AKT1 mediates multiple phosphorylation events that functionally promote HSF1 activation

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Running Title: AKT1-mediated activation of HSF1

Keywords: HSF1, AKT1, heat shock, mTOR, MEK1, p38, CDK9, TFIIB

Abstract

The heat stress response activates the transcription factor heat shock factor 1 (HSF1), which subsequently upregulates heat shock proteins to maintain the integrity of the proteome. HSF1 activity requires nuclear localization, trimerization, DNA binding, phosphorylation, and gene transactivation. Phosphorylation at S326 is an important regulator of HSF1 transcriptional activity. Phosphorylation at S326 is mediated by AKT1, mTOR, p38, and MEK1. mTOR, p38, and MEK1 all phosphorylated S326 but AKT1 was the more potent activator. Mass spectrometry showed that AKT1 phosphorylated HSF1 at T142, S230, and T527 in addition to S326 whereas the other kinases did not. Subsequent investigation revealed that phosphorylation at T142 is necessary for HSF1 trimerization and that S230, S326, and T527 are required for HSF1 gene transactivation and recruitment of TFIIB and CDK9. This study suggests that HSF1 activity is regulated by phosphorylation at specific residues that promote different stages of HSF1 activation. Furthermore, this is the first study to identify the functional role of these phosphorylation events.

Introduction

Attenuation of cellular stressors is necessary for cell viability in unicellular and multicellular organisms. The discovery of the cellular heat stress response was first observed in 1962 (1). Over two decades later, it was discovered that heat shock factor 1 (HSF1) is the master regulator of the heat shock response (2,3). Prior to heat stress-induced activation, HSF1 is sequestered in the cytoplasm within an inhibitory protein complex comprised of several molecular chaperone proteins (4-7). With the onset of heat stress, this complex dissociates and the HSF1 protein undergoes nuclear localization, trimerization, and phosphorylation leading to DNA binding at heat shock elements (HSEs) and gene transactivation (8-13). HSF1 target genes following heat stress include a large panel of chaperone proteins that safeguard the proteome from heat-induced denaturation (3,14).

Dysregulation of HSF1 is associated with several disease phenotypes. Elevated HSF1 activity in multiple cancer types is a frequent occurrence (14-18). Loss of HSF1 delays or prevents formation of multiple cancer types (19-22). HSF1 appears to promote cancer by regulating genes distinct from its heat stress-induced gene targets (14) but has multiple functions to support tumor growth and progression (23). Conversely, loss of HSF1 is associated with neurodegenerative diseases, such as Huntington's and Alzheimer's diseases among others (24-31).

The majority of the functions of HSF1, both physiological and pathological, require a transcriptionally active HSF1. Transcriptionally active HSF1 exists in a trimer complex in the nucleus that is capable of binding to HSEs (8-13). HSF1 phosphorylation appears to strongly regulate the transcriptional activity of HSF1, particularly at S326 (8). Phosphorylation at S326 is routinely used as a marker of active HSF1 (8,15,23,32-38). Since the initial observation that S326 phosphorylation affects heat stress-induced HSF1 activity (8), four kinases have been observed to phosphorylate S326 including mTORC1 (32,36), MEK1 (37), p38 (33), and AKT1 (15). However, the functional mechanism for how S326 phosphorylation promotes HSF1 transcriptional activity is not well understood. Furthermore, the relative contribution to HSF1 activity by these kinases is unknown. Our results indicate that AKT1 activates HSF1 more potently than other kinases that phosphorylate S326 has the unique ability to phosphorylate additional residues that greatly enhance different steps in the process of HSF1-driven.

Results

AKT1 enhances HSF1 transcriptional activity mTORC1, MEK1, or p38.

HSF1 transcriptional activity has previously been shown to be regulated by phosphorylation at S326 (8). Our work, and others, previously identified that HSF1 is phosphorylated at S326 by AKT1, mTORC1, p38, and MEK1 leading to HSF1 transcriptional activity (15,32,33,36,37). However, the relative contribution to HSF1 transcriptional activity of these kinases is unknown. Our previous results indicate AKT1 can phosphorylate S326 using an in vitro kinase assay (15). MEK1, mTORC1, and p38 all were also able to phosphorylate S326 using this assay (**Fig 1A**). Furthermore, introduction of any of these kinases into cells increased S326 phosphorylation of HSF1 (**Fig 1B**). Interestingly, however, AKT1 was more potent than the others in activating HSF1 transcriptional activity (**Fig 1C**). To further assess the correlation between these kinases and HSF1 activity, gene set enrichment analysis (GSEA) was performed (39,40). This analysis was performed using TCGA datasets for breast cancer (BRCA) and colorectal adenocarcinoma (COADREAD) wherein patients for each dataset were scored for HSF1 activity using the HSF1 CaSig (14). The patients in each dataset were then separated into high HSF1 activity (upper 50%) or low HSF1 activity (lower 50%) based on HSF1 signature score. Enrichment in these two groups of HSF1 activity were performed using signatures for activity of AKT1 (41,42), p38 (40), mTORC1 (43), and MEK1 (44). Results indicated that signatures for AKT1 and mTORC1 were significantly enriched in high HSF1 activity tumors (**Fig 2A-H**), suggesting a strong correlation between the activity of these kinases and HSF1 activity in living tissues. There was no significant enrichment toward either high or low HSF1 activity for p38 or MEK1 signatures. Taken together, these data indicate a lack of consistency between HSF1 phosphorylation at S326 by this group of kinases and the resulting HSF1 transcriptional activity.

AKT1 phosphorylates additional residues aside from S326 that are critical for HSF1 activity.

AKT1, mTOR, MEK1, and p38 can phosphorylate HSF1 at S326, its canonical activating phosphorylation event (8). However, phosphorylation by these different kinases resulted in varying degrees of HSF1 transcriptional activity with AKT1 promoting higher activity than the other kinases (**Figs. 1-2**). To resolve this apparent contradiction, we reasoned that AKT1 may be phosphorylating additional residues that support HSF1 activation. To test this hypothesis, recombinant HSF1 was subjected to a kinase assay with either AKT1, p38, mTOR, or MEK1 followed by mass spectrometry to identify novel phosphorylation sites (**Fig 3A**). Results from this analysis are summarized in **Fig 3B-C**, which illustrates all of the sites phosphorylated by each kinase outside of the known target of S326. Interestingly, AKT1 phosphorylated T142, S230, and T527. These sites were entirely unique to AKT1 as only MEK1 was seen to phosphorylate any of these sites. All four of these sites, including S326, are conserved across several mammalian species (**Fig 3D**). Phosphorylation at T142 has only been reported once and this was mediated by CK2 (45). Phosphorylation at S230 has been previously reported and was shown to promote inducible HSF1 activity (46). Phosphorylation at T527 has never been reported. Phospho-null mutations at each of these sites individually resulted in no substantial effect or a weak inhibitory effect on HSF1 activity (**Fig 4A**). However, when all four of these sites were phospho-null, HSF1 no longer responded to heat stress (**Fig 4B**). These results indicate that AKT1 modifies HSF1 in a unique pattern by phosphorylating T142, S230, and T527 in addition to S326. This unique pattern of phosphorylation may mechanistically explain why AKT1 promotes HSF1 activity more potently than the other kinases tested.

Phosphorylation at T142 enhances trimerization of HSF1.

AKT1 phosphorylates HSF1 at multiple residues in addition to S326 (**Fig 3**). Should these additional phosphorylation sites by AKT1 mediate the additional HSF1 activity observed in the presence of AKT1,

it stands to reason that these sites functionally participate in the activation process of HSF1. Therefore, the role of each of these four phosphorylation sites in the process of HSF1 activation was investigated. Threonine 142 sits in the first leucine-zipper domain, also referred to as the heptad repeat domain (47) and this domain is responsible for trimerization of HSF1 (11,13,48). Considering T142 is located in this domain, it was tested whether phosphorylation at this site affected trimerization. Introduction of AKT1 with wild type (WT)-HSF1 resulted in an increase in HSF1 trimerization (**Fig. 5A**). However, HSF1 trimerization did not occur in the T142A phospho-null mutant in the presence of AKT1 (**Fig 5A**). To test whether T142 regulation of trimerization was AKT1-specific, HSF1 trimerization was also tested in the presence of CK2, which has previously been observed to phosphorylate T142 (45). In the presence of CK2, WT-HSF1 also showed a significant increase in trimerization but T142A-HSF1 did not undergo trimerization in the presence of CK2 (**Fig 5B**). These results likely indicate that phosphorylation at T142 is an important regulatory step of HSF1 trimerization and is not AKT1-specific. Binding of HSF1 to DNA requires trimerization (49-52). Therefore, if T142 phosphorylation regulates trimerization, it should have downstream effects on HSF1 DNA binding. To test this hypothesis, FLAG-tagged WT- or T142A-HSF1 were expressed in cells with or without heat shock and subjected to chromatin immunoprecipitation (ChIP). Results indicate that T142A-HSF1 had reduced DNA binding to multiple putative HSF1 target gene promoters compared to WT-HSF1 in response to heat stress (**Fig 5C**). These results suggest that phosphorylation at T142 is necessary for effective trimerization and DNA binding of HSF1.

Phosphorylation at S230, S326, and T527 enhances HSF1 association with general transcriptional factors.

The human HSF1 protein has 529 residues, of which 103 are residues potentially capable of phosphorylation by being tyrosine (n=8), serine (n=70), or threonine (n=25). A total of 39 of these residues have been previously shown to be phosphorylated either by non-biased screening using mass spectrometry or low-throughput methods (53). Our analysis identified T527 as a phosphorylated residue in the presence of AKT1 (**Fig 3**), which is a novel phosphorylation site never previously reported. Since this residue resides in the transactivation domain (TAD) of HSF1, it was hypothesized that T527 may contribute to the function of the TAD in recruiting transcriptional machinery for initiation of transcription. HSF1 has previously been shown to have physical association with CDK9 as part of the p-TEFb complex, TFIIB, and TATA binding protein (TBP) (54-58). To initially screen if phosphorylation at T527 affects HSF1 interaction with these proteins, either WT- or T527A-HSF1 were expressed in cells in the presence of CDK9, TFIIB, or TBP followed by assessment of HSF1 activity. HSF1 activity was significantly reduced with T527A phospho-null mutant in the presence of CDK9 and TFIIB but not TBP (**Fig 6A-C**), suggesting this phosphorylation site may be involved in interaction with CDK9 and TFIIB. Co-immunoprecipitation revealed a loss of interaction between T527A-HSF1 with CDK9 and TFIIB (**Fig 6D-E**), indicating phosphorylation at this site is critical for this protein complex formation.

Phosphorylation at S326 to upregulate HSF1 activity is well documented (8,15,32,33,36,37). Phosphorylation at S230 has also been observed to promote HSF1 transcriptional activity (46). However, the precise function of these phosphorylation events in HSF1 activation is unclear. Both S230 and S326 sit in the regulatory domain of HSF1, which has previously been shown to be a region for many post-translational modifications (8,59). Additionally, it is apparent that the regulatory domain, and S326 specifically, do not regulate nuclear localization or DNA binding of HSF1 (59,60). Since this domain also does not participate in trimerization, it was tested whether S230 and S326 regulate gene transactivation by interaction with transcriptional machinery. Similar to T527, phospho-null mutants of S230 and S326 had decreased transcriptional activity in the presence of CDK9 (**Fig 7A**). However, only the S326 phospho-null reduced HSF1 activity in the presence of TFIIB (**Fig 7B**). Similar to T527, neither site had any effect on HSF1 activity in the presence of TBP (**Fig 7C**). It was also observed that the S326A mutant had decreased protein-protein interaction with CDK9 and TFIIB (**Fig 7D-E**) and the S230A mutant had decreased interaction with CDK9 (**Fig 7F**). Collectively, these results indicate that S230 and S326

phopshorylation contribute to HSF1 interaction and recruitment of general transcription factors CDK9 and TFIIB. Phosphorylation at these sites and the promotion of these activating events likely contributes to the increased potency of HSF1 activation by AKT1.

Discussion

The heat shock response is primarily regulated by increasing the transcriptional activity of HSF1 in response to heat stress. The regulation of HSF1 activity by phosphorylation has been debated but what is generally accepted is that S326 phosphorylation is important for HSF1 activity (8). Phosphorylation at S326 has been shown to be mediated by several kinases including mTORC1 (32,36), AKT1 (15), MEK1 (37), and p38 (33). The involvement of these kinases appears to be context-dependent. The current study indicated that the context-dependent nature of these kinases may stem from the unique pattern of residues phosphorylated by each kinase. This study indicates mTORC1, MEK1, and p38 phosphorylate HSF1 primarily at S326 as well as S363, a known inhibitory site (61,62), S221, also thought to be an inhibitory site (60), or at S241 and S344, which are two novel phosphorylation sites with unknown function. However, AKT1 appeared to be a more potent inducer of HSF1 activity that likely stems from its ability to phosphorylate an additional three residues identified here that functionally promote HSF1 activation. The PI3K pathway has been linked to HSF1 activity as far back as 2000 (63,64) but whether this was an indirect regulation or a direct regulation was unclear until our previous study indicating AKT1 directly activates HSF1 (15). Collectively, this study indicates for the first time the functional role in HSF1-induced transcription for these phosphorylation events by enhancing trimerization and gene transactivation and AKT1 targeting these residues likely explains its ability to promote higher levels of HSF1 activity.

Upon heat stress, HSF1 can be observed to undergo hyperphosphorylation. Hyperphosphorylation was initially thought to be a part of the HSF1 activation process (10,12,65). A more recent study indicated that an HSF1 mutant wherein all of the serine residues within the regulatory domain are phospho-null has greater activity compared to WT-HSF1 and this mutant did not undergo classic hyperphosphorylation (60), suggesting that hyperphosphorylation is actually suppressive to HSF1 activity. There were also early indications that extensive phosphorylation can suppress HSF1 activity (66). However, results from the current study indicate that phosphorylation on specific residues enhance HSF1 transcriptional activity by mediating specific steps in the process of activation. Thus, it seems that the model for HSF1 activation involves phosphorylation at specific residues but then hyperphosphorylation leads to inactivation of HSF1. This model would presume that activating phosphorylation events occur prior to hyperphosphorylation, although there is currently no published evidence for this temporal relationship. This model is also supported by the numerous studies indicating that specific phosphorylation on S326 is important for HSF1 activity. While this model is intriguing as a potential negative feedback on HSF1, the mechanism for hyperphosphorylation, and the kinases involved, remain to be uncovered.

Phosphorylation at S326 has been used as a biomarker for transcriptionally active HSF1 in many studies since its identification. The current study confirms previous studies that multiple kinases can phosphorylate S326 and this site is associated with increased HSF1 activity. However, the current study also identified that AKT1 was a stronger inducer of HSF1 activity despite S326 being phosphorylated by all kinases tested. This apparent paradox prompts the question of whether S326 phosphorylation is a reliable marker for HSF1 activity. The current study indicates S326 phosphorylation actively participates in HSF1-induced gene transactivation giving support that it can serve as a marker for activity. Other studies have also observed S326 phosphorylation can still be used as a marker in disease contexts, such as S326 phosphorylation serving as a biomarker for ovarian cancer (38). Thus, based on the current and previous studies, it is likely that S326 phosphorylation is a marker for transcriptionally-competent HSF1 but may not fully indicate the intensity of activity.

The potency for AKT1-mediated activation of HSF1 observed in the current study advances our understanding of this relationship in disease settings wherein HSF1 is dysregulated. In particular, HSF1 is well known to be hyperactivated in several cancer types (14,15,17-20,22,35,37,38,67). Hyperactivation of HSF1 in cancers likely stems from the presence of several cellular stressors in cancer cells and the tumor

microenvironment as well as dysregulation of many kinases. While AKT1, mTORC1, MEK1, and p38 can all activate HSF1, the current study indicates that activity of only AKT1 and mTORC1 maintain a strong association with HSF1 activity in tumors. It is likely that AKT1 activity maintains a strong association with HSF1 activity due to the mechanisms uncovered in this study. The likely explanation for the association between HSF1 activity and mTORC1 activity in these cancer datasets is that HSF1 has been shown to enhance mTORC1 activity via suppression of JNK (36). This would explain the stronger association between HSF1 and mTORC1 if HSF1 is acting upstream of mTORC1.

These studies together indicate that HSF1 activity is regulated by phosphorylation at specific residues. In particular, phosphorylation at the canonical site S326 promotes gene transactivation by enhancing the association between HSF1 and CDK9/TFIIB. Furthermore, AKT1 can phosphorylate additional residues that further enhance HSF1 activation including T142, S230, and T527. We show here that T142 phosphorylation enhances trimerization of HSF1 in response to AKT1 or CK2. These data demonstrate that S230 phosphorylation by AKT1 enhances HSF1 association with CDK9 to promote transcription. Phosphorylation at T527 has never been reported and we observed AKT1-mediated phosphorylation of T527 enhances the association between HSF1 and CDK9/TFIIB to promote transcription. These studies illuminate further the complex regulation of HSF1 activation and, in concert with previous reports, likely indicate that HSF1 is activated by phosphorylation at a few specific residues. Additionally, a greater understanding of HSF1 post-translational modifications in response to various cellular stressors would likely indicate key events required for consistent HSF1 activation.

Experimental Procedures and Data Availability

Cell culture and reagents

Human embryonic kidney cells (HEK293) used in this study were obtained from ATCC (Manassas, VA, USA), and maintained according to ATCC's instructions. All chemicals were purchased from Sigma (St Louis, MO, USA) unless otherwise stated. All transfections were performed with cells in exponential growth using X-tremeGENE HP (Roche, Indianapolis, IN, USA).

Plasmids, transfection and luciferase assay

The FLAG-HSF1 plasmid was purchased from Addgene (ID 32537, Cambridge MA, USA), which was originally established by Dr Stuart Calderwood (68). The pcDNA3 Flag HA plasmid was purchased from Addgene (ID 10792), which was originally established by Dr William Sellers. The pcDNA3 Myr HA AKT1 plasmid was purchased from Addgene (ID 9008), which was originally established by Dr William Sellers (69). The pZW6 (CK2alpha) plasmid was purchased from Addgene (ID 27086), which was originally established by Dr David Litchfield (70). The pcDNA 3-CDK9 HA plasmid was purchased from Addgene (ID 14640), which was originally established by Dr Matija Peterlin (71). The pEGFP-C1-TFIIB plasmid was purchased from Addgene (ID 26675), which was originally established by Dr Sui Huang (72).

The heat shock element (HSE) firefly luciferase reporter was purchased from Promega (Madison, WI) and has two copies of the HSE promoter driving the luc2P gene. A Renilla luciferase expression vector, pRL-SV40P, was used to control for transfection efficiency. The pRL-SV40P was purchased from Addgene (ID 27163), which was originally established by Dr. Ron Prywes (73). 48hr after transfection, the cells were lysed and luciferase activity was measured using the Firefly and Renilla Luciferase Assay Kit (Promega). Relative promoter activity was computed by normalizing the Firefly luciferase activity against that of the Renilla luciferase. Experiments were performed using three independent biological replicates.

Immunoblotting and Immunoprecipitation (IP)

This was performed as we described previously (15,67). Briefly, protein was isolated using RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 1% SDS) for immunoblotting or with IP lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1% Triton X-100) for lysate to be used for IP. Lysates were subjected to SDS-PAGE and subjected to immunoblotting with indicated antibodies. Antibodies used for immunoblotting included p-HSF1 (S326) (Abcam #76076), HSF1 (CST #4356), AKT1 (CST #2938), AKT2 (CST #3063), AKT3 (CST #3788), beta-actin (BD Biosciences #612656), GAPDH (CST #2118), GST (CST #2625), Flag (Sigma #3165), CDK9 (CST #2316), and GFP (CST #2956). Antibodies used for IP were FLAG resin beads (Sigma #A2220) and control mouse IgG beads (Sigma #A0919).

Kinase Assay and Mass Spectrometry

Recombinant AKT1, recombinant AKT2, recombinant HSF-1, recombinant p38, recombinant mTOR, recombinant MEK and recombinant ERK were purchased from Sigma. Indicated amounts of recombinant GST-HSF-1 (0.1–0.4 mg) was incubated with or without 0.1 mg recombinant kinases for up to 120 min at 30°C in the presence of ATP in kinase assay buffer (100 mM HEPES, 50 mM $MgCl_2$, 50 mM $MnCl_2$). Samples were then boiled and subjected to SDS–PAGE and WB with indicated antibodies or subjected to mass spectrometry for detection of post-translational modifications.

Mass spectrometry for post-translational modifications was performed at the Laboratory for Biological Mass Spectrometry at Indiana University (Bloomington, IN). Protein samples were dissolved in 8M urea, 100mM ammonia bicarbonate (pH8.0) solution. Samples were incubated for 1 hr at 56°C with 10 mM TCEP to reduce cysteine residue side chains. The alkylation of cysteine residue side chains was proceeded for 45 min at room temperature in the dark with 20 mM iodoacetamide. For chymotrypsin digestion, 1 μg of chymotrypsin was added to the diluted sample and the samples were digested at 25°C

overnight. Desalted peptides were injected into an Easy-nLC 100 HPLC system coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific, Bremen, Germany). Peptide samples were loaded onto an Acclaim PepMapTM 100 C18 trap column (75 µm × 20 mm, 3 µm, 100 Å) in 0.1% formic acid. The peptides were separated using an Acclaim PepMapTM RSLC C18 analytical column (75 µm × 150 mm, 2 µm, 100 Å) using an acetonitrile-based gradient (Solvent A: 0% acetonitrile, 0.1% formic acid; Solvent B: 80% acetonitrile, 0.1% formic acid) at a flow rate of 300 nl/min. A 30 min gradient was as follows: 0-0.5 min, 2-8% B; 0.5-24 min, 7-38% B; 24-26 min, 40-100% B; 26-30 min, 100% B, followed by re-equilibration to 2% B. The electrospray ionization was carried out with a nanoESI source at a 260 °C capillary temperature and 1.8 kV spray voltage. The mass spectrometer was operated in data-dependent acquisition mode with mass range 350 to 2000 m/z. The precursor ions were selected for tandem mass (MS/MS) analysis in Orbitrap with 3 sec cycle time using HCD at 30% collision energy. Intensity threshold was set at 2.5e4. The dynamic exclusion was set with a repeat count of 1 and exclusion duration of 30 s. The resulting data was searched in Protein Prospector (http://prospector.ucsf.edu/prospector/mshome.htm) against Human HSF1 sequence (Q00613). Carbamidomethylation of cysteine residues was set as a fixed modification. Protein N-terminal acetylation, oxidation of methionine, protein N-terminal methionine loss, pyroglutamine formation, phosphorylation on STY were set as variable modifications. A total of two variable modifications were allowed. Chymotrypsin digestion specificity with two missed cleavage was allowed. The mass tolerance for precursor and fragment ions was set to 5 ppm and 10 ppm respectively. Peptide and protein identification cut-off scores were set to 15 and 22, respectively.

HSF1 Trimerization

Cells were transfected with HSF1-Flag for 48 hrs. Cells were then fixed by adding 1 mM ethylene glycol bis-succinimidylsuccinate (Thermo Scientific, Waltham, MA) for 30 min at room temperature (RT) for crosslinking. The crosslinking reaction was quenched by addition of 10 mM Tris, pH 7.5, for 15 min at RT. Protein was isolated using RIPA buffer and the resulting lysate underwent SDS-PAGE and subjected immunoblotting with antibodies to HSF1.

Chromatin immunoprecipitation (ChIP)

This was performed using a ChIP Assay Kit (Millipore-Upstate, Billerica, MA, USA) as we described previously (15). Rabbit polyclonal HSF1 antibody was used for IP (CST #4356). Primer sequences for ChIP-qPCR are listed in **Supplemental Table 1**.

Gene Set Enrichment Analysis (GSEA)

GSEA was done as previously described (40,67). Gene Cluster Text file (.gct) was generated from the TCGA BRCA or TCGA COADREAD datasets. Categorical class file (.cls) was generated based on a signature for HSF1 activity (14). The Gene Matrix file (.gmx) was generated using published gene signatures for AKT1 (74), AKT2 (74), p38 (40), mTORC1 (40), and MEK (44). The number of permutations was set to 1000 and the chip platform for TCGA gene lists was used. Normalized enrichment scores (NES) and adjusted p-values are reported.

Mutagenesis

Generation of mutant HSF1-T142A, HSF1-S230A, HSF1-S326A and HSF1-T527A were carried out using a QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA) as per the manufacturer's instructions. Primers used for mutagenesis are listed in **Supplemental Table 1**. Mutation was confirmed by sequencing.

Statistical analysis

Data are presented as mean ± SEM. Unpaired *t*-tests and ANOVA with Tukey's post-hoc test were used for statistical analysis where appropriate. A p value of <0.05 was considered statistically significant.

Acknowledgements

This work was supported by funds from the National Cancer Institute (K22CA207575, RLC). We would like to acknowledge the Laboratory for Biological Mass Spectrometry at Indiana University for help with performing the experiments using mass spectrometry and data analysis.

Author Contributions

RLC conceived the idea and supervised the project. WCL collected and analyzed data for trimerization, ChIP, co-immunoprecipitation, and some reporter assay experiments as well as generated all mutant constructs used in this study. RO and HR performed all kinase assays as well as some immunoblotting and reporter assay experiments. All authors assisted with data discussion and manuscript preparation. WCL and RLC wrote the manuscript.

Funding and Additional Information:

This work was supported by funds from the National Cancer Institute (K22CA207575, RLC).

Conflicts of Interest:

The authors declare that they have no conflicts of interest with the contents of this article.

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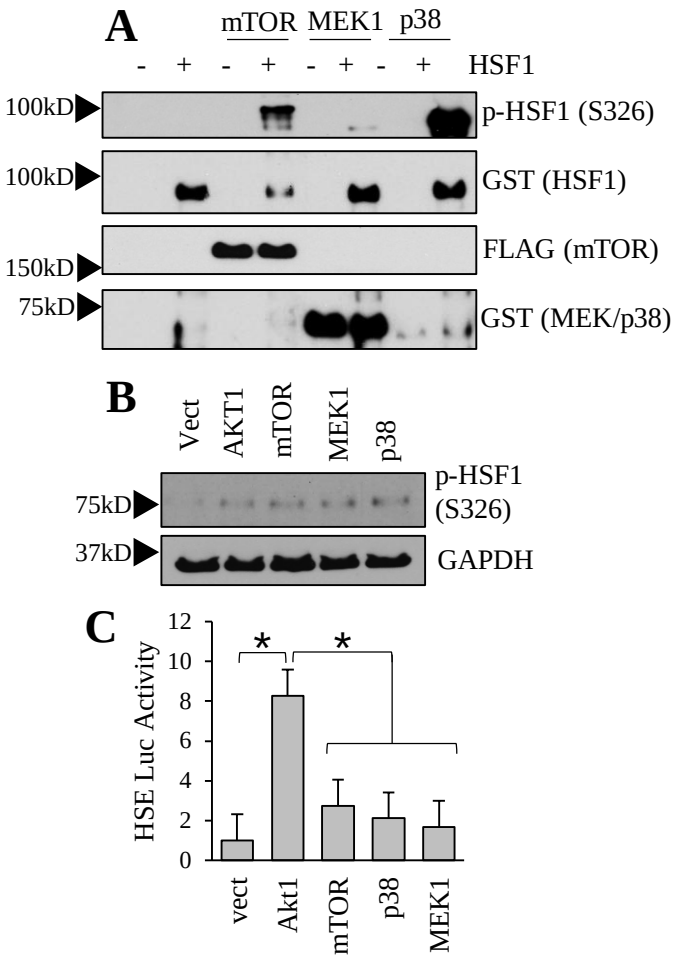

A
mTOR
MEK1
p38
- + - + - + - +
HSF1
100kD
p-HSF1 (S326)
100kD
GST (HSF1)
150kD
FLAG (mTOR)
75kD
GST (MEK/p38)
B
Vect
AKT1
mTOR
MEK1
p38
75kD
p-HSF1
(S326)
37kD
GAPDH
C
HSE Luc Activity
12
10
8
6
4
2
0
*
*
vect
Akt1
mTOR
p38
MEK1

Figure 1

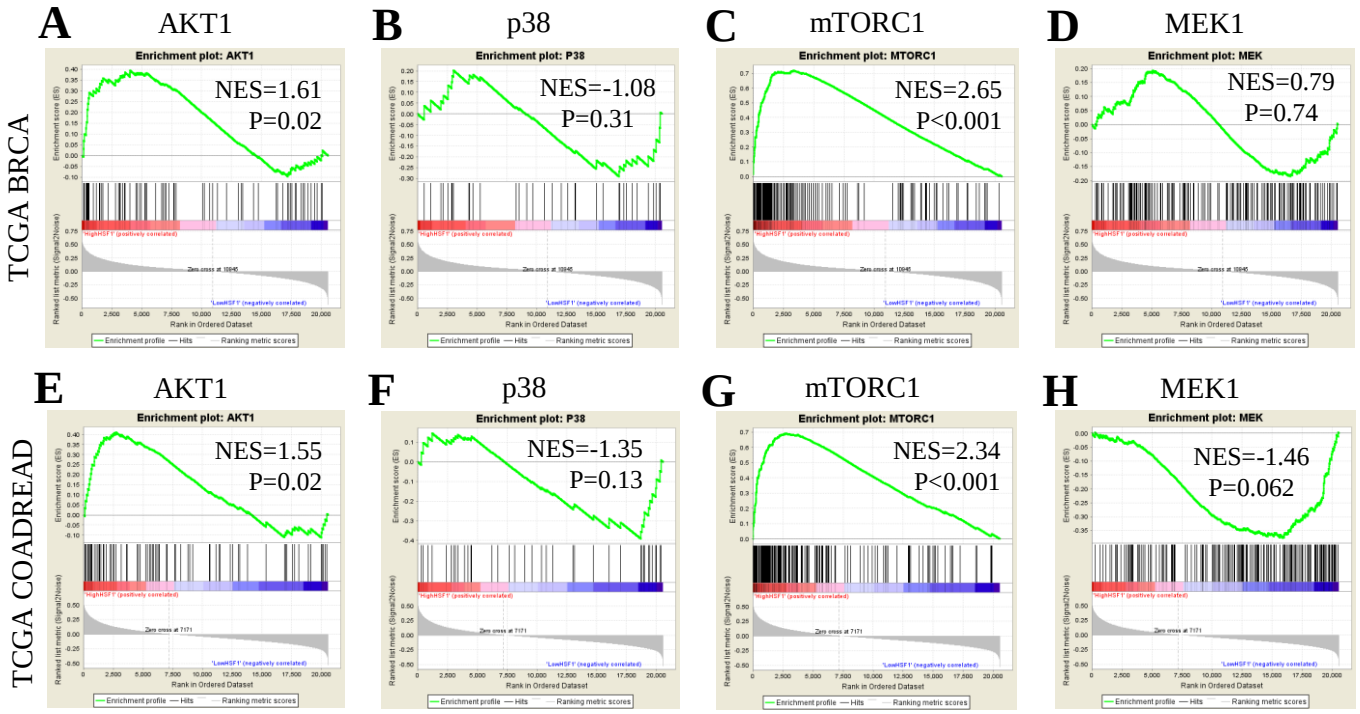
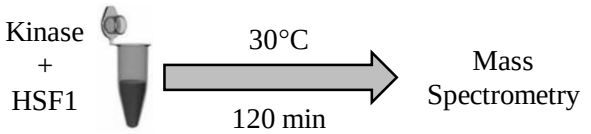

A
AKT1
Enrichment plot: AKT1
NES=1.61
P=0.02
B
p38
Enrichment plot: P38
NES=-1.08
P=0.31
C
mTORC1
Enrichment plot: MTORC1
NES=2.65
P<0.001
D
MEK1
Enrichment plot: MEK
NES=0.79
P=0.74
TCGA BRCA
E
AKT1
Enrichment plot: AKT1
NES=1.55
P=0.02
F
p38
Enrichment plot: P38
NES=-1.35
P=0.13
G
mTORC1
Enrichment plot: MTORC1
NES=2.34
P<0.001
H
MEK1
Enrichment plot: MEK
NES=-1.46
P=0.062
TCGA COADREAD

Figure 2

A



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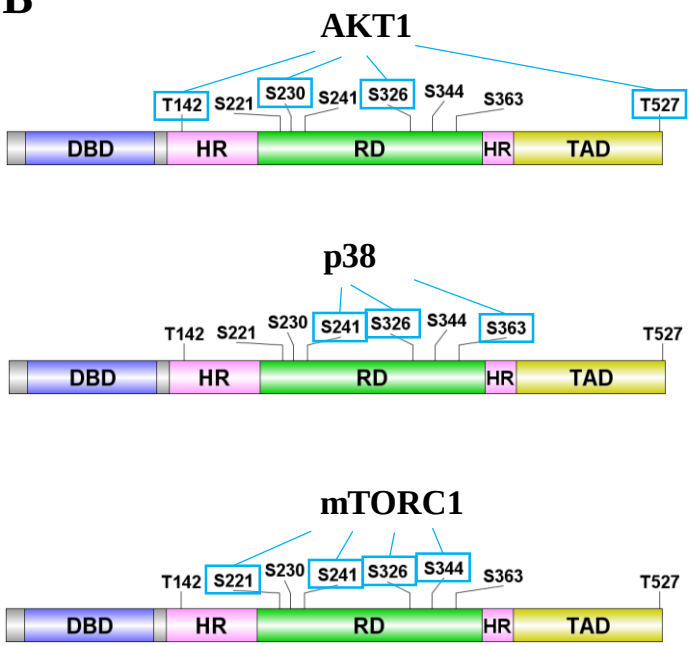


C

Kinase	HSF1 Peptide	Peptide Start	P-site
AKT1	SVTKLL**p**TDVQLM	136	T142
	pSLEHVHGSGPY	230	S230
	TGSEPPKAKDP**p**TVS	516	T527
AKT2	**p**SAPSPAYSSSSLY	241	S241
	TDARGHTDTEGRPP**p**SPPPTSTPEKCL	349	S363
P38	pSAPSPAYSSSSLY	241	S241
	TDARGHTDTEGRPPpSPPPTSTPEKCL	349	S363
mTOR	MLNDSGSAHpSMPKYSRQF	212	S221
	pSAPSPAYSSSSLY	241	S241
	IDSILRESEPAPApSVTAL	331	S344
MEK1	TGSEPPKAKDPpTVS	516	T527

D

```
          142         230         326         527
Human     KLLTDVQ...SRQFSLEH...DTLLSPTA...AKDPTVS
Mouse     RLLTDVQ...GRQYSLEH...DTPLSPTA...AKDPTVS
Bovine    KLLTDVQ...GRQYSLEH...ETPLSPTT...AKDPTVS
Rat       RLLTDVQ...GRQYSLEH...DTPLSPTA...AKDPTVS
Dog       KLLTDVQ...GRQYSLEH...EIPLSPTA...AKDPTVS
Pig       KLLTDVQ...GRQYSLEH...ETPLSPTA...AKDPTVS
Macaque   KLLTDVQ...GRQFSLEH...DTLLSPTA...AKDPTVS
```

Figure 3

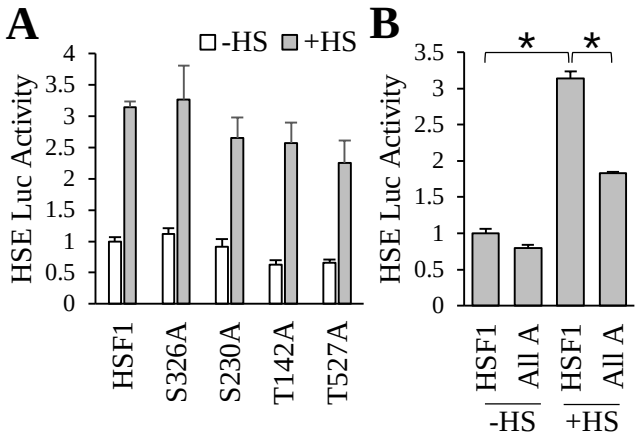
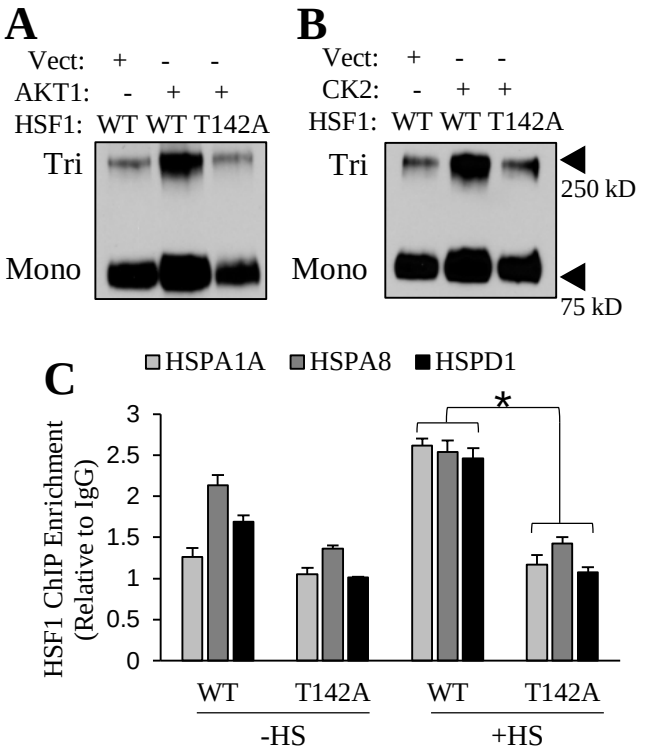

A
-HS
+HS
HSE Luc Activity
4
3.5
3
2.5
2
1.5
1
0.5
0
HSF1
S326A
S230A
T142A
T527A
B
*
*
HSE Luc Activity
3.5
3
2.5
2
1.5
1
0.5
0
HSF1
All A
HSF1
All A
-HS
+HS

Figure 4


A
Vect: + - -
AKT1: - + +
HSF1: WT WT T142A
Tri
Mono
B
Vect: + - -
CK2: - + +
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Tri
Mono
250 kD
75 kD
C
HSPA1A
HSPA8
HSPD1
HSF1 ChIP Enrichment (Relative to IgG)
3
2.5
2
1.5
1
0.5
0
*
WT
T142A
WT
T142A
-HS
+HS

Figure 5

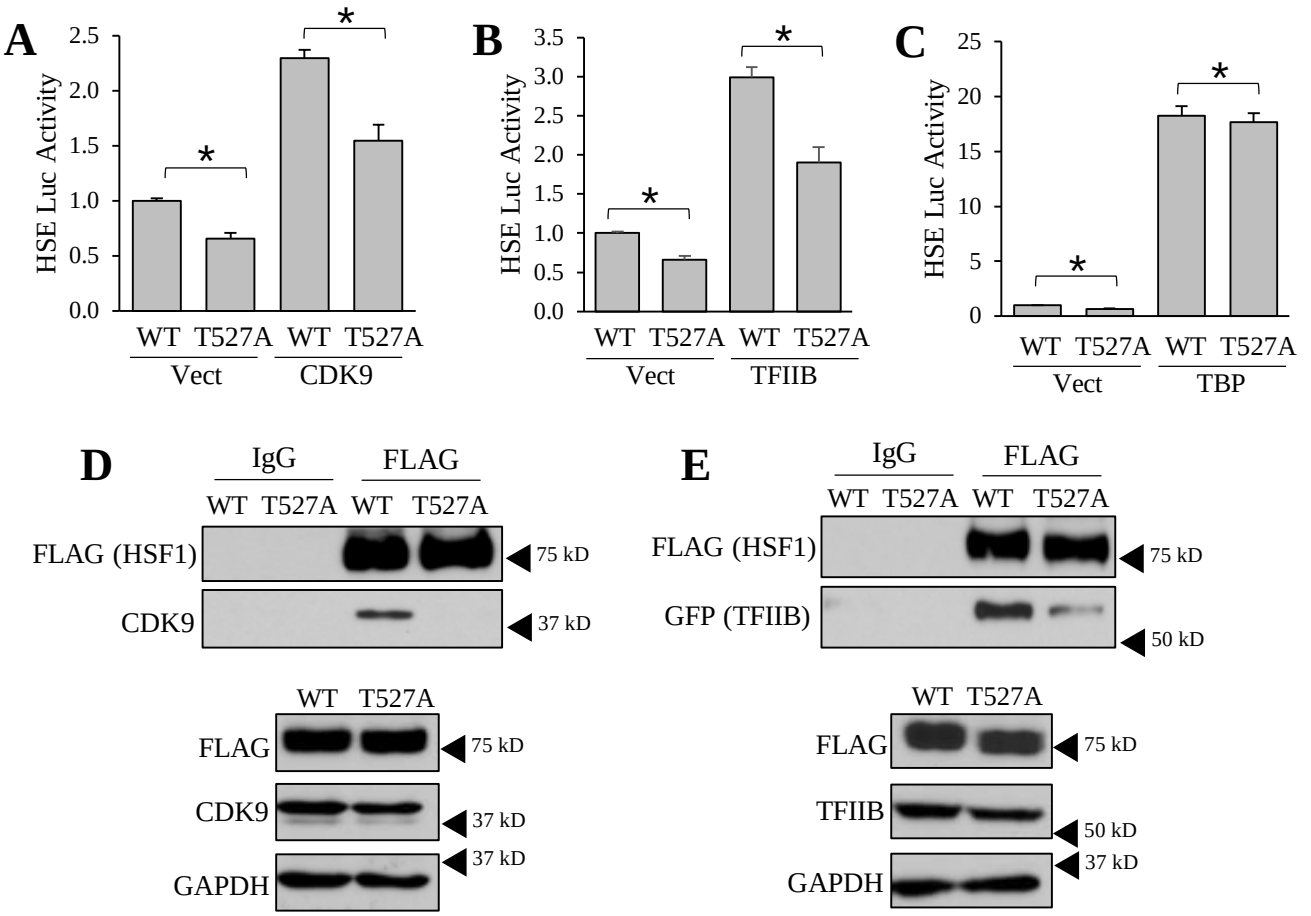
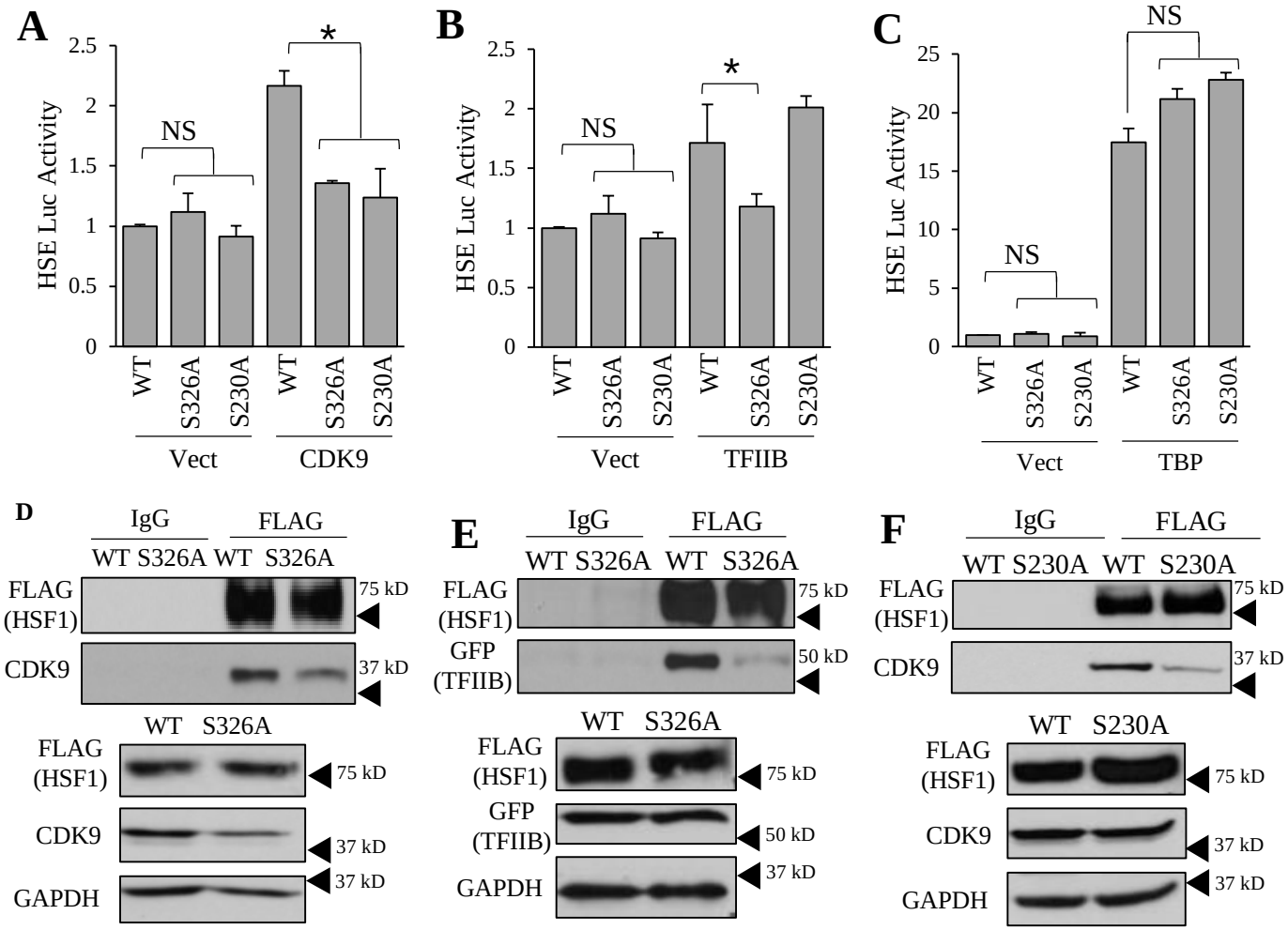

A
HSE Luc Activity
2.5
2.0
1.5
1.0
0.5
0.0
*
*
WT T527A WT T527A
Vect CDK9
B
HSE Luc Activity
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
WT T527A WT T527A
Vect TFIIB
C
HSE Luc Activity
25
20
15
10
5
0
*
*
WT T527A WT T527A
Vect TBP
D
IgG FLAG
WT T527A WT T527A
FLAG (HSF1)
75 kD
CDK9
37 kD
WT T527A
FLAG
75 kD
CDK9
37 kD
GAPDH
37 kD
E
IgG FLAG
WT T527A WT T527A
FLAG (HSF1)
75 kD
GFP (TFIIB)
50 kD
WT T527A
FLAG
75 kD
TFIIB
50 kD
GAPDH
37 kD

Figure 6


A
HSE Luc Activity
2.5
2
1.5
1
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0
NS
*
WT
S326A
S230A
WT
S326A
S230A
Vect
CDK9
B
HSE Luc Activity
NS
*
Vect
TFIIB
C
HSE Luc Activity
25
20
15
10
5
0
NS
NS
Vect
TBP
D
IgG
FLAG
WT S326A WT S326A
FLAG (HSF1)
75 kD
CDK9
37 kD
WT S326A
FLAG (HSF1)
75 kD
CDK9
37 kD
GAPDH
37 kD
E
IgG
FLAG
WT S326A WT S326A
FLAG (HSF1)
75 kD
GFP (TFIIB)
50 kD
WT S326A
FLAG (HSF1)
75 kD
GFP (TFIIB)
50 kD
GAPDH
37 kD
F
IgG
FLAG
WT S230A WT S230A
FLAG (HSF1)
75 kD
CDK9
37 kD
WT S230A
FLAG (HSF1)
75 kD
CDK9
37 kD
GAPDH
37 kD

Figure 7

Figure Legends

Figure 1 – AKT1 induced HSF1 activity stronger than other known activating kinases despite similar induction of S326 phosphorylation. A) Indicated recombinant proteins were incubated at 30°C for 2 hrs in the presence of ATP and subsequently subjected to SDS-PAGE and immunoblotting with indicated antibodies. **B, C)** HEK-293 cells were transfected with vector, AKT1, mTOR, MEK1, or p38 for 48 hrs. Cells were collected and subjected to immunoblotting with indicated antibodies (B) or reporter luciferase assay (C). Experiments were completed in triplicate and analyzed using one-way ANOVA and Tukey's post-hoc test where applicable. Data is presented as mean ± SEM. *P<0.05.

Figure 2 – Activity of AKT1 and mTORC1 are associated with HSF1 activity. Gene set enrichment analysis (GSEA) was performed using data from TCGA for breast (A-D) and colorectal cancers (E-H). Patients from these datasets were separated into high and low HSF1 activity groups using previously published gene signature for HSF1 activity. GSEA was then performed on these two groups using activity signatures for AKT1, p38, mTORC1, or MEK1. NES= normalized enrichment score.

Figure 3 – AKT1 phosphorylates four residues of HSF1 unique from other kinases. A) Workflow for identification of kinase-specific phosphorylation sites. **B-C)** Pattern of HSF1 phosphorylation for each kinase tested via mass spectrometry. **D)** Conservation of four sites phosphorylated by AKT1 across mammalian species.

Figure 4 – Loss of phosphorylation at T142, S230, S326, and T527 prevents HSF1 from responding to heat stress. A) HEK-293 cells were transfected with WT-HSF1 or indicated phospho-null HSF1 mutants along with the HSE reporter for 48 hrs followed by heat shock at 42°C for 1 hr. Firefly/Renilla ratios were compared across groups. **B)** HEK-293 cells were transfected with WT-HSF1 or an HSF1 mutant with all four sites (T142A, S230A, S326A, T527A) with phospho-null mutations for 48 hrs followed by incubation at 42°C for 1 hr. Firefly/Renilla ratios were compared across groups. Experiments were completed in triplicate and analyzed using one-way ANOVA and Tukey's post-hoc test. Data is presented as mean ± SEM. *P<0.05.

Figure 5 – Phosphorylation at T142 enhances HSF1 trimerization. A, B) HEK-293 cells were transfected with WT-HSF1 or T142A-HSF1 in the presence or absence of AKT1 (A) or CK2 (B) for 48 hrs. Cells were fixed with 1 mM EGS for 30 min and lysates were subjected to SDS-PAGE and immunoblotting for HSF1 trimers. **C)** HEK-293 cells were transfected with WT-HSF1 or T142A-HSF1 for 48 hrs followed by heat shock at 42°C for 1 hr. Cells were then fixed with formaldehyde, lysed, and sonicated prior to ChIP with either control IgG or HSF1 antibodies. ChIP product was subjected to qPCR for indicated gene promoters. Data is presented relative to IgG signal. Experiments were completed in triplicate and analyzed using one-way ANOVA and Tukey's post-hoc test. Data is presented as mean ± SEM. *P<0.05.

Figure 6 – Phosphorylation at T527 enhances HSF1 association with general transcription factors. A-C) HEK-293 cells were transfected with WT-HSF1 or T527A-HSF1 in the presence or absence of CDK9 (A), TFIIB (B), or TBP (C) along with the HSE luciferase reporter for 48 hrs. Firefly/Renilla ratios were compared across groups. **D-E)** HEK-293 cells were transfected with FLAG-tagged WT-HSF1 or T527A-HSF1 for 48 hrs. Cell lysates were subjected to immunoprecipitation with control IgG or FLAG antibodies and subjected to immunoblotting with indicated antibodies.

Figure 7 – Phosphorylation at S230 and S326 regulate HSF1 interaction with CDK9 and TFIIB. A-C) HEK-293 cells were transfected with WT-HSF1, S326A-HSF1, or S230A-HSF1 in the presence or absence of CDK9 (A), TFIIB (B), or TBP (C) along with the HSE luciferase reporter for 48 hrs. Firefly/Renilla ratios were compared across groups. **D-E)** HEK-293 cells were transfected with FLAG-

tagged WT-HSF1, S326A-HSF1, or S230A-HSF1 for 48 hrs. Cell lysates were subjected to immunoprecipitation with control IgG or FLAG antibodies and subjected to immunoblotting with indicated antibodies. Data is presented relative to IgG signal. Experiments were completed in triplicate and analyzed using one-way ANOVA and Tukey's post-hoc test. Data is presented as mean ± SEM. *$P<0.05$.