The protein chaperone HSP90 can facilitate the divergence of gene duplicates

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Running title: HSP90 and duplicate gene divergence

Key words: chaperone, paralog evolution, brassinosteroid pathway, cryptic genetic variation, robustness

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Abstract

The heat shock protein 90 (HSP90) acts as a chaperone by ensuring proper maturation and folding of its client proteins. The HSP90 capacitor hypothesis holds that interactions with HSP90 allow proteins to accumulate mutations while maintaining function. Following this logic, HSP90 clients would be predicted to show relaxed selection compared with non-clients. In this study, we identify a new HSP90 client in the plant steroid hormone pathway: the transcription factor BES1. Its closest paralog, BZR1, is not an HSP90 client. This difference in HSP90 client status in two highly similar proteins enabled a direct test of the capacitor hypothesis. We find that BES1 shows relaxed selection compared to BZR1, hallmarks of neo- and subfunctionalization, and dynamic HSP90 client status across independent evolutionary paths. These results suggested that HSP90’s influence on gene evolution may be detectable if we compare gene duplicates, because duplicates share most other properties influencing evolutionary rate that might otherwise conceal the chaperone’s effect. We test this hypothesis using systematically-identified HSP90 clients in yeast, and observe a significant trend of HSP90 clients evolving faster than their non-client paralogs. This trend was not detected when yeast clients and non-clients were compared without considering paralog status. Our data provide evidence that HSP90 influences selection on genes encoding its clients and facilitates divergence between gene duplicates.
Introduction

The phenotypic capacitor HSP90 is thought to influence evolutionary processes through its ability to both conceal and release genetic variation (JAROSZ and LINDQUIST 2010; QUEITSCH et al. 2002; RUTHERFORD and LINDQUIST 1998; YEYATI et al. 2007). Perturbation of this conserved and essential chaperone reveals cryptic genetic and epigenetic variation in flies, plants, fish, and yeast (JAROSZ and LINDQUIST 2010; QUEITSCH et al. 2002; RUTHERFORD and LINDQUIST 1998; SOLLARS et al. 2003; YEYATI et al. 2007). In worms, HSP90 affects the penetrance of partial loss of function mutations (BURGA et al. 2011). As expected under the capacitor hypothesis, worms with naturally lower HSP90 levels show significantly higher mutation penetrance (CASANUEVA et al. 2012). HSP90-dependent variation can be revealed by moderate environmental stress alone, providing a plausible release mechanism for this concealed variation in nature (JAROSZ and LINDQUIST 2010; QUEITSCH et al. 2002; RUTHERFORD and LINDQUIST 1998). We showed previously that HSP90-dependent variation is common in natural plant populations, implicating the chaperone as an important player in shaping phenotype and evolutionary trajectories (SANGSTER et al. 2008a; SANGSTER et al. 2008b). Together, these findings have prompted a longstanding debate about the importance of HSP90 in evolutionary processes and the magnitude of its effect (BERGMAN and SIEGAL 2003; MEIKLEJOHN and HARTL 2002; RANDO and VERSTREPEN 2007).

It is well-established that HSP90 recognizes metastable proteins and facilitates their folding and stability (TAIPALE et al. 2010). Recent studies demonstrate that protein stability is a major constraint on protein evolution (BLOOM et al. 2006; PENA et al. 2010). Stable proteins tend to evolve faster as they can explore greater sequence space without losing function (BLOOM et al. 2006; PENA et al. 2010).
In prokaryotes, overexpression of the chaperonin GroEL/ES allows the evolution of a far greater number of highly active enzyme variants by compensating for their reduced stability (TOKURIKI and TAWFIK 2009). We hypothesized that in eukaryotes HSP90 may facilitate gene divergence by similarly relaxing constraints on protein stability. If so, HSP90 clients should show greater tolerance to mutations and evolve faster than non-clients facing similar evolutionary pressures. To address HSP90’s role in gene evolution in this context, we focused on recent gene duplicates, which initially face similar selection pressures and encode proteins of similar stability. We used the brassinosteroid (BR) pathway in Arabidopsis thaliana as an experimental model because every step in BR signaling is encoded by gene families (KIM and WANG 2010) and previous studies suggested that the BR pathway may require HSP90, although no HSP90 client had been identified (SANGSTER et al. 2007; SANGSTER and QUEITSCH 2005).

We demonstrate here that only one of two paralogous transcription factors in the BR pathway is an HSP90 client and that its encoding gene shows relaxed purifying selection compared to its non-client paralog. Gene duplicates diverge through sub- and neofunctionalization. Consistent with subfunctionalization, only the HSP90 client is temperature sensitive; consistent with neofunctionalization, the gene encoding the HSP90 client contains a novel exon and non-synonymous polymorphisms in divergent A. thaliana strains. HSP90-facilitated divergence of gene duplicates is widespread, because in the yeast Saccharomyces cerevisiae, genes encoding HSP90 clients tended to evolve faster than those encoding their non-client paralogs. Together, our data provide strong evidence for HSP90-facilitated evolution in extant genomes and hence strong support for the capacitor hypothesis.
Materials and Methods Summary

Plant growth conditions and treatments. Columbia-0 (Col-0) was used as wild type (WT). *bes1-D, bzrl-D, DWF4-ox, bin2-1, BRII-ox, and bes1-2 (WiscDsLox246D02)* were in the Columbia-0 (Col-0) background. The *bes1-D* mutant is a recapitulation line using a transgene to constitutively express the mutant form of *bes1-D* in a Col-0 background. Seedlings were grown for seven days on media with DMSO (mock) or geldanamycin, brassinolide, and brassinazole, dissolved in DMSO. Statistical significance of response of hypocotyl length of seedlings from 2-4 replicates of 10-60 seedlings was determined using standard least square linear regression.

Biochemistry. For western blot, seven-day-old seedlings, grown in red LED light, were ground in liquid nitrogen. Buffer (0.15M Tris pH 6.8) was added, and extracted protein was quantified using Bradford’s assay. Proteins were resolved using SDS-PAGE, transferred to nitrocellulose and probed with anti-BES1 antibody. For co-immunoprecipitation, ground rosette tissue was used. Extracted protein was incubated with Protein L Agarose, which was pre-incubated with anti-HSP90 3G3 antibody. Beads were pelleted and washed in buffer. Anti-BES1 antibody was used to detect BES1 in the input and pellet.

Phylogenetic tree and dN/dS analysis. Sequences for *BZR/BEH* family members in available sequenced plants were acquired from http://phytozome.net v5.0 from a BLAST search for gene families with similarity to *BES1*. MUSCLE 3.7 was used for amino acid alignment of the identified sequences, and Gblocks was used to remove regions with poor conservation. The remaining 86 sequences were re-aligned and neighbor-joining was used to create a distance tree. For the *BZR/BEH* tree, the outgroup was identified as a *BZR/BEH* family member that was closely related, but an outgroup to all *A. thaliana* *BZR/BEH* family members. Sequences were aligned in MUSCLE 3.7 and PhyML was used for maximum likelihood tree (GUINDON and
For dN/dS analysis, codeml from PAMLv4.4b was run using models 0, 1, and 2 (BIEŁAWSKI and YANG 2003).

**Yeast data analyses.** Published HSP90 interactors were used (ZHÁO et al. 2005). The branch length of HSP90 interactors in three-member and two-member families was obtained from Ensembl Compara (release 61). Supplementary Materials and Methods can be found in File S1.

**Results**

**BES1 IS AN HSP90 CLIENT**

Inhibition of HSP90 yields a wide variety of morphological phenotypes in *A. thaliana* plants (MCLELLAN et al. 2007; QUEITSCH et al. 2002; SANGSTER et al. 2007; WHITESSELL et al. 1994). Among these phenotypes, we previously noted severely dwarfed plants, which closely resembled known BR mutants. To directly test whether the BR pathway requires HSP90 function, we grew seedlings in the presence of exogenous BR (brassinolide, the most biologically active BR) with and without the highly specific HSP90 inhibitor geldanamycin (GdA) (QUEITSCH et al. 2002). Inhibition of HSP90 function significantly interfered with response to BRs (Figure S1A, \( R^2=0.68, p<0.0001 \), linear regression model, standard least square fit). Consistent with this finding, GdA also reduced seedling response to brassinazole, an inhibitor of BR biosynthesis (ASAMI et al. 2000), (Figure S1B, red light, \( R^2=0.76, p<0.0001 \); figure S1C, dark \( R^2=0.81, p<0.0001 \)).

We next addressed what step in the BR signaling pathway was most responsive to a loss of HSP90 function. The best-characterized HSP90 clients are the mammalian steroid hormone receptors and kinases (PICARD et al. 1990; TAIPALE et al. 2010; WHITESSELL et al. 1994). The
most common clients are transcription factors (TAIPALE et al. 2010). In A. thaliana, only a few clients are known, none of which function in the BR pathway (HUBERT et al. 2003; IKI et al. 2010; ISHIGURO S 2002; TAKAHASHI et al. 2003). As HSP90 clients do not share a common sequence or structural motif, client status is typically determined by a combination of genetic and biochemical analyses. Here, we took advantage of several well-characterized mutants in the BR pathway to test their response to HSP90 inhibition. We focused on the most likely clients: the steroid hormone receptor kinase BR INSENSITIVE1 (BRI1) and the transcription factors BES1 and BZR1 (WANG et al. 2001). To distinguish between HSP90 effects on BR signaling versus BR synthesis, we included a mutant in DWARF4 (DWF4), an enzyme that catalyzes a rate-limiting step of BR biosynthesis. Well-characterized gain-of-function mutants were used to bypass the extensive redundancy in the BR pathway. As had been shown previously, each mutant significantly increased hypocotyl length (HE et al. 2005) (Figure 1A, B). Upon inhibition of HSP90 with 0.5 µM GdA, BRI1-ox, DWF4-ox, and bzr1-D seedlings responded like wild-type seedlings (BRI1-ox, p=0.22; DWF4-ox, p=0.99, bzr1-D, p=0.6273), whereas bes1-D seedlings showed significant hypersensitivity to HSP90 inhibition (p<0.0001, table S1, figure 1A-C). bes1-D hypersensitivity to GdA suggests that BES1 may be an HSP90 client, while the wild-type response of bzr1-D mutants to GdA argues against an HSP90 client status for BZR1. Notably, the dominant bes1-D and bzr1-D mutants carry the identical amino acid change from proline to leucine (ALBRECHT et al. 2008; GUO et al. 2009; LI et al. 2009; TANG et al. 2011; WANG et al. 2002; YIN et al. 2002). The bes1-D mutant used here is expressed from a constitutive 35S CaMV promoter, providing an alternative explanation for the increased GdA sensitivity of bes1-D. Further increases in GdA levels yielded a significant response in BRI1-ox, DWF4-ox (BRI1-ox, p<0.0001; DWF4-ox, p=0.0003), but not in bzr1-D seedlings (p=0.5918, table S1), suggesting
that there may be additional HSP90 targets upstream or alongside the transcription factors tested
here (Figure 1A, B). Similar results were obtained in the dark (Figure S2A, B). Together these
data suggest that BES1, but not BZR1, is an HSP90 client.

Another criterion for HSP90 client status is proof of physical interaction (Taipale et al. 2010).
Using a co-immunoprecipitation assay with an HSP90-specific antibody, we found that BES1
physically interacts with HSP90 in plants (Figure 1D). In the absence of BRs, BES1 is negatively
regulated by BIN2 and several related kinases (Vert and Chory 2006). Cellular perception of
BRs triggers inhibition of BIN2 and activates BES1. Hypophosphorylated, active BES1 can be
detected as a fast mobility band on western blots (Yin et al. 2002) (Figure 1E). In this form,
BES1 interacts with other transcription factors, binds DNA, and promotes plant growth (Yin et
al. 2005). In our studies, BES1 can interact with HSP90 independent of phosphorylation, as we
were able to pull-down faster and slower mobility BES1 bands (Figure 1D).

While BRs and GdA have opposite effects on plant growth, and likely on BES1 function,
inhibition of HSP90 produced a similar shift in BES1 mobility as BR treatment (Figure 1E). Like
other well-established clients, such as the Drosophila Argonaute Piwi and the human
transcription factor HSF, BES1 was not degraded upon HSP90 inhibition (Figure 1E)
(Gangaraju et al. 2011; Zou et al. 1998). It appears that while the hypophosphorylated form of
BES1 accumulated upon HSP90 inhibition, it is non-functional (Figure 1A-D). Our data suggest
that BES1 may require HSP90 for its activation, perhaps by facilitating BES1 dimerization,
promoting nuclear translocation, or interfering with phosphorylation by BIN2. If GdA indeed
reduced the functional pool of BES1, we predicted that any increase in BIN2 function would
sensitize plants to inhibition of HSP90. \textit{bin2-1} mutants are semi-dominant hypermorphs with increased levels of phosphorylated BES1 leading to strongly reduced BES1 activity and repressed BR signaling (Kim and Wang 2010). As predicted, loss of HSP90 activity sensitized seedlings to a gain of BIN2 activity, as evidenced by a dramatically increased proportion of severely dwarfed seedlings in a segregating population of \textit{bin2-1} mutant seedlings (Figure S2C). These results also suggest that BIN2 does not require HSP90 activity for its function; such a scenario would lead to suppression not enhancement of the \textit{bin2-1} phenotype.

\textbf{HSP90 FACILITATES THE DIVERGENCE OF GENE DUPLICATES}

If HSP90 indeed allows its clients to explore a wider range of sequence space, the \textit{BES1} gene would be predicted to show evidence of relaxed selection compared to the \textit{BZR1} gene. We created a phylogenetic tree of the \textit{A. thaliana BZR/BEH} gene family using a gene from \textit{Aquilegia coerulea} as an outgroup (Figure 2A, S3). \textit{BES1} and \textit{BZR1} are the most recently diverged paralogs among the six \textit{A. thaliana BZR/BEH} family members, with 88\% amino acid identity (Wang et al. 2002). We determined the ratio of the rate of non-synonymous substitutions to the rate of synonymous substitutions (dN/dS) for \textit{BES1} and \textit{BZR1}, under a model allowing all branches of the \textit{A. thaliana BZR/BEH} tree to evolve at different rates. Consistent with our prediction, the gene encoding the HSP90 client \textit{BES1} (dN/dS=0.09) shows relaxed purifying selection compared to the gene encoding the non-client \textit{BZR1} (dN/dS=0.04) (Figure 2A). As \textit{BES1} and \textit{BZR1} diverged recently and only differ in a small number of amino acids, their difference in dN/dS was not significant. However, we took advantage of the entire \textit{BEH} gene family to test whether \textit{BES1} shows a different evolutionary rate compared to other family members. Using a maximum likelihood approach (Bielawski and Yang 2003), we showed that
BES1 indeed exhibits a significantly different evolutionary rate, if all other branches are assumed to evolve at the same rate ($2\delta=5.232, \text{df}=1, p=0.0222$). In contrast, no significant difference was found for the evolutionary rate of BZRI under the same assumptions ($2\delta=3.244, \text{df}=1, p=0.0717$).

To address whether HSP90 clients generally evolve faster, we analyzed a data set of systematically identified *Saccharomyces cerevisiae* HSP90 clients (ZHAO et al. 2005). Proteins that physically interact with HSP90 by tandem affinity purification-tagged (TAP) mass spectrometry (ZHAO et al. 2005) were considered as likely HSP90 clients. Likely HSP90 co-chaperones, identified by the TPR domain (WEGELE et al. 2004), were removed from the analysis. Unlike clients, co-chaperones interact with HSP90 through the TPR domain and modulate HSP90 activity. As expected, the evolutionary rates of HSP90 clients did not differ significantly from all other yeast genes (Figure S4A). Factors such as differences in selection pressure, protein stability, or codon bias, among others, likely obscure any impact on evolutionary rate by HSP90. To compare genes well-matched for these factors, we identified gene duplicates in which one paralog encoded a likely HSP90 client. Consistent with our results for BES1, genes encoding yeast HSP90 clients showed significantly longer branch length than their respective closest paralog (Figure 2B, table S2, $n=13$, 95% confidence interval 1.02-1.64, $p=0.002$, one-sample Wilcoxon test, testing the deviation from the expected ratio of client/non-client branch length of 1). Likely non-clients, identified by synthetic genetic interaction with an *hsp90* mutation (SGI), did not show this trend (Figure S4B, $n=27$, $p=0.97$). We then tested a more stringent situation. In cases in which yeast HSP90 interaction status was the derived state (i.e., not present in a common ancestor), three out of four HSP90 clients showed longer branch length...
than their respective closest paralog (Figure 2B, three-member families). Next, we addressed whether these differences in evolutionary rate were due to expression differences between clients and their respective non-client paralogs. Genes that evolve faster tend to be expressed at a lower level (Drummond et al. 2005). In contrast, many clients are significantly higher expressed than their non-client paralogs across nearly 200 environmental conditions (Swarbreck et al. 2008) (Table S2, figure S5). We observed no correlation of expression levels and branch lengths between clients and their respective paralogs (Figure S4C, figure S5, table S2). Taken together, our data suggest that HSP90 can facilitate the divergence of gene duplicates in yeast and plants.

HSP90 CLIENT BES1 SHOWS HALLMARKS OF SUB-AND NEOFUNCTIONALIZATION
Evolutionary theory holds that after gene duplication, one copy dies off quickly or changes function (Conant and Wolfe 2008). A surviving gene copy can retain part of the ancestral gene function, such as expression in fewer tissues or under certain environmental conditions (subfunctionalization) and/or acquire a novel beneficial function (neofunctionalization) (Conant and Wolfe 2008). An obvious subfunctionalization path for an HSP90 client is loss of function under environmental conditions that challenge HSP90 chaperone activity, such as increased temperature. HSP90 clients are typically less stable than other proteins and hence lose function at increased temperature despite induced HSP90 expression (Taipale et al. 2010). We grew seedlings at 27°C, a temperature known to challenge HSP90 function but not induce heat stress in A. thaliana (Queitsch et al. 2002). The temperature response of Bri1-ox, DWF4-ox, and bzr1-D mutants closely resembled the response of wild-type seedlings (Figure 3D). In contrast, not one of more than 100 bes1-D seeds in multiple independent experiments germinated at 27°C (Figure 3D, E). This germination phenotype was completely suppressed at standard growth
conditions (22°C) (Figure 3D, E, figure S2D). The loss of BES1 function at moderately elevated temperature, likely a direct result of challenged HSP90 function, is strong support for subfunctionalization.

If genes encoding HSP90 clients evolve faster than their non-client paralogs, neofunctionalization may be facilitated. Although there appears to be extensive redundancy between the HSP90 client BES1 and the non-client BZR1 (Yin et al. 2005), we found evidence of BES1 neofunctionalization. First, the major BES1 splice variant At1g19350.3 encodes a novel exon not found in BZR1 or any of the other BEH/BZR family genes (Figure 3A). This exon shares significant homology with intergenic regions on chromosomes 3 and 5 (Figure 3B). This exon together with adjacent intron sequence has additional matches to the 5’UTR BZR1 sequence and to another intergenic region (Figure 3C). Gene chimeras are a hallmark of neofunctionalization (Hahn 2009), and these findings suggest that distant genomic regions may have contributed to the novel BES1 exon.

Second, we found that BES1 polymorphisms across divergent A. thaliana accessions were significantly associated with the phenotypic variation these strains showed in response to HSP90 inhibition. Wild A. thaliana accessions harbor considerable genetic variation, yet due to A. thaliana’s inbreeding life-style, individual accessions are nearly isogenic. Sensitivity to HSP90 inhibition varied dramatically among accessions (Figure 4A). Some accessions grouped with the hypersensitive bes1-D mutant (Figure 4A, red bar), whereas others responded very little. Accessions that grouped with the hypersensitive bes1-D mutant contained three intronic polymorphisms; but this association was not significant (Figure 4A, B, light-blue). In contrast,
we found significant associations between decreased sensitivity to HSP90 inhibition and two other BES1 polymorphisms (Figure 4B, figure S6A). The first polymorphism is a frameshift mutation in the strains Zdr-6 and Tottarp-2 that results in an early stop codon (Figure 4A, black bars, figure 1E). Consistent with a severely truncated BES1 protein, these strains responded little to HSP90 inhibition. The second polymorphism was found in four different strains (Figure 4A, green bars). This synonymous polymorphism in the 5’ end of BES1 alters the preferred codon for alanine to a rarely used codon (WRIGHT et al. 2004). Changes in codon usage can alter translation efficiency and protein folding kinetics, potentially making stabilization by HSP90 superfluous. A similar change in codon usage has been observed for evolutions of viral proteins under conditions of reduced HSP90 in mammalian cells (VAUGHAN et al. 2010). Thus, in strong support of the capacitor hypothesis, HSP90 inhibition revealed phenotypic differences among divergent A. thaliana strains that are associated with BES1 polymorphisms. Moreover, HSP90 client status appears to be highly dynamic with BES1 losing HSP90 dependence in some strains.

Another non-synonymous polymorphism in BES1 did not correlate with response to HSP90 inhibition (Figure 4B). Consistent with HSP90 facilitating BES1 evolution, this polymorphism may represent a step towards a novel phenotype through acquisition of a second mutation, with which it interacts epistatically (Figure 4B) (SALVERDA et al. 2011). Neutral non-synonymous mutations, such as mutations that increase protein stability without affecting function, can facilitate ascent to new fitness optimum (TOKURIKI and TAWFIK 2009). In contrast, all twenty BZR1 polymorphisms were synonymous (Figure 4B), consistent with BZR1’s lower evolutionary rate compared to BES1. None of these were associated with sensitivity to HSP90 inhibition, supporting BZR1’s non-client status (Figure 4B, figure S6B). Eight BZR1 polymorphisms were
unique to the strain Uod-7, which carries a large intronic insertion in addition to other intronic polymorphisms (Figure 4B). These polymorphisms may lead to mis-regulation of BZR1 (Le Hir et al. 2003), thereby increasing the need for BES1. This scenario is consistent with Uod-7’s hypersensitivity to GdA (Figure 4A). Taken together; our data suggest that HSP90 facilitates divergence of gene duplicates by promoting sub-functionalization through temperature sensitivity of its clients and neofunctionalization through their increased tolerance of mutations.

Discussion

With BES1, we have identified a novel HSP90 client in a crucial plant growth pathway (Figure 1). By necessity, plant growth must be finely tuned to the environment. The temperature sensitivity of BES1 tightly links BR pathway function to the ambient environment. We and others showed previously that HSP90 plays an important role in defenses against herbivores and microbial pathogens, (Hubert et al. 2003; Sangster et al. 2007; Sangster and Queitsch 2005; Takahashi et al. 2003), as well as the timing of flowering (Sangster et al. 2007). Our findings add an important layer of complexity to known hormone-environment interactions (Nemhauser 2008; Robert-Seilaniantz et al. 2011), especially in light of HSP90’s known role in resource allocation among defense and growth pathways. Our data also add to the emerging evidence of functional divergence between BES1 and BZR1—the critical downstream targets of BR signaling. For example, recent genome-wide chromatin immunoprecipitation experiments show only partial overlap of gene targets in BES1 and BZR1 (Sun et al. 2010; Yu et al. 2011). This partial overlap highlighted key unresolved questions about BR transcriptional responses. Specifically, what distinguishes BES1- or BZR1-specific targets from targets regulated by both proteins, and what determines whether BES1 and BZR1 act as repressors or activators? As BES1, but not BZR1, is an HSP90 client, we speculate that interaction with the
chaperone may facilitate association of BES1 with specific partner proteins, resulting in BES1-specific functions (Yin et al. 2005).

In addition to identifying a novel plant HSP90 client, our study provides support for the capacitor hypothesis by garnering evidence of HSP90-facilitated evolution in extant genomes. When we compared paralogs, we found a significant trend that genes encoding diverse HSP90 clients evolved faster than non-clients (Figure 2). In contrast, when we compared diverse yeast HSP90 clients and non-clients as aggregate groups, we could not detect any significant difference in evolution rate (Figure S4B). This result is consistent with a similar analysis comparing prokaryotic genes encoding diverse clients of the bacterial chaperonin GroEL/ES and non-clients (Williams and Fares 2010). On the contrary, within the superfamily of mammalian kinases, strong HSP90 clients carry more non-synonymous mutations than non-clients (Taipale et al. 2012). These different results are not surprising because many other factors or gene properties influence evolutionary rate, most of which will differ for a diverse set of genes. Combined, these factors can outweigh and conceal effects of HSP90 and GroEL/ES client status on evolutionary rate. By focusing on gene duplicates of diverse HSP90 clients, which share many properties influencing evolutionary rate, we found support for HSP90’s previously hypothesized effect on gene evolution. As overexpression of GroEL/ES also increases the evolutionary rates of its clients (Tokuriki and Tawfik 2009), our study supports an ancient and conserved role for protein chaperones in gene evolution.

Our findings also help resolve an apparent paradox about the fate of gene duplicates. Gene duplicates are functionally redundant immediately after duplication, rendering one copy
superfluous or even harmful (LYNCH and CONERY 2000; PAPP et al. 2003). Evolutionary theory predicts non-functionalization— one gene copy is silenced and subsequently lost—as the fate of most duplicated genes. Recent studies have challenged this view by revealing that gene duplicates with partially redundant function are maintained much longer than expected (CONANT and WOLFE 2008; DEAN et al. 2008; DELUNA et al. 2008; LYNCH and CONERY 2000; MAERE et al. 2005). Moreover, in yeast, plants, insects, and humans, genes that can be maintained in duplicate show strong functional bias, with transcription factors and kinases significantly over-represented (AURY et al. 2006; CONANT and WOLFE 2008; GUAN et al. 2007; MAERE et al. 2005; WAPINSKI et al. 2007). Both observations raise questions about the molecular mechanism(s) that aid the initial preservation, continued maintenance, and eventual divergence of a specific subset of gene duplicates. Our data are consistent with a model in which acquisition of HSP90 client status is a molecular mechanism aiding each of these steps. Acquisition of HSP90 client status can occur through a single mutational step (CITRI et al. 2006; TAIPALE et al. 2010). A new HSP90 client will be subject to immediate environmental subfunctionalization. As we observed for BES1, HSP90 clients are exquisitely sensitive to environmental conditions affecting protein folding (NATHAN et al. 1997). Immediate and efficient subfunctionalization will counteract deleterious dosage effects and foster long-term maintenance of gene duplicates, and hence provide opportunity for their functional divergence. In fact, bes1-D mutants were more sensitive to a temperature increase than to the HSP90 inhibition, which is consistent with the fact that temperature change is a more complex perturbation compared to the specific inhibition of a single chaperone. In addition to providing evidence for HSP90-faciliated subfunctionalization, we show that HSP90 client status correlates with hallmarks of neofunctionalization and increased evolutionary rates of the genes encoding them. HSP90 recognizes metastable signal transduction
proteins, most of which are transcription factors and kinases (TAIPALE et al. 2010). We speculate that HSP90’s specificity for these substrates contributes to the observed functional bias among gene duplicates. Additional support for HSP90’s role in gene duplicate divergence comes from our observation that HSP90 client status is dynamic in the BZR/BEH family and in yeast gene families, with frequent gains and losses (Figure 4A, B). Dynamic client status is also observed in the mammalian kinome (TAIPALE et al. 2012). In fact, even across wild A. thaliana accessions BES1 HSP90 client status itself appears to be dynamic.

The presence of HSP90 clients and non-clients in gene families with partially redundant function has important implications for another aspect of HSP90-mediated capacitance. Inhibition of HSP90 increases phenotypic variation even in the absence of genetic variation (QUEITSCH et al. 2002; SANGSTER et al. 2008a; SANGSTER et al. 2008b). This increase of phenotypic variation in isogenic lines has been attributed to an increased frequency of stochastic events in development or a greater sensitivity to microenvironments. As recently shown, significant phenotypic variation arises in isogenic worms due to the loss of functional redundancy between paralogs (BURGA et al. 2011; CASANUEVA et al. 2012). Under conditions that challenge HSP90 function, HSP90-dependent paralogs will become inactive, thereby reducing functional redundancy and increasing phenotypic variation. We suggest a general role for HSP90 in maintaining a reservoir of phenotypic variation through facilitating conditional functionality of gene duplicates.
Acknowledgements

This work was supported by grants from the National Human Genome Research Institute Interdisciplinary Training in Genomic Sciences (T32 HG00035) (JL), National Science Foundation Graduation Research Fellowship (DGE-0718124) (JL), National Institute of General Medical Sciences Academic Research Enhancement Award R15-GM086822 (PJMM), and National Science Foundation Grant IOS-0919021 (JLN).

JL, PJMM, JLN, and CQ conceived and designed experiments. JL performed experiments. JL, TL, JHT, and CQ conceived and designed computational analyses. JL and TL performed computational analyses. JL, JLN, and CQ wrote the paper. JLN and CQ should be considered co-corresponding authors on this paper.

We thank members of the Queitsch and Nemhauser groups for useful discussions, Orrin Stone for experimental assistance, Yanhai Yin for anti-BES1 antibody, Elhanan Borenstein, Maitreya Dunham, Stanley Fields, and Harmit Malik for helpful comments, Magnus Nordborg and Scott Hodges for use of unpublished Aquilegia sequences, and Neeraj Salathia for manual curation of TPR domain containing proteins.
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**Figure 1** BES1 is an HSP90 client.

(A) Seedlings with increased BR signaling through overexpression of DWF4 (*DWF4-ox*) or overexpression of BRI1 (*BRI1-ox*) showed similar sensitivity to HSP90-inhibition by GdA compared to WT. In contrast, seedlings with constitutive activation of BES1 (*bes1-D*) were significantly more sensitive than WT in red light. Standard error is shown.

(B) In contrast to the dramatic GdA hypersensitivity of *bes1-D* mutants, *bzr1-D* mutants respond like WT in red light.

(C) Representative WT and *bes1-D* seedling phenotypes in red light.

(D) BES1 interacts physically with HSP90. BES1 was immunoprecipitated (IP) with an HSP90 antibody in WT, but not in loss-of-function *bes1-2* mutants, confirming that this antibody is specific to BES1. Input and IP are shown for both.
GdA treatment caused a shift of BES1 mobility. Unlike a similar shift caused by brassinolide (BL) treatment, the GdA-induced mobility shift was associated with decreased hypocotyl length. Zdr-6 and Tottarp-2 show reduced levels of BES1; detected protein is presumably due to the presence of other splice forms. Coomassie Brilliant Blue (CBB) is shown as a loading control.
Figure 2  HSP90 clients show relaxed selection compared to their paralogs.

(A) The branch leading to BES1 has a larger dN/dS ratio than the branch leading to BZR1. dN/dS ratios are in italics. Branch lengths represent the difference in the number of amino acid substitutions among family members from an unrooted tree. The fraction of 100 bootstraps supporting each branch are shown in bold.

(B) S. cerevisiae HSP90 clients tend to evolve faster than their paralogs in two-member and three-member families. Clients are dark-grey circles; non-clients are light-grey squares. Star denotes significance. Significance was determined by using a one-sample Wilcoxon test, which tests the deviation from the expected ratio of client/non-client branch length of 1, n=13, 95% confidence interval 1.02-1.64, p=0.002.
Figure 3  BES1 shows evidence of neo- and subfunctionalization.

(A) The major BES1 splice variant, BES1.3, encodes a novel exon. Grey boxes are exons, black lines are introns, and white boxes are UTRs.

(B) A BLASTn search for regions with homology to the novel BES1.3 exon identifies intergenic loci.

(C) A BLASTn search for regions with homology to the novel exon and first intron of BES1.3 identifies BZR1 5’ UTR sequence in addition to intergenic loci.
(D) At 27°C, the \textit{besl-D} mutant failed to germinate, while all other mutants showed a wild-type response.

(E) Representative \textit{besl-D} seedlings and seeds at 22°C and 27°C, respectively. Enlarged image of \textit{besl-D} (inset) shows a seed that failed to germinate at 27°C.
Figure 4  BES1 HSP90 client status is dynamic.

(A) Hypocotyl length GdA sensitivity of divergent *A. thaliana* strains and *bes1-D* (red). *BES1* and *BZR1* were sequenced in strains highlighted in grey. Strains with frameshift mutations are in black; strains with *BES1*-synonymous polymorphisms are green. Both polymorphisms are significantly associated with GdA sensitivity. Strains with *BES1* intronic polymorphisms are light blue.

(B) *BES1.3* and *BZR1.1* polymorphisms. Grey boxes are exons, black lines are introns, and white boxes are UTRs. The octagon marks the frameshift mutation, the green triangle marks the synonymous SNP, the blue triangle marks the nonsynonymous polymorphism, white triangles mark non-coding or synonymous polymorphisms, light blue triangles mark intronic polymorphisms in *BES1*, and stars mark the *bzr1-D* and *bes1-D* dominant mutations. The domains are N-nuclear localization signal, D-DNA binding domain, P-phosphorylation domain, PEST-PP2A interaction domain.
(C) A protein that exists in a free energy minimum (function a) acquires mutations that render it metastable and thus recognized by HSP90. As an HSP90 client, the protein can visit a greater sequence space, increasing the chance of reaching another free energy minimum associated with a novel function (function b) and loss of client status.