsp90 interaction (Supplemental figures). Table 1 shows the Genbank accession numbers of orthologs identified in the different species. We do not know whether many of these co-chaperones are expressed and/or functional. For those species lacking a clear ortholog, the letters na (not apparent) appears. Orthologs were only included in this alignment if the reverse blast of the designated protein returned the target co-chaperone. For example, a BLAST search of human Hop identified a potential ortholog, XP_955669 in *E. cuniculi*. However, the multiple sequence alignment showed that this protein contains only one TPR domain (Hop has three) and alignment of that sequence against the entire human genome revealed that XP_955669 has greater homology to a TPR-repeat containing protein other than Hop.

*Prevalence of individual co-chaperones* Somewhat surprisingly, none of the co-chaperones was present in all species (Table 1). In addition, there was a surprising amount of species-specific differences in individual co-chaperone domain structure, primarily with regard to the presence or absence of TPR domains (Fig. 1 and Supplemental Figures).

*Hop* Hop interacts with Hsp70 and Hsp90 in vertebrates and *S. cerevisiae* and associates with a wide range of Hsp90 client proteins. Hop may also modulate retinal proliferation and cell death (Pearl and Prodromou 2006). Hop was present in 18 out of 19 species and absent only from *E. cuniculi*. Consistent with prior studies that noted differences in the domain structure of Hop, three species examined appear to encode truncated forms of Hop that lack TPR1 and DP1, *C. elegans*, *T. pseudonana*, and *C. parvum*, while *D. melanogaster* lacks DP1 (Supplemental figures). These proteins likely maintain the ability to interact with Hsp70 through TRP2B (Carrigan et al. 2005; Flom et al. 2007).

*PP5* Mammalian PP5 and yeast Ppt1 interact with Hsp90 (Riggs et al. 2004; Wandinger et al. 2006). Orthologs have been characterized in *T. brucei* and tomato, and tomato PP5

interacts with a plant protein that confers resistance against a fungal pathogen (de la Fuente van Bentem et al. 2005; Jones et al. 2008). Two species analyzed, *E. cuniculi and T. pseudonana*, contained proteins with conserved phosphatase domains, but these lack TPR domains and thus are not presumed to interact with Hsp90.

*p23* The interaction of mammalian and *S. cerevisiae* p23 (Sba1) with Hsp90 has been demonstrated (Pearl and Prodromou 2006), and the p23 of *S. pombe* (Wos2) is important in the control of mitotic division (Munoz et al. 1999). p23 modulates the activity of telomerase in yeast and mammals (Holt et al. 1999; Toogun et al. 2007). Consistent with a critical role in regulating Hsp90 function, the mouse p23 is essential for perinatal survival (Grad et al. 2006). Only two species, *E. cuniculi* and *E. histolytica*, do not contain recognizable orthologs of p23.

*Aha1* Despite evidence that Aha1 is a powerful regulator of Hsp90 function, only a few in vivo functions of Aha1 have been identified. In mammalian cells, Aha1 downregulation specifically rescued misfolding of mutant CFTR (Wang et al. 2006). In addition, Aha1 of the silkworm *Bombyx mori* was proposed to play a role during spermatogenesis (Miyagawa et al. 2005). *S. cerevisiae* contains a homolog of Aha1, Hch1, which contains the part of Aha1 that binds Hsp90. Prior database searches revealed that orthologs of Aha1 are conserved from yeast to man, but Hch1 was restricted to lower eukaryotes like *S. cerevisiae* and *C. ablicans* (Pearl and Prodromou 2006). Two species, *T. annulata* and *T. pseudonana*, did not contain Aha1 orthologs. The genome of *E. histolytica,* encodes a protein (XP_653657.1) that has homology to the second half of Aha1 but was not included in this list since it appears to lack the Hsp90-binding domain.

*Sgt1* Sgt1 interacts with Hsp90 in yeast, mammals, and plants and may have similar functions in mammalian and plant innate immune responses (Bhattarai et al. 2007; Boter et al. 2007; Mayor et al. 2007). Sgt1 was highly conserved among these disparate eukaryotes, with only three species lacking clear orthologs. Multiple Sgt1 orthologs (*D. melanogaster*, *C. elegans*, *L. major*, *E. cuniculi*, *T. brucei*, *G. lamblia*, *P. falciparum*, and *E. histolytica*) lack the amino-terminal TPR domain (Supplemental figures). However, these proteins likely retain the ability to bind Hsp90 since the isolated p23 domain of yeast Sgt1 was able to bind Hsp90, and alteration of the MEEVD residues at the carboxy-terminus of Hsp90 did not disrupt the interaction between Sgt1 and Hsp90 (Catlett and Kaplan 2006).

*FKBP52* FKBP52 was identified in complexes with Hsp90 and vertebrate steroid receptors (Riggs et al. 2004). Male FKBP52-deficient mice exhibit partial androgen insensitivity, and females display characteristics of progesterone insensitivity (Cox et al. 2007). FKBP52 orthologs in *C. elegans* and *A. thaliana* have been described, but their in vivo functions are unknown (Aviezer-Hagai et al. 2007; Richardson et al. 2007). Three species, *T. annulata*, *T. pseudonana*, and *T. thermophila* contain proteins that may be classified as 'dual immunophilins', with distinct FKBP and cyclophilin domains separated by TPR repeats, a type of protein first recognized in *Toxoplasma gondii* (Barik 2006). The dual immunophilin in *T. thermophila* is part of a larger protein (EAR86074, 1134 amino acids) that also contains sequences homologous to a subunit of mitochondrial ATP synthase. Orthologs of FKBP52 were not identified in four species.

*Cns1* Little is known about the in vivo functions of yeast Cns1, although it is essential in yeast. The *Drosophila* ortholog Dpit47 is a nucleoplasmic protein that interacts with DNA polymerase alpha (Crevel et al. 2001). The human ortholog, TTC4, was recently shown to be a nucleoplasmic protein that interacts with Hsp70 and Hsp90 and the replication protein Cdc6 (Crevel et al. 2008). Point mutations in TTC4 may be associated with the progression of malignant melanoma (Poetsch et al. 2000). All but four species contained orthologs of Cns1.

*Cyp40* The most well-known interactions of Cyp40 are with steroid receptors, such as the glucocorticoid receptor and the androgen receptor (Riggs et al. 2004). As noted above for FKBP52, three species, *T. annulata*, *T. thermophila*, and *T. pseudonana* appear to express Cyp40 as a dual immunophilin (Barik 2006), but experimental evidence will be required to demonstrate that these proteins interact with Hsp90. Six species did not contain apparent orthologs of Cyp40.

*Pih1* In *S. cerevisiae*, Pih1 interacts physically and functionally with the DNA helicases Rvb1/Rvb2 and cooperates with Hsp90 in the accumulation of small nucleolar RNAs (Zhao et al. 2005; Zhao et al. 2008). Recent evidence suggests that Pih1 and Hsp90 are also required for ribonucleoprotein biogenesis in both *S. cerevisiae* and humans (Boulon et al. 2008). Nine species lacked clear Pih1 orthologs.

*Cdc37* Cdc37 is required for the folding and stabilization of multiple mammalian kinases and a significant proportion of protein kinases in *S. cerevisiae*. Two recent reviews describe the role of Cdc37 in a variety of signaling cascades as well as what is known about the interactions of Cdc37 and Hsp90 with oncogenic kinases (Caplan et al. 2007; Whitesell and Lindquist 2005). Given the critical role of Cdc37 in kinase maturation, it was surprising to find that ten species lack clear orthologs of Cdc37.

*The genomes of organisms with the smallest proteomes encode fewer co-chaperone proteins* From Table 1, it is clear that all species contain genes encoding at least three co-chaperones, and thus, the presence of co-chaperones for eukaryotic cytosolic Hsp90 appears universal. However, no single co-chaperone or group of co-chaperones was present in all species, indicating that there is not a core group of co-chaperones critical for Hsp90 function. The organisms examined vary both in the overall size of the genome and the number of distinct isoforms of cytosolic Hsp90 expressed (Table 2). The number of Hsp90 isoforms appears unrelated to the number of co-chaperones expressed. Since these organisms vary in the size of the proteome, or number of predicted protein coding genes, the number of co-chaperones was compared to the overall number of proteins expressed in each organism (Abrahamsen et al. 2004; Armbrust et al. 2004; Eisen et al. 2006; Merchant et al. 2007; Morrison et al. 2007; Pain et al. 2005). The two species that have the fewest examined co-chaperones, *E. cuniculi* and *T. annulata*, also have the smallest proteomes of the species examined. The genome of *E. cuniculi* contains 1,997 predicted proteins, including three co-chaperones, while *T. annulata* contains 3,792 predicted proteins, including six co-chaperones. This result suggests that there is a correlation between client diversity and the complexity of the Hsp90 co-chaperone machine.

Strikingly, the organisms with the fewest number of co-chaperones, *E. cuniculi* and *T. annulata*, contain different subsets of co-chaperones. *E. cuniculi* contains orthologs of Aha1, Cdc37, and Sgt1, while *T. annulata* contains orthologs of p23, Cdc37, Ppt1, Sti1, plus a dual immunophilin (Table 1). Both these species contain Cdc37, which, along with Sti1, is able to inhibit the ATPase activity of Hsp90. However, only *E. cuniculi* contains an ortholog of Aha1, which is able to stimulate the ATPase activity of Hsp90 (Pearl and Prodromou 2006).

*The parasite E. cuniculi lacks genes encoding TPR containing Hsp90 co-chaperones and contains alterations in the TPR binding site at the carboxy-terminus of Hsp90 E. cuniculi* lacked orthologs of Hop, PP5, Cns1, Cyp40, and FKBP52. *E. cuniculi* also encodes the shorter form of Sgt1, which lacks the TPR domain. Co-chaperones containing TPR domains competitively interact with the carboxy-terminal 12 kDa fragment of Hsp90, which ends in the highly conserved MEEVD sequence (Riggs et al. 2004; Young et al. 1998). A comparison of Hsp90 sequences revealed differences between the Hsp90 of *E. cuniculi* and other species examined, particularly in the carboxy-terminal 12 kDa (Fig. 2). A prior report noted that the MEEVD is not strictly conserved (Chen et al. 2006), and as shown in Fig. 2, Hsp90 from *D. discoideum*, *T. thermophila*, *T. pseudonana*, and *L. major* all contain a single amino acid alteration in this sequence. Hsp90 from *G. lamblia* and *E. cuniculi* each contain two amino acid alterations in the MEEVD. Notably, the terminal amino acid in *E. cuniculi* is glutamine rather than aspartic acid. In addition, Hsp90 from *E. cuniculi* contains an additional deletion of 20–30 amino acids within the carboxy-terminal domain and additional alterations in residues conserved throughout other species.

**Table 2** Correlation between proteome size and number of Hsp90 co-chaperone

| Organism | Description | Genome size (Mb) | No. of total genes | No. of cytosolic Hsp90s | Co-chaperones |
|---|---|---|---|---|---|
| *Encephalitozoon cuniculi* | Amitochondriate parasite | 2.5 | 1,997 (Abrahamsem) | 1 | 3 |
| *Theileria annulata* | Obligate intracellular parasite | 8.4 | 3,792 (Pain) | 1 | 6 |
| *Cryptosporidium parvum* | Obligate intracellular parasite | 9.1 | 3,807 (Abrahamsem) | 1 | 6 |
| *Schizosaccharomyces pombe* | yeast | 12.5 | 4,929 (Abrahamsem) | 2 | 8 |
| *Plasmodium falciparum* | Obligate intracellular parasite | 22.9 | 5268 (Morrison) | 1 | 8 |
| *Cyanidioschyzon merolae* | Red algae | 16.5 | 5331 (Merchant) | 1 | 6 |
| *Giardia lamblia* | Amitochondria parasite | 11.7 | 6470 (Morrison) | 1 | 6 |
| *Saccharomyces cerevisiae* | Yeast | 13 | 6,561 (Eisen) | 2 | 9 |
| *Leishmania major* | Obligate intracellular parasite | 32.8 | 8,272 (Morrison) | 1 | 9 |
| *Trypanosoma brucei* | Protozoan extracellular parasite | 26.1 | 9,068 (Morrison) | 1 | 9 |
| *Entamoeba histolytica* | Amitochondria parasite | 24 | 9,938 (Morrison) | 3 | 5 |
| *Thalassiosira pseudonana* | Diatom | 34.5 | 11,242 (Armbrust) | 1 | 7 |
| *Dictyostelium discoideum* | Slime mold, free living amoeba | 34 | 12,500 (Eisen) | 1 | 9 |
| *Dictyostelium melanogaster* | Fruit fly | 180 | 13,679 (Eisen) | 1 | 10 |
| *Chlamydomonas reinhardtii* | Green algae | 121 | 15,143 (Merchant) | 1 | 8 |
| *Caenorhabditis elegans* | Nematode | 103 | 19,971 (Eisen) | 1 | 9 |
| *Arabidopsis thaliana* | Plant, mouse ear cress | 120 | 26,207 (Eisen) | 4 | 8 |
| *Tetrahymena thermophila* | Unicellular ciliate | 104 | 27,424(Eisen) | 1 | 9 |
| *Home sapiens* | Humans | 2,851 | 35,845 (Eisen) | 2 | 10 |

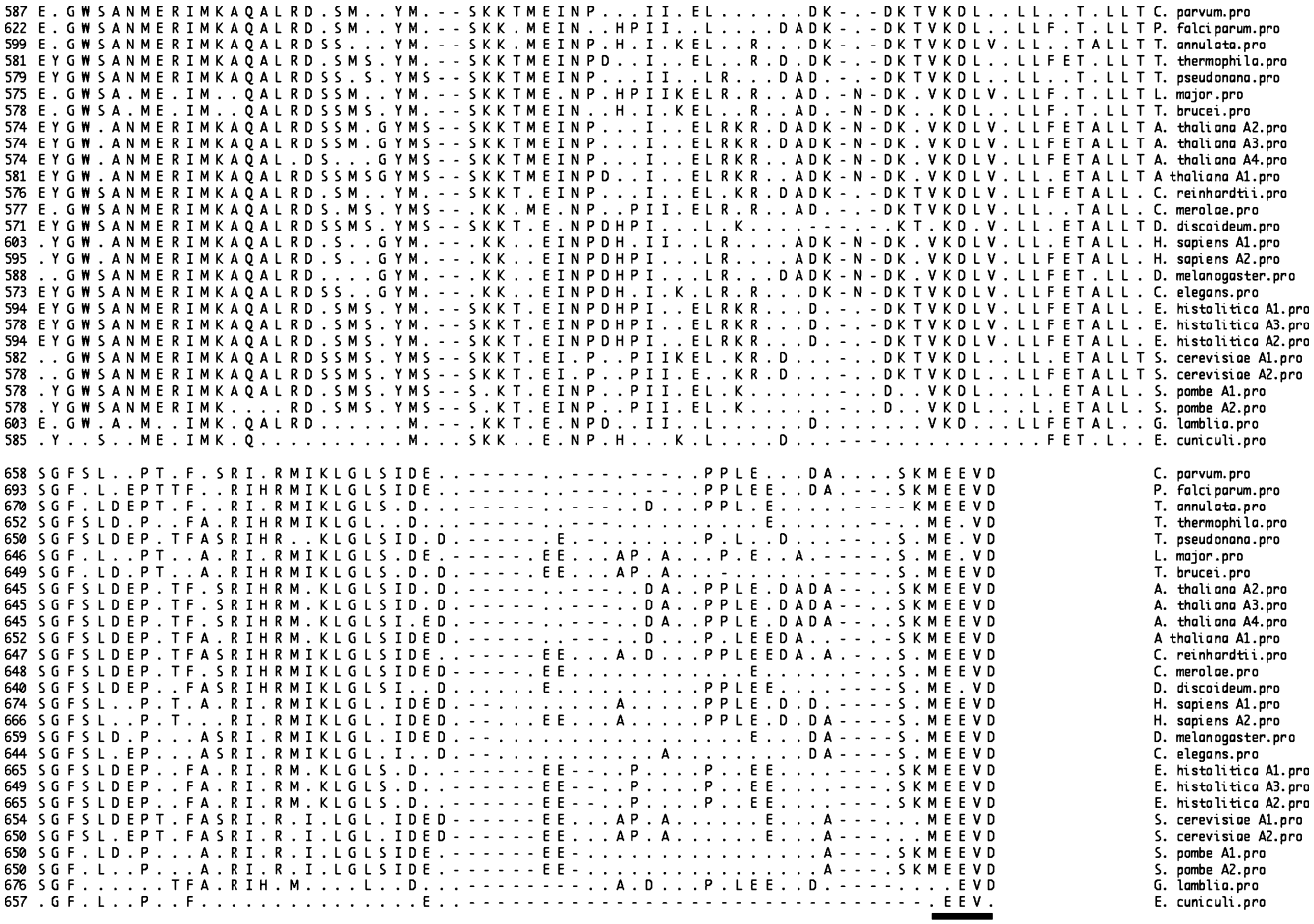

**Fig. 2** The carboxy-terminus of Hsp90 interacts with TPR-containing co-chaperones such as Hop, Cyp40, FKBP52, PP5 and Cns1. The carboxy-terminus of Hsp90 from *E. cuniculi*, which does not contain orthologs of any of those co-chaperones contains alterations in conserved residues, including the terminal MEEVD sequence (underlined). The Genbank accession numbers of Hsp90 orthologs used in this alignment are listed in the "Materials and methods" section. A dash (-) represents a gap in the sequence whereas a period (.) represents an amino acid that differs from the consensus

Further tests are needed to demonstrate that Hsp90 from *E. cuniculi* exhibits reduced binding to proteins with TPR domains. However, it is tempting to speculate that sequence differences in Hsp90 from *E. cuniculi* resulted in reduced functionality of TPR-containing co-chaperones, resulting in loss of genes encoding those proteins during genome compaction (Katinka et al. 2001).

We examined Hsp90 sequences from the different organisms to determine whether additional co-chaperone binding sites are less conserved in species that lack genes encoding those co-chaperones. The Aha1 binding site within Hsp90 appears to be conserved even in those species that lack Aha1, but this is not surprising since Aha1 binds a loop in Hsp90 that is critical for ATPase activity (Meyer et al. 2004). Cdc37 interacts with a 'lid' segment of the amino-terminus of Hsp90 that closes over bound nucleotide (Roe et al. 2004). In most of the species that lack orthologs of Cdc37, hydrophobic residues involved in Hsp90–Cdc37 interaction are well conserved. An exception is that in *E. histolytica*, which also lacks Cdc37, three of those hydrophobic residues encode polar or charged residues (not shown). Finally, two of the contact sites between p23/Sba1 and the amino-termini of Hsp90 are residues 12–21 and 151–155 of yeast Hsp90 (Ali et al. 2006). The corresponding sequence of Hsp90 from *E. cuniculi*, which lacks a p23 ortholog, exhibits multiple amino acid changes within both of these regions (not shown). Together, these results suggest that in most cases, co-chaperone binding sites are conserved even when genes expressing the corresponding co-chaperone are not apparent.

## Discussion

Although Hsp90 is frequently described as a 'molecular chaperone machine', there is no evidence that Hsp90 exists as one large complex containing Hsp90 and all co-chaperones.

Instead, many co-chaperones exhibit mutually exclusive binding (Harst et al. 2005; Pearl and Prodromou 2006; Pratt et al. 2004). Differences in the types of co-chaperones present in divergent organisms provide further support for the hypothesis that the Hsp90 molecular chaperone machine is actually a spectrum of varied Hsp90 complexes.

One of the more surprising aspects of this study was that that no single co-chaperone or group of co-chaperones was present in all species. The function of some co-chaperones may be restricted to specific subsets of client proteins, be required for client protein activation in a species-dependent manner, or be redundant with other co-chaperones. However, it was unexpected that co-chaperones that directly regulate the essential ATPase activity of Hsp90 are not absolutely conserved. All species examined contain orthologs of either Hop or Cdc37, the two co-chaperones that have been shown to inhibit the ATPase activity of Hsp90. However, three species lack orthologs of Aha1, which is the only co-chaperone known to activate ATPase activity. Differences in the ATPase rates of yeast and mammalian Hsp90 have been observed (Wegele et al. 2004), and it will be interesting to determine whether Hsp90 from species that lack Aha1 have higher intrinsic ATPase activity, which might reduce the requirement for Aha1 in vivo.

*Cdc37 is restricted to certain organisms* Another surprising finding was that Cdc37 was the co-chaperone absent from the most species. Cdc37 has a critical importance in kinase stability and activation in *Drosophila* and mammals. A recent study demonstrated that alteration of Cdc37, which is essential in *S. cerevisiae*, disrupted the stability of 51 out of 65 kinases examined (Mandal et al. 2007). Protein kinases represent approximately 2% of the eukaryotic genome, and the catalytic domain of protein kinases is highly conserved (Caplan et al. 2007). However, Cdc37 orthologs were identified in only nine out of nineteen species examined (Fig. 3). One likely possibility is that Hop and Cdc37 have overlapping functions in stabilizing kinases (Lee et al. 2004). Another possibility is that some organisms encode families of kinases that have reduced dependency on Cdc37 and/or Hsp90. In support of this hypothesis, the ratios of different types of kinases encoded by *Giardia*, which lacks Cdc37, appear different than in yeast or mammalian cells (Morrison et al. 2007).

*Co-evolution of the Hsp90 molecular chaperone machine* From this analysis, it is evident that co-chaperones are present in all examined branches of eukaryotes. This suggests that the entire set of co-chaperones may have been present in an ancestral organism or there has been lateral gene transfer

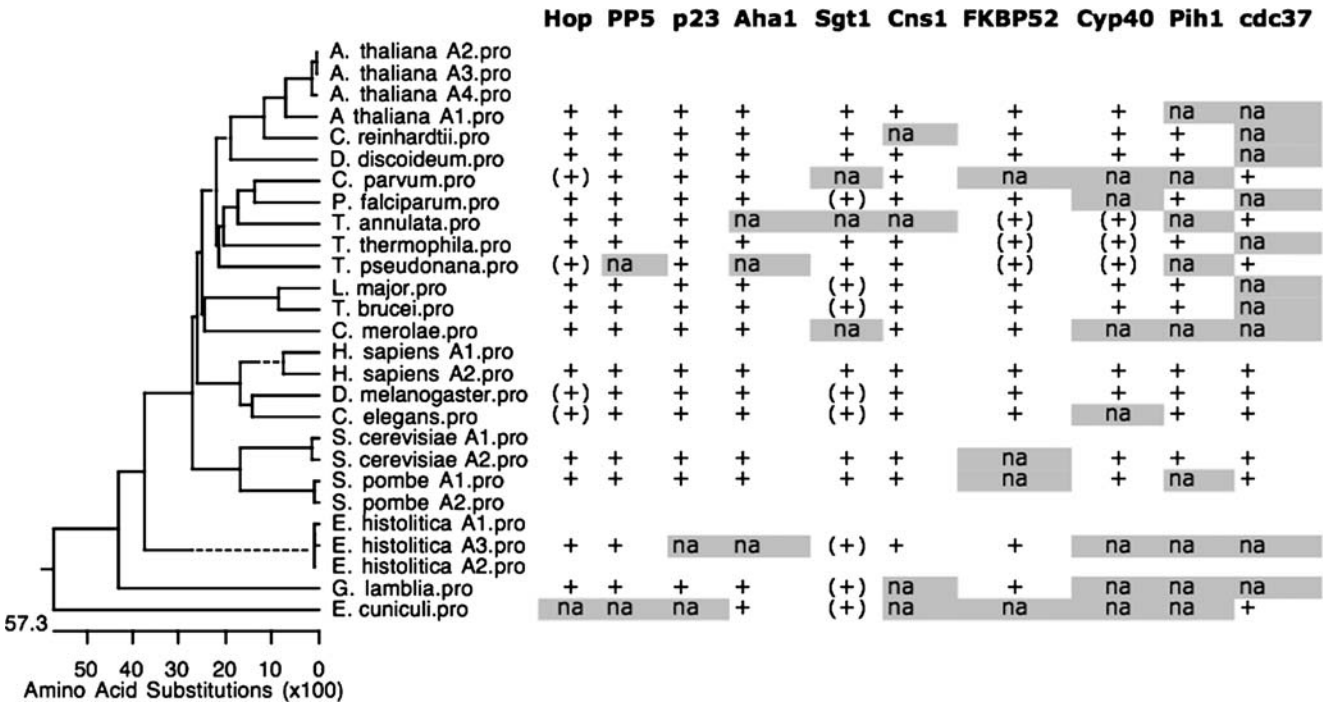


**Fig. 3** Conservation of Hsp90 and co-chaperones across divergent species. A phylogenetic tree of Hsp90 sequences inferred using UPGMA (Megalign, Lasergene) is shown. The presence or absence of co-chaperone orthologs is noted as + (identified) or na (not apparent). Slight differences in the trees were obtained with different methods (see Materials and Methods): *D. discoideum* clusters with the multicellular eukaryotes in the parsimony analysis, whereas *C. merolae* clusters with the plants and *T. pseudonana* clusters with *T. brucei* and *L. major* in the neighbor joining analysis. Observed differences in domain structure are noted as follows: Hop (+) denotes the apparent lack of TPR1 and/or DP1 domains; Sgt1 (+) denotes the apparent lack of the TPR domain; and FKBP52 (+), and Cyp40 (+) denotes the apparent combination of these co-chaperones in one 'dual immunophilin'

between organisms. The organisms with the smallest proteomes may have lost nonessential co-chaperones that were retained in organisms with more complex folding requirements. Further duplication and divergence of specific co-chaperones, such as FKBP52 and Cyp40 or other TPR-containing co-chaperones, likely enhanced the ability of Hsp90 to function in the stability, regulation, and activation of an even larger range of client proteins.

The co-chaperones are widely dispersed throughout the eukaryotic tree of life (Wickstead and Gull 2006), and each co-chaperone displays unique patterns of occurrence (Fig. 3). Most co-chaperones are present in all Metazoa, with the exception that *C. elegans* lacks Cyp40. The consistent pattern among Plantae is that they all contain Hop, PP5, p23, Aha1, and FKBP52 yet lack an obvious Cdc37 homolog. In general, parasites contain fewer co-chaperones than non-parasites. The obligate intracellular parasites contain Hop, PP5, p23, and Aha1 but additional co-chaperones are present in a species-specific manner. As a group, the only consistent pattern among the amitochondriates was that they lacked Cyp40 and Pih1 and contained the shortened version of Sgt1. The lack of a greater correlation between the presence or absence of specific co-chaperones and the phylogenetic tree suggests that within divergent organisms, loss of any individual co-chaperone was tolerated as long as overall Hsp90 function was maintained.

The essential cycle of Hsp90 interaction with co-chaperones results in functional and structural dependencies, and the co-evolution of an Hsp70-Hop-Hsp90 system has been described (Travers and Fares 2007). In light of the level of cooperation between Hsp90 and co-chaperones required for folding a diverse array of client proteins, it was surprising to observe differences in the composition of individual co-chaperones, particularly with regard to TPR domains. These differences suggest that the specific functions of co-chaperones may vary between species, adding another source of flexibility to the chaperone machine.

Another intriguing aspect of co-chaperone function is that cytosolic Hsp90 appears to be the only form of Hsp90 that requires co-chaperones for function. As yet, co-chaperones of other forms of Hsp90, including HtpG of *E. coli*, Grp94 of the endoplasmic reticulum, and TRAP1 of mitochondria, have not been identified (Richter and Buchner 2006). *Giardia* is considered to be among the earliest of existing eukaryotes, and many multiprotein complexes in *Giardia* contain fewer and more basic subunits relative to other eukaryotes. For example, *Giardia* contains orthologs of only four of the 12 transcription initiation factors and five of the 25 proteins required for polyadenylation in *S. cerevisiae* (Morrison et al. 2007). However, *Giardia* contains orthologs of six of nine co-chaperones relative to *S. cerevisiae*. The finding that divergent eukaryotic lineages contain multiple co-chaperones solidifies the importance of co-chaperones in regulating Hsp90 function. Further studies will be required to establish whether the co-chaperone requirement reflects differences in the mechanics of Hsp90 function or differences in the types of proteins that require Hsp90 for function.

*How do alterations in the Hsp90 chaperone machine affect the range of interacting clients?* Hsp90 and co-chaperones regulate key clients in a variety of biological processes, including reproduction, the immune response, cell growth, and differentiation. More recent studies point to a critical role for plant Hsp90 and co-chaperones in resistance to pest and disease. Environmental conditions and the growth state of cells may result in changes in Hsp90 functions and co-chaperone interactions. A recent study demonstrated that, under normal conditions, Hsp90 is important for the function of the secretory pathway and cellular transport, but under environmental stress, Hsp90 is required for the cell cycle, meiosis, and cytokinesis (McClellan et al. 2007). Changes in the growth state of the cell may also specifically alter Hsp90-co-chaperone interaction. For instance, compared with normal tissues, a greater proportion of Hsp90 from tumor cells is complexed with co-chaperone proteins (Kamal et al. 2003), and some cytosolic Hsp90 appears to localize to the mitochondria in tumor cells, cooperating with endogenous TRAP1 to regulate mitochondrial integrity (Kang et al. 2007).

It is remarkable that Hsp90 is able to mediate client activation in divergent species using different combinations of co-chaperone proteins. Is the absence of co-chaperones from particular species tolerated because particular client proteins are absent or not critical for survival? As more is learned about the specificity of co-chaperone interactions, it may be possible to distinguish whether specific clients must have access to the same co-chaperones in disparate species or whether a client that requires one set of co-chaperones in mammalian cells is dependent on a different set of co-chaperones in another species. It is also possible that clients become dependent on Hsp90 in a species-specific manner and that orthologs of mammalian clients do not require Hsp90 for stability or activation. In either case, it appears that the Hsp90 molecular chaperone machine is highly adaptive, able to function with a changing set of partner co-chaperone proteins as it mediates the folding requirements of divergent eukaryotic species.

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