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Approaches for Defining the Hsp90-dependent Proteome

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Abstract

Hsp90 is the target of ongoing drug discovery studies seeking new compounds to treat cancer, neurodegenerative diseases, and protein folding disorders. To better understand Hsp90's roles in cellular pathologies and in normal cells, numerous studies have utilized proteomics assays and related high-throughput tools to characterize its physical and functional protein partnerships. This review surveys these studies, and summarizes the strengths and limitations of the individual attacks. We also include downloadable spreadsheets compiling all of the Hsp90-interacting proteins identified in more than 23 studies. These tools include cross-references among gene aliases, human homologues of yeast Hsp90-interacting proteins, hyperlinks to database entries, summaries of canonical pathways that are enriched in the Hsp90 interactome, and additional bioinformatic annotations. In addition to summarizing Hsp90 proteomics studies performed to date and the insights they have provided, we identify gaps in our current understanding of Hsp90-mediated proteostasis.

Keywords

Hsp90-interactome; Proteomics; LC-MS/MS; High-throughput screens; Hsp90 Inhibitors

1. Introduction

Hsp90 is a molecular chaperone that is required for the viability of eukaryotic cells [1, 2]. It is frequently described as the core component of a multimeric chaperone machine that functions in the folding, maturation, stabilization and activation of other proteins. Hsp90 function requires the binding and hydrolysis of ATP, which drives it through its reaction cycle that appears to involve approximately four distinct conformations [3–5]. Hsp90 works in concert with a cohort of co-chaperones that modulate its binding and hydrolysis of ATP and its interaction with protein substrates (a.k.a. "clients") [6–9]. Since these topics are covered in depth in other submissions to this special edition, we will forgo a detailed discussion of Hsp90's reaction cycle and the regulatory roles of its co-chaperones.

Most of the 200 plus proteins that have been found to interact with Hsp90 were discovered to do so serendipitously, with Hsp90 and the client co-purifying as a complex, or co-precipitating in antibody pull-down assays. The discovery that geldanamycin [10] and other

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compounds are highly specific Hsp90 inhibitors further advanced the field, and small molecule Hsp90 inhibitors have become invaluable tools for dissecting the functional significance of Hsp90's interactions with other proteins [7, 11–14].

Because a comprehensive list of Hsp90's client proteins and regulatory subunits is available at the web site maintained by Dr. Didier Picard (http://www.picard.ch/downloads/downloads.htm), we will not attempt to duplicate information available therein. Rather, this review will concentrate on just those studies that have utilized high throughput (HTP[1]) approaches to define the Hsp90 interacting proteome, in the absence of a priori targets. Much of this discussion will center on proteomics techniques, reflecting their potential and prominence in assessing proteomes and protein interactions. Our discussion will also include findings from powerful yeast studies directed toward understanding the breadth and depth of Hsp90's roles in supporting the cellular proteome. Although mass spectrometry has been used to identify posttranslational modifications on specific individual proteins, this review will only encompass proteomics studies, defined here as studies directed toward whole sets of proteins. Below, we will introduce the HTP techniques that have been used to study Hsp90, touching upon the general strengths and weaknesses of each approach. We will then briefly survey results from studies that have used these techniques. Finally, we will attempt a synthesis of these findings and a description of how these studies have changed our perceptions of this fascinating protein. The reader's attention is also directed toward the Hsp90 interaction mapping initiatives from the laboratories of Houry [15–17], Frydman [18], and Picard [19].

2. Overview of Techniques

2.1 Hsp90 interactions

Most HTP studies of Hsp90 function are directed at identifying Hsp90-interacting genes or gene products. However, this very term "Hsp90-interacting" merits consideration. An Hsp90 interaction can be a functional interaction, wherein manipulation of Hsp90 function impacts the Hsp90-interacting gene product or gene function. On one end of the spectrum, this functional impact might be direct, wherein compromised Hsp90 function manifests as a change in the expression of an Hsp90-interacting protein (e.g., compromised expression of that protein on a Western blot) or in some direct measure of its function (e.g., cellular phosphotyrosine content). On the other end of the spectrum, a functional impact might be further removed, manifesting as alterations in cellular growth, cell survival, or the phenotype of the organism or cell. In this manifestation, "Hsp90 interaction" would describe a genetic interaction between Hsp90 and the Hsp90-interacting gene/gene product. Typically, a detailed biochemical dissection is required to determine the extent to which functional interactions between Hsp90 and other genes or gene products are direct, versus indirect interactions that are mediated by intermediary Hsp90-dependent gene products.

Alternatively, "Hsp90 interaction" might describe the direct physical association of Hsp90 with a polypeptide. Physical interactions with Hsp90 should probably be regarded as weak evidence that Hsp90 modulates the function of a gene product (or vice versa), because protein-protein interactions are notoriously promiscuous in vitro. Exacerbating this

[1]**Abbreviations:** 17-AAG, 17-allylamino-17-demethoxygeldanamycin; 17-DMAG, 17-desmethoxy-17-N, N-dimethylaminoethylaminogeldanamycin; 2-D PAGE, two-dimensional polyacrylamide gel electrophoresis using sodium dodecyl sulfate in the second dimension; DIGE, difference gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; HTP, high throughput; ICAT, isotope-coded affinity tag; iTRAQ, isobaric tags for relative and absolute quantitation; LC-MS/MS, liquid chromatography eluting directly into an electrospray tandem mass spectrometer; MALDI-TOF, matrix-assisted laser desorbtion/ionization time-of-flight mass spectrometry; MRM, multiple reaction monitoring; MS, mass spectrometry or mass spectrometer; MS/MS, tandem mass spectrometry or tandem mass spectrometer; RNP, ribonucleoprotein; SGA, synthetic genetic array; SIM, selected ion monitoring; SRM, selected reaction monitoring; TAP, tandem affinity purification.

limitation, Hsp90 displays a highly charged surface capable of binding a wide range of proteins, and Hsp90 is notoriously "sticky," in that it binds non-specifically to a wide range of inert chromatographic pull-down media. These facets of Hsp90 biochemistry place certain burdens on studies assaying binding to Hsp90 in the absence of evidence for a functional interaction. None-the-less, Hsp90 binding is an extremely important part of the larger puzzle, because it addresses the question of intermediary functional interactions raised above. Throughout this review, we will use the term "Hsp90-interacting" to describe both physical and functional associations with Hsp90.

An additional complication in studying interactions with Hsp90 is the challenge of determining whether a given interaction reflects Hsp90's chaperoning of a client substrate, versus an Hsp90 interaction with a protein (e.g., a co-chaperone) that regulates Hsp90 function. Traditional criteria for making this distinction appear below, elsewhere in this issue, and in the literature [9, 20].

2.2 High throughput genetic screens

The first attempts to identify novel Hsp90-interacting partners were carried out by Susan Linquist's group, who screened *Saccharomyces cerevisiae* for mutations that were synthetically lethal when combined with Hsp90 mutants [21], or for genes that functioned as multi-copy suppressors of Hsp90 deficiencies [22]. The first approach screens for gene products whose function is required for cell growth or viability under conditions where Hsp90's function is compromised, while the latter approach screens for gene products whose overexpression restores the growth of cells that are Hsp90 deficient.

Extending these studies, Hsp90's functional interactions have been exhaustively mapped using synthetic genetic arrays (SGA), and chemical-genetic screens for gene deletions that create hypersensitivity to Hsp90 inhibition in yeast [16, 18]. These genetic screens are based on the logic that if a gene becomes essential when the function of Hsp90 is compromised, Hsp90 may be required for the proper folding of a protein whose function overlaps with that of the deleted gene [16]. While these assays do not readily fit a classification as "proteomics," any discussion of proteomics assessments of Hsp90 function would be incomplete without them.

In the SGA approach, the Houry group utilized a haploid query strain expressing a temperature sensitive allele of Hsp90 [16]. This strain was crossed against approximately 4,000 haploid strains, each bearing a single deletion of a non-essential gene. Double-mutant haploid progeny strains were then assessed for a combinatorial synthetic growth defect, thus reporting a functional interaction between Hsp90 and the deleted gene/gene product.

In the chemical-genetic approach, both the lab of Houry and the lab of Frydman each propagated libraries of approximately 4,000 viable bar-coded yeast deletion mutants in the presence of the Hsp90 inhibitors geldanamycin or macbecin II (respectively) [16, 18]. After passage, DNA from surviving strains was isolated and assayed by microarray detection of the PCR-amplified gene-embedded bar codes. In this assay for deletion-induced sensitivity, a genetic interaction with Hsp90 results in the loss of the Hsp90-interacting strains from the population, with this loss being evident in loss of signal on the bar-code microarray assay.

Several criteria are used to validate the "hit lists" of candidate Hsp90-interacting genes identified in these HTP genetic screens. One criterion is the re-identification of previously characterized Hsp90 co-chaperone partners and known client families or pathways, thus validating the assay's ability to identify Hsp90 interactions. A second criteria is the identification of multiple components of an individual cellular pathway or process, validating the conclusion that Hsp90 functions in that pathway. A third criteria is that results

from discrete assays (performed in separate labs or using orthogonal genetic techniques) demonstrate significant overlaps in their lists of Hsp90-interacting genes, validating the individual overlapping genes as high-confidence subsets within the larger HTP data. As a fourth criterion, subsequent detailed characterizations have confirmed Hsp90's interactions with individual novel genes identified in the screen. Other more subtle bioinformatic criteria have also been presented [18].

Though similar HTP genetic techniques have not been used to assess Hsp90 function in human cells, highly specific small-molecule inhibitors of Hsp90 have proven to have similar potential. In this approach, cells are treated with Hsp90 inhibitor and subsequently analyzed using proteomics techniques, thereby identifying sets of proteins whose expression is governed by Hsp90 function. This approach is analogous to functional assays in Hsp90-deficient yeast, but assesses direct changes in protein expression rather than genetic deficiencies that compromise growth.

Several criteria are available to validate results from inhibitor-based proteomics studies of Hsp90 function. Based on well-established precedents, we should expect that an N-terminal Hsp90 inhibitor should increase the expression of Hsp70 and (to a more limited extent) Hsp90. Since both Hsp90 and Hsp70 are abundant and readily detected, this is an easily achievable validating benchmark. Similar expectations extrapolate to any other gene products regulated by HSF1, if they are apparent in the proteomics data set.

As another validator, N-terminal Hsp90 inhibitors deplete Hsp90 client proteins from treated cells. This seminal finding [10] has been duplicated for nearly every Hsp90 client protein studied in detail, and is a dogmatic hallmark of Hsp90 dependency. However, this criterion is a difficult benchmark for proteomics assays of Hsp90 function: while most Hsp90 clients are expressed at levels that are easily assayed by Western blotting, it is much more difficult to detect them using mass spectrometry. None-the-less, we might reasonably anticipate that when known Hsp90 clients are detected, they should be down-regulated by Hsp90 inhibitors that target Hsp90's N-terminus. In these cases, and those above, it is important to assess the whole dataset for these behaviors, and to avoid cherry picking data that support the study's validity whilst ignoring those that question it.

2.3 High throughput interaction screens

The strategies above focus on Hsp90 function, but physical interactions between proteins are often the first evidence of a functional interaction. Thus, substantial effort has been directed toward identifying proteins that bind to Hsp90. These studies often utilize affinity purification of Hsp90, subsequently using one or more mass spectrometry-based assays to identify co-purifying proteins. Hsp90 has been affinity purified using antibodies that directly bind Hsp90, and by expressing affinity-tagged Hsp90 gene constructs. This second scenario creates challenges with regards the importance of Hsp90 N- and C-termini, and with regards the large amounts of endogenous Hsp90 expressed in normal cells. Affinity approaches have also utilized immobilized recombinant Hsp90 or Hsp90 domain constructs, to "fish" Hsp90-binding proteins from cell lysates, and biotinylated-geldanamycin to capture of Hsp90 complexes.

The criteria for validating hit lists of proteins that co-purify with Hsp90 are similar to those described above to assess functional interactions. As an additional validating criterion, control adsorptions performed with inert affinity media are required to address the specificity the co-adsorptions; "Hsp90-binding" proteins should be much less abundant in control adsorptions. Unfortunately, the importance of using control resins to quantify non-specific binding is often overlooked.

Large-scale yeast two-hybrid screens have also been carried out to identify novel Hsp90-binding proteins [16, 23, 24]. While the yeast two-hybrid assay utilizes a genetic approach rather than mass spectrometry, it is fundamentally a proteomics technique, in that it is used to answer questions about the functions and interactions of sets of proteins. Yeast two-hybrid assays are typically validated using empty bait vectors and other controls specific to the technique, and by confirming interactions using one of the co-adsorbing assays described above.

It is reasonable to expect that a functional Hsp90 partner, either client or regulatory subunit, should also show a physical interaction with Hsp90. Paired physical and functional interactions are apparent in the large body of traditional studies, and represent the gold standard for demonstrating an Hsp90 partnership. Thus, one might similarly expect a given protein to appear in hit lists both from HTP assessments of Hsp90 binding and in hit lists from assessments of Hsp90 function. This expectation is not always readily met, however, due to the quantitative limitations discussed above, and due to experimental challenges (discussed below). When observed, however, HTP evidence for both physical and functional interactions can propel an Hsp90-associated gene product to center stage.

2.3.1 Fractionation methodologies for identifying interacting proteins—In addition to understanding the rationale behind HTP assays of Hsp90 interactions, it is important to appreciate how the strengths and weaknesses of the downstream proteomics analyzes influence our perception of Hsp90 function. Two-dimensional polyacylamide gel electrophoresis (2D-PAGE) has frequently been utilized to separate, detect, and quantify cellular responses to Hsp90 inhibition, and to assay the Hsp90 interactome [25, 26]. 2-D PAGE is a technically challenging technique [27], and it can be difficult to achieve the reproducibility required to accurately align gels. However, researchers from diverse backgrounds can readily assess 2-D PAGE images of the Hsp90-associated proteome. Thus, published images of Hsp90-associated proteomes typically speak for themselves, and gel displays of Hsp90-interacting proteomes published to date have generally been quite good.

However, only finite amounts of material can be loaded onto 2-D PAGE gels [28], hence these assays only detect the most abundant proteins in the Hsp90-dependent proteome or the Hsp90-binding interactome. This limit predicts that gel-based assays of Hsp90 function or Hsp90 inhibition are probably incapable of detecting Hsp90 clients. Similarly, gel-loading limits probably make them incapable of detecting rare Hsp90 subunits. It is also important to recall that some proteins (e.g., membrane proteins) are not readily tractable to analysis by 2-D PAGE [28–30]. These limitations argue that gel-based studies performed to date are unlikely to have fully probed the Hsp90 interactome.

We should also appreciate that gel based assays may understate the biological range of variances in assays of Hsp90's associations. This caveat acknowledges the poor quantitative response of silver staining [31], a technique frequently used to visualize the Hsp90-associated proteome. Modern fluorescent stains address these limits [31], but these are recent arrivals relative the body of Hsp90 studies discussed below. Difference gel electrophoresis, or DIGE [32], has also been used to assess Hsp90 function, providing a large dynamic response range and addressing many of the problems with gel-to-gel irreproducibility. This addresses the dynamic range compression characteristic of silver-stained images, and provides a particularly strong quantitative assessment.

2.3.2 Mass spectrometry methods for identifying protein interactors—In addition to detecting and quantifying proteins on gels, it is also necessary to identify them. In the past two decades, mass spectrometry (MS) techniques have almost wholly replaced

other protein identification methods such as Edman protein sequencing and Cleveland mapping [33].

Several Hsp90 proteomics studies have utilized the MS technique of peptide mass fingerprinting to identify unknown proteins [15, 16, 25, 34–36]. This somewhat outdated approach is capable of identifying proteins that are well-resolved from each other, e.g., 2-D PAGE spots [27, 37–41]. However, this technique requires careful, skeptical evaluation of each bioinformatic protein "identification." Fortunately, Hsp90 studies utilizing this technique show the hallmarks of conservative interpretation, and the proteins thus identified are consistent with our understanding of Hsp90.

Due to its weaknesses, peptide mass fingerprinting has largely been replaced by approaches that utilize tandem mass spectrometry (MS/MS). Protein identifications by MS/MS are more confident than those obtained by peptide mass fingerprinting. MS/MS approaches also eliminate the need that proteins be perfectly resolved from each other [41].

The ability of MS/MS to directly assay protein mixtures has also given advent to gel-free chromatographic proteomics approaches [42, 43]. These approaches are well capable of identifying hundreds to thousands of Hsp90-associated proteins in a single protein sample. Gel-free approaches have also been coupled to sophisticated strategies in which separate Hsp90-associated proteomes (e.g., inhibitor-treated vs. untreated) are covalently labeled with different isotopic tags (e.g., ICAT [44] or iTRAQ [45]), then pooled prior for analysis by LC-MS/MS. Like the DIGE approach above, this reduces the impacts of technical variability.

It is also important to appreciate that the advent of gel-free chromatographic proteomics strategies leaves us with two very different scenarios with regards to a protein's identification by MS/MS. Protein identifications based upon the MS/MS fragmentation of numerous unique peptides from each protein are typically regarded with a high degree of confidence, regardless of whether the protein was isolated from a gel or not. On the other hand, chromatographic approaches may also yield protein identifications based upon only a few peptides from the parent protein, potentially diminishing confidence. Identifications based upon only a single peptide ("one hit wonders") have traditionally been regarded with skepticism, though this prejudice may be unjustified [46].

Most proteomics experiments are discovery surveys, wherein mass spectrometry is used to identify unknown proteins and to detect changes in protein expression in the absence of a priori targets. In these MS techniques, the mass spectrometer is programmed to scan the whole peptide population, and to use sophisticated real-time data-dependent strategies to select predominant peptides for MS/MS fragmentation [47].

In contrast, mass spectrometers can also be programmed with foreknowledge of the proteins of interest [48]. Examples include selected ion monitoring (SIM) [49, 50], selected reaction monitoring (SRM) [48], and multiple reaction monitoring techniques (MRM) [51]. In these assays, the mass spectrometer is "blinded," being programmed to look only for specific peptide ion targets and their fragments. This turns the mass spectrometer into an exquisitely specific chromatography detector, yielding chromatographic peaks that can be used to quantify Hsp90-associated proteins. Because this specialized technique cannot discover changes in the proteome in the absences of a priori targets, it is reserved for mature proteomics questions.

Proteomics assays of Hsp90 function also face bioinformatic challenges. Due to lack of uniformity in database indexing and protein nomenclature, it can be challenging to compare proteins identified from different databases or different model organisms. Even in a single

database, a given protein sequence is typically represented by more than one name and accession number. An even more difficult challenge is created by true biological complexity within an individual species' proteome. A "single" organism's genome (and hence its sequence databases) often includes multiple protein homologues, point mutants, splice isoforms, pseudogenes, and sequencing errors, making it challenging to correctly assign a single gene product to the "correct" representative sequence in the database [52, 53].

An authentic biological mixture of related gene products creates similar challenges [54]. Mass spectrometry data are not always capable of distinguishing between multiple protein isoforms. 2-D PAGE can be useful in resolving such mixes, but can also be compromised by degradation or covalent derivatization *in vitro*. To be fair, the issue of protein isoforms is certainly not specific to mass spectrometry techniques: most antibodies and many gene probes face similar challenges. None-the-less, it is helpful to remember that mass spectrometers do not actually sequence proteins, and that "identifications" of Hsp90-interacting proteins are bioinformatic conclusions. Thus, it is sometimes helpful to consider an "identified" protein as a protein archetype or an operational definition.

As is the case with a popular and rapidly evolving technique, diverse early proteomics studies varied widely in their quality. In response, Carr et al. [55] proposed general standards for the publication of proteomics research. In 2006, an international group refined the Carr standards to produce the "Paris" guidelines [56]. To keep abreast of technical developments and data archiving resources, the Paris guidelines were further revised in 2009, to produce the Philadelphia guidelines [57]. These standards or their journal-specific variants are now widely regarded as essential practices in the field of proteomics. Like other disciplines, the body of published Hsp90 proteomics studies show numerous gaps relative to these modern standards, with the impacts of these gaps ranging from trivial to consequential.

3. Studies assessing Hsp90's physical interactions with proteins

Below, we briefly survey studies of the Hsp90-binding proteome. These studies are summarized in Table 1. We have also complied a master list that includes most of the proteins identified in these studies (Supplementary Table 1A), with an emphasis on the human Hsp90 interactome. Where appropriate, we have attempted to identify human homologues of yeast Hsp90-binding proteins. From a total of 289 Hsp90-binding proteins thus compiled, 56 have been found in more than one study, of which 28 have been previously described as known or putative Hsp90 associates. We also include the interactomes of some of Hsp90's better-characterized co-chaperones (Supplemental Table 1D). We also call the readers attention to Avrom Caplan's study in which a yeast strain expressing mutant Cdc37 was used to identify 51 Cdc37-dependent protein kinases [58].

Te et al [25] utilized the 8D3 monoclonal anti-Hsp90 antibody to purify Hsp90 chaperone machinery from Dounce homogenates of human Jurkat lymphona cells, displayed these co-adsorptions on 1-D and 2-D PAGE gels and identified individual proteins by peptide mass fingerprinting. Irrelevant antibody was used to validate the specificity of the adsorptions. They also used a truncated Hsp90 C-terminus to fish Hsp90-binding proteins from cell lysates in vitro. They identified 23 Hsp90-interacting proteins, including 6 novel interactions. The high prevalence of known Hsp90 co-chaperones likely reflects limitations in gel loading and the sensitivity of colloidal Coomassie Blue staining utilized. They also observed human homologs of Pih1, RVB1L, and RVB2L, suggesting that the Hsp90 complex previously described in yeast [16] was conserved in human cells. Their Hsp90 interactome also included RPAP3/hSpagh (FLJ21908), which was subsequently shown to be a co-chaperone involved in the Hsp90-dependent assembly of RNA polymerase II complexes [59], and to function in an snRNP assembly complex [60]. The presence of

Hsp90-bound tubulin further suggested interactions between Hsp90 and microtubule machineries.

Skarra and co-workers [61] utilized Flag affinity-tagged Hsp90 to isolate Hsp90-bound proteins in HEK293 cells. The negative control consisted of Flag-tagged pull downs of extracts from cells expressing the Flag-tag alone. Their Hsp90 interactome was analyzed by direct solution trypsinolysis and LC-MS/MS analysis. As quantitative criteria, they conducted statistical analysis of spectral count data, incorporating control purification data to calculate a probability score for a true interaction between two proteins [61]. Results identified 27 Hsp90-binding proteins, consisting primarily of Hsp90 co-chaperone partners and various isoforms of tubulin. Unique gene products identified in the screen include: PDRG1, a p53 regulated gene; SSB1; GIGYF2, mutants of which are associated with Parkinson's disease (Park11); and IRS4.

Wang et al [26] used immobilized 9D2 anti-Hsp90α monoclonal antibody to co-immunoadsorb Hsp90 complexes from HCT-116 colon cancer cell lysates. Control resins consisted of mock-coupled agarose. The Hsp90-binding proteome was displayed by 2-D PAGE, and 43 Hsp90-associated proteins were identified by mass spectrometry (although supporting proteomic/bioinformatic parameters were not provided). In addition to the expected identification of Hsp90 associated co-chaperones, they identified protein components of the cytoskeleton, proteins involved in RNA processing and proteins modulating protein turnover via ubiquitination and proteasome degradation.

Falsone et al [62] used the AC88 monoclonal antibody to isolate Hsp90-binding proteins from HEK297 cells. Naïve protein-G agarose resin was used as the negative control. They identified 40 candidate Hsp90-binding proteins, 29 of which were novel, including metabolic enzymes, cytoskeleton components and components of the cell's protein synthesis apparatus. Only three of Hsp90's co-chaperones were co-adsorbed (Hsp70, Hsp105 and TCP-1), and only five of their novel interactors were apparent in other studies. As the authors note, this may reflect properties of the AC88 antibody, whose epitope is masked in most previously described Hsp90 complexes.

Tsaytler and co-workers [63] used three complementary proteomic approaches to identify novel Hsp90 interactors: co-immunoadsorption with F-8 anti-Hsp90 monoclonal antibody, fishing in lysates using immobilized recombinant Hsp90β, and, affinity capture of Hsp90 with biotinylated-geldanamycin. Control resins consisted of irrelevant HA-tag antibody bound to protein-G agarose, mock coupled resin, and naïve NeutAvadin beads, respectively. Co-adsorbing proteins were separated on 10% SDS-PAGE gels, which were subsequently cut into 20 slices for in-gel trypsinolysis, and identified by nano-spray MS/MS. Thirteen of the 44 identified proteins were identified by more than one approach, 18 of the 44 were novel associations, and 12 of the 44 were known co-chaperones. Cytoskeletal components, and components of the protein synthesis and RNA processing machineries were also prominent in the study.

Gano and Simon [64] utilized tandem affinity purification of N- and C-terminal tagged Hsp90 from HEK293T cell lysates, tagging both wild-type Hsp90α and an Hsp90 mutant capable of binding, but not hydrolyzing ATP. Hsp90 was captured in the presence of an ATP, ADP, or geldanamycin. Lysates from cells transfected with empty vector were used as negative controls. The Hsp90-bound proteomes were assayed using the quantitative spectrum counting technique, thus identifying statistically significant changes in the Hsp90 conformations to which the proteins bound. They identified 37 known Hsp90 partners, 20 of which were co-chaperones, as well as 28 novel associations. Seven of the proteins contained tetratricopeptide repeat (TPR) domains, and seven contained CHORD-Sgt (CS) domains,

both of which are established Hsp90-interacting motifs. Eight Hsp70 family members were also identified. This study demonstrated the specificity of protein interactions for particular ligand-bound forms (conformations) of Hsp90, providing considerable elaboration to our understanding of the Hsp90 reaction cycle. The interaction of 52 proteins (84% of those identified) with Hsp90 was ligand-dependent, with 43 being significantly over-represented in one ligand group versus the others [64]. Their proteomics assays demonstrated that the previously described Hsp70/BAG2/DNAJC7/ST13(HIP) machineries preferentially associated with geldanamycin-bound Hsp90. They also observed that Hsp90 co-chaperones CDC37L1, RPAP3 and PIH1D1 interacted preferentially with geldanamycin–bound Hsp90. In contrast, the co-chaperones AHA1, p23, NUDC, CACYBP and UNC45A associated preferentially with ATP-bound mutant HSP90, while CHORD1 interacted with ADP-bound Hsp90. Of additional interest is the observation that a number of known and putative Hsp90 clients either preferred to bind to geldanamycin-bound Hsp90 (MFL2, YTHDC2 and NR3C1/glucocorticoid receptor) or the ATP-bound conformation of the mutant Hsp90 (CHUK/IKKalpha, IKBKG, DYNC1H1, CALD1, MAP3K7IP1/2, LLRIQ2, SCRIB and CNOT6). The authors note that seven of the 15 proteins, whose interactions with Hsp90 were specifically enhanced by geldanamycin, are involved in RNA metabolism, linking Hsp90 function to RNA synthesis and processing [64].

Millson and co-workers [23] conducted a two hybrid screen utilizing as bait an Hsp82(E33A) mutant that binds but does not hydrolyze ATP, in an effort to stabilize Hsp90's interactions with clients. They identified 177 gene products that interact with Hsp90, 46 of which have putative human homologues (see Supplementary Table 1A). The Hsp90 interactome thus identified includes co-chaperone partners, metabolic enzymes, proteins involved in vesicular/protein transport, protein synthesis, and signal transducers.

Zhao and co-workers [16] similarly conducted a two-hybrid screen using Hsp90 as bait and a library of yeast genes as prey. In addition, they used TAP-tag pull downs from yeast, in conjunction with solution digests and LC-MS/MS analysis of the Hsp90-binding proteome. This study also included synthetic lethal and chemical-synthetic lethal screens (discussed below). They identified a total 90 Hsp90-binding proteins via the two-hybrid screen and 118 via TAP-tag pull down, but only 10 of these interactions were common to both sets of proteins. These overlapping proteins included Hsp82, Hsc82 and 5 co-chaperones. The proteins identified via the two-hybrid screen in this study had minimal overlap with those identified by Millson et al [23] (4 genes with human homologues), perhaps reflecting the different reporter constructs utilized to carry out the screens. Other novel Hsp90 associations identified in this study suggest roles for Hsp90 going far beyond chaperoning signal transduction, implicating Hsp90 in ribosome biogenesis, regulation of chromatin structure, RNA processing and vesicular/protein transport.

In an ambitious follow up study, Gong et al. [15] mapped protein interactions with yeast molecular chaperones using a variety of TAP-tagged chaperones as bait. The study identified 1,154 Hsp90-binding proteins: 433 binding to Hsc82 and 878 binding to Hsp82. However, only 157 proteins are common to both sets, indicating that discrete Hsp90 isoforms have distinct functions. Due to the large number of candidate protein interactions discovered in these surveys, we have not included their human counterparts in the list compiled in Supplementary Table 1. However, we have included these yeast gene lists in Supplementary Table 2A and B, adding our own bioinformatic analyses and links. As originally noted by Gong et al. [15] and McClellan et al. [18], results indicate that Hsp90 is involved nearly every major physiological process in yeast, including chromatin remodeling and DNA repair, RNA processing, and vesicle-mediated transport. Gong et al. [15] suggest that Hsp90 function may be delivered to these cellular processes by three known Hsp90 chaperone modules, and by 11 novel Hsp90-containing chaperone modules.

For completeness here we include references to studies carried out in Plasmodium falciparum and Toxoplasma gondii [65–67]. However, the findings of these studies will not be discussed here as they are the subject of another submission to this issue. We also include here a reference to a proteomic study carried out to identify in vivo Hsp90-interacting proteins in psychrophilic bacteria [68], since very few clients of bacterial HtpG have yet to be identified.

4. Studies assessing Hsp90's functional interactions with proteins

As mentioned in the introduction, highly specific small-molecule inhibitors of Hsp90 can be used to assess the Hsp90 dependent proteome in mammalian cells. Below, we briefly survey studies utilizing this approach (Table 2). A list of genes products that were found to functionally interact with Hsp90 in these studies is given in Supplemental Table 1B.

Maloney et al. [34] used a gel-based proteomics approach to demonstrate that 3% of the detectable A2780 ovarian cancer cell proteome was sensitive to 17-AAG. Cellular responses to Hsp90 inhibition included the up-regulation of the chaperone cassette regulated by HSF1. Among the novel responses observed (see Supplemental Table 1B), the authors went on to demonstrate that the down-regulation of histone acetyltransferase and arginine methyl transferase observed in the proteomics data were also apparent on Western blots, and were accompanied by deficiencies in cellular acetylation. A physical interaction between PRMT5 with Hsp90 was demonstrated, and subsequently, PRMT5 was confirmed to be slowly depleted from BT20 breast cancer cells treated with novel Hsp90 inhibitors [34].

Yao et al. [36] used silver-stained 2-D PAGE gels to assess ARPE-19 retinal pigment epithelial cell cultures treated for 16 hr with 3 μM 17-AAG. Spots showing apparent changes in expression of more than 1.5-fold were identified by peptide mass fingerprinting. Although Hsp90 and Hsp70 were not identifiable in the study, 94 other proteins showed altered expression, and 87 of these were identifiable. Although signal transducers were not readily apparent, the inhibitor-sensitive sub-proteome included components of pathways for glycolysis/gluconeogenesis, ubiquitinylation/proteosome degradation, oxidative stress, and cytoskeleton function.

Muroi et al. [35] used a gel-based approach to identify 20 HeLa proteins whose expression was altered by geldanamycin. This study is noteworthy due to its statistical assessment of changes in protein expression, analyzed by DIGE assays of biological replicates. However, the most important aspect of this study is its demonstration that proteomics fingerprints could be used to organize 21 different compounds into clusters based upon their impacts on the proteome. In this hierarchical cluster, radicicol and geldanamycin segregated into a distinct branch from the other compounds. Furthermore, the MG-132 inhibitor of the proteasome segregated into a different branch of the same hierarchical tree as Hsp90 inhibitors, reflecting both similarities and differences in their effects on cells. This successful and accurate classification of compounds into mechanistic families is an important proof of the principle that drug mechanisms can be elucidated on the basis of their impacts on the cellular proteome.

Falsone et al. [69] utilized another novel gel-based approach to study Hsp90 function in HeLa cells. They analyzed the impacts of dual treatments with proteasome inhibitors and radicicol, identifying 48 ubiquitinylated proteins that were recruited to the aggresome in response to radicicol and MG132. Among these proteins, 12 had been previously implicated in physical or function interactions with Hsp90, making the other 36 proteins high-probability candidates as physical or functional partners of Hsp90.

Schumacher et al. [70] used a gel-free isotope-coding approach (ICAT) to investigate the impact of geldanamycin in anaplastic large cell lymphoma (ALCL) cells. Based upon a single-pass analysis, the authors concluded that 68% of the quantifiable ALCL proteome was impacted by Hsp90 inhibition. At first glance, this impact seems inconsistent with current models for Hsp90 as a highly specific chaperone, and with results obtained in other studies. Similarly, there are several examples within their data set that conflict with previous reports of the effects of Hsp90 inhibitors. This may reflect protein misidentifications, since their false-discovery rate (7%) was fairly high by modern standards. However, it is also important to note that the affinity-selective ICAT approach appeared to sample a subset of the proteome, because several abundant proteins were not apparent in the dataset. Hence the relatively large drug impacts that they observed may have reflected a non-uniform sampling of the proteome by the affinity tag ICAT reagent. Moreover, the authors used Western blotting to confirm the drug-induced up-regulation of four of their proteins (USP9, GAP1, PCLN1, and STCN) and the down-regulation of four others (TNKS, SOCS4, NEMO, OPG). Their ICAT data also suggested the depletion of four kinases, and the somewhat surprising induction of three others. Moreover, certain themes in this protein data set are echoed in other proteomics studies. Also notable is that this study is one of the few proteomics assays to address very early cellular responses to Hsp90 inhibition, utilizing 12 hr drug treatments rather than the overnight drug treatments that are more typically employed.

Song et al. [71] used a gel-free isotope coding approach (iTRAQ) to characterize the effects of IPI-504 in a mouse xenograft model of tumor cell biology. In two different explant tumors, assays demonstrated that 2% of the detectable cellular proteome was depleted by drug treatment, while 4% was upregulated. Two of the proteins depleted (GOLPH2 and MHC class IB protein) and two of those induced (bigycan and galectin-3) were validated by immunohistochemical staining and by manual inspection of reporter ions in the primary MS/MS spectra. The most dramatic insight from this study was indications that tumor explants may respond to Hsp90 inhibition quite differently than the same cells cultured in vitro. Eleven protein kinases were quantified in their MS data, but showed no consistent response to the in vivo drug regimen. The only HSF1 cassette protein induced in vivo was Hsp70. Western blotting confirmed that several protein kinases (Her-2, p70S6K, AKT, MAPK) were not depleted in explants treated in vivo, but the same tumor cells cultured in vitro showed classic inhibitor-induced down regulation of these Hsp90 client proteins. Thus, both the proteomics assays and the traditional assays suggest cellular responses in vivo that challenge models derived from Hsp90 inhibition in cultured cells.

Zhao et al. [16] employed SGA and chemical-genetic screens of Hsp90 function to identify 451 candidate interactions with Hsp90, 49 of which were common to both functional assays. These overlapping 49 genes represent an especially high-confidence data subset. Surprisingly, however, no Hsp90-interacting proteins were identified as common to all four screens from the Houry group (two functional screens and two physical screens). None-the-less, when their aggregate set of functional interactions was compared to the aggregate set of physical interactions, 22 genes/gene products were capable of both binding to Hsp90 and compromising yeast growth.

McClellan et al. [18] also conducted a chemical-genetic screen for Hsp90-interacting proteins, winnowing their large data sets by focusing on the 5% of their mutant strains that showed the greatest growth defects. Additionally, they conducted their screens at both 30 degrees C and 37 degrees C, observing dramatic temperature-dependent differences in Hsp90's functional associations. Higher temperatures enrich Hsp90's interactions with stress proteins, signal transducers and cell cycle regulators, some metabolic proteins, and Hsp90's associations with protein components of the MTOC, cytokinesis, and bud assemblies. A comparison of the GS screens from the labs of Frydman [18] and Houry [16] shows that 78

of 316 total chemical-genetic hits were common to both labs. Other insights gained from these yeast screens are discussed more fully below.

Remily-Wood et al. [51] used multiple reaction monitoring (MRM) assays to measure the induction of selected heat shock proteins in RPMI-8226 multiple myeloma cells treated with 0.1 μM 17-DMAG. Peptides representing each protein were quantified to high degrees of precision and accuracy, and the results were compared to ELISA-based assays directed against the same target. Their assays demonstrated the drug-induced up-regulation of several isoforms of Hsp90 and Hsp70. This study is notable in several regards, and is discussed in more detail in our concluding remarks.

5. Compilation and annotation of Hsp90-interacting proteins

In order to assess proteomics contributions to our understanding of the Hsp90 interactome, we have complied data from the studies cited above. These compilations appear in the Supplementary Materials. The compiled tables are designed to be working tools for the reader, and include cross-references to gene aliases and hyperlinks to various database resources. Supplementary Table 1A lists gene products that bind to Hsp90 or Hsp90 heterocomplexes. Included in this list are human gene homologues of Hsp90-binding proteins that were identified in yeast. Supplementary Table 1B lists gene products identified to interact functionally with Hsp90. Included in this list are human gene homologues of yeast genes that induce SGA or chemical-genetic defects. Supplementary Table 1C lists the genes that demonstrate both physical and functional interactions. Similarly, we provide a compilation of proteins that interact with select Hsp90 co-chaperone partners (studies listed in Table 1B, [24, 61, 65, 72, 73]) in Supplementary Table 1D. Supplementary Tables 1E and 1F summarize canonical pathways that are enriched within the population of Hsp90-binding and Hsp90-dependent gene products, respectively. Supplementary Table 1G summarizes enriched GO biological process ontologies and pathways for human Hsp90 interactors.

Supplementary Table 2A and B contain lists of the gene products found to interact with TAP-tagged Hsc82 and Hsp82, respectively, and is largely identical to the lists originally published by Gong et al. [15]. However, we have added functional annotations for this yeast Hsp90-binding proteome, linking each gene product to the Sacchaomyces Genome Database (http://www.yeastgenome.org/). Furthermore, enriched GO Ontologies for Hsc82 and Hsp82 Interactors ($p<0.5$) are given in Supplementary Tables 2C and D, respectively. Enriched MIPS complexes interacting with Hsc/Hsp82 that were identified by Gong et al [15] are given in Supplemental Table 2E. A table summarizing the general physical properties, and the top ten protein Pfam domains and GO slim biological processes of Hsc/Hsp82 interactors derived by Gong et al [15] are given in Supplemental table 2F. Proteins that interacted with both Hsc82 and Hsp82 are collated in Supplemental Table 2G.

6. Discussion

6.1 Results may vary

Perhaps the most striking feature apparent in the human studies described above is the failure to re-discover numerous well-documented Hsp90 clients. This limitation is consistent with our appreciation that proteomics techniques usually assay only the most abundant proteins, that co-chaperone partners dominate the Hsp90 interactome, and that Hsp90 clients are expressed at low levels. Consistent with the apparent limitation in the human data, yeast genetic screens have yielded more than 1,000 Hsp90-binding proteins and more than 200 high-quality functional interactions.

The shallow depth of our current attempts in human cells could be predicted to amplify minor differences in assay performance, generating the appearance of disagreement between studies. Consistent with this interpretation, the greater coverage of the yeast Hsp90 interactome also yields much more overlap among yeast studies than has been obtained among human studies. Thus, the shallow coverage typical of human studies is one explanation for lack of congruence between these studies.

Another potential source of in variability between studies is that isoforms of Hsp90 are not functionally equivalent. The proteomics assays of Gong et al. [15] show that discrete yeast Hsp90 isoforms have distinct interactomes. The chemical genetic assays of McClellan et al. [18] demonstrate that deletion of heat-inducible isoform of yeast Hsp90 compromises growth in media containing Hsp90 inhibitor, while deletion of the yeast Hsp90 cognate does not. These distinctions are also consistent with findings that the human Hsp90 co-chaperone GCUNC45A can differentiate Hsp90α from Hsp90β [74], that recombinant mice deleted in specific Hsp90 isoforms manifest isoform-specific developmental defects [75], and with other evidence hinting at isoform-specific functions [76–79]. Thus, isolation of mammalian complexes containing Hsp90α versus Hsp90β are unlikely to give equivalent results, even when the complexes are isolated from the same cell line, yet studies in mammalian systems have not been designed to address the Hsp90 isoforms.

An additional source of variability is indicated by the findings of Gano and Simon [64], showing that the nucleotide-bound state of Hsp90 profoundly affects its interactions with co-chaperone partners and client proteins. However, the issue of Hsp90's alternative conformations has typically been ignored when assessing and discussing the Hsp90 interactome. Because the cellular ATP charge is almost immediately lost upon cell lysis, the Gano and Simon study predicts that most cell lysis protocols would be incapable of yielding the full complement of Hsp90 chaperone machinery, i.e., past studies may be biased toward identifying proteins that preferentially interact with ADP-bound or nucleotide-free conformations of Hsp90. Future probes of the Hsp90 interactome should acknowledge and accommodate Hsp90's complex conformational lifestyle.

To what extent do the incongruities apparent between studies reflect the different cell types assayed? While there have been numerous studies documenting changes in the expression of Hsp90 during development, there are few studies examining changes in co-chaperone expression. Certainly, we can reasonably predict that different tissues will show different panels of Hps90 clients, but are Hsp90's co-chaperone coteries regulated by development? Precedents hint that the answer is "yes." An analysis of the genomes of 19 disparate eukaryotic organisms indicates that they differ in the arrays of co-chaperones that they express [20]. Squirrel monkeys are glucocorticoid resistant because they express high levels of FKBP51, which reduces glucocorticoid receptor binding affinity [80, 81]. Changes in FKBP52 expression have been observed during differentiation of male germ cells [82]. Hsp90, Hsp70, STIP1/HOP and p23 levels change in reticulocytes during their maturation to erythrocytes [83]. Cancer cells are often observed to over-express chaperones, including Hsp90 and its subunits. McDowell and coworkers [84] examined the expression of Hsp90 chaperone proteins in 17 different tumor types versus normal tissue, finding that levels of expression of Hsp90α and β, Aha1, p23 and Tpr2 varied markedly between normal and tumor tissue. These points do not mean that proteomics assays have failed to deliver a congruent picture. Rather they argue that Hsp90 function encompasses degrees of complexity that we have only begun to appreciate.

Subtleties in experimental design and execution are another potential source of variability. Cellular responses to Hsp90 inhibition can readily be predicted to depend on the status of the cell population. Thus, sub-culturing routines, identity and doses of Hsp90 inhibitors applied,

and duration of drug exposure all have the potential to impact the Hsp90-dependent proteome. More dramatically, responses to Hsp90 inhibitor in vitro may vary considerably from responses in vivo [71], perhaps reflecting the surrounding matrix [85].

Some differences almost certainly reflect the different proteomics techniques used. In future initiatives, the potential impacts of technical differences can best be addressed by careful descriptions of all aspects of the experiment. This recommendation lies at the heart of modern proteomics guidelines.

6.2 Insights into Hsp90 function

Our compilation of Hsp90's interactions with the cellular proteome suggests that Hsp90 should no longer be viewed as just the "signal transduction" chaperone. Rather, Hsp90 appears to contribute to diverse cellular processes and pathways. This is consistent with Hsp90's emerging role in the function of complex proteins machines such as those involved in RNA processing, RNP assembly, and chromatin remodeling (see additional articles in this issue). However, it is worth noting that proteomics assays first revealed Hsp90's involvement therein. Stories that have emerged from yeast studies are also apparent in the human Hsp90 interactome. (Table 3). While disruptions in signal transduction pathways remain a potential explanation for the wider impacts discussed below, we begin to suspect more direct roles for Hsp90 in these processes.

6.2.1 Hsp90 and glucose metabolism—An analysis of canonical cellular pathways that contain protein components that interact directly or functionally with Hsp90 reveals highly significant enrichment in enzymes involved in glycolysis and gluconeogenesis. Cancer cells are well known to have increased aerobic glycolysis, a phenomenon known as the Warburg effect [86, 87]. Cancer cells also commonly over-express Hsp90, suggesting that such over-expression might contribute to the Warburg effect. This contribution could be either through direct interactions between Hsp90 and glycolytic enzymes, or via indirect impacts on glucose metabolism due to altered signal transduction. In this vein, the well characterized Hsp90-dependent proteins Akt, HIF1α, receptor tyrosine kinases (e.g., erbB2) and Src have been linked to the Warburg effect through their ability to enhance the expression and/or activity of glucose transporters and glycolytic enzymes [86, 87]. A recent report indicates that treatment of ARPE-19 cells with 17-AAG leads to a decrease in the levels of key glycolytic enzymes [36]. This effect can be hypothesized to be a result of the loss of Akt and HIF1α, leading to the transcriptional suppression of the genes encoding these enzymes. Alternatively, Hsp90 may interact more directly with the glycolytic enzymes compiled in the Supplement. Regardless of the finer mechanisms, evidence that Hsp90 interacts with the cellular machinery involved in glycolysis and gluconeogenesis suggests that some of the tumoricidal activity of Hsp90 may be due to diminution of glycolysis.

6.2.2 Hsp90, the cytoskeleton and intracellular transport—Proteomics assays and genetic HTP analyses indicate that Hsp90 interacts with intracellular trafficking machineries. In yeast, the Hsp90 interactome is significantly enriched in genes/gene products involved in intracellular vesicle trafficking and Golgi transport [16, 18]. As previously noted [18], these Hsp90-interacting gene products represent virtually all exocytic and endocytic secretory pathways in yeast, including ER quality control, ubiquitin-regulated protein trafficking components, and in ESCRT multivesicular sorting pathway. In support of these conclusions drawn from proteomic data, Okiyoneda et al. [88] have recently characterized the components of the Hsp90 chaperone machine that compose part of the peripheral quality control system involved in the degradation of damaged plasma membrane proteins. Furthermore, a role for Hsp90-regulated quality control and translocation of transmembrane ion channels (CTFR and hERG, [89–91]) and receptors (MC4R, [92]) at the

ER is now established. Similarly, the protein:protein interaction networks of steroid hormone receptors and Hsp90 chaperone machineries intersect at genes encoding intracellular trafficking proteins [93]. Hsp90's role in intracellular trafficking is also apparent in the Sba1/p23 protein interaction network proposed by Brian Freeman [94]. Here, our compilation of human and yeast Hsp90-interacting proteins implicates 62 human trafficking proteins and 43 human cytoskeleton proteins as part of the human Hsp90 interactome. Thus, it is highly likely that roles for yeast Hsp90 in trafficking and cytoskeleton functions are conserved in human cells.

Because mechanisms behind Hsp90's role in these processes are largely unknown, we have grouped them into a single concept of trafficking/cytoskeleton. This conceptual grouping acknowledges the potential for overlaps between these cellular networks. The overlap between these networks is supported by past and current work from the laboratories of William Pratt and Mario Galigniana that has established a role for cytoskeletal components in the Hsp90-dependent nucleo-cytoplasmic trafficking of steroid hormone receptors (reviewed in [95] and [96]). A number of mechanisms could account for how Hsp90, constituents of the cytoskeleton and vesicular trafficking proteins could be found to functionally interact. For instance, inhibition of Hsp90 could directly inhibit cytoskeleton function, subsequently contributing to synthetic lethality when combined with a deletion in a vesicle trafficking gene product. In this regard, Hsp90's interaction with the mammalian NudC protein is noteworthy [25, 64], because NudC is widely suspected to chaperone the assembly of dynein motor complexes [97–102]. Hsp90's role in chaperoning myosin motors is similarly noteworthy [103–105]. It is also important to appreciate that the endoplasmic reticulum-specific Hsp90 isoform GRP-94/endoplasmin binds to most, if not all, Hsp90 inhibitors [106–108]. However, yeast do not express recognizable homologs of NudC or GRP94, suggesting alternative mechanisms in yeast, e.g., Hsp90 chaperoning of molecular motors or indirect dysregulation due to titration of Hsp90's role in signal transduction. While Hsp90's roles in supporting cytoskeleton and trafficking processes are largely unknown, proteomics assays to date provide strong evidence for an Hsp90 connection.

6.3 Future Prospects

Proteomics is often described as a hypothesis-generating discipline, and studies to date have delivered several new hypothetical functions for Hsp90 and new models for its regulation. Many of these hypotheses will require a deeper qualitative and quantitative understanding of the human Hsp90 interactome, and the interactomes of its co-chaperone partners. Most pressing, perhaps, is the need for proteomics assays capable of mining Hsp90's clientele.

This depth may be obtainable using orthogonal fractions of the Hsp90 interactome (e.g., MuDPIT or GeLC-MS), or using techniques such as ion mobility mass spectrometry to further fractionate the ion stream. Because such fractionations can induce technical variability, they will likely require isotope-coding strategies that allow samples to be mixed prior to chromatographic separation. MS instrumentation and proteomics software themselves continue to advance at a breathtaking pace (e.g., [109]), and will certainly enhance our penetration into the Hsp90 interactome. Despite the exact technologies utilized, however, the next generation of Hsp90 proteomics studies will require enhanced quantitative rigor, featuring the binding controls, biological replicates, and statistical validations necessary to firmly differentiate meaningful cellular responses from technical noise.

One of the main challenges now raised is that of differentiating direct relationships from indirect relationships. In many HTP datasets, it is not possible to differentiate direct relationships (e.g., co-chaperones and Hsp90:client relationships) from indirect relationships (e.g., transcriptional impacts and Hsp90-dependent nodes within larger pathways). To date, this differentiation has required traditional, tightly focused characterizations of individual

Hsp90-associated gene products. An HTP method for making this distinction is greatly needed.

Proteomics assays of Hsp90 function also hold promise in addressing pharmacodynamic discrepancies observed between tumors in vivo vs. tumor cell cultures [71, 85], and the poor predictive potential of peripheral blood mononuclear cells currently serving as pharmacodynamic proxies *in vivo* [110]. This promise is apparent in the work of Remily-Wood et al. [51], demonstrating that it should be wholly feasible to monitor tumor biopsy specimens for specific *in vivo* responses to Hsp90 inhibition. Targeted high-sensitivity MS-based assays hold similar promise for other questions that require efficient assessment of limited numbers of cells, e.g., microdissections of tumors, tumor stem cells, and tumor specimen banks. Achieving this promise will require initial surveys to discover high-confidence, readily detectable peptides representing proteins that respond to Hsp90 inhibition, followed by rigorous validation of these peptides as quantitative pharmocodynamic reporters.

Researchers utilizing a variety of proteomics techniques have provided an illuminating picture how Hsp90 and its regulatory subunits support protein folding in vivo. None-the-less, a great deal of opportunity remains. Additional proteomics assays of Hsp90's functions and partners have the potential to answer mysteries old and new, but will require careful appreciation of the pivotal parameters identified in studies performed to date.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Summary of findings from past proteomic studies examining the Hsp90-interactome.
- Critical assessment of methodologies used in past Hsp90 proteomic studies.
- Discussion of new insights gained from proteomic studies into Hsp90 function.
- Future perspectives on studies of Hsp90 proteomics: reaching the next level.

Table 1

Identification of proteins that interact directly with the Hsp90 machine.

Table 1A: ***Summary of studies to identify Hsp90 interacting proteins***

Studies	Method	Separation	Identification	Cell System
Falsone et al [62]	Co-IP, anti-Hsp90 antibody	1D SDS-PAGE	Nano-HPLC–MS/MS	HEK293
Gano & Simon [64]	TAP-tagged Hsp90α	Solution digest	Nano-HPLC–MS/MS with spectral counting	HEK293T
Zhao et a [16]l	TAP-tagged Hsc82 bait 2-Hybrid screen Hsp82 bait	1D SDS-PAGE[1] Solution digest[2]	Peptide mass fingerprinting MALDI- TOF[1] Nano-HPLC–MS/MS[2]	S. cerevisiae
Millson et al [23]	2-Hybrid screen Hsp82-bait			S. cerevisiae
Skarra et a [61]l	Flag-Hsp90α pull down	Solution digest	Nano-HPLC–MS/MS with spectral counting	HEK293
Te et al [25]	Co-IP, anti-Hsp90 antibody Hsp90α CT-agarose capture	1D SDS-PAGE 2D PAGE gels	Peptide mass fingerprinting MALDI-TOF	Jurkat
Tsaytler et al [63]	Co-IP, anti-Hsp90 antibody Hsp90β-agarose pull down Biotin-geldanamycin capture	1D SDS-PAGE	Nano-HPLC–MS/MS	A431 human epidermoid carcinoma cells
Wang et a [26]l	Co-IP anti-Hsp90α antibody	2-D PAGE gels	MS	HCT-116
Gong et a [15]l	TAP-tagged Hsp90 or other molecular chaperones	1D SDS-PAGE[1] Solution digest[2]	Peptide mass fingerprinting MALDI-TOF[1] Nano-HPLC–MS/MS[2]	S. cerevisiae

Table 1B. ***Summary of studies to identify proteins that interact with Hsp90 co-chaperones***

Studies	Method & cochaperone target	Separation	Identification	Cell System
Millson et al [24]	2-Hybrid screen: Cdc37, CNS1, Sba1/p23			S. cerevisiae
Park et al [72]	TAP-tagged Cyclophilin 40	1D SDS- PAGE	Nano-HPLC–MS/MS	HeLa
Echeverria et al [65]	Co-IP, anti-p23 antibody	1D SDS- PAGE	Nano-HPLC–MS/MS	T. gondii
Skarra et al. [61]	Flag-PP5 pull down	Solution digest	Nano-HPLC–MS/MS with spectral counting	HEK293
Song et al [73]	Co-IP, anti-Sti1/HOP antibody	1D SDS- PAGE	Nano-HPLC–MS/MS	C. elegans

Studies utilizing affinity purification techniques to isolate Hsp90 (A) or co-chaperone complexes are listed above together with studies using two-hybrid screens to identify interacting proteins. The table summarizes the methods used to capture the complexes (Co-IP, co-immunoprecipitation; TAP, tandem affinity purification), separate components of the complexes, identify those components and the organism or cell line used for the study.

Table 2

High Throughput Assays of Functional Interactions with Hsp90.

Study	Separation	Quantitation	Protein Identification	System
Malony et al. [34]	2-D PAGE	silver	MS	17-AAG in A2780 cultures
Muroi et al. [35]	2-D PAGE	DIGE	MS	GA and radicicol in HeLa cultures
Falsone et al. [69]	2-D PAGE	Coomassie R- 250	MS/MS	MG132 vs. aggresome in HeLa cultures
Shumacher et al. [70]	orthogonal chromatography	ICAT	MS/MS	GA in ALCL cultures
Song et al. [71]	orthogonal chromatography	iTRAQ	MS/MS	IPI-504 in vivo
Yao et al. [36]	2D-PAGE	silver	MS	17-AAG in ARPE-19 cells
Remily-Wood et al. [51]	SDS-PAGE	multiple reaction monitoring	LC-MRM	17-DMAG in cancer cell lines
McClellan et al. [18]	Not applicable	Not applicable	Chemical genetic screen	S. cerevisiae
Zhao et a.. [16]	Not applicable	Not applicable	Synthetic lethal & chemical genetic screens	S. cerevisiae

Studies using screens to identify gene products that interact functionally with Hsp90 are listed above. The methods by which those components were separated, quantitated, and identified, together with the cell line used for the study are summarized. For definition of acronyms see “Abbreviations”.

Table 3

Enriched GO terms and pathways for human Hsp90 interacting proteins presented in Supplementary Table 1

Enriched Biological Process GO Terms for Human Direct and functional interactors[1]			
GO Term BP	**Genes**	**GO Term BP**	**Genes**
protein folding	33	RNA splicing	18
mitotic cell cycle	42	post-translational protein modification	50
regulation of ubiquitin-protein ligase activity	19	mitochondrial outer membrane translocase complex assembly	3
response to unfolded protein	16	cellular localization	40
cell cycle	52	epidermal growth factor receptor signaling pathway	5
glycolysis	12	response to stress	64
organelle organization	72	regulation of protein complex disassembly	6
translation	26	nuclear export	6
regulation of molecular function	52	somatic cell DNA recombination	4
regulation of programmed cell death	44	cell proliferation	20
cellular macromolecular complex assembly	23	telomere maintenance	4
cytoskeleton organization	28	cytoskeleton-dependent intracellular transport	5
intracellular transport	36	protein targeting to mitochondrion	4
organelle fission	17	post-Golgi vesicle-mediated transport	5
microtubule-based process	18	DNA replication	10
RNA transport	10	regulation of MAP kinase activity	9
Enriched Pathways			
KEGG_PATHWAY Sublist Term	**Genes**	**Sublist Term**	**Genes**
Proteasome	15	Prion diseases	6
Glycolysis/Gluconeogenesis	13	Pancreatic cancer	8
Antigen processing and presentation	13	Adherens junction	8
Pyruvate metabolism	9	Propanoate metabolism	5
Gap junction	13	NOD-like receptor signaling pathway	7
Ribosome	12	Glioma	7
Pentose phosphate pathway	6	Tight junction	11
DNA replication	7	Insulin signaling pathway	11
Spliceosome	13	Epithelial cell signaling in Helicobacter pylori infection	7
MAPK signaling pathway	21	Regulation of actin cytoskeleton	15

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***Enriched Biological Process GO Terms for Human Direct and functional interactors*[1]**

GO Term BP	**Genes**	**GO Term BP**	**Genes**
Mismatch repair	5	Cell cycle	10
Panther Pathway Sublist Term	**Genes**	**Sublist Term**	**Genes**
P00049:Parkinson disease	15	P00024:Glycolysis	5
P00060:Ubiquitin proteasome pathway	12	P00029:Huntington disease	12
P02776:Serine glycine biosynthesis	3	P00016:Cytoskeletal regulation by Rho GTPase	9
P00006:Apoptosis signaling pathway	12	P00025:Hedgehog signaling pathway	5
Reactome Pathway Sublist Term	**Genes**	**Sublist Term**	**Genes**
REACT_11045:Signaling by Wnt	20	REACT_578:Apoptosis	21
REACT_13635:Regulation of activated PAK-2p34 by proteasome	16	REACT_6185:HIV Infection	24
REACT_383:DNA Replication	22	REACT_13:Metabolism of amino acids	22
REACT_6850:Cdc20:Phospho-APC/C mediated degradation of Cyclin A	18	REACT_1762:3′-UTR-mediated translational regulation	17
REACT_152:Cell Cycle, Mitotic	41	REACT_6167:Influenza Infection	20
REACT_9035:APC/C:Cdh1-mediated degradation of Skp2	17	REACT_71:Gene Expression	34
REACT_1538:Cell Cycle Checkpoints	22	REACT_12508:Metabolism of nitric oxide	4
REACT_17015:Metabolism of proteins	30	REACT_1505:Integration of energy metabolism	20
REACT_474:Metabolism of carbohydrates	18	REACT_15380:Diabetes pathways	22

Human genes were compiled from Supplementary Tables 1A and B, and analyzed for enrichment in GO biological processes, and KEGG, Panther and Reactome Pathways utilizing DAVID Bioinformatics Resources 6.7 (NIAID).

[1]Selected GO terms are listed to remove redundancy and highlight major enriched biological processes (from Supplemental Table 1G)