AKT PHOSPHORYLATION OF *DROSOPHILA* HEAT-SHOCK FACTOR: A SIGNATURE FOR STRESS RESISTANCE

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ABSTRACT

The heat-shock response is vital to cellular homeostasis. *Drosophila melanogaster* heat-shock factor (dHSF) is the primary transcriptional activator in the stress response pathway for induction of heat-shock-mediated gene transcription. This work investigates the potential for dHSF to undergo post-translational modification by phosphorylation and lysine tagging, specifically, direct phosphorylation by kinases and covalent-lysine tagging by ubiquitin, acetyl, and SUMO groups. Direct phosphorylation of, and binding to, dHSF was demonstrated by Akt/PKB kinase. Knock-down of this kinase by RNAi resulted in a heat-shock phenotype for dHSF and the acquired DNA-binding ability characteristic of activated transcription factor. Site-directed mutagenesis of lysines within a putative nuclear localization sequence (NLS) revealed two potential sites for regulation of dHSF activation by post-translational modification. The functional consequences of synergistic Akt phosphorylation and lysine modifications are discussed – this work implicates a role for direct kinase phosphorylation in regulating the stability of dHSF.

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CHAPTER 1: Introduction

The heat-shock response (HSR) is the concerted stimulation of *heat shock protein* (hsp) gene transcription by Heat-Shock Transcription Factor (HSF) in response to elevated environmental temperatures. Stress response pathways, such as the HSR, are essential for maintaining cellular homeostasis during temperature, nutrient, and chemical stresses. Transcription factor regulation by kinase phosphorylation and post-translational lysine (and arginine) modifications allows for control of multi-step signaling cascades involved in stress-mediated transcriptional activation and/or repression.

In this review, the heat stress response is detailed, alongside that of the Akt/FOXO insulin-signaling stress pathway. The mechanisms of post-translational modification by ubiquitylation, acetylation, and sumoylation are discussed, and RNAi is presented as a methodology for reducing gene expression in cell culture.

Heat-shock factor and the heat-shock (HS) response

Under heat stress conditions, heat-shock transcription factor (HSF) binds the heat-shock promoter element (HSE) (Fig 1A, B). *Drosophila* HSF (dHSF) is the 691-amino-acid primary transcriptional activator of the heat-shock response in *Drosophila melanogaster* (Fig 1A). In the *Dm* heat-shock (HS) response (Fig 1B), HSF remains a monomer in the non-shocked (NS) state throughout the nucleus [1]. Upon HS, it spontaneously trimerizes and is localized to sub-nuclear granules [2], where it binds HSEs upstream of *hsp* genes, one of them the temperature-regulated *hsp70*, a crucial HS chaperone protein [3, 4].

The *hsp* genes are among the most highly conserved [5, 6], are rapidly synthesized in response to heat stress, and range in size from 20-140 kDa. Sudden, significant (10°C) changes in temperature conditions from the basal growth temperature are sufficient to activate the HS pathway [7]. *Hsp* transcription can also be induced by oxidative stress, viral infection, and chemical stressors [8]. Genome-wide proteomics analysis of HSF-mediated stress responses have

revealed target genes involved in protein folding and transport, cell signaling, and cellular metabolism and energy generation [9], and include ubiquitin- and proteolysis-related genes. There is some evidence for HSF to act as a direct thermometer [10], and it responds to changes in temperature from cell-specific standard growth conditions [11, 12].

HSFs can be characterized according to distinct regions of similar functionality, yet share little primary sequence homology [13, 14]. Two domains exist within all HSFs: an amino terminal DNA-binding domain (DBD) of approximately 100 amino acids, followed by a region of hydrophobic heptad (leucine/isoleucine) repeats (LIR) that mediate HSF oligomerization [15]. *Drosophila*, plant, and vertebrate HSFs also have an isoform-specific, C-terminal LIR (the HR-C), and deletion and/or mutation of this region results in constitutive HSF trimerization and DNA- binding, pointing to an inhibitory role in the monomer-trimer transition [16]. *Saccharomyces cerevisiae* HSF, which does not contain a HR-C, trimerizes and constitutively binds DNA [1, 9]. In contrast, *S. pombe*, which contains a region homologous to the HR-C, has inducible DNA- binding that is only partially- regulated by the presence of this region [17]. The multiple chicken and tomato HSF isoforms (both with and without this HR-C) dimerize during heat-induced activation [1].

The most highly conserved region of HSF is the heat-shock element (HSE), a region upstream of heat-shock gene promoters consisting of inverted repeats of the sequence NGAAN; these residues provide the necessary contacts for heat-induced transcriptional activation [18]. The conformation of HSF-bound HSE renders it sensitive to proteolysis. Accessibility of heat-shock gene promoters to HSFs is dictated by protein cofactor docking and sequence elements of flanking regions, such as the presence of paused RNApII and the positions of the GAGA element and TFIID in the case of the *Dm hsp70* gene promoter [19].

While there is only a single *Drosophila* HSF, there are three mammalian homologs of HSF: HSF1, HSF2, and HSF4 [1]. Several mammalian HSF homologs may have evolved in

response to the complexity of higher-organism-specific responses [19]. Individual isoforms carry out specialized functions, whether they are temporally or compartmentally- defined. HSF1, (like dHSF, a monomer in non-HS conditions) is heat-stress- induced, while HSF2 (a dimer under non-HS conditions), responds during basic cell differentiation [20]. As a group, human HSF isoforms heterotrimerize to enhance activation of the *hsp70* gene promoter, allowing for measured gene transcription in a cell-line-specific manner [21]. It has been noted that HSF1 is activated during muscle injury, as suppression of HSF1 activity results in activation of the pro-inflammatory pathway that includes the NF-κB and AP-1 genes [22]. Knock-out of HSF1 has been shown to inhibit induction of *hsp70* in response to heat- shock [23-25]. However, since HSF1 is not responsible for induction of all heat-shock-related gene transcription, other *hsp* transcripts are still present during heat stress. HSF2 plays the most prominent role in regulating embryonic development, but unlike HSF1, is not activated for DNA- binding during heat stress. HSF1 and HSF4 have been linked to formation and maintenance of cells in the olfactory and visual systems [26]. The single *Dm* HSF may encompass the functions of the three HSF isoforms in vertebrate systems. Recently, four alternately spliced forms of dHSF were identified that modulate the amplitude of the transcriptional response to heat and cold stress [27], and the same has been found for vertebrate HSF1 [28-30], pointing to pre-translational regulation of HSF activity.

There is limited structural data for full-length HSFs; currently, only the DBD has undergone NMR and x-ray analysis, revealing similarities to the helix-turn-helix motif class of DNA-binding proteins [31]; up to this point, evolutionary/phylogenetic hypotheses have been based strictly on domain architecture. Studies of *S. cerevisiae* HSF have yielded the most data concerning protein-DNA interactions in this pathway. Analysis of heat- shock element-bound dHSF has identified residue contacts essential for binding and activation of the HS response [18, 32]. In *S. cerevisiae*, HSF1 and the Skn7 oxidative stress regulator share structural homology, namely within the DBDs and tandem coiled-coil structural domains [33]; in addition, Skn7 can

bind yeast HSEs, demonstrating induction of heat-shock gene transcription as a result of non-stress conditions, for example, oxidative stress. Structural study of the yeast HSF DBD through cross-linking experiments have revealed highly flexible N- and C-terminal domains, allowing for transcriptional activity in these regions to proceed unhindered [34].

Heat- shock proteins are synthesized in response to activation of the heat- stress pathway

There are several heat-shock proteins whose gene transcription is induced by temperature stress: Hsp60, 70, 83, 26, and 27, among others.

Hsp70 gene induction is a result of environmental stress, cell growth and developmental regulation, or disease. A basal level of *hsp70* transcription is noted at normal growth temperatures, but rapid transcriptional activation is achieved as a result of *hsp70* promoter-paused RNA polymerase II (RNApII) molecules that have recently synthesized 25-nucleotide transcripts in their active sites, ready for heat- shock-associated transcription [19, 35, 36].

Unlike heat-induced activation of *hsp70*, *hsp83* is a tissue- and developmental-stage-specific gene that is induced during normal development of *Dm*, and this role is distinct from hsp83's response to heat stress [37]. Maximal induction of *hsp83* gene transcription takes place at 33-35°C vs. 37°C for *hsp70* [38].

The heat shock chaperone proteins are essential for mediating protein folding – defects in this regulatory function for hsps can lead to disease. Diseases characterized by accumulation of misfolded proteins range from Parkinson's to familial neurohypophyseal diabetes insipidus [39, 40].

The Akt/FOXO stress pathway is the primary insulin-signaling stress cascade

The key regulators of stress-dependent responses that modify lifespan are forkhead transcription factors (FKHR), the most notable being the Akt/FOXO pathway. FKHRs are key components in proliferative cell responses and regulate genes involved in metabolism, apoptosis,

and oxidative stress [41]. FOXO, while not a prime candidate for drug targeting due to its ubiquitous nature, may benefit from cell-specific regulation, for example, in pancreatic β-cells to control diabetes [42].

FKHRs are characterized by a conserved DNA-binding domain consisting of three α-helices and two large loops [41]. Daf-16/FOXO1, a member of the FOXO sub-group of FKHRs that regulates insulin signaling in *C. elegans*, plays a dual role as both transcriptional activator and repressor [43]. Daf-16 is negatively regulated by phosphorylation by Akt/Protein Kinase B (Akt/PKB), repressing FOXO-related gene transcription by disrupting the interaction of FOXO with cofactors necessary for transcriptional activation. FOXO1 binds the major groove of DNA as a monomer at the consensus DNA-binding sequence TTGTTTAC [44].

Akt plays a role in the regulation of nucleocytoplasmic shuttling and transcriptional activity of FOXO [45], however, the mechanisms by which Akt is activated are not yet fully understood. Akt is both nuclear and cytosolic (personal observation). In the presence of insulin, (PI3K) generates 3'-phosphatidylinositol lipids, which act as secondary messengers and bind the pleckstrin-homology (PH) domains of Akt [8, 46]. PH domains mediate transport of Akt to the plasma membrane, where it is phosphorylated at Thr308 and Ser473 [47]. In humans, this activated form of Akt can now phosphorylate FOXO *in vivo* at 3 of 5 possible residues: Thr24, Ser256, and Ser319. Ser256 is located in the basic region of the DNA-binding domain [48]; phosphorylation at this site is critical for nuclear exclusion of FOXO1 and its subsequent transcriptional inactivation, and may have major effects on DNA-binding [49].

The PI3K/Akt signaling pathway is conserved in *Drosophila* [50]. The single *Drosophila* FOXO homolog (dFOXO) is 613aa [51] (Fig 1C), while *Dm* Akt (dAkt) exists in two forms: a 66 kDa protein (530 amino acids), and a longer, less- expressed, 85 kDa form (611 amino acids), a result of transcription from an upstream initiation codon that contains several proline residues in the N-terminal region [52]. DAkt is activated at S505 and T342 (S473 and T308 on the mammalian homologs, respectively), and targets residues Thr44, Ser190, and Ser259 of dFOXO

[51]. DAkt can phosphorylate transcription factors involved in stress signaling cascades, as well as cellular developmental processes- for example, tracheal system development [46].

Components of the Akt pathway, such as the 14-3-3 proteins [53], enhance pathogenic protein aggregation, often found in human neurodegenerative disorders. Several diseases are characterized by long, polyglutamine tracts in essential proteins, like *Spinocerebellar ataxia type 1* [54].

RNAi: A methodology for the dissection of signaling pathways by knock-down of protein expression

Injection of antisense RNA is the basis of the ground-breaking knock-down method of endogenous genes in *C. elegans*, and now several other organisms [55-58]. RNA interference (RNAi) is a form of post-transcriptional gene silencing by introduction of RNA molecules complementary to the mRNA of the target protein [57]; both endogenous and exogenous gene expression have been effectively silenced by this method [56]. RNAi has been successfully utilized in classifying genes essential for cell culture, as well as upstream effectors of signal transduction cascades, such as regulators of Down Syndrome proteins [59] and cell growth and division.

RNAi evolved as a defense mechanism against retroviral invasion. RNAi, as it is referred to in *C. elegans* and *Drosophila*, is known as post-transcriptional gene silencing (PTEG), while in the fungi *Neospora crassa*, it is known as "quelling" [60].

In *Drosophila* cells, the enzyme Dicer digests exogenous (or invading) double-stranded RNA (dsRNA) into 21-23 nucleotide segments [61]. The RNA-induced silencing complex (RISC) unwinds the double-stranded fragment, and stores the antisense strand. Complementary mRNA hybridizes to this stored strand and this hybrid nucleic acid is degraded and unable to translate into protein. Exon sequences are required to silence genes via RNAi; early experiments using intron and promoter sequences were unable to induce RNAi on targeted genes [60]. In *Dm*

cell culture, addition of long, unprocessed dsRNA is sufficient to deplete the targeted gene's endogenous RNA levels due to the absence of an interferon response in cell culture, and the injection of long dsRNA into *C. elegans* or *Dm* flies acts systemically to carry out the same process. In the mouse and zebrafish embryos [28], stable lentiviral vectors and transfection reagents [60] are required for dsRNA to permeate the cell membrane, and may need to be processed into the smaller, 21-23 nucleotide double-stranded fragments for transfection. Some success has been achieved with RNAi in mammalian cell culture [56, 62] as well as in whole model organisms [63].

RNAi is a powerful new tool for elucidating gene function within complex networks. We target Akt by RNAi in *Drosophila* S2 culture to reduce protein levels as a means to assay the regulatory function of this kinase on the activation of dHSF.

The nuclear localization signal (NLS) dictates cellular localization of proteins

Proteins containing a nuclear localization signal sequence (NLS) are targeted to the nucleus after their synthesis in the cytosol [64-67]. Improper/incorrect tagging of nuclear proteins has been noted in neurodegenerative diseases. A characteristic of Huntington's disease is nuclear, rather than cytosolic, inclusions of mutant huntingtin protein in neuroblasts [68], possibly a result of incorrect nucleocytoplasmic protein transport.

Three classes of NLS exist: monopartite, bipartite, and a third that shares homology with the NLS of the c-Myc oncoprotein [69]. A monopartite sequence is a single cluster of basic residues, while bipartite sequences consist of two basic residue clusters, the N-terminal cluster making a significant contribution to the binding energy of the NLS [67]. Lysine positioning within the NLS is crucial for its signaling function. The SV40 large T-antigen (PKKKRKV) is a classical monopartite NLS that requires lysine at position 3, while the c-Myc NLS (PAAKRVKLD) requires lysine at position 4, as well as residues P at position 1 and LD at 8 and 9 [70-72].

The NLS is recognized by the import receptor complex, composed of importin proteins α and β, which shuttle NLS-containing proteins into the nucleus [70]. DHSF has diffuse nuclear localization in the absence of temperature stress, and its subcellular location is dictated by developmental cues. DHSF cannot enter the nucleus during heat shock in early embryos until developmental cycle 14, at which time it migrates between sub-cellular chambers, such as from the nucleus to the cytoplasm during oogenesis [73]. However, dHSF nuclear localization is a requirement for transcription of the *hsp70* genes, and is achieved beginning in/during developmental stage 13 upon synthesis of the *Drosophila* Kap-α3 karyopherin transport protein [74].

Studies have shown that cytosolic proteins tagged with an NLS can also be translocated to the nucleus [75], indicating that synthetic NLS motifs that are appended to proteins are sufficient to signal nuclear localization of a non-nuclear protein. In this work, we took note of a mutant dHSF (ΔNLS) where deletion of a previously identified nuclear localization signal resulted in excess protein levels in the cytosol. We conducted site-directed mutagenesis within this region at lysine residues (Chapter 3) to find the minimal mutation necessary for this phenotype. The SV40 NLS was used to target cytosolically retained mutants, identified by mutation, to the nucleus to determine the purpose/nature of the mutation. We also note a novel mutant, ΔD, which exhibits differences in DNA-binding function when mutated at S256.

Post-translational lysine (K) modifications: Lysine modification by ubiquitylation can signal proteolysis and/or regulate protein function

Probing the ubiquitin-proteasome-mediated degradation of *Drosophila* proteins has become commonplace; recent examples include the clock proteins dPER and dTIM) [35, 76]. Several disease states are associated with incomplete or inefficient processing of misfolded proteins that compromise cell toxicity/health, primarily neurodegenerative diseases like Parkinson's Syndrome, Huntington's, and Alzheimer's [77].

Ubiquitin (Ub) is a highly conserved, 76 amino acid (8.6 kDa) protein that exists in both bacteria and eukaryotes. Target proteins are covalently tagged at lysine (K) residues to signal their destruction or regulate their localization or function [75].

The ATP-dependent ubiquitin-proteasome pathway (UPP) is responsible for the proteolysis of ubiquitin-tagged proteins, and the primary mechanism for signaling protein degradation in the cytosol is through this covalent tagging [78, 79]. This three-step, catalytic process requires ATP to activate ubiquitin for transfer to one of its several ubiquitin-conjugating enzymes (E2). A ubiquitin ligase (E3) recruits the target protein to its designated E2 to catalyze Ub-transfer to lysine residues, in turn forming Ub chains that target the protein to the 26S proteasome complex [80]. The 26S proteasome complex consists of one, barrel-shaped 20S-, and two 19S- sub-complexes, one on either side of the 20S; the 19S regulatory complexes recognize the Ub-tagged substrate and process it for destruction within the 20S proteasome core [75, 81]. A limited number of proteins are preferentially degraded by the 26S proteasome without the need for ubiquitin tagging.

Two recognized signaling regions exist for ubiquitin-tagging proteins – 1) an N-terminal degron [82], and 2) a PEST domain [83]. Specificity is conferred by the E3 ligase, which recognizes the degron of a target protein, one of the signaling modules for activating its degradation. It is unknown whether the PEST is recognized by E3 ligases for purposes of ubiquitin tagging.

In addition to UPP-targeting of misfolded or denatured proteins, overexpressed, cytotoxic mutant proteins or excess protein-complex subunits are tagged for degradation to maintain cell vitality and this multimer complex stoichiometry [84]. Defects in the UPP are linked to several neurodegenerative disorders, such as Huntington's, characterized by protein aggregates as a result of overexpressed mutant huntingtin protein (exon 1) carrying polyglutamine tracts [85].

The specific linkage of lysine residues between ubiquitin-chain subunits determines whether the substrate is targeted for degradation or Ub-tagged for activation or to alter its

function. Initially, a single Ub monomer is transferred to lysines of the target protein. Polyubiquitylation is the multi-Ub chain formation of four or more subunits that are linked via K48 of the first Ub unit for signaling proteolysis [86]. Fewer than four Ub subunits, or linkage through K63 of Ub, tags the target protein to modify its function. This is referred to as monoubiquitylation, as these Ub chains range from one to three subunits [87, 88] and can regulate diverse processes, from receptor endocytosis [89, 90] to protein translocation or conformation. Some transcription factors require their own polyubiquitylation for transcriptional activation [91].

The heat-shock chaperone proteins, hsp70 and hsp60, protect newly synthesized proteins during protein folding [3]. Previous studies have found a requirement for both chaperones and proteases for protein degradation in *E. coli* and eukaryotes [92, 93]. Theories range from a cofactor mechanism whereby chaperones maintain misfolded proteins in a proteolytically sensitive environment, to possible "signaling" to proteasomes by association with difficult-to-fold or partially folded protein [84]. Understanding how HSF is regulated by ubiquitin for degradation or monoubiquitylation may give insight into the mechanisms underlying the roles of heat-shock proteins' cell defense system as a result of the stability and activation functions of dHSF.

Acetylation, sumoylation, and methylation of lysine residues regulate protein function

In addition to ubiquitylation, lysines are also prime targets for post-translational modification by acetylation [94, 95], sumoylation [96, 97], and methylation [94].

Reversible acetylation of histones has been well- documented as an integral regulator of chromatin remodeling and transcriptional activity [98, 99]. Lysine acetylation can also regulate the function of protein components in the cell cycle, cell differentiation, and DNA replication and repair pathways. In this process, acetyltransferases transfer acetyl groups from acetyl-coenzymeA to the ε-amino groups of lysines or the α-amino group of N-terminal residues [100], and proteins/substrates are deacetylated as necessary.

The 101aa, small ubiquitin-like modifier (SUMO) has similar tertiary structure to ubiquitin [96]. However, sumoylation serves to target transcription factors to specialized cellular compartments to regulate their transactivation potential rather than tag them for entry into the UPP [101]. Sumo has been conjugated to a number of transcription factors [96], and recognizes lysines within the motif ΨKxE, where Ψ is a hydrophobic and x is any residue. The fly NF-κB homolog Dorsal is sumoylated in order to translocate it into the nucleus and enhance its activation, and may have a potential role in the *Drosophila* innate immune response [102].

There have been limited studies of lysine and arginine methylation [103]. N-terminal histone lysine methylation is a dynamically regulated process that controls transcriptional activation and repression [104]. Methylation has recently been reported as a method of chemical modification in the crystallization of otherwise soluble protein species [105]. Currently, the only reliable method for identifying methylation has been through mass spectrometry analysis [98].

We conducted site-directed mutagenesis of lysine residues within the nuclear localization signal of dHSF to determine whether the strict cytosolic localization of a ΔNLS protein is a result of modification by acetylation or sumoylation. Due to the difficulty of identifying covalent modification by methylation, we do not investigate this mechanism in our study of post-translational modification.

In this work

Clearly, the interdependence of cellular defense pathways is important for targeting drug discovery efforts against disease states that may result from improper activation of stress responses and its consequences. In the HSR, an example of this is misregulation of the heat-shock chaperone protein expression and/or function.

In this work, we explore the potential for dHSF activation by post-translational modification. We postulate that dHSF phosphorylation by Akt kinase maintains it in an inactive state in the absence of heat stress. At heat-shock temperatures, dHSF becomes dephosphorylated

for activation and induction of heat-shock-mediated gene expression. Chapter 2 investigates the direct interaction of dHSF with Akt, an important regulator of the FOXO-stress-related pathway, by direct binding studies, functional kinase/phosphorylation assays, and site-directed mutagenesis of the Akt consensus site Serine 256. Chapter 3 details the potential modification of lysine residues within the NLS of dHSF for DNA-binding activation and protein degradation functions. Chapter 4 puts this all into perspective: We delve into the possible interdependence of dHSF regulation by Akt and its consequences on the stability of dHSF.

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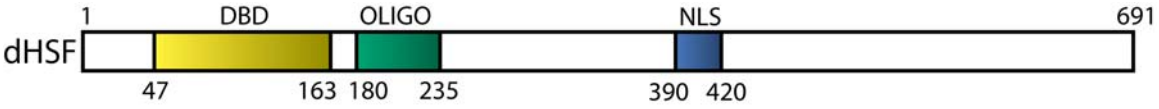
Figures
Figure 1-1

A



B

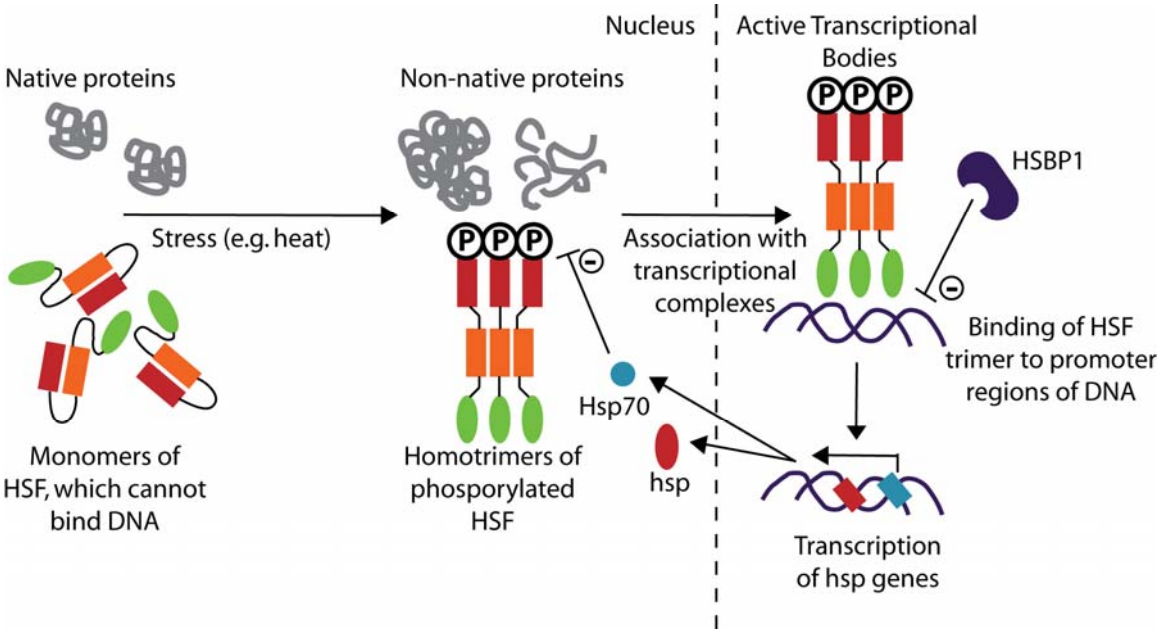


Figure 1-1

A – *Drosophila* heat -shock transcription factor (dHSF) is the 691-aa primary transcriptional activator of the heat-shock pathway. The DNA-binding domain (DBD) resides from residues 47-163, followed by the oligomerization domain (residues 180-235). A putative NLS was found at residues 390-420 by Zandi et al. Adapted from Zandi et al., 1997.

B- The heat-shock response (HSR) pathway. DHSF resides in the nucleus as a monomer that is unable to bind DNA. Upon heat-shock, it trimerizes and binds to heat shock elements (HSE) in active transcriptional complexes, for induction of heat-shock mediated gene transcription. Adapted from Pockley, 2001.

Figure 1-2

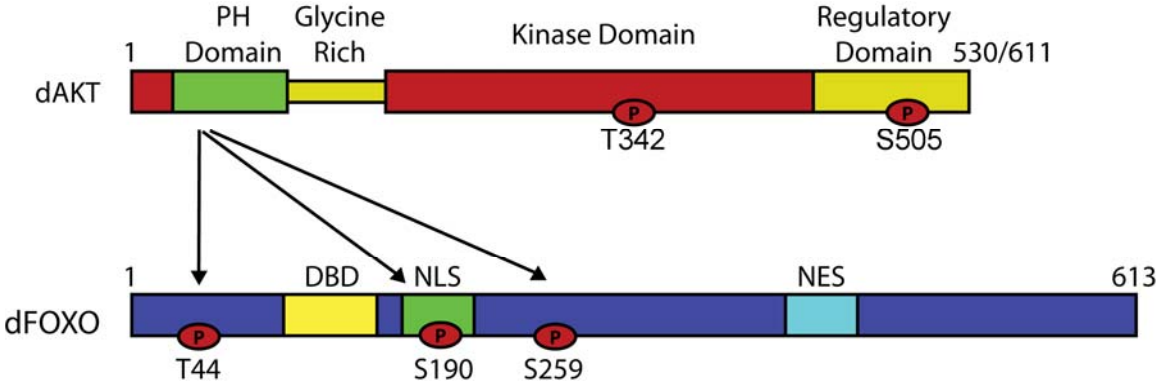

PH
Domain
Glycine
Rich
Kinase Domain
Regulatory
Domain
1
530/611
dAKT
P
T342
P
S505
1
DBD
NLS
NES
613
dFOXO
P
T44
P
S190
P
S259

Figure 1-2

The Akt/FOXO insulin-signaling pathway. DAkt is activated by phosphorylation at T342 and S505. DAkt activates dFOXO by phosphorylation at T44, S190, and S259.

CHAPTER 2: Akt negatively regulates dHSF by phosphorylation of S256

Introduction

Stress pathways are activated in response to a variety of cellular environments, such as during nutrient deficiency, metal and chemical toxicity, and temperature changes [1-4]. Initiation of stress-dependent signaling cascades results in accelerated stress-protein synthesis [3, 5], the transcription and activation of cofactors regulating protein folding, such as the heat-shock chaperone proteins (hsp) [3], and cell cycle arrest [2, 6, 7]. A number of key stress pathway protein regulators are members of the Serine-Threonine Kinase family [8-10] that function to phosphorylate Ser/Thr residues of target proteins.

The Akt/FOXO pathway is the key insulin-signaling cascade in vertebrates

The insulin-signaling pathway is known for mediating stress resistance and longevity in many metazoan and vertebrate species [10]. Recent investigations have shown that one of the primary initiators in this cascade is Protein Kinase B (Akt), a regulator of members of the forkhead transcription factor family (FOXO) [11, 12]. During non-nutrient-limiting conditions, *Drosophila* Akt phosphorylates *Dm* FOXO (dFOXO) to inhibit its activity. However, under nutrient-deficient conditions, dFOXO is dephosphorylated and translocated to the nucleus, inducing the transcription of FOXO-related genes, which include regulators of cell cycle arrest [2, 7].

During heat shock, the primary transcriptional activator *Drosophila* heat-shock factor (dHSF) initiates transcription of the *hsp* genes, including a strictly temperature-regulated form of *hsp70* [3, 5, 13]. In addition, there is evidence to suggest transactivation of *hsp* gene induction via an alternative pathway. Daf-16/FOXO in *C. elegans* may directly activate hsp synthesis based on the results of HSF1 knock-out studies [14]. However, there is no direct biochemical evidence linking direct modification of HSF by Akt/PKB. HSF activation has been noted in several organisms under glucose-deficient conditions [15]; in the yeast *S. cerevisiae*, ScHSF is activated

by the metabolic regulating kinase Snf1, for DNA-binding and HSF-mediated gene transcription [15]. Studies have also shown that overexpression of *C. elegans* HSF1 extends organismal lifespan [14], while its inhibition results in decreased longevity [16, 17].

There is evidence that Akt, the primary kinase in the insulin-signaling response involving FOXO, may be regulated directly or indirectly by hsps [18]. Hsp27 can bind to and inactivate the pro-apoptotic caspase proteins [19], inhibit Fas-mediated apoptosis [20], and regulate Akt activity to control apoptosis of human neutrophils [18]. Proper regulation of Akt and its upstream activators is essential for preventing the onset of disease and the progression of oncogenesis.

The heat-shock response is regulated by activation of a dynamically phosphorylated heat-shock factor

Reversible, multi-site phosphorylation facilitates regulatory function and DNA-binding activity as both a binary switch and a dynamic, measured response [21]. In the absence of stress, dHSF does not undergo changes in its phosphorylation state, and the relative distribution of phosphorylated HSF isoforms remains the same for the duration of all developmental stages [22]. Upon heat stress, dHSF becomes highly phosphorylated; at the same time its concentration increases several-fold. The question arises whether this phosphorylation process is a direct regulatory step in the activation of dHSF, a consequence of activation, or a combination of both of these possibilities.

To analyze the activity of Akt on dHSF, RNA interference (RNAi) will be utilized to reduce protein levels of this kinase in S2 cells. RNAi is a powerful tool to knock-down specifically targeted gene expression. Previous studies using RNAi to reduce expression of potential regulators of the FOXO/Akt pathway, for example, the insulin receptor substrate CHICO and the PI3K phosphatase PTEN, have confirmed their roles in regulating either Akt transactivation or repression, respectively [23].

In our lab, a process was devised for simultaneous knock-down (KD) of endogenous dHSF and knock-in (KI) of exogenous mutant dHSF (A.B. Hicks) to assay the functionality of mutant dHSF. By specifically targeting the 3' and 5' untranslated region (UTR) of endogenous dHSF with complementary double-stranded RNA, expression levels below 10% of the endogenous factor were achieved in the S2 cell line. This potent method allows assessment of mutant dHSF activity with minimal contributions from endogenous wild-type (wt) factor at the level of DNA-binding and transcriptional activation.

_Dm_ HSF may undergo phosphorylation-dependent regulation by Akt kinase similar to dFOXO

Analysis of the dHSF sequence revealed a consensus Akt phosphorylation site at residue S256 that may be phosphorylated by Akt (Fig 4A). A direct interaction between *Dm* Akt and dHSF may link dHSF function to the Akt/FOXO pathway. The phosphorylation of dHSF by Akt may act as a negative regulatory step, similar to the regulation of FOXO. This putative Akt recognition site is conserved in HSFs among several species (Table 1), and is located adjacent to the oligomerization domain (residues 180-235). Modifications at this position may play a role in DNA binding, possibly by altering the oligomerization state. Limited structural data exists for dHSF, however, NMR studies of the dHSF DNA-binding domain (DBD) point to similarities between itself and that of the Forkhead protein family members [24] via a winged helix-turn-helix motif that is present in both transcription factors. RNAi was used to reduce Akt levels in S2 cells, and examine the effect of this knock-down on the heat-shock response. These studies will determine the role of potential dHSF modification by Akt in the heat-shock response.

Results

Akt directly interacts with and phosphorylates dHSF

To determine whether dHSF is a substrate of Akt, immunoprecipitated endogenous and transfected wild type (wt)-dHSF from non-shocked (NS) and heat-shocked (HS) cell extracts were probed with an α-phospho-Akt-substrate antibody. Comparison of NS and HS dHSF shows a significantly greater signal for NS dHSF as a phosphorylated Akt substrate when probed by this antibody (Fig 1A). Direct association of Akt with dHSF was determined by additional immunoprecipitation experiments using wt- and endogenous dHSF (Fig 1B). Antibodies reactive against Akt reveal association of Akt with both NS and HS factor. Akt is active in both NS and HS cell extracts (Fig 1C), and is not heat-shock concentration-dependent - its concentration does not change upon HS, while dHSF concentration increases several-fold during these conditions.

Akt is a negative regulator of FOXO by directly phosphorylating this transcription factor at three residues [25, 26]. It is necessary to establish the phosphorylation state of dHSF prior to and after HS to determine if Akt is acting by the same mechanism to regulate dHSF. Several methods were used to establish the *in vivo* phosphorylation state of dHSF at S256. First, an *in vitro* inverse kinase assay was performed on wt and endogenous dHSF. As shown in Fig 2A, immunoprecipitated dHSF from heat-shocked cells can be phosphorylated by Akt (lane 2); these results showed NS dHSF as a significantly poorer substrate than HS, suggesting that reactivity of S256 to recombinant Akt (for radio- labeling) in this assay is due to a dephosphorylation step during HS. Recombinant (GST-tagged) wt-dHSF (wt-rdHSF) can be phosphorylated by recombinant Akt kinase, as noted by radioactive labeling (Fig 2A, lanes 3 and 4, bottom), though it is not as sensitive a substrate as endogenous dHSF.

Wt-rdHSF can bind heat-shock elements (HSEs) (Fig 4D); it does not exhibit the same lack of HSE-DNA-binding in the NS *in vivo* expressed protein, indicating the possibility that protein cofactors are needed to properly regulate its function. In addition, inverse kinase assay on internal deletions of rdHSF indicate that the Akt kinase site is between residues 241-270 (Fig 2A,

lanes 5-8). This biochemical data for the direct interaction, and substrate specificity, between dHSF and Akt, is evidence for a direct link between the insulin-signaling and heat-stress response pathways. Specifically, that phosphorylation of non-shocked/inactive dHSF is removed upon heat-shock.

Akt affects the cellular localization of dHSF

In vivo RNAi knock-down of Akt (AKD) was performed in S2 cells, and cell extracts were analyzed for changes in the localization and DNA-binding ability of dHSF. Wt-dHSF tagged with enhanced GFP (EGFP) forms nuclear punctate bodies when Akt expression levels are knocked-down by RNAi in the absence of HS (Fig 3A). This is the first visual evidence suggesting Akt maintains dHSF in a non-induced state, and removal of this kinase allows association with the active transcriptional bodies that are seen during HS-induced activation. This observation was supported by cytosolic (S10)- and nuclear (NE)- fractionated cell extracts that showed an increase in the amount of nuclearly fractionated dHSF in AKD cells while still under NS conditions (Fig 3B). In this Akt knock-down background, NS dHSF bound to DNA (Fig 3C), the same as is observed under heat-shocked conditions. This functional data for dHSF activity in the absence of Akt supports the idea that Akt is a negative regulator of dHSF.

Cells were transfected with wt-dHSF-EGFP and treated with LY294002, an inhibitor of PI3K (the upstream activator of Akt), under NS conditions. Mirroring the Akt KD experiment, the distribution of EGFP-dHSF revealed a punctuate pattern of non-shocked dHSF similar to what is observed under HS conditions (data not shown). Since LY294002 is a general inhibitor of PI3K (an indirect regulator of Akt), as opposed to a specific Akt inhibitor, there is an overall reduction in the number of phosphorylated Akt substrates in *Dm* cell extracts (Fig. 3D). This is likely to be the result of non-specific inhibition of several proteins that may be modified by both PI3K and Akt. In comparison, direct KD of Akt does not significantly reduce the overall number

of phosphorylated Akt substrates, and would be a more revealing indicator of direct or secondary effects of Akt on its participating pathways.

Treatment of endogenous NS dHSF with LY294002 results in dHSF acquiring DNA-binding ability (data not shown), in confirmation with HSE-binding data from AKD studies. Similarly, cells treated with a specific Akt inhibitor (Calbiochem) revealed an increase in the proportion of active, DNA-binding molecules in the nuclear fraction as the concentration of inhibitor was progressively increased (Fig 3E). The data from chemical inhibition of Akt corroborates the Akt knock-down data, suggesting Akt imparts a negative regulatory function on dHSF activation.

Interestingly, when immunoprecipitated dHSF from Akt knock-down cell extracts was probed with an α-phospho-Akt-substrate antibody, a larger population of phosphorylated dHSF was found in heat-shocked AKD cells than from NS AKD or NS and HS S2 cells (Fig 3F). The specificity of this antibody for S256 of dHSF may come into question; it could be recognizing a pseudo-Akt-kinase motif, like the one located at S35. During Akt knock-down, a second kinase may be modifying dHSF at the Akt site, possibly GSK-3β [27, 28], which is also active in the NS and HS states in *Dm* S2 cells (Fig 1C). While active Akt is present under NS and HS conditions, it may be only conditionally regulating dHSF.

Site-directed mutagenesis of dHSF reveals a negative regulatory role for phosphorylation at S256

In order to assess the probability that dHSF is a substrate for Akt, sequence analysis via a computational motif search program revealed a putative Akt binding site at S256 (251-RARTTS-256) (PROSITE, Fig 4A). Site-directed mutagenesis of S256 to Ala (S/A) or Asp (S/D) of EGFP-fusion dHSF did not yield any differences from wt-dHSF in overall protein expression levels or transcriptional activation (Fig 4B). However, there was a difference in protein localization and DNA-binding activity. According to fractionation studies, protein distribution of dHSF in non-

shocked S10- and nuclear-cell fractions show higher levels of nuclearly fractionated S/A-dHSF, while S/D-dHSF has a larger proportion that is fractionated into the cytosol (Fig 4C). This is visually observed in EGFP-tagged mutant dHSFs; S/A is present in the nucleus, while wt- and S/D-dHSF protein distributions are more diffuse, and a portion may exist in the cytosol. DNA binding of S/D-dHSF was reduced during HS conditions in S2 cells with endogenous dHSF knocked-down by RNAi (Fig 4D). There is minimal DNA-binding of non-shocked wt-, S/A-, and S/D-dHSF. This replacement of serine at 256 with a negatively- charged residue (D) exhibits changes in protein distribution within the cell, and compromised DNA-binding ability of heat-shocked protein, possibly resulting from phosphorylation at this site.

It has been noted that in some instances, for serine residues to be Akt-modified, prior post-transcriptional modifications need to be carried out, such as lysine methylation or acetylation [21, 29, 30]. This prompted a closer look at mutants from our collection of internally deleted and C-terminal-deletion dHSF constructs containing several modifiable lysines (K). One mutant (Δ), missing the previously identified nuclear localization signal [31] (Fig 5A), has 100-fold wt protein levels *in vivo* (Fig 5A and 5B), and constitutive DNA binding in the NS and HS states. A Δ mutant in conjunction with the S-to-D mutation to mimic a permanently phosphorylated serine and yield a negative-charge at the Akt binding site - ΔD - exhibited abolished DNA-binding activity, while ΔA (like Δ) bound HSE-DNA constitutively in the NS and HS states (Fig 5C). Both (Δ+S256)-mutant dHSF constructs, like Δ, were cytosolically localized when observed under fluorescence. It has been noted that S256D in the human FOXO DBD limits its binding activity, and a negative charge at this site may be sufficient to limit transactivation by FOXO proteins [32]. Protein levels of ΔD are similar to those of Δ and ΔA (Fig 5C) - clearly, a negative charge at S256 is sufficient to disrupt the normal mechanism of dHSF activation. Oligomerization of mutant dHSF (ΔA and ΔD) was not hindered by replacement of S256 or deletion of the 393-420 residue region (Fig 5D); this may be a result of high protein levels activating oligomerization and DNA-binding functions, even when HSEs are inaccessible (cytosol vs. nucleus).

Recombinant dHSF also oligomerizes normally (Fig 5E) and as previously noted, has DNA-binding ability in the NS and HS states, much like HS wt-dHSF. Cryptically, transcriptional assay of simultaneous [endogenous dHSF knock-down/ (Δ+S256)-mutant-dHSF knock-in] indicated levels of *hsp70* transcription under HS conditions (Fig 5F), likely a result of incomplete endogenous HSF knock-down. So, while oligomerization and transcriptional activation of Δ-mutant-dHSF is not hindered, ΔD results in a mutant that is non-functional for DNA-binding.

Discussion

Akt phosphorylation at S256 may repress dHSF activation

Independently functioning domains of transcription factors determine DNA-binding and transcriptional-activating functions. These are well characterized for proteins such as the yeast activator GAL4 [33], among others. As DNA-binding is a prerequisite for transcriptional activation, determining whether phosphorylation within the Akt binding site alters the DNA-binding activity of dHSF implicates a role for Akt in regulating the HS response. Though there were no significant changes in dHSF function as a result of site-directed mutagenesis of S256, there were notable differences in its subcellular localization and a reduction in the population of DNA-binding S/D-dHSF. These data demonstrate dynamic phosphorylation as a means to sequester proteins throughout the cell for site-specific function. DHSF is nuclearly localized as a single large body, but upon HS, forms tiny, punctuate bodies that are tightly associated with active transcriptional complexes. In the NS state, dHSF can be fractionated into the cytosol as a monomer, as it is not bound to DNA. The replacement of serine 256 of dHSF with a non-phosphorylatable residue (alanine, A) allows it to remain in the nuclear compartment, as seen by fluorescent imaging of EGFP-tagged protein. The phosphorylated "mimic" (S256D) exhibits some nucleo-cytoplasmic protein migration. S256A mutants possess properties akin to the HS-form of HSF in that they preferentially fractionate within the nuclear extract, whereas S256D

mutants, like NS wt-dHSF, readily fractionate into the cytosol. The same mutation in a cytosolically retained protein lacking a NLS produces a non-binding mutant when serine is mutated to aspartic acid (D). There is no enhancement of DNA-binding in S256-mutated dHSF with a functional NLS, leading to the conclusion that the abolishment of ΔD DNA-binding activity is a synergistic effect of both the S/D and ΔNLS mutation. It is necessary to investigate mutations in residues within the NLS that could work in conjunction with this putative Akt phosphorylation site to alter the *Dm* HS response.

Post-translational modification of transcription factors results in their specific targeting to cellular compartments, leading to transcriptional activation in the nucleus, or lack of activation if localized to the cytosol [29, 34]. In the event that post-translational modification of dHSF includes phosphorylation by Akt at S256, identifying the location of Akt in the cell during heat shock may reveal the phosphorylation state of S256 under non-shocked and heat-shocked conditions. Akt is purportedly cytosolic during its participation in the PI3K pathway [4, 26, 35]; however, it is unknown whether it ever traverses the nuclear membrane. In order to observe its location in response to heat shock, Akt-EGFP was transfected into S2 cells, and monitored with a fluorescent microscope under NS and HS conditions. Preliminary observations show Akt uniformly distributed throughout the cytosol and nucleus (data not shown). Cytosolic compartmentalization of Akt would render it unable to regulate dHSF by phosphorylation during HS, since dHSF remains in the nucleus and then binds HSEs to activate the heat shock pathway. The fact that Akt is not retained in one cellular fraction/area during NS and HS conditions in *Dm* cells supports conditional regulation of dHSF by Akt kinase. Cytosolic and nuclear cell extract fractionation and subsequent assay by Western blot will reveal the sub-cellular location of Akt or the possibility of Akt moving freely between the cytosol and the nucleus.

These data show direct interaction of endogenous Akt to dHSF by immunoprecipitation experiments. In addition, recombinant and *in vivo* expressed wt-dHSF were able to be phosphorylated by recombinant Akt. The Akt phosphorylation site is within residues 241-270,

while the kinase binding site may overlap between sequences defined by the internal deletions. The ability of Akt to modify recombinant factor suggests that: 1) dHSF does not need to undergo additional modifications to be phosphorylated at this kinase site, or 2) cofactors that may prevent Akt from modifying dHSF under *in vivo* conditions are not present, and cannot interfere with *in vitro* wt-rdHSF phosphorylation. Recombinant dHSF is a more sensitive substrate to phosphorylation by Akt kinase, as demonstrated in the *in vitro* kinase assay (Fig 2A, lanes 3 and 4). The constitutive DNA-binding that results from spontaneous oligomerization of this factor suggests that dHSF that is activated for oligomerization, if not also DNA binding, provides the optimal substrate conditions for Akt phosphorylation.

RNAi was successfully used to remove a large population of Akt from *Drosophila* cultured cells. Fluorescent imaging of wt-dHSF and assay by Western blot revealed that dHSF was stably localized within the nucleus and in a punctuate pattern characteristic of heat-stressed cells. Along the same lines, chemical inhibition of Akt and assay of HSE-DNA-binding confirmed the increase in molecules in the nucleus that have acquired DNA-binding ability. Interestingly, when Akt expression levels were knocked-down, there was an increase in the amount of HS dHSF recognized as an Akt substrate. The possibility of a second kinase, GSK-3β, which has an overlapping recognition motif that includes S256, may indicate that shared kinase sites are now accessible and may have enhanced phosphorylation. Akt may compete with other kinases by possessing high affinity for S256 of dHSF, yet have low efficiency of phosphorylation activity. Additional studies will assay for GSK-3β activity during Akt knock-down. In a similar fashion to knockdown of Akt, removal of other, Akt/FOXO-associated kinases, such as GSK-3β and SGK will allow for more precise identification of the conditions under which Akt participates in dHSF regulation. It is known that Akt functions to regulate glycogen synthesis by inactivation of GSK-3α and -3β [27]. In studies of human HSF1, simultaneous overexpression of GSK-3β and ERK1 has been shown to halt transcription and decrease HSF1 in heat-stress induced granules

during the recovery stage [36]; similar to FOXO, HSF1 is negatively regulated by phosphorylation.

DHSF and dFOXO may have cofactor roles in activating both the Akt/FOXO and HS pathways

It was previously reported that a Daf-16(FOXO):GFP fusion protein was found systemically throughout *C. elegans*, and transported into the nucleus during environmental stresses, including heat stress [9]. To assay for localization in *Dm* cells, dFOXO tagged with DsRed6 under control of a constitutive promoter will be expressed in S2 cells. Based on current knowledge, it will be nuclearly-localized under both NS and HS conditions. Co-expression of dHSF:EGFP and dFOXO:DsRed6, and subsequent assay of DNA binding with NS and HS cell fractions may suggest either: 1) that activation of this Daf-2/Daf-16 pathway initiates non-heat-induced activation of the HSF-mediated pathway; or 2) that HSF activation is independent of FOXO activation or that of its downstream targets.

The possibility of activating *hsp* gene transcription in a non-heat-induced state could be a result of the binding of dFOXO, rather than dHSF, at HSEs. A similar phenomenon is observed in yeast, with the oxidation protein Skn7 able to bind HSEs, a region previously believed to be occupied solely by ScHSF [37]. Constructing an *in vivo luciferase/*transcription assay using an HSE promoter-driven template, and assay of gene transcription by dFOXO and dHSF (as a control) may give insight into the transactivation potential of dFOXO in the HS response. Chromatin ip (CHip) assays might also be informative [38]. Previous reports have shown that removal of HSF1 in *C. elegans* did not reduce *hsp70* and *hsp90* transcript levels [14]. *Hsp* gene induction by a partner protein that can bind HSEs and transactivate *hsp* gene transcription implicates HSEs may be secondary, minor downstream targets of FOXO1 by necessity. This evidence suggests that *hsp* gene induction can be compensated for by alternate transcription factors in the event of a non-functional HSF.

Recently, the *S. cerevisiae* Akt homolog, SCH9, has been shown to influence lifespan and longevity in the presence of stress-resistance transcription factors [6, 39-41] and functional Sod2, a mitochondrial superoxide dismutase that, when overexpressed, extends lifespan by 30% [41]. Deletion of SCH9 is synonymous with calorie restriction and increased longevity [6]. Expression of the yeast HSF, as well as the three mammalian HSF homologs, in *Dm* S2 cells may provide insight into how this regulatory mechanism is conserved between species and reveal species-specific complexity within this nutrient-sensing pathway. While there is only a single yeast HSF, mammalian HSF1, HSF2, and HSF4 respond in stress-specific manners, as only HSF1 is activated by heat stress [42, 43]; isolating each homolog, and assaying for its ability to activate the HS response, will reveal the HSF isoform-specific stress response with which the dAkt/dFOXO pathway may cooperate in order to modulate the aging pathway/response. In this manner, we may also be able to distinguish between the cells' response to the Akt/FOXO pathway and to the dHSF heat shock response.

The aim of this study was to elucidate the biochemical mechanism for HSF activation/repression by dAkt, and to investigate direct Akt-dHSF interaction and phosphorylation of dHSF by this kinase. These data show mutants with properties similar to non-shocked dHSF as a result of modification at a consensus Akt binding motif. Deletion of the dHSF nuclear localization sequence in combination with S256D substitution rendered this mutant non-functional for DNA binding. These data point to a negative charge at S256 by phosphorylation as a means to prevent activation of *Dm* Heat Shock Factor.

Materials and Methods

Cell line maintenance. S2 Schneider cells [44] were maintained with S2 cell media (Gibco) supplemented with 10% fetal bovine serum (FBS) and split 1:3 every 4-5 days.

Plasmid constructs and protein expression. DHSF was inserted into pAc 5.1A/V5-His (Invitrogen) with a C-terminal enhanced GFP (EGFP) tag (BD Biosciences). EGFP from pEGFP (BD Biosciences) was subcloned into the plasmid pAc/5.1A/V5-His, generating pAc 5.1A/EGFP-V5-His. Recombinant dHSF constructs were cloned into pET11b (NEB) for expression in inducible DE3 cells (Invitrogen). Site-directed mutagenesis via PCR was carried out according to protocols provided by the Mayo Lab (Caltech), and the resulting reaction was transformed into DH5α cells (Invitrogen). Transformed colonies were selected with ampicillin and the DNA isolated from cells using Qiagen Miniprep columns. Transfections were performed with FuGene6 (Roche) transfection reagent into S2 cells. Briefly, 1 μg construct DNA was diluted in ddH_2O and combined with 12 μL Fugene6 for 30 minutes at 25°C. This solution was added to 2 x 10^6 cells in 900 μL FBS-deficient media. After 24 hours, 1 mL complete media and 100 μL FBS was added to each transfection. Cells were harvested 24-72 hours post transfection.

Antibodies and Chemical Inhibitors. Monoclonal anti-dHSF (line 17H8) and anti-α-3 (line E43), used as a control, were obtained from [31]. Anti-V5 antibody was obtained from Serotec. Anti-phospho-Akt-substrate, anti-Akt, anti-phospho-S473, and anti-GSK3-β antibodies were purchased from Cell Signaling Technology. Cells were incubated with the following chemical inhibitors: LY294002 (Sigma) at a concentration of 30 μM for 6-12 hours, and Akt Inhibitor V/Tricibine (Calbiochem) at a concentration of 40 μM for 2 hours.

Whole cell extract preparation, cytosolic and nuclear fractionation, and Western blotting. S2 Schneider whole cell extracts (WCE) and cytosolic/nuclear fractions were prepared in lysis

(A) buffer (15 mM HEPES (pH 7.6), 10 mM KCl, .1 mM EDTA, 5 mM $MgCl_2$, .2% NP40, and 1 mM PMSF) according to [31]. Western blotting analysis was performed with whole cell extracts or ips and combined with 2X Laemmli sample buffer (LSB), heated to 95°C for 5 min. Samples were resolved by SDS-PAGE and blotted onto nitrocellulose. After incubation with the indicated antibody, bands were analyzed by chemiluminescence (Pierce).

Immunoprecipitation. Immunoprecipitation of dHSF was carried out with either αV5-PGS (Roche) X-linked, α17-PGS X-linked, or nickel agarose (NiArg) beads (Qiagen) as noted, and incubated with whole cell extracts for 2-14 hours at 4°C. Beads were washed 3 times and resuspended in D buffer (25 mM HEPES (pH 7.6), 10% glycerol, .1 mM EDTA, .1% NP40, 10 mM KCl). Bead-bound proteins were fractionated by SDS-PAGE and analyzed by Western blot with the appropriate antibody.

Binding assays. Binding assays were performed using the dsHSE γ-^{32}P labeled HSE (5'-GCGCGCCTCGAATGTTCGCGAAAAGA-3') in binding cocktail (100 microg/mL BSA, 100 microg/mL poly(dI-dC) and 5% Ficoll in HGE buffer (25mM Hepes, 100% glycerol, .1 mM EDTA)). WCE or ips were incubated in binding cocktail and end-labeled HSE at room temperature for 30 minutes, then analyzed by 10%/1X TBE gel and autoradiography.

Primer extension assays. The oligonucleotide sequences used for primer extension analysis were (5'-GGTTACTTTTAATTGATTCAC-3') for *hsp70* and (5'-CCTTTCCACTAGTTTTCGGA-3') for the quantitative control *H2b*. The oligonucleotides were 5' γ-^{32}P end-labeled with T4 Polynucleotide kinase (NEB) and 5 ng were hybridized to 8 μg total RNA in TEK (10 mM Tris-Cl, 1 mM EDTA, pH 7.4, and 200 mM KCl.) by heating to 65°C for 15 minutes and slowly cooling 4°C. Avian myeloblastosis virus (AMV) reverse transcriptase was added to the

RNA:oligonucleotide hybrid mixture and incubated at 37°C for 90 minutes. Glycogen and 95% EtOH/.1M NaOAc was added to the reaction, incubated on dry ice for 5 minutes, and clarified by centrifugation. Samples were resuspended in formamide and XCFF dye, heated to 70°C for 5 minutes, and run on a 10% acrylamide-6M urea gel in 1X TBE, 300V for 20 minutes. The gel was soaked in ddH_2O to remove urea, and imaged by autoradiography.

Akt KO via RNAi. Cloning and IVT. DAkt was obtained from a *Dm* embryonic cDNA library [31], cloned into pAc5.1A, and the plasmid linearized with EcoRI (NEB). T7 RNA polymerase (T7RNAp) start sites were attached to the ends of the first 500 bp of the coding region of Akt by PCR to form the template for *in vitro* transcription. Primers for construction of ssRNA to endogenous dHSF proceeded in a similar manner. ssRNA was synthesized by *in vitro* transcription with the PCR product as template. Briefly, 3 μg template, 1X RNA secure (Roche), 16 mM rNTPs (Roche), and 20 μL 5X transcription buffer (200 mM Tris-HCl (pH 7.9 @ 25°C), 30 mM $MgCl_2$, 50 mM DTT, 50 mM NaCl, and 10 mM spermidine) were mixed with water to a total reaction volume of 99 μL. This was heated to 65°C for 15 minutes, cooled, and 1 μL T7RNAp was added and incubated at 3 °C for 5 hours. ssRNA was redissolved with .5M EDTA, phenol/chloroform extracted, and re-precipitated from the aqueous phase with .1 volume 3M NaOAc/2.5 volumes 95% EtOH for 15-30 minutes on ice. RNA was pelleted and washed with 70% EtOH, dried by speedvac, and resuspended in Tris-EDTA pH 7.4. dsRNA was formed by heating RNA to 70°C and slowly cooling to anneal strands.

Akt KO – cell manipulation. 2 x 10^6 cells were left to adhere several hours in 6- well plates. Growth media was removed and replaced with 900 μL FBS-deficient media, and 40 μg dsRNA was added to each well and incubated for 2 hours. Cells were then supplemented with 100 μL

FBS. After 24 hours, 1 mL complete media was added and cells grown at 25°C for an additional 72 hours for optimal RNA knockdown.

Inverse Kinase Assay. Inverse Kinase assay was performed with immunoprecipitated endogenous dHSF or rdHSF using anti-dHSF (17) antibody. Ips were washed two times with kinase buffer (20 mM Tris-HCl, 10mM $MgCl_2$, 5 mM DTT (pH 7.5)). Beads were incubated 1:1 (v/v) with γ-^{32}P-[ATP] reaction solution containing rAkt (S. Milward, Caltech) for 1 hour at 30°C. Reactions were stopped by addition of 2x LSB, and analyzed by SDS-PAGE and autoradiography.

Oligomerization assay. Proteins were oligomerized by addition of 30 μM EGS to WCE for 30 minutes RT. The reactions were stopped by 10 minutes incubation with 340 μM Lysine. Samples were mixed with 2XLSB and run on a 7.5% SDS-PAGE gel for 90 minutes at 200V for analysis by Western blot.

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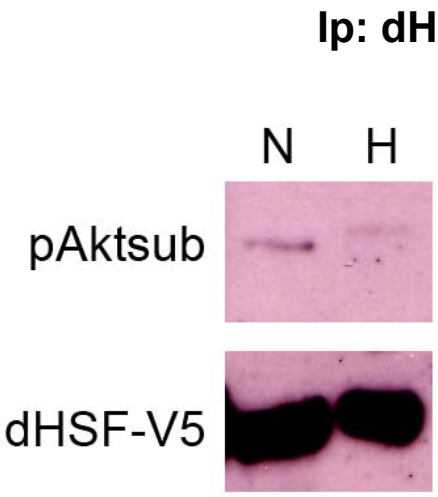
Figures

Figure 2-1

A



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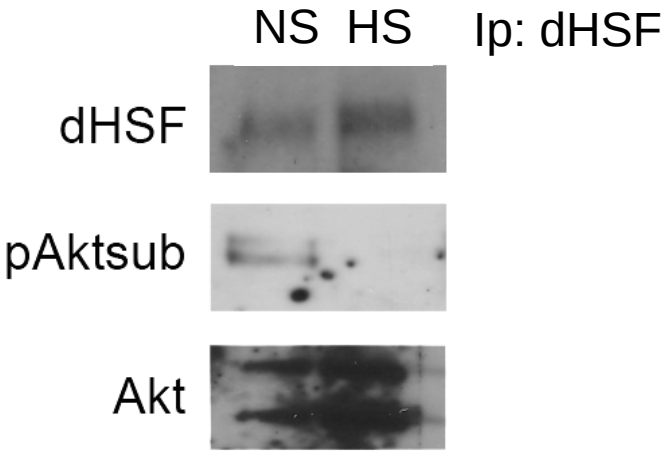

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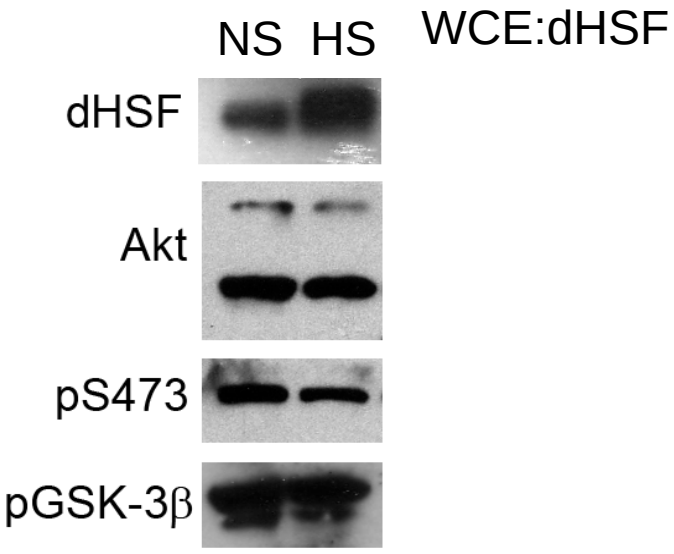


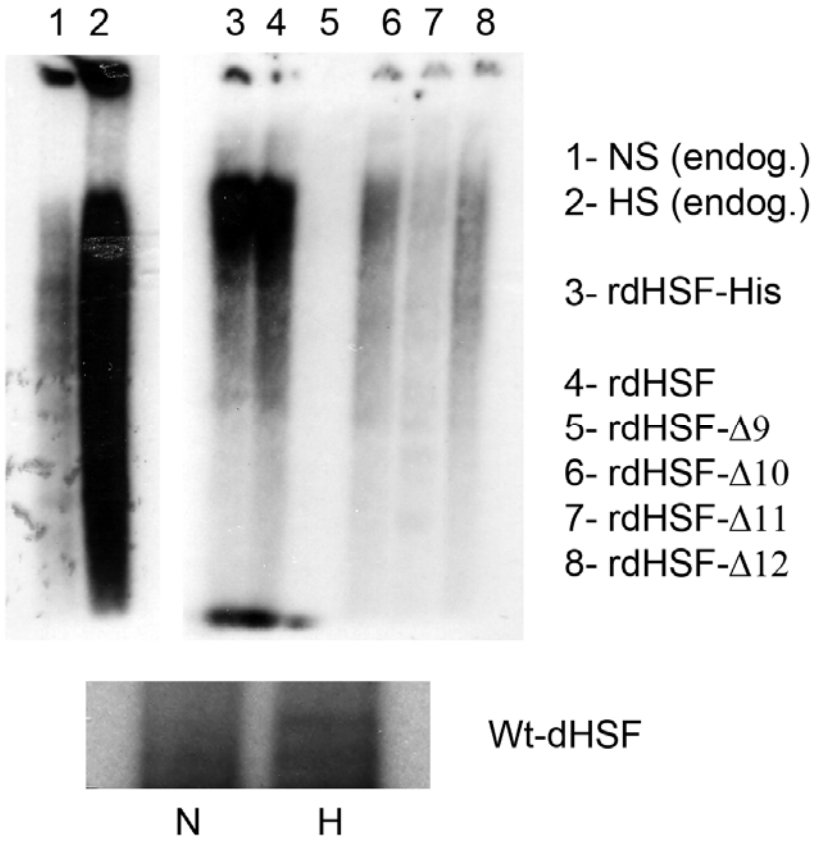
Figure 2-1

A- Non-shocked (NS) dHSF is phosphorylated by Akt kinase. Immunoprecipitated wt-dHSF-V5-EGFP was isolated from NS and HS S2 cell extracts. Comparable levels of immunoprecipitated protein were resolved by SDS-PAGE, and blotted onto nitrocellulose, followed by incubation in αV5 antibody.

B- Akt kinase binds to and phosphorylates NS endogenous dHSF. Immunoprecipitated endogenous dHSF from NS and HS S2 cell extracts were analyzed by Western blot and incubated in α17 (dHSF), αV5, or α Akt antibodies.

C- Akt levels remain the same in the NS and HS states. WCE from S2 cells were analyzed by Western blot and incubated in α17 (dHSF), αAkt, αpS473, and αpGSK-3β antibodies. DHSF levels increase as a result of heat stress, while Akt levels do not change. Akt phosphorylated at S473 is recognized in both NS and HS extracts, as is phosphorylated GSK-3β.

Figure 2-2



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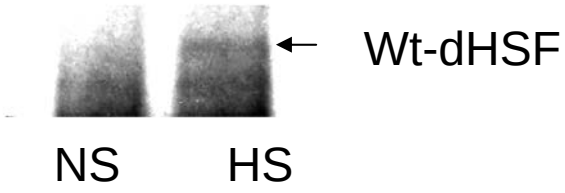
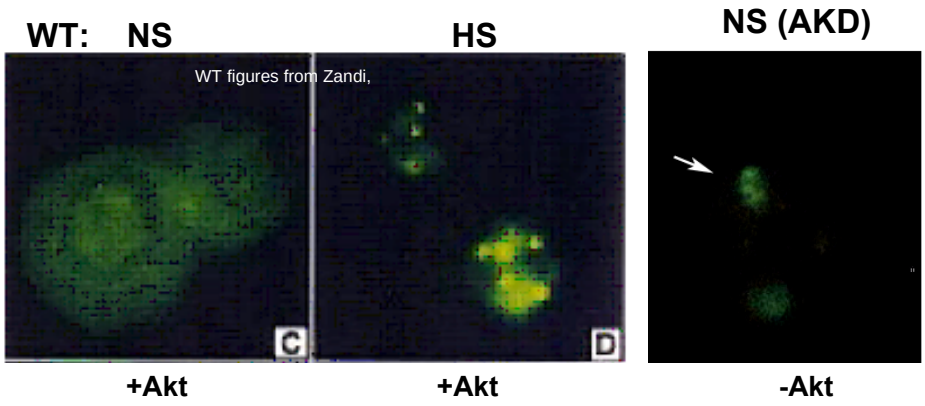


Figure 2-2

Immunoprecipitated NS endogenous and recombinant dHSF can be phosphoryated by Akt in an *in vitro* kinase assay. Immunoprecipitated endogenous dHSF from NS (lane 1) and HS (lane 2) S2 cell extracts were incubated in an *in vitro* kinase assay (Materials/methods). Recombinant dHSF that is GST-tagged was also able to be phosphorylated by this method. In lanes 3 and 4 – wt-rdHSF. In lane 5 – wt-rdHSF containing an internal deletion of residues 241-270 (Δ9). Lane 6- Δ10 (missing residues 271-300). Lane 7- Δ11 (missing residues 301-330). Lane 8- Δ12 (missing residues 331-360). Samples were run on SDS-PAGE and visualized by autoradiography.

Figure 2-3

A



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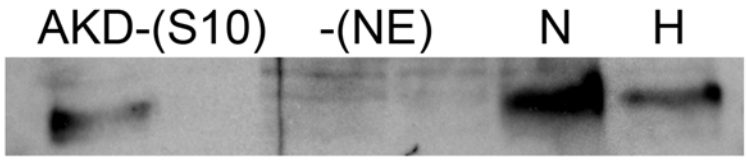

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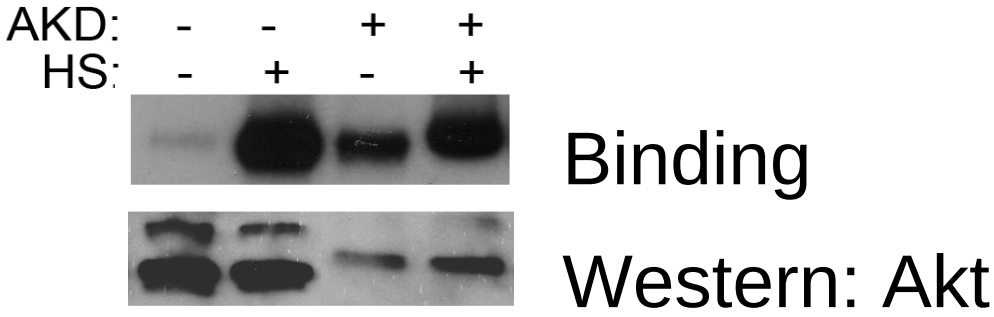

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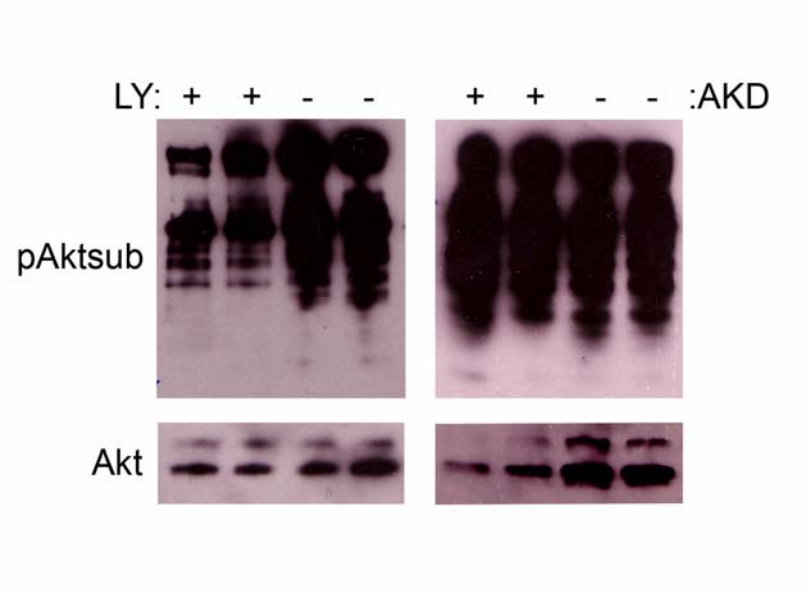
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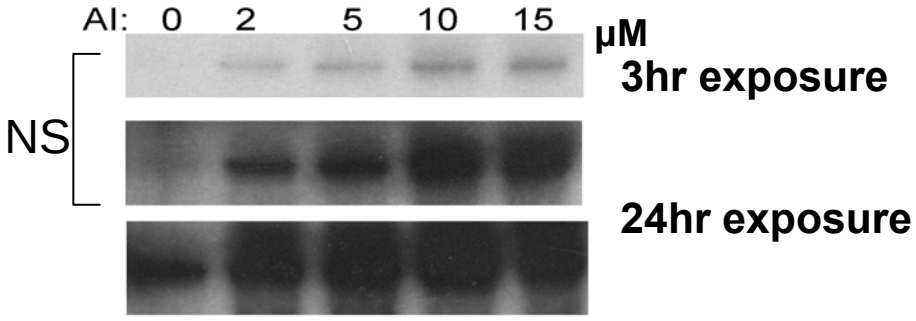
pAktsub
Akt

E


AI: 0 2 5 10 15
μM
3hr exposure
NS
24hr exposure

F

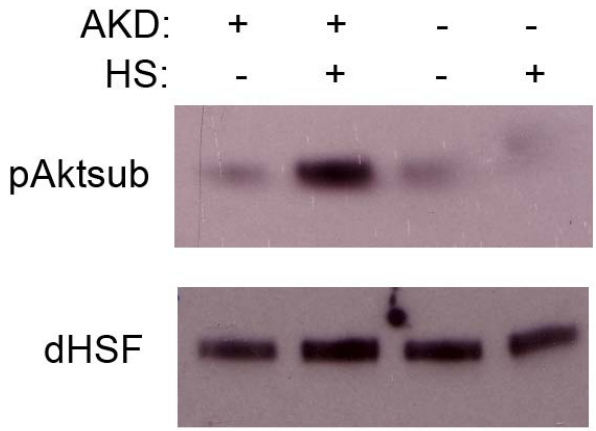


Figure 2-3

A- RNAi-mediated knock-down of Akt results in a nuclear punctuate pattern of dHSF-EGFP. Akt knock-down by RNAi resulted in a punctuate pattern of dHSF-EGFP, similar to the phenotype observed in HS dHSF. S2 cells were treated with dsRNA (Akt) to lower endogenous Akt expression levels, then transfected with wt-dHSF-EGFP and observed under fluorescence. Wt-dHSF is a monomer in a diffuse body in the nucleus, which forms punctuate bodies upon heat stress. Images of NS and HS S2 cells were taken from Zandi et al., 1997.

B- DHSF preferentially fractionates into the nuclear extract during Akt knock-down S2 cells. Cytosolic (S10) and nuclear (NE) fractions were run on Western blot and incubated with α17 (dHSF) antibody.

C- DHSF acquires DNA-binding ability in Akt knock-down S2 cells. Binding assays were conducted with comparable levels of endogenous dHSF to assay for DNA- binding to a double-stranded HSE. Samples were run on a native polyacrylamide gel and imaged by autoradiography.

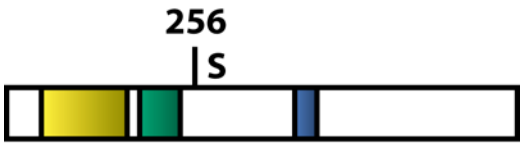
D- Substrate recognition by αphospho-Akt-substrate antibody in LY294002-treated and Akt knock-down cell extracts. NS and HS WCE were probed with αpAsub antibody to reveal phosphorylated Akt substrates. Note the reduction in overall number of substrates recognized in LY294002 cells as compared to Akt knock-down cells. Akt levels are reduced in Akt knock-down cells, while they remain the same in cells treated with LY294002.

E- DNA-binding assay of Akt inhibitor-treated S2 cells. S2 cells were treated with increasing levels of a specific Akt Inhibitor (Calbiochem) and fractionated into S10 and NE extracts. DNA-binding assay on NE extracts indicated acquired DNA binding as a result of Akt inhibition.

F- DHSF may be phosphorylated by a second kinase in Akt knock-down cells upon HS. Immunoprecipitated wt-dHSF from Akt knock-down cells were probed with αpAsub antibody to reveal a more highly phosphorylated population of dHSF from HS Akt knock-down cells.

Figure 2-4

A

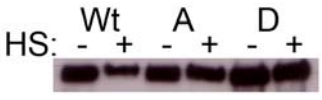


Akt Phophorylation motif:

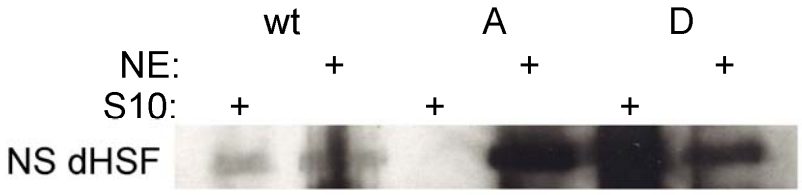
	RXRXXS/T
Dm dHSF	**R**A**RTTS** (**S**256)
Dm dFOXO	**R**A**RS**N**T** (**T**44)
	RRRAA**S** (**S**190)
	RQ**R**A**SS** (**S**259)
C. elegans DAF-16	**R**D**R**CN**T** (**T**54)
	RTRE**RS** (**S**240)
	RE**RS**N**T** (**S**242)
	RP**RT**Q**S** (**S**314)

B

	251	256
dHSF WT:	RARTT	**S**
S/A:	RARTT	**A**
S/D:	RARTT	**D**



C



D

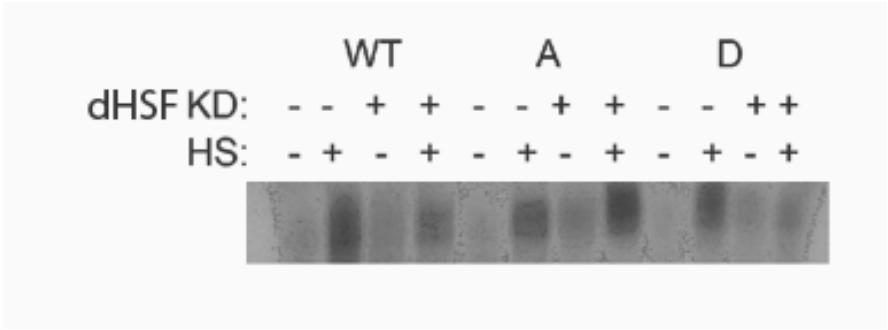


Figure 2-4

A- Schematic of Akt Kinase motifs. Survey of Akt kinase sites in dHSF, dFOXO, and DAF-16. The Akt consensus site exists in dHSF at residues 251-256.

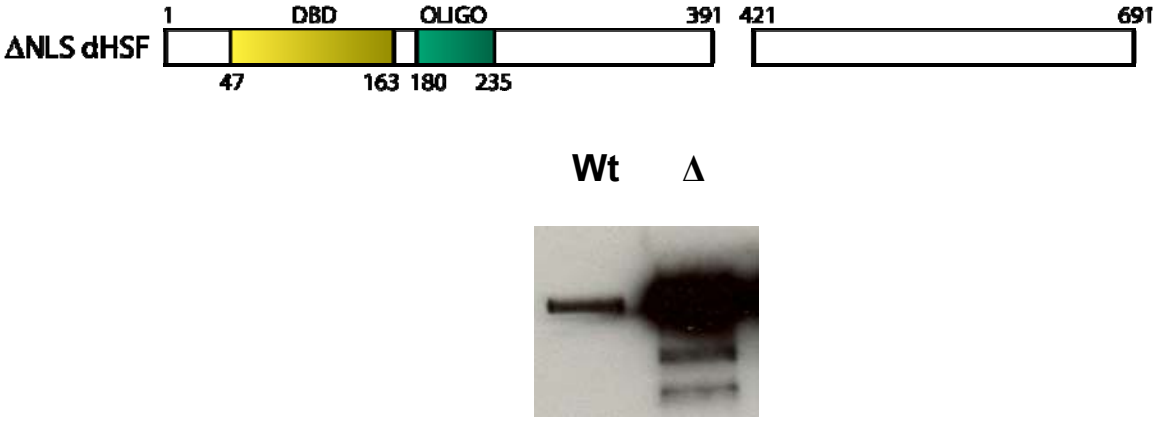
B- Site-directed mutagenesis of S256 in dHSF. Mutagenesis of S256 to A and D reveal no differences in protein levels as a result of mutation at this site.

C- Cellular fractionation of S256 mutant dHSF. S/A (A) mutation results in a higher population of mutant dHSF that fractionates into the nuclear extract, while S/D (D) mutation exhibits similar S10 and NE fractionated levels as wt-dHSF.

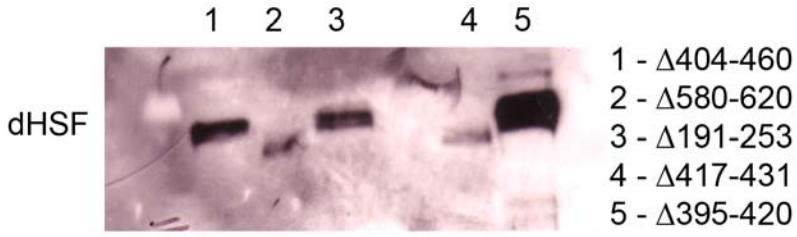
D- DNA binding of S256 mutant dHSF. S/A (A) mutation results in slightly increased DNA- binding ability compared to wt-dHSF, while S/D (D) exhibits compromised DNA-binding ability. Endogenous dHSF was removed to prevent heterotrimerization with mutant dHSF that may show up as binding artifacts.

Figure 2-5

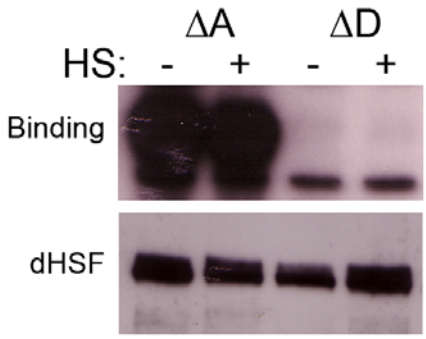
A


ΔNLS dHSF
1
DBD
OLIGO
391
421
691
47
163
180
235
Wt
Δ

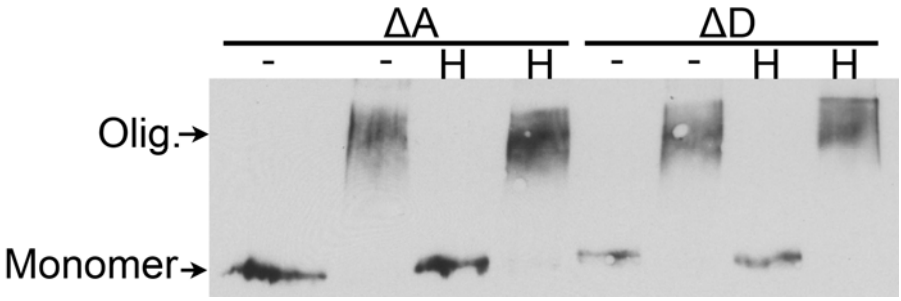
B


1 2 3 4 5
dHSF
1 - Δ404-460
2 - Δ580-620
3 - Δ191-253
4 - Δ417-431
5 - Δ395-420

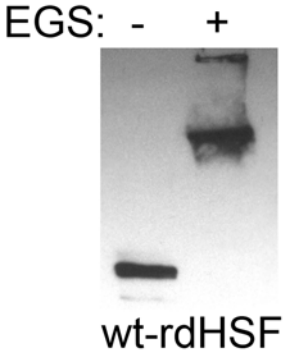
C



D



E



F

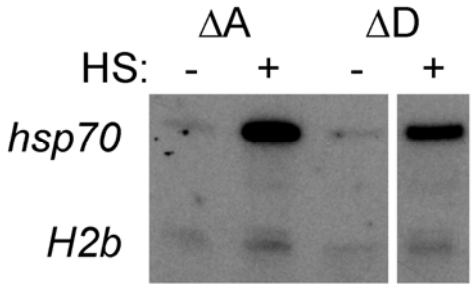


Figure 2-5

A- Schematic of ΔNLS-dHSF. This mutant is lacking a nuclear localization sequence at residues 390-420; identified in Zandi et al., 1997. Western blot reveals differences in protein levels upon removal of the NLS.

B- Internal deletions of dHSF reveal overexpressed Δ dHSF levels. Mutant internally-deleted dHSF were analyzed by Western blot and incubated with α17 antibody.

C- DNA-binding of Δ dHSF with mutations at S256. DNA-binding assay reveals minimal binding for the ΔD mutant, while ΔA bind DNA-constitutively. Western blot show comparable levels of mutant dHSF as probed by α17 antibody.

D- Oligomerization of mutant dHSF. The Δ/S256 combination mutants were not hindered in oligomerization ability as a result of mutation. Cross-linking with EGS and subsequent assay by Western blot revealed the presence of oligomerized factor in NS and HS mutant protein.

E- Recombinant dHSF can oligomerize spontaneously. Recombinant wt-dHSF can oligomerize when cross-linked by EGS and assayed by Western blot.

F- Transcriptional activity of Δ/S256 combination mutants. Primer extension assays were performed on standardized levels of total RNA isolated from mutant-dHSF transfected S2 cells. *Hsp70* transcripts were still synthesized in ΔD-dHSF transfected cells. *H2b* internal control transcripts were used as a control.

Table 2-1

HSF Kinase	*D. melanogaster*	*S. cerevisiae*	*H. sapiens*	*M. musculus*	*C. elegans*	*K lactis*
AKT	S256	T388	S230	S230	S309	S299
CKII	S17	T580		T503		
DNA PK	S223, T442				T328	6 sites
ATM		T694, S608			S559, T328	S575
ERK1	S378	S655, T555	S363, 4 more sites	S303, S440	T662, S551	T434
Calmodulin	T513		S136, S230	S230, S136	S559, S196	S229, T569

Table 2-1

Survey of putative Akt kinase sites in heat-shock factor from several model organisms.

Putative Akt consensus sequence sites that are present in HSF from several species point to a possible conserved mechanism for Akt modification of heat-shock factor.

CHAPTER 3: Post-translational modification of dHSF within the nuclear localization sequence

Introduction

Lysine post-translational modification is a crucial modulator of protein function. Lysines can be tagged with ubiquitin or SUMO, as well as acetylated or methylated, and regulate processes as diverse as transcription and protein degradation [1, 2]. Ubiquitin tags target the protein to the 26S proteasome [1], while lysines that undergo other post-translational modifications can finely tune cellular responses by altering protein function.

The ubiquitin proteasome pathway signals degradation or changes in regulatory function of its target protein

The ubiquitin proteasome pathway (UPP) handles degradation of misfolded, denatured, or potentially cytotoxic proteins by covalently tagging them with ubiquitin (Ub). Heat-shock chaperone proteins (hsp) are necessary cofactors for proteases during proteolytic processing [3]. Examples also exist of proteins associating with hsps to increase their own half-life and stability [4].

Two recognized signaling regions exist for ubiquitin-tagging proteins: an N-terminal degron and a PEST domain. Recently, there is evidence for a third putative degradation-signaling domain within a hydrophobic core, that becomes unmasked during degradation and/or misfolding (Parker lab, unpublished results). Currently, most degrons that have been characterized lie within the transcriptional activation domain [5], a phenomenon that intertwines the degradation and transcriptional activating functions observed in several transcription factors.

Ubiquitin-tagging of transcription factors can regulate both their destruction and their nuclear translocation for the transcriptional activation of tightly regulated processes. As degradation is an energetically unfavorable process, it has been postulated that dHSF may be

recycled rather than degraded, and previous studies have suggested a conversion between activated/DNA-binding dHSF and its inactive form in the same molecule [6].

Ubiquitin substrate tagging is a concerted process whereby E1 modifies Ub into a reactive state and associates with an E2 that catalyzes the attachment of Ub to a target protein. This target protein is recognized and recruited to the E2 by a substrate-specific E3 [7]. Ub attachment consists of either chains (polyubiquitylation) or single monoubiquitin subunits. Ubiquitin is a stress protein exhibiting heat-stress induced *ub* gene transcription. This in turn alters cell protein profiles as a result of ubiquitin-mediated degradation [8]. During HS, polyubiquitin gene expression is induced, and increases for the duration of temperature stress, while no differences are observed in monoubiquitin gene expression under the same conditions [8]. Proteins synthesized prior to heat stress accounted for the majority of proteins that were ubiquitylated during HS. Studies have shown that one of the principal heat shock pathway components, hsp70, is rapidly turned over and degraded within the UPP [8].

Several transcriptional activator proteins are polyubiquitylated to induce their transcriptional activation [9], and are destroyed as a result of this covalent tagging. Meanwhile, monoubiquitylation can alter protein function without signaling protein turnover, as seen in histone H2B [10, 11]. In some cases, such as the yeast activator GAL4, polyubiquitin-tagging and subsequent proteolysis of the factor is a requirement for its transcriptional activation [12]. Recently, Salghetti [13] and colleagues found that ubiquitylation of the (*S. cerevisiae)* VP16 transcriptional activation domain (TAD) signals both TAD activation and protein degradation of a TAD:transcription factor fusion protein, uncoupling the degradation and ubiquitylation functions. Many ubiquitin ligases are proto-oncogenes, and mutations in these enzymes, their substrates, and ubiquitin itself are noted in human disease pathogenesis [1, 14, 15]. Elucidating the process of ubiquitylation as a regulator of transcriptional activation/repression will play a prominent role in understanding disease states.

Non-ubiquitin post-translational modification can modulate the activity of a target protein

Acetylation is another method for regulating transcriptional activation. Examples exist of modulating transcriptional activity by site-specific acetylation of lysines in relation to the DNA-binding domain (DBD), where acetylation outside the DBD enhances transcriptional activity, and acetylation within this domain diminishes it [16]. The p53 tumor suppressor protein is activated by phosphorylation and subsequently acetylated at several candidate lysines throughout the transcription factor [17, 18]. Post-translational modifications within the DNA-binding domain regulate cellular apoptosis [17, 19, 20], while modifications to serines and lysines at the extreme N- and C-terminal regions mediate DNA-binding and the induction of p53 transcriptional activity [21].

In contrast to ubiquitylation, sumoylation does not dictate degradation of proteins tagged by the small ubiquitin-related modifier (SUMO), but can direct nucleo-cytoplasmic transport and transcriptional regulatory functions, among others [22, 23]. Methylation at lysine and arginine residues of histone tails by multiple methyl groups directs chromatin accessibility to transcriptional activators and repressors [24].

DHSF may be post-translationally modified for degradation and/or regulation of its activating function

In dHSF, deletion of the putative NLS resulted in extremely high protein levels (Chapter 2), suggesting there exist modifiable residues within, and flanking, this region. Mutation of these residues to arginine exhibited extremely high levels of protein that are retained in the cytosol. These mutants are degradation-deficient (within a given test period) according to pulse-chase analysis. Experiments were designed to determine whether this region (Δ) acts as a degron to promote degradation or is post-translationally modified by ubiquitin, acetylation, or sumoylation at lysine residues within this nuclear localization sequence. Identifying the nature of the

modifications within the NLS may distinguish between the functions of the signaling and targeting domains of dHSF.

Results

A nuclear localization signal in dHSF contains lysines that may be modified by ubiquitin

In order to identify putative sites of degradation and/or the degron, C-terminal deletions of EGFP-tagged dHSF were expressed in S2 cells and their sub-cellular localization was surveyed (Fig 1). During this process, the region between residues 405 and 435 was found to play a role in nuclear (N-435) vs. cytosolic (N-405) partitioning (Hicks, A.B.). Internal deletions and sequence analysis of this region implicate a putative nuclear localization signal (NLS) between residues 393 and 420 [25]. This protein (Δ) exhibits 100-fold excess protein levels *in vivo* and is cytosolic when exogenously expressed (in pAc) (Chapter 2, Fig 5A). Stability of this mutant is persistent when assayed via S^{35} pulse-chase analysis (Fig 2E), which suggests that this region may be a degron or a region containing the lysines targeted by the ubiquitylation mechanism. Further supporting this theory, an internal deletion of residues 404–460 had protein levels comparable to ΔNLS and constitutive DNA-binding for NS and HS dHSF (unpublished). Examination of the lysines in, and surrounding, this region may reveal post-translational lysine modifications and their role(s) in the ubiquitin-mediated proteolysis of dHSF, and the nuclear-dependent degradation of this transcription factor.

Drosophila HSF contains 32 lysines- three of these are within the Δ-deleted region (K400, 405, and 409) (Fig 2A). Site-directed mutagenesis of lysine (K) to arginine (R) to yield charge-conservative mutations in all three Ks, singly and in combination (K3R), revealed wild-type (wt)-dHSF expression levels (Fig 2B) and sub-cellular distribution. However, mutation of K432R in conjunction with K3R (K4R) resulted in strict cytosolic localization, dramatically swollen nuclei, and 20-fold wt-dHSF expression levels, like that of Δ (Fig 2B, Fig 2C). DNA-binding assays revealed constitutive binding to the heat shock element by K4R, while K3R had

only wt-dHSF DNA-binding activity (Fig 2D). K432R alone had no effect on binding, localization, or expression levels. Additional K/R mutations to K4R (addition of K454R to make K5R) did not further increase the levels of protein; instead, these mutations seemed to limit the amount of protein produced to 3-4X wt. Pulse-chase analysis by metabolic-labeling of methionine shows that K5R is a stable mutant (Fig 2E).

Endogenous dHSF can be degraded, as observed during treatment with the reversible proteasome inhibitor MG132. The presence of trapped, higher molecular weight complexes suggests Ub-tagging of dHSF (Fig 2F). To assess whether K-mutated dHSF was degraded, and in a proteasome-dependent manner, lysine-mutated dHSF was treated with MG132. K4R did not exhibit an increase in protein levels, suggesting it has already exited the proteasome pathway, possibly by virtue of K/R mutagenesis (Fig 2G). There was no enhanced DNA-binding activity due to these mutations, (though there may be an increase in the DNA-binding ability of K5R). A time course of K5R mutant protein treated with MG132 showed a shift within a 20 minute heat-shock, consistent with wt-dHSF shifting in response to HS, but no change in protein expression levels (Fig 2H). This mutant is still able to undergo modification, either at other lysines in the factor, or by phosphorylation, during HS conditions.

To determine if dHSF and K-mutated dHSF are able to be ubiquitin-tagged *in vivo*, cells were co-transfected with wt- or K-mutant dHSF and a ubiquitin construct under control of a metallothionein promoter. Ubiquitin expression was induced following proteasome inhibition by MG132. Tagging ubiquitin to wt- and mutant-dHSF resulted in higher-order complexes (Fig 3A). Endogenous dHSF was also able to be tagged *in vivo,* when assayed by HSF-immunopreciptation and Western blot probed with α17 dHSF antibody. However, the EGFP tag on dHSF can also be Ub-tagged for degradation, and this may interfere with recognition of appropriate substrates of ubiquitin/E3 ligase. An *in vitro* ubiquitylation reaction on dHSF ips utilizing recombinant ubiquitin (rUb) resulted in higher- order complexes as assayed by Western blot (Fig 3B). Recombinant dHSF is unable to be rUb-tagged *in vitro* in this reaction, suggesting that either

post-translational modification or cofactor recruitment is necessary for ubiquitylation. This preliminary data encourages further study of direct dHSF ubiquitylation.

A "synthetic" SV40 NLS can force cytosolically retained mutant dHSF into the nucleus

The SV40 large T-antigen NLS can be tagged at the C-terminus of a target protein to force its nuclear entry. Strictly cytosolic dHSF mutants also exhibiting overproduction of the protein were tagged with a C-terminal SV40 nuclear localization sequence to force them into the nucleus to observe any differences in subcellular localization or protein turnover. K4R, while still overproduced, can now enter the nucleus and form punctuate bodies during HS – suggesting these lysines are not essential for degradation; rather they may be post-translationally modified (other than by polyubiquitylation) for proper sequestration in the cell (data not shown). K5R-SV40 is also overexpressed but nuclearly localized. While this localization does not necessarily indicate HS-inducibility, K5R-SV40 dHSF does exhibit the punctuate pattern characteristic of heat-stressed protein under HS conditions. ΔNLS-SV40 is also nuclear, but has a protein haze throughout the cytosol (also seen in EGFP-tagged mutant dHSF), as if the cell were attempting to control/handle degradation of excess protein.

Other post-translational modifications of dHSF reveal two lysine residues/sites that may control dHSF activation

To assay the possibility of other lysine modifications to dHSF, full-length factor was ip-ed and probed with commercial antibodies to acetylated-lysine and sumoylated-lysine. DHSF is acetylated in the NS and HS states (Fig 4A). It is also sumoylated by sumo-1 and sumo-2/3 (responsible for poly-sumo-chain modification). Sumoylation has been noted in human HSF1 and HSF2 [26], and the motif is present for 3 lysine residues in dHSF (Fig 4B).

Conservative lysine-to-arginine mutation did not result in degradation-resistant mutants (according to SV40-tagging dHSF data), so combinations of lysine to alanine (K/A) mutants were

constructed to investigate the possibility of acetyl or SUMO modification of the lysines within the NLS. A lysine-to-alanine mutation substitutes a constitutively acetylated lysine (A) in place of a modifiable lysine, while a non-acetylated lysine would be "mimicked" by an arginine (R). Two double mutants were cytosolically localized: K409/432A (9/2A) and K409/454A (9/4A) (schematic - Fig 2A). Replacement of the lysines in pairs at these three positions is sufficient to target dHSF to a different cellular compartment in the NS state, in this case the cytosol. In the HS state, 9/4A is concentrated in nuclear bodies, while 9/2A is still overexpressed and in the nuclear periphery. Analysis of these double mutants by Western blot revealed a pattern of heat shock molecular weight shifts for the 9/4A mutant characteristic of wt-dHSF upon HS, but that is absent in the 9/2A mutant (Fig 4C). Mutant 9/2A is compromised as a transcription factor, as primer extension assay of 9/2A *hsp70* gene transcripts reveals a reduction in activity, compared to the transcriptional ability of wt- and 9/2R-dHSF (Fig 4D). When tagged with SV40, the 9/2A mutant still remains as an engorged phenotype in the cytosol during NS and HS, and could not enter the nucleus for further processing. K409R/432R (9/2R) does not differ from wt-dHSF in phenotype, but mirrors a non-acetylated form of dHSF at these two lysine residues. From this data, it is evident that modification at both K409 and K432 can affect the sub-cellular distribution, and possibly a regulatory step, in dHSF activation.

Discussion

Dissecting the individual contributions of modified lysines to the elaborate dynamics of transcriptional activation has proven difficult; overlapping motifs and reversible modifications may affect the same sets of lysines at distinct regulatory steps. This phenomenon is seen during NF-κB activation, as a result of ubiquitylation and sumoylation of its regulatory oncoprotein Tax [27]. Mutations in C-terminal lysine residues of the human p53 tumor suppressor protein, that are normally acetylated for proper regulation, interfere with the efficient ubiquitylation of the protein [18].

DHSF can be ubiquitylated and degraded *in vivo* and *in vitro*

These data present point mutations within a characteristic nuclear localization sequence that confer stability to dHSF. More than one destruction-signaling sequence can exist in a protein. This study examined the possibility that the NLS acts as a degron or contains ubiquitin- or post-translationally-tagged lysine residues. Many examples exist of lysine modification as a way to target transcription factors to the nucleus, therefore cytosolically localized K/R mutants were expected. As these lysines can no longer be modified, they would be unable to enter the nucleus to be activated themselves or to activate other protein components. Upon insertion of a C-terminal NLS (SV40-tag), the mutants were able to enter the nucleus and their intracellular protein levels decreased (when observed by fluorescent imaging), though they were not necessarily punctuate, a visual cue in EGFP tagged protein for activated heat-shock factor. Pulse-chase assays show these K/R mutants have extended half-lives, far longer than wt, yet they may still get degraded by Ub tagging of lysines elsewhere in the factor. MG132 treatment results in a decrease in degradation by-products, and consistent levels of K5R-dHSF with and without MG132 suggest its exit from the ubiquitin-proteasome pathway. These SV40 data are observations of EGFP-tagged protein; data from Western blot of protein expression levels will determine if the amount of protein changes, or if it is simply translocated into the nuclear compartment. Δ-SV40 has excess dHSF throughout the cell as a diffuse cloud/ haze found in the nucleus with some residual protein levels in the cytosol. Since Δ spontaneously binds HSE-DNA, the phenotype of ΔSV40 suggests that the cell is attempting to degrade this excess protein. Taken together, these data may indicate that DNA binding of dHSF results in its subsequent degradation. The overflow of protein in the nucleus may result from an excess of Δ to heat-shock element. Future work will include isolation of dHSF from various SV40-tagged mutants followed by an assay of their DNA-binding ability.

The possibility still exists for the NLS to act as a degron to signal the destruction of dHSF. These lysines may not be polyubiquitin-tagged, but these data do not rule out the

possibility of monoubiquitylation. Preliminary data show dHSF can be ubiquitin-tagged, both *in vivo* and *in vitro*, but the presence of an EGFP tag (with its own degron) makes it difficult to distinguish between the Ub- tagging of mutant dHSF vs. EGFP. Follow-up experiments will need to be conducted with an unambiguous ubiquitin-tagging protocol, ideally utilizing a mutant-Ub construct and a minimally tagged dHSF. K48R-Ub will bind to Ks preferentially tagged for degradation, while K63R-Ub [28, 29] will attach to sites that are monoubiquitylated. Co-expression of K/R-Ub with K-mutant dHSF will reveal the location of Ub-tagging and its purpose at that site (as a result of the Ub construct expressed). The absence or shortening of a rUb-dHSF ladder (protein-ubiquitin conjugates) points to the mutated lysines as the residues being modified in wt-dHSF.

The PEST sequence can also signal degradation [30]. There exist three regions containing PEST-like sequences in dHSF – residues 17-30, 256-266, and 361-390. The first two were identified computationally (PROSITE). Very recently, it was found that EGFP-tagged deletions of these regions under control of a constitutive promoter have higher intracellular protein levels. Further characterization of these regions to test for stability by pulse-chase analysis and MG132 treatment may reveal whether these areas, either singly or in tandem, act as the degron and are able to signal the proteasome-dependent degradation of dHSF.

The mutants 9/2A and 9/2A-SV40, which have cytosolic distribution and cannot enter the nucleus even when tagged with a C-terminal SV40 tag, will be studied further. Comparison of 9/2A and 9/4A showed differences in electrophoretic mobility, with 9/4A exhibiting wt-dHSF characteristics, while 9/2A had no mobility shift upon HS. Clearly, this shift is a result of mutation of K432A vs. K454A. K409A (as a member of K3A), is unable to localize preferentially to the cytosol, the same being true for K432A, K454A, and K432R mutants (data not shown). These single mutants are unable to alter sub-cellular localization, suggesting this phenotype is a synergistic effect of several lysine mutations.

Based on the finding that Δ is an overexpressed, DNA-binding mutant with limited proteolysis, it will be insightful to ask whether the nuclear localization sequence has the ability to function as an autonomous degron. An "autonomous" degron exists as a fragment containing the lysines that are Ub tagged to signal its own proteolysis. Mutated lysines within this region will render the mutant protein unable to promote its own degradation. To test this theory, the region of dHSF between residues 375-450 will be inserted it into pAc/5.1A-V5-His, and expressed in S2 cells. The lysines in this segment (K 400, 405, 409, and 432) will be mutated to arginine to prevent post-translational tagging of ubiquitin to these residues. Indirect immunofluorescence will reveal localization of the factor without contribution of a degron from EGFP (which could destroy the NLS-containing fragment by polyub- tagging of itself). If these lysines are ubiquitylated, there will be overexpressed intracellular protein levels. If so, this will be the first instance of an NLS containing overlapping domains for signaling destruction and the K-sites for degradation-promoting ubiquitylation.

DHSF undergoes non-ubiquitin Lysine modification

Identifying non-ubiquitin lysine modifications in dHSF is a more difficult proposition, as several modifications may be responsible for temporal or transient regulation of cellular processes. In the case of sumoylation, the preliminary evidence suggests S1, S2 and S3 sumoylation of dHSF, but it still remains to be seen what role and to what extent sumoylation plays in regulating dHSF. While only wild-type, full-length dHSF has been probed for sumoylation, it will be useful to identify sumoylation-deficient mutants by K/R substitutions and Western blotting with α-S1 and α-S2/S3 antibodies will reveal whether these lysines are tagged for sumoylation *in vivo*. Another motif, a highly conserved, bipartite sumoylation sequence (ΨKxExxSP), has been found that requires phosphorylation of serine prior to SUMO tagging [26]. Based on sequence analysis, there exist three potential K sites for sumoylation (K191, K454, and more poorly, K162), but no sequence homology to the phosphorylation-dependent motif was

found within dHSF. Mammalian HSF homologs HSF1 and the splice variant HSF4b both contain the phosphorylation-dependent motif, and sumoylation of the lysine in each of these proteins effectively represses their transactivation [26].

To identify acetylated residues, general acetylated lysine antibodies are available, but antibodies reactive against specifically acetylated lysines are rare, and for our data a commercially available antibody that was reactive to our protein was difficult to obtain. We were able to identify a possible candidate for acetylation in 9/2A, an acetylated-lysine mutant that is spatially targeted, in contrast to the 9/2R non-acteylated mutant that retains wt-dHSF activity. Functional data reveals the transcriptional activity of 9/2A is partially compromised, suggesting it is unable to activate *hsp70* gene transcription at wt-dHSF levels due to an inhibitory role of lysine modification. Aside from site-directed mutagenesis of individual lysines, determining the number of acetylated lysine residues has been best tackled via mass spectrometry (MALDI) [2], or a less-sensitive ^{3}H-acetate labeling assay.

DHSF may undergo post-translation modification upon HS - this is seen as a shift/change in the distribution of dHSF from the lower band of a doublet to the upper band; we note the absence of this shift in the 9/2A mutant. To determine if there is an inability to carry out other modifications/regulatory changes upon HS, the dHSF doublet can be probed with antibodies to Sumo, acetylated-lysine, and ubiquitin to reveal the possibility of, and relative distribution of, lysine-modified dHSF in each of these bands.

These studies analyze lysine mutations within the nuclear localization sequence of dHSF. A K/R mutation in four lysine residues within this region results in a cytosolically localized, highly expressed dHSF that constitutively binds DNA and has a longer half-life than wt factor. A K/A mutation in two of these lysines results in the same defect in both nuclear-localization and protein expression level. DHSF can be tagged by ubiquitin *in vivo* and *in vitro*, and the full-length protein is acetylated and sumoylated. We find that dHSF undergoes post-translational modification, and likely, several at once at lysines within the NLS for regulation of dHSF

activity. Identifying the exact modification at each lysine residue within this region will reveal the functional consequences of post-translational modification on dHSF activation function.

Materials and Methods

Antibodies and Proteasome Inhibitor. Anti-dHSF (17H8) and anti-α-3 antibodies were obtained as noted in Chapter 2. Anti-V5 antibody was obtained from Serotec. Anti-ubiquitin (P4D1), anti-AcK, anti-S1, and anti-S2/3 antibodies were purchased from Cell Signaling Technology. MG132 proteasome inhibitor (Sigma) was added to cells at a concentration of 50 μM for 90-120 minutes.

Site-directed mutagenesis. C-terminal dHSF deletions and ΔNLS-dHSF were constructed according to [25]. A C-terminal SV40 tag was inserted into pAc 5.1A/EGFP-V5-His to make pAc5.1A/SV40-EGFP-V5-His. Site-directed mutagenesis was carried out according to protocols provided by the Mayo Lab (Caltech). Ubiquitin (Ub) was cloned into pAc 5.1A/V5-His. Recombinant Ub was cloned into pET15b (NEB).

Pulse-chase assays. Pulse-chase and immunoprecipitation analysis were modified for S2 cells from ([13] or online at http://tanseylab.cshl.edu). In brief, S2M3 cells (S2 cells acclimated to M3 media) grown in M3 medium were transfected with wt and mutant dHSF under control of a pAc constitutive promoter or pMT inducible metallothionein promoter [31]. Eight mL cells were rinsed twice with 1.5 mL labeling medium (without ^{35}S-methionine) supplemented with 10% dialyzed FBS, then incubated one hour in 1.5 mL of the same labeling medium. Media was removed, and incubated with ^{35}S labeling medium (150 μL of 5 mCi ^{35}S-methionine in 3 mL media) for 30 minutes. Labeling medium was removed, and 3 mL chase media (supplemented with 10X excess methionine and 10% undialyzed FBS) was added to the flask. Cells were aliquoted and WCE were isolated at designated time points. DHSF was immunoprecipitated with

NiArg beads, washed with D buffer and 10 mM imidazole, and fractionated by SDS-PAGE for later analysis by autoradiography.

Binding assays. Binding assays were performed using the dsHSE γ-^{32}P labeled HSE (5'-GCGCGCCTCGAATGTTCGCGAAAAGA-3') in binding cocktail (100 microg/mL BSA, 100 microg/mL poly(dI-dC) and 5% Ficoll in HGE buffer (25 mM Hepes, 100% glycerol, .1 mM EDTA)). WCE or ips were incubated in binding cocktail and end-labeled HSE at RT for 30 minutes, then analyzed by 10%, 1X TBE gel and autoradiography.

***In vitro* ubiquitylation reactions.** Ubiquitylation reactions were carried out with 1 μg rUb in reaction buffer (10 mM $MgCl_2$ and 1 mM γ-^{32}P-[ATP] in HGEN) with α17- or αV5- antibody-bead immunoprecipitations for 60 minutes at 25°C. Samples were mixed with 2X LSB and run by SDS-PAGE for autoradiography.

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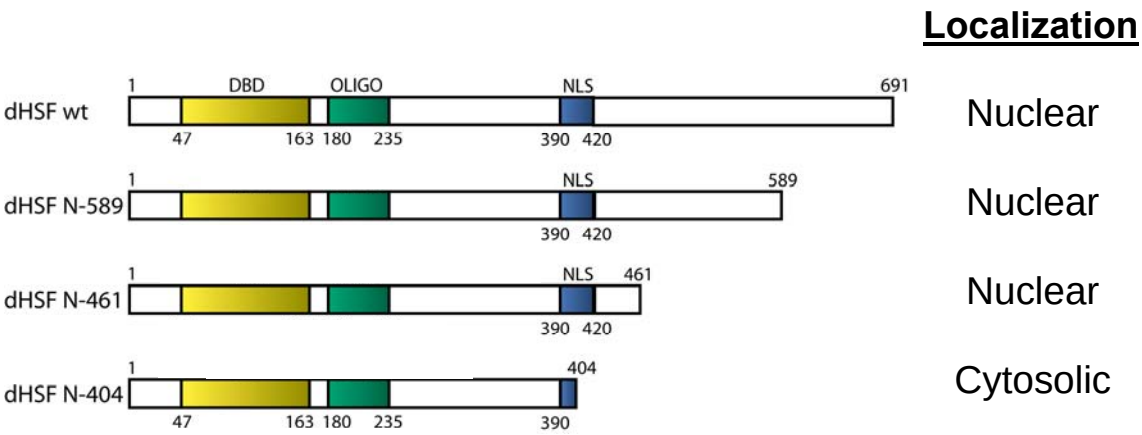
Figures

Figure 3-1



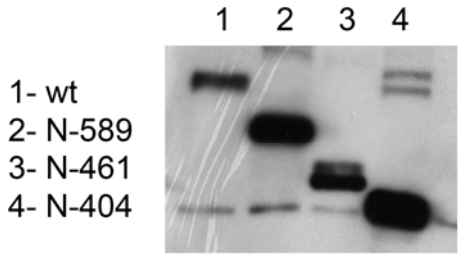


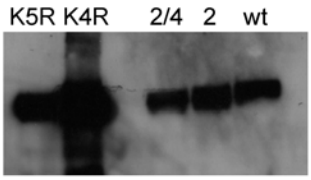
Figure 3-1

Schematic of C-terminal deletions in dHSF-EGFP. C-terminal deletions constructed by Zandi et al. were used to assay overall protein expression levels by Western blot with α17 antibody and cellular localization by fluorescence imaging. N and C represent nuclear and cytosolic/S10-fractionated protein. The increase in protein expression levels between N-461-dHSF and N-404 dHSF may be a result of loss of the NLS.

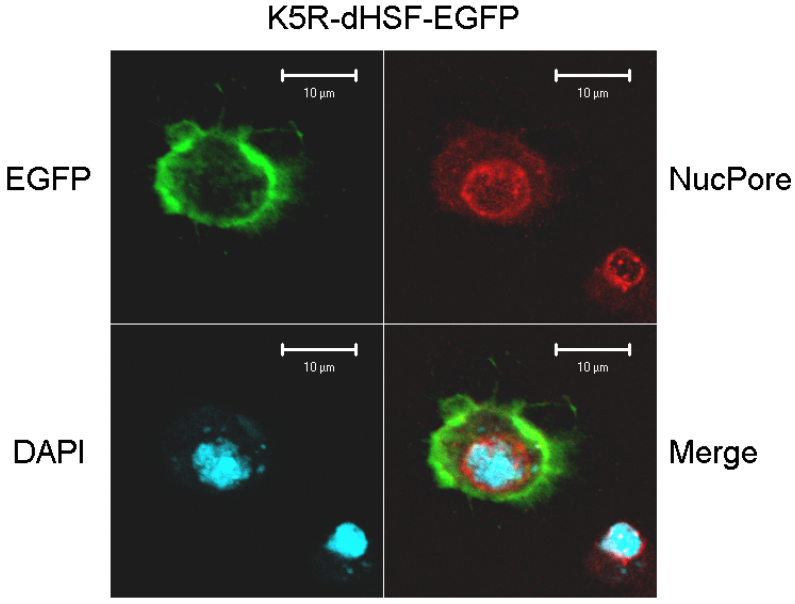
Figure 3-2
A

	395	400	405	409	420	432	454
dHSF WT:	PMVR [EQEQQ**K**	RQQL**K**ENN**K**L	…VILI]	AGD…F**K**A…			MI**K**S..
K3R:		R	R	R			
K4R:		R	R	R		R	
K5R:		R	R	R		R	R
9/2A:				A		A	
9/4A:				A			A

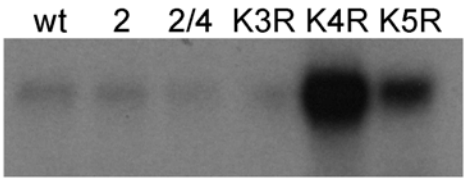
B



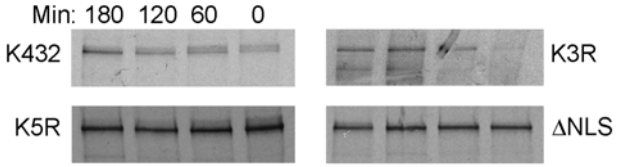
C



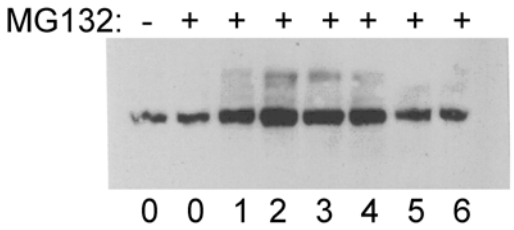
D


wt 2 2/4 K3R K4R K5R

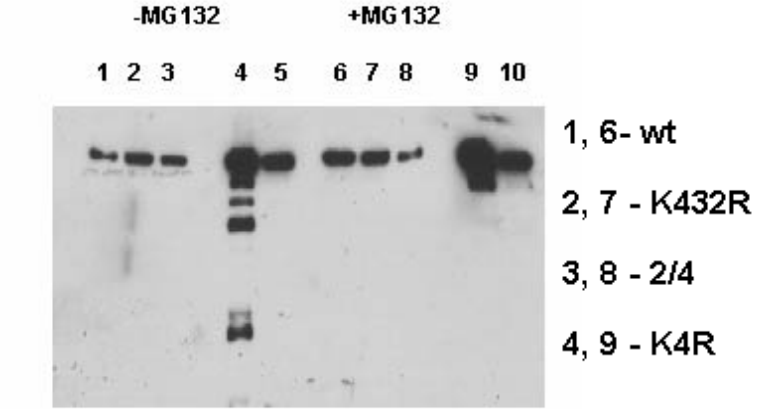
E


Min: 180 120 60 0
K432
K3R
K5R
ΔNLS

F


MG132: - + + + + + + +
0 0 1 2 3 4 5 6

G


-MG132
+MG132
1 2 3 4 5 6 7 8 9 10
1, 6- wt
2, 7 - K432R
3, 8 - 2/4
4, 9 - K4R

H

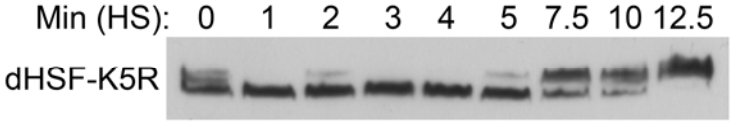

Min (HS): 0 1 2 3 4 5 7.5 10 12.5
dHSF-K5R

Figure 3-2

A- Schematic of lysine residues in the nuclear localization sequence. K/R mutant dHSF-EGFP (Materials/Methods) was transfected into S2 cells for observation of cellular localization and analysis of protein expression levels by Western blot.

B- Protein expression levels of K/R mutation within the NLS. WCE were assayed by Western blot and incubation with α17 antibody.

C- Site-directed mutation of 5 lysine residues results in cytosolic localization of mutant dHSF. Images were taken for DAPI nuclear staining (blue), fluorescent antibody reactive against nuclear pore proteins (red), and the EGFP dHSF construct. K4R exhibits the same protein localization pattern.

D- DNA-binding ability of K/R mutations in the NLS. Constitutive binding of K4R and K5R mutant protein correlates to sub-cellular localization. K4R and K5R mutants were cytosolically-localized, while K3R and single or double mutants from (K400, 405, 409) did not exhibit differences from wt-dHSF. WCE were run in a standard DNA-binding assay.

E- Metabolic- labeling in K/R mutant dHSF. Pulse-chase analysis was performed on K/R mutants over the course of three hours as described in Materials/Methods. Persistent mutations as a result of K/R mutation were observed in K5R, while Δ-dHSF also exhibited stability in this assay.

F- Endogenous tagging of dHSF *in vivo* observed after treatment with proteasome inhibitor MG132

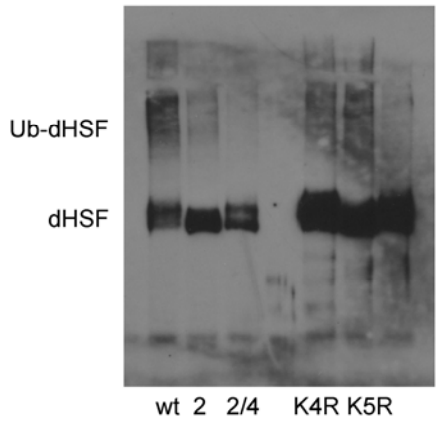
G- MG132 treatment of K/R mutant dHSF. Mutant dHSF transfected S2 cells were treated with MG132 as described in Materials/Methods. Samples were analyzed by western blot and incubated with α17 antibody.

H- MG132 treatment of K5R dHSF indicates it can be further modified as a result of heat stress. MG132 treatment was performed as described. WCE were taken at

representative time points and analyzed by Western blot.

Figure 3-3

A


Ub-dHSF
dHSF
wt 2 2/4 K4R K5R

B

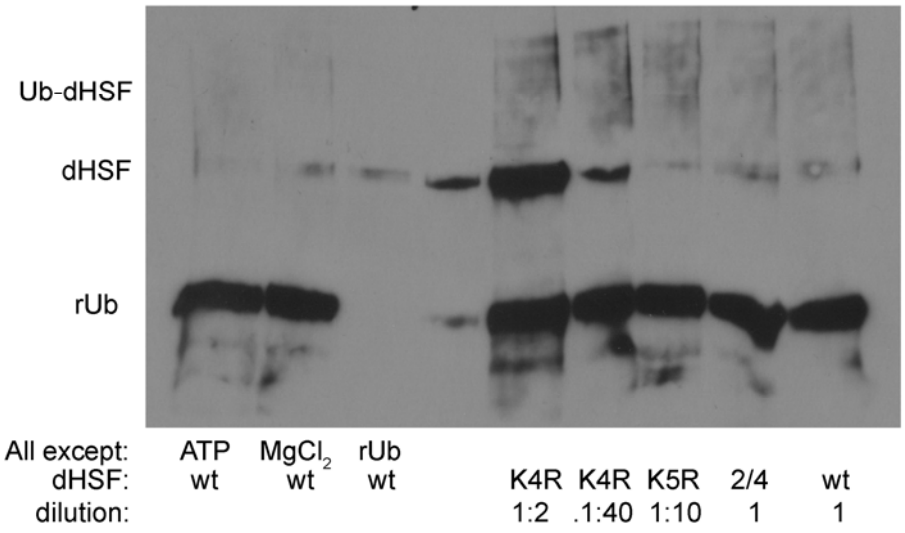

Ub-dHSF
dHSF
rUb
All except: ATP MgCl2 rUb
dHSF: wt wt wt K4R K4R K5R 2/4 wt
dilution: 1:2 .1:40 1:10 1 1

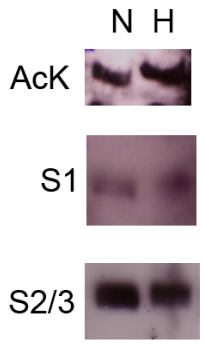
Figure 3-3

A- DHSF can be tagged *in vivo* by ubiquitin. Wt- and mutant dHSF were co-transfected with a Ub-construct and non-shocked WCE were analyzed by Western blot and incubation with α17 antibody.

B- DHSF can be modified by ubiquitin in an *in vitro* ubiquitylation reaction. Wt and mutant dHSF were incubated with rUb and ATP in reaction buffer (Materials/Methods). The left lanes are controls for the reaction buffer minus a single component.

Figure 3-4

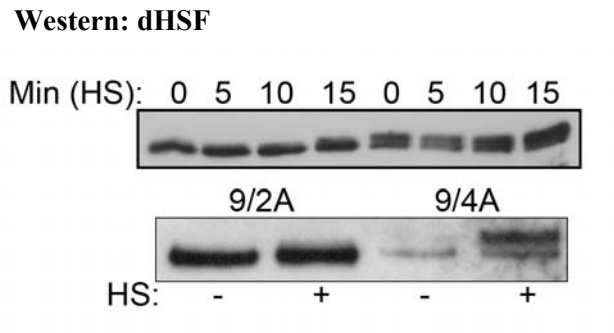
A



B

SUMO consensus motif: ΨKX D/E
dHSF wt: **K191: M K QE**
K454: I K SE
K162: L K PE

C



D

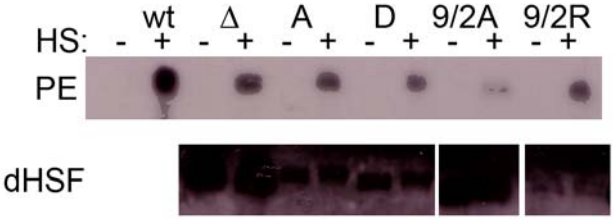


Figure 3-4

A- DHSF is acetylated and sumoylated. Immunoprecipitated NS and HS dHSF were run on SDS-PAGE and analyzed by Western blot. Antibodies utilized were reactive against acetylated lysine (AcK) and single-(S1) and multi- (S2/3) chain sumoylation.

B- DHSF contains 3 putative sumoylation motifs. K191, K454, and K162 that may fall within a sumoylation consensus motif. Hydrophobic residues are denoted by Ψ, X is any residue.

C- DHSF double K/A mutants point to key residues within the NLS for cellular localization. Site-directed mutants (9/2A, 9/4A) were analyzed by Western blot and observed by fluorescence imaging. Comparison of NS and HS WCE of dHSF mutants and a time course of 15 minute heat shock revealed differences in electrophoretic mobility, as assayed by Western blot and α17 antibody.

D- Functionality of 9/2R and 9/2A mutants. Double K/A mutants in dHSF exhibited extremely high protein levels as compared to wt and 9/2R-dHSF. Transcriptional analysis revealed dramatic differences in *hsp70* gene transcript levels as assayed by primer extension (Materials/Methods).

CHAPTER 4: Akt phosphorylation of *Drosophila* heat-shock factor – a signature for stress resistance

The heat-shock response is vital to cellular homeostasis. *Drosophila melanogaster* heat-shock factor (dHSF) is the primary transcriptional activator in the stress-response pathway for transcription of protective heat-shock chaperone proteins. Understanding the regulatory mechanism underlying dHSF activation will reveal how this process maintains a cellular environment that can combat environmental stresses while being conducive to proper cell growth and development.

This work investigates the potential for dHSF to undergo post-translational modification by phosphorylation and lysine tagging, specifically, phosphorylation by Akt kinase and covalent lysine tagging by ubiquitin, acetyl, and SUMO groups. Here, the findings of this study are summarized, and comparisons are made between the regulation of dHSF activation and that of dFOXO. Detailed below, a discussion of the functional consequences of synergistic phosphorylation and lysine modification – this work may implicate a prominent role for Akt phosphorylation in regulating the stability of dHSF.

The dHSF and dFOXO stress-related transcription factors are negatively regulated in a similar fashion by Akt kinase

This study shows that the direct association of *Dm* heat-shock factor by Akt/Protein Kinase B and the phosphorylation of dHSF at Serine 256 by Akt kinase inhibits its activation function. This negative regulatory mechanism (Fig 1) follows that of the primary insulin-signaling transcription factor in *Dm*, dFOXO. Phosphorylation by Akt at three sites on dFOXO (one primary, two secondary), serves to inhibit its transcriptional activity when insulin levels are maintained in the cell [1]. During nutrient deficiency, Akt relieves the inhibitory phosphorylation of dFOXO, allowing for its translocation and the transcription of stress-related proteins involved

in apoptosis, metabolism, and oxidative stress [2, 3]. Several major findings have demonstrated that dFOXO can confer longevity as an essential regulator of organismal lifespan [3].

These observations show that dHSF is inhibited by phosphorylation at S256 by Akt, in a similar fashion as dFOXO. In this phosphorylated state, dHSF is unable to activate its DNA-binding and transcription functions. When heat stressed, Akt relieves this inhibitory phosphorylation for the multi-step transcriptional activation of dHSF. Removal of Akt by RNAi or inhibition by chemical agents in S2 cells (Chapter 2) results in dHSF activation at the level of DNA binding. Fluorescence studies confirmed that in the absence of Akt, dHSF exhibits a phenotype consistent with that of heat-shocked activated factor. Site-directed mutagenesis of S256 revealed aspects of dHSF phenotype and activity that mimicked those of the non-shocked (S/D) or heat-shocked (S/A) states of dHSF.

These findings suggest that the dHSF (and dFOXO) stress-related transcription factors are negatively regulated by the same inhibitory phosphorylation mechanism of Akt kinase.

DHSF may be degraded through a phosphorylation-dependent mechanism by Akt

Recently, Aoki et al. have reported the phosphorylation-dependent degradation of FOXO1 by either Akt or the PI3K homolog, P3k [4]. The upregulation of Akt and subsequent loss of FOXO1 transcriptional regulation results in the oncogenic transformation of chicken embryo fibroblasts.

Does dHSF undergo similar proteasome-mediated degradation as a result of phosphorylation (or de-phosphorylation) by Akt? In one theory, under non-shocked (NS) conditions Akt phosphorylation may promote the degradation of inactive dHSF to maintain the basal level of dHSF that is present in the nucleus prior to heat shock. Upon heat stress, Akt relieves phosphorylation of dHSF at S256 for its association into transcriptional complexes for HSE-DNA binding and transcriptional activation (Fig 2). In a second scenario, contrary to the previous model, dephosphorylation of dHSF at the Akt kinase site may result in the upregulation

of dHSF activity, and only then permit the subsequent degradation of active dHSF participating in protein-DNA interactions (Fig 3).

Transient modification of dHSF by Akt kinase may comprise both a phosphorylation and a dephosphorylation step as a single event. A small population of dHSF may be regulated at any given time by Akt. In the phosphorylation-dependent degradation of FOXO, Akt phosphorylation of FOXO keeps it in an inactive state, and its subsequent degradation maintains steady protein levels [4]. DHSF already exists at a basal level within the nucleus as a soluble species that fractionates preferentially to the cytosol. This nuclear localization allows access to heat-shock elements for rapid activation of the HS response. If the model for phosphorylation and degradation of dHSF follows that of FOXO, phosphorylation by Akt in the absence of heat stress would maintain baseline levels of dHSF by also promoting its degradation (Fig 2).

The degradation of dHSF under non-shocked conditions is readily observed (Chapter 3). During heat shock, overall cellular protein degradation levels increase as polyubiquitylation function is upregulated. However, proteins that are poly-Ub tagged during heat shock (HS) are synthesized prior to temperature stress [5]. When dHSF levels dramatically increase in response to HS, newly synthesized dHSF would not comprise a significant portion of the proteins that undergo proteolysis. The population of dHSF that can bind DNA for the activation of *hsp* gene transcription may get subsequently degraded, but only as a result of this DNA-binding event/activation step. During standard NS conditions, a small amount of dHSF (that may be dephosphorylated at S256) is bound to HSEs for baseline transcriptional activity and ongoing synthesis of dHSF. If DNA binding is a prerequisite for degradation, this small percentage of active dHSF will undergo proteolysis. The increase in dHSF levels upon HS would result in a larger population of activated DNA-binding molecules, however, this species would not be poly-ubiquitylated for degradation. This theory is in direct contrast to what is observed for regulating proteasomal degradation of FOXO by Akt: upon dephosphorylation of dHSF at the Akt kinase

site, dHSF becomes activated for DNA binding. Only after carrying out DNA-binding function can the dephosphorylated species get degraded by a proteasome-mediated process (Fig 3).

Regulation of dHSF activity is modulated by several levels of post-translational modification

Other possibilities exist for dHSF regulation at the levels of Akt kinase phosphorylation, post-translational lysine modification, and protein degradation, in concert and individually.

Domains that signal degradation (degron, PEST) and domains that are substrates for polyubiquitylation are usually found as separate entities within a protein; some proteins contain more than one of these degradation-signaling regions. Sequence analysis of dHSF reveals homology to a ubiquitin interacting motif (UIM) at residues 249-259. A UIM consists of the motif eeeXΨXXAXXXSXXe, where e is a charged residue, X is any residue and Ψ is a hydrophobic residue, to promote protein ubiquitylation. The UIM itself can be bound by polyubiquitin chains [6, 7]. In dHSF, this region overlaps the Akt kinase site, and may be where the Akt-regulating and ubiquitylation pathways converge. The orientation of the UIM in relation to the polyubiquitylation sites is integral to Ub tagging at these lysines -many UIMs are C-terminal to the lysines in question. If the putative UIM in dHSF follows this model, the lysines to be tagged would be located N-terminal to the 249-259 region. The data presented here (Chapter 3) suggest that the dHSF NLS may act as a degron, however, its constituent lysines may not be Ub tagged. There is a possibility that the lysines tagged for degradation lay in the 17-30 reside region (PEST) yet to be investigated.

Careful regulation of dHSF proteolysis is essential for cell vitality, but in oncogenic cell types, dHSF may not undergo normal protein turnover. This has been noted in Myc, where a non-proteolytic form of the protein is found in tumor cells [8]. If DNA binding is a requirement for subsequent proteolysis of a factor, an inability to signal the destruction of a transcriptional activator yields an abundance of bound (or able to be re-bound) dHSF that is trapped in an

activated state with DNA-binding ability. This results in extremely high levels of heat-shock proteins and upregulation of dHSF's own protein levels. The influx of excess protein in the UPP ties up the proteolytic machinery and hinders the "waste-disposal" process, in turn activating pro-apoptotic cascades and associated stress pathways, possibly by Akt phosphorylation-dependent degradation.

The location within the cell of the dHSF phosphorylation step by Akt kinase is yet to be determined. Protein translation occurs in the cytosol and dHSF localization may be dynamic, similar to its migration between cellular compartments in the NS state during early development [9]. Assessing the phosphorylation state of cytosolically localized mutant dHSF may reveal if dHSF is modified in the cytosol prior to nuclear entry, where the factor will eventually reside. However, there is the possibility that mutant protein, if processed inefficiently, may get phosphorylated by Akt in order to be polyubiquitylated and degraded. Two stress pathways would then be initiated: Akt may be activated for non-specific phosphorylation in response to an increase in the activity of the ubiquitin proteasome pathway. Kim et al. [10] have shown that inhibition of proteasomal degradation by treatment with MG132 induces *hsp* transcription and HSF1 hyperphosphorylation. However, in cells treated with MG132 and the general protein kinase inhibitor wortmannin (for PI3K and DNA-PK), MG132 treatment still hyperphosphorylates HSF1. Based on the activity of proteasome inhibitors, treatment with this reagent would retain proteins that were originally targeted for destruction and that may have already undergone activation. This supports the theory of negative regulation of dHSF by Akt: initially, MG132 treatment activates the heat-shock response [11, personal observations], resulting in the hyperphosphorylation event. Treatment with this proteasome inhibitor alone traps activated dHSF, which can continue *hsp* gene transcription. Subsequent addition of the Akt inhibitor does not modify the highly phosphorylated dHSF species. Note that the hyperphosphorylated shift mirrors the mobility shift seen in HS dHSF. Taken together, it is not a phosphorylation event by Akt (or by PI3K pathway components) that contributes to this observed

hyperphosphorylation of HSF once it has been activated (by MG132, as previously noted). Rather, a dephosphorylation event at Akt kinase consensus site S256 may have initiated this response.

There are distinct examples of post-translational lysine modifications that regulate transcription factor activation [12]. Several modifications may be simultaneously responsible for the resulting effect. These steps may raise or lower the sensitivity of dHSF to phosphorylation by Akt, or be entirely independent of the Akt/FOXO regulating mechanism. In the case of ubiquitylation, these data suggest recombinant dHSF cannot be modified by ubiquitin, but ongoing research into the possibility of sumoylation, acetylation, and methylation of recombinant protein is necessary. If there is pathway overlap, Akt phosphorylation may be a necessary step prior to, instead of a result of, post-translational modification to activate the DNA binding of dHSF.

Akt phosphorylation of *Drosophila* Heat Shock Factor – a signature for stress-resistance

Elucidating the activation of the *Drosophila* heat-shock response (dHSR) as a result of Akt phosphorylation provides a link between two key stress-signaling pathways: the HSR and the FOXO-mediated insulin-signaling pathway. Characterizing the nature of dHSF activation may reveal the HSR as a parallel and/or cooperating mechanism alongside the Akt/FOXO cascade in conferring stress resistance, and potentially, longevity.

From this work, we extrapolate that Akt phosphorylation is a negative regulatory mechanism in the activation of the heat-shock factor-mediated response, and *Dm* heat-shock factor stabilization may be a consequence of dHSF activation. Implications for this work in understanding disease states and identifying targets for drug delivery are far reaching, as misregulation of the HS pathway could lead to the onset of disease and, as a result, oncogenic cell transformation.

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Figures

Figure 4-1

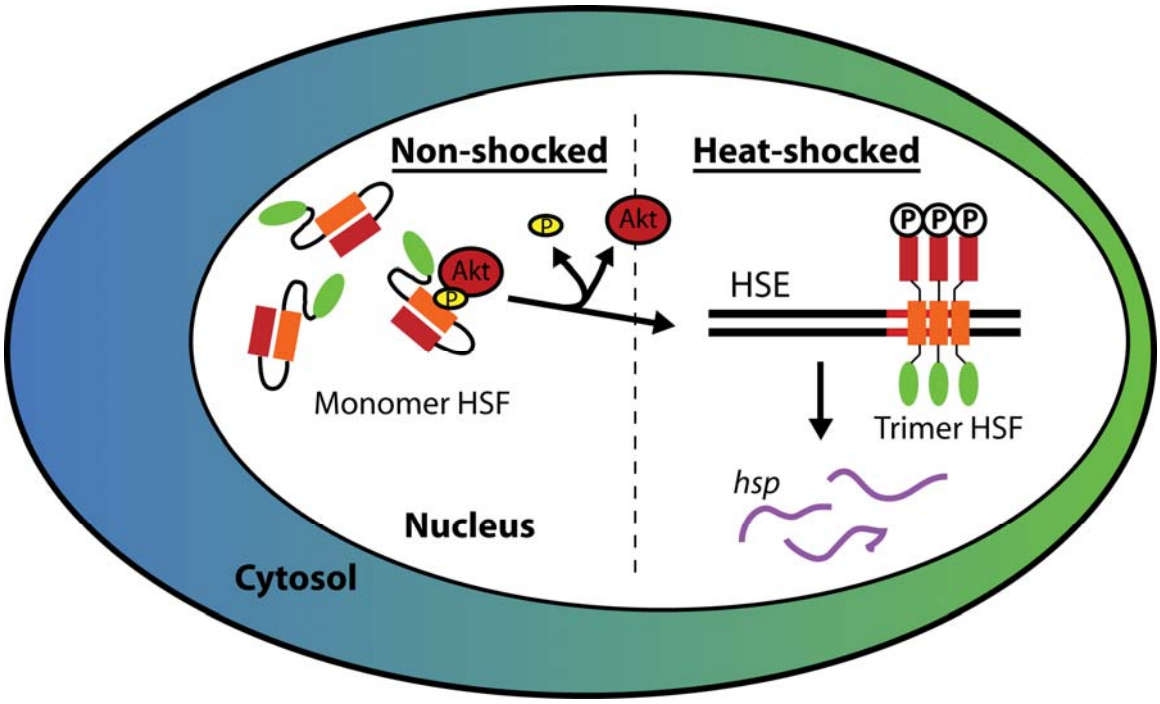

Figure 4-1

DHSF is phosphorylated by dAkt at S256 in a negative regulatory mechanism. DHSF is a monomer in the nucleus, where it is maintained in an inactive state by dAkt phosphorylation at S256 of dHSF. Upon HS, dHSF becomes dephosphorylated for association into active transcriptional complexes for heat-shock mediated gene transcription.

Figure 4-2

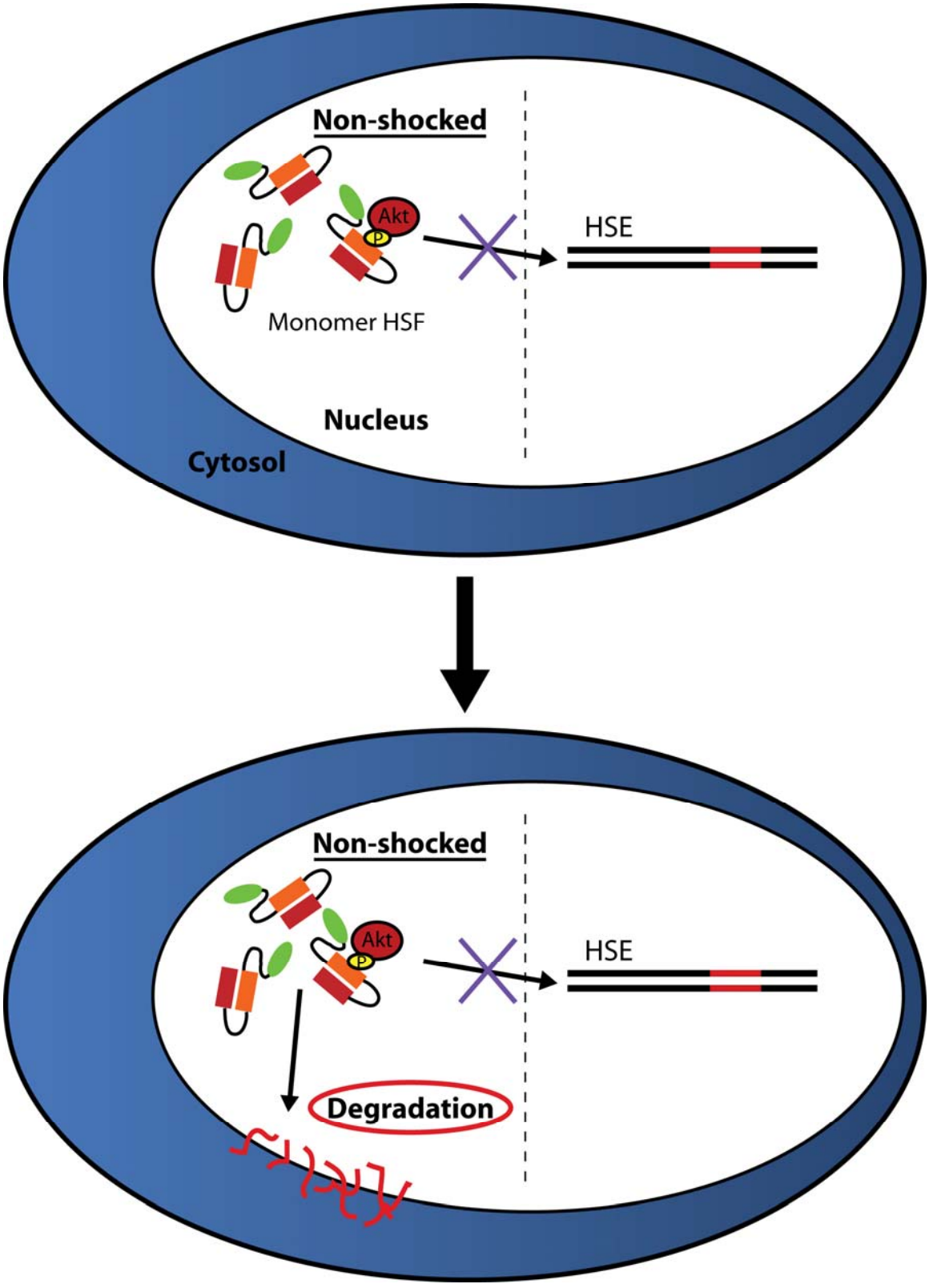
Non-shocked
Akt
P
HSE
Monomer HSF
Nucleus
Cytosol
Non-shocked
Akt
P
HSE
Degradation

Figure 4-2

DHSF may be degraded through a phosphorylation-dependent mechanism by Akt:

Scenario 1. DHSF is maintained in an inactive state by phosphorylation by Akt at S256, which also promotes proteasome-mediated dHSF degradation.

Figure 4-3

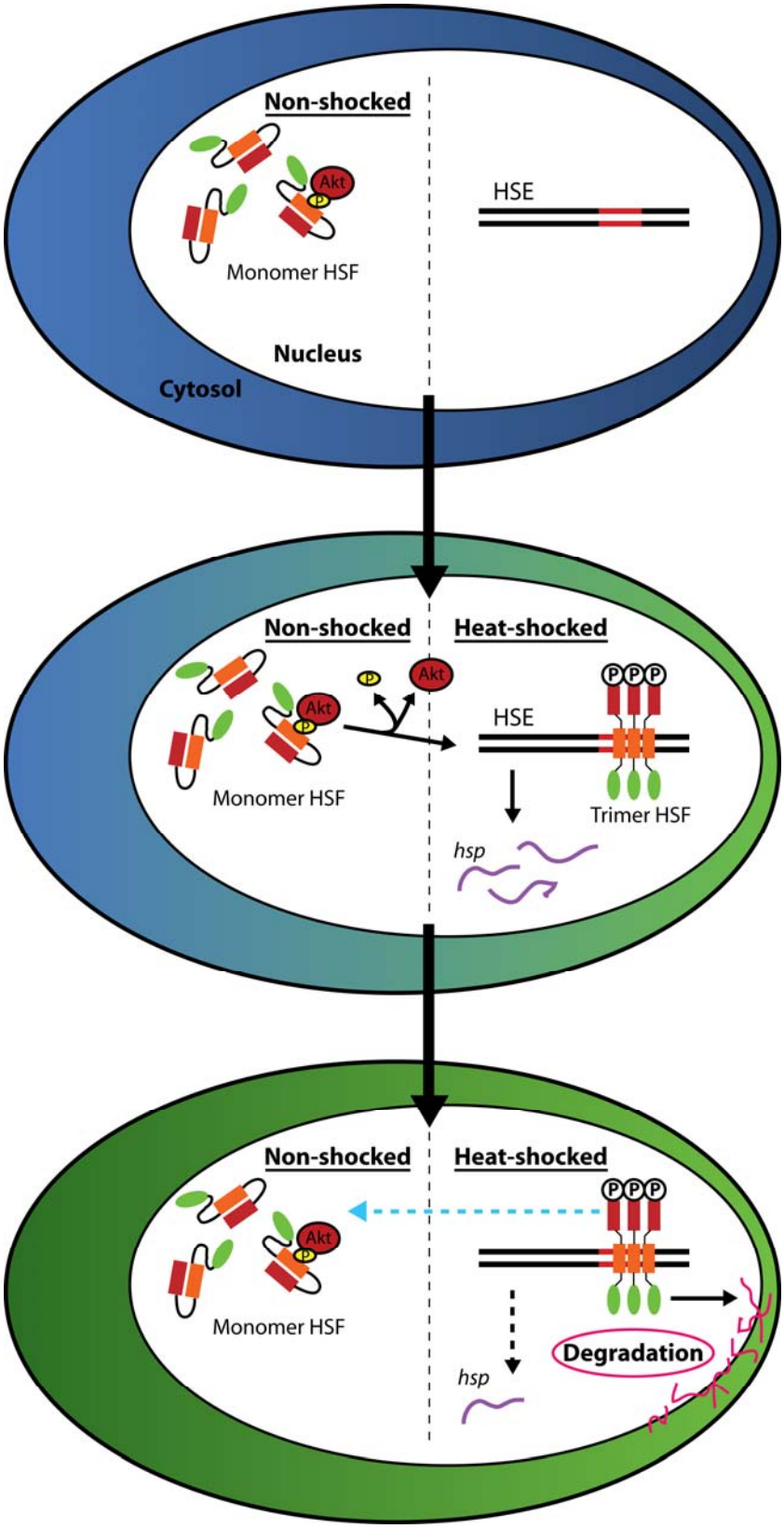
Non-shocked
Akt
P
Monomer HSF
HSE
Nucleus
Cytosol
Non-shocked
Heat-shocked
Akt
P
Monomer HSF
HSE
P P P
Trimer HSF
hsp
Non-shocked
Heat-shocked
Akt
P
Monomer HSF
P P P
Degradation
hsp

Figure 4-3

DHSF may be degraded through a phosphorylation-dependent mechanism by Akt:

Scenario 2. Upon dephosphorylation at the Akt kinase site, dHSF becomes activated for DNA-binding. Following DNA-binding, the dephosphorylated species gets degraded in a proteasome-mediated process. Recovery of dHSF from the activated state is represented by a dotted line.