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Navigating the Chaperone Network: An Integrative Map of Physical and Genetic Interactions Mediated by the Hsp90 Chaperone

Resource

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Summary

Physical, genetic, and chemical-genetic interactions centered on the conserved chaperone Hsp90 were mapped at high resolution in yeast using systematic proteomic and genomic methods. Physical interactions were identified using genome-wide two hybrid screens combined with large-scale affinity purification of Hsp90-containing protein complexes. Genetic interactions were uncovered using synthetic genetic array technology and by a microarray-based chemical-genetic screen of a set of about 4700 viable yeast gene deletion mutants for hypersensitivity to the Hsp90 inhibitor geldanamycin. An extended network, consisting of 198 putative physical interactions and 451 putative genetic and chemical-genetic interactions, was found to connect Hsp90 to cofactors and substrates involved in a wide range of cellular functions. Two novel Hsp90 cofactors, Tah1 (YCR060W) and Pih1 (YHR034C), were also identified. These cofactors interact physically and functionally with the conserved AAA$^+$-type DNA helicases Rvb1/Rvb2, which are key components of several chromatin remodeling factors, thereby linking Hsp90 to epigenetic gene regulation.

Introduction

Hsp90 is a ubiquitous molecular chaperone found in eubacteria and all branches of eukarya. It plays a central role in cellular signaling since it is essential for maintaining the activity of key signaling factors, including steroid hormone receptors and protein kinases. In eukaryotes, Hsp90 is essential for cell viability (Borkovich et al., 1989). In the budding yeast *Saccharomyces cerevisiae*, there are two virtually identical isoforms of cytoplasmic Hsp90; one, Hsp82, is heat shock induced, while the other, Hsc82, is constitutively expressed. The global role of Hsp90 in normal protein homeostasis has been revealed by studies showing that Hsp90 can buffer phenotypic variations in different organisms, either directly by masking the phenotypic effects of mutant polypeptides as a result of facilitating their folding into an active state or indirectly by regulating the activity of signal transduction pathways (Rutherford, 2003).

A large number of chaperones facilitate protein folding in the cell (Houry, 2001; Young et al., 2003). Hsp90 is, however, distinct from other chaperone systems in two main aspects. First, Hsp90 does not act to fold nonnative proteins but rather binds to substrate proteins at a late stage of folding and thus at a near-native state (Pearl and Prodromou, 2002). Second, Hsp90 seems to be a specialized chaperone, targeting specific client proteins involved mainly in signal transduction. Hsp90 functions as an ATP-dependent dimeric molecular chaperone, which typically forms the core of large macromolecular complexes that include other cochaperones, cofactors, and substrates. The complete set of Hsp90-interacting proteins is unknown.

Sequence conservation and proteolysis studies of Hsp90 have indicated the presence of at least three structural domains. The N-terminal domain (residues 1–220 in yeast Hsp82) is the site of ATP binding and hydrolysis. This domain is essential for the ATP-dependent function of the chaperone in vivo and in vitro. Its structure has been solved for the yeast (Prodromou et al., 1997) and human (Stebbins et al., 1997) isoforms and contains a unique ATP binding site, termed the Bergerat fold, found in a growing superfamily of proteins (Dutta and Inouye, 2000). Inhibitory drugs, such as geldanamycin, specifically target Hsp90 by binding to this unique ATP binding site on the chaperone (Pearl and Prodromou, 2000). The N-terminal domain is connected through a small, highly charged linker region to a middle domain (residues 255–599 in yeast Hsp82). This domain is a site of cofactor binding as well as a possible site for substrate and ATP binding (Garnier et al., 2002), although the latter has not been firmly established. Finally, the C-terminal domain (residues 600–709 in yeast Hsp82) provides a strong dimerization interface, which is essential for Hsp90 activity (Prodromou et al., 2000).

The in vivo and in vitro activity of Hsp90 depends on its association with a variety of cochaperones and cofactors, which form large Hsp90-containing multiprotein complexes involved in folding client proteins. Cochaperones include Hsp70 and Hsp40. The formation of a complex between Hsp70 and Hsp90 is mediated through the association of both chaperones to an adaptor protein termed Hop/Sti1 (Hernandez et al., 2002). Hop is composed of multiple 34 amino acid helix-turn-helix tetratricopeptide repeat (TPR) motifs and does not function as a chaperone on its own (Frydman and Hohfeld, 1997). Hop has eight to nine TPR motifs, forming three TPR domains. It is proposed that the N-terminal domain of Hop binds to the C-terminal EEVD motif of Hsp70, while the two C-terminal TPR domains bind the C-terminal EEVD motif of Hsp90 (Scheufler et al., 2000; Brinker et al., 2002). The Hsp90-Hop-Hsp70

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complex is considered to be the minimal Hsp90 core complex (Prodromou et al., 1999). Other yeast Hsp90 cofactors include p23/Sba1, which stabilizes the ATP bound state of Hsp90 (Young and Hartl, 2000), Aha1, which enhance the Hsp90 ATPase activity (Panaretou et al., 2002; Lotz et al., 2003), p50/Cdc37, which can link Hsp90 to client protein kinases (Grammatikakis et al., 1999), as well as the cyclophilins Cpr6 and Cpr7 and the immunophilin Cns1 (Panaretou et al., 2002). The cyclophilins and immunophilins contain TPR domains.

In order to understand how Hsp90 imposes its expansive protective effect on cells, we undertook an integrated, large-scale proteomic and genomic study in *S. cerevisiae* aimed at identifying the global set of Hsp90-interacting protein partners and substrates. Yeast is an ideal model system to investigate Hsp90 function due to the availability of high-throughput functional genomics methods (Zhu et al., 2003). Furthermore, many fundamental processes are conserved from yeast to human (Guarente and Bermingham-McDonogh, 1992). Our efforts were based on a combination of four complementary experimental strategies. First, we carried out systematic genome-wide screens for protein-protein interactions mediated by Hsp90 using a two hybrid technique based on ordered strain arrays (Uetz et al., 2000). Second, we used mass spectrometry to identify proteins that copurify with Hsp90 in tandem affinity purification procedures. Third, we screened for synthetic lethal interactions between a specific mutant allele of Hsp90 and members of a panel of ~4700 single yeast gene deletion strains using the synthetic genetic array (SGA) technology (Tong et al., 2001). Fourth, we screened the deletion mutant strains for differential hypersensitivity to the Hsp90 inhibitor geldanamycin in liquid culture using a microarray-based readout (Giaever et al., 2002). Collectively, these efforts identified 627 putative Hsp90 substrates and cofactors, representing about 10% of the yeast proteome, many of which suggest possible links of Hsp90 to previously unrecognized roles in transcriptional regulation, cell cycle and DNA processing, and cellular transport, among others. Lastly, we have studied the protein products of two previously uncharacterized open reading frames (ORFs), YCR060W and YHR034C, which interact physically and functionally with Hsp90 and with each other and demonstrated that they are novel cofactors which interact with two essential helicases linked to chromatin remodeling.

Results

Mapping the Physical Interaction Network of Hsp90

Protein interaction assays can provide insight into functionally relevant targets of the Hsp90 chaperone system because physical association of proteins is a strong indicator of related functions (Marcotte et al., 1999). In order to comprehensively identify polypeptides that physically interact with Hsp90 in yeast, we performed systematic genome-wide two hybrid (2H) screens as well as large-scale proteomic analysis involving tandem affinity purification (TAP) of Hsp90-containing native protein complexes.

For the 2H screens, yeast strains expressing either full-length Hsp82 (residues 1–709) or the N-terminal (residues 1–220), middle (M; residues 271–599), C-terminal (C; residues 599–709), or M+C (residues 271–709) domains fused to the C terminus of *GAL4* DNA binding domain were mated with an ordered array of 6084 yeast colonies expressing activation domain fusions to single ORFs and screened for interacting partners as described (Uetz et al., 2000). A strain bearing a *GAL4* empty vector was screened as a negative control. To reduce the rate of false discovery, each of the screens was repeated six times. Ninety ORFs which specifically and reproducibly interacted with either full-length Hsp90 or with a subset of the Hsp90 domain fusions were identified (see Table S1 in the Supplemental Data available with this article online). Based on colony size and reproducibility, the most strongly interacting ORFs bound to the middle and C-terminal regions of Hsp90. Several of these proteins represented established Hsp90 cofactors (e.g., Cns1, Cpr6, Cpr7, and Ppt1) known to bind to the C terminus of Hsp90 through TPR domains, confirming the general effectiveness of our screening procedure. The reliability of these results was further established by several additional criteria. First, only 1.5% of the 2H preys were detected as Hsp90-interacting partners. Second, the screen was carried out using high concentrations of 3-amino-1,2,4-triazole, a competitive inhibitor of the *HIS3* reporter gene (see Experimental Procedures). Third, the results were fully reconfirmed for a subset (~15%) of the fusion protein plasmids by isolating and retransforming them into a virgin reporter strain, indicating an absence of spurious background.

For the proteomic (TAP) method, we created (Krogan et al., 2003) or obtained (Ghaemmaghami et al., 2003) a total of ~4,000 yeast strains each expressing a single ORF fused to a C-terminal TAP tag at the endogenous genomic locus, including both Hsp82 and Hsc82. This aspect of the study is a component of a larger ongoing collaborative effort aimed at characterizing the entire yeast interactome (N.K., G.C., M.D., E. O'Shea, J. Weissman, J.G., and A.E., unpublished data). To avoid disrupting protein partners that might interact with the important C-terminal EEVD motif of Hsp90, which binds to TPR domains (Scheufler et al., 2000), we also constructed a strain in which the *HSP82* locus was deleted and *HSC82* was expressed as an N-terminal TAP-tagged fusion under the control of its endogenous promoter (strain R0009, see Experimental Procedures). Native protein complexes were then purified 10^6-fold to virtual homogeneity from log phase cultures of each of these strains by sequential affinity chromatography on immunoglobulin and calmodulin beads. Purified proteins were identified either from SDS-PAGE gels by MALDI-ToF or from solution by LC-MS after digestion with trypsin (see Experimental Procedures). The latter approach allows improved detection coverage of lower abundance interacting proteins.

Although interactions of Hsp90 with its target substrates are predicted to be highly dynamic in vivo, we identified Hsp90 as a putative binding partner of 118 distinct proteins (Table S2). Eighty-three of these interacting proteins were identified when Hsp90 was TAP tagged, while the rest copurified with detectable levels

of Hsp90 as TAP-tagged protein baits. The cochaperones Ssa1 and Sti1 copurified with near-apparent stoichiometry with N-terminally TAP-tagged Hsc82, whereas most of the other interacting partners were present at low, substoichiometric levels (data not shown), suggesting that Hsp90-Sti1-Ssa1 forms the core Hsp90 complex (Chang and Lindquist, 1994). The specificity of these interactions is suggested by the fact that Hsp90 was detected with only a small fraction (<1%) of all the TAP-tagged baits analyzed to date. Moreover, several of the identified interacting partner proteins are well-known Hsp90 interactors (e.g., Aha1, Cdc37, Ssa1, and Sti1).

Mapping the Genetic Interaction Network of Hsp90

In an effort to elucidate essential functional targets of Hsp90, we used SGA technology (Tong et al., 2001) to systematically screen for genetic interactions, synthetic lethal, and synthetic slow growth, among a large array of engineered yeast double mutants bearing a defective Hsp90 allele. A yeast haploid *MAT*α query strain, with a marker-substituted *hsc82* deletion and a conditional, temperature-sensitive hypomorphic G170D allele of *hsp82*^{ts} (strain R0013, see Experimental Procedures) was crossed to a panel of ~4700 haploid strains of the opposite mating type, each of which had a single deletion of a nonessential ORF (Giaever et al., 2002). The diploids were sporulated and plated on media to select for triple mutant haploid meiotic progeny. Growth rates at a semipermissive temperature of 35°C were monitored by visual inspection and by computerized image analysis of colony sizes (Tong et al., 2004). A total of 300 nonviable (synthetic lethal) or slow-growing (synthetic sick) colonies, which should be indicative of a functional relationship between Hsp90 and the respective ORF, were scored (Table S3). These putative genetic interaction partners were highly specific as most (>90%) were not detected in previous full-genome SGA screens with more than 100 other unrelated query genes (Tong et al., 2004). Furthermore, tetrad analysis of a randomly selected subset of 30 putative interactors confirmed 23 as synthetic lethal with impaired Hsp90 alleles (data not shown), indicating a modest rate of false positives (i.e., <25% of the dataset).

In parallel, we used a novel microarray-based chemical-genetic screening methodology (Parsons et al., 2004) to survey a pool of bar coded yeast single gene deletion strains (Giaever et al., 2002) for mutants that are hypersensitive to geldanamycin, a potent and specific inhibitor of Hsp90 that binds the N-terminal domain of the chaperone (Whitesell et al., 1994). A liquid culture of pooled haploid bar coded deletion mutants was treated with geldanamycin or mock treated. The viability levels of the deletion mutants were measured by PCR amplification of the unique bar codes and subsequent hybridization onto high-density oligonucleotide yeast arrays. The geldanamycin screen (GS) was repeated four times, leading to the identification of a total of 200 ORFs that reproducibly depend on Hsp90 for efficient cell growth (Table S4).

Overview of the Data

The combined 2H, TAP, SGA, and GS screens resulted in the identification of a total of 627 candidate ORFs interacting with Hsp90 (Table S5). Collectively, these data provide a holistic framework for evaluating the relationships among the specific biochemical pathways linked to Hsp90. It should be noted that neither the SGA screen nor the GS screen provide hits to essential genes, since these are not present in the mutant strain panels. Figure 1A shows a Venn diagram demonstrating the overlap of ORFs obtained using the four different methods. Since ORFs identified using the 2H method are more likely to represent direct interactors with Hsp90, whereas ORFs identified using the TAP method are part of larger macromolecular complexes containing Hsp90, one might not expect much overlap between these two datasets. Indeed, only 10 proteins (Ade1, Aha1, Bet3, Bni4, Cns1, Cpr6, Cpr7, Pat1, Ppt1, and YDR533Cp) were detected in common. On the other hand, the genetic and chemical-genetic interaction screens (SGA and GS) shared a significantly larger overlap of 49 common ORFs (Figure 1A and Figure 5). These two screens are based on the same principle of synthetic lethality, and a high overlap is expected. We consider this set of 49 genes to be a high-fidelity dataset (discussed further below).

Twenty-two of the proteins that interact physically with Hsp90 (in 2H or TAP) also interacted genetically with the chaperone (SGA or GS; Figure 1B). Two plausible scenarios implied by this unique property are as follows: (1) since Hsp90 itself is essential in yeast, these proteins may be cofactors of the Hsp90 system which become essential for chaperone function when the activity of the core Hsp90 subunit is reduced, or (2) these proteins are direct substrates of Hsp90, the loss of which results in synthetic lethality when combined with deletions of other proteins that likewise depend on Hsp90 for maturation. Cpr6, Cpr7, and Sti1 are established Hsp90 cofactors and possibly represent proteins satisfying the first scenario, while the other proteins listed in Figure 1B might satisfy the second scenario and, hence, represent putative substrates of Hsp90.

The interaction datasets were subsorted into functional categories based on annotation in the MIPS database (Mewes et al., 2002) and protein subcellular localization (Huh et al., 2003). Statistical enrichment (p value < 0.01) within these categories was assessed using a hypergeometric distribution function (Robinson et al., 2002). While the ORFs detected in each of the four screening methods were distributed across all major functional categories, the Hsp90 interactors were specifically enriched in proteins involved in cellular fate/organization, cellular transport, metabolism, protein fate, and transcription (Figure 1C). In terms of subcellular localization (Figure 1D), Hsp90 physical interactors (TAP) were enriched for proteins localized in the cytoplasm and bud neck, while the Hsp90 genetic interactors (SGA and GS) were enriched for proteins localized in the vacuole, vacuolar membranes, and punctate composites (mainly Golgi). In contrast, there were no significant differences in molecular weight, pI, hydrophobicity (GRAVY plot), codon adaptation index, domain motif composition, predicted secondary structure, or tertiary structure (CATH classification) between the four different datasets and the total yeast proteome, consistent with the global role that Hsp90 plays in the cell.

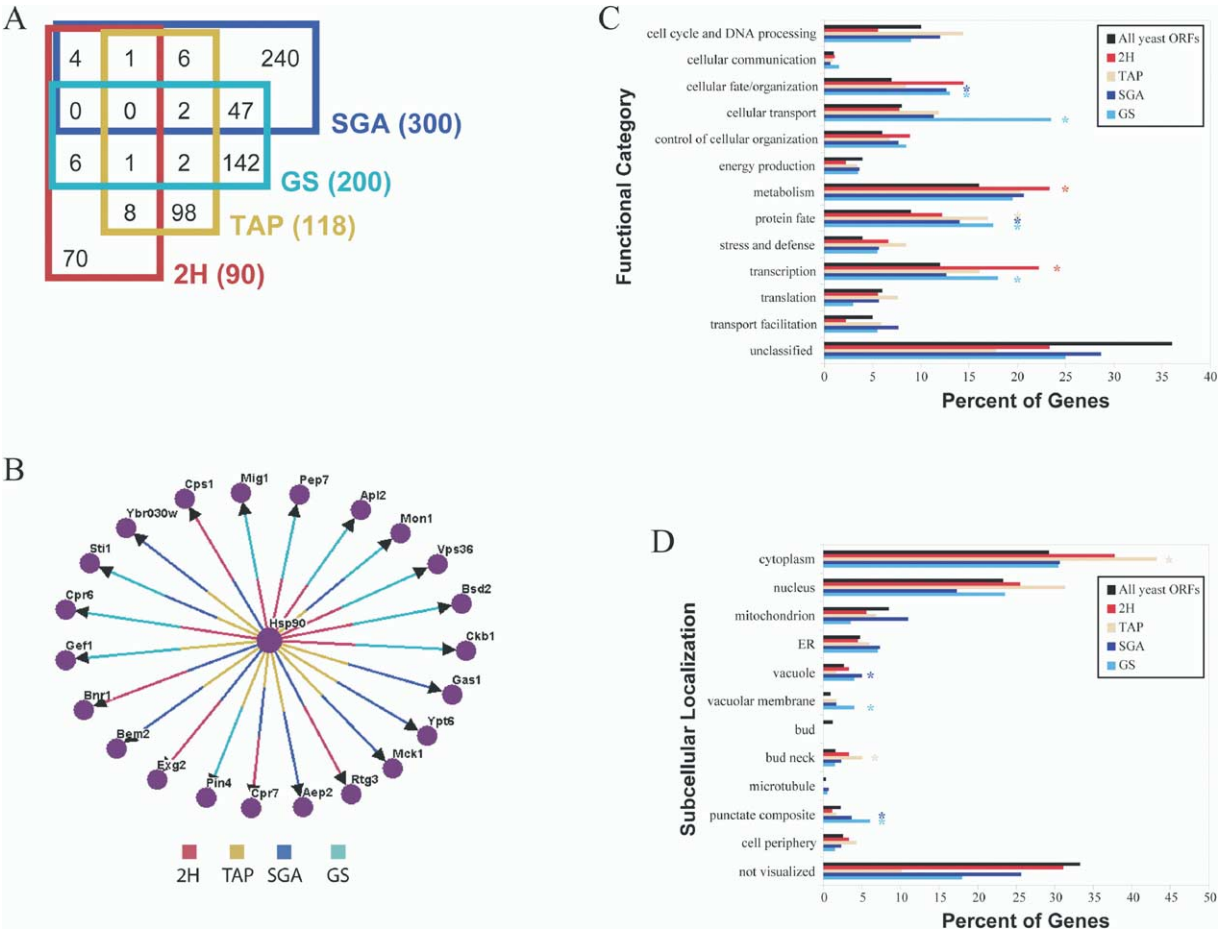


Figure 1. Overview of the Hsp90 Interaction Data

(A) Venn diagram showing the overlap among proteins found to physically or genetically interact with Hsp90 using 2H, TAP, SGA, and GS screens.

(B) A network overview using Osprey showing the 22 proteins that physically (2H or TAP) *and* genetically (SGA or GS) interacted with Hsp90. Arrows connecting the proteins to Hsp90 are color coded according to the screening method(s) used.

(C) Functional distribution of Hsp90 interactors using MIPS functional categories compared to that of total yeast proteins.

(D) Localization of Hsp90 interactors compared to total yeast proteins using the experimental data of Huh et al. (2003).

In (C) and (D), stars refer to statistical enrichment of certain types of proteins in a given screening method as compared to total yeast proteins as assessed by FunSpec (Robinson et al., 2002).

Linking Hsp90 to Specific Cellular Complexes and Processes

A clearer effect of Hsp90 on specific cellular pathways was apparent once the datasets were manually combined into well-established protein complexes, functional modules, and pathways. The KEGG and AmiGO databases were initially used to functionally classify the different proteins, and then the information was verified using the published literature. A genetic or physical interaction between Hsp90 and more than one component in a given complex provides a reliable indication of a role for Hsp90 in the folding/maturation/function of that complex. Examples are given for protein complexes (Table 1A), metabolic pathways (Table 1B), and certain protein classes (Table 1C) that have been found to be genetically or physically linked to Hsp90.

Although the screens have been designed to be as comprehensive and as reliable as feasible, there are several cofactors and substrates that have not been detected. A notable missing cofactor is Sba1; however, it is known that the binding of this cofactor to Hsp90 depends on the presence of nucleotides which, for example, were not added in our TAP tag pull-downs (Prodromou and Pearl, 2003). Notable missing substrates include Ste11 (Louvion et al., 1998), Hap1 (Lan et al., 2004), Ctf13/Skp1 (Stemmann et al., 2002), and regulatory particle non-ATPase subunits, Rpn components, of the proteasome (Imai et al., 2003). Varied reasons can be given to explain the absence of these hits from our screens; nevertheless, they provide an example of false negative hits and indicate that there are still more putative Hsp90 substrates and cofactors to be identified by varying the buffer conditions of the screens.

Two Novel Cofactors of Hsp90

Interestingly, 133 uncharacterized ORFs were identified as Hsp90 interactors. As part of this study, we have functionally and biochemically characterized two of

Table 1. Effect of Hsp90 on Specific Complexes and Processes

A. Example of Protein Complexes that Interact with Hsp90

SAGA Complex	Swi/Snf Complex	Ap-3 Adaptor Complex	Retromer Complex	Retrograde Complex	Vacuolar ATPase Complex
Ada2 (GS)	Swi1 (2H)	Apl5 (SGA, GS)	Vps35 (GS)	Cog5 (GS)	Vma10 (SGA)
Gcn5 (GS)	Snf2 (SGA)	Apl6 (GS)	Vps29 (GS)	Cog6 (SGA, GS)	Vma13 (SGA)
Spt3 (SGA, GS)	Snf11 (GS)	Apm3 (GS)	Pep8 (GS)	Cog7 (GS)	Vph1 (SGA, GS)
		Aps3 (GS)		Cog8 (SGA, GS)	Ppa1 (SGA)
Spt4 (SGA, GS)					
Spt15 (TAP)					Rav1 (GS)
					Rav2 (GS)

B. Example of Specific Metabolic Pathways that Interact with Hsp90

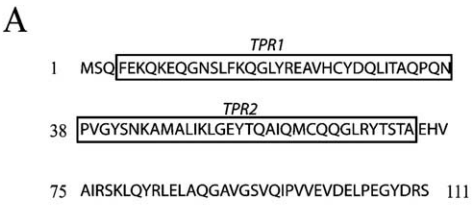
Sphingolipid Biosynthesis	Ergosterol Biosynthesis	Inorganic Phosphate Transport
Sur1 (SGA)	Erg2 (GS)	Gtr1 (GS)
Sur2 (GS)	Erg5 (GS)	Pho4 (GS)
Sur4 (GS)	Erg6 (GS)	Pho84 (GS)
	Erg28 (SGA)	Pho86 (GS)
		Pho88 (TAP)

C. Example of Protein Classes that Interact with Hsp90

Transcription Factors	Protein Kinases	Chaperones and Cochaperones
Adr1 (2H)	Cdc15 (2H)	Aha1 (2H, TAP)
Aft2 (SGA)	Cka2 (GS)	Cct8 (2H)
Bas1 (SGA)	Ckb1 (2H, GS)	Cdc37 (TAP)
Cha4 (SGA)	Cla4 (SGA)	Cns1 (2H, TAP)
Cst6 (GS)	Ctk1 (SGA)	Cpr6 (2H, TAP, GS)
Gat1 (TAP)	Ctk2 (TAP)	Cpr7 (2H, TAP, SGA)
Gis2 (SGA)	Dbf2 (SGA)	Gim3 (SGA)
Gln3 (SGA)	Elm1 (SGA)	Gim4 (SGA)
Hcm1 (2H)	Fab1 (SGA)	Hch1 (TAP)
Mot3 (TAP)	Hxk2 (GS)	Hsp12 (GS)
Pdr1 (GS)	Kcc4 (TAP)	Ppt1 (2H, TAP)
Pho4 (GS)	Kcs1 (SGA)	Scj1 (SGA)
Rim101 (GS)	Mck1 (TAP, SGA)	Ssa1 (TAP)
Rrn10 (SGA)	Pgk1 (TAP)	Ssa2 (TAP)
Rtg3 (2H, SGA)	Pkh1 (TAP)	Ssb1 (TAP)
Spt15 (TAP)	Pro1 (2H)	Sse1 (TAP)
Swi1 (2H)	Prr1 (SGA)	Ssz1 (TAP)
Yap3 (2H)	Prs3 (GS)	Sti1 (TAP, SGA, GS)
YER028C (2H)	Prs5 (GS)	Xdj1 (SGA)
	Smk1 (2H)	Ydj1 (GS)
	Snf1 (SGA)	
	Ssk2 (TAP)	YCR060W/Tah1 (2H)
	Ssn3 (GS)	YHR034C/Pih1 (2H)
	Ssn8 (GS)	
	Tom1 (TAP)	
	Tpk3 (GS)	
	Ypk1 (SGA, GS)	

these ORFs, YCR060W and YHR034C, and have determined that they represent novel cofactors of Hsp90. YCR060Wp is a small protein of 111 amino acids (12.5 kDa) that contains, according to SMART database, a single TPR domain with at least two well-defined TPR motifs (Figure 2A). We have termed the protein Tah1 for *T*PR-containing protein *a*ssociated with *H*sp90. Tah1 bound tightly to the Hsp90 C terminus (residues 599–709) in the 2H screen (Table S1). Based on BLAST analysis, Tah1 is most closely similar to the TPR2B domain of the Hsp90 cofactor Sti1 (YOR027Wp, a 589 amino acid protein; Scheufler et al., 2000) and to the TPR motifs in Sgt2, a 346 amino acid glutamine-rich TPR-containing protein of unknown function, while the closest human homolog is human SGT2 (also called SGTB, NP_061945, 304 amino acids). As with several other Hsp90 cofactors, deletion of *TAH1* in either the S288C or W303 strain backgrounds did not confer any obvious growth defects (data not shown).

YHR034Cp is a medium-sized protein of 344 amino acids (39.5 kDa) that contains no recognizable motifs. We termed the protein Pih1 for *p*rotein *i*nteracting with *H*sp90 as it strongly interacted with the C terminus of Hsp90 in the 2H screens (Table S1). While there are no clear yeast paralogs of Pih1, a close human ortholog (E value 10^{-41}) of similar length but unknown function, NP_060386.1 (hypothetical protein FLJ20643, 290 amino acids), was detected. We observed that an S288C-derived strain deleted for the *PIH1* gene was slow growing, proliferating, at 28°C in rich medium, roughly half as quickly as wild-type cells (data not shown), although this slow-growth phenotype was not



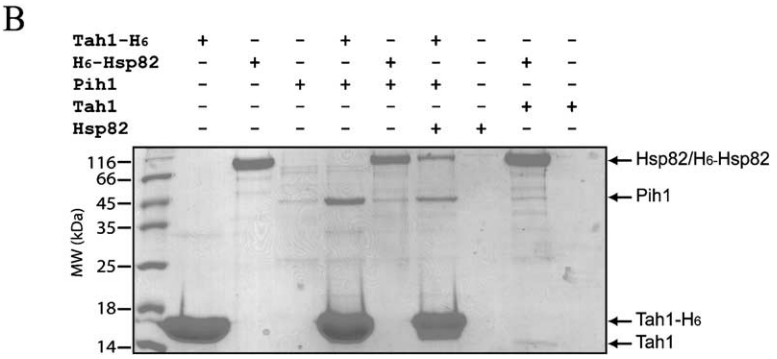


Figure 2. The Binding of Novel Cofactors to Hsp90

(A) TPR Motifs in Tah1 (YCR060W) are shown as identified by SMART database.

(B) Pull-down experiments on Ni-NTA resin using purified recombinant Tah1-H_6 with Pih1, or with Pih1 and Hsp82. Alternatively, the pull-downs were carried out using H_6-Hsp82 with Pih1 or with Tah1. The gel shown was Coomassie stained.

observed in a W303 background strain (see below). While the reason for this difference is unknown, the fact that S288C *pih1Δ* deletion mutants exhibit a small cell size (whi) phenotype (Jorgensen et al., 2002) suggests an important role for this protein in cell growth and/or division.

Tah1 has previously been reported to bind to Pih1 by 2H screen (Ito et al., 2001). Consequently, we performed in vitro binding assays to confirm the putative physical interactions between purified recombinant Hsp82, Tah1, and Pih1. When His-tagged Tah1, Tah1-H_6, was added to Pih1, both proteins were recovered on Ni-NTA resin (Figure 2B). Also, H_6-Hsp82 significantly interacted with Tah1 in the pull-down experiments, but no significant binding was detected between H_6-Hsp82 and Pih1 (Figure 2B). However, when Tah1-H_6 was added to recombinant Pih1 and Hsp82, all three proteins were recovered by Ni-NTA pull-down (Figure 2B). This suggests that the interaction between Hsp82 and Pih1 observed by 2H is probably mediated by Tah1, with the three proteins possibly forming a ternary complex. It should be noted that we did not detect any effect of Tah1 or Pih1 on the ATPase activity of Hsp82 under the conditions used (data not shown).

The binding data and the presence of TPR motifs in Tah1 strongly suggested that Tah1 and Pih1 are novel cofactors of the Hsp90 chaperone. We therefore used an in vivo assay to determine whether the knockout of the *TAH1* or *PIH1* genes affects the ability of Hsp90 to properly fold two well-established Hsp90 model substrates, glucocorticoid receptor (GR; a transcription factor) and vSrc (a protein tyrosine kinase). Wild-type W303 cells and strains deleted of *TAH1* or *PIH1* had similar growth rates under the conditions used for these experiments. For the GR maturation assay, the strains were transformed with a plasmid expressing GR under the control of a constitutive promoter along with a reporter plasmid in which the expression of *lacZ* reporter gene was placed under the control of a GR-response element (GRE) (Riggs et al., 2003). The addition of the hormone deoxycorticosterone (DOC) results in GR maturation; the receptor, subsequently, binds GRE, driving the expression of LacZ. As seen in Figure 3A, when either *TAH1* or *PIH1* was knocked out, GR maturation was significantly impaired as reflected by reduced LacZ activity (~60% the level of wild-type), whereas the expression levels of GR were not perturbed. Tah1 and Pih1 have no direct effect on LacZ activity since LacZ expressed from a *CYC1* constitutive promoter in wild-type strain had similar activity to that expressed in strains deleted of *TAH1* or *PIH1* (Figure 3B). The effect of Tah1 and Pih1 on GR maturation is particularly striking considering that the cellular levels of those two proteins are estimated to be less than 1% that of Hsp90 (R.Z. and W.A.H., unpublished data; Ghaemmaghami et al., 2003); in comparison, Hsp90 represents around 1%–2% of total soluble proteins in nonstressed yeast cells (Borkovich et al., 1989). Furthermore, the majority of Tah1 and Pih1 seems to be localized mainly to the nucleus (Huh et al., 2003), suggesting a nuclear-related functional target.

Similar effects were observed for vSrc maturation, which is highly dependent on Hsp90 for folding (Nathan et al., 1997). Ectopically expressed vSrc promiscuously phosphorylates a broad spectrum of yeast proteins (Figure 3C); deletion of either *TAH1* or *PIH1* did not sig-

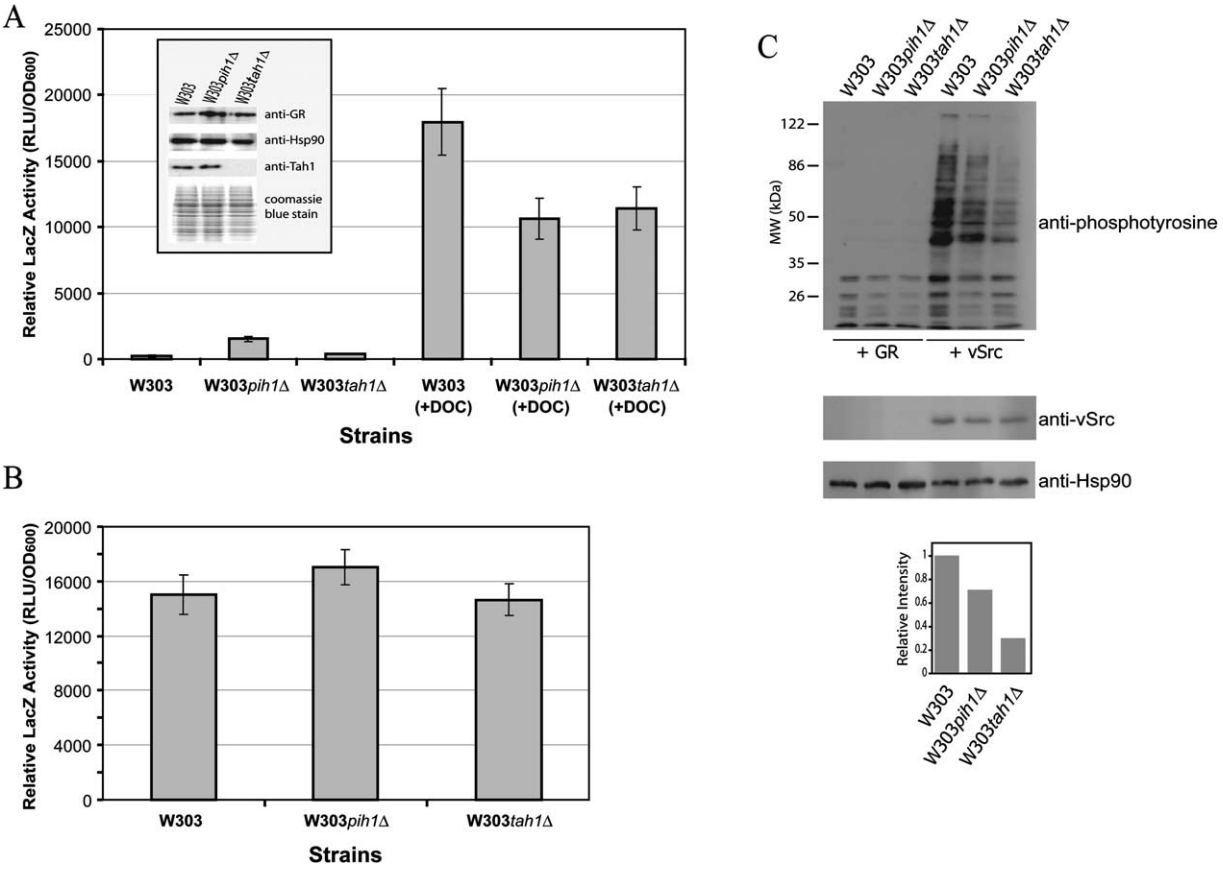


Figure 3. Tah1 and Pih1 Affect the in Vivo Function of Hsp90

(A) Wild-type yeast strain, W303, and strains deleted of *PIH1*, W303 *pih1⊿*, and *TAH1*, W303 *tah1⊿*, were transformed with GR expression plasmid and GR reporter plasmid in which LacZ expression is under the control of a GR response element. The proper maturation of GR upon addition of the hormone DOC was assessed by measuring LacZ activity at 28°C. Inset shows samples immunoblotted with antibodies against GR, Hsp82, and Tah1. Error bars represent standard deviations from the mean of at least six independent experiments.

(B) W303, W303 *pih1⊿*, and W303 *tah1⊿* were transformed with plasmid expressing LacZ under *CYC1* constitutive promoter. LacZ activity was then measured as in (A).

(C) The upper panel shows Western blot analysis to measure yeast protein phosphotyrosine levels following induction of vSrc in different yeast cells at 28°C. Also shown are control Western blots of cells expressing GR grown under similar conditions. The levels of vSrc, second panel from top, and of Hsp90, third panel from top, are similar in the different strains. The lowest panel shows the intensity of the phosphotyrosine proteins (top panel) normalized against the amount of vSrc present in the cells (second panel from top). Experiments have been repeated multiple times and similar results have been obtained.

nificantly affect the expression levels of vSrc but resulted in a much reduced level of protein tyrosine phosphorylation as compared to wild-type cells (Figure 3C), indicating a role for Tah1 and Pih1 in Hsp90-dependent maturation of vSrc. The effect of perturbed Tah1 and Pih1 function on Hsp90 activity in vivo is similar to that observed for other established Hsp90 cofactors, such as Aha1, Cns1, Hch1, and p23.

Tah1 and Pih1 Link Hsp90 to Chromatin Remodeling

From the in vitro binding experiments and the in vivo maturation assays, we concluded that Tah1 and Pih1 are novel cofactors of Hsp90 and that these two proteins probably form a ternary complex with the chaperone. Since it is assumed that different Hsp90 cofactors target the chaperone to different client proteins (Pearl and Prodromou, 2002), we next attempted to identify putative substrates of Tah1 and Pih1. To this end, we performed TAP affinity purifications using yeast strains in which either *TAH1* or *PIH1* were C-terminally TAP tagged. Strikingly, Tah1 and Pih1 copurified not only with each other but also with stoichiometric amounts of Rvb1 and Rvb2 (Figure 4A), two closely related RuvB-like AAA$^+$ DNA helicases that have been linked to chromatin remodeling in yeast (SGD database). In the Tah1-TAP purification, polypeptide bands corresponding to Pih1, Rvb1, and Rvb2 were visible by SDS-PAGE; these same proteins were also observed in the Pih1-TAP purification, although Tah1 was only detected by LC-MS, presumably due to the higher sensitivity of this method. Hsp90 was not observed in these pull-downs probably because the chaperone is involved with many other interactions simultaneously and its levels in this complex, if any, are below our detection limits; however, see below and Figure 4B.

Rvb1/2 are essential components of the Ino80 chro-

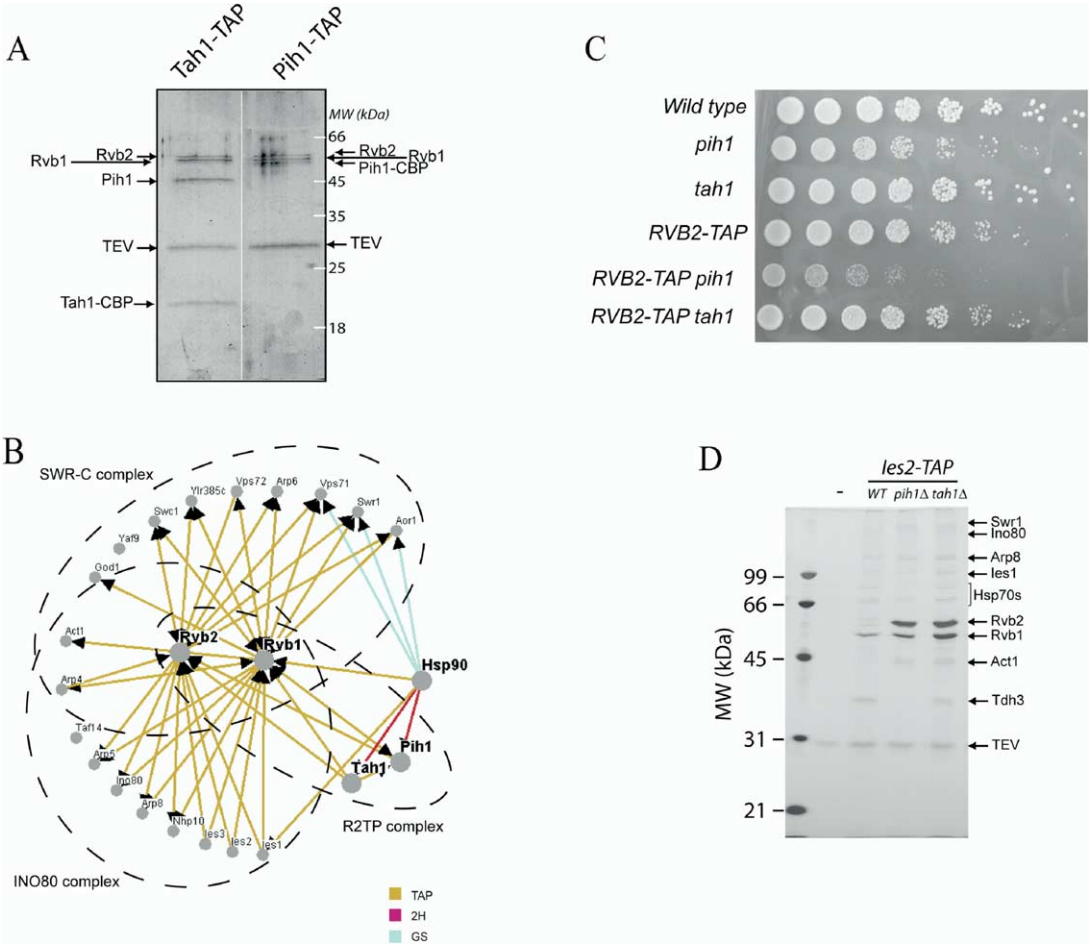


Figure 4. Tah1 and Pih1 Provide a Link to the Chromatin Remodeling Machinery

(A) Tandem affinity purifications of R2TP complex were carried out on strains with either endogenous Tah1 or Pih1 C-terminally TAP-tagged. Proteins were then separated on SDS-PAGE gel, silver stained, and subsequently identified by MALDI-ToF. In the Pih1-TAP pull-down, Tah1 was not observed on the gel and was only detected by LC-MS.

(B) The interaction network of Hsp90 with the R2TP, Ino80, and SWR-C complexes is shown using the 2H, TAP, and GS data obtained in our screens and from the ongoing yeast interactome project. The diagram was drawn using Osprey. For clarity, proteins of the Ino80 and SWR-C complexes are shown connected to Rvb1/2 only; TAP-based interactions among proteins in each complex are not shown.

(C) Approximately equal number of cells of the indicated genotype in an S288C background were subjected to a 5-fold serial dilution, spotted onto YPD plates (from left to right), and incubated at 30°C for 2 days.

(D) Tandem affinity purifications of the Ino80 protein complex using Ies2-TAP were carried out on wild-type strain (WT) or strains deleted of *TAH1* or *PIH1*. A negative control purification was carried on an untagged strain (–). Components of the Ino80 complex were then separated by SDS-PAGE, silver stained, and, subsequently, identified by mass spectrometry. Ies2 was not detected on the gel probably due to poor staining but was detected by LC-MS.

matin remodeling complex linked to the transcriptional regulation of 5% of yeast genes (Jonsson et al., 2001). They are also core subunits of a recently discovered 13 protein chromatin remodeling complex, SWR-C, which regulates gene transcription near silent heterochromatin by facilitating recruitment of the histone H2A variant, Htz1, into chromatin (Krogan et al., 2003; Mizuguchi et al., 2004; Kobor et al., 2004). In this regard, Hsp90 was found to physically interact in our TAP screens (Table S2) with TAP-tagged Rvb1, Htz1, as well as with Ies1, another component of the Ino80 complex. These interactions are likely bridged by Tah1 and Pih1. Furthermore, in the GS screen, Hsp90 showed chemical-genetic interactions with Vps1, Swr1, and Aor1 (Table S4), which are core components of the SWR-C complex (Figure 4B). We have termed the complex of Rvb1-Rvb2-Tah1-Pih1 the R2TP complex (Figure 4B).

These data suggest that Tah1 and Pih1, and by extension Hsp90, play a joint role in mediating the folding and/or assembly of Rvb1/2 into the Ino80 and/or SWR-C transcriptional regulatory complexes. Consistent with this notion, we observed impaired cell growth when *PIH1* was deleted in a strain background in which *RVB2* was also C-terminally TAP tagged (Figure 4C), indicating a functional linkage. No phenotype was observed with *RVB2-TAP tah1Δ* cells. This implies that Pih1 is an important component of the R2TP complex that is likely required for proper helicase function. It

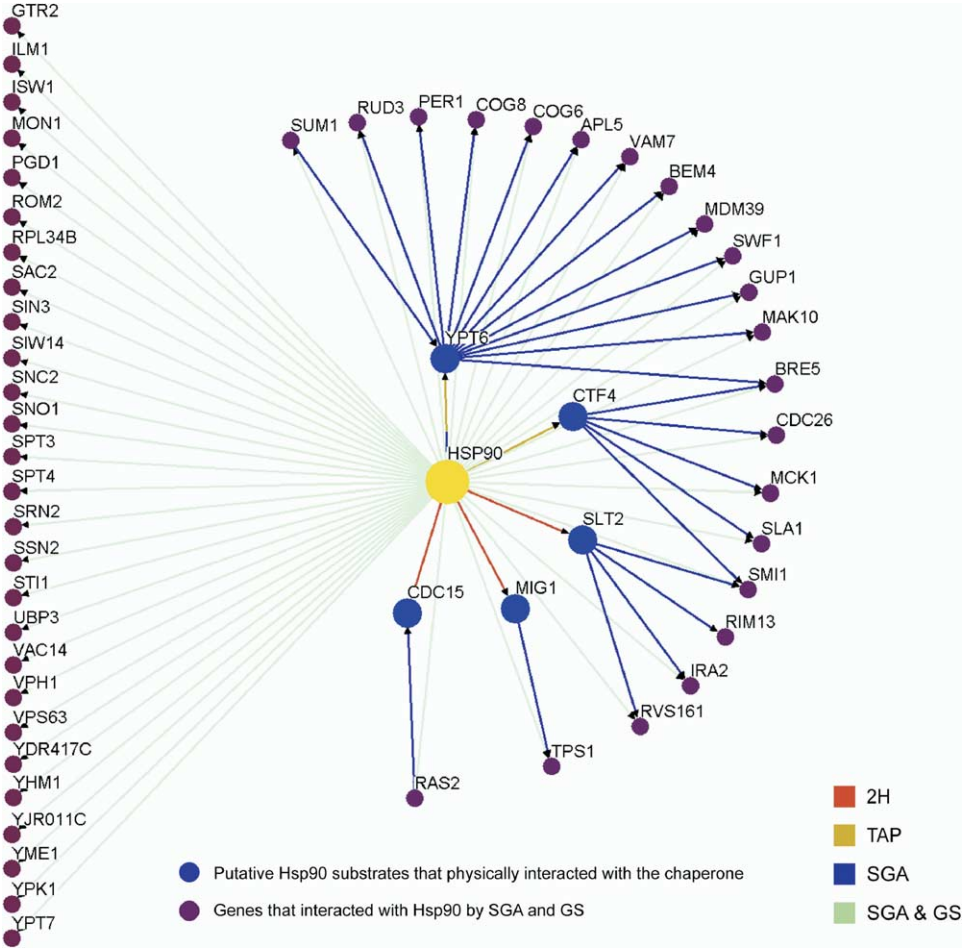


Figure 5. Integrating the Genetic and Physical Interaction Data

Forty-nine ORFs were found to genetically interact with Hsp90 by SGA and GS. Twenty-two of these ORFs were also found to be synthetic lethal, according to the GRID database, with proteins that physically interact with Hsp90 by 2H or TAP. The other 27 ORFs are listed on the left.

should be emphasized that since neither Tah1 nor Pih1 have been detected as components of the Ino80 or SWR-C complexes, this suggests that the action of Hsp90/Tah1/Pih1 on Rvb1/2 is transient and possibly related to proper folding or incorporation of Rvb1/2 into larger chromatin remodeling assemblies.

As further evidence of a role for Tah1 and Pih1 in modulating the activity of Rvb1/2, we found a substantial increase in the apparent levels of both of these helicases in association with affinity-purified preparations of the Ino80 complex upon deletion of either *TAH1* or *PIH1*. For example, when the Ies2 subunit was TAP tagged, the Ino80 complex obtained from *TAH1* or *PIH1* deletion mutants had several-fold higher levels of Rvb1/2 as compared to that obtained from a wild-type control strain (Figure 4D). Given that Tah1 and Pih1 are detected in both nuclear and cytoplasmic fractions while Hsp90 is exclusively cytoplasmic in yeast (Huh et al., 2003), one interpretation of these results is that Hsp90 and R2TP act as a transient cytoplasmic folding/maturation machinery that holds Rvb1/2 helicases in a poised state until a certain signal is sensed; subsequently, the folded/matured helicases are transported via R2TP into the nucleus where they partition into the Ino80 and SWR-C complexes. The absence of proper Tah1, Pih1, or Hsp90 function would therefore be predicted to alter chromatin remodeling complex assembly and activity, thereby perturbing gene expression. Consistent with this, preliminary studies of an Ino80-responsive reporter gene in *TAH1* or *PIH1* deletion mutants indicate a major deficiency in transcriptional regulation (data not shown). This proposed scenario would be similar to the effect of Hsp90 on GR maturation (Buchner, 1999). Hsp90 holds the receptor until the cognate hormone enters the cell; upon binding the hormone, GR is released from Hsp90 and is translocated into the nucleus, where it binds specific DNA elements and activates transcription. The detailed aspects of the role of Tah1 and Pih1 in modulating Hsp90 and Rvb1/2 activities are currently being investigated.

Discussion

Integrating the Genetic and Physical Interaction Networks

We have performed four complementary genome-wide screens to systematically examine physical and functional interactions involving Hsp90. The combination of

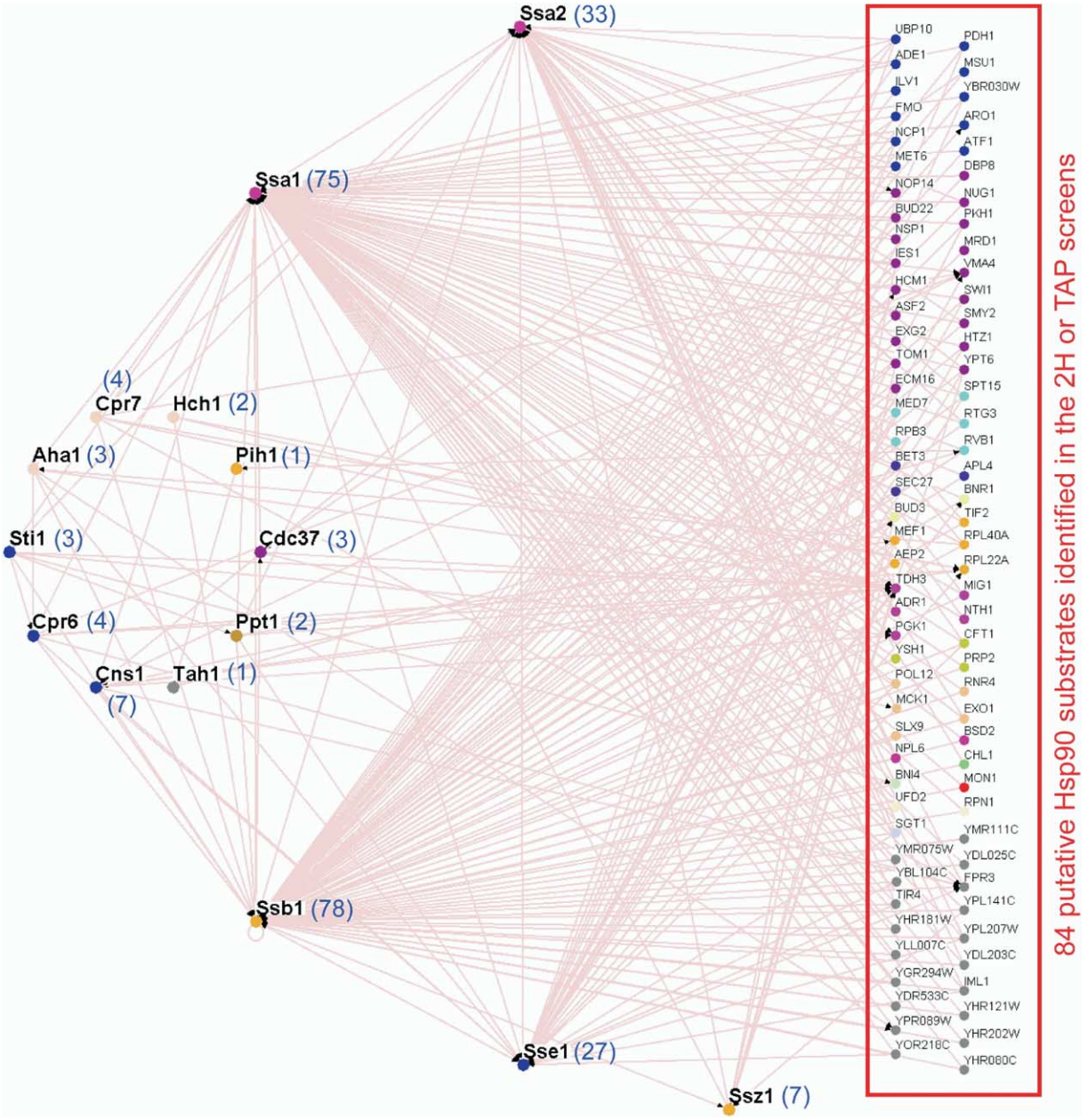


Figure 6. Mapping the Interaction of Hsp90 with Its Putative Substrates/Cofactors through Cochaperones and Cofactors

Eighty-four putative Hsp90 substrates/cofactors identified in the 2H or TAP screens were also found to interact with at least one of the 15 Hsp90 cochaperones and cofactors in TAP tag pull-downs. The putative Hsp90 substrates/cofactors are grouped according to GO terms and are placed in the rectangle. Lines refer to TAP-based interactions. The numbers in brackets refer to the number of putative substrates/cofactors in the rectangle that interact with a given cochaperone or cofactor. The interactions are listed in Table S6.

four independent datasets, two based on physical interactions and two based on genetic/chemical-genetic interactions, provides for a unique perspective on the varied physiological roles of Hsp90. Collectively, the data indicate that Hsp90 lies at the heart of an extended network of genetic and physical interactions connecting a wide array of cellular pathways and functions (Figure 1C).

Indeed, we can use our combined interaction datasets to explain the origin of synthetic lethality of certain ORFs with Hsp90. For instance, if a gene becomes essential when Hsp90 function is compromised, one plausible explanation is that Hsp90 facilitates the correct folding of another protein that functionally overlaps with that same gene. Such an explanation can be provided for at least 22 of the 49 ORFs in the high-fidelity dataset (Figure 5) since we were able to identify five proteins that interact physically with Hsp90 and that are

synthetic lethal with at least one or more of these 22 ORFs. This exercise further verifies these five proteins (blue nodes in Figure 5) as likely substrates of Hsp90.

Mapping the Interaction of Hsp90 with Its Substrates through Cochaperones and Cofactors

198 proteins were found to interact with Hsp90 in our 2H or TAP screens (Figure 1A). Many of these proteins also represent putative Hsp90 substrates that probably interact with the chaperone through association with one or more cochaperones or cofactors (such as Tah1/Pih1). Consequently, using the TAP detection method, we found that 15 known Hsp90 cochaperones/cofactors (Table 1C) interacted with 84 of the 198 putative Hsp90 substrates (Figure 6 and Table S6). This further establishes the 84 proteins as candidate Hsp90 substrates. It is interesting to note that many of these interactions are mediated by Ssa1 and Ssb1, indicating a pivotal role for the Hsp70 system in mediating substrate interactions with Hsp90.

We suspect that a small fraction of these 84 proteins might also be cofactors of Hsp90 and one or more of the cochaperones. Sgt1, a highly conserved protein required for both SCF (Skp1p/Cdc53p-cullin-F box)-mediated ubiquitination and kinetochore function in yeast (Kitagawa et al., 1999), might represent such a protein since it physically interacts with the Hsp70 chaperones Ssa1, Ssa2, Ssb1, and Sse1 in addition to Hsp90. Moreover, it has a p23-like CHORD domain, which is known to bind to Hsp90, as well as a TPR domain that could possibly bind to the C terminus of Hsp90 and Hsp70 family members.

Linking Hsp90 to Transcriptional Regulatory Complexes

Hsp90 appears to physically or genetically interact with at least 10% of the yeast proteome. As such, it can be considered the master regulator of multiple essential metabolic and cellular processes. The chaperone, therefore, seems to play two important but distinct roles. On the one hand, Hsp90 has a well-established ability to directly influence the folding of critical signaling proteins, protein kinases, and transcription factors (Pratt and Toft, 2003). On the other hand, based on our results with Tah1/Pih1, which imply an association between Hsp90 and chromatin remodeling, as well as based on the results of recent studies that have linked the cofactor p23/Sba1 to promoter remodeling (Freeman and Yamamoto, 2002), it seems that Hsp90 can also indirectly affect cell physiology through its influence on global patterns of gene expression. Consistent with this notion, a direct interaction between Hsp90 and the core histones had been suggested earlier in the literature (Csermely et al., 1994). Given the broad overall evolutionary conservation of the Hsp90 system and given its critical involvement in the emergence of the tumor cell phenotype, it will be of great interest to investigate the interplay between these two roles.

Experimental Procedures

Yeast Strains

The yeast strains R0009 (*MATα can1Δ::MFA1pr-HIS3 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lys2Δ0 hsp82Δ::LEU2 TAP-HSC82*) and R0013 (*MATα can1Δ::MFA1pr-HIS3 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lys2Δ0 hsc82Δ::LEU2 hsp82^{ts}-URA3*) were constructed from Y3068 strain background. The *hsp82^{ts}* gene, containing the G170D mutation, was isolated from plasmid pTGPD-Hsp82^{ts} (Nathan and Lindquist, 1995).

2H Screen

HSP82 gene fragments spanning residues 1–220, 271–599, 271–709, 599–709, and 1–709 were ligated into pODB2 plasmid (Uetz et al., 2000), which has the *GAL4* DNA binding domain under ADH promoter to generate the *GAL4-HSP82_fragment* bait fusions, and then transformed into strain pj694α to generate the bait strains. The bait strains were mated with an ordered activation domain fusion array as described (Uetz et al., 2000). Positive interaction between bait and prey drives the expression of *HIS3* reporter gene. The array was a generous gift from Dr. Stanley Fields (University of Washington). The diploid strains were initially grown on synthetic media supplemented with the appropriate amino acids with the exception of tryptophan and leucine. The strains were then reselected on synthetic media supplemented with 5 mM 3-amino-1,2,4-triazole but lacking tryptophan, leucine, and histidine. Colonies were scored after 5 days of incubation. Appropriate positive and negative controls were carried out. The screen was repeated six times. A random set of positive hits were reconfirmed by extracting and verifying the plasmid by restriction digest, followed by retransformation into the reporter yeast strain and reperforming the 2H screen.

Protein Complex Purification and Identification

TAP purification procedure was carried out essentially as described (Krogan et al., 2003). A fraction of the purified protein preparations were separated on SDS-PAGE gels and proteins visualized by silver staining were identified by MALDI-ToF mass spectrometry. A second fraction was concentrated by TCA precipitation prior to analysis by tandem mass spectrometry. The precipitated proteins were resuspended and digested overnight at 37°C with trypsin. The peptide mixture was fractionated on a 5 cm reverse phase C18 capillary (100 μm I.D.) column and eluted online into a Thermo Finnigan LCQ-Deca ion trap tandem mass spectrometer. Peptide spectra were searched against the complete yeast protein database (SGD; 6/2002) using the SEQUEST computer algorithm.

Systematic Genetic Analysis

SGA analysis using the ordered deletion array was carried out as previously described (Tong et al., 2001). The R0013 strain was used as the query strain. Mating and screening for haploids were carried out at 26°C. To identify genes synthetic lethal with *hsp82^{ts}*, the haploid triple mutants (*hsc82Δ::LEU2 hsp82^{ts}-URA3 yfgΔ::KAN*, *yfgΔ* indicates a single gene deletion) were pinned in haploid selection medium and incubated at 35°C for 2 days. The sizes of the resulting colonies were measured. Tetrad analysis on a random subset of the resulting diploid mutants was carried out to verify the observed synthetic lethality.

Geldanamycin Screen

Bar coded *MAT***a** haploid deletion mutants (~4800) were obtained from Research Genetics and pinned onto YPD agar plates. Strains were diluted to OD_{600} = 0.025 and incubated at 30°C in the presence of 20 μg/ml geldanamycin (from NCI) or 1% DMSO (solvent control) while shaking. Cells were harvested for genomic DNA extraction at two different time points. The UPTAG and DOWNTAG bar codes were amplified and analyzed as previuosly described (Giaever et al., 2002). The ratio of control signal to treated-sample signal corresponding to each barcode was averaged for two fluor-reversed chips and reported as a $\log_2$ ratio. $\log_2$ ratios greater than 1.58 (greater than 3× under representation of signal from the drug-treated pool) were considered significant. The experiment was repeated four times.

Recombinant Protein Expression and Purification

Proteins were cloned and expressed from p11 plasmid (Savchenko et al., 2003) which introduces an N-terminal His6 tag followed by a

tobacco etch virus (TEV) protease cleavage site. Proteins were purified using Ni-NTA resin (Qiagen) according to manufacturer's protocols. Subsequently, the His6 tag was cleaved using TEV protease. All proteins were more than 95% pure as judged by SDS-PAGE analysis. Rabbit polyclonal antibodies against Hsp82 and Tah1 were generated at the Division of Comparative Medicine at the University of Toronto using purified proteins.

Binding Assays

Yeast lysates from strain Y3068 or R0009 were prepared according to the procedure described above for the TAP tag pull-downs. Yeast lysate (2 ml) was incubated with 100 μg of Tah1-H_6 for 1 hr at 4°C. Bound proteins were isolated using Ni-NTA resin. For binding between purified proteins, typically, 1 mg of each protein was used. All mixtures were incubated for 1 hr at 4°C and then passed over 100 μl Ni-NTA resin.

GR Maturation Assays

Hormone induction assays were conducted according to Riggs et al. (2003). Control experiments were carried out using plasmid pLG312 bearing a *URA3* marker and expressing LacZ under *CYC1* promoter (generous gift from Dr. Jacqueline Segall, University of Toronto).

vSrc Maturation Assays

Yeast strains transformed with YpRS314vSrc (Murphy et al., 1993) expressing the chicken vSrc under the *GAL1-10* promoter were grown in 10 ml liquid minimal medium at 28°C with dextrose supplied as carbon source until the culture reached midlog phase. Cells were then centrifuged and placed in 10 ml minimal medium supplemented with galactose to induce the expression of vSrc. After induction for 6 hr, cells were collected, washed, and then lysed using bead beating. Cellular proteins were separated on 10% SDS-PAGE and subjected to Western blot analysis using antibodies against phosphotyrosine (4G10, from UpState Biotech) or Src (ab4816, from Abcam). Images were scanned and the signal intensities for phosphotyrosine proteins were quantified using ImageQuant (Molecular Dynamics) and then normalized against the amounts of vSrc.

Supplemental Data

Supplemental Data include six tables and can be found with this article online at http://www.cell.com/cgi/content/full/120/5/715/DC1/. In the supplemental tables, protein function description is based on SGD, March, 2004 version.

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Note Added in Proof

While our paper was under review, a paper by Jónsson et al. was published describing a complex of Rvb1, Rvb2, and Pih1 of unknown function (Jónsson, C.O., Jha, S., Wohlschlegel, J.A., and Dutta, A. [2004]. Rvb1p/Rvb2p recruit Arp5p and assemble a functional Ino80 chromatin remodeling complex. Mol. Cell, *16*, 465–477).