1   HSP90 inhibitors disrupt a transient HSP90-HSF1 interaction and identify a noncanonical model

2   of HSP90-mediated HSF1 regulation

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1   **Abstract**

2   Heat shock factor 1 (HSF1) initiates a broad transcriptional response to proteotoxic stress while

3   also mediating a cancer-specific transcriptional program. HSF1 is thought to be regulated by

4   molecular chaperones, including Heat Shock Protein 90 (HSP90). HSP90 is proposed to

5   sequester HSF1 in unstressed cells, but visualization of this interaction *in vivo* requires protein

6   crosslinking. In this report, we show that HSP90 binding to HSF1 depends on HSP90

7   conformation and is only readily visualized for the ATP-dependent, N-domain dimerized

8   chaperone, a conformation only rarely sampled by mammalian HSP90. We have used this

9   mutationally fixed conformation to map HSP90 binding sites on HSF1. Further, we show that

10   ATP-competitive, N-domain targeted HSP90 inhibitors disrupt this interaction, resulting in the

11   increased duration of HSF1 occupancy of the *hsp70* promoter and significant prolongation of

12   both the constitutive and heat-induced HSF1 transcriptional activity. While our data do not

13   support a role for HSP90 in sequestering HSF1 monomers to suppress HSF1 transcriptional

14   activity, our findings do identify a noncanonical role for HSP90 in providing dynamic

15   modulation of HSF1 activity by participating in removal of HSF1 trimers from heat shock

16   elements in DNA, thus terminating the heat shock response.

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1 **Introduction**

2 Heat shock factor 1 (HSF1) is an evolutionarily conserved transcription factor that initiates the

3 cytoprotective heat shock response (HSR). Found throughout the eukaryotic kingdom, HSF1

4 allows for the cellular adaptation to proteotoxic stress [1]. Through an incompletely defined

5 mechanism, mammalian HSF1 monomers in cytosol are activated and form trimers, translocate

6 into the nucleus, and bind sequences of DNA known as heat shock elements (HSE), ideally

7 represented as nGAAnnTTCnnGAAn [2,3]. Throughout this process HSF1 is heavily post-

8 translationally modified and interacts with numerous cellular components. The binding of HSF1

9 trimers to HSE induces the transcription of a specialized set of genes known as molecular

10 chaperones while also repressing the expression of other genes [4,5], although the repressive effect

11 of HSF1 is controversial [6]. Once expressed, these molecular chaperones (or Heat Shock Proteins,

12 HSPs) act to stabilize the three-dimensional structure of numerous cellular proteins, thus helping

13 to maintain cellular proteostasis. HSP90 and HSP70 are ATP-dependent HSPs that interact with

14 a large sector of the eukaryotic proteome while also modulating HSF1 transcriptional activity [7,8]

15 [9]. The relationship between HSF1 and one or more components of the cellular proteostasis

16 network is thought to represent a primary axis in the control of the HSR [7,10].

17 In cancer, HSF1 enables malignant cell growth and is overexpressed in a number of tumor types

18 associated with poor prognosis [11,12,13]. Although HSF1 does not initiate oncogenic

19 transformation, tumors become addicted to HSF1 activity as their microenvironments become

20 increasingly toxic and as they require higher levels of HSPs to maintain proteostasis [14].

21 Moreover, many oncogenes that drive tumorigenesis are metastable and rely on HSPs to sustain

22 their activity. This is particularly true for mutated or overexpressed kinases and transcription

23 factors that interact with HSP90 [15]. HSF1 also promotes a cancer-specific transcriptional

24 program that supports malignancy through the expression of genes for proliferation, anabolic

3

1   metabolism, metastasis and apoptosis prevention. Comprised of over 500 genes, this cancer-

2   specific HSF1 transcriptome is associated with poor clinical outcomes [11].

3   The human *HSF1* gene is encoded on chromosome 8q24 by 14 exons that produce two splice

4   variants. The largest variant, which is described in this report, is translated into 529 amino acids.

5   HSF1 has a predicted molecular weight of 57 kDa, yet migrates at approximately 75 kDa on

6   SDS-PAGE due to a large number of post-translational modifications (PTMs), including

7   phosphorylation, acetylation and sumoylation [16] [17]. The overall structure of HSF1 is mostly

8   disordered except for the evolutionarily conserved N-terminal DNA-binding domain (DBD) that

9   forms a winged helix-turn-helix structure [18] [19]. The rest of HSF1 is predicted not to maintain a

10   stable tertiary structure, a feature observed for many proteins involved in transcription and

11   cellular regulation [20]. Following the DBD and a linker region is the set of heptad repeats (HR-

12   A/B) that form the leucine zippers that allow for HSF1 trimerization [21]. Adjacent to the HR-A/B,

13   the unstructured regulatory domain (RD) is the molecular region understood to be capable of

14   sensing heat and initiating the HSR [22]. The RD contains numerous phosphorylation sites [23] and

15   functions, along with a portion of HR-A/B, to repress the transcriptional activity of NF-IL6 [24].

16   Another heptad repeat (HR-C), C-terminal to the RD, is understood to sequester HR-A/B in an

17   intramolecular interaction that suppresses spontaneous HSF1 trimerization [21]. Most recently,

18   Hentze et al have shown that HSP90β interacts with the HR-C domain in solution to promote its

19   temperature-sensitive dissociation from HR-A/B, thus reducing the temperature at which HSF1

20   is able to trimerize [25]. The C-terminal transactivation domain (TAD) is highly disordered and is

21   required for robust HSF1 transcriptional activity in response to stress [26]. The TAD is also a site

22   of HSP70 binding, which negatively regulates HSF1 transcriptional activity [27].

23   While the interaction of HSP70 with HSF1 in unstressed cells is readily observed by co-

24   immunoprecipitation [27], interaction of endogenous HSP90 and HSF1 is not readily detectable

25   and requires cross-linking to stabilize the association [7]. These data argue against a frequently

1 cited model postulating sequestration of HSF1 monomers by HSP90 under non-stress conditions

2 (7), but they would support such a role for HSP70, as has recently been proposed in yeast [28].

3 Chaperone binding to HSF1 monomers is thought to prevent spontaneous trimerization. In such

4 a model, HSF1 is released from chaperone suppression and allowed to trimerize when cells

5 experience severe proteotoxic stress that generates a large population of denatured client proteins

6 and an increased demand for chaperones [29]. Other studies have shown that HSP90 can also bind

7 the trimerized DNA-bound state of HSF1 and that this interaction attenuates HSF1

8 transcriptional activity [8]. Thus, HSP90 has variously been proposed to function as a sensor,

9 repressor and/or attenuator of the HSR.

10 Determining the mechanism by which HSP90 affects the HSR is of medical significance.

11 Currently, HSP90 is the only HSP whose targeting by small molecules has been extensively

12 evaluated in the clinic as a strategy to treat cancer [30]. However, although HSP90 inhibitors have

13 shown great promise in pre-clinical studies due to their ability to disrupt proteostasis and to

14 promote the degradation of oncogenic kinases and other pro-growth components [31], their single

15 agent activity in patients has not been impressive [32][33]. This discrepancy has been ascribed, in

16 part, to the observation that ATP-competitive HSP90 inhibitors promote the HSR and result in

17 the increased expression of HSP70, HSP27, and other pro-survival components [34][35], possibly

18 offsetting any anti-cancer benefit of these drugs. Moreover, HSP90 inhibitors may enhance the

19 recently identified cancer-specific HSF1 transcriptional program [11]. How this is mechanistically

20 achieved in the absence of robust HSP90/HSF1 interaction has remained obscure.

21 In this report, we define the interactions of HSF1 with both nuclear-cytosolic paralogs of HSP90,

22 stress-inducible HSP90α and constitutively expressed HSP90β, and with endogenous HSP70.

23 Remarkably, we readily observed interaction of HSF1 with mutant HSP90 trapped in an ATP-

24 dependent "closed" conformation (unable to hydrolyze ATP), and we found this interaction to be

25 disrupted by N-terminal HSP90 inhibitors that are currently being clinically evaluated. To

5

1   determine the domains in HSF1 that bind HSP90 and HSP70 we made a series of HSF1 deletion

2   mutants and analyzed their interactions by immunoprecipitation-western blot. We observed that

3   the binding of HSP90α, HSP90β and HSP70 uniquely map across HSF1. This was

4   complemented by analysis of the effects of these deletion constructs on HSF1 trimerization and

5   HSR reporter activity. Finally, we found that HSP90 inhibitors disrupt association of "closed"

6   HSP90 proteins with HSF1, while concomitantly increasing the duration of HSF1 binding to

7   *hsp70* promoter and extending the duration of heat-induced HSF1 transcriptional activity. While

8   our data do not support a role for HSP90 in sequestering HSF1 monomers, our findings reveal

9   that HSP90 inhibitors interfere with a noncanonical role for HSP90 in providing dynamic

10   modulation of HSF1 activity by removing HSF1 trimers from heat shock elements in DNA.

11   **Results**

12   **Wild type HSF1 readily interacts with N-domain dimerized ("closed" conformation)**

13   **HSP90**

14   Previous studies have suggested that the intracellular interaction of HSF1 and HSP90 is weak

15   and transient, and requires chemical crosslinking for visualization [7]. We showed recently that the

16   strength of this interaction may be influenced by HSP90 conformation [36]. Binding of ATP to N-

17   terminal domains of HSP90 promotes their transient dimerization and induces a "closed"

18   conformation that is poised for ATP hydrolysis. ATP binding and hydrolysis, in turn, drive the

19   structural rearrangements in HSP90 that support chaperone function. Upon release of ADP, or in

20   the presence of ATP-competitive HSP90 inhibitors, HSP90 N-domains revert to an undimerized

21   state ("open" conformation) (Figure 1A).

22   Mutation of key residues within the N-terminal domain of HSP90 stabilize either the "closed"

23   (HSP90α E47A, HSP90β E42A; allow ATP binding but prevent hydrolysis) or "open" (HSP90α

24   D93A, HSP90β D88A; prevent ATP binding) conformations. We co-transfected HEK293 cells

1 with plasmids encoding FLAG-tagged wild type or conformationally restricted HSP90 proteins

2 together with wild type HA-HSF1, harvested the cells the following day, and observed the

3 protein-protein interactions by anti-FLAG immunoprecipitation-western blot (IP-WB) analysis

4 (Figure 1B, C). The relative expression of HSF1 in transfected and untransfected cells is shown

5 in Supplemental Figure 1A. We found that wild type HSF1 strongly interacts, without the need

6 for crosslinking, with both "closed" HSP90 mutants but not with the pair of "open" HSP90

7 mutants. Importantly, the results with transfected HSF1 were confirmed by co-

8 immunoprecipitation of "closed" HSP90 with endogenous HSF1 in HEK293 cells (Supplemental

9 Figure 1B). As previously reported [7], interaction of wild type HSP90, which samples all

10 available conformational states, with full-length HSF1 is not detectable in the absence of protein

11 crosslinking. This result is consistent with the observation that human HSP90, in contrast to the

12 yeast chaperone, is most likely to be found in the "open" conformation, even when ATP is bound

13 [37]. Moreover, we observed that HSP90α E47A bound more avidly to HSF1 than did HSP90β

14 E42A, suggesting that the stress-inducible HSP90α isoform may be a stronger negative regulator

15 of HSF1 activity compared to the constitutively expressed HSP90β isoform, as previously

16 proposed [36].

17 To further assess the interaction between wild type HSF1 and the "closed" HSP90 mutants, we

18 repeated the reciprocal experiment in the absence or presence of the anion molybdate (MoO$_4$)

19 Molybdate has been reported to stabilize a high affinity client binding state of HSP90 [38]. Target

20 proteins were pulled down via either FLAG-HSP90 or FLAG-HSF1 (Figure 1C). Although, the

21 HSF1/ "closed" HSP90 interaction was confirmed, molybdate only moderately increased the

22 interaction of the "closed" HSP90 mutants with no discernable effect on interaction of wild type

23 HSP90 with HSF1, indicating that HSP90 interacts with HSF1 in a manner that is likely unique

24 when compared to other clients such as protein kinases [38] [39].

7

1   Administration of HSP90 inhibitors often results in apparent induction or augmentation of the

2   HSR. Currently, there are two general classes of inhibitors. The more clinically advanced N-

3   terminal inhibitors competitively bind to the ATP pocket and prevent or disrupt nucleotide-

4   dependent N-domain dimerization [40]. These inhibitors include 17AAG (tanespimycin), STA9090

5   (ganetespib) and SNX2112, among others [30]. A second class of inhibitors, based on the antibiotic

6   novobiocin, binds a putative nucleotide-binding domain in the C-terminal portion of HSP90

7   (adjacent to the C-terminal dimerization domain) and inhibits the chaperone in a distinct manner

8   [41]. The synthetic novobiocin derivative KU32 is representative of this class of inhibitors [42]. To

9   explore how N- and C-terminal inhibitors might affect HSF1/HSP90 interaction, we co-

10   transfected HEK293 cells with wild type HA-HSF1 and FLAG-HSP90α E47A, briefly treated

11   cells with inhibitors the following day, harvested and analyzed protein-protein interactions by IP-

12   WB (Figure 1D, Supplemental Figure 1C). Each of the three N-terminal inhibitors disrupted the

13   interaction between HSF1 and HSP90α E47A, consistent with their disruption of N-domain

14   dimerization. In contrast, KU32 did not disrupt the interaction between HSF1 and "closed"

15   HSP90, indicating that this is a unique property of ATP-competitive, N-terminal HSP90

16   inhibitors.

17   **Mapping of HSP90 and HSP70 interactions across HSF1**

18   To investigate the interactions of HSP90 and HSP70 with HSF1 in the absence of a crosslinking

19   agent, we constructed and transiently expressed in HEK293 cells a series of HSF1 truncation and

20   internal deletion mutants, and we analyzed them for chaperone interaction by IP-WB (Figure 2A

21   and B). Of note, N195, an N-terminal truncation mutant lacking the DBD and most of the HR-

22   A/B domain, expressed very poorly (see blot in Figure 2B) and data obtained using this mutant

23   should be considered in this context. These HSF1 constructs were used to initially query the

24   interaction of HSP90 and HSP70 across the TAD, HR-C, RD, HR-A/B and DBD domains of

25   HSF1 (Supplemental Figure 2A, B).

1   Based on these preliminary data obtained with the closed HSP90 mutants, a subpanel of HSF1

2   truncation and deletion mutants were selected to assess association of HSP90α E47A.

3   Pulldowns were repeated in triplicate, resulting blots were subjected to densitometry to generate

4   mean values +/- standard deviations and p values to assess significance (compared to association

5   of HSP90α E47A with full length HSF1) were obtained. These data are shown in Figure 2,

6   panels C and D. Analysis of HSF1 C-terminal truncations revealed a distinct interaction motif for

7   "closed" HSP90 E47A (Figure 2C).   The "closed" mutant bound most intensely to HSF1

8   proteins containing the HR-A/B domain and a portion of the heat-sensing RD, but lacking the

9   HR-C and TAD. The clear difference in HSP90 interaction between the C442 and C383 HSF1

10 truncation mutants suggests that presence of the HSF1 HR-C + TAD domain inhibits HSP90

11 interaction. Although HSP90 E47A bound weakly to more extensive C-terminal truncations,

12 binding was not significantly different from wild type HSF1. The dual importance of both the

13 HR-A/B and RD domains for HSP90 binding is supported by the observation that neither the

14 isolated HR-A/B domain (N120-C205, Figure 2D and Supplemental Figure 2B) nor the isolated

15 RD domain (N262-C383, Supplemental Figure 2B) display binding to HSP90.

16 Importantly, deletion of the HR-C domain dramatically improves the binding of both "closed"

17 and endogenous HSP90, suggesting that HR-C intermolecular binding to HR-A/B (characteristic

18 of inactive HSF1 monomers) interferes with HSP90 binding and providing further evidence that

19 is inconsistent with a role for HSP90 in sequestering monomeric HSF1. Finally, the HSF1 HR-

20 A/B internal deletion construct DEL142-169, where amino acids 142-169 were removed, bound

21 "closed" HSP90 while the HR-A/B internal deletion construct DEL183-214 did not (Figure 2D

22 and Supplemental Figure 2D), suggesting that the C-terminal half of the HR-A/B is necessary for

23 HSP90 binding.

24 While the binding pattern of endogenous HSP90 generally mirrors that of "closed" HSP90

25 (although, in contrast to "closed" HSP90, detectable binding of endogenous HSP90 requires

9

1   deletion of the HR-C domain, see Supplemental Figure 2A, B), endogenous HSP90 also uniquely

2   binds the HSF1 constructs N195 and N262 but not the equivalent HSF1 mutants lacking the HR-

3   C and TAD domains (N195-C383 or N262-C383, see Supplemental Figure 2B). These findings

4   are consistent with the interaction of endogenous HSP90 (primarily existing in an N-domain

5   undimerized conformation) with the HR-C domain, as recently reported by Mayer and

6   colleagues for HSP90β. Importantly, these authors demonstrated that HSP90 binding to the

7   HSF1 HR-C domain promotes its temperature-sensitive dissociation from HR-A/B, thus

8   facilitating HSF1 trimerization [25]. Like our data, these findings are also not consistent with a role

9   for HSP90 in suppressing HSF1 trimerization.

10   In contrast to the binding profile of HSP90, endogenous HSP70 interacts robustly with full-

11   length HSF1 as well as with most of the C-terminal truncation and deletion mutants

12   (Supplemental Figure 2A and B), with the exception of those mutants containing increasingly

13   truncated RD. Endogenous HSP70 also bound poorly to N120-C205 (isolated HR-A/B domain)

14   despite ample expression of this HSF1 protein fragment. Taken together, these data identify the

15   RD as being sufficient for HSP70 binding in unstressed cells, although HSP70 binding to other

16   domains of HSF1 has also been reported [26][27].

17   To further compare the role of the HR-A/B domain in HSF1 trimerization and in HSP90

18   interaction, we made a set of alanine scanning mutants ($145A_4$, $151A_5$, $161A_5$) between amino

19   acids142-169 (see Methods) and compared them in the experiments below with the internal

20   deletion constructs DEL142-169 and DEL183-214. Using IP-WB analysis of co-transfected WT

21   HA-HSF1 and mutant FLAG-HSF1, we observed that the HR-A/B domain alanine mutants did

22   not disrupt HSF1 intermolecular interaction, in distinct contrast to the HR-A/B deletion mutants

23   DEL142-169 and DEL182-214 (Supplemental Figure 2C). In contrast, these alanine mutants

24   bound "closed" HSP90α E47A to a greater extent compared to WT HSF1 (but similarly to

25   DEL142-169 (Supplemental Figure 2D and Figure 2D). These data are consistent with the

1   hypothesis that the HR-A/B region contributing to HSF1 trimerization is distinct from the HR-

2   A/B region that is required for HSP90 binding. Further, our findings emphasize that the presence

3   or absence of HSP90 binding has no impact on HSF1 trimerization (see Figure 3B, D and

4   Supplemental Figure 5). Finally, the enhanced HSP90 binding of the HSF1 truncation and

5   deletion mutants shown in Figure 2C, D does not involve possible heterotrimerization of the

6   transfected HSF1 constructs with endogenous HSF1, as similar results are obtained when the

7   HSF1 truncation mutants are transfected into HEK293 cells stably knocked down for

8   endogenous HSF1 (Supplemental Figure 2E).

9   **The TAD and DBD are essential for HSF1 transcriptional activity while the HR-A/B**

10   **domain is required for dominant negative activity of truncated HSF1**

11   To further explore potential relationships between the ability of HSF1 to (1) form intermolecular

12   trimers, (2) activate transcription, and (3) interact with HSP90, we examined the impact of HSF1

13   N/C-truncations and internal deletions on *hsp70* promoter-reporter activity as a readout of the

14   HSR, and we complemented these observations with IP-WB analysis of HSF1 trimerization and

15   HSF1-HSP90 interaction. Overexpression of WT HSF1 increased *hsp70*-reporter activity in

16   response to heat shock by nearly 2-fold above a pcDNA control. Brief pretreatment of cells with

17   17AAG prior to heat shock increased *hsp70*-reporter activity in pcDNA control by 3.5-fold

18   compared to heat shock alone, and a similar fold increase in reporter activity was observed upon

19   17AAG exposure prior to heat shock in cells transfected with WT HSF1 (Figure 3A). For

20   reference, reporter activity in response to 17AAG alone, compared to heat shock and heat shock

21   + 17AAG is shown in Supplemental Figure 3A. Both heat-induced and 17AAG + heat-induced

22   *hsp70*-reporter activity were progressively impacted by increasing truncation of the TAD (C-

23   terminal deletion constructs C509, C477 and C442), consistent with the importance of this

24   domain in modulating HSF1 activation [43]. Complete removal of TAD, HR-C and RD domains

25   (constructs C383 through C205) fully repressed *hsp70*-reporter activity even with 17AAG

11

1   pretreatment, suggesting that the presence of the HR-A/B domain of these mutants is essential

2   for dominant-negative activity, as a consequence of disrupting endogenous HSF1 trimers. Indeed,

3   truncation through the C-terminal portion of the HR-A/B domain (C182) restored *hsp70*-reporter

4   response to that seen after treating pcDNA control cells with heat and heat + 17AAG. Consistent

5   with this result, HSF1 C182 is unable to form an intermolecular interaction with wild type HSF1,

6   as are additional HR-A/B truncations (Figure 3D). Unexpectedly, further truncation (C167, C146

7   and C129) through the HR-A/B, increased *hsp70*-reporter activity above that of the pcDNA

8   control, suggesting the presence of a repressing motif located between amino acids 167 and 182

9   of HSF1 (Figure 3A).

10   Our previous observation of HSP90 binding to a series of RD-containing C-terminal truncation

11   mutants (C383, C339, C311, Figure 2C) suggested that HSP90 binding might be involved in

12   repressing HSF1-driven *hsp70*-reporter activity. To address this possibility, we used IP-WB to

13   investigate the effect of 17AAG on the interaction of HSP90α E47A with these HSF1 RD

14   truncation mutants since these mutants repressed *hsp70*-reporter activity even in the presence of

15   17AAG (Figure 3A). Consistent with the data in Figure 2C, we observed increased binding of

16   HSP90α E47A to the RD truncation mutants C383, C339 and C311 when compared to wild type

17   HSF1. However, upon addition of 17AAG, HSF1-HSP90α E47A interaction was fully disrupted

18   (Figure 3B), while *hsp70*-reporter activity remained repressed (Figure 3A). These data indicate

19   that robust HSP90 interaction with HSF1 is not responsible for the dominant negative activity of

20   these mutants. Consistent with this hypothesis, the RD truncation mutant C287 bound poorly to

21   HSP90α E47A, yet displayed dominant negative activity (see Figures 3A and B). Taken together,

22   our findings support the hypothesis that the dominant negative activity of HSF1 C-terminal

23   truncations retaining an intact HR-A/B domain is due to disruption of endogenous full length

24   HSF1 trimers necessary for transcriptional activity.

12

1   Next, we assessed the effects of another set of HSF1 N/C-terminal deletion constructs on *hsp70-*

2   reporter activity (Figure 3C). Here we observed that the deletion constructs N120, N120-C383,

3   N120-C325 and N120-C287, all lacking the DBD but including an intact HR-A/B domain,

4   repressed *hsp70*-reporter activity below the level seen with empty plasmid, consistent with

5   dominant negative inhibition of endogenous HSF1. Removal of the HR-A/B domain (N195,

6   N262) relieved this repression to some extent, even when the TAD and RD were removed

7   (N195-C383, N195-C325, N262-C383, N262-C325). Finally, the internal HR-A/B deletion

8   mutants DEL142-169 and DEL182-214 supported *hsp70*-reporter activity at a level similar to

9   that of empty plasmid, confirming that an intact HR-A/B domain is required for dominant

10 negative activity. The statistical significance of the changes in reporter activity can be found in

11 Supplemental Figure 3B. Of note, these data are in contrast to the impact of these two HR-A/B

12 internal deletions on HSP90 binding (Figure 2D, right two lanes).

13 To profile the ability of these deletion constructs to affect HSF1 intermolecular interaction

14 (indicative of trimerization capability), we analyzed by IP-WB the interaction of co-transfected

15 wild type HA-HSF1 with various FLAG-HSF1 deletion mutants. We found that FLAG-tagged

16 deletion mutants (C383, C287, C233, C205 and N120) that dominant negatively repressed

17 *hsp70*-reporter activity interacted efficiently (equivalent or enhanced interaction compared to

18 FLAG-tagged wild type HSF1) with wild type HA-HSF1. In contrast, deletion mutants (C182,

19 C167, C146, C129, DEL142-169, DEL183-214) that did not strongly repress *hsp70*-reporter

20 activity did not interact with WT HA-HSF1 (Figure 3D). We excluded N195 from this analysis

21 due to its poor expression as shown in Figures 2B and 3D. Likewise, the dominant negative

22 HSF1 mutants N120, N120-C383, N120-C325 and N120-C287 strongly interacted with full

23 length HSF1, while N195-C383 (which showed no dominant negative activity) did not

24 (Supplemental Figure 3C and D). Combined with our previous observations, these data further

25 confirm that HSF1 mutant-mediated dominant negative repression of endogenous HSF1-driven

13

1   transcription depends on the ability to form an intermolecular interaction with wild type HSF1.

2   A cartoon summarizing these data is provided in Figure 3E.

3   **Impact of RD phosphorylation state on HSP90 inhibitor effect on HSF1 activity and**

4   **mobility shift in SDS-PAGE, and on HSF1 interaction with HSP90**

5   Stress-inducible phosphorylation of the HSF1 RD coincides with acquisition of transactivation

6   capacity, although a functional requirement of RD phosphorylation for HSF1 activation has not

7   been demonstrated. Indeed, a recent report dissociates RD phosphorylation from HSF1 activation

8   and suggests that RD phosphorylation may serve as a rheostat to modulate the degree of

9   activation [23]. Since N-terminally targeted HSP90 inhibitors also cause a distinct,

10   phosphorylation-dependent mobility shift of HSF1 coincident with disrupting its association with

11   conformationally restricted HSP90 proteins (see Figure 1D, Supplemental Figure 1C and

12   Supplemental Figure 4), we wished to determine whether these drugs promoted phosphorylation

13   of sites only within the RD and whether RD phosphorylation was a prerequisite for HSP90

14   inhibitor-enhanced HSF1 activity. For this purpose, we used the delta PRD (dPRD) HSF1

15   construct previously described, in which all phosphorylation sites within the RD have been

16   mutated to alanine (see Figure 4A) [23]. Using the *hsp70*-reporter assay, we confirmed that dPRD

17   HSF1 was more sensitive to heat shock, and to the combination of heat shock and 17AAG,

18   compared to wild type HSF1 (Figure 4B). Unexpectedly, the mobility of HSF1 dPRD was also

19   shifted when challenged with three N-domain HSP90 inhibitors (Figure 4C), strongly suggesting

20   that HSP90 inhibitor-induced HSF1 phosphorylation is not restricted to site(s) within the RD

21   (Supplemental Figure 4). Importantly, lack of phosphorylation within the RD domain does not

22   affect trimerization of either full length HSF1 or the various HSF1 deletion mutants discussed

23   above (Supplemental Figure 3E).

24   **HSP90 inhibition increases duration of HSF1 binding to *hsp70* promoter and prolongs**

25   **duration of HSF1 transcriptional activity in response to heat shock**

14

1   To assess the outcome of combining HSP90 inhibition with heat shock compared to either

2   inhibitor or heat shock alone, we monitored the extent and duration of HSF1 occupancy of the

3   *hsp70* promoter by chromatin immunoprecipitation (ChIP) with antibody to HSF1. HEK293 cells

4   were pretreated for 1 hour at 37°C with 10 μM 17AAG or left untreated, and then exposed to

5   heat shock (42°C for 30 minutes). After recovery for varying times at 37°C (no recovery, 2 hours,

6   4 hours, 6 hours), HSF1 ChIP was performed and pulldown of associated *hsp70* promoter

7   sequences was assessed by PCR. An additional group exposed to 17AAG but in the absence of

8   heat shock was also subjected to ChIP analysis at the indicated times (based on recovery time

9   after heat shock). The top panel in Figure 5A shows a representative experiment in which the

10   expected PCR fragments were run on an agarose gel. In the bottom panel, PCR data reflecting

11   the extent and duration of HSF1 occupancy of the *hsp70* promoter were quantified by

12   densitometry (3 independent experiments +/- standard deviation), normalized to input and

13   graphed as shown (Figure 5A, values obtained in the absence of both heat shock and 17AAG

14   pretreatment were arbitrarily set to 1). In the absence of 17AAG, HSF1 binding to the *hsp70*

15   promoter was highest immediately after heat shock (no recovery). Promoter occupancy declined

16   below baseline by 2 hours and remained below baseline for the duration of the experiment. In

17   contrast, 1-hour pre-exposure of cells to 17AAG prior to heat shock resulted in an increased

18   duration of HSF1 binding to the *hsp70* promoter (significantly above baseline at 2 hours of

19   recovery and significantly above the heat shock only value at later time points). Importantly, the

20   17AAG alone samples displayed the same maximal level of HSF1 binding to *hsp70* promoter as

21   did both the heat shock alone samples and the heat shock + 17AAG samples. However, in

22   contrast to cells treated with heat shock alone, HSF1 promoter occupancy did not decline as

23   rapidly in cells treated with 17AAG alone or in combination with heat shock. Thus, the level of

24   HSF1 binding to DNA in these groups remained significantly above heat shock only values for

25   the full 6-hour recovery period (at least 4 hours longer than after exposure to heat shock alone).

15

1   Additionally, we assessed HSF1 transcriptional activity in HEK293 cells after heat shock alone,

2   17AAG alone, or after pre-exposure to 17AAG followed by heat shock, using the same

3   experimental conditions as for the ChIP analysis above (Figure 5B). For this purpose, we

4   examined endogenous *HSP70* gene expression by RT-qPCR. Similar to the impact of heat shock

5   on HSF1 promoter occupancy, relative *HSP70* mRNA expression peaked after 2 hours of

6   recovery from heat shock and declined thereafter to below baseline by 6 hours. In contrast,

7   17AAG alone promoted a modest increase in *HSP70* mRNA (although below the peak seen after

8   heat shock) that remained elevated at 2 and 6 hours. After combined 17AAG treatment and heat

9   shock, *HSP70* mRNA level increased on par with exposure to heat shock alone (similar values at

10   2 hours), but continued to increase to a peak value at 4 hours (25 % higher than the heat shock

11   alone peak value) that was maintained at 6 hours. Importantly, the 4- and 6-hour increase in

12   *HSP70* mRNA after combination treatment was significantly higher (2 – 2.5-fold) compared to

13   HSP90 inhibitor alone.

14   **Discussion**

15   In this study, we assessed the importance of HSP90 conformation for binding to HSF1 and we

16   found that only the ATP-dependent "closed" (e.g., N-domain dimerized) conformational mutants

17   E47A (HSP90α) and E42A (HSP90β) readily co-precipitate full-length HSF1, with HSP90α

18   displaying the stronger interaction. As HSP90α is stress-inducible and transcriptionally regulated

19   by HSF1, this provides an appealing feedback control mechanism to attenuate the HSR,

20   consistent with a model first proposed nearly twenty years ago [8,44]. Indeed, our previous finding

21   that transient over-expression of HSP90α reduced HSF1 transcriptional activity supports this

22   possibility [36]. In the absence of these conformationally restrictive mutations, mammalian HSP90

23   only rarely and transiently occupies the "closed" conformation, even in the presence of ATP,

24   with the bulk of the protein at steady-state occupying an N-domain undimerized conformation [37].

16

1   This is inconsistent with a role for HSP90 in binding to and sequestering HSF1 monomers to

2   prevent trimerization, as has also been proposed [7].

3   To identify chaperone binding sites in HSF1, we used a series of Flag-tagged HSF1 truncation

4   and internal deletion mutants to interrogate binding of HSP90 and HSP70, including the binding

5   of transiently expressed HSP90αE47A or HSP90β E42A. Using this approach, we identified a

6   region in HSF1 that associates most strongly with "closed" HSP90 (Supplemental Figure 5). This

7   motif is comprised of the HR-A/B trimerization domain in the context of a portion of the RD.

8   Binding of "closed" HSP90 to this motif is dramatically enhanced upon deletion of the HSF1

9   HR-C and TAD domains. Since the HR-C domain interacts with the HR-A/B domain to retard

10   HSF1 trimerization under basal conditions, these data are consistent with the preferential binding

11   of "closed" HSP90 to HSF1 trimers. Residues in the C-terminal half of the HR-A/B domain

12   (amino acids 183-214) are necessary and sufficient for binding. Of note, while endogenous

13   HSP90 shares the HR-A/B + RD binding motif, visualization of endogenous HSP90 binding

14   requires deletion of HR-C and TAD domains, very likely due to the weaker affinity of

15   endogenous HSP90 (primarily existing in an "open" conformation) for client interactors. Taken

16   together, these findings are not consistent with a role for HSP90 in repressing HSF1

17   trimerization.

18   In contrast to HSP90, endogenous HSP70 binds to the isolated RD as well as other domains in

19   HSF1. Only the isolated HR-A/B and DBD domains display weak interaction with HSP70.

20   These data build upon earlier work by Shi et al demonstrating repression of HSF1 transcriptional

21   activity as a consequence of HSP70 binding to the TAD [26,27]. Our observation that HSP70 also

22   binds to the stress sensing RD may be relevant to the recent proposal that HSP70 provides an

23   on/off switch for dynamic regulation of stress-induced HSF1 activation in yeast [28]. Whether

24   HSP70 plays a similar role in regulating HSF1 activation in mammalian cells remains to be

25   explored.

17

1   We used these HSF1 truncation and deletion mutants to identify the HR-A/B domain as

2   necessary for dominant negative activity of HSF1 mutants lacking the TAD or the DBD.

3   Importantly, and consistent with our model, we found that HSP90 binding to the HR-A/B is not

4   required for dominant-negative activity as it is not reversed by 17AAG. Instead, dominant-

5   negative activity depends on the ability to form an intermolecular interaction with other HSF1

6   monomers, suggesting that each monomer of an HSF1 trimer must be intact in order to attain

7   optimal HSF1 transcriptional activity.

8   A panel of clinically evaluated N-terminal HSP90 inhibitors uniformly enhance HSF1

9   phosphorylation coincident with dissociation of HSF1 from HSP90. One or more drug-sensitive

10   phosphorylation sites must lie outside of the RD, as an HSF1 protein with all putative RD

11   phosphorylation sites mutated to alanine (dPRD HSF1) still underwent a phosphorylation-

12   dependent mobility shift upon HSP90 inhibitor treatment. We are currently exploring the

13   relevance of this observation to the activity of both wild type and dPRD HSF1, as well as the

14   mechanistic impact of HSP90 inhibitor-mediated HSF1 phosphorylation on its transcriptional

15   activity.

16   HSP90 ATP-competitive N-terminal inhibitors efficiently dissociate HSF1 from HSP90 "closed"

17   mutants, likely as a consequence of ATP displacement and subsequent disruption of N-domain

18   dimerization [40]. If ATP-bound, transiently N-domain dimerized HSP90 (including stress-

19   inducible HSP90α) provides feedback control of the HSR by removing HSF1 trimers bound to

20   heat shock promoter elements, HSP90 inhibition and subsequent dissociation from HSF1 would

21   interfere with this process and would be predicted to prolong and/or intensify the HSR.

22   Consistent with this model, HSF1-CHiP data show that HSP90 inhibition alone and in

23   combination with a brief heat shock significantly enhanced the duration of HSF1 binding to

24   *HSP70* promoter, compared to heat shock alone. When we assessed HSF1 transcriptional activity

25   after these treatments (monitoring endogenous *HSP70* mRNA levels by RT-qPCR), we found

18

1   that pre-exposure to HSP90 inhibitor markedly enhanced the degree and significantly extended

2   the duration of *HSP70* mRNA increase during recovery from a brief heat shock. HSP90

3   inhibition alone caused a moderate increase in *HSP70* transcription, likely due to disruption of

4   the dynamic regulation of constitutive HSF1 activity in transformed cells, a phenomenon

5   recently described by Lindquist and colleagues [11] [12].

6   In summary, this report increases our understanding of the molecular mechanisms that regulate

7   HSF1 and the unique contribution of molecular chaperones, including HSP90 and HSP70, to this

8   process. While not precluding other possibilities, our findings are consistent with a role for

9   HSP90 in attenuating the HSR in an ATP-dependent and conformation-specific manner, a

10   process that is antagonized by ATP-competitive HSP90 inhibitors. A role for HSP90 in

11   disassembling transcriptional regulatory complexes has been described previously [45]. Our data

12   further suggest that, in contrast to HSP90, the readily observable interaction of endogenous

13   HSP70 with HSF1, and our observation that the RD represents a major interaction motif for this

14   chaperone, support the possibility that HSP70 may provide constitutive, stress-sensitive

15   repression of the HSR in mammalian cells.

16

1   **Materials and Methods**

2   **Cell culture:** HEK293 cells (American Type Culture Collection) were maintained in DMEM

3   supplemented with 10% FBS. Cells were grown in 6-well or 96-well plates to 50% confluency

4   and then transfected for 16-24 hours with X-tremeGENE 9 transfection reagent (Roche)

5   according to the provided protocol. HEK293 cells stably expressing an shRNA targeting HSF1

6   was obtained from Dr. Chengkai Dai (Center for Cancer Research, NCI) and has been described

7   previously [46].

8   **Reagents:** 17AAG was obtained from the Developmental Therapeutics Program, NCI. STA9090

9   (ganetespib) and SNX2112 were obtained from Synta Pharmaceuticals and Esanex, respectively.

10   The C-terminal inhibitor KU32 was kindly provided by Dr. Brian Blagg (The University of

11   Kansas, Lawrence, KS). MG132 and molybdate were purchased from Sigma-Aldrich. All drugs

12   were used at 10 μM for IP-WB or *hsp70*-reporter assays.

13   **Plasmids:** Sets of ATPase mutants of pcDNA-FLAG-HSP90α/β and pcDNA-HA-HSP90α/β

14   were made by the Quickchange method to alter designated amino acids. The series of C-terminal

15   deletion constructs of pcDNA-FLAG-HSF1 were made by inserting a terminal stop codon into

16   designated regions by the Quickchange method. The series of N-terminal deletion constructs of

17   pcDNA-FLAG-HSF1 were made by ligating truncated HSF1 insert with pcDNA-FLAG

18   backbone by Gibson assembly according to the provided protocol (New England Biolabs).

19   **Immunoprecipitation and western blotting:** Cells were lysed with TGNET buffer [50 mM Tris

20   HCl pH7.5, 5% Glycerol, 100 mM NaCl, 2 mM EDTA, 0.5% Triton X-100] containing protease

21   inhibitor cocktail and phosphatase inhibitor cocktail (Roche). Lysates were centrifuged at

22   maximum speed for 15 minutes at 4°C. Supernatants were subjected to BCA protein assay for

23   normalization, and then 40 μg of protein were used as input lysate to confirm protein expression.

24   From remaining lysates, 700 μg of protein were subjected to immunoprecipitation by addition of

20

1   40 µl of Anti-FLAG M2 affinity beads (Sigma-Aldrich, A2220) or HA affinity beads (clone HA-

2   7, Sigma-Aldrich, A2095) followed by incubation with rotation for 2 hours at 4°C. After

3   centrifugation, beads were washed 4 times with TGNET buffer. The proteins were then eluted

4   with 30 µl of 2X SDS sample buffer by boiling at 95°C for 5 minutes. Subsequently, samples

5   were subjected to SDS-PAGE followed by western blotting. The following antibodies were used

6   for western blot: HSF1 (Santa Cruz, sc-13516), HSP90 (Enzo Life Science, ADI-SPA-835),

7   HSP70 (Enzo Life Sciences, ADI-SPA-810), HA (Rockland, 600-401-384), and FLAG (Sigma-

8   Aldrich, A8592).

9   Additional Methods may be found in Supplemental Information. Materials and data generated in

10   this study are available upon request.

1   **Acknowledgments**

6   **Competing Interests**

7   None of the authors report competing financial interests with relevance to this work.

8   **Author Contributions**

9   T.K. designed experiments, performed experiments and helped write the paper; T.L.P. designed

10   experiments, performed experiments, interpreted the data and helped write the paper; M.L.T.,

11   K.H.Y., H.S., K.B. performed experiments and interpreted the data; S.L. and J.B.T. performed

12   experiments, interpreted the data and helped write the paper; M.A.B. and L.S. provided reagents,

13   interpreted data and helped write the paper; H.W. and S.C. interpreted data and helped write the

14   paper; L.N. oversaw the whole project, devised experiments, interpreted data and wrote the paper.

15   **Data Availability**

16   Materials and data generated in this study are available upon request to the corresponding

17   authors (T.L.P. and L.N.).

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1   **Figure Legends**

2   **Figure 1. HSF1 is bound by HSP90 in the "closed" conformation**

3   A. HSP90 ATP-driven conformational cycle. HSP90α E47A/HSP90β E42A mutants bind ATP

4   but cannot hydrolyze it and remain trapped in the "closed" N-domain dimerized conformation.

5   Conversely, HSP90α D93A/HSP90β D88A mutants do not bind ATP and remain trapped in an

6   "open" N-domain undimerized conformation.

7   B. HSF1 interaction with HSP90 "closed" and "open" mutants. HEK293 cells were co-

8   transfected with HA-HSF1 and with each FLAG-HSP90 construct, harvested and analyzed for

9   HSF1/HSP90 association by anti-FLAG IP - HSF1 WB. Pull-down experiments were repeated at

10   least twice.

11   C. Reproducible and reciprocal HSF1 interaction with "closed" HSP90α E47A and HSP90β

12   E42A mutants in the presence and absence of MoO4. Interactions were assessed by anti-FLAG

13   (HSP90) IP - HSF1 WB (top panel) or anti-FLAG (HSF1) IP – HA (HSP90) WB (bottom panel).

14   Pull-down experiments were repeated at least twice.

15   D. Effect of HSP90 inhibitors on HSF1-HSP90α E47A interaction. HEK293 cells were

16   transfected with HA-HSF1 along with FLAG-HSP90α E47A, treated with N-terminal or C-

17   terminal HSP90 inhibitors for 4 hours and analyzed by anti-FLAG (HSP90) IP- HSF1 WB. Pull

18   down experiments were repeated at least twice.

19   Full blots used to create cropped figure panels are shown in Supplementary Figure 6.

20

21   **Figure 2: Mapping interaction sites of "closed" HSP90 on HSF1**

22   A. Schema of FLAG-HSF1 C-terminal truncation constructs and their expression in HEK293

23   cells. The associated blot shows expression of each construct in HEK293 cells. Equal amounts of

24   DNA were used for transfection. For construct C477, the upper band represents the intact

25   truncation (designated by an asterisk), while the lower band is likely a proteolytic fragment.

26   B. Schema of FLAG-HSF1 N-terminal truncation and internal deletion constructs and their

27   expression in HEK293 cells. The associated blot shows expression of each construct in HEK293

28   cells. Equal amounts of DNA were used for each transfection. Note the poor expression of N195.

29   For N195-C383, the lower band represents the intact construct (designated by an asterisk); the

30   upper band may reflect a post translationally modified peptide.

28

1 C. Interaction of selected FLAG-HSF1 C-terminal truncation constructs with HA-tagged

2 HSP90α E47A. HEK293 cells were transfected with either FLAG-HSF1 constructs or FLAG-

3 pcDNA control and HA-HSP90α E47A, and then analyzed by anti-FLAG (HSF1) IP followed by

4 HA (HSP90α E47A) WB. Pull downs were performed in triplicate and western blots analyzed by

5 densitometry. All HA IP bands were normalized to their respective FLAG IP signals (see

6 Supplemental Figure 6, compilation of full blots) and then normalized to the WT signal. A one-

7 way ANOVA utilizing multiple comparisons was performed to determine significant differences

8 in HSP90 interaction between truncated HSF1 constructs and F-HSF1 WT. A representative blot

9 is shown above the bar graph. Significance is indicated by asterisks (** indicates $p<0.01$). The p-

10 value for the difference between C339 and WT interaction was 0.0534.

11 D. Interaction of FLAG-HSF1 N-terminal truncation and internal deletion constructs with HA-

12 tagged HSP90α E47A: HEK293 cells were transfected and analyzed as in panel C. Significance

13 is indicated by asterisks (** indicates $p<0.01$ and **** indicates $p<0.0001$).

14 Full blots used to create cropped figure panels and for densitometry analysis for the bar graphs

15 are

16 shown in Supplementary Figure 6.

17

18 **Figure 3: Effects of HSF1 truncation and internal deletion mutants on hsp70 promoter**

19 **reporter activity and HSF1 dimerization.**

20 A. Expression of TAD-deleted HSF1 constructs inhibits endogenous HSF1 driven *hsp70*

21 promoter-

22 reporter activity, while removal of the HR-A/B domain abrogates dominant negative activity.

23 HEK293 cells were transfected with *hsp70* promoter-reporter construct and HSF1 C-terminal

24 truncation constructs, treated with DMSO or 17AAG for 1 hour, heat shocked at $42^{O}C$ for 30

25 minutes and allowed to recover for 4 hours before harvesting.

26 B. Disruption of HA-tagged HSP90α E47A interaction with HSF1 C-terminal RD truncations by

27 17AAG. HEK293 cells were transfected with HA-HSP90α E47A and FLAG-HSF1 RD

28 truncation mutants, then analyzed by anti-FLAG (HSF1) IP followed by HA (HSP90α E47A)

29 WB. Pull-down experiments were repeated at least twice.

30 C. Deletion of the DBD and retention of the HR-A/B domain inhibits *hsp70*-reporter activity

29

1 driven by endogenous HSF1. HEK293 cells were transfected, treated and processed as described

2 in panel A.

3 D. Deletion of the HR-A/B domain disrupts HSF1 dimerization. HEK293 cells were transfected

4 with WT HA-HSF1 and FLAG-HSF1 deletion constructs, then analyzed by anti-FLAG IP and

5 HA WB. Pull-down experiments were repeated at least twice.

6 E. Summary of the effects of HSF1 domain deletions on *hsp70*-reporter activity.

7 Full blots used to create cropped figure panels are shown in Supplementary Figure 6.

8

9 **Figure 4: Impact of RD phosphorylation on HSP90 inhibitor effect on HSF1 activity and**

10 **mobility shift in SDS-PAGE, and on HSF1 interaction with HSP90**

11 A. Schema of WT and dPRD FLAG-HSF1 constructs depicting RD phosphorylation sites

12 mutated to alanine in dPRD-HSF1.

13 B. The N-terminal HSP90 inhibitor 17AAG enhances transcriptional activity of dPRD HSF1.

14 HEK293 cells were transfected with *hsp70*-reporter construct and HSF1 C-terminal truncation

15 constructs, treated with DMSO or 17AAG for 1 hour, heat shocked at 42°C for 30 minutes and

16 allowed to recover for 4 hours before harvesting. This experiment was repeated three times and

17 values represent mean +/- S.D.

18 C. N-terminal HSP90 inhibitors induce a mobility shift in dPRD HSF1 similarly to that seen with

19 WT-HSF1. HEK293 cells were transfected with WT or dPRD HSF1, treated with the three N-

20 terminal HSP90 inhibitors as shown, and harvested and HSF1 was analyzed by WB.

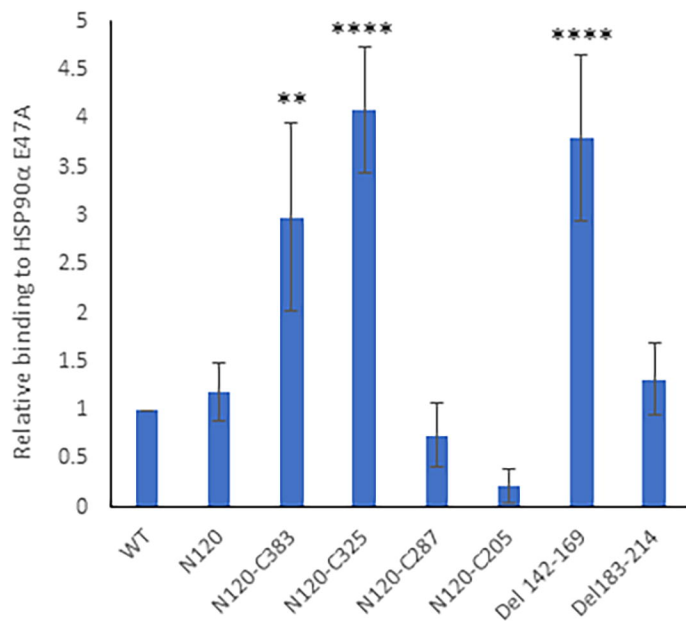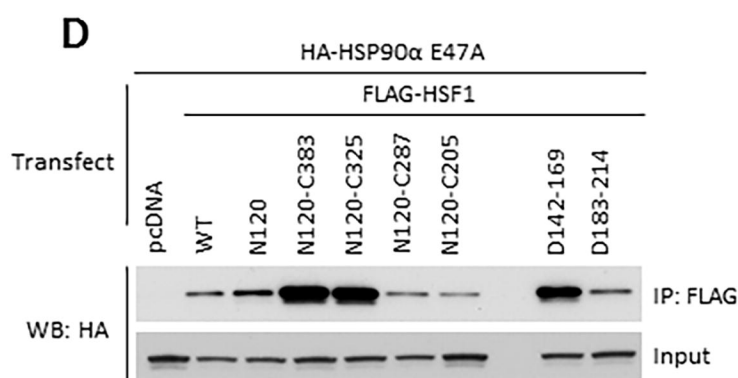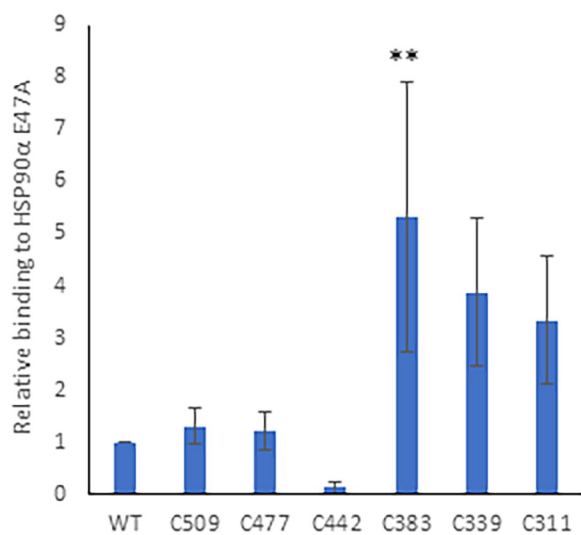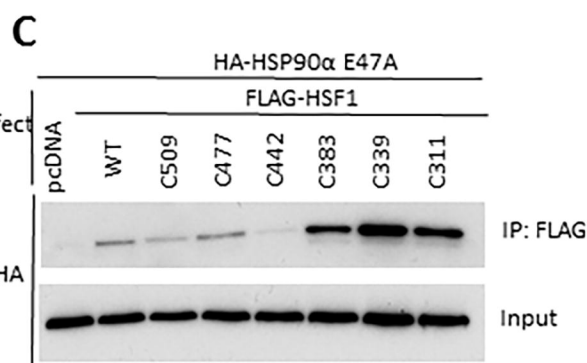21 Full blots used to create cropped figure panels are shown in Supplementary Figure 6.

22

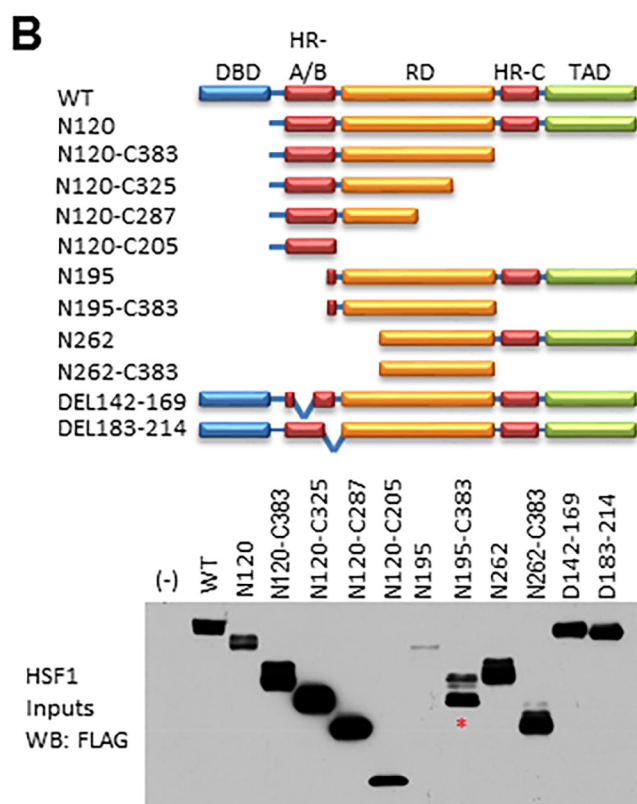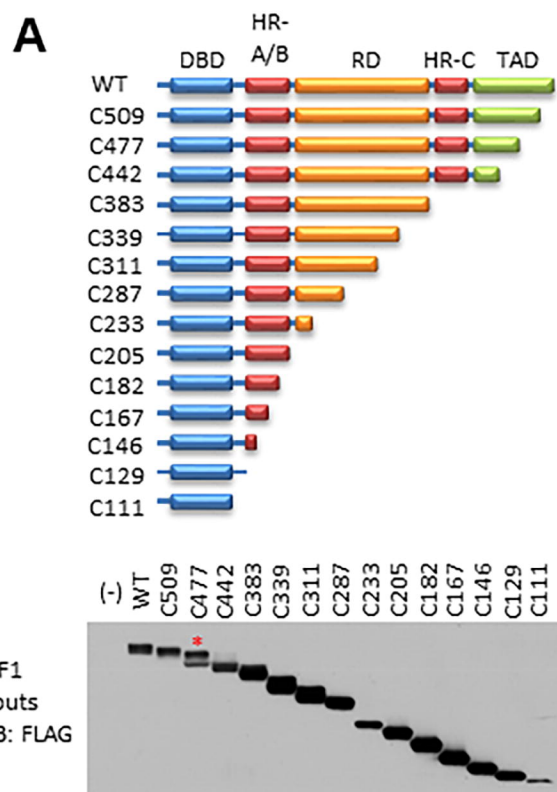23 **Figure 5: HSP90 inhibition increases duration, but not extent, of HSF1 binding to the**

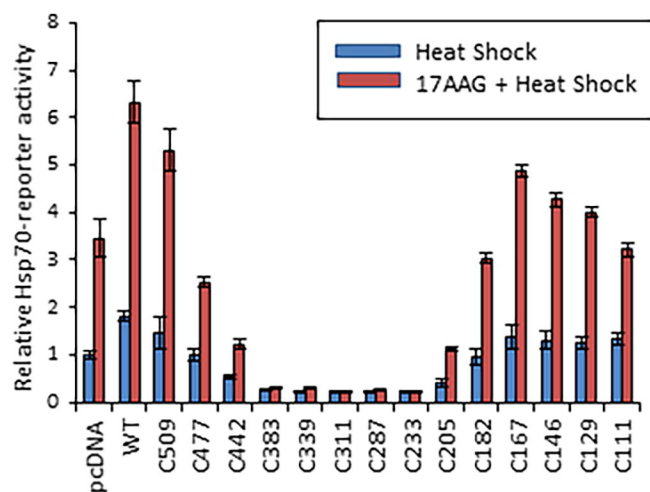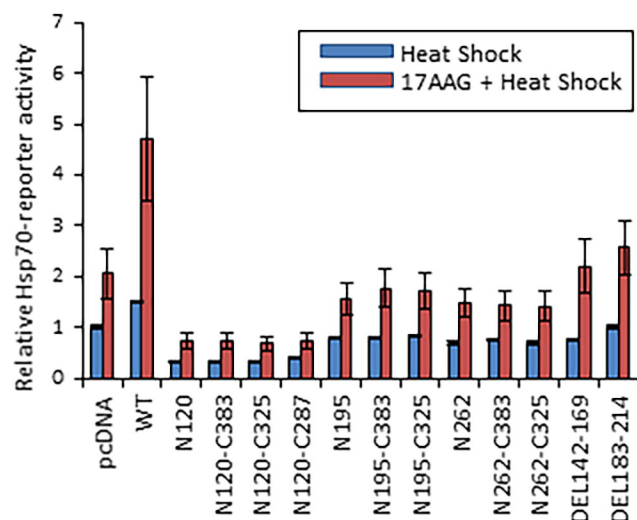24 **hsp70 promoter and enhances HSF1 transcriptional activity in response to heat shock**
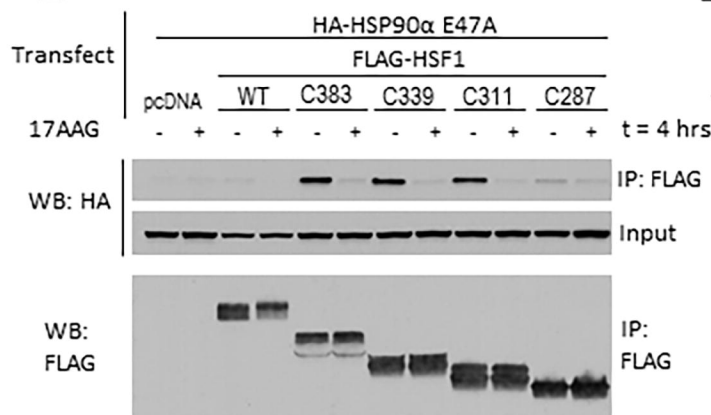
25 A. Chromatin immunoprecipitation using HSF1 antibody followed by PCR amplification of the

26 *hsp70* promoter sequence (top, representative DNA gel; bottom, graphical display of band optical

27 density data from 3 independent experiments +/- S.D.). HEK293 cells were either subjected to

28 heat shock (42°C/30 minutes), treated with 17AAG, or both. The 17AAG groups were pre-

29 exposed to the HSP90 inhibitor (10 μM, 1 hour) prior to heat shock. Samples were then put back

30 in a 37°C incubator to recover for the indicated times. Values for the 'no drug, no HS' control

30

1 (first lane) were set to 1, and remaining values are expressed as fold above or below this baseline

2 value. This experiment was repeated three times and values represent mean +/- S.D. A two-way

3 ANOVA was used to analyze the samples for statistically significant differences compared to
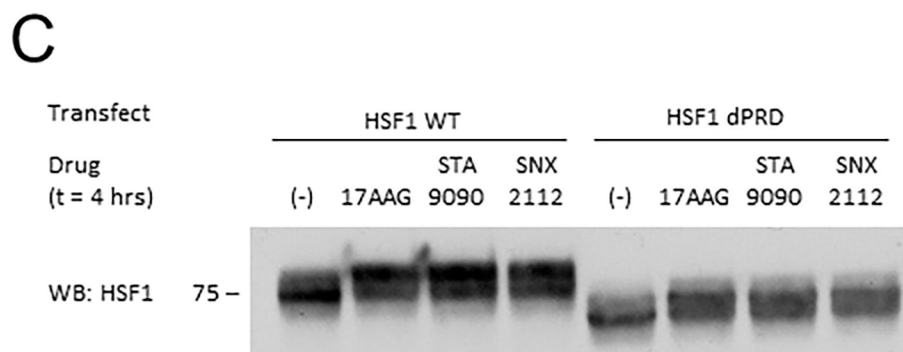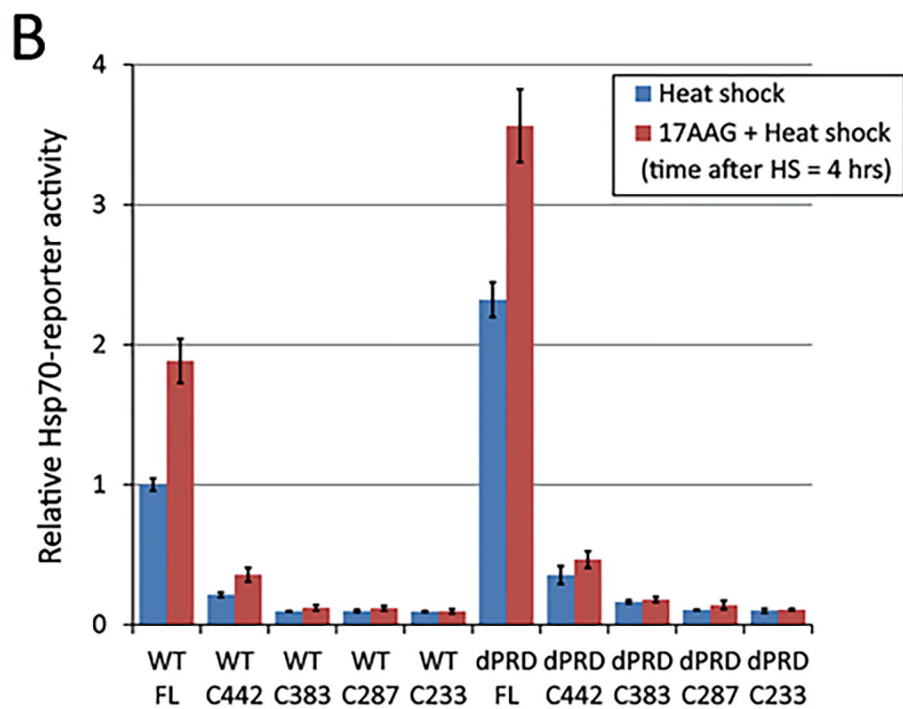
4 other samples at a given time. Significant difference compared to 'no drug, no HS' is indicated

5 by asterisks (*** indicates $p<0.001$, ** indicates $p<0.01$, and * indicates $p<0.05$); '17AAG + HS'

6 is also significantly lower than '17AAG' alone at 6hr (## - $p<0.01$) and significantly higher than

7 'HS' alone at 2 and 4hr (+++ indicates $p<0.001$, + indicates $p<0.05$).

8 B. Endogenous HSP70 mRNA was quantified by RT-qPCR in HEK293 cells subjected to heat

9 shock alone, 17AAG alone, or heat shock after pre-exposure to 17AAG, using identical

10 experimental conditions as in panel A. This experiment was performed in triplicate and values

11 represent mean +/- S.D. A two-way ANOVA was used to analyze for statistically significant

12 differences. Notably, there was a statistically significant difference between '17AAG alone' and

13 '17AAG + HS' samples at 4 and 6 hours (*** indicates $p<0.001$ and **** indicates $p<0.0001$).

14

A

Relative Hsp70-reporter activity

Legend: Heat Shock (blue), 17AAG + Heat Shock (red)

X-axis: pcDNA, WT, C509, C477, C442, C383, C339, C311, C287, C233, C205, C182, C167, C146, C129, C111

B

Transfect: HA-HSP90α E47A / FLAG-HSF1

| | pcDNA | WT | C383 | C339 | C311 | C287 |
17AAG: - + - + - + - + - + - +   t = 4 hrs

WB: HA — IP: FLAG
Input

WB: FLAG — IP: FLAG

C

Relative Hsp70-reporter activity

Legend: Heat Shock (blue), 17AAG + Heat Shock (red)

X-axis: pcDNA, WT, N120, N120-C383, N120-C325, N120-C287, N195, N195-C383, N195-C325, N262, N262-C383, N262-C325, DEL142-169, DEL183-214

D

Transfect: HA-HSF1 WT / FLAG-HSF1

pcDNA, WT, C383, C287, C233, C205, C182, C167, C146, C129, N120, N195, D142-169, D183-214

WB: HA — IP: FLAG
Input

WB: FLAG — IP: FLAG

E

Functional HSF1
Constructs: WT, C509

Truncated/deleted Transactivation domain
Constructs: C477, C442, C383, C339, C311, C287, C233, C205

Weak DNA binding
Constructs: N120, N120-C383, N120-C325, N120-C287

No Trimerization (Activation by endogenous HSF1)
Constructs: C182, C167, C146, C129, C111, N195, N262, N195-C383, N195-C325, N262-C383, N262-C325, DEL 142-169, DEL 183-214

A

DBD HR-A/B RD HR-C TAD

WT-FL
WT-C442
WT-C383
WT-C287
WT-C233
dPRD-FL
dPRD-C442
dPRD-C383
dPRD-C287
dPRD-C233

○ Phosphorylation sites in RD
○ Mutated to Alanine

B

Relative Hsp70-reporter activity

■ Heat shock
■ 17AAG + Heat shock
(time after HS = 4 hrs)

WT FL WT C442 WT C383 WT C287 WT C233 dPRD FL dPRD C442 dPRD C383 dPRD C287 dPRD C233

C

Transfect	HSF1 WT				HSF1 dPRD			
Drug (t = 4 hrs)	(−)	17AAG	STA 9090	SNX 2112	(−)	17AAG	STA 9090	SNX 2112

WB: HSF1   75 −

A

17AAG pretreatment (1hr)

| HS (30min) | – | + | + | + | + | – | – | – | – | + | + | + | + | Mock |
| Time after HS (hr) | 0 | 0 | 2 | 4 | 6 | 0 | 2 | 4 | 6 | 0 | 2 | 4 | 6 | IP |

ChIP: HSF1

Input

B