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Role of Heat Shock Transcription Factor 1 in Ovarian Cancer Epithelial-Mesenchymal Transition and Drug Sensitivity

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by

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DEDICATION

I would like to dedicate this work to my wife, Anne T Powell, for all of her support, patience, and love. To my children Elise, Madeleine, Aidan, and Ethan for the encouragement and inspiration they have provided me. To my parents Christina and Chris Simcox for their continued belief in me. And lastly, to my mentor Sandy D. Westerheide for allowing me the opportunity to learn.

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ABSTRACT

The heat shock response (HSR) is a robust cellular reaction to mitigate protein damage from heat and other challenges to the proteome. This protective molecular program in humans is controlled by heat shock transcription factor 1 (HSF1). Activation of HSF1 leads to the induction of an array of cytoprotective genes, many of which code for chaperones. These chaperones, known as heat shock proteins (HSPs), are responsible for maintaining the functional integrity of the proteome. HSPs achieve this by promoting proper folding and assembly of nascent proteins, refolding denatured proteins, and processing for degradation proteins and aggregates which cannot be returned to a functional conformation. The powerful ability of the heat shock response to promote cell survival makes its master regulator, HSF1, an important point of research. To garner a better understanding of HSF1, we reviewed the role of the highly dynamic HSF1 protein structure and investigated how HSF1 affects cancer cell behavior and drug response.

Cancers can be characterized in part by abhorrent replication, self-sufficient growth signaling, invasion, and evasion of apoptosis. HSF1 has been found to promote proliferation, invasion, and drug resistance in several types of cancer; including lung and ovarian cancer. Ovarian cancer has elevated levels of HSF1, but the role of HSF1 in ovarian cancer behavior had not been previously examined. Researching the role of HSF1 in ovarian cancer is merited, because treatment outcomes are poor due to the high frequency of late stage detection and drug resistance. We hypothesized that HSF1 is important in the malignant growth and drug resistance of ovarian cancer.

We have created ovarian cancer cell lines with inducible knockdown of HSF1 to investigate how HSF1 contributes to the behavior of ovarian cancer. This allowed us to examine the behavior of cells in the absence HSF1. Both 2D and 3D spheroid tissue culture models were used to study how HSF1 contributes to the growth and invasion of ovarian cancer cells after treatment with the transforming growth factor β (TGFβ) cytokine. Additionally, we studied how HSF1 reduction modulates the response to multiple therapeutic drugs. Our research shows that HSF1 induces epithelial-mesenchymal transition (EMT) in a 3D growth model. Our work also demonstrates that reduction of HSF1 sensitizes ovarian cancer cells to multiple drugs.

CHAPTER ONE: INTRODUCTION

Discovery of the Heat Shock Response

The discovery of the heat shock response (HSR) came by way of a fortuitous accident in the early 1960s. The Italian geneticist, Ferruccio Ritossa, was researching nucleic acid synthesis associated with chromosomal puffs in *Drosophila* polytene salivary gland cells. These chromosomal puffing patterns offered a simple visual indication of gene transcription. During these studies a fellow researcher increased the temperature of the incubator by mistake [1].

When Ritossa observed the *Drosophila* that had been exposed to higher temperatures, he discovered a completely distinct puffing pattern. This change in chromosomal puffing represented one of the strongest examples of environmentally-induced changes in gene expression known at the time. Ritossa found that the response only took 2 – 3 minutes to occur, and that it was present in different tissues, developmental stages, and species of *Drosophila* [2, 3]. These observations led him to believe that the response he was observing was of importance. However, his work was not well received by the scientific community for many years.

In the 1970s, the study of the heat shock response focused on the nature of the response and the role of the induced proteins. It was established by protein and mRNA radiolabeling that a very specific set of mRNAs and corresponding proteins were being produced during the HSR. Simultaneously, basal protein production was halted [4]. These proteins induced by the heat shock response were called heat shock proteins (HSPs). The HSPs were named based upon the proteins size in kilo Daltons. Of the HSPs, HSP70 was found to be produced in the most abundance in *Drosophila* after heat shock [4]. As researchers sought to characterize HSPs, it was discovered that they were well conserved across *E. coli, Drosophila,* and many other organisms

[5-7]. While the homology across kingdoms suggested that HSPs were involved in foundational cell processes, it was not until the late 1980s that HSPs were understood to be molecular chaperones [8, 9].

To understand the regulation of the heat shock response, researchers studied the promoter of the rapidly inducible *hsp70* gene to investigate how the system is regulated. The critical region for heat shock induction of *Drosophila hsp70* was determined by creating promoter deletions and detecting gene activation by employing a S1 nuclease protection assay [10]. A short repeated sequence was found to be necessary for the HSR induction of *hsp70* and many other HSPs within a GC rich promoter region [10-12]. This promoter element was dubbed the heat shock element (HSE). The HSE is generally comprised of three contiguous inverted repeats: nTTCnnGAAnnTTCn [13, 14]. Shortly after the discovery of the HSE, promoter footprint analysis was used to discover a unique RNA polymerase II transcription factor which bound HSEs [15, 16]. This transcription factor was subsequently named heat shock transcription factor 1 (HSF1). HSF1 was shown to be required for HSR gene induction in *Drosophila* and human cells [17]. This research established the foundational understanding of the heat shock response. HSF1 binds HSEs in the promoters of target genes and strongly induces their transcription during HSR activation.

Regulation of the Heat Shock Response by HSF1

Overview of Heat Shock Response Regulation by HSF1

The heat shock response is presided over by the heat shock transcription factor (HSF) family of proteins in eukaryotes. While *C. elegans, S. cerevisiae* and *D. melanogaster* each have a single HSF, mammals possess 6 HSF family members [18]. Of these, heat shock factor 1 (HSF1) serves as the master regulator of HSR in mammals (Figure 1.1). This critical role in activating the heat shock response is demonstrated by the inability of *hsf*-/- mice and derived cell lines to undergo a heat shock response [19, 20].

HSF1 is constitutively expressed at low levels and is present in both the nucleus and cytoplasm as an inactive monomer. Upon activating stress, HSF1 forms trimers and accumulates in the nucleus where it aggregates as a part of nuclear stress bodies [21, 22]. HSF1 is concurrently hyper-phosphorylated and binds heat shock elements in the promoters of target genes [23-25]. There are often many HSEs within the promoters of strongly induced genes. After binding HSEs, the HSF1 trimer activates robust gene induction. Following stress, attenuation occurs due in part to acetylation of the HSF1 DNA binding domain and negative feedback from HSPs [26, 27].

Activation of the Heat Shock Response

HSF1 protein is constitutively expressed and has a long half-life of approximately 13 -20 hours [28]. HSF1 is an inactive monomer during normal conditions. HSF1 is kept in an inactive monomer state by both intermolecular and intramolecular mechanisms. At the intramolecular level, HSF1 is stabilized in the monomer state by interactions between the hydrophobic repeat domains HR-A/B and HR-C (Fig 2.2). This interaction creates a coiled-coil structure which tethers the N and C termini together. The result is a semi stable monomer [29]. At the intermolecular level, HSF1 is repressed by interaction with the HSP90 complex, HSP70 and TRiC/CCT [27, 30, 31]. These chaperones bind HSF1 and maintain the inactive monomer state. When activated, HSF1 forms a homotrimer. Trimerization is generally induced by two mechanisms. It can be promoted by the loss of HSF1 associated chaperones to denatured proteins during ongoing stress. Alternately, it can be induced by elevated temperatures which cause the unfolding of the inhibited HSF1 monomer. While trimerization is required for HSF1 activation, it is not alone sufficient [32].

The next step in HSF1 activation is extensive phosphorylation. This occurs to such a degree that a marked shift in electrophoresis mobility occurs.[22] Some degree of phosphorylation is believed to be required for HSF1 transcription, because trimerization and DNA binding do not always result in transcriptional activity. This is demonstrated by the ability of salicylic acid to

induce trimerization and DNA binding, but not active transcription or phosphorylation [33]. The extensive phosphorylation occurs primarily on serine residues in the regulatory domain (Fig 2.2). Additionally, some studies have shown a small degree of threonine phosphorylation occurring [34-36]. Phosphorylation of ser326 by mTOR or p38 MAPK strongly supports activation [37, 38]. The phosphorylation of ser320 by Protein Kinase A leads to the nuclear accumulation HSF1 prompting activation [39, 40]. Many other serines are also known to be phosphorylated in HSF1 during the heat shock response: Ser121, Ser230, Ser292, Ser303, Ser307, Ser314, Ser319, Ser344, Ser363, Ser419, and Ser444. Interestingly, point mutation analysis of these sites showed none of them are individually critical for HSF1 transcriptional activity [36, 41]. It is reasonable to assume that these sites serve as a mechanism to finely modulate HSF1 activity, or interactions with its partners.

Concurrent with extensive phosphorylation, active HSF1 trimers accumulate in the nucleus. Transport into the nucleus is driven by a strong bipartite nuclear localization signal [21]. This signal is recognized by importin-alpha/beta [21]. Import into the nucleus occurs under basal conditions, but does not entirely accumulate in the nucleus due to export by 14-3-3 ε [42]. In unstressed cells, HSF1 location exists in an equilibrium between the cytoplasm and the nucleus. The primary location in unstressed cells varies. However, a literature review shows 31 of 38 studies found the nucleus is the primary location under basal conditions [21]. During stress, complete nuclear accumulation is achieved by the cessation of nuclear export. The rate of nuclear export by 14-3-3 ε is controlled by the phosphorylation of Ser303 and S307. Phosphorylation of both these site is required for export. These sites are phosphorylated by ERK, GSK3β, and possibly other kinases [43]. Regulation of nuclear export allows for fine tuning of HSF1 activity.

Active HSF1 accumulates in the nucleus and quickly congregates in nuclear stress bodies. These bodies form rapidly and range from 0.3 – 3 μm in size [44]. Assembly of nuclear stress bodies is directed by blocks of satellite III DNA [44]. HSF1 binds these regions and recruits CREB binding protein which leads to chromatin remodeling and active transcription [44].

The discreet chromosomal locations of satellite III DNA sequences results in fixed locations for stress body formation. The resultant Satellite III RNA interacts with HSF1 and becomes part of the nuclear stress bodies. There HSF1 binds several splicing cofactors and suppresses translation of some non-heat shock proteins [45]. The full purpose of nuclear stress bodies is not fully understood. However, it is thought that the specific localization of nuclear stress bodies may serve to further direct and enhance transcription [46]. Surprisingly, nuclear stress bodies do not form in rodent cells, which suggests recent evolution.

Active HSF1 binds within the promoter regions of genes which are regulated during heat shock. HSF1 promoter binding activates transcriptions in all but few cases [47, 48] Promoter binding is mediated by the N-terminal DNA binding domain which interacts with the major groove and phosphate backbone [49, 50]. This binding is directed by a short sequence of DNA consisting of the sequence nGAAn, usually in three inverted repeats [51]. This is known as the Heat Shock Element (HSE) and was first identified in the *hsp70* promoter [11]. The multiple repeats of the nGAAn sequence serve to bind the three DNA binding domains in the active HSF1 trimer [46]. The number, spacing and sequence of the HSEs can vary some from the consensus sequence and still be recognized by HSF1. This is due in part to the co-operative nature of the DNA binding domain which interacts synergistically with other HSF1 DNA binding domains via a 'winged' structure [50]. The co-operative nature of HSEs and the HSF1 DNA binding domain allow HSF1 affinity to be greatly regulated by the strength, location and repetition of HSEs [52].

Repression of HSF1

HSF1 activation is repressed through multiple mechanisms. The primary mechanism is believed to be negative feedback by heat shock proteins. Many of the HSPs expressed during heat shock inhibit HSF1 activation. These inhibitory HSPs include the HSP90 and its associated complex, HSP70, and TRiC [31, 53-55]. These inhibitory heat shock proteins achieve this by

binding and stabilizing HSF1. It is believed that these chaperones bind HSF1 in a repressed monomeric state. During damage to the proteome, denatured proteins are assumed to titrate away HSPs, alleviating inhibition. Multiple studies have found that HSF1 interacts with HSP90 and associated complex members including p23, Hip, and Hop [53]. Interestingly, co-immunoprecipitation research showing this interaction required cross-linking in both cases, suggesting that the interaction is of limited strength. Surprisingly, recent research has shown that HSP90 does not support or promote the monomeric state of HSF1 in *in vitro* studies. Instead, HSP90 facilitates transition to the active trimer form [29]. This suggests that HSP90 is important in the regulation of activation, but alone does not inhibit HSF1 activation. *In vitro* studies of HSF1 binding with TRiC and HSP70 found a stronger affinity which didn't require cross linking to examine [31, 56].

The ability of HSF1 to bind DNA can be reduced by post translational modification. This serves as another means by which to attenuate the heat shock response. Within the DNA binding domain lysine 80 and 118 are acetylated by p300 and other histone acetyltransferases [26, 57, 58]. This leads to the reduction of DNA binding affinity, presumably due to the loss of positive charges which facilitate interaction with negatively charged DNA. Acetylation which reduces HSF1 activity can be alleviated by the SIRT1 deacetylase [26].

HSF1 activity can also be regulated by changes in the available HSF1 protein itself. Active HSF1 can be targeted for degradation by the ubiquitin-proteasome system, which leads to the repression of the heat shock response. Multiple lysines can be ubiquitinated by NEDD4 and possibly other ubiquitin E3 ligases [59]. This leads to FILIP-1L mediated transport to the 19s proteasome subunit [60]. Surprisingly, HSF1 proteasome degradation can be inhibited by the acetylation of lysine residues by p300 and other acetyltransferases [57]. While p300 acetylation within the HSF1 DNA binding domain reduces HSF1 activity, the acetylation of lysine residues elsewhere prevents their ubiquitination and there by prevents degradation.

Additional means of HSF1 repression can be modulated by phosphorylation and sumoylation. While phosphorylation is a hallmark of HSF1 activation, multiple phosphorylation events serve to hamper activity. Phosphorylation of 303 and 307 by GSK3B leads to increased 14-3-3 ε mediated nuclear export [43, 61]. Phosphorylation can also inhibit transcriptional activity. Phosphorylation of serine 121 by MAPK activated protein kinase 2 reduces transcriptional activity and promotes HSP90 binding [55]. A reduction in transcriptional activity also occurs by sumoylation at lysine 289. Sumoylation of HSF1 requires phosphorylation of serine 203 as prerequisite [62]. The conjugation of SUMO is mediated by HSP27 oligomers [63].

The HSF1 Regulated Response.

Heat Shock Proteins

HSP27. The small heat shock protein HSP27 functions primarily by binding and stabilizing unfolded protein intermediates [64]. This indirectly promotes the successful refolding or clearing of misfolded poly peptides. HSP27 works in a range of forms from monomers to large homo complexes [65]. These complexes passively stabilizes clients, as HSF27 has no ATPase function [66]. The co-operative aggregation is driven by the α-crystallin domain [67].

In addition to its chaperone function, HSP27 plays a role in cytoskeletal organization and inhibits apoptosis. HSP27 interacts with actin to form cap ends which can inhibit actin polymerization [68]. This inhibition of actin polymerization is promoted the phosphorylation of HSP27 at multiple sites [69]. In the non-phosphorylated form HSP27 does not interact with actin and instead form oligomers which facilitate its chaperone function. HSP27 inhibits apoptosis via interacting pro-caspase 3 and inhibiting its activation [70].

HSP40. Acts as a co-chaperone and has two critical functions. It directs clients to HSP70 and it also controls the ATPase activity of HSP70 [71]. HSP70 has very weak ATPase activity alone and is dependent on HSP40 for its ATP dependent functions [72]. The J domain of

HSP40 is critical for HSP70 related functions. Outside of its role with HSP70, some members of the HSP40 family have functions in processing aggregates and inhibiting apoptosis [73].

HSP70. Of the heat shock proteins, the HSP70 family is one of the most highly induced proteins during heat shock response [13]. HSP70 functions in a large array of chaperone processes including, folding nascent proteins, complex assembly, and processing misfolded proteins for degradation. Hsp70 is composed of two domains, a N-terminal nucleotide-binding domain that regulates client interactions and a C-terminal substrate-binding domain, which recognizes exposed hydrophobic stretches in the client proteins [74]. HSP70 has a large number of clients. Because of this, HSP70 can affect many cellular processes including cells signaling, apoptosis and immune response [75, 76]. The client interactions are largely mediated by HSP40 which both shepherds client proteins and promotes HSP70 ATPase function.

HSP90. HSP90 processes larger proteins and protein complexes. It functions as a homodimer assisted by a bevy of co-factors which allows HSP90 to deal with large, complex clients. The large number of co-factors and client proteins make the HSP90 complex serve as a signaling hub in addition to a chaperone. This is illustrated by its role in regulating hormone receptor complexes, protein kinases and transcription factors functions [77, 78]. For these reasons, HSP90 in critical in the folding, activation and assembly of proteins and also ligand receptor binding interactions [79].

HSP110. The large HSP110 generally acts as a cochaperone. It is loosely related to HSP70 but possesses an extended loop structure within the C-terminus which allows interactions with larger clients [80]. Because of the similarity, HSP110 is often considered to be part of the HSP70 super family [81]. It works both by intrinsically stabilizing denatured proteins and directing clients to HSP70. Due in part to its large size, HSP110 excels at preventing irreversible aggregation [82]. It also has the ability to bind non-protein ligands, such as pathogen-associated molecules, and is implicated in immune response modulation [83].

Functioning as a co-chaperone, HSP110 assists in the activity of HSP70 by escorting client proteins and acting as a nucleotide exchange factor, similar to HSP40 [84].

Other cytoprotective functions

HSF1 is capable of directing cellular responses through mechanisms outside of heat shock protein induction and their direct effects. HSF1 is able to modulate a variety of cellular functions including development, cell division, energy production, cytoskeletal organization, and vesicular transport [20, 85-89]. This is achieved through HSF1 direct interactions, alternative roles for expressed HSPs, and the expression of non-HSP genes.

In addition to activating transcription, HSF1 can inhibit genes under certain circumstances. Most notably, HSF1 is able to inhibit inflammatory response genes during a lipopolysaccharide-induced acute immune response [90-92]. This is achieved by both facilitating promoter interactions of transcriptional inhibitors and by direct inflammatory transcription factor inhibition [93, 94]. A variety of genes involved in the inflammation and immune response contain heat shock elements within their promoters, but are not strongly expressed during heat shock [48, 95]. Examples include multiple CXC chemokines, interleukin 6 (IL6) and tumor necrosis factor (TNF) genes [48, 93, 95]. In these instances, HSF1 is believed to be actively binding the promoter and directing the interaction of other transcription modulators. During heat shock, HSF1 has been shown to bind the promoter and facilitate an inhibitory effect on IL6, TNF, and CXCL5 [48, 92, 96, 97]. HSF1 is also able to affect the inflammatory response by directly binding and inhibiting the transcription factor CCAAT/enhancer binding protein beta (C/EBP-β), also known as nuclear factor of interleukin 6 (NF-IL6). C/EBP-β is a key mediator of metabolic and inflammatory responses, and promotes expression of interleukins such as IL1-β and IL6 along with other cytokines [94, 98-100]. The trimerization and regulatory domain HSF1 binds the basic leucine zipper domain of C/EBP-β [94]. This leads to the inhibition of both transcription factors [94]. During

heat shock, C/EBP-β inhibition by HSF1 leads to reduced induction of IL1-β and G-CSF [101, 102].

Some of the heat shock proteins expressed during heat shock have protective functions outside of protein folding. HSP70 has a variety of specific functions and interactions which stave off apoptosis. HSP70 blocks mitochondrial translocation and activation of BCL-2 family member BAX, partially via direct interaction [103]. This prevents the mitochondrial release of pro-apoptotic factors [104]. HSP70 also directly inhibits apoptosis protease-activating factor 1 (Apaf-1), apoptosis-inducing factor (AIF), and death receptors DR4 and DR5 [105-107]. HSP27 is able to inhibit apoptotic signal by binding multiple apoptosis mediators including caspase-3, cytochrome c, and death-domain-associated protein (DAXX) [108-110].

HSF1 has been shown to directly and indirectly regulate expression of multiple non-HSP genes in order to promote survival. This is achieved through various means such as inhibiting apoptosis, enhancing autophagy, and regulating cell cycle. HSF1 mediated expression of Bcl-2-associated athanogene domain 3 (BAG3) leads to the stabilization of Bcl-2 family proteins which inhibit apoptosis [111, 112]. Similarly, HSF1 can regulate second mitochondria-derived activator of caspase (SMAC) which in turn affects apoptosis [113]. Activation of HSF1 can also promote autophagy, which enhances cell survival during metabolic stress [114]. This is accomplished by HSF1-dependent activation of the autophagy-related protein 7 (ATG7) gene and indirect activation of p62 [115, 116]. HSF1 has also been shown to regulate the cell cycle. Research suggests that HSF1 is required for proper mitotic progression, as is demonstrated by the increased rate of mitotic disturbances in *hsf*1-/- cell lines [85, 117]. Active HSF1 promotes FOXM1 expression which regulates G2/M cell cycle progression [118]. Additionally, HSF1 directly interacts with CDC20 and can modulate metaphase to anaphase transition [119].

Role of HSF1 in Cancer

As the master regulator of the heat shock response, HSF1 has been postulated to be an important factor in oncogenesis. HSF1 plays many roles which could be beneficial to the progression and maintenance of the cancer phenotype. In addition to the canonical role in controlling the heat shock response, HSF1 is also involved in development, ageing, angiogenesis, inflammatory response, cell cycle signaling, metabolism, and translation [120-124]. Research over the last two decades has supported this idea and shown that HSF1 is important in cancer biology. HSF1 and many of its regulated targets are often overexpressed in cancers and support malignant behavior and survival.

Heat shock factor 1 and many associated HSPs are elevated in cancer cells compared to normal cells, which supports the theory that HSF1 can facilitate cancer development and survival. High HSF1 expression has been found in a wide array of cancers. These include breast, hepatocellular, ovarian, colorectal, multiple myeloma, glioma, oral squamous cell, and prostate cancers [125-132]. In addition to elevated levels of HSF1, many of these cancers also have increased nuclear accumulation of HSF1, which is indicative of activation [133]. Further, these elevated HSF1 levels correlate with poor outcomes in patients with breast cancer and hepatocellular carcinoma [129, 134]. Similarly, elevated levels of HSPs are found in many cancers including ovarian, breast, hepatocellular, and prostate cancer [135].

Continuing research has elucidated some of the ways HSF1 supports oncogenic behavior. Initial studies using *hsf-/-* mice and their derived cell lines demonstrated that HSF1 is required for RAS and mutant p53-induced transformation [136]. Subsequent studies determined that HSF1 supports cancers in a variety of ways. In many cases, HSF1 can improve survival of proteotoxic damage created by the malignant state [121, 137]. This is achieved through the general cytoprotective functions of the HSR. These functions include the anti-apoptotic effects of many HSPs and improved drug tolerance from increased efflux by ABC transporters [138, 139]. Other studies have found that HSF1 activation in cancer promotes malignant characteristics due to

effects outside of the classic HSR. In breast cancer and hepatocellular cancer, increased HSF1 is required to maintain abhorrent signaling pathways including HER2 and MAPK pathways [140, 141]. This HSF1-driven maintenance of vital signaling pathways in cancer is thought to be supported in part by elevated levels of HSP90,because HSP90 serves many client proteins that are key in signal transduction [142]. The methods by which HSF1 facilitates cancer progression continue to be elucidated. Research using high-throughput techniques have found that in cancer, HSF1 can modulate many cell processes including energy metabolism, cell cycle signaling, DNA repair, apoptosis, cell adhesion, extracellular matrix formation, and translation [133].

Ovarian Cancer

Ovarian cancer is the leading cause of cancer related deaths among gynecological malignancies [143]. It is projected that there will be 22,440 new cases of ovarian cancer and 14,080 ovarian cancer related deaths in 2017 [143]. Outcomes are typically poor; ovarian cancer has a low 46.5% 5 year survival rate. This high morbidity rate is due to the combination of late stage diagnosis coupled with a high rate of drug resistant recurrence. Failure to detect the disease in early stages is due in part to generalized symptoms such as abdominal and back pain, irregular vaginal discharge and pelvic pressure or bloat. Additionally, there is a lack of reliable diagnostic markers or tests. As a result, the vast majority of women are diagnosed at advanced stages III and IV [144]. Currently, there is work to establish diagnostic markers; however, this work has not yet substantially changed early stage detection rates [145].

Types of Ovarian Cancer

There are multiple histological subtypes of ovarian cancer. Of these, epithelial ovarian cancers are the most common and account for about 90% of ovarian cancer cases [146]. Other less common histological subtypes include germ cell and stromal, which make up the remainder of cases. Epithelial ovarian cancer can be further divided into serous, endometrioid, clear cell,

and mucinous carcinoma subtypes [147]. The clear cell carcinoma and endometrioid subtypes are believed to come from endometriosis [148]. Of the types of epithelial ovarian cancer, serous accounts for the majority of cases [146]. Serous epithelial ovarian cancer can be further divided into high and low grade. Both types of serous ovarian cancer have traditionally been thought to come from the ovarian surface epithelium. There has been a recent debate over the origin of high grade serous, and current research suggests that it may actually originate from the fallopian tube [149]. High grade serous generally has an aggressive phenotype and is more common [146]. Low grade serous is less aggressive and sometimes has oncogenic drivers such as mutated KRAS, BRCA, and PTEN [148]. Both types are characterized by high genomic instability and frequent loss of p53 function [150, 151].

Treatments

First line ovarian cancer treatment generally consists of surgical debulking and a combination of platinum and taxane chemotherapies. Initial response to these therapies is generally good, especially if resection was complete. Unfortunately, greater than 80% of patients will experience a recurrence, and most of these will be resistant to platinum and taxane therapies [146]. Other agents, notably bevacizumab and doxorubicin, are used in resistant cases. While these can increase progression-free survival, they very rarely achieve remission [152].

Epithelial to Mesenchymal Transition

Epithelial to mesenchymal transition (EMT) is the shift of epithelial cells toward more mesenchymal characteristics. Cells which have undergone EMT have distinct changes in gene expression patterns which lead to the loss of epithelial morphology, and the gain of mesenchymal traits [153]. These include increased migratory behavior and stem cell like properties [154]. This mesenchymal like cell behavior facilitates migration through the surrounding extracellular matrix and the establishment of new growth. This behavior makes EMT an important part of early

development and wound healing [155]. However, it can also support aberrant conditions including cancer and fibrosis [153].

Epithelial to mesenchymal transition is believed to be an important step in the establishment of metastases because it promotes dissemination and invasive behavior [156]. In ovarian cancer, EMT is thought to be a critical step in progression for several reasons. The surface ovarian epithelium cells which serve as the source of most ovarian cancers, have a high degree of plasticity and naturally exhibit some mesenchymal properties [157]. This propensity to undergo EMT likely facilitates the detachment and dissemination with in the peritoneal cavity [158]. This is a primary route which ovarian cancer spreads. Additionally, ascites fluids contain elevated levels of inflammatory and growth stimulating factors which drive the EMT process [159, 160]. For these reasons understanding EMT is an important part of understanding ovarian cancer.

On the molecular level EMT can be identified largely in part by the changes in cell to cell junctions and gene expression. This shift in cell interaction is driven by a handful of transcription factors which can serve as EMT markers [153]. During EMT there is reduction of E-Cadherin which destabilizes adherens junctions. Similarly claudins and occludins are reduced which weakens tight junctions [161]. N-cadherin is elevated, which promotes interaction with mesenchymal cells, increased invasion and migration, and disassociation with epithelial cells [162]. The extracellular matrix protein fibronectin is also more abundantly produced. This leads to further remodeling of the extracellular matrix and correlates with migration [163]. These changes in protein expression are driven principally by the transcription factors SNAIL1, SLUG (SNAIL2), TWIST and ZEB. SNAIL1 and SLUG are both zinc finger transcriptional repressors and function by binding E-box sequences within promoters and recruiting other repressors [164]. TWIST is a bHLH (basic helix-loop-helix) transcription factor which can activate or repress genes by directing histone modifications [164]. ZEB recognizes the E-box sequence motif, similar to SNAIL1 and SLUG, and can act as both a transcriptional activator and repressor depending on associated co-factors [165].

Spheroid Models to Study EMT. Standard tissue culture involves growing cells in a monolayer. This reinforces the cell polarity because the treated petri dish serves as the basal surface. This limits the usefulness of standard 2D cultures in studying EMT because the cells are not able to fully reorganize their interactions and transition away from the epithelial organization. Multiple studies have shown that culturing cells as 3 dimensional spheroids facilitates the signaling and gene expression changes that indicate EMT. Spheroid culture has been shown to increase TGFβ1 and multiple growth factor levels as compared to 2D culture [166]. Additionally, the EMT transcription factors SLUG, SNAIL, and TWIST are elevated in spheroids versus 2D cultures [167]. In ovarian cancer patient ascites-derived cells, formation of spheroids is accompanied by increases the EMT transcription factors SNAIL, TWIST, and ZEB2 [168]. For these reasons, spheroid cell culture is an apt model for studying EMT and offers advantages over standard 2D culture.

Transforming Growth Factor β

While many cytokines can promote EMT, transforming growth factor β (TGFβ) is the principal driver of EMT in ovarian cancer [169, 170]. It is commonly overexpressed in cancer tissue, plasma and peritoneal fluid of ovarian cancer patients [171]. The TGFβ pathway controls multiple cell processes such as differentiation, apoptosis, migration and immune response [172]. TGFβ can act as either a tumor suppressor or promoter depending on cellular conditions. In primary and precancerous ovarian epithelial cells TGFβ induces cell death, but in ovarian cancer cells it promotes EMT [173]. Of the 3 forms of TGFβ, TGFβI is most commonly associated with EMT in cancer [172].

The TGFβ pathway is activated by TGFβ ligand – receptor binding which triggers downstream changes in gene expression via the SMAD proteins (Fig 1.2). Extracellular TGFβ binds the TGFβRII (TGFβ receptor II) and is incorporated into a hetero-tetramer receptor complex consisting of 2 TGFβRII and 2 TGFβRI receptors [174]. This activates the receptors' serine/threonine kinase activity resulting in the phosphorylation of receptor SMADs (R-SMADs)

in the cytoplasm. The activated R-SMADs associate with co-SMADs and accumulate in the nucleus. The SMAD complexes interact with various transcription factors and control expression of a wide set of genes [175]. . In addition to the canonical SMAD-mediated effects, TGFβ can activate the PI3K and MAPK pathways which can support EMT and proliferation [176, 177].

Studies

To better understand the role of HSF1 in ovarian cancer we have done a review of the HSF family and associated chaperones, and completed studies to elucidate how HSF1 contributes to ovarian cancer cell behavior and drug resistance. Chapter 2 reviews the HSF and chaperone families with a focus on their general intrinsically disordered structure, which has implications for how they may function. Chapter 3 describes our work investigating how HSF1 induces ovarian cancer epithelial-mesenchymal transition, particularly in a spheroid growth model. Chapter 4 describes how HSF1 levels affect drug response in ovarian cancer cell lines to multiple agents. Chapter 5 discusses the implication of this work and future directions, including how HSF1 could translate to a potential drug target or prognostic marker.

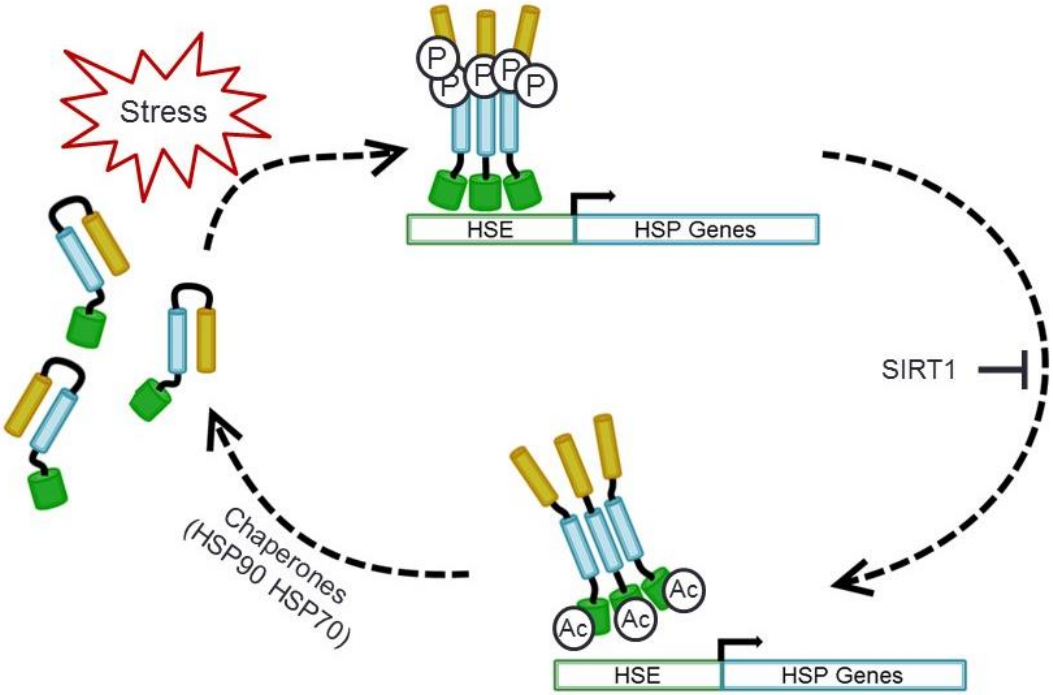

Figure 1.1. Overview of Heat Shock Regulation by HSF1. HSF1 forms trimers upon activating stress and is concurrently hyperphosphorylated. It accumulates in the nucleus, where it bind HSEs within the promoters of regulated gene. The response is shut off by negative feedback from HSPs and acetylation of the DNA binding domain. The DNA binding ability can be modulated by the deacetylase SIRT1.

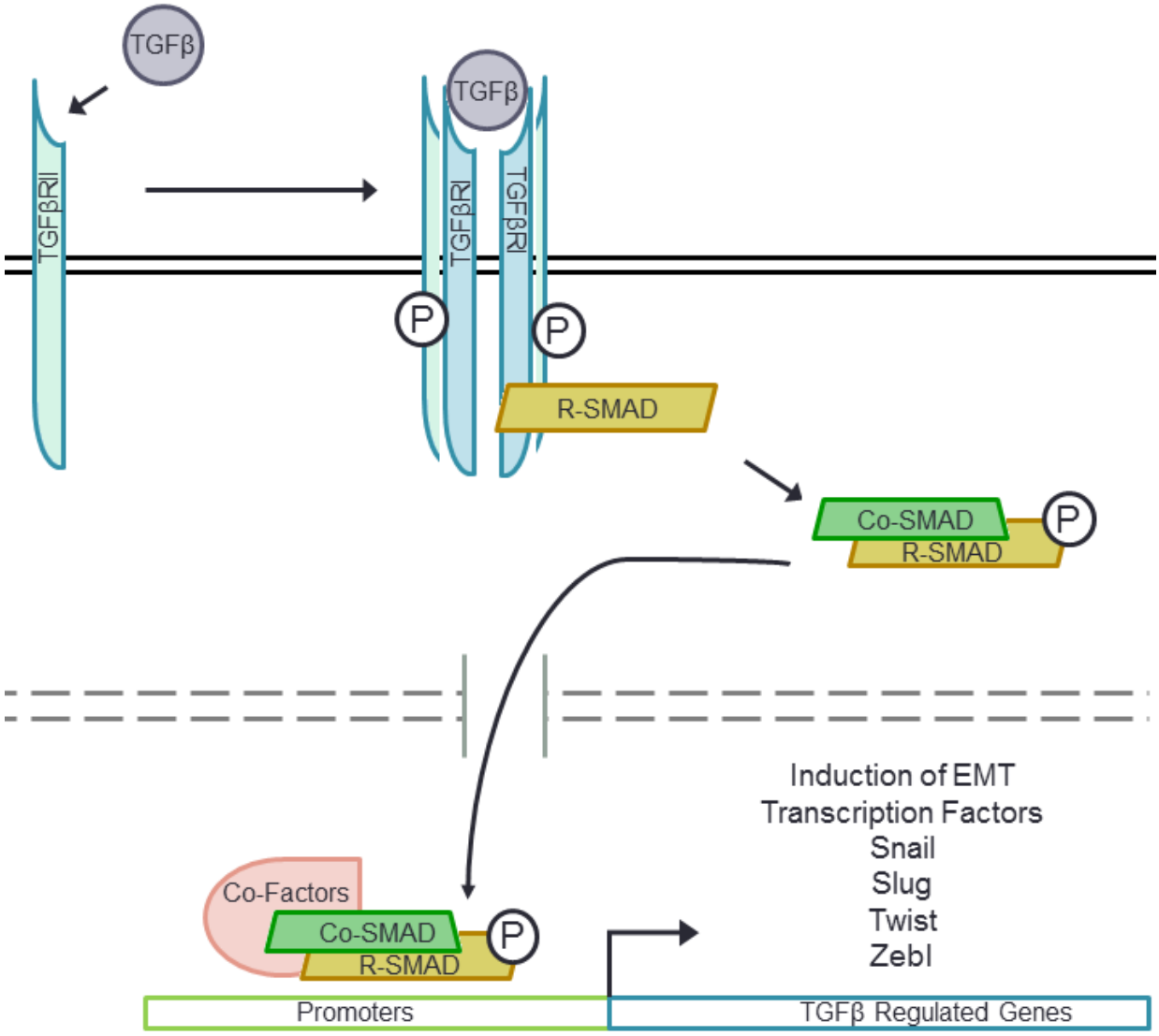

Figure 1.2. TGFβ Pathway. Active TGFβ binds the TGFβ receptor type II (TGFβRII) which results in the formation of the heteromeric complex with TGFβ receptor type I (TGFβRI) and autophosphorylation. This is followed by the phosphorylation of receptor-regulated SMAD (mothers against decapentaplegic homologue). R-SMAD forms complexes with co-mediator SMAD (Co-SMAD) and is translocated to the nucleus. The SMAD complex associates with DNA binding co-factors and transcriptional co-activators. These co-factors are largely responsible for directing the outcome of TGFβ signaling. In many ovarian cancers the TGFβ pathway leads to the induction of transcription factors which promote epithelial to mesenchymal transition (EMT).

CHAPTER TWO: INTRINSIC DISORDER IN THE HSF TRANSCRIPTION FACTOR FAMILY AND MOLECULAR CHAPERONS

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Authors contributed equally to the text. Background research and literature review performed by S. Westerheide, R. Raynes and C. Powell. Sequence and structure analysis was done by B. Xue and V. Uversky. See appendix D for copyright permissions.

Abstract

Intrinsically disordered proteins are highly abundant in all kingdoms of life, and several protein functional classes, such as transcription factors, transcriptional regulators, hub and scaffold proteins, signaling proteins, and chaperones are especially enriched in intrinsic disorder. One of the unique cellular reactions to protein damaging stress is the so- called heat shock response that results in the upregulation of heat shock proteins including molecular chaperones. This molecular protective mechanism is conserved from prokaryotes to eukaryotes and allows an organism to respond to various proteotoxic stressors, such as heat shock, oxidative stress, exposure to heavy metals, and drugs. The heat shock response-related proteins can be expressed during normal conditions (e.g., during cell growth and development) or can be induced by various pathological conditions, such as infection, inflammation, and protein conformation diseases. The initiation of the heat shock response is manifested by the activation of the heat

shock transcription factor 1 (HSF1), part of a family of related HSF transcription factors. This review analyzes the abundance and functional roles of intrinsic disorder in various heat shock transcription factors and clearly shows that the heat shock response requires HSF flexibility to be more efficient.

Intrinsically Disordered Proteins: General Overview

Research over the last decade or so made it absolutely clear that in addition to well-folded and highly structured transmembrane, globular and fibrous proteins, the protein universe includes intrinsically disordered proteins (IDPs) and proteins with intrinsically disordered regions (IDRs). These IDPs and IDRs are biologically active and yet fail to form specific 3D structure, existing instead as collapsed or extended dynamically mobile conformational ensembles [178-184]. These floppy proteins and regions are known as pliable, rheomorphic [185], flexible [186], mobile [187], partially folded [188], natively denatured [189], natively unfolded [180, 190], natively disordered [183], intrinsically unstructured [179, 182], intrinsically denatured, [189] intrinsically unfolded [190], intrinsically disordered [191], vulnerable [192], chameleon [193], malleable [194], 4D [195], protein clouds [196], and dancing proteins [197], among several other terms. The variability of terms used to describe such proteins and regions is a simple reflection of their highly dynamic nature and the lack of unique 3-D structure. None of these terms or their combinations is completely appropriate, as the majority of them have been borrowed from fields such as protein folding or crystallography, which are not directly related to the biologically active proteins that normally exist as structural ensembles.

Since these proteins are highly abundant in any given proteome [198], the role of disorder in determining protein functionality in organisms can no longer be ignored. Native biologically active proteins were conceptualized as parts of the "protein trinity" [181] or the “protein quartet” [199] models, where functional protein might exist in one of several conformations – ordered, collapsed-disordered (molten globule-like), partially collapsed-disordered (pre-molten globule-

like) or extended-disordered (coil-like), and protein function might be derived from any one of these states and/or from the transitions between them. Disordered proteins are typically involved in regulation, signaling and control pathways [200-202], which complement the functional repertoire of ordered proteins, which have evolved mainly to carry out efficient catalysis [203]. It is also important to remember that sites of posttranslational modifications (acetylation, hydroxylation, ubiquitination, methylation, phosphorylation, etc.) and sites of regulatory proteolytic attack are frequently associated with regions of intrinsic disorder [191].

Because of the fact that IDPs play crucial roles in numerous biological processes, it was not too surprising to find that many of them are involved in human diseases [204]. Originally, this hypothesis was based on numerous case studies where a particular IDP was shown to be associated with a particular disease. For example, the presence of disorder has been directly observed in several cancer-associated proteins, including p53 [205], $p57^{kip2}$ [206], Bcl-X_L and Bcl-2 [207], c-Fos [208], thyroid cancer associated protein, TC-1 [209], and many others. Some other maladies associated with IDPs includes Alzheimer's disease (deposition of amyloid-β, tau-protein, α-synuclein fragment NAC [210-213]), Niemann-Pick disease type C, subacute sclerosing panencephalitis, argyrophilic grain disease, myotonic dystrophy, and motor neuron disease with neurofibrillary tangles (accumulation of tau-protein in the form of neurofibrillary tangles [212]); Down's syndrome (nonfilamentous amyloid-β deposits [214]); Parkinson's disease, dementia with Lewy body, diffuse Lewy body disease, Lewy body variant of Alzheimer's disease, multiple system atrophy and Hallervorden-Spatz disease (deposition of α-synuclein in a form of Lewy body, or Lewy neuritis [215]); prion diseases (deposition of PrP^{SC} [216]); and a family of polyQ diseases, a group of neurodegenerative disorders caused by expansion of CAG trinucleotide repeats coding for polyQ in the gene products [217].

Three computational and bioinformatics approaches have been elaborated to estimate the abundance of IDPs in various pathological conditions. The first approach was based on the assembly of specific datasets of proteins associated with a given disease and the computational

analysis of these datasets using a number of disorder predictors [200, 204, 218-221]. In essence, this was an analysis of individual proteins extended to a set of independent proteins. Using this approach, a prevalence of intrinsic disorder was detected in proteins associated with cancer [200], cardiovascular disease [219], neurodegenerative diseases [220, 222], various amyloidoses [223] and diabetes [204]. A second approach utilized the diseasome, a network of genetic diseases where the related proteins are interlinked within one disease and between different diseases [224]. A third approach was based on the evaluation of the association between a particular protein function (including the disease-specific functional keywords) with the level of intrinsic disorder in a set of proteins known to carry out this function [225-227]. Based on the fact that IDPs and proteins with long IDRs were commonly found in various diseases, the "disorder in disorders" or D^2 concept was introduced to summarize work in this area [204] and the concepts of the disease-related unfoldome and unfoldomics were developed [228].

Intrinsic Disorder and Transcription Regulation

Several protein functional classes (e.g., transcription factors [196, 229-231], transcriptional regulators [194, 232, 233], hub proteins [201, 233-238], scaffold proteins [239-242], signaling proteins [200], chaperones [243-250], etc.) were shown to be enriched in intrinsic disorder. Recent studies suggested that eukaryotic proteomes are highly enriched in IDPs relative to bacterial and archaeal proteomes [251-253], which may reflect the greater need for signaling and transcriptional regulation in nucleated cells [191, 200, 254]. Transcription factors (TFs) act through the recognition of specific DNA sequences and recruitment and assembly of the transcription machinery. Therefore, both protein-DNA and protein-protein recognition are central processes in TF function. It has been reported that protein-protein and protein-DNA interaction are often accompanied by a local folding in a protein molecule [255]. One of the important biological implications of this coupled binding and folding scenario is that protein backbone mobility may play an important role in the early stages of a binding event [256], where the specific

signal from the complex of protein with its binding partner emerges only after appropriate conformational changes take place [257].

Comprehensive computational analysis of several transcription factor datasets revealed that from 94.13% to 82.63% of TFs possess long regions of intrinsic disorder, and ≥70% proteins in the TF datasets were predicted to be wholly disordered [229]. Furthermore, the degree of disorder in eukaryotic TFs was shown to be significantly higher than in prokaryotic proteins. Eukaryotes have a well-developed gene transcription system, which probably requires a great deal of flexibility and plasticity. The intrinsically disordered TFs or partially unstructured regions can offer significant advantages in response to different molecular targets, allowing one protein to interact with multiple cellular partners and allowing fine control over binding affinity [229]. This analysis also revealed the existence of two distinct classes of DNA-binding domains (DBDs) in TFs. In one class, the DBDs are well-structured and specifically recognize DNA using the molecular surfaces they present to the environment. In another class, various DBDs such as basic domains and AT-hooks, are likely to be highly unstructured in isolation, and presumably undergo a disorder-to-order transition upon binding to specific DNA sequence. [229]. The high prevalence of intrinsic disorder in TFs suggested that it may play a critical role in the primary functions of TFs, which are molecular recognition, DNA binding, and transcriptional regulation.

This hypothesis is in a good agreement with several recent findings showing that eukaryotic TFs contain a variety of structural motifs that interact with specific DNA sequences [258] and are involved in activating the transcription. For example, based on the analysis of binding of multiple zinc fingers to cognate DNA a ‘snap-lock’ model has been recently introduced [259, 260]. According to this model, C_2H_2-type zinc finger domains consist of well-folded modules connected by highly conserved linker sequences that are mobile and unstructured in the absence of the cognate DNA. NMR analysis revealed that upon binding to the correct DNA sequence, the linker becomes highly structured and locks adjacent fingers in the correct orientations in the major groove [259, 260]. Furthermore, it has been shown that many alterations of this linker disrupt the

conformation of the bound linker, increase its flexibility, and impair DNA binding, thereby altering both the biological function and sub-nuclear localization of the protein [259, 260]. This model illuminates the sophisticated relationship between the function of a TF, its domain structure, and intrinsic disorder.

It is known that two totally disordered types of DBDs, AT-hook and basic domain, act as a versatile minor groove tether to anchor TFs to particular DNA sites. In addition to having sequence-specific DNA-binding activity, many TFs contain a region involved in activating the transcription of the gene whose promoters or enhancers they have bound. Usually, this trans-activating region enables the TF to interact with a protein involved in binding RNA polymerase. Many trans-activating domains were predicted either unstructured or partly structured [229]. This was in agreement with the accumulated experimental data that many trans-activating domains are significantly disordered in an unbound form and their interactions with their targets involve coupled folding and binding events [200, 252, 254, 261]. Recently, the kinase-inducible activation domain of CREB (cAMP response element binding protein) [262], the trans-activation domain of p53 [263], and the acidic activation domain of herpes simplex virus VP16 [264] were comprehensively examined. These studies revealed that the activation domains remained mostly unstructured in their normal unbound states, and form a helix or helices upon binding to the target proteins.

In another elegant computational study, the abundance and functional roles of intrinsic disorder in human TFs were investigated [230]. The authors emphasized that eukaryotic TFs, especially so-called *trans*-acting factors such as activators, repressors or enhancer-binding factors that specifically bind DNA *cis* elements, were noticeably larger than prokaryotic TFs. In fact, the average sequence of human TFs was more than twice as long as length of the TFs in prokaryotes. Furthermore, the fractions of sequence aligned to domains of known structure were 31% and 72% in human and bacterial TFs, respectively [230]. Therefore, as a rule, human TFs were long and poorly annotated. Analysis revealed that as high as 49% of the entire sequence of

human TFs was occupied by IDRs, and that more than half of the human TFs consisted of a small DBD and a set of long IDRs. In general, IDRs were shown to occupy a high fraction of TFs from eukaryotes, but not prokaryotes [230].

An Overview of Intrinsic Disorder in Chaperones

Generally, a polypeptide chain of a protein contains all the information required to achieve functional conformation [265, 266]. Although this principle is generally correct for many proteins, the information contained in some potentially foldable proteins is not complete enough to guarantee the formation of a functionally active 3-D structure. Many such potentially foldable proteins cannot fold spontaneously and require the help of molecular chaperones; i.e., cellular proteins which act to ensure that the folding of certain polypeptide chains and their assembly into oligomeric structures occurs correctly [8]. Chaperones are an important part of the cellular quality control system, maintaining an intricate balance between protein synthesis and degradation and protecting cells from the devastating consequences of uncontrolled protein aggregation. In addition to chaperones, this system includes the ubiquitin-proteasome system and the autophagy-lysosome system. Molecular chaperones protect cells from apoptosis induced by toxic oligomers. There are several mechanisms by which chaperones fight devastating consequences of misfolding and aggregation. These mechanisms can be grouped into three major classes of action: prevention, reversal and elimination. At the prevention stage, chaperones bind to unfolded stretches in proteins and keep them in a folding-competent state while preventing aggregation. In the reversal mechanism, chaperones act as disaggregating and unfolding machines which help dissolve aggregates and give a misfolded protein a second chance for folding correctly. At the elimination step, chaperones target misfolded proteins for degradation by the ubiquitin-proteasome system and/or the autophagy-lysosome system.

The principal heat-shock proteins that have chaperone activity belong to five conserved classes: Hsp33, Hsp60, Hsp70, Hsp90, Hsp100, and the small heat-shock proteins. Molecular

chaperones have been divided into three functional subclasses based on their mechanism of action. "Folding" chaperones (e.g., DnaK and GroEL in prokaryotes, and Hsp60 and Hsp70 as well as the HspB group of Hsp including Hsp27 and HspB1 in eukaryotes) rely on ATP-dependent conformational changes to mediate the net refolding/unfolding of their substrates. "Holding" chaperones (e.g., Hsp33 and Hsp31) bind partially folded proteins and maintain these substrates on their surface to await the availability of "folding" chaperones. “Disaggregating” chaperones (e.g., ClpB in prokaryotes and Hsp104 in eukaryotes) promote the solubilization of proteins that have become aggregated as a result of stress.

Molecular chaperones are classified as either inducible or constitutively expressed according to their expression mechanisms. Both types of chaperones act by the selective binding of solvent-exposed hydrophobic segments of non-folded polypeptides, and, through multiple binding-release cycles, bring about the folding, transport, and assembly of the target polypeptides [267-269]. Some chaperones are ATPases; i.e., they use free-energy from ATP binding and/or hydrolysis to perform work on their substrates.

The concentration of inducible chaperones, also known as heat shock proteins (HSP), increases as a response to stress conditions. These molecular chaperones prevent and reverse the misfolding and aggregation of proteins that occur as a consequence of stress [270, 271]. On the other hand, constitutively expressed chaperones, also known as heat shock cognate proteins (Hscs), facilitate protein translation, help newly synthesized proteins fold, promote the assembly of proteins into functional complexes, and assist the translocation of proteins into cellular compartments such as mitochondria and chloroplasts [268, 272]. In the Hsp70 family of proteins, in addition to the inducible Hsp70 form, there is a constitutively expressed form, the heat shock cognate protein 70 (Hsc70), which has 85% identity with human Hsp70 and binds to nascent polypeptides to facilitate correct folding.

Molecular chaperones have evolved to protect proteins from misfolding and aggregation regardless of their classification as inducible or constitutively expressed. One important feature

of chaperones is that, although they assist the non-covalent folding/unfolding and the assembly/disassembly of other macromolecular structures, they do not occur in these structures when the latter are performing their normal biological functions. Generally, molecular chaperones have no effect on a protein's folding rate. Of course, apparent folding and assembly rates can be increased by the elimination of non-productive oligomer/aggregate formation. Furthermore, by binding to partially folded species and preventing their aggregation, chaperones increase the yield of functional folded/assembled proteins. However, these actions do not affect intramolecular folding rates. On the other hand, there is a last class of proteins - helpers which assist protein folding and are not present in the final folded/assembled functional form of a protein-substrate. Therefore, these helpers, known as foldases, belong to the family of chaperones. Contrary to the typical chaperones considered so far, foldases have evolved to catalyze the folding process by directly accelerating the protein folding rate-limiting steps. Well-known foldases include eukaryotic protein disulfide isomerase [273-275], peptidyl-prolyl cis/trans isomerase [275, 276], and lipase-specific foldases, Lifs, found in the periplasm of Gram-negative bacteria [275, 277].

Earlier, the prevalence of functional regions without a well-defined 3-D structure in RNA and protein chaperones was emphasized [250]. The analysis revealed a high proportion of predicted IDRs in chaperones, with 54% of residues of RNA chaperones falling into IDRs and with 40% of their residues being located within the disordered regions longer than 30 consecutive residues. Intrinsic disorder was also to be abundant in protein chaperones, for which corresponding values were 37% and 15%, respectively [250]. Based on the analysis of several individual cases it has been concluded that the IDRs in chaperones might function as molecular recognition elements solubilizing or locally loosening the structure of the kinetically trapped folding intermediate via transient binding to facilitate its conformational search. An "entropy transfer" model was proposed to account for the mechanistic role of structural disorder in chaperone function. According to this model, the binding of the disordered chaperone to the misfolded

substrate was proposed to be accompanied by ordering of the chaperone with a concomitant unfolding of the substrate [250].

Recently, it was confirmed that intrinsic disorder is highly abundant in protein chaperones and plays a number of important roles in the action of these intricate machines. IDRs determine the promiscuity of chaperones, act as pliable molecular recognition elements, wrap misfolded chains and participate in disaggregation and local unfolding of the aggregated and misfolded species. The functions of chaperones, co-chaperones and decorating proteins are precisely orchestrated. These proteins often act as large chaperone machines. They communicate with each other and form sophisticated chaperone networks. Protein intrinsic disorder plays a crucial role in the coordination and regulation of these chaperone machines and networks, thus helping form a flexible net of malleable guardians [243].

The Heat Shock Response

The heat shock response is the cell's molecular reaction to protein damaging stress and results in the upregulation of heat shock proteins including molecular chaperones (reviewed in [13]). This response is conserved from prokaryotes to eukaryotes and allows an organism to respond to proteotoxic stressors, including heat shock, oxidative stress, heavy metals, and drugs, as well as cell growth and developmental conditions [278]. Pathology, such as inflammation or infection, may also induce the heat shock response, in addition to protein conformation diseases, such as Alzheimer's disease [278]. Upon initiation of the heat shock response, heat shock transcription factors (HSFs) are activated through trimerization and post-translational modifications [13]. A single, essential HSF1 gene exists in *Saccharomyces cerevisiae* and in *Drosophila melanogaster,* where this gene is responsible for both constitutive and heat-inducible heat shock gene expression [279-281]. Vertebrate species express a family of HSF proteins with diverse cellular roles.

HSF1- The Master Heat Shock Response Regulator

HSF1 is the HSF family member responsible for stress-induced expression of heat shock proteins. Once the heat shock response is induced, HSF1 trimerizes, accumulates in the nucleus, and binds the heat shock elements (HSEs) located in the promoter regions of *hsp* genes (Figure 2.1). The HSEs consists of several adjacent and inverted repeats of the pentamer sequence nGAAn [282]. Activation of transcription occurs upon HSF1 hyperphosphorylation [34].

Attenuation of the heat shock response is important because the overexpression of HSPs is deleterious to the cell. Attenuation is regulated by a dual mechanism involving negative feedback inhibition from HSPs [27] and acetylation at a critical lysine residue within the DNA binding domain of HSF1 causing loss of affinity for DNA [26]. SIRT1 is a NAD-dependent histone deacetylase that deacetylates HSF1, thus promoting stress-induced HSF1 DNA binding ability and increased HSP expression [26]. The end result of the heat shock response is the upregulation of HSPs, thus promoting the cell's ability to cope with denaturing stress.

Domains of HSF1

Similar to many eukaryotic TFs, HSF1 possesses a complex modular structure with several functional domains shown schematically in Figure 2.1a. These functional domains of HSF1 have been mapped by mutational analyses [281, 283-285]. Figure 2.1b represents the distribution of intrinsic disorder propensity within the human HSF1 sequence as estimated by two established disorder predictors, PONDR® VLXT (red line) and PONDR-FIT (black line). According to this analysis, more than a half of the protein is predicted to be disordered. In fact, only two of the HSF1 functional domains, DNA-binding domain (DBD, residues 15-120) and hydrophobic repeat HR-A/B domain (residues 130-203) are predicted to be ordered. Figure 1 also shows that the HR region C (which is located at the beginning of the transactivation domain (TAD), residues 384-409) is predicted to be partially ordered. These two domains are connected by a flexible

linker, and the remainder of the protein, including two important functional domains, the regulatory domain (RD, residues 221-310) and TAD (residues 371-529) and their linker, is mostly disordered.

DNA Binding Domain (DBD)

The DBD is the most conserved domain of HSF1 from *Caenorhabditis elegans* to humans (Figure 2.4). Both the crystal structure of the DBD for *Kluyveromyces lactis* HSF and the solution structure of the DBDs for *K. lactis* and *D. melanogaster* HSF1 show that the DBD is a helix-turn-helix motif [49, 286, 287].

The helix-turn-helix structure contains 3 alpha helices, 4 beta strands, and an exposed loop. Upon trimerization, the DBD of each HSF1 monomer recognizes an inverted nGAAn repeat of the HSE [288]. The domains make contact with the major groove and with the DNA phosphate backbone by helix 3 of domain [50, 289]. Helix 3 is a region rich in positive charge and is the most conserved region of the DBD. Unlike other helix-turn-helix proteins, the loop in the HSF1 DBD does not bind DNA. Instead, the loop forms an interaction with the surface between adjacent HSF monomers [50].

The characteristic helix-turn-helix structure of the HSF1 DBD is shown in Figure 2.2a, b, and e representing both solution and crystal structures of DBDs from non-mammalian HSFs. Comparison of the crystal and NMR structures of *K. lactis* HSF (Figure 2.2b and c) revealed that DBD preserved the overall topology in solution. In fact, both structures contained a 4-stranded antiparallel beta-sheet and a 3-helix bundle [49, 286]. Seeing in the crystal structure an irregular segment defined as an α-helical bulge in helix H2 was consistent with the NMR results. Furthermore, in the solution structure, the ill-defined loop connecting β-strands B3 and B4 was consistent with the results of the X-ray model, which showed larger B-factors in this region than in the rest of the structure, and the absence of electron density for residues 76-79 [49, 286]. These data are also consistent with the result of disorder prediction for the *K. lactis* HSF. In fact, Figure 2.2a clearly shows that the C-terminal part of the DBD is predicted to have significant amount of

intrinsically disordered residues. Comparison of Figure 2.2b, c, and e also shows that the overall structure of the DBD is conserved between *K. lactis* and *D. melanogaster*, but the DBD of the *D. melanogaster* HSF is bit less flexible in solution in comparison with the domain from *K. lactis*. Once again, this is consistent with the results of disorder predictions for these two domains, since according to Figure 2.2a and d, *D. melanogaster's* DBD is expected to have less disorder propensity than its homologue from the yeast.

Trimerization Domains

The HSF1 trimerization domain is composed of three arrays of hydrophobic heptad repeats (HR-A/B) [285]. HSF1 trimerization is unusual in that when the monomers come together, they form a leucine zipper, an artifact typically seen in dimerization. Trimerization is constitutive and is actively suppressed by the hydrophobic heptad repeat HR-C [290, 291]. When a mutation is introduced into the HR-C domain, trimerization and binding to the DNA is able to persist. *S. cerevisiae* and *K. lactis* HSFs have constitutive DNA-binding activity. In these HSFs, the HR-C is poorly conserved and does not actively suppress trimerization. Mutating the HR-C does not affect the oligomerization status of HSF in these organisms [292].

Transactivation Domains (TAD)

Transcriptional elongation of *hsp* genes is regulated by the transactivation domains (TAD) of HSF1. In the absence of HSF1, RNA polymerase pauses at 46-49 bases downstream from the transcription start site even in the presence of excess free nucleotides [293]. The HSF1 transactivation domain can be divided into two distinct regions rich in hydrophobic and acidic residues: activation domain I and activation domain II, spanning amino acids 371-430 and 431-505, respectively [283, 294, 295]. These domains have been shown to equivalently stimulate both initiation and elongation of transcription [296]. This redundancy may be in effect due to the importance of heat shock proteins in maintaining protein homeostasis.

Regulatory Domain (RD)

The regulatory activity of HSF1 is located between amino acids 221 and 310 and is functionally conserved between mammalian *hsf1* genes, but not between other members of the HSF family [283]. Upon non-stress conditions in which protein homeostasis is preserved, the transactivator activity of TAD I and TAD II is repressed by the regulatory domain and an exposure to stress leads to a reversal of this repression [283, 284]. The regulatory domain of HSF1 contains several serine residues that are responsive to protein kinases and lead to repression of the TADs upon phosphorylation. Phosphorylation by the MAP kinase ERK is responsible for this transactivator repression and mutation of these phosphorylation sites abolishes the suppression of the transactivation domains even in the absence of stress [297].

Conservation of HSF Across Different Species

Figure 2.4 represents alignment of amino acid sequences of HSF from different species and clearly shows that the DBD and HR-A/B domains are highly conserved during evolution, whereas other functional domains of HSFs are much less conserved. Interestingly, two non-vertebrate HSFs possess long and poorly conserved N-terminal extensions. Figure 2.5 shows the conservation of the distribution of intrinsic disorder propensity in the HSF family. In agreement with sequence alignment data, Figure 2.5 shows that there is a great conservation of order propensity in the N-terminal parts of HSFs; i.e., in their DBDs and HR-A/B domains, with evolutionarily more advanced organisms typically showing very similar disorder patterns in this region Figure 2.5a and b. On the contrary, the C-terminal parts of these proteins generally do not show noticeable conservation except for the fact that all of them are predicted to be highly disordered. On the other hand, Figure 2.5c shows that the C-terminal fragments of these HSFs possessed relatively similar amino acid compositions, and also emphasizes that these regions, in general, have some features of typical disordered proteins, being depleted in major order-promoting residues and showing enrichment in major disorder-promoting residues.

Alternative Splicing of HSF1

In mammalian cells, there are two distinct HSF1 mRNA isoforms that are produced via alternative splicing of the HSF1 pre-mRNA [298]. For example, in mouse, the two HSF1 mRNA isoforms differ by a single 66 bp exon of the HSF1 gene, which is present in the HSF1-α mRNA isoform but is skipped in the HSF1-β mRNA isoform. The extra 22 amino acid sequence in the HSF1-α isoform is inserted immediately adjacent to a C-terminal leucine zipper motif [298]. The levels of the two HSF1 isoforms were shown to be regulated in a tissue dependent manner, with testis expressing higher levels of the HSF1-β isoform, and with heart and brain expressing higher levels of the HSF1-α isoform [298]. In human, the shorter alternatively spliced isoform of HSF1 is different from the canonical isoform by lacking the last 40 residues and by in the shorter form and by changing the canonical residues $GKQLVHYTAQPLFLLDPGSVDTGSNDLP_{462\text{-}489}$ to the alternative sequence $AGALHSAAAVPAGPRLRGHREQRPAGAV_{462\text{-}489}$. Earlier, it has been pointed out that alternative splicing occurs mostly in regions of RNA that code for the disordered protein regions [299]. In agreement with these earlier findings, Figure 2.3 clearly shows that in both mouse and human HSF1 proteins, the regions affected by alternative splicing are predicted to be mostly disordered.

Post-Translational Modifications Regulate HSF1 Transcriptional Activity

HSF1 is extensively modified by post-translational modifications including phosphorylation, sumoylation and acetylation (Figure 2.7). This extensive modification may form a code to allow fine-tuning of the activity of HSF1 to the precise needs of the cell.

Phosphorylation

HSF is phosphorylated at several residues in order to promote or inhibit transactivation. HSF was first reported to display a reduction in electrophoretic mobility as a result of phosphorylation in *S. cerevisiae* [280]. Mammalian HSF1 also exhibits a similar electrophoretic shift due to

hyperphosphorylation upon activation of the heat shock response. Phosphorylation regulates transcriptional activity and not DNA binding, as some inducers of heat shock, such as the anti-inflammatory agent sodium salicylate, are capable of inducing HSF1 to bind to the DNA, but do not cause hyperphosphorylation [32]. Another example of the distinction between these two activities is observed in yeast. In *S. cerevisiae*, HSF exists as a trimer with constitutive DNA-binding activity. However, transcriptional activation only occurs in the presence of increased phosphorylation [280, 300, 301].

Through phosphoamino acid analyses and mass spectrometry, several phosphorylation sites within HSF1 have been mapped. While phosphorylation has been primarily mapped to serine residues, a low level of threonine phosphorylation has also been detected [34, 35, 297, 302, 303]. Of the first phosphorylation sites to be characterized are serine residues S303, S307, and S363 located within the C-terminal region of the regulatory domain. These sites are proline-directed and typically experience phosphorylation in the absence of stress. Upon stress, the repressive activity of these residues is superseded by the activation of HSF1 [34, 297, 304]. An HSF1 phosphorylation site that leads to transcriptional activation is serine residue 230, located within the N-terminal region of the regulatory domain [35]. Many sites within HSF1 can be phosphorylated, and a mass spectrometry analysis of HSF1 activated by heat shock found phosphorylation on Ser121, Ser230, Ser292, Ser303, Ser307, Ser314, Ser319, Ser326, Ser344, Ser363, Ser419, and Ser444 [41]. Phosphorylation of Ser326, but none of the other serine residues, was found to contribute significantly to activation of HSF1 upon stress in this study [41]. While the implications of HSF1 phosphorylation have yet to be fully characterized, it is clear that dynamic phosphorylation modulates HSF1 activity in both a positive and negative manner.

HSF1 phosphorylation is regulated by a number of kinases with several of the residues suspected to undergo modification by more than one kinase. MAP kinases have been shown to negatively regulate HSF1 activity and the HSF1 Serine residues S303, 307 and 363 have been found to be MAP kinase targets. The MAP kinase ERK1 has been shown to phosphorylate S307,

after which S303 is receptive to phosphorylation by glycogen synthase-3 kinase (GSK-3) [34, 297, 304]. The MAP kinase p38 has also been reported to phosphorylate S303 and/or S307, resulting in basal repression of HSF1 [305]. JNK, another MAP kinase family member, and the PKC isoforms alpha and zeta have been suggested to phosphorylate S363, there by contributing to the maintenance of HSF1 in an inert state [306, 307]. Inhibition has also been shown to occur through the phosphorylation of S121 by the proinflammatory protein kinase MK2, which may lead to an increase in affinity for Hsp90 [308]. Various kinases suspected of activating HSF1 have also been discovered. For instance, the Ca2+/calmodulin-dependent kinase II (CaMK II) phosphorylates S230 and promotes transcriptional activity [35]. Given the broad range of stimuli that induce the heat shock response and the vast number of HSF1 phosphorylation sites, it may be that different kinases regulate HSF1 depending on the nature of the stress. As an example, in yeast Snf1 protein kinase has been shown to regulate HSF1 in low glucose conditions, but does not phosphorylate HSF upon heat shock [309].

Sumoylation

Upon stress, HSF1 is modified by SUMO-1 at the well-conserved lysine residue 298 within the N-terminal region of the regulatory domain [62]. In order for K298 to become sumoylated, S303 must first undergo phosphorylation, which has a repressive effect on HSF1 activation [62]. There may be a regulatory interplay of modifications at this residue as residue K298 is also an acetylation site [110].

Acetylation

While the kinetics of phosphorylation and sumolyation are brought on rapidly upon stress, acetylation is delayed and coincides with a reduction of HSF1 DNA-binding activity at the decline of the heat shock response [26]. Acetylation of HSF1 by the histone acetyltransferase p300 occurs to attenuate HSF1 off of the DNA. The deacetylation of HSF1 is regulated by the NAD-

dependent deacetylase SIRT1. When SIRT1 is inhibited by siRNA, not only are the levels of heat shock-induced *hsp70* mRNA significantly decreased, but the ability of HSF1 to bind to the *hsp70* promoter region is also greatly reduced [26].

A Correlation Between intrinsic Disorder and Post Translational Modifications

In the past, a correlation between intrinsic disorder and PTM sites was found. In fact, in a study of the functions associated with more than 100 long disordered regions, many were found to contain sites of protein posttranslational modifications (PTMs) [310, 311]. These PTMs included phosphorylation, acetylation, fatty acylation, methylation, glycosylation, ubiquitination, and ADP-ribosylation suggesting the possibility that protein modifications commonly occur in regions of disorder. A particular advantage of disorder for regulatory and signaling regions is that changes, such as protein modification, lead to large-scale disorder-to-order structural transitions: such large-scale structural changes are not subtle and so could be an advantage for signaling and regulation as compared to the much smaller changes that would be expected from the decoration of an ordered protein structure.

Protein phosphorylation and dephosphorylation are crucial for signaling. Indeed, about one-third of eukaryotic proteins are phosphorylated [312]. Many sites of protein phosphorylation were found to be in regions structurally characterized as intrinsically disordered [310, 311]. This conclusion was based on several lines of evidence, such as: very small number of PDB structures for both the un-phosphorylated and phosphorylated forms of the same protein [313, 314]; the fact that the residues of the phosphorylation site often have extended, irregular conformation consistent with disordered structure [314]; the fact that the segments containing phosphorylation site not only lack secondary structure but are held in place by side chain burial and also by backbone hydrogen bonds to the surrounding kinase side chains [315-320]; the fact that regions flanking the sites of phosphorylation are enriched in the disorder-promoting amino acids;[314] the fact that the sequence complexity distribution of the residues flanking phosphorylation sites

matches almost exactly the complexity distribution obtained for IDPs [314]. and the fact there is a high correspondence between the prediction of disorder and the occurrence of phosphorylation [314].

Ubiquitination, the reversible modification of proteins by the covalent attachment of ubiquitin, is implicated in the regulation of a variety of cellular processes and is involved in many diseases. Recently, 141 new ubiquitination sites were identified using a combination of liquid chromatography, mass spectrometry, and mutant yeast strains [321]. The detailed analysis of the sequence biases and structural preferences around known ubiquitination sites indicated that the properties of these sites were similar to those of IDPRs. In agreement with this computational study, structural information about the ubiquitination sites is sparse. In fact, despite the large size of PDB, only 7% of currently known ubiquitination sites in yeast could be confidently mapped to protein structures. The analysis of 3D structures of 32 homologous protein chains (with 15 of them being 100% identical with query proteins) containing 28 ubiquitination sites revealed that 10 ubiquitination sites were in crystal or interchain/intrachain contacts, and therefore the assignment of these sites to a specific structural element should be made with caution. Of the 18 sites that could be confidently assigned to ordered regions, 11 were located within coils (two of which were close to the observed disordered regions), four within helices, and three within strands. The majority of the sites within coils and helices were surface exposed and had high B-factor values indicating high flexibility [321]. The authors also pointed out that along with the lack of structural information for the majority of experimentally detected ubiquitination sites, there were several examples of ubiquitination sites located in the experimentally confirmed disordered regions [321]. Based on these observations it has been concluded that the involvement of flexible and disordered protein regions into various aspects of ubiquitination process provides a strong support for the functional importance of such regions. In addition to protease digestion, ubiquitination, and phosphorylation, several other types of PTMs, such as acetylation, fatty acid acylation, and methylation, have also been observed to occur in regions of intrinsic disorder [227, 311].

In agreement with all these earlier finding, Figure 2.1c shows that human HFS1 is not an exception and most of its experimentally verified PTM sites are located in the regions predicted to be disordered. From these findings, it is tempting to suggest that sites of protein modification in eukaryotic cells in general, and in HSF in particular, universally or at least very commonly exhibit a preference for intrinsically disordered regions. The likely explanation for this behavior is the fact that the modifying enzyme has to bind to and modify similar sites in a wide variety of proteins. If all the regions flanking these sites are disordered before binding to the modifying enzyme, it is easy to understand how a single enzyme could bind to and modify a wide variety of protein targets.

HSF1 as an Interaction Hub

As expected for such an important and conserved transcription factor, HSF1 interacts with many molecules to allow it to carry out its diverse functions over the course of its activity cycle, including transcription factors, mRNA processing factors, molecular chaperones, and modifying enzymes. These interactions are transient and dynamic due to need for precise regulation under many stressful conditions.

During the resting state, chaperones including Hsp70 and Hsp90 contribute towards keeping HSF1 in an inactive state by preventing trimerization [27, 30, 53, 54]. Stress-induced activation of HSF1 may occur in part due to chaperones being titrated away from HSF1 by accumulating unfolded proteins, thus relieving the negative inhibition and allowing HSF1 to trimerize. In addition, heat actively promotes the trimerization of HSF1 through a heat-sensitive RNA called HSFR1 which has been proposed to act as a thermosensor [322]. Heat changes the conformation of HSFR1, and together with the translation elongation factor eEF1A, HSFR1 promotes the trimerization of HSF1 [322].

In order for HSF1 to be transcriptionally active, HSF1 must be hyperphosphorylated through transient interactions with the kinases described in the previous section.

Hyperphosphorylation may allow recruitment of basal transcription factors and coactivators required for transcription. HSF1 target promoters frequently contain a paused DNA polymerase that is poised to activate transcription rapidly upon stress. *D. melanogaster* HSF1 recruits elongation factors including pTEFb that allow release of the paused polymerase [323]. In addition, chromatin remodeling factors are recruited to target promoters by HSF1. The SWI/SNF chromatin-remodeling complex interacts with the activation domain of human HSF1, mediating the chromatin architecture changes required for proper Pol II elongation [324, 325]. *D. melanogaster* HSF1 also recruits the Mediator complex and the FACT complex to facilitate chromatin remodeling and promote transcription [326, 327]. Human HSF1 has been shown to interact with coactivators such as activating signal co-integrator 2 (ASC-2) to activate transcription on specific target promoters [328]. Interestingly, HSF1 is also linked to the recruitment of mRNA processing factors as evidenced by the binding of symplekin to human HSF1 [329]. Inhibition of this interaction inhibits *hsp70* mRNA polyadenylation, suggesting that HSF1 is involved in cotranscriptional mRNA processing.

During the attenuation phase, HSF1 is under negative control by its own targets including Hsp70 and Hsp90. Hsp70 and its cochaperone Hdj1/Hsp40 bind to the activation domain of HSF1 trimers and interfere with its function [27, 54, 330]. HSF1 trimers also interact with the Hsp90-immunophilin (FKBP52)-p23 complex, resulting in a loss of transcriptional activity [331]. Additionally, acetylation of HSF1 at a conserved lysine residue within the DNA binding domain promotes this phase [26]. HSF1 can be acetylated by p300 and deacetylated by SIRT1 [26], so interactions with these factors have important implications in the regulation of the heat shock response. The unstructured regions of HSF1 likely facilitate the multitude of interactions and modifications that HSF1 participates in.

Other Heat Shock Transcription Family Members

The mammalian HSF family is composed of HSF1, 2, 3, 4, 5, Y and X [332]. While there is some redundancy, the HSF family members maintain different functionalities. Mammalian HSF1 is the ortholog of the single, essential HSF that is present in *S. cerevisiae* and *D. melanogaster*. HSF1 is the best characterized member of the HSF family and the factor responsible for the heat shock response. HSF2 and HSF4, on the other hand, have various roles in development and differentiation [333]. Mammalian HSF3, 5, Y and X have not yet been well-characterized.

HSF2

HSF2 has been shown to recruit to satellite III repeats in nuclear stress bodies (NSBs) along with HSF1 [334]. At this colocalization site, HSF2 has been indicated to play a role in the heat shock response by modulating HSF1 in a heterocomplex formation [335]. HSF2 was originally discovered in tandem with HSF1 and similarly was shown to stimulate HSE-dependent transcription *in vitro* [336, 337]. While HSF2 is capable of inducing the transcription of heat shock proteins, it is not activated by heat and therefore has not been associated with the induction of the heat shock response [337, 338]. However, HSF2 has been shown to be involved in development and cellular differentiation [338-342].

Despite low amino acid sequence homology between HSF1 and HSF2, the DNA binding domain, as well as the N-terminal and C-terminal trimerization domains, are conserved. Figure 2.6a represents the distribution of disorder propensity within the human HSF2 sequence evaluated by PONDR® VLXT (red line) and PONDR-FIT (black line). Similar to human HSF1, more than a half of HSF2 is predicted to be disordered. Similar to HSF1, HSF2 possesses several ordered functional domains, DBD (residues 7-112), HR-A/B domain (residues 119-192), and HR-C (residues 360-385). The remainder of HSF2 is mostly disordered. Figure 2.6b compares PONDR® VLXT predictions for HSF2 (red line) and HFS1 (blue line) and shows that despite

relatively low sequence homology, these two proteins possess remarkably similar disorder profiles. However, the manner of DNA-binding differs between the two proteins. HSF2 appears to regulate a different set of target genes compared to HSF1 and also experiences variable expression patterns in different tissues and cell types [338, 342]. The DNA binding-specificity of HSF1 is determined by the loop within the DNA binding domain. Chimeric HSF2 containing the HSF1 DBD loop is capable of binding to HSF1 target genes upon heat shock stress [343]. In addition, the transactivation activity of HSF2 is considerably weaker than HSF1, possibly as a result of a dispersed AD [344]. As mentioned previously, in addition to the role of HSF2 in development, evidence has indicated that HSF2 may act as a modulator of HSF1 transcription. While HSF2 has not been shown to directly regulate the heat shock response, HSF2 has been shown to interact with HSF1, is recruited to nuclear stress bodies along with HSF1, and has been shown to stimulate HSF1-mediated transcription upon heat shock stress [345].

HSF4

HSF4 exhibits limited sequence homology to the other HSF family members, but regions of similarity correspond to the DNA binding domain and the N-terminal hydrophobic repeats (HR-A/B). Like other HSF family members, HSF4 is able to bind to HSEs. However, when HSF4 is co-transfected into Cos7 cells along with a transcriptional HSE-reporter construct, HSF4 is unable to induce transcription and is therefore not a typical activator for the transcription of *hsp* genes [346]. In addition, HSF4 lacks the *cis*-regulatory domain that represses HSF1 under non-stress conditions [283].

HSF4 has also been shown to exhibit crosstalk with HSF1. Together, HSF1 and HSF4 are involved in the maintenance of sensory organs and are critical during lens development [179]. HSF4 expression is specific to the brain and lungs and has been indicated to play a role in lens development and quality control [347, 348]. Mutations of HSF4 have been shown to lead to cataractogenesis and the breakdown of the lens microarchitecture [349, 350]. Two HSF4

missense mutations have been identified by screening age-related cataract patients [351]. These mutations appear to have an effect on HSF4 DNA-binding to HSEs resulting in an under-expression of heat shock proteins in the lens, which consequently lead to an increase in protein aggregates that cause cataracts.

HSF3, 5, X and Y

HSF3 was first identified in chicken, where it is the HSF that is essential for activation of the heat shock response in this species [352]. Mammalian HSF3 has only recently been identified in mouse, and is capable of activating non-classical heat-shock genes during heat shock by binding to the PDZ domain-containing 3 (Pdzk3) promoter [175]. Although sequences related to HSF3 have also been found in the orthologous region of the human genome, this sequence is thought to be a pseudogene as no transcripts corresponding to this gene have been found [332].

HSF5 has recently been discovered as part of a large gene characterization project [180] and has been identified as a potential transcription factor within the HSF family, but has not undergone characterization. HSFY and HSFX are the only members of the HSF family found on the sex chromosomes. HSFY is present on the Y chromosome as well as murine chromosome 2. It is predominantly expressed in the testes and may potentially have a role in spermatogenesis [181-183]. Even less has been characterized regarding HSFX, but both HSFs have been found to exist as two identical copies [183].

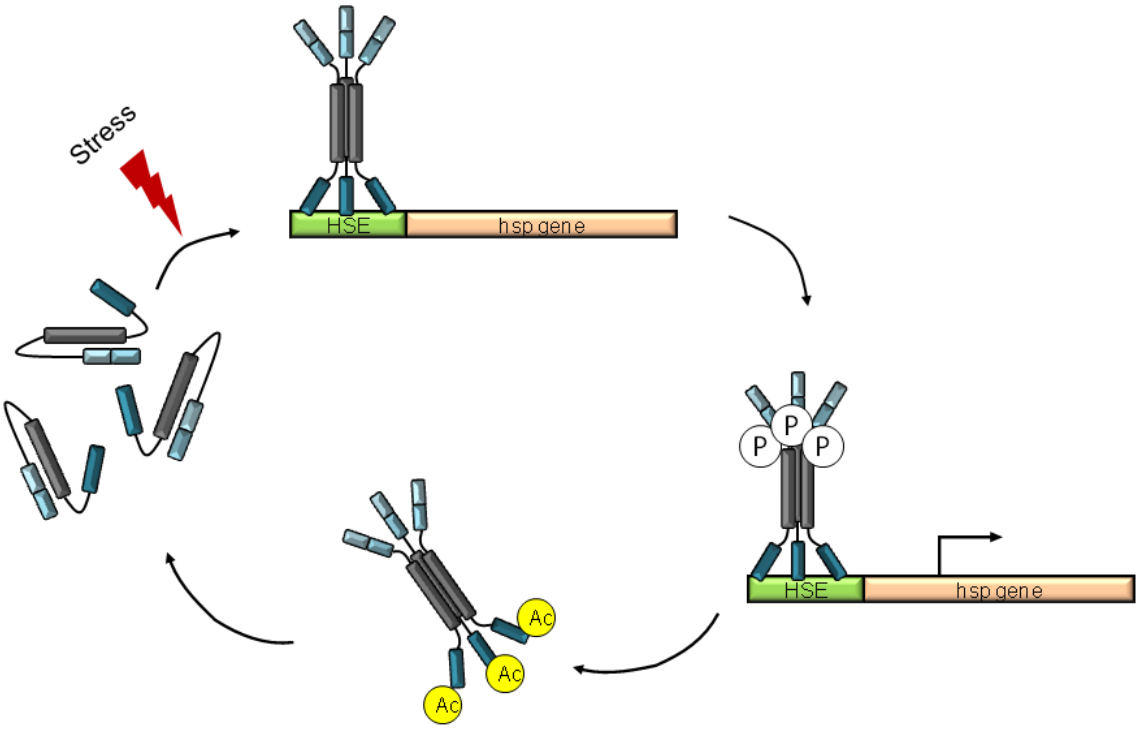

Figure 2.1. The HSF1 Activity Cycle. HSF1 in unstressed cells is a monomer in both the cytoplasm and nucleus. Following heat shock, HSF1 accumulates in the nucleus in a trimeric form and is capable of binding to heat shock elements (HSE). Transcriptional activity requires hyperphosphorylation of HSF1 by various kinases. Attenuation of the cycle involves negative feedback by chaperones as well as acetylation of a conserved lysine within the DNA binding domain. P and Ac reflects the hyperphosphorylation and acetylation sites.

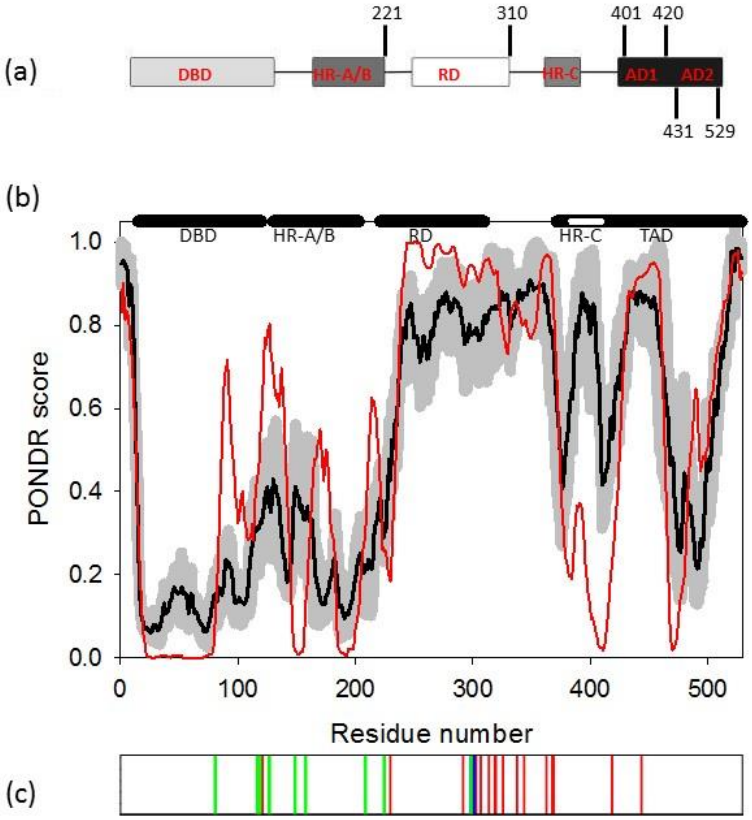

Figure 2.2. Structural Characterization of Human HSF1. (a) Domain structure of human HSF1. (b) Intrinsic disorder propensity evaluated by PONDR® VLXT (red line) and PONDR-FIT (black line). Gray shadow represents standard errors of disorder prediction by PONDR-FIT. Thick bars on the top of this plot represent localization of the major domains. PONDR scores above 0.5 correspond to predicted disordered residues. (c) Localization of PTM sites within the human HSF1 sequence. Phosphorylation, acetylation and SUMOylation sites are shown by red, green and blue bars, respectively. DBD, HR-A/B, RD, HR-C, AD1, and AD2 correspond to the DNA-binding domain, hydrophobic heptad repeat regions A/B, regulatory domain, hydrophobic heptad repeat region C, activation domain 1 and activation domain 2, respectively.

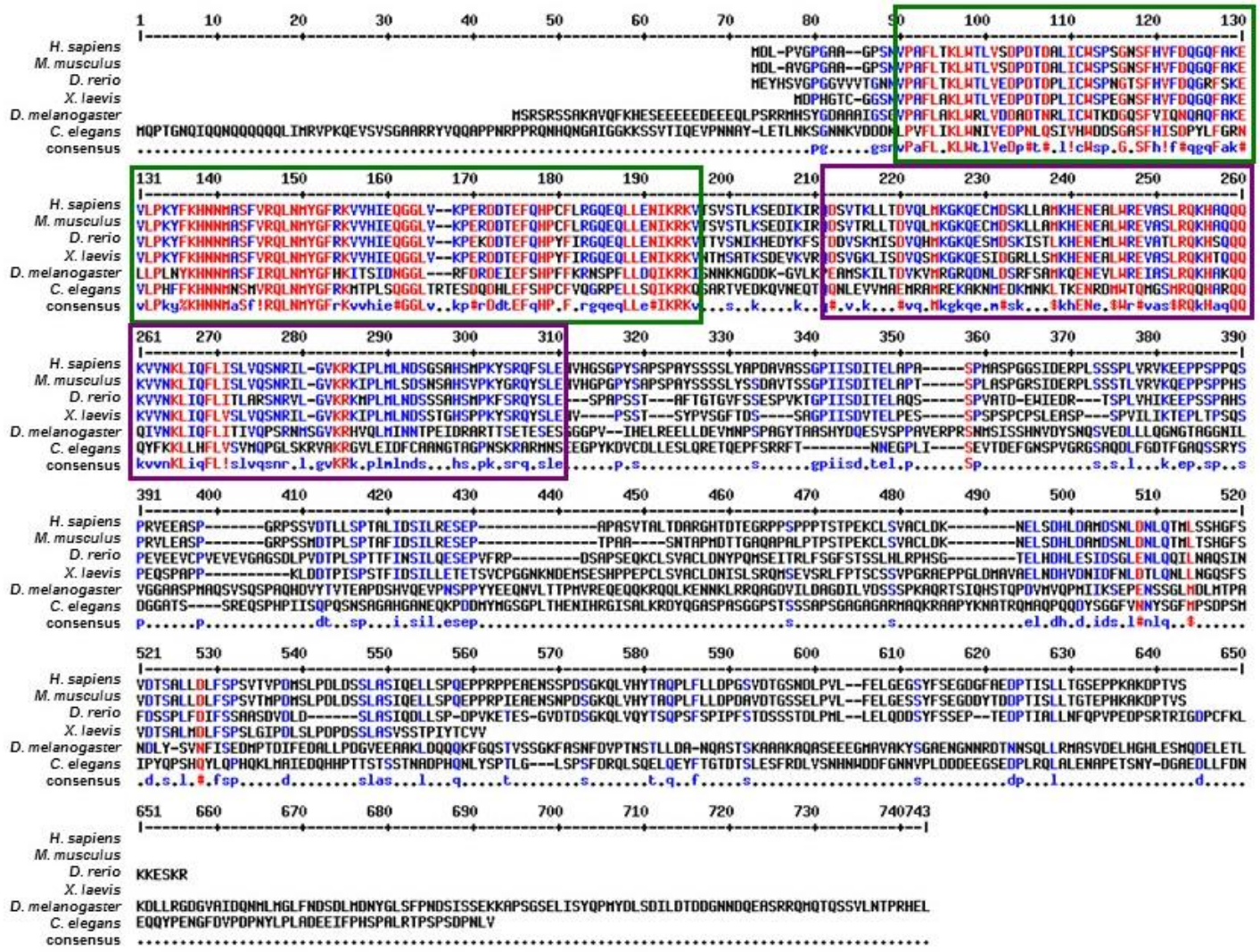

Figure 2.3. Sequence Alignment of HSF1s from Different Organisms Using BLAST. Green and violet rectangles correspond to the DBD and HRA/B, respectively.

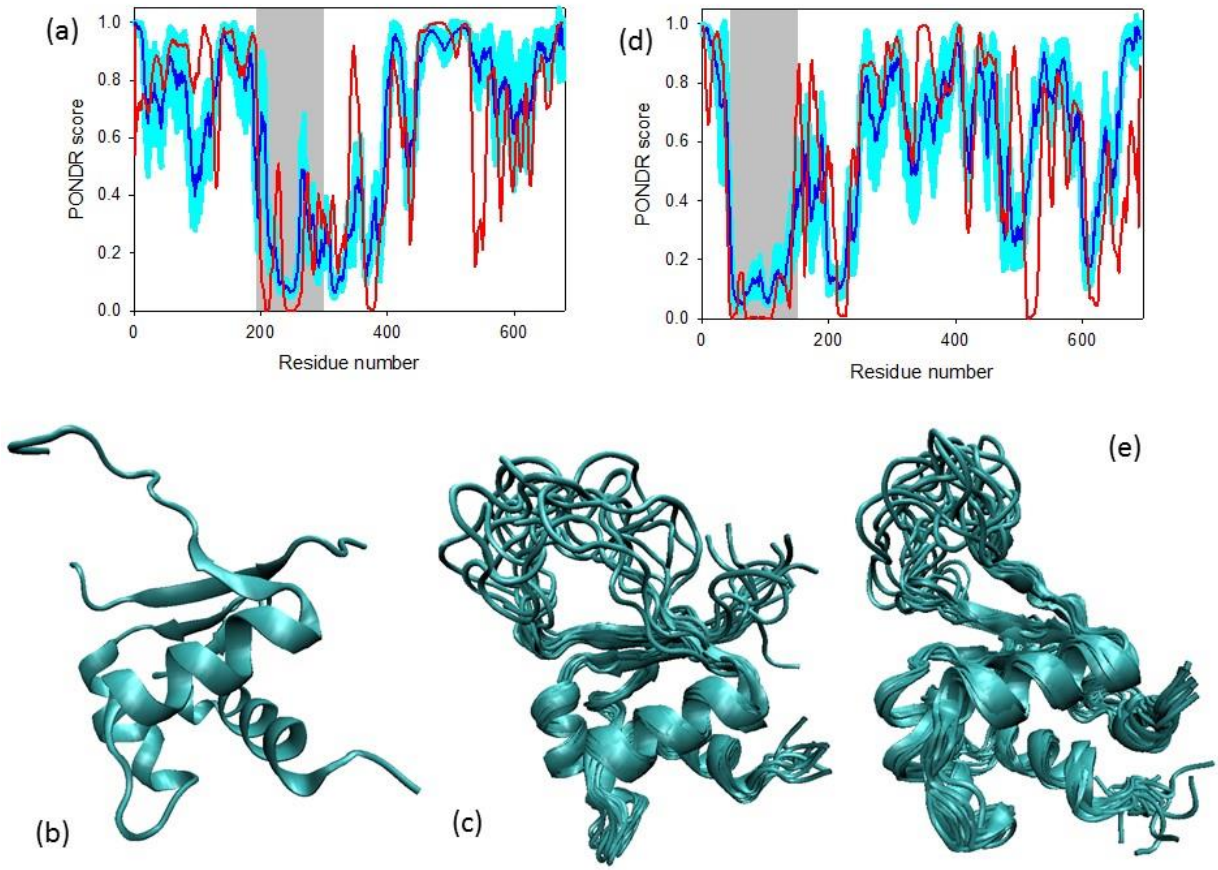

Figure 2.4. Structural Characterization of the DBDs from *Kluyveromyces lactis* (plots (a), (b) and (c)) and from *Drosophila melanogaster* (plots (d) and (e)). (a) and (d) Intrinsic disorder propensity evaluated by PONDR® VLXT (red lines) [190, 191] and PONDR-FIT (blue lines) [192]. Cyan shadow represents standard errors of disorder prediction by PONDR-FIT. DBDs are shown as shaded gray areas. (b) and (c) Crystal and solution structures of DBD of the K. lactis HSF, respectively. (e) Solution structure of the D. melanogaster HSF1 DBD.

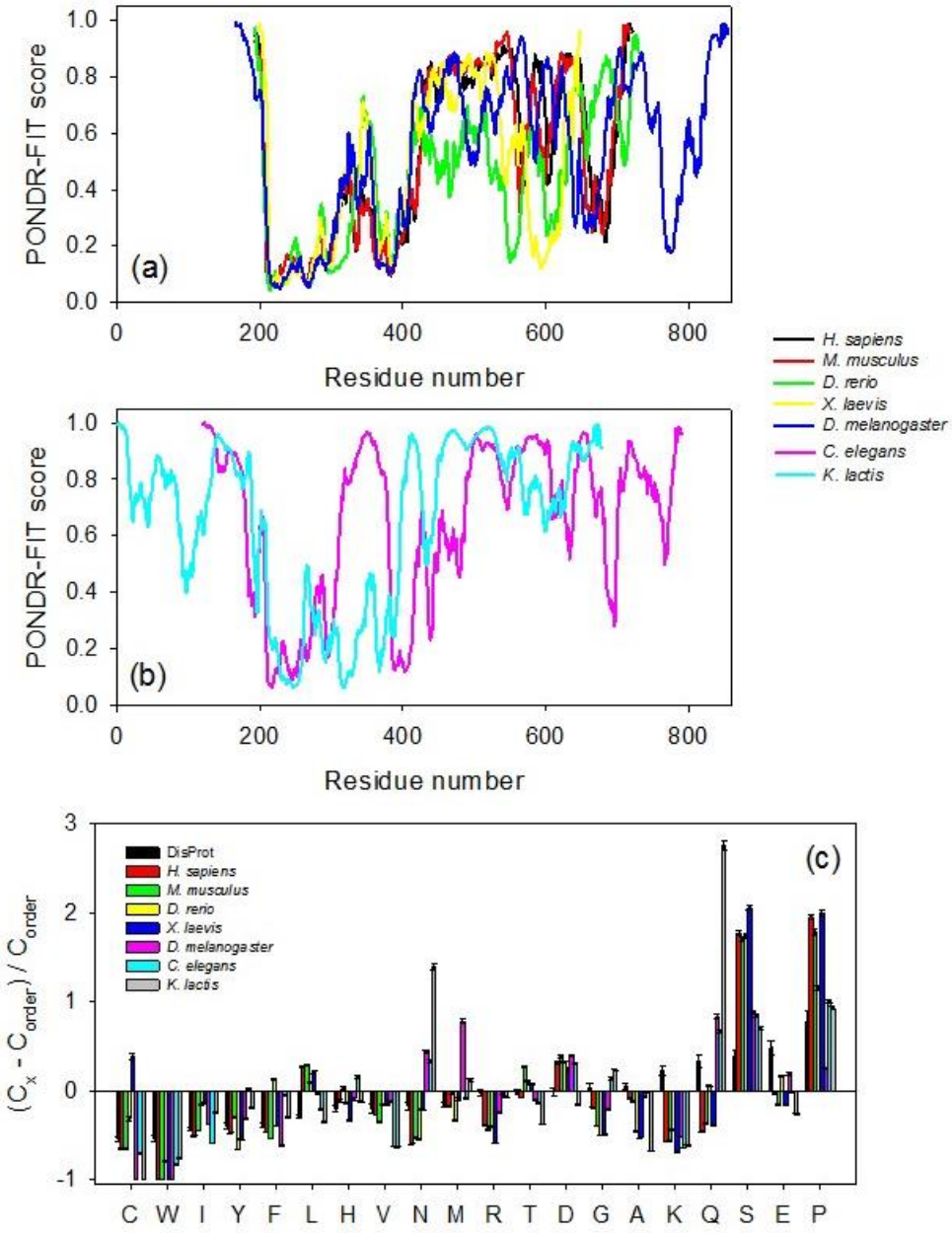

Figure 2.5. Conservation of Intrinsic Disorder in HSFs from Different Species. (a) PONDR-FIT plots for the evolutionary more-advanced organisms. (b) PONDR-FIT profiles of the evolutionary less-advanced organisms. (c) Compositional profiling of the C-terminal fragments of HSFs. The bar for a given amino acid represents the fractional difference in composition between a given protein and a set of ordered proteins. The fractional difference is calculated as (CX-Cordered)/Cordered, where CX is the content of a given amino acid in a given protein, and Cordered is the corresponding content in a set of ordered reference proteins and plotted for each amino acid [14, 193]. The amino acid residues are arranged according to the abundance of residues in a set of well-characterized IDPs from the DisProt database [194, 195]. Negative values indicate residues that a given protein has less than the reference set, positive values correspond to residues that are more abundant in a given protein in comparison with the reference set.

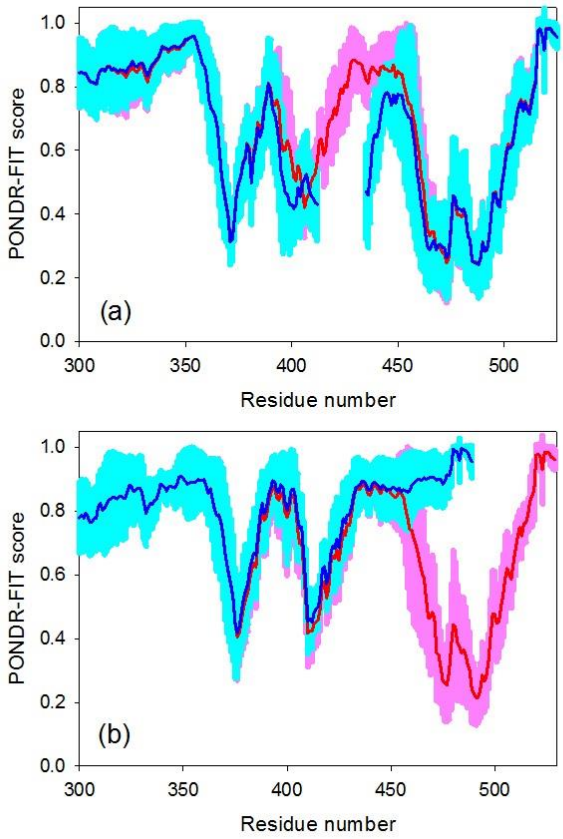

Figure 2.6. Effect of Alternative Splicing on Disorder Profiles of the C-terminal Regions of Mouse (a) and Human (b) HSF1 Proteins. Propensity for intrinsic disorder was evaluated by PONDR-FIT for canonical (red) and short (blue) isoforms of these proteins. Standard errors of disorder predictions for long and short forms are shown as red and cyan shadows, respectively.

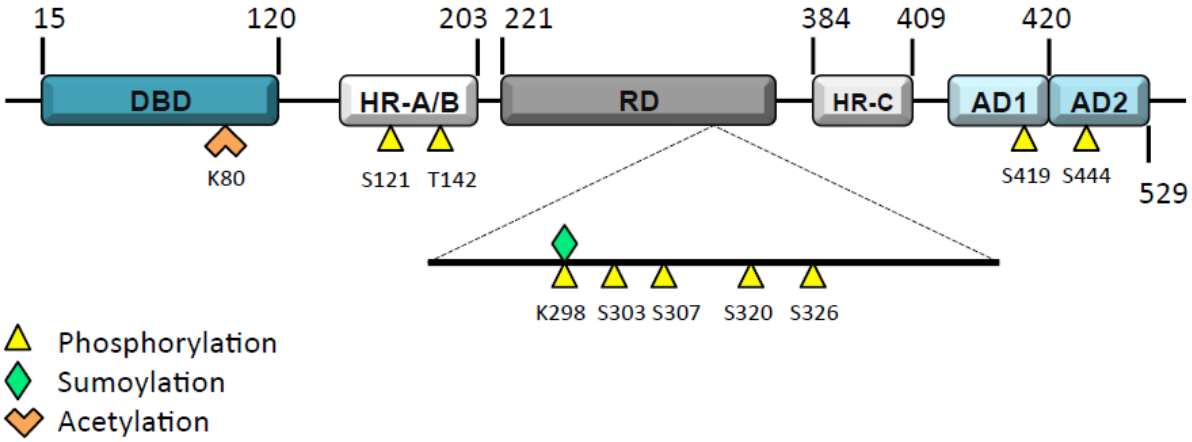

Figure 2.7. Post-Translational Modification Sites for HSF1. In addition to these sites, additional phosphorylation sites include serine residues 97, 230, 292, 314, 319, 344, and 363 [137]. While K80 is a critical lysine residue that when acetylated will cause a loss of affinity for DNA, other uncharacterized sites for acetylation include lysine residues 116, 118, 126, 148, 157, 208, 224, and 298 [110].

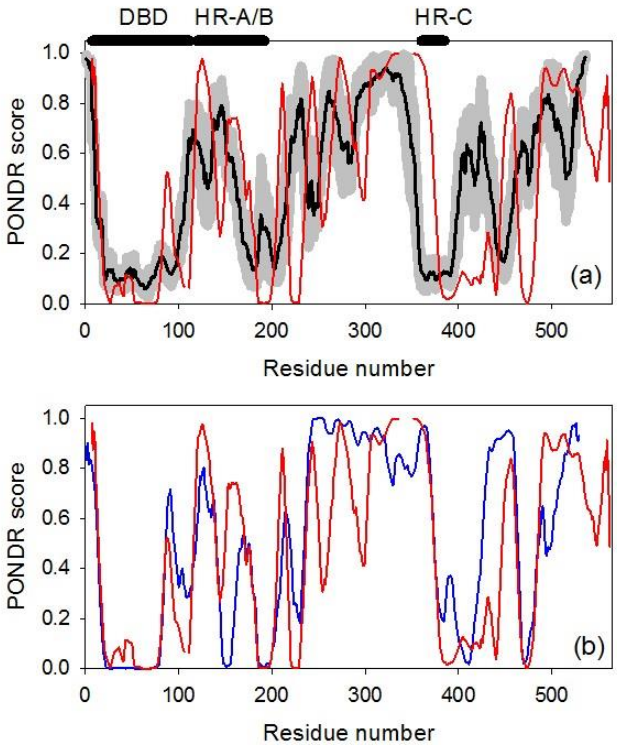

Figure 2.8. Evaluating the Intrinsic Disorder Propensity of Human HSF2. (a) Per residue intrinsic disorder propensities evaluated by PONDR® VLXT (red line) and PONDR-FIT (black line). Gray shadow represents standard errors of disorder prediction by PONDR-FIT. Thick bars on the top of this plot represent localization of the major domains. (b) Conservation of intrinsic disorder in human HSF2 (red line) and HSF1 (blue line) as evaluated by PONDR® VLXT.

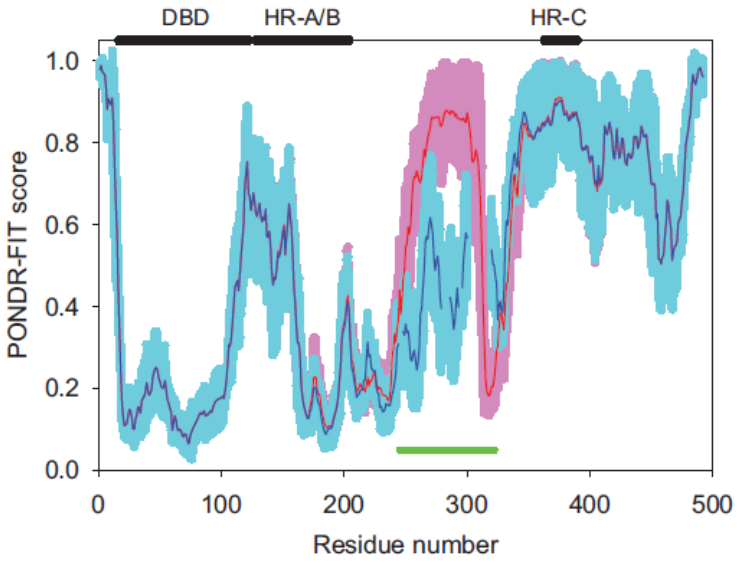

Figure 2.9. Evaluating Disorder Propensity Distribution in Human HSF4 by PONDR-FIT for Canonical (red) and Alternatively Spliced Isoforms (blue). Standard errors of disorder predictions for long and short forms are shown as red and cyan shadows, respectively.

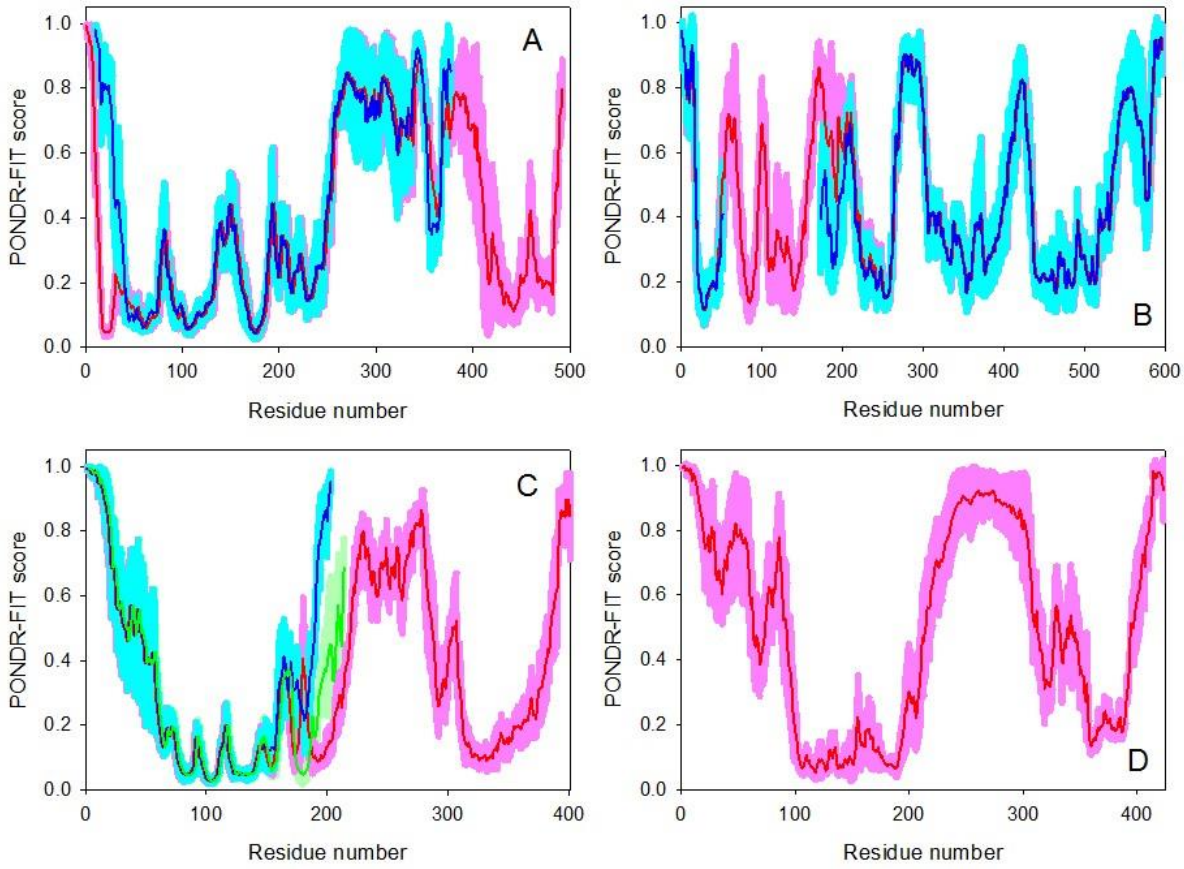

Figure 2.10. Evaluating the Intrinsic Disorder Propensity of Human HSF3 (a), HSF5 (b), HSFY (c) and HSFX (d). Propensity for intrinsic disorder was evaluated by PONDR-FIT for canonical (red) and short (blue) isoforms of these proteins. Alternatively spiced isoforms of HSFX were not described as of yet, whereas HSFY has two short alternatively spliced isoforms (shown by blue and green lines in plot (c)). Standard errors of disorder predictions for long and short forms are shown as red and cyan (and light green) shadows, respectively.

CHAPTER THREE: THE HEAT SHOCK TRANSCTIPTION FACTOR HSF1 INDUCES OVARIAN CANCER EPITHELIAL-MESENCHYMAL TRANSITION IN A 3D SPHEROID GROWTH MODEL

Authored by Chase D. Powell, Trillitye Paullin, Candice Aoisa, Christopher Menzie, Ashley Ubaldini, and Sandy D. Westerheide

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Experimental design created by C. Powell. All experiments and analysis, with the exception of qRT-PCR, were performed by C. Powell with the assistance of A. Ubaldini. T. Paullin, C. Aoisa and C Menzie performed and analyzed qRT-PCR experiment. The manuscript was written by S. Westerheide, C. Powell, and T. Paullin. See appendix D for copyright permissions.

Abstract

Ovarian cancer is the most lethal gynecological cancer, with over 200,000 women diagnosed each year and over half of those cases leading to death. The proteotoxic stress-responsive transcription factor HSF1 is frequently overexpressed in a variety of cancers and is vital to cellular proliferation and invasion in some cancers. Upon analysis of various patient data sets, we find that HSF1 is frequently overexpressed in ovarian tumor samples. In order to determine the role of HSF1 in ovarian cancer, inducible HSF1 knockdown cell lines were created. Knockdown of HSF1 in SKOV3 and HEY ovarian cancer cell lines attenuates the epithelial-to-mesenchymal transition (EMT) in cells treated with TGFβ, as determined by western blot and

quantitative RT-PCR analysis of multiple EMT markers. To further explore the role of HSF1 in ovarian cancer EMT, we cultured multicellular spheroids in a non-adherent environment to simulate early avascular tumors. In the spheroid model, cells more readily undergo EMT; however, EMT inhibition by HSF1 becomes more pronounced in the spheroid model. These findings suggest that HSF1 is important in the ovarian cancer TGFβ response and in EMT.

Introduction

Ovarian cancer is the number one cause of death related to gynecological malignancies [353]. This is partially due to a lack of physical symptoms during early cancer stages as well as shortcomings in screening techniques. In fact, a majority of newly diagnosed ovarian cancer cases present with stage III and IV disease [354]. Recent advances in surgery and chemotherapy treatment have led to improvement in short-term survival of ovarian cancer patients, however long-term survival remains bleak [355]. Conventional chemotherapy agents used to treat ovarian cancer include platinum and taxol-based drugs. While these agents are largely effective upon initial treatment, the patient commonly develops resistance to the drugs, yielding them inefficient should the patient relapse [356]. In addition, agents such as cisplatin can be toxic to the patient's organs, such as the kidneys and gastrointestinal tract, indicating a need for more efficient, as well as safer, treatment options [357].

The heat shock response (HSR), driven by the heat shock transcription factor HSF1, is a cytoprotective response to proteotoxic stressors, including heat shock, that results in the induction of various genes including molecular chaperones essential for recovery from cellular damage [278]. Chaperones function to guide protein folding and protect cells against proteotoxic stress [84]. The HSR is regulated at the transcriptional level by the heat shock transcription factor 1 (HSF1) [278].

Multiple lines of evidence suggest that HSF1 is important in promoting tumorigenesis. For instance, studies in HSF1 null mice show they are refractory to chemically-induced tumors, and HSF1 -/- mouse embryonic fibroblasts resist oncogene-induced transformation [136]. In cancer, HSF1 controls many genes that may support the transformed phenotype, including genes involved in cell-cycle regulation, signaling, metabolism, adhesion and translation [133]. HSF1 is elevated in breast, colon, lung and hepatocellular cancers, and activated or elevated HSF1 often couples with poor cancer prognosis [129, 133].

The dissemination of primary tumors occurs through a multi-step process called the epithelial-to-mesenchymal transition (EMT). EMT consists of detachment of primary tumor cells, infiltration of local stroma, spread through cavities or vascular and lymphatic vessels, and adhesion followed by colonization at distant sites [358]. Sweeping changes are made in the cytoskeleton and extracellular matrix during EMT, and cells develop a spindle-like morphology. TGFβ inhibits proliferation in normal tissues, but this effect is lost in advanced cancer where it strongly promotes EMT [359]. The expression of a number of transcription factors are induced by TGFβ and support the EMT process, including SNAI2/SLUG, SNAI1/SNAIL, TWIST1 and ZEB1 [358]. Once the mesenchymal-like cell has migrated into a new organ, it can then undergo the reverse mesenchymal-to-epithelial transition (MET) and begin to form a secondary tumor [360].

Here, we have established two ovarian cancer inducible HSF1 knockdown cell lines to study the effect of HSF1 on ovarian cancer. We show that HSF1 knockdown inhibits colony formation, wound healing, migration and the induction of FN1/fibronectin, a protein important in the EMT process. We also show that the induction of EMT markers by TGFβ is enhanced when cells are grown as 3D spheroid cultures vs. 2D monolayer cultures. Upon 3D culturing, there is a marked effect of HSF1 on the induction of transcription factors known to promote EMT. HSF1 knockdown also alters spheroid morphology. Thus, we conclude that HSF1 plays a striking role in regulating the EMT process under 3D growth conditions.

Materials and Methods

HSF1 Copy Number, Expression Determination and Survival Analysis

Data comparing HSF1 copy number across multiple cancers with GISTIC analysis was obtained from The Cancer Genome Atlas (TCGA) via the cBio portal [361, 362]. HSF1 expression levels across multiple cancers were assessed from TCGA RNA seq V2 data via the cBio portal. Data for the comparison of ovarian cancer and normal ovarian tissue were obtained from GEO and the TCGA. The datasets analyzed were: GSE18520, consisting of 10 normal ovary and 53 ovarian cancer samples assayed on Affymetrix HG-U133 Plus 2.0 GeneChips, and TCGA data, consisting of 8 normal ovary and 568 ovarian cancer samples assayed on Affymetrix HG-U133A GeneChips. Gene intensity was compared by one-sided unpaired T-test.

Cell Culture and Treatments

HEY, SKOV3 and T80 cells were authenticated using short tandem repeat (STR) DNA profiling (Genetica, Inc.) and comparing profiles to ATCC profiles and other previously published profiles [363]. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO) and 1% Pen-Strep-Glutamine (CellGro) in a humidified incubator at 37^{o}C with 5% CO_2. Heat shock treatment was performed by wrapping plates in parafilm and submerging them in a 42^{o}C circulating water bath for designated times. Cells were treated as indicated with 1 µg/ml doxycycline (Sigma-Aldrich) and 5 ng/mL TGFβ1 (Thermo Fisher).

Lentiviral Creation and Infection for Stable, Inducible shRNA-mediated HSF1 Knockdown

To allow for inducible knockdown of HSF1, we utilized the doxycycline-inducible TRIPZ shRNAmir system (Thermo Scientific). Two shRNA sequences targeting HSF1 were obtained from the RNAi codex database [364]: CGCAGCTCCTTGAGAACATCAA (shHSF1A) and CCCACAGAGATACACAGATATA (shHSF1B). These two sequences were cloned into the pTRIPZ vector. For lentiviral packaging, a 2nd generation lentiviral system was used with the pCGP packaging and pVSVG envelope plasmids (Addgene). HEK293T cells, cultured in RPMI medium, were used as the packaging cell line. Transfection was achieved using Polyfect Transfection Reagent (Qiagen) according to the manufacturer's protocol using a 1:1:1 ratio of lentiviral vectors. 24 hours post-transfection, medium with transfection reagent was removed and replaced with fresh RPMI. Medium containing viral stock from the HEK293T cells was harvested 48 hours post-transfection. A 0.45 micron PVDF filter was used to filter viral stock and infection of the HEY and SKOV3 cell lines was performed in a single round with the addition of 8 µg/mL of hexadimethrinebromide (Sigma-Aldrich). Selection of stable HEY and SKOV3 cells was achieved with 1 µg/ml and 0.5 µg/ml of puromycin (Thermo Fisher) for the HEY and SKOV3 cell lines, respectively. Infection was verified via immunoblotting analysis for knockdown of HSF1 after doxycycline-induced expression of the shRNAs.

Protein Isolation, SDS-PAGE, and Western Analysis

Cells were washed once and scraped in chilled PBS. After pelleting the cells, protein was extracted using the M-PER lysis buffer (Thermo Scientific) with a protease inhibitor cocktail (Halt^{TM} Protease Inhibitors, Thermo Scientific). A Bio-Rad Protein Assay was then utilized to quantify protein concentrations. 20 µg of lysate was resolved on 8% to 12% sodium dodecyl sulphate

polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to Immun-Blot® 0.2µm PVDF Membrane with a Trans-Blot semi-dry transfer cell (Bio-Rad). Membranes were blocked in 2% w/v non-fat milk in TBS with 0.1% Tween (TBST milk). Blots were probed with primary and secondary antibodies before incubation in ECL Prime Western Blotting Detection System (Amersham™) and film exposure. Primary antibodies used were: HSF1 (Assay Designs), HSF1 P-S326 (Abcam) fibronectin (BD Biosciences), HSP90 (Cell Signaling), HSP70 (Cell Signaling) and Actin (Santa Cruz). HRP-conjugated secondary antibodies were from Millipore and Jackson ImmunoResearch.

Cell Viability Assay

Cells at a concentration of 2 x 10^5 cells/ml were seeded in a 96-well plate at 100 µl per well with eight replicates for each test condition. The cells were then incubated either with or without doxycycline treatment for 72 hours. After incubation, 10 µl of PrestoBlue® Cell Viability Reagent (Invitrogen) was added to each well and incubated for 1 hour at 37°C. The reduction of the reagent was measured by fluorescence (excitation 570 nm, emission 600 nm) using a microplate reader (BioTek®). Mean percent viability and standard error were then plotted.

Clonogenic Assay

Cells were seeded at 500 cells per well in 6-well plates and were treated with or without 1 µg/ml doxycycline to induce HSF1 knockdown. Treated wells were given an additional treatment with 1 µg/ml doxycycline on day 4 to maintain doxycycline levels. After 8 days, colonies were stained with 1% crystal violet (w/v) in methanol and rinsed 3X in deionized water. Stained colonies were subsequently photographed and counted.

Wound Healing Assay

Cells were plated at 3 x 10^5 cells per well in a 6-well plate, and then either treated with 1 μg/ml doxycycline 48 hours prior to the assay or left untreated. Once the cells reached confluency, a 2 μl pipet tip was used to scrape the cells in 2 vertical and 2 horizontal lines yielding 4 intersections per well. Cells were washed twice with PBS to remove debris and serum-free medium was added. Pictures were taken immediately and again 12 hours after the creation of the wound, using an EVOS inverted microscope (Advanced Microscopy Group). The experiment was performed in triplicate and wound closure was determined using TScratch software [365]. Significant differences were calculated by ANOVA and Bonferroni post-hoc tests.

Cell Migration

For the transwell migration assay, cells were treated with or without 1 μg/ml doxycycline 48 hours prior to the assay to induce HSF1 knockdown, and cells were then serum-starved 24 hours before performing the assay. Cells were then resuspended in serum-free medium, and seeded at 2.5 x 10^4 cells per upper chamber. 400 μL of complete medium containing FBS was added as a chemoattractant to the lower chamber. After a 16-hour incubation, non-migrating cells on the upper surface of the filter were removed by scrubbing with a cotton swab. The remaining cells on the lower surface were fixed and stained with 1% (w/v) crystal violet in methanol. Migrated cells were counted from 10 random fields of view from each well and each condition was performed with triplicate samples. Statistical analysis done by paired t-test.

Spheroid Formation

The hanging drop method was utilized to form spheroids [366]. Briefly, cells released with trypsin were resuspended at 1 X 10^6 cells/mL in RPMI medium, supplemented as described

above. Cell suspension droplets of 25 µl were placed on the plate lids, which were then inverted and put back on plates containing phosphate buffered saline (PBS) and incubated for 48 hours. Upon incubation, cells aggregated into spheroids. Prior to plating the cells, TGFβ1 was added to the suspension as indicated at a final concentration of 5 ng/mL. Following aggregation for 48 hours, spheroids were collected in 1X PBS. Pictures were obtained using an EVOS (Advanced Microscopy Group) inverted microscope.

Quantitative RT-PCR

Cells were harvested in cold 1X PBS and RNA extraction was completed utilizing the TRIzol reagent (Thermo Fisher) according to standard protocol. Reverse transcription reactions of the RNA were performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem), as per the manufacturer's protocol. The cDNA samples were then used as a template for qRT-PCR. Applied Biosystem's Step One Plus Real-time PCR machine was used with BioRad's iTaq™ Fast SYBR® Green Supermix with ROX according to the manufacturer's protocol. The primer sets used for each gene can be found in Supplementary Table 1. GAPDH was used as the endogenous reference control. Statistical significance was measured by Student's T-test.

Results

HSF1 is Overexpressed in Ovarian Cancer

We analyzed data from The Cancer Genome Atlas (TCGA) database to compare HSF1 levels across multiple cancer types. Interestingly, we find that HSF1 gene duplication is more common in ovarian cancer than in any other cancer type in this database by a large margin (Fig. 3.1A). Additionally, we find that HSF1 mRNA transcripts are elevated in ovarian cancer tumor

tissue vs. normal epithelial tissue from matched patient samples (Fig. 3.1B). Other cancers with high HSF1 mRNA levels include liver cancer, head and neck cancer, and breast cancer (Fig. 3.1B). Two distinct data sets of matched ovarian tumor tissue vs. normal tissue show that HSF1 mRNA expression is significantly higher in tumor tissue (Fig. 3.1C-D). Given this data, we postulate that HSF1 may drive ovarian cancer progression. We thus sought to study the effect of HSF1 knockdown in ovarian cancer cell lines.

Establishment of SKOV3 and HEY Inducible HSF1 Knockdown Ovarian Cancer Cell Lines

We chose two epithelial ovarian cancer cell lines for our studies, SKOV3 and HEY. These cell lines were authenticated by using short tandem repeat (STR) DNA profiling (Genetica, Inc.) and comparing the profiles to ATCC profiles and other previously published profiles [363]. We first wanted to test whether the cell lines we selected exhibited a normal response to heat, including the characteristic activation of HSF1 and induction of chaperones. We find that both SKOV3 and HEY cells exhibit multiple hallmarks of activation of the heat shock response (Figure 3.2A).

Upon treatment with a 42°C heat shock over a 6 hour timecourse, we observe stress-induced hyperphosphorylation of HSF1 followed by a return to the hypophosphorylated state. This result is characteristic of HSF1 activation by heat shock and can be readily detected by electrophoretic retardation on SDS-PAGE and Western blot analysis [367]. Interestingly, while SKOV3 cells contain a similar level of basal and activated HSF1 as compared to normal ovarian epithelial T80 cells, HEY cells express higher levels of HSF1, corresponding to the higher levels of HSF1 expression we identified in ovarian cancer patient databases. Upon heat shock, we also observed that both SKOV3 and HEY cells show HSF1 phosphorylation at serine 326, a marker of activated HSF1 [41]. Additionally, the chaperone HSP70 was induced by heat shock in both

SKOV3 and HEY cells, and HSP90 was induced in HEY cells. Overall, we conclude that both SKOV3 and HEY cells express HSF1 and respond to heat shock, validating the choice of these two ovarian cancer cell lines for our studies.

We next wanted to generate HSF1 knockdown SKOV3 and HEY cell lines. Our initial attempts to create stable HSF1 knockdown in these cell lines were not successful, perhaps due to selective pressure for the cancer cells to re-express HSF1. We therefore employed a doxycycline-inducible shHSF1 system (pTRIPZ vector, Open Biosystems). To ensure that doxycycline treatment alone would not alter HSF1 levels or activity, we treated SKOV3 and HEY cells with both 0.5 and 2.0 μg/ml of doxycycline for 48 hours and found no changes in HSF1 levels or hyperphosphorylation status (Figure S.1). We also found no change in HSP90 levels (Figure S.1). We therefore concluded that a doxycycline-inducible system would be a viable option for HSF1 knockdown in our studies.

We used two shHSF1 sequences obtained from the RNAi codex database [364], shHSF1A (CGCAGCTCCTTGAGAACATCAA) and shHSF1B (CCCACAGAGATACACAGATATA), as well as a control sequence that is non-targeting, to create SKOV3.shControl, SKOV3.shHSF1A, SKOV3.shHSF1B, HEY.shControl, HEYshHSF1A and HEY.shHSF1B stable cell lines (Figure 3.2B).

The shHSF1A sequence knocks down HSF1 expression by about 75%, while shHSF1B knocks down HSF1 expression more completely. Knockdown of HSF1 resulted in only a marginal reduction of cell viability in the SKOV3 or HEY cell lines over a 72 hour doxycycline treatment time course (Figure 3.2C). We thus have established an effective means of knocking down HSF1 to varying degrees in two different ovarian cancer cell lines.

HSF1 Knockdown Inhibits Colony Formation, Wound healing, Cell Migration and Fibronectin Expression

We then assayed our HSF1 knockdown cell lines to determine whether HSF1 is important for ovarian cancer tumorigenicity. As a measure of the ability of HSF1 to allow cell survival and growth upon plating at a low cell density, clonogenic assays were performed (Fig. 3). SKOV3.shControl, SKOV3.shHSF1B, HEY.shControl and HEY.shHSF1B stable cells were treated with or without doxycycline to induce HSF1 knockdown and then plated at 250 cells per well in 6-well plates in triplicate. Colonies, stained after 8 days, show that HSF1 knockdown strongly inhibits colony formation in both HEY and SKOV3 cells.

To assess the ability of HSF1 to affect cellular motility, we used a wound healing assay as well as a cell migration assay. For the wound healing assay, cells were seeded in equal numbers into 6-well plates and grown to approximately 80% confluence prior to introducing scratches in straight lines through the monolayers. TScratch software was then used to automatically analyze wound healing rates (Figure 3.4A).

HSF1 knockdown in SKOV3 and HEY cells inhibits wound-healing ability by 25% and 28%, respectively. Next, cell migration assays were employed to assess the ability of cells to pass through a matrigel-coated transwell membrane (Fig. 4B). Cells were seeded in equal numbers into the insert of a transwell plate, with no cells in the lower chamber. The number of cells that passed through the membrane were then calculated and plotted after 48 hrs. HSF1 knockdown was found to inhibit cell migration by 29% in SKOV3 cells and 33% in HEY cells. These experiments in sum support a role for HSF1 in promoting cell motility in ovarian cancer.

We next wanted to test whether HSF1 knockdown can suppress the EMT process. Fibronectin, a mesenchymal marker, is upregulated during EMT and plays a crucial role in altering cell adhesion and migration processes, allowing for transition to the mesenchymal state [368].

We tested protein expression levels of fibronectin using Western blot analysis of SKOV3.shHSF1B and HEY.shHSF1B cells treated with and without doxycycline and with and without the EMT inducer TGFβ (Figure 3.4C). As expected, TGFβ treatment induces fibronectin expression (Figure 3.4C, compare lanes 1 with lanes 3). Interestingly, HSF1 knockdown in both SKOV3 and HEY cells reduces both the basal expression levels of fibronectin (Figure 3.4C, compare lanes 1 and 2) as well as the TGFβ-induced levels of fibronectin (Figure 3.4C, compare lanes 3 and 4). Therefore, HSF1 may promote the EMT process by enhancing TGFβ-induced fibronectin expression.

The induction of Fibronectin by TGFβ is Enhanced in 3D Cultures as Compared to 2D Cultures

As ovarian cancer cells typically spread throughout the peritoneal cavity in the form of 3D spheroids, we cultured cells in 3D culture using the hanging drop method [369] in order to create a more biologically-relevant *in vitro* system for our studies. We first tested whether the induction of fibronectin by TGFβ is altered in 3D cultures as compared to 2D cultures.

In both monolayer and spheroid SKOV3 cells, TGFβ increased fibronectin expression (Figure 3.5). Surprisingly, this effect was enhanced in the SKOV3 spheroid model as compared to monolayer cells (Figure 3.5, compare lanes 2 and 4). The HEY cells also showed enhanced fibronectin expression upon 3D growth, although this effect was not enhanced by TGFβ. Therefore, we conclude that 3D culturing enhances fibronectin expression.

3D Culturing Reveals a Marked Effect of HSF1 on the Induction of EMT Transcription Factors

Various transcription factors, including snail, slug, twist, and zeb, help to coordinate the EMT process [358]. We tested whether 3D growth affected the expression of these genes (Fig.

6). We find that 3D growth enhances TGFβ induction of these transcription factors as shown by qRT-PCR (Fig. 6, compare lanes 2 and 4). We wondered whether HSF1 may regulate the expression of these EMT transcription factors. We thus tested our HSF1 knockdown cell lines, grown under both 2D and 3D conditions, to test for effects on the expression of *SNAIL, TWIST1, SLUG* and *ZEB1* mRNAs (Figure 3.6). SKOV3.shControl, SKOV3.shHSF1B, HEY.shControl, and HEY.shHSF1B stable cell lines, grown both as 2D and 3D cultures, were treated with and without doxycycline treatment to induce HSF1 knockdown. We find that HSF1 knockdown in most cases slightly inhibits the expression of EMT transcription factors in SKOV3 and HEY cells grown in 2D (Fig. 6, compare lanes 2 with lanes 3). Interestingly, the effect of HSF1 knockdown on the expression of these genes is magnified for most of the genes upon growth in 3D conditions (Figure 3.6, compare lanes 4 with lanes 5). Therefore, using a 3D ovarian cancer culturing system, we have uncovered a positive effect of HSF1 on the ability of TGFβ to induce EMT genes. We thus conclude that HSF1 promotes EMT in ovarian cancer 3D spheroids at least in part through regulating the levels of EMT-inducing transcription factors.

Discussion

As ovarian cancer is highly lethal and has few treatment options, identifying new therapeutic targets for this disease is highly important. Through mining patient data, we find that HSF1 DNA levels are most highly amplified in ovarian cancer as compared to other cancers, and also that ovarian cancer is one of the top cancer types with amplified HSF1 mRNA levels. A previous study of 37 malignant vs. benign ovarian tumors has shown that HSF1 expression is higher in the malignant tumors [132]. Our findings thus add to this data and suggest that HSF1 may be an important therapeutic target for ovarian cancer.

We have identified HSF1 as a critical player in promoting ovarian cancer tumorigenicity by multiple measures in both SKOV3 and HEY ovarian cancer cells. Via HSF1 knockdown and

colony formation assays, we show that HSF1 promotes the ability of cells to grow under conditions of low cell density, a hallmark of cancer cells. Cell motility is another characteristic of cancer cells. Previous work has shown that cell motility is inhibited in immortalized mouse embryonic fibroblast cells derived from *hsf1* -/- mice [370]. In addition, HSF1 knockdown reduces the invasiveness of multiple types of tumor cells [129, 371-374]. Consistent with these findings, we show that HSF1 knockdown inhibits wound healing and cell migration in SKOV3 and HEY ovarian cancer cell lines. Our results thus add further evidence that HSF1 enhances tumorigenicity in multiple types of cancer.

EMT is essential for cell migration and is a key rate-limiting step in metastasis. Previous studies have shown that HSF1 promotes EMT in breast cancer cells through a mechanism that requires HER2 [140, 375]. As ovarian cancer cells typically spread throughout the peritoneal cavity in the form of 3D spheroids [376], culturing ovarian cancer cells as spheroids is likely to better mimic the *in vivo* growth conditions as compared to conventional 2D culturing conditions. Here, we show that HSF1 knockdown reduces the ability of TGFβ to induce EMT. Interestingly, we find that this effect is stronger upon growth in 3D spheroids. We also show that HSF1 is required for the compact morphological structure of spheroid growth.

Our data suggests that HSF1, either directly or indirectly, controls the expression of transcription factors that are important for the EMT process. Interestingly, upon promoter analysis, we find consensus heat shock element (HSE) sequences containing three inverted arrays of the sequence nGAAn [377] in the promoters of the EMT transcription factor genes *SNAIL, ZEB* and *TWIST1* (Table S2). Putative HSEs are also present in the *FN1* (*fibronectin*)*, VIM* (*vimentin*)*, and CDH2* (*N-cadherin*) promoters, additional genes that are associated with EMT (Table S2). Future experiments will be required to determine whether any of these genes are direct HSF1 targets. This is plausible given that HSF1 was recently found to bind to the *SLUG* promoter through an imperfect HSE motif [375].

In summary, we have identified HSF1 as a critical player in ovarian cancer progression, and have identified EMT as a process that is promoted by HSF1. The effects for HSF1 are more striking when cells are grown as 3D spheroids, which more closely mimic the *in vivo* growth conditions of ovarian cancer. Therefore, HSF1 deserves further research and development as a promising anticancer strategy for ovarian cancer.

Acknowledgements

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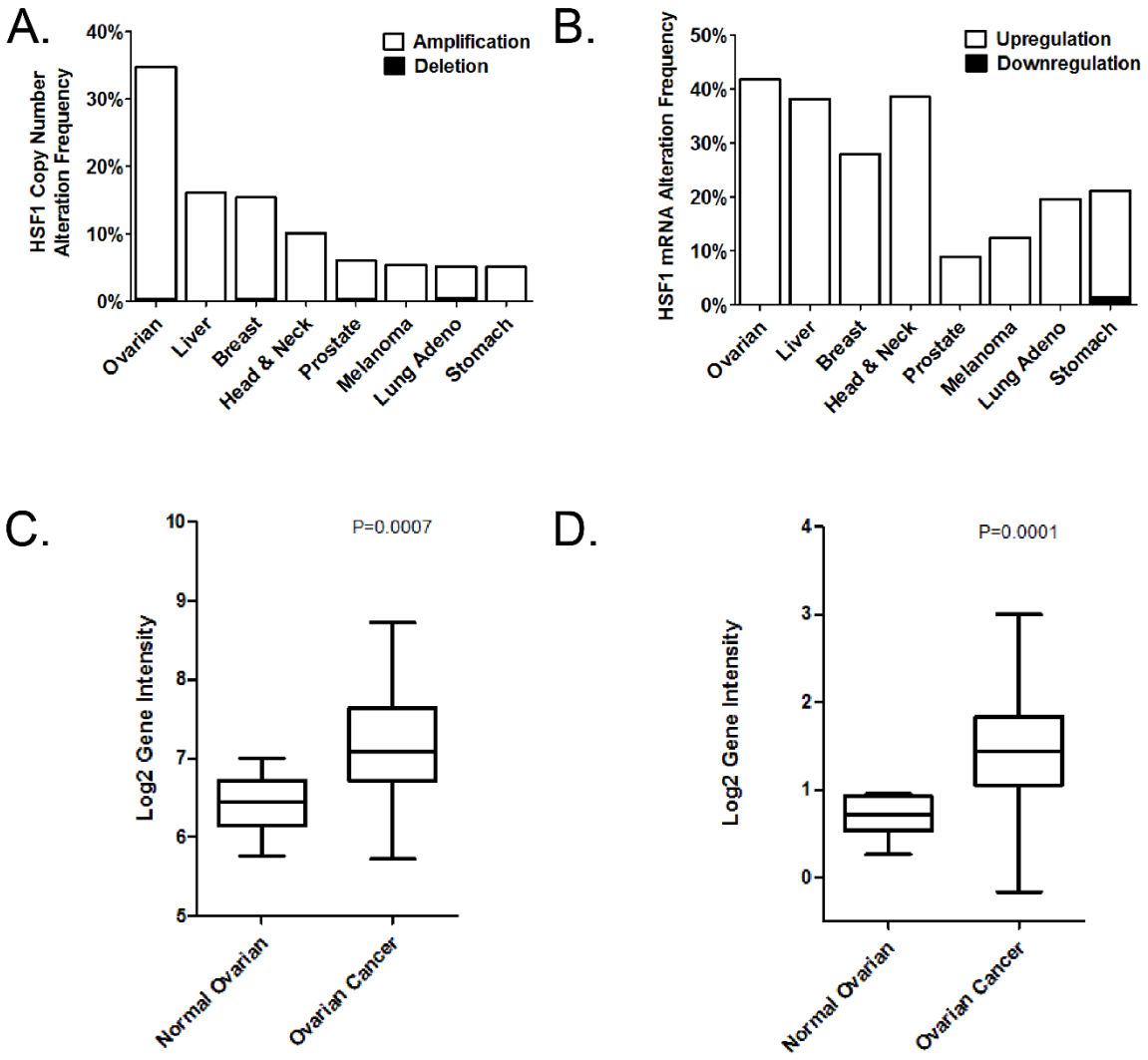

Figure 3.1. HSF1 Levels are Elevated in Ovarian Cancer Patient Samples. A, HSF1 copy number is increased most frequently in ovarian cancer. HSF1 copy number was analyzed in a variety of cancers using TCGA data and GISTIC analysis with a threshold CNA change of +/-2. B, HSF1 transcripts are elevated in a variety of cancers. Samples from tumor tissue and matched normal tissue were compared in the TCGA database using RNA Seq V2 RSEM data with a z-score threshold of +/-2. C, HSF1 is increased at the mRNA level in an ovarian cancer data set GSE18520 consisting of 10 normal ovarian samples and 53 late stage, primary site, high grade ovarian cancer samples. D, HSF1 is increased at the mRNA level in a TCGA ovarian cancer data set consisting of 8 normal ovarian samples and 568 ovarian cancer samples.

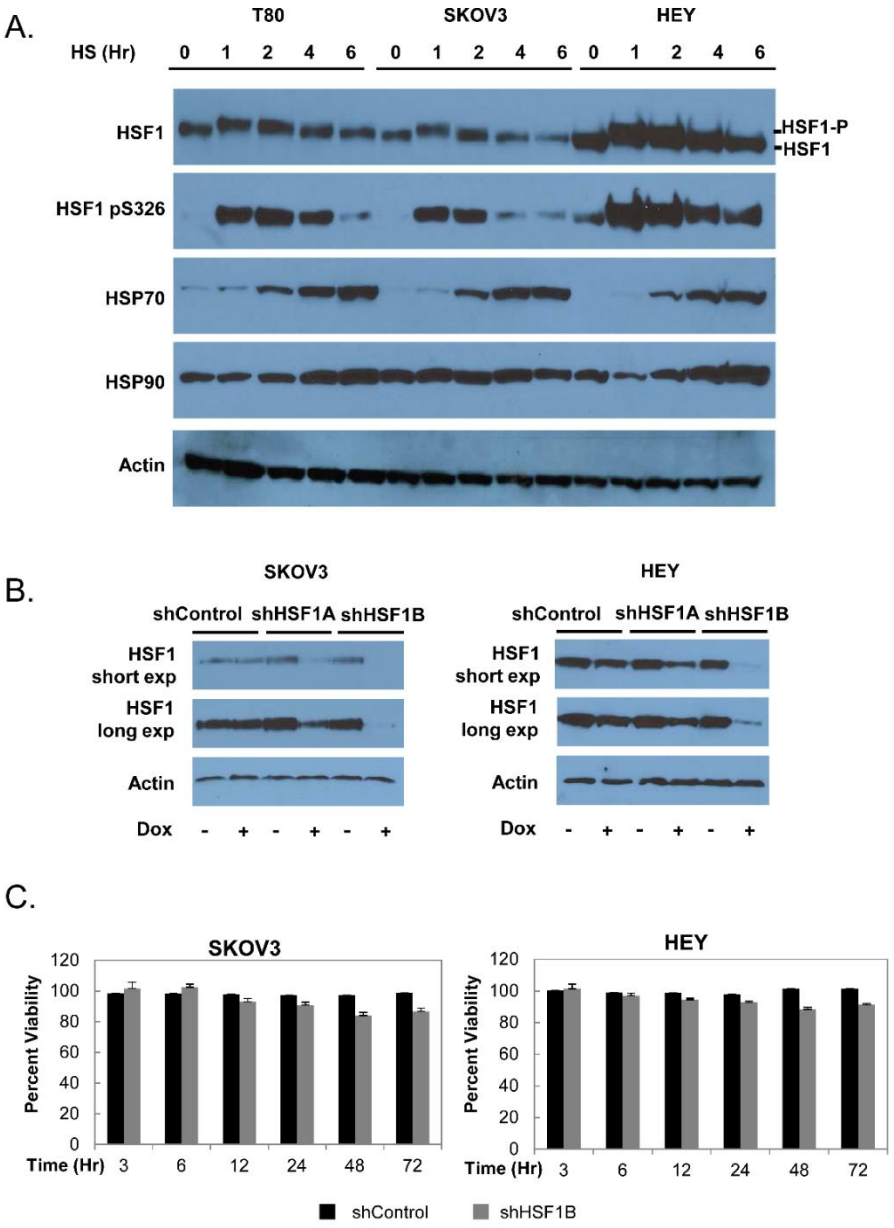

Figure 3.2. Validation of Inducible HSF1 Knockdown Ovarian Cancer Cell lines. A, The heat shock response in the epithelial ovarian carcinoma cell lines SKOV3 and HEY as compared to normal ovarian epithelial T80 cells. T80, SKOV3, and HEY cells were treated with a 42°C heat shock for the indicated times and harvested immediately after. Cell lysates were subjected to Western blot analysis using antibodies recognizing HSF1, HSF1 phosphorylated at S326, HSP90, HSP70, and actin as a loading control. B, The pTRIPZ system was used to create the doxycycline-inducible HSF1 knockdown cell lines SKOV3.shHSF1A, SKOV3.shHSF1B, HEY.shHSF1A and HEY.shHSF1B. After treatment with 1 µg/ml doxycycline for 48 hours, cell lysates were subjected to Western blot analysis using antibodies recognizing HSF1 and actin as a loading control. Both short and long exposures are shown for the HSF1 blot. C, HSF1 knockdown does not cause a large decrease in cell viability. The viability of the SKOV3.shHSF1B and HEY.shHSF1B cell lines as compared to shControl cells was assessed after treatment with 1 µg/ml doxycycline for the indicated times using the PrestoBlue cell viability assay. Mean percent viability (n=8) and standard error is shown.

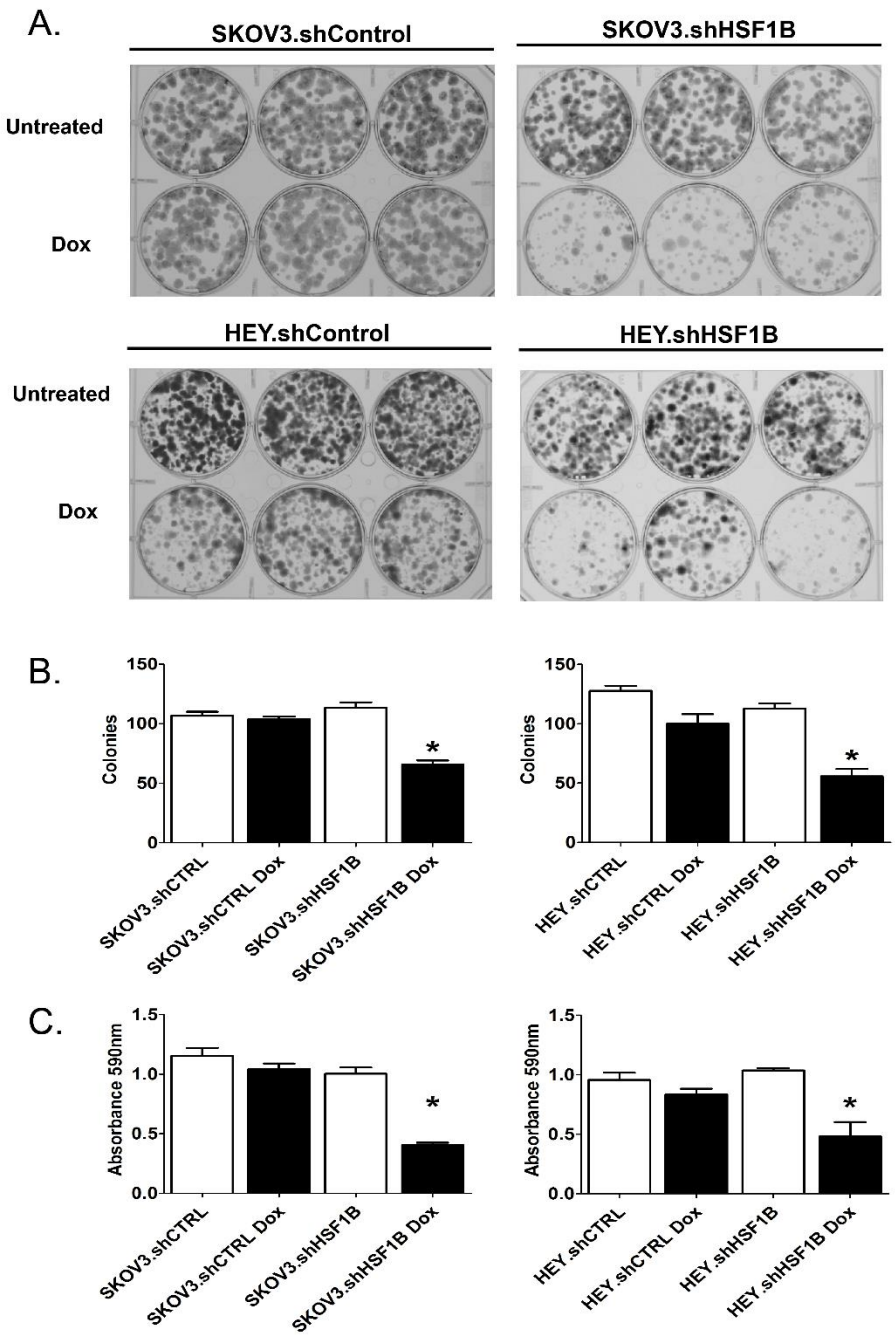

Figure 3.3. HSF1 Knockdown Reduces Colony Formation. SKOV3.shHSF1B, HEY.shHSF1B and control cell lines were plated 250 cells per well in 6-well plates in triplicate. Cell were treated with or without 1 µg/ml doxycycline (Dox) to induce HSF1 knockdown and were given an additional dose after 4 days. Cells were stained with crystal violet after 8 days to visualize colonies.

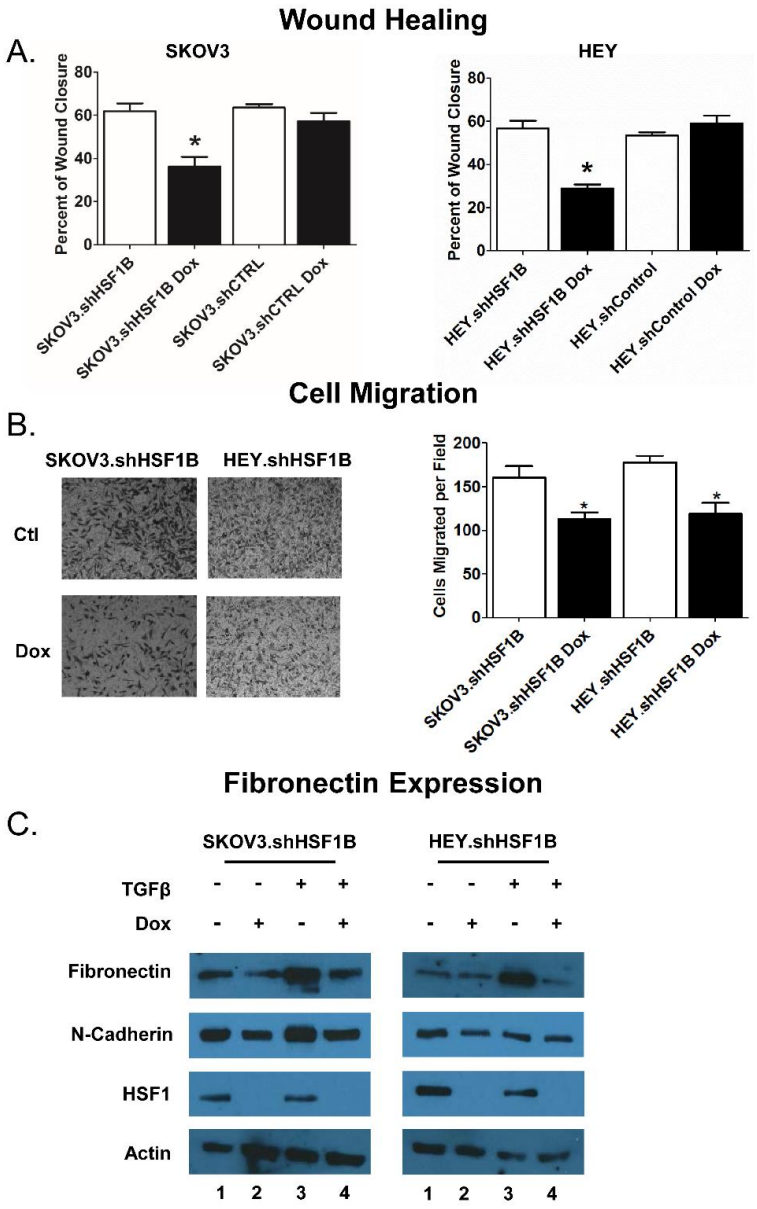

Figure 3.4. HSF1 Knockdown Inhibits Wound Healing, Migration and Induction of Fibronectin. A, HSF1 knockdown reduces wound closure. Cells treated with or without 1 µg/ml doxycycline were grown in 6-well plates to confluency. Cells were scraped to create wounds, the cells were washed and serum-free media was added. The intersections of perpendicular scratches were photographed immediately and 12 hours after and analyzed using Tscratch software. Asterisk denotes significant difference from all other samples calculated by ANOVA ($P < 0.05$). B, HSF1 knockdown reduces migration. After treatment with or without 1 µg/ml doxycycline and 12 hours of serum starvation, cells were added to a Boyden chamber at 2.5 x 104 cells per chamber. Serum was used as the chemoattractant in the lower chamber. After 16 hours, nonmigrating cells were scrubbed and cells which had migrated stained. The experiment was done in triplicate and analysis done by paired t-test. Asterisk marks significant difference ($P < 0.05$). C, HSF1 KD reduces TGFβ-induced expression of fibronectin. SKOV3.shHSF1B and HEY.shHSF1B were treated with 1ug/ml doxycycline, 10 ng/µl TGFβ, or both, and cell lysates were harvested for immunoblotting. Cell lysates were subjected to Western blot analysis using antibodies recognizing fibronectin, HSF1, and actin as a loading control.

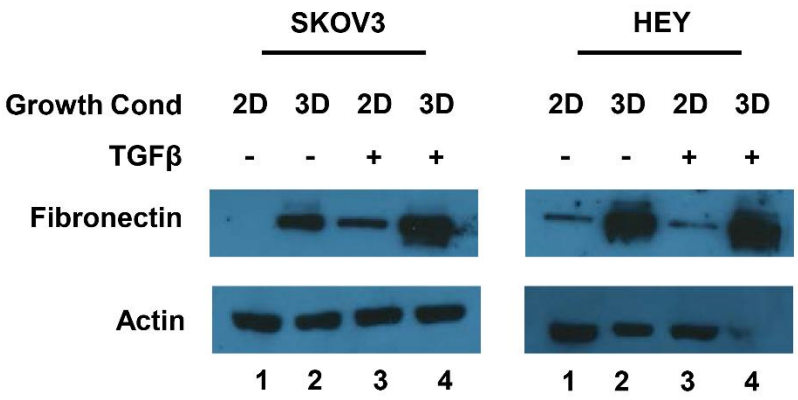

Figure 3.5. Fibronectin Expression is Induced by 3D Growth. SKOV3 and HEY cells were cultured under 2D or 3D conditions, with or without TGFβ, as indicated. Cell lysates were subjected to Western blot analysis using antibodies recognizing fibronectin, and actin as a loading control.

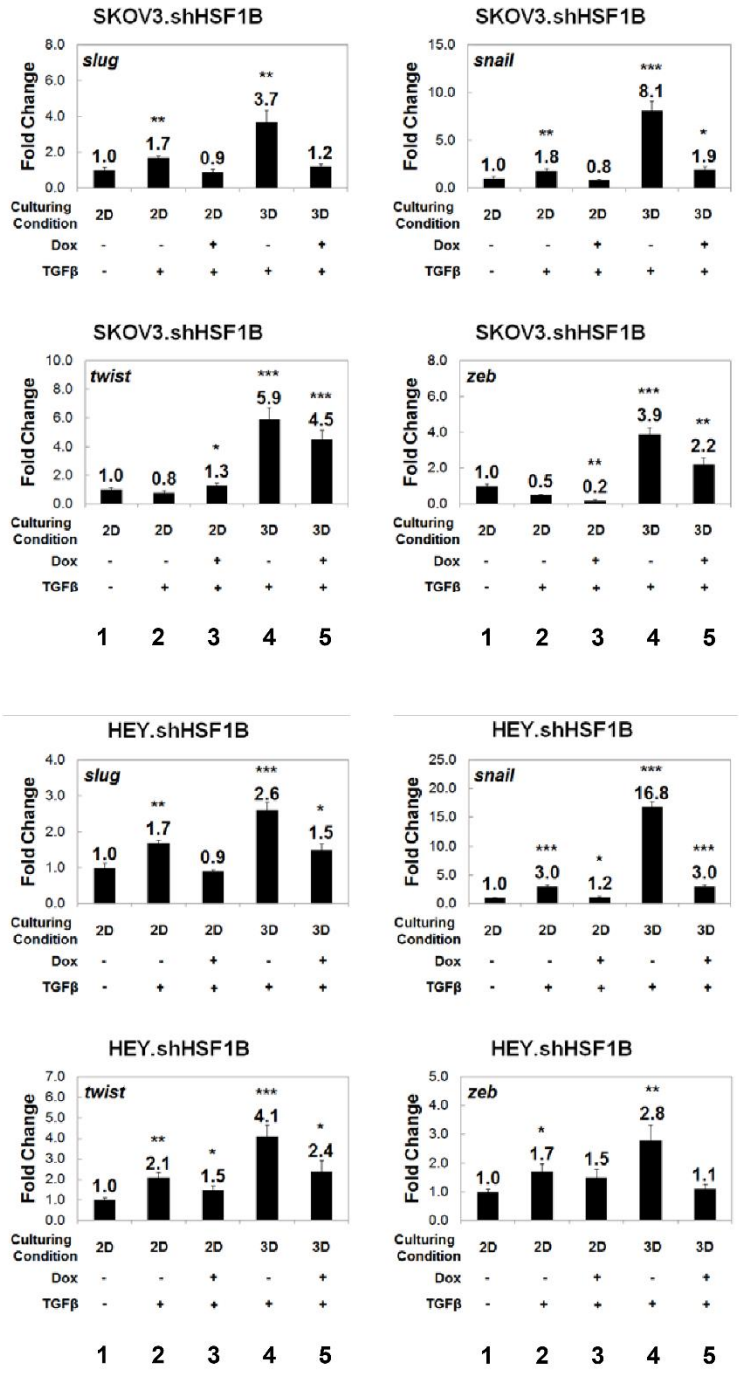

Figure 3.6. TGFβ Induction of EMT Master-Switch Transcription Factors are Reduced Upon HSF1 Knockdown, and the Effect is Enhanced Upon 3D Culturing. Quantitative real-time polymerase chain reaction (qRT-PCR) of selected genes shows that the EMT master-switch transcription factors *SNAI1/SNAIL, TWIST1, ZEB1, and SNAI2/SLUG* are upregulated when HSF1 inducible knockdown SKOV3.shHSF1B and HEY.shHSF1B cells are cultured as 3D spheroids. This effect is significantly reduced upon knockdown of HSF1 via doxycycline treatment. Gene expression was normalized to the housekeeping gene *GAPDH*, and fold change was calculated relative to monolayer non-treated conditions. Statistical significance was measured by Student's t test as compared to untreated monolayer cell culture (*p<0.05; **p<0.01 ; ***p<0.001).

CHAPTER FOUR: MODULATION OF HEAT SHOCK TRANSCRIPTION FACTOR HSF1 AFFECTS RESPONSE TO MULTIPLE DRUGS

Introduction

The heat shock transcription factor HSF1 is an important component in the cellular survival response to a multitude of stressors. HSF1 acts as the master controller for the HSR, which mitigates damage from heat and a variety of other stresses. Examples include proteasome inhibition, translational inhibition, heavy metals, ischemia, osmotic pressure and protein aggregation [378-381]. The HSR promotes survival in part through increased expression of molecular chaperones including HSP25, HSP70, and HSP90 [13]. These HSPs are capable of conferring resistance to multiple treatments in *in vitro* studies, including doxorubicin and paclitaxel [382-384]. In addition, HSF1 is required for the some aberrant signaling pathways that are vital in highly malignant cancers, such as HER2, BRAF, RAS, and AKT signaling [133, 136, 372, 385, 386].

HSF1 has become an attractive target for the treatment of cancer because it is implicated in facilitating aberrant oncogenic pathways and drug resistance in multiple cancers such as breast and hepatocellular cancers. Previous studies have found that HSF1 promotes resistance to doxorubicin, paclitaxel, and HSP90 inhibitors in hepatocellular, breast, and melanoma cancers [115, 139, 387] . High levels of HSF1 are common in approximately a third of ovarian cancer cases, which suggests that HSF1 may play an important role in the drug resistance of ovarian cancer [388]. We performed a knockdown study on the HSF1 in two ovarian cancer cell lines, SKOV-3 and HEY. We determined how the knock down of HSF1 affected the cell survival

response to cisplatin, paclitaxel, doxorubicin, curcumin, 17-AAG, and ganetespib. These drugs are either commonly used chemotherapeutic agents or prospective chemotherapeutic drugs.

Overview of Drugs Tested

Cisplatin. Cisplatin is the general name for cis-diamminedichloroplatinum(II) and is a very stable planar platinum coordination compound (Figure 4.1). It was discovered to have cytotoxic properties in the 1960s and in 1978 became the first FDA approved metallic based cancer therapy [357]. The primary mechanism of action is through DNA damage. This is accomplished mostly through the interaction with purine bases creating interstrand and intrastrand DNA-DNA crosslinks and also through the creation of DNA-protein crosslinks [389]. Of these DNA adducts, intrastand adducts are believe to be the source of most damage. While these nuclear lesions affect DNA synthesis, it is the triggering of the DNA damage response which ultimately leads to cell cycle arrest and apoptosis [389]. Cisplatin and other platinum therapies are the first line treatment for ovarian cancer. While initially effective in the majority of cases, resistance usually develops with cancer recurrence [390].

Paclitaxel. The compound paclitaxel was originally derived the pacific yew tree, *T. brevifolia* and was found to have cytotoxic effects in the late 1970s (Figure 4.2) [391]. It was the first of the taxol family to be discovered and was FDA approved for the treatment of refractory ovarian cancer [392]. Paclitaxel functions by stabilizing the microtubule assembly. This stabilization results in cellular arrest during mitosis at high doses [393]. At low doses, cell death occurs after undergoing irregular mitosis [394]. Paclitaxel is often combined with platinum based therapies as first line chemotherapy regime [395].

Doxorubicin. Doxorubicin is the most commonly used of the anthracycline class of drugs (Figure 4.3). It was originally derived from daunorubicin, an antibiotic from *Streptomyces peucetius*. Doxorubicin is very potent and is used in the treatment of a variety of cancers. It is

limited in application by its toxicity profile, in particular its cardiotoxicity [396]. The primary mechanism of action is through the inhibition of topoisomerase II [397]. This is accomplished by the formation of a doxorubicin-DNA-topoisomerase II ternary complex, stabilized in part by the intercalation of the DNA minor groove by the planar ring system [398]. Doxorubicin is not typically a first line chemotherapy agent. It is usually employed to treat recurrent cases of ovarian cancer that are resistant to platinum therapies [399].

Curcumin. Curcumin (diferuloylmethane) is a polyphenol derived from *Curcuma longa*, which is commonly known as turmeric. The chemical structure for curcumin is shown in figure 4.4. Its recent use in clinical trials is proceeded by use in traditional Chinese and Indian medicines [400]. Curcumin has antioxidant and anti-inflammatory properties. It induces functions through multiple means including the inhibition of proliferation, invasion, and angiogenesis [401]. This is accomplished by the inhibition of signaling proteins such as NF-κB, AP-1 and STAT3 [402]. The potential for clinical use of curcumin is hindered by its low aqueous solubility and poor bioavailability. However, analogues and different delivery strategies are being created to circumvent this. Curcumin as a single agent and in combination has advanced to phase II trials for treating colorectal and pancreatic cancer [403]. Curcumin was included in this study due to a significant correlation between HSF1 transcript levels and curcumin LD50 values in the NCI-60 cell line panel. This was found using the CellMiner suite of tools [404].

17-AAG (Tanespimycin). 17-AAG is a benzoquinone ansamycin antibiotic (Figure 4.5). It was developed a less toxic analogue geldanamycin, which was limited by its high liver toxicity. It interrupts many oncogenic signaling pathways through inhibition of cytosolic HSP90 function [405]. Inhibition is achieved through affinity with the ATPase N-terminal domain of the HSP90 complex, which prevent ATP dependent client refolding by HSP90 [406]. This has the effect of interrupting the critical oncogenic signal pathways HER2, EGFR, MEK, Akt, Src, and many others [407].

Ganetespib. Ganetespib was developed as a second generation HSP90 inhibitor with a substantially different chemical structure. The critical difference is the absence of the benzoquinone moiety which is the cause of the high liver toxicity that hampered first generation HSP90 inhibitors in clinical trials (Figure 4.6) [408]. Despite the difference in chemical structure, ganetespib functions by inhibiting the N-terminal ATPase domain like earlier HSP90 inhibitors. In addition to having a better pharmacological profile, ganetespib is roughly 20 times more potent than 17-AAG [409]. Ganetespib has made it as far as phase II clinical trials when included in combinatorial therapy for treatment of hepatocellular, pancreatic, and prostate cancers [410-412]. The biggest challenge for the development of treatment with ganetespib is identifying patients most likely to respond to the therapy. Ganetespib works best on cases were the cancer is reliant on constitutively active kinases (including c-KIT, EGFR, and B-RAF).

Material and Methods

Cell Culture

The HEY and SKOV3 cell lines with inducible HSF1 knockdown were created as previously described in Chapter 2. The doxycycline-inducible TRIPZ shRNAmir system (Thermo Scientific) was used in conjunction with shRNA targeting HSF1. The shRNA sequences for targeting HSF1 were obtained from the RNAi codex database [364]. Two sequences were cloned into the pTRIPZ vector and tested for efficacy: CGCAGCTCCTTGAGAACATCAA (shHSF1A) and CCCACAGAGATACACAGATATA (shHSF1B). Infection was done using a 2nd generation lentiviral system with pCGP packaging and pVSVG envelope plasmids (Addgene). Packaging was done with HEK293 cells cultured in RPMI medium. Transfection was performed using Polyfect Tranfection Reagent (Qiagen) based on the manufacturer's suggested protocol. After a single round of infection, stable HEY and SKOV3 cells were selected with 1 µg/ml and 0.5 µg/ml

puromycin (Thermo Fisher) respectively. Knockdown efficacy was tested by western blot after a 48 hour treatment with 1 µg/ml doxycycline (Figure 2.2B). In all subsequent experiments, HSF1 knockdown was achieved with a 1 µg/ml doxycycline treatment 48 hours prior.

Protein Isolation, SDS-PAGE, and Western Analysis

Treated cells were washed and released by scraping in chilled PBS. Cells were then pelleted and protein was extracted using M-PER lysis buffer (Thermo Scientific) containing Halt™ Protease Inhibitors (Thermo Scientific). Protein concentration was determined using Pierce™ 660nm protein assay (Thermo Scientific) following the manufacturers recommended procedures. 15 µg of cell lysate run on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred using a Trans-Blot semi-dry transfer cell (Bio-Rad) to 0.2 µm Immun-Blot® PVDF membrane. The membranes were blocked with 1% w/v non-fat milk in TBS with 0.1% Tween. Blots were probed with primary overnight, followed by secondary the next day. Blots were developed using ECL Prime Western Blotting Detection System (Amersham™) and film exposure. The primary antibodies used were: Actin (Santa-Cruz), HSF1 (Cell Signaling), HSF1 P-S326 (Abcam) and HSP70 (Cell Signaling). Secondary HRP-conjugated antibodies were from Millipore and Jackson ImmunoResearch.

Viability Assay

Cells at a concentration 1.5 x 10^5 cell/ml were plated in clear bottom, black walled 96-well plates at 100 µl per well. After a 6 hour incubation to allow cell to adhere, cells were treated with drugs or vehicle control in triplicate. DMSO was used as vehicle control for all drug treatments with the exception of cisplatin, which used saline water. The drugs used were: cisplatin (Tocris), 17-AAG (Selleck Chemical), paclitaxel (ACROS), doxorubicin (Fisher), curcumin (Sigma-Aldrich) and ganetespib (Selleck Chemical). After a 16 hour treatment, cells were washed 3 times with

PBS, followed by the addition of 50 µl RPMI medium. After rinsing, 5 µl of PrestoBlue® Cell Viability reagent (Invitrogen) was added to each well followed by a 45 minute incubation at 37ºC. Fluorescence (excitation 570nm, emission 600nm) was measured using a microplate reader (BioTek). Viability was determined by comparing treated and control samples after subtracting a dead cell control reading. Dead cell control was generated by treatment with lethal dose of cycloheximide (Fisher). Curve calculations were done using variable slope linear regression analysis with GraphPad prism® 7 software (GraphPad Software, Inc.).

Results

Doxycycline Treatment Does Not Effect Drug Sensitivity

Doxycycline is known to have a variety of effects on cancer cells when used at higher doses, such as the inhibition of Protease-Activated Receptor 1 (PAR1) and Matrix Metaloproteinases (MMPs) [413, 414]. To determine if the use of doxycycline changed the drug response, HEY.shControl and SKOV3.shControl cells were treated with and without 1 µg/ml doxycycline for 48 hours. This was followed by a drug response assay. Treatments were performed in triplicate for doses previously determined to be IC50 values. The results of the viability assay show that doxycycline does not substantially effect the sensitivity of HEY.shControl or SKOV3.shControl cell lines to any of the drugs tested (Figure 4.7).

HSF1 Knockdown Sensitizes Cells to Multiple Chemotherapeutic Agents.

Previous studies have indicated that HSF1 levels can increase cancer cell tolerance to therapeutic agents [139]. Elevated HSF1 levels have been found to raise melanoma and breast cancer cell line resistance to doxorubicin, paclitaxel, trastuzumab, and carboplatin [115, 139, 415]. We used HEY.shHSF1B and SKOV3.shHSF1B ovarian carcinoma cells lines with inducible HSF1

knockdown to test if HSF1 contributed to ovarian cancer cell survival when challenged with drug treatment. After pretreatment with or without 1 µg/ml doxycycline for 48 hours, HEY.shHSF1B and SKOV3.shHSF1B cells were treated with different drugs for 16 hours. These included cisplatin, paclitaxel, doxorubicin, curcumin, 17-AAG, and ganetispib. Response curves were generated and the IC50 values compared (Figure 4.8 – 4.10). Knockdown of HSF1 in SKOV3.shHSF1B cells significantly reduced the IC50 values for treatment with paclitaxel, doxorubicin, 17-AAG, and ganetespib based on an extra sum of squares F test (P=0.01). Similarly, knockdown of HSF1 reduced tolerance in HEY.shHSF1B cells for paclitaxel, doxorubicin, 17-AAG, and ganetespib, but not cisplatin and curcumin (Figure 4.9 and 4.10). While the sensitivity of SKOV3.shHSF1B cells to cisplatin was increased in the absence of HSF1, the change in response slope results in the significance being undetermined.

Drug Treatment Does Not Induce Robust Heat Shock Response.

Some previous reports have suggested that some cancer therapeutics elicit the HSR, or at least prime it by promoting the trimerization and phosphorylation of HSF1 [115, 139, 416]. HSP90 inhibitors are especially known to be HSR activators. This is due to feedback which activates HSF1 to replenish the pool of functional HSP90 [417]. To determine if the compounds being tested activated the HSR, SKOV3 cells were treated with semi-lethal doses of cisplatin, paxlitaxel, doxorubicin, curcumin, 17-AAG, and ganetespib. Treatments were performed for 16 hours and activation of the HSR was determined by western blot analysis (Figure 4.11). Exposure to a 42ºC heat shock followed by a 2 hour recovery resulted HSF1 phosphorylation at S326 and expression of HSP70. Surprisingly, none of the drug treatments induced a heat shock response.

Discussion

The heat shock response results in a robust increase of molecular chaperones including HSP27, HSP70, and HSP90. Given that these chaperones individually can promote survival

under stress, we sought to determine if HSF1, the master regulator of the HSR, supported resistance to therapeutic agents in ovarian cancer cells. We found that the knockdown of HSF1 sensitizes both HEY and SKOV3 ovarian carcinoma cells to paclitaxel, doxorubicin, 17-AAG, and ganetespib. Our results also showed that HSF1 knockdown did not affect cisplatin or curcumin sensitivity. Additionally, we found that treatment with the selected drugs does not activate HSF1 or induce HSR at the interval and doses used.

HSF1 knockdown reduces tolerance to all drugs tested to some degree. While the reduction in tolerance to cisplatin and curcumin was not as pronounced as other treatments, knockdown of HSF1 did lower the IC50 dose. In particular, the reduction of HSF1 appears to sensitize cells to low doses as evident by the change in slope (Figure 4.8 and 4.9). Low, non-lethal doses result in a broader curve during HSF1 knock down which suggests the cells may struggle to deal with minor stressors. This shift is most evident in the response curves of SKOV3.shHSF1B to cisplatin and curcumin, and HEY.shHSF1B to cisplatin, doxorubicin, and 17-AAG.

The increase in drug sensitivity during HSF1 knock down is reasonable given the protective and wide ranging roles HSF1 plays in cellular processes. In addition to controlling chaperone levels, HSF1 has been shown to promote cell survival under stress in other ways. For example, HSF1 can attenuate apoptosis through indirect stabilization of the anti-apoptotic Bcl-2 protein family [111]. HSF1 has also been shown facilitate drug efflux of doxorubicin and paclitaxel when over expressed in melanoma cells [139]. Interestingly, that study reported a 2 – 3 fold increase in IC50 values when HSF1 is overexpressed. Conversely, our studies show a 2 – 4 fold decrease when HSF1 is reduced. The HSF1-driven increase in drug efflux was found to be caused by an increase in ABC transporters.

The ability of HSF1 to increase chaperone levels was assumed to mitigate HSP90 inhibitors shortly after their discovery. Experiments using *hsf*1-/- mouse embryonic fibroblast cells

showed a modest increase in HSP90 inhibitor sensitivity compared to control cells [418]. A later study also found the same conclusions using hepatocellular carcinoma cell lines [387]. Interestingly, the mechanism by which HSF1 mitigates the effects of HSP90 inhibitors is dependent in part on the expression of DEDD proteins. This demonstrates that the protection conferred by the activation of HSF1 is not based solely on elevating HSP90 levels. Our findings are consistent with previous research in demonstrating that HSF1 attenuates the effects of HSP90 inhibitors such as 17-AAG and ganetespib. There is also some evidence to suggest that the degree to which HSF1 knockdown sensitizes cells correlates with HSF1 levels. The HEY cells, which express higher levels of HSF1, had a slightly larger increase in HSP90 inhibitor sensitivity after treatment with doxycycline compared to the SKOV3 cells.

The drug treatments used in this study did not induce HSF1 activation as determined by western blot analysis. This deviates from previous findings that HSP90 inhibition activates the heat shock response [373, 387, 419]. There are multiple possible explanations for this. The most likely cause is the duration of treatment used. Our experiments used a 16 hour dose time while other studies used 2 – 5 day treatment durations. Another possibility is that the HSR elicited by HSP90 inhibitors is weak compared to a traditional heat shock treatment, causing the results to appear negative. Lastly, the dose of inhibitor may have been too high or low. This is a possibility that cannot be ruled out since only a single dose of each HSP90 inhibitor was tested.

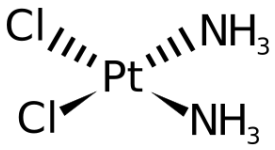

Figure 4.1. Cisplatin Chemical Structure. Cisplatin, also known as CDDP, is a DNA damage inducing alkylating agent. It is usually used in combination with a taxane as first round treatment.

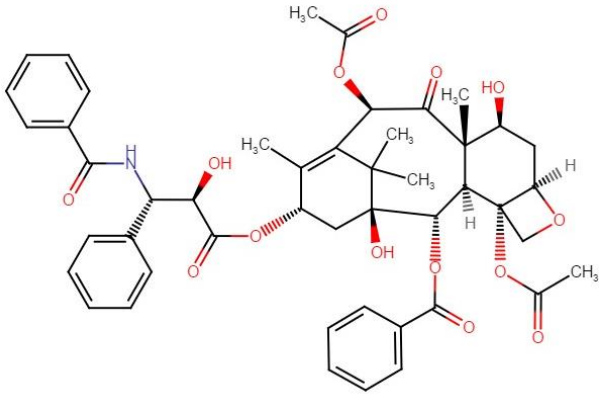

Figure 4.2. Paclitaxel Chemical Structure. Paclitaxel, a plant derived alkaloid, was the first member of taxane family. It functions as an anti-microtubule agent and is usually combined with platinum therapies as a first line of treatment.

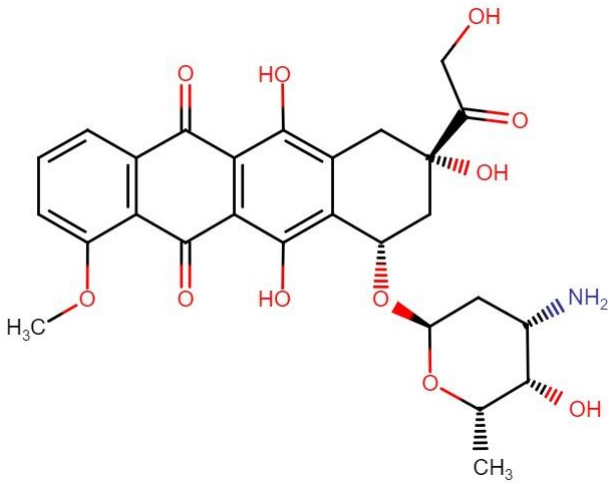

Figure 4.3. Doxorubicin Chemical Structure. Doxorubicin is an anthracycline antibiotic which is commonly used to treat platinum-resistant recurrence.

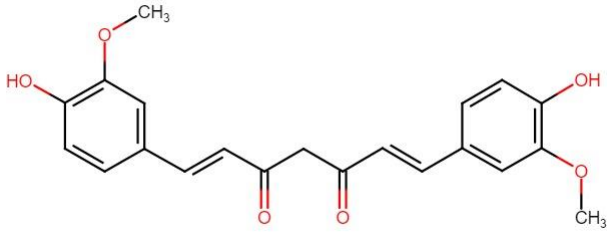

Figure 4.4. Curcumin Chemical Structure. Curcumin is a polyphenol which has anticarcinogenic effects. It has been used in combination with traditional therapies in clinical trials.

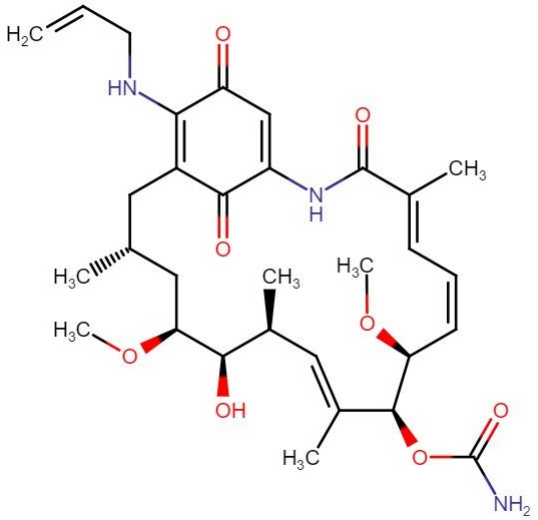

Figure 4.5. 17-AAG Chemical Structure. 17-AAG is a derivative of the antibiotic geldanamycin, which acts as a HSP90 inhibitor. It has been used in clinical trials for solid tumors and leukemia.

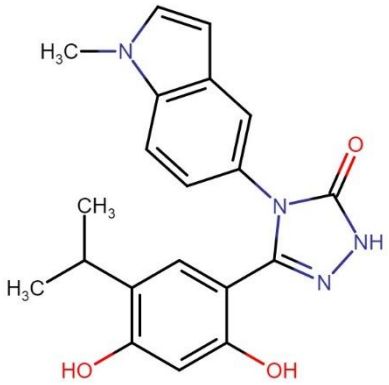

Figure 4.6. Ganetespib Chemical Structure. Ganetespib is a HSP90 inhibitor with lower toxicity and greater potency than earlier inhibitors. It is currently being used in phase II ovarian cancer trials.

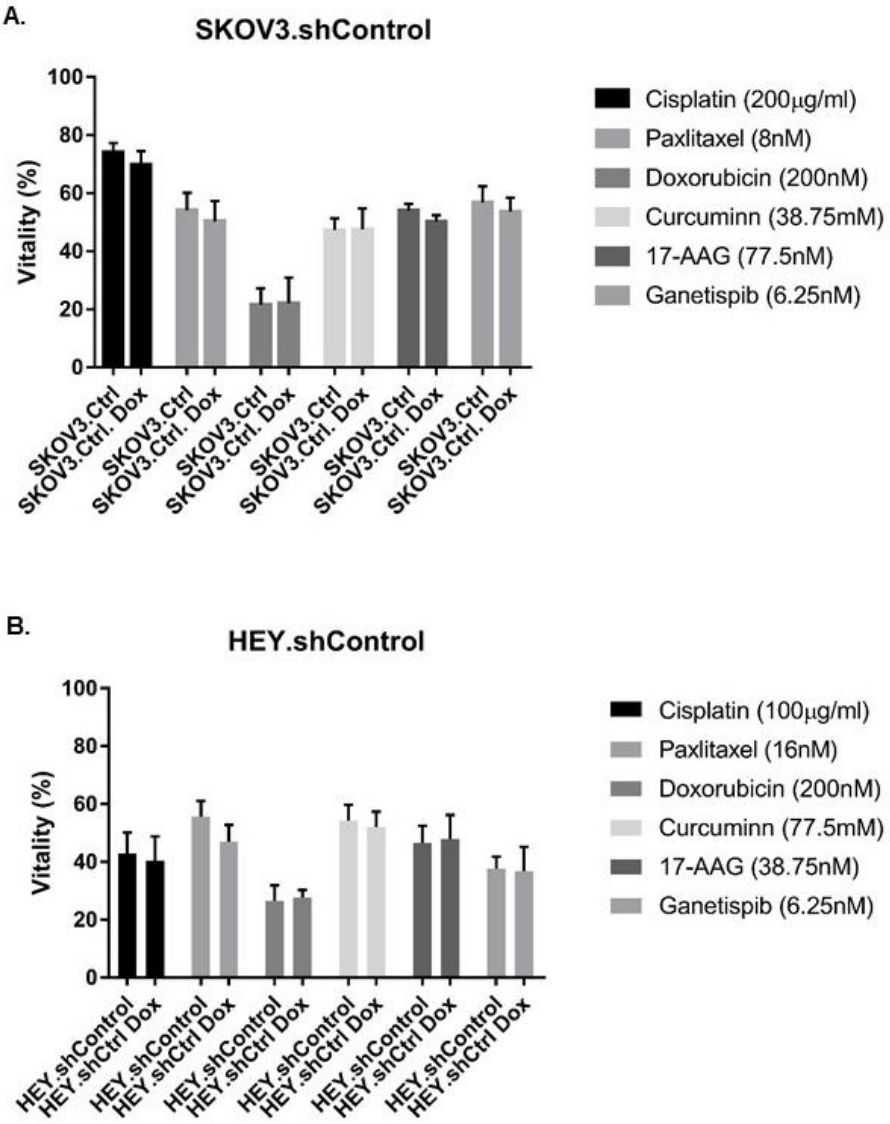

Figure 4.7. Doxycycline Does Not Affect Drug Response in Control Cells. SKOV3.shControl and HEY.shControl cells were treated with or without doxycycline in combination with a variety of drugs to determine if doxycycline affects IC50 values. Test was performed in triplicate using previously determined IC50 treatment values. Treatment with doxycycline did not significantly change the SKOV3.shControl and HEY.shControl IC50 values for any of the drugs tested. Statistical analysis was performed using a paired t-test.

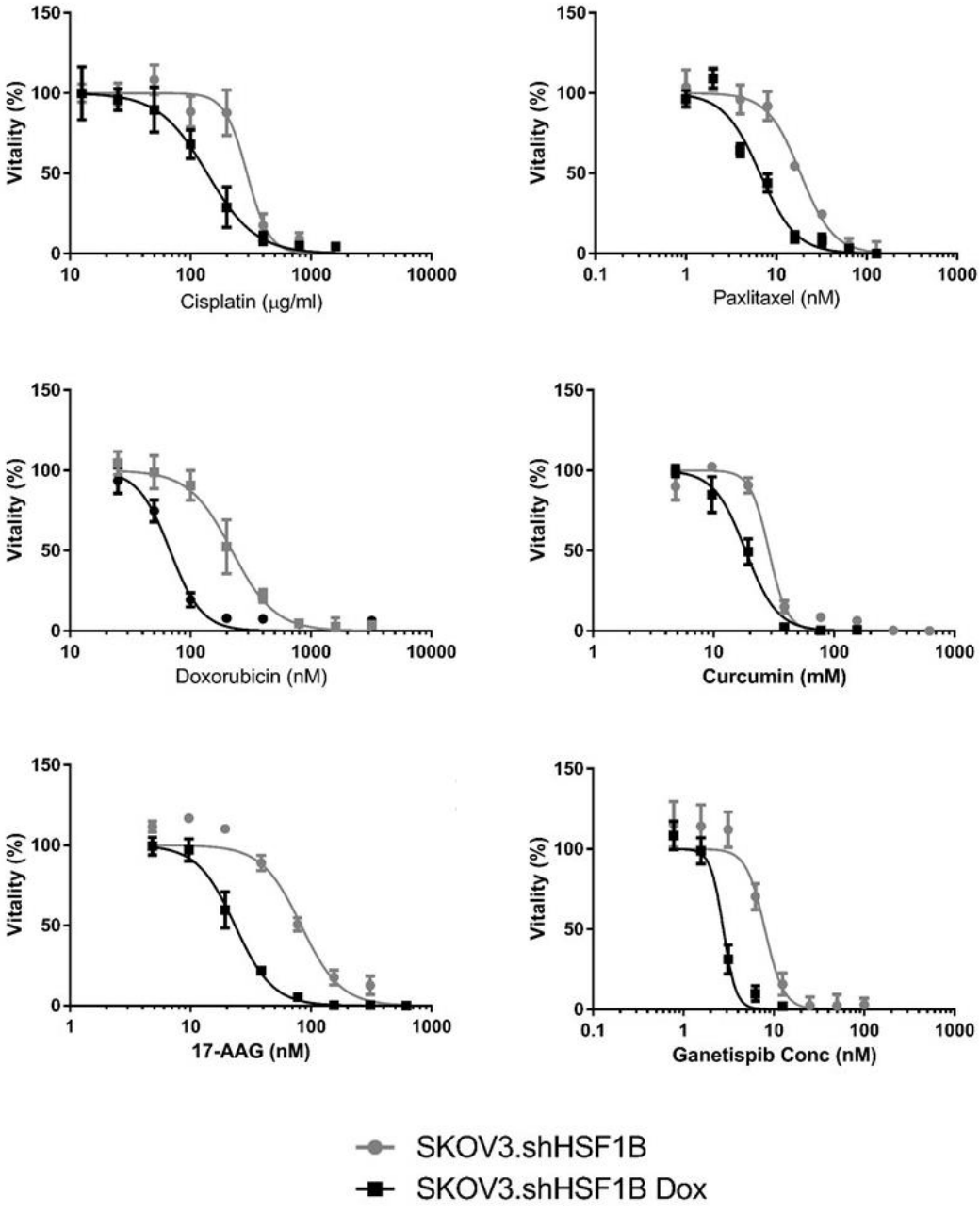

Figure 4.8. Effect of HSF1 Knockdown on SKOV3.shHSF1B Dose Response. SKOV3.shHSF1B cells were treated with or without doxycycline and then treated with serial dilutions of different drugs. After a 16 hour incubation, viability of the cells was assessed by PrestoBlue® assay.

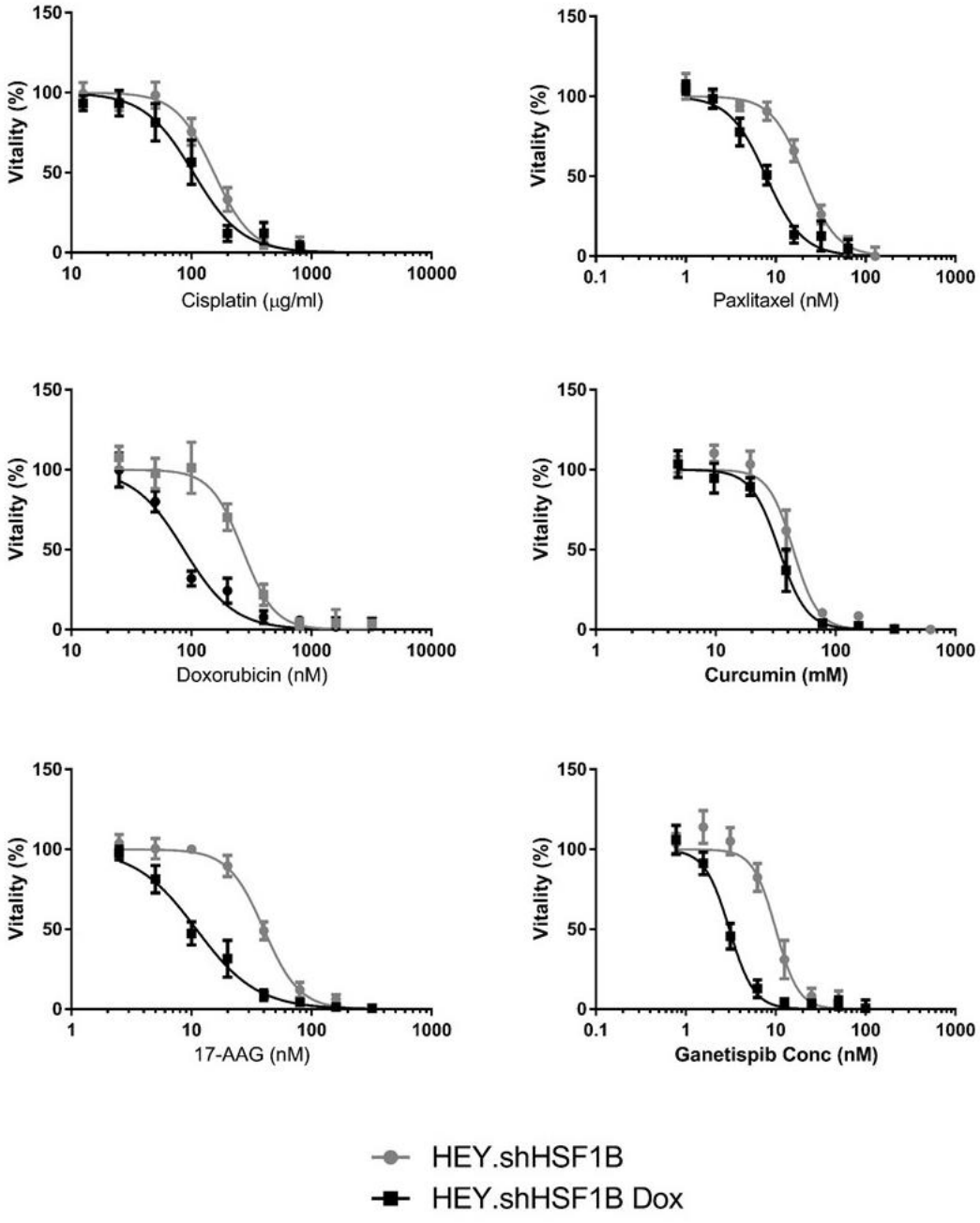

Figure 4.9. Effect of HSF1 Knockdown on HEY.shHSF1B Dose Response. HEY.shHSF1B cells were treated with or without doxycycline and then treated with serial dilutions of different drugs. After a 16 hour incubation, viability of the cells was assessed by PrestoBlue® assay.

Table 4.1. IC50 Values with and without HSF1 Knockdown

	HEY.shHSF1B		SKOV3.shHSF1B	
Dox	-	+	-	+
Cisplatin	153.6µg/ml	103.4µg/ml	294.4µg/ml	137.7µg/ml
*Paxlitaxel	20.8nM	7.8nM	18.5nM	6.6nM
*Doxorubicin	267.1nM	86.7nM	221.1nM	67.7nM
Curcumin	44.3mM	33.7mM	28.7mM	18.5mM
*17-AAG	40.07nM	10.98nM	82.72nM	23.4nM
*Ganetespib	10nM	3.05nM	7.96nM	2.76nM

*****Denotes sigificant difference between doxycycline treated and untreated as determined by extra sum-of-squares F test (P<0.01).

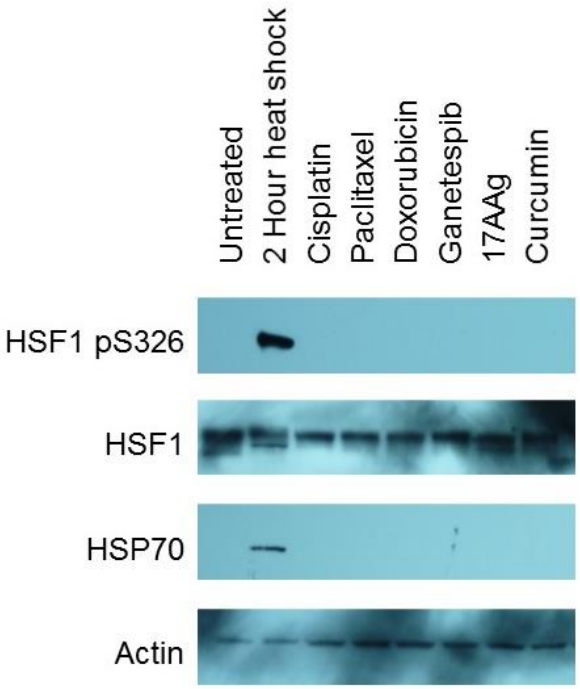

Figure 4.11. Drug Treatment Does Not Induce HSR. SKOV3 cells were treated with half the IC50 value for a variety of drugs. After a 16 hour treatment, cells were collected and analyzed by western blot analysis. Untreated cells, and cells that were heat shocked at 42ºC for 2 hours followed by a 2 hour recovery were included as controls.

CHAPTER FIVE: IMPLICATIONS AND FUTURE DIRECTIONS

Implications for Disorder in HSF Protein Family and Chaperones Studies

Role of Disorder and Structure in HSF1 Function

The activation of human HSF1 is a multistep process which includes the conversion of the monomer to a trimer. The trimerization of HSF1 can be induced by elevated temperatures without any cofactors *in vitro* [290]. This serves as the primary step in the activation of HSF1. It was proposed that the HR-A/B and HR-C hydrophobic repeats form a coiled-coil interaction. This is known to stabilize the monomer but dissolves at activating temperatures. This model was supported by initial studies, and was proposed to act as a thermodynamic switch to activate HSF1 [420]. Further studies showed transfected human HSF1 in *Drosophila* cells formed trimers at temperatures below the 42°C activation threshold [421]. This led to the conclusion that trimer formation was not controlled by temperature-dependent unfolding of the inactive HSF1 monomer. Subsequently, a thermally activated model of trimer formation was considered not plausible. More recent research has returned to a model which includes temperature-dependent conformation changes and trimerization [29]. Notably, it was discovered that disassociation of the HR-A/B and HR-C domains occurs slowly at basal temperatures, but usually re-associates before the complete unfolding of the monomer occurs. This is due to the high apparent concentration of the intramolecular interaction. The proposed model based on these findings has the monomer concentration and stability of the unfolded or intermediate forms as limiting factors in the

conversion to trimer. This model allows for both temperature-induced trimerization, and concentration/interaction dependent trimerization.

During activation HSF1 is hyperphosphorylated. However, the role of most of these post-translational modifications in activation is poorly understood. Point mutation studies have shown that most of sites are not individually significant [36]. Assuming trimerization occurs as proposed by Hentze *et al*., it is possible that the many phosphorylation sites with no known function collectively serve to promote the unfolding of the monomer form, presumably because the hyper-phosphorylation is substantial enough to greatly increase the hydrophobicity of the regulatory domain. The increased hydrophobicity in the regulatory domain, which is located between the HR-A/B and HR-C domains, would be expected to promote the unfolded, disordered state. As demonstrated in our work, most of the regulatory domain tends towards disorder based on *in situ* analysis (Figure 2.2). It is reasonable to assume the increased hydrophobicity and charge repulsion would shift the conformation equilibrium toward the unfolded, disordered state. In the Hentze model of trimerization, this would promote the formation of trimers.

Potential Benefit of HSF1 Conformation Changes in Activation

HSF1 conformation and oligomer status is a critical part of its function and regulation in higher organisms. The transition from a monomer to a trimer serves as a primary step in heat shock response activation; however, transition from a monomer to a trimer is not found in lower organisms. In *S. cerevisiae* and *K. lactis,* HSF1 is constitutively a trimer, whereas in metazoans HSF1 trimerization is the first activation step [300, 422]. This suggests that the regulation of HSF1 trimerization is a more recently evolved step of HSF1 activation. This is further supported by the absence of a conserved HR-C hydrophobic repeat domain which is critical in the control of the HSF1 oligomer state (Figure 2.5) [292]. The regulation provided by this additional step allows for further tuning of the response in a way that responds to temperature changes in the environment.

Interestingly, this coincides with the ability of organisms to move and seek environments with amendable temperatures. It is possible that organisms with a better ability to choose the environmental temperatures they are exposed to could benefit from a heat shock response with a more specific activation temperature. Possessing a heat shock response with a biological thermo-sensor would allow cells to react directly to heat stress instead of reacting to damage created by heat stress. This would be especially beneficial in organisms which regulate body temperature and have a narrow temperature threshold in which proteome damage begins to occur when exposed to heat stress [423].

Implications for HSF1 in Ovarian Cancer

Origin of HSF1 Gene Duplications

Our work illustrates that HSF1 gene duplications are common in serous ovarian cancer (Figure 3.1). The reason the HSF1 gene copy number is higher than most other genes is not known. The selection of sub populations with specific gene duplications is driven by the advantage those duplications confer [424]. Our study and others show that HSF1 acts to shift the cellular programs toward survival and invasion in cancer, but does not generally act as a prototypical proliferation-promoting oncogene [380, 425-427]. Myc, a well-established oncogene that promotes proliferation, is near the HSF1 locus and might be an implicating factor for the increase in HSF1 gene copy number [428-430]. Myc gene duplication often occurs by translocation [431]. Myc is located at 8q.24.21 and HSF1 is located at 8q24.3. Duplication of Myc by translocation of the distal chromosome 8q arm will subsequently result in HSF1 gene duplication [432]. This notion is supported by the elevated copy number of the distal chromosome 8q arm up to the Myc locus (Figure S2). While it might be happenstance that HSF1 is duplicated in this scenario, our work demonstrates that it substantially modifies serous ovarian cancer behavior.

Spheroids as a Model to Study EMT

The hanging drop spheroid culture method has been established for over a decade but has seen only limited use [369]. It has primarily been proposed as a model for studying rudimentary angiogenesis and drug penetration [433, 434]. More recent bodies of work demonstrate spheroids are useful in studying EMT [168, 435]. Our work augments this and shows that spheroids are a good model for studying EMT in serous ovarian cancer cell lines. This is demonstrated by increased levels of EMT markers in 3D vs 2D cultures (Figure 3.5 and 3.6). The mRNA levels for the *SNAIL, TWIST1, ZEB1*, and *SLUG* transcription factors, which in part drive EMT, were elevated in spheroid cultures. The increased potential of spheroids to undergo EMT may allow future studies to more robustly examine EMT and uncover aspects not apparent in 2D culture. Future work should continue to establish spheroids as a sound model for emulating the tumor microenvironment and studying EMT. This will allow the simple and economical spheroid model to bridge the gap between 2D culture and animal models.

Role of HSF1 in Cancer Treatment

The ultimate goal for studies examining the role of HSF1 in drug response are finding applications in medicine. There are two ways in which HSF1 could be relevant to cancer treatment. It is possible that HSF1 could be used as a biomarker to guide which therapies are selected. Additionally, HSF1-targeted treatments could be used to sensitize cancers to other chemotherapeutic agents. Currently there are multiple ways HSF1 might be targeted, though none have been tested clinically. HSF1 could be targeted by therapeutic siRNA. However, despite great progress, there are huge challenges that still need to be overcome before siRNA therapy is a plausible treatment [130]. Another approach is through use of small molecule HSF1 inhibitors. There are currently multiple inhibitors already available on the market for research use. These include quercetin, KNK437, triplotide, and MTOR inhibitors which work indirectly to inhibit HSF1

[31, 436, 437]. Unfortunately, these inhibitors all have low specificity and multiple off target affects. Both siRNA and molecular inhibitor methods have shown efficacy in cell culture and xenograft mouse models, but are still far from clinical testing. Multiple studies, including our own, have established that the reduction or inhibition of HSF1 sensitizes cancer to multiple treatments. Research should continue to find a HSF1 inhibitor with relevant clinical applications for cancer patients [115, 139, 438, 439].

Given the strong affect HSF1 has on ovarian cancer cells, it is possible that HSF1 could be a biomarker to predict the behavior of a given cancer case. This has already been established for some cancers; elevated HSF1 is correlated with poor prognosis in esophageal, breast, non-small cell lung, and hepatocellular cancers [140, 440, 441]. While predicting outcomes for individual cases is valuable, it doesn't improve treatment. A more valuable use of HSF1 as a biomarker would be to guide treatment choices for personalized medicine. Our work adds to previous findings that HSF1 plays a role in the sensitivity to multiple drugs [115, 139, 438, 439]. It is possible that HSF1 protein or mRNA levels could be used to better select treatment options.

HSP90 inhibitors are the most likely drug for which HSF1 levels may significantly predict treatment response. Based on our research and that of others, reduced levels of HSF1 increase sensitivity to HSP90 inhibitors 2 – 3 fold (Table 4.1) [419, 442, 443]. It has been reported that HSF1 is activated by HSP90 inhibitors; thereby creating a feedback mechanism to compensate for the loss of chaperone function [387]. Interestingly, our data did not corroborate robust HSF1 activation by HSP90 inhibition. This suggests there may be other or additional causes for the increase in sensitivity. Regardless of the mechanism, cancers with low levels of HSF1 should be more sensitive to HSP90 inhibitor given our findings. In theory, this would make cases with low HSF1 expression good candidates for treatment with HSP90 inhibitors. Surprisingly, phase II clinical trials for HSP90 inhibitors have not selected for cases with low levels of HSF1. This is

most likely because HSP90 inhibitors have been touted as a broad spectrum approach, due to their ability to disrupt a variety of oncogenic signaling pathways [444, 445].

Future Studies

Further HSF1 Structure Studies

The mechanism by which HSF1 converts from the active trimer back into the monomer is not known. Understanding this step would give a more complete idea about how HSF1 is regulated. It would also offer a point for potential therapeutic regulation. Multiple studies have found that formation of the human HSF1 homotrimer is irreversible [29, 290]. This suggests that the active trimer form is more stable and has lower enthalpy. To test this, differential scanning calorimetry should be used to find enthalpy changes between the monomer and trimer states. This would allow the stability of each form to be assessed. I would expect the homotrimer has lower enthalpy and is therefore more stable. This would explain why the trimer will not spontaneously convert to the monomer at lower temperatures. Additionally it would make sense the active form is more stable, as the trimer must be functional during heat stress.

If the differential scanning calorimetry results reveal that reverting from the trimer to monomer form is an energy-intensive process, then it could be assumed that it is an active process *in vitro* requiring ATP, or alternately HSF1 trimers are degraded by the proteasome [59, 60]. To test if the HSF1 trimer is converted back into the monomer within cells, [^{35}S] methionine labeling may be required. Cells would be treated with [^{35}S]methionine, subsequently heat shocked, and then allowed to recover. Protein lysate would then be separated by native polyacrylamide gel electrophoresis, and the oligomer status of HSF1 determined. If there is any monomeric radiolabeled HSF1 it would indicate the trimer to monomer conversion occurs *in vitro*.

If it is verified that active HSF1 is returned back the monomer, the next step would be to determine which proteins are responsible for converting the trimer to the monomeric form. The most likely candidates are the HSP90 and TRiC chaperone complexes. Both of these chaperone complexes are involved in actively refolding proteins [446, 447]. Additionally, inhibition of these chaperones leads to HSF1 activation [31, 418]. For these reasons, it is possible that chaperones not only provide negative feedback by binding the inactive monomer, but by also actively dismantling HSF1 trimers.

Mechanism of HSF1 Effect on EMT

Modulation of β Catenin and Wnt Signaling. HSF1 promotes higher β Catenin levels, thereby supporting the Wnt/β Catenin pathway, and subsequently EMT [448]. This occurs via the HSF1 activation of ELAVL1 gene, not by direct activation of the β Catenin gene. The ELAVL1 protein then escalates β Catenin translation. Wnt/β Catenin signaling has been shown to promote EMT in ovarian cancer [449]. This suggests that it is possible that HSF1 supports EMT indirectly through regulation of β Catenin. Future studies could determine if β Catenin protein levels are reduced after HSF1 knockdown in ovarian cancer cell lines. If so, ELAVL1 mRNA levels should then be assessed under the same conditions to determine if HSF1 knockdown also has reduced ELAVL1 levels. HSF1 ChIP analysis of the ELAVL1 promoter could then be done to verify if the effect occurs by the previously elucidated pathway. Finally, β Catenin could be overexpressed during HSF1 knockdown to determine if HSF1 knockdown effects on EMT can be rescued by β Catenin.

Direct Activation of EMT Transcription Factors. Multiple EMT transcription factors have putative heat shock elements (Table S2). It is possible that HSF1 directly facilitates EMT behavior after TGFβ treatment. While these genes are not upregulated during a classic heat shock response, studies have shown that unique gene sets can be regulated by HSF1 in cancer under

non heat shock conditions [133]. One such EMT related gene, *slug*, has previously been found to be regulated by HSF1 during EMT despite the presence of only a weak heat shock element [450]. To explore this possibility, HSF1 ChIP could be performed to determine if HSF1 is in fact binding to these putative HSEs. These possible EMT-associated HSF1 targets include *fibronectin, vimentin, SNAIL, N-cadherin, ZEB, and TWIST* (Table S2).

Direct Activation of Interleukin Genes

A less often discussed target of HSF1 and the heat shock response is the interleukin family of cytokines. These cytokines regulate the inflammatory and immune response [451]. These cytokines were first discovered in leukocytes, but have since been found to be produced by a wide variety of cells. Interleukins have been shown to have a significant and complicated role in cancer biology [452]. While some members of the interleukin family can be used to treat tumors, others are associated with tumorigenesis and poor patient outcomes [452]. Among interleukins involved in cancer, IL-8 and IL-6 are well understood to promote oncogenic behavior in ovarian cancer [453, 454]. IL-8 promotes anchorage-independent growth, while IL-6 promotes STAT3-driven proliferation [455, 456]. HSF1 has previously been reported to co-activate the IL-8 and IL-6 genes, and may be doing so during EMT [48, 120, 456]. To investigate this, qPCR could be used to determine if TGFβ induces IL-8 and IL-6 in SKOV-3 and HEY ovarian cancer cell lines. If IL-8 and IL-6 are induced, CHIP could be used to see if HSF1 is binding the HSEs within the IL-8 and IL-6 promoters during TGFβ-induced EMT.

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APPENDICES

Appendix A: Supplementary Figures

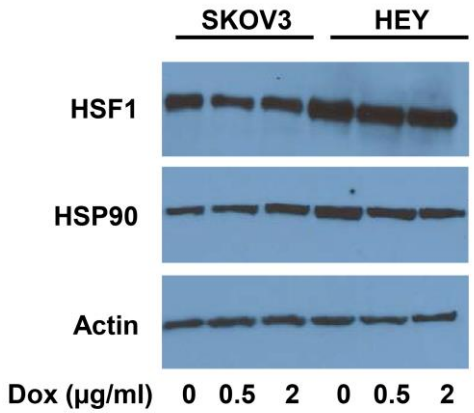

Figure S.1. Doxycycline Treatment Alone Does Not Alter HSF1 Levels or Induce HSP90 Expression in Ovarian Cancer Cell Lines. SKOV-3 and HEY cells were treated with 0–2 µg/ml doxycycline, as indicated, for 48 hours. Cell lysates were subjected to Western blot analysis using antibodies recognizing HSF1, HSP90, and actin as a loading control.

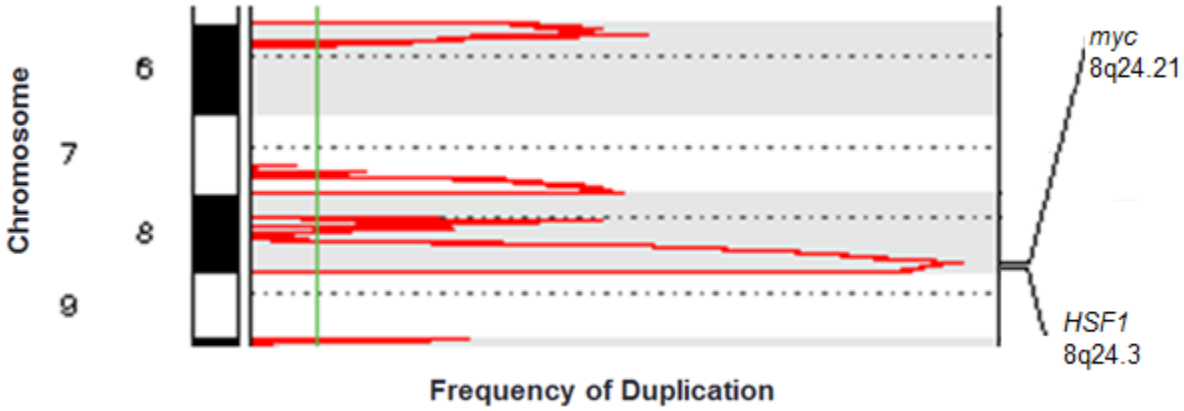

Figure S.2. HSF1 Gene Locus is Highly Amplified in Serous Ovarian Cancer. Data is based on GISTIC2 copy number analysis of 579 tumor samples from TCGA. Graphic adapted from Broad Institute TCGA Genome Data Analysis Center doi:10.7908/C1P84B9Q.

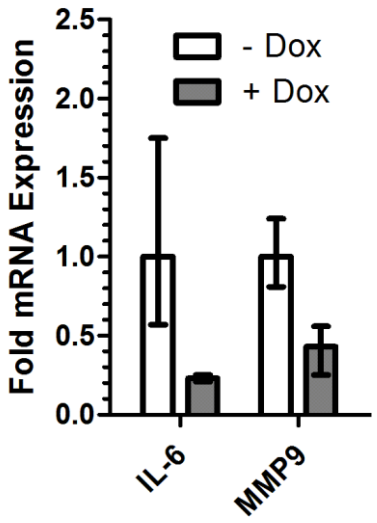

Figure S.3. Knockdown of HSF1 Reduces IL-6 and MMP9 mRNA induction During TGFβ Treatment in SKOV-3.shHSF1B Cells. SKOV-3.shHSF1b cells were treated with and without doxocycline for 48 hours, follow by 10 ng/ml TGFβ for 48 hr. qPCR was used to assess changes in mRNA levels compared to GAPDH.

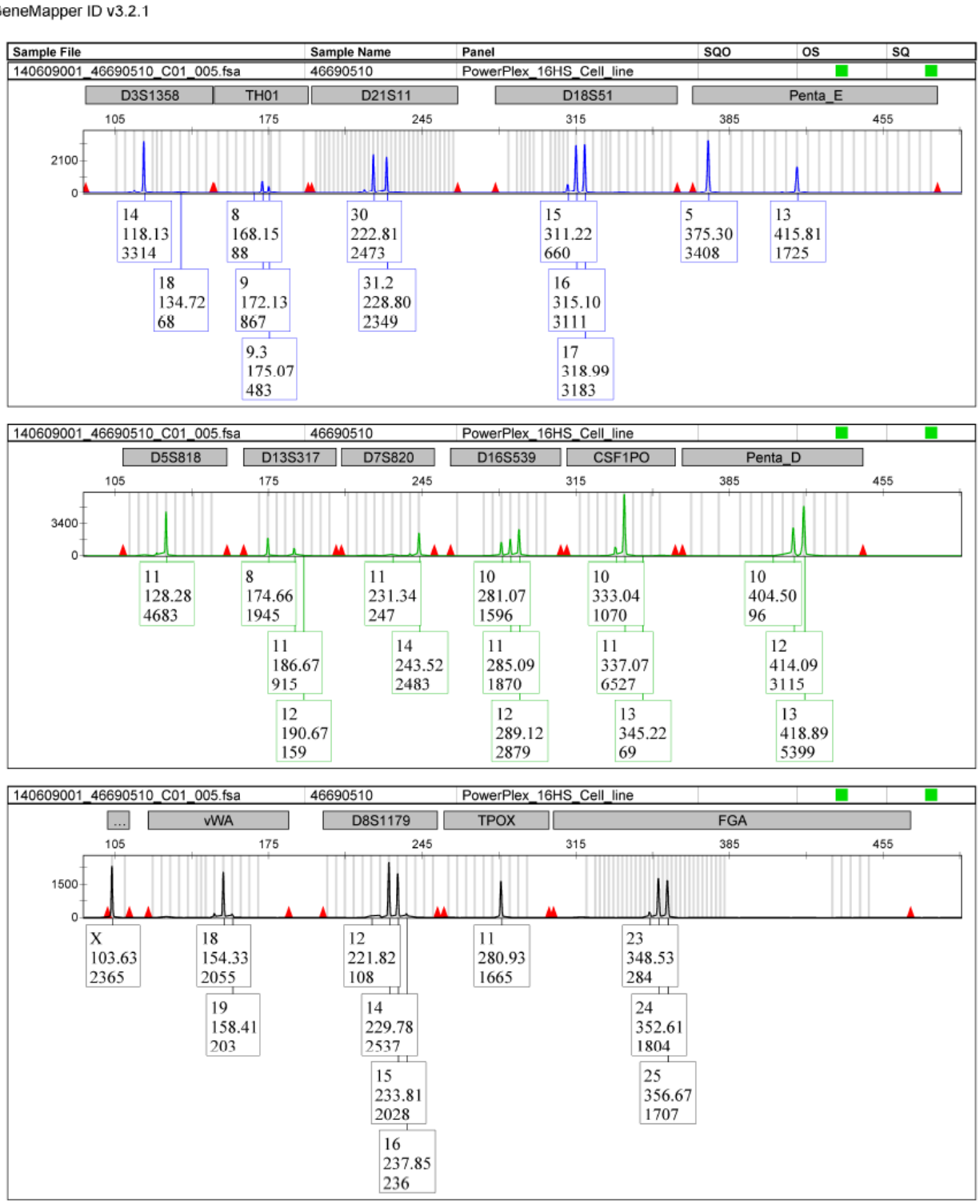

Figure S.4. SKOV-3 Short Tandem Repeat Analysis. Genomic DNA was purified by phenol chloroform extraction and used to verify cell line authenticity. Short tandem repeat analysis was performed using PowerPlex® 16 HS System. Major peaks were found to match SKOV-3 with over 90% identity using American Type Culture Collection (ATCC) database.

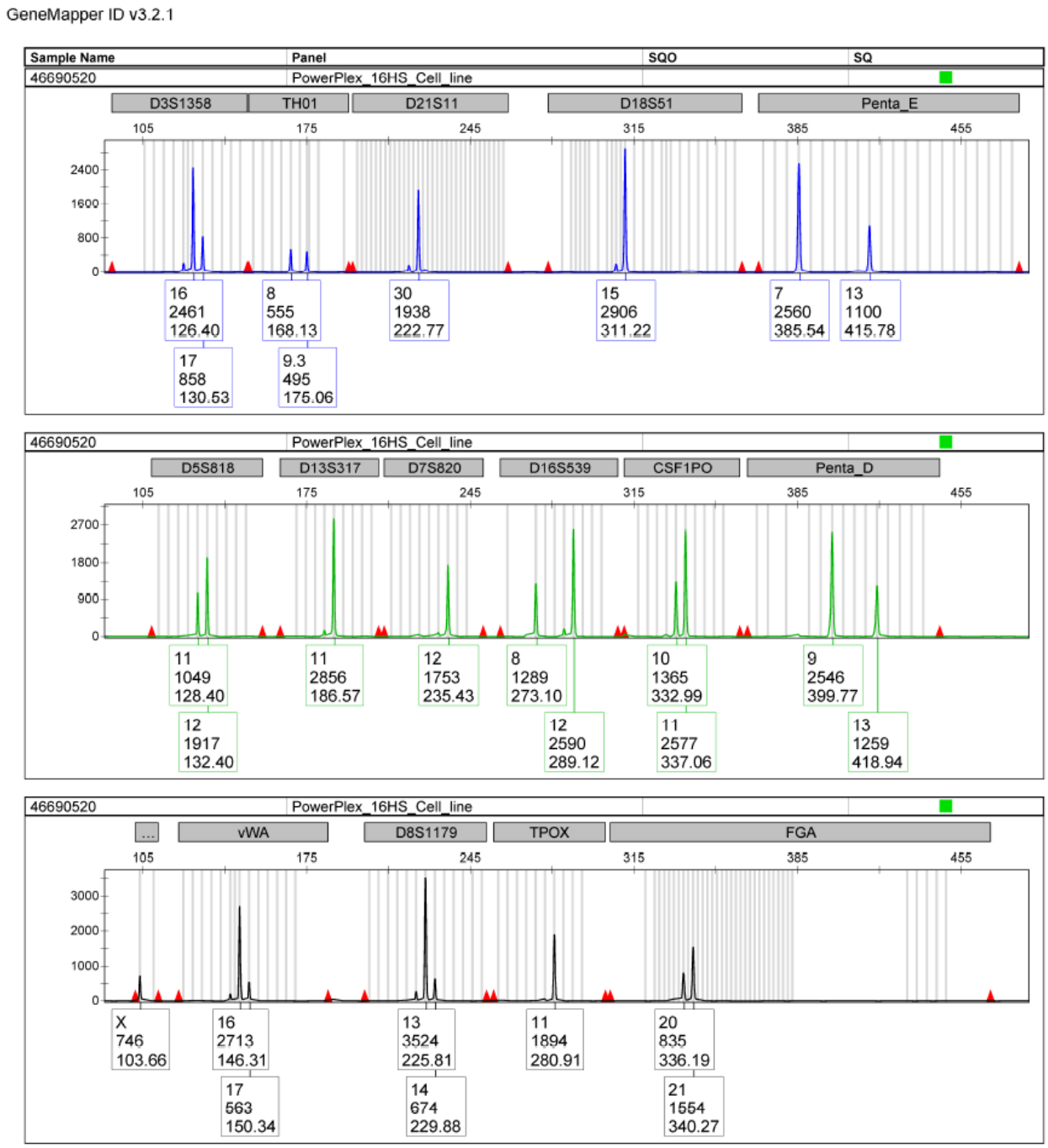

Figure S.5. HEY Short Tandem Repeat Analysis. Genomic DNA was purified by phenol chloroform extraction and used to verify cell line authenticity. Short tandem repeat analysis was performed using PowerPlex® 16 HS System. Major peaks were found to strongly match HEY results previously published [363].

Appendix B: Supplementary Tables

Table S.1. List of Primers Used in Quantitative RT-PCR.

Gene Name	Common Name	Sequence
GAPDH	*GAPDH*	F: 5' - CCACTCCTCCACTTTGAC - 3' R: 5' - ACCCTGTTGCTGTAGCCA - 3'
SNAI1	*SNAIL*	F: 5' - TCCCAGATGAGCATTGGCAG - 3' R: 5' - CGCGCTCTTTCCTCGTCAG - 3'
SNAI2	*SLUG*	F: 5' - TTCGGACCCACACATTACCT - 3' R: 5' - GCAGTGAGGGCAAGAAAAAG - 3'
TWIST1	*TWIST*	F: 5' - GGAGTCCGCAGTCTTACGAG - 3' R: 5' - TCTGGAGGACCTGGTAGAGG - 3'
ZEB1	*ZEB*	F: 5' - CAATACCGTCATCCTCAGCA - 3' R: 5' - CCAATCCCAGGAGGAAAAAC - 3'

Table S.2. Location of HSEs in EMT Genes.

Gene Name	Common Name	Location from CDS	Sequence
FN1	*fibronectin*	-3254	**TTC**TGC**AA**CT**TTC**A
VIM	*vimentin*	-3754	**TTC**CA**GAA**GG**TT**AA
SNAI1	*SNAIL*	-3201	**TTC**TA**GAA**GC**TTC**A
		-3207	**TTC**TA**GAA**TT**TT**GG
CDH2	*N-cadherin*	-4429	**TTC**TG**G**G**A**AG**TTC**C
		-2183	**TTC**CG**GAA**CC**TT**TT
		-2177	**TTC**CG**GAA**AA**TT**TA
		-2544	**TTC**CT**G**G**A**TT**TTC**T
ZEB1	*ZEB*	-289	**TTC**ACT**AA**CT**TTC**C
TWIST1	*TWIST*	-1301	**TTC**GA**G**C**A**CC**TTC**C
Consensus			**TTC**nn**GAA**nn**TTC**n

Table S.3. Antibody Dilutions and Incubation Times

Target	Manufacturer (cat#)	Dilution	Species	Location	Notes
Actin	Santa Cruz sc1616R	1:5000	Rabbit	4˚C	4C Overnight
Beta-tubulin	Cell Sig Tech 2128	1:1000	Rabbit	-20˚C	4C Overnight
HSF1 Mono	Assay Design spa950	1:1000	Rat	-20˚C	4C Overnight
HSP70	Cell Sig 4872	1:1000	Rabbit	-20˚C	4C Overnight ; Detects all HSP70 and HSC70
Vimentin	Cell Sig 5741	1:5000	Rabbit	-20˚C	RT 2 hours; Very strong - 5% BSA 0.1% tween recommended
Fibronectin	Thermo ms165	1:1000	Mouse	4˚C	RT 2 hours; Not working?
N-Cadherin	BD 610920	1:5000	Mouse	-20˚C	4C Overnight; Very strong (RT 2 Hours maybe better?)
HSF1 pS326	Epitomics 2092-1	1:5000	Rabbit	-20˚C	4C Overnight; maybe weak?
HSP90 Alpha	Cell Sig 8156	1:2000	Rabbit	-20˚C	4C Overnight; (Heat shock 90 only) 5%BSA 0.1%Tween recommended
HSP70	Thermo ma3009	1:1000	Mouse	-20˚C	4C Overnight; only for heat shock hsp70, maybe weak?
HSP27	Thermo MS-101-P	1:1000	Mouse	4˚C	4C Overnight; recognized HSP27 variants unkown
Slug	Thermo MA5-41881	1:1000	Mouse	-20˚C	4C Overnight
Fibronectin (new)	Thermo MS 1351	1:5000	Mouse	4˚C	4C Overnight
Fibronectin	BD 610077	1:1000	Mouse	-20˚C	4C Overnight, Long Exp on PVDF, 2 Hour transfer Required

Appendix C: Detailed Methods

Thawing Cells

1. Remove the cryo-vial containing the frozen cells in DMSO freezing medium from liquid nitrogen storage and immediately place it into a 37ºC water bath.
2. Quickly thaw the cells (< 1 minute) by gently swirling the vial in the 37ºC water bath until there is little to no ice left in the vial.
3. Transfer the vial into a laminar flow hood. Before opening, thoroughly wipe the outside of the vial with 70% ethanol and dry.
4. Using a pipette, gently transfer thawed cells to a 60 mm culture dish with 5 ml warm medium. Rock plate to distribute cells.
5. The following morning, aspirate medium to remove residual DMSO and replace with fresh medium.

Creation of pTripZ Constructs

Primers were created so that the shRNA targeting sequences CGCAGCTCCTTGAGAACATCAA (shHSF1A) and CCCACAGAGATACACAGATATA (shHSF1B) with the addition of the 5' EcoRI and 3' XhoI would be created upon annealing and extension of the primers. This was done to conserve costs. Alternatively the entire double stranded sequence could be ordered.

Generate Double Stranded Hairpin DNA

1. Mix primers constituting the region to be cloned in a 1:1 ratio, heat to 95ºC and slowly cool. This allows double stranded DNA formation.
2. Extend duplexed primers via PCR with standard TAQ polymerase using the maximum recommended primer concentrations for a single cycle. This extends the double stranded DNA sequence to the ends. Set up the reaction using 2.5 µl 10x Taq polymerase Buffer, 1µg DNA template, 0.5 µl dNTPs, 0.125 µl Taq polymerase. Bring to a final volume of 20 µl.
3. Extend using a single 20 minute step at 72ºC.

Digest PCR Product Directly in the PCR Tube

1. Add 5 µl 10X compatible NEB buffer, 2 µl of each restriction enzyme, 21 µl nuclease free water.
2. Digest for 1 – 2 hours at 37ºC

Purify Digested DNA Using Qiagen PCR Clean Up

1. Add 250 µl buffer PB1.
2. Add to column, spin at 13,000 rpm for 1 minute; discard flow through.
3. Wash with 750 µl buffer PE, spin, discard flow through.
4. Spin once more, then transfer column to a clean 1.5 ml tube.
5. Add 30 µl buffer EB and let stand for 1 minute.
6. Spin to collect eluent.
7. Determine concentration using UV spectrophotometer.

Prepare Plasmid by Digest (pTripZ was digested with EcoRI and XhoI)

1. Add 2 µl of 10X compatible NEB buffer, 2 µg of DNA, and 2 µl of each restriction enzyme. 1 µg may be used if quantity is limited or the conc. is < 160 ng/µl.
2. Add Nuclease free water to 20 µl total volume.
3. Incubate for 2 hours at 37ºC.

Separate Digested Plasmid Backbone via Agarose Gel Electrophoresis

1. Pour a 0.8% agarose gel with 1 µl ethidium bromide.
2. Add 4ul of 6X dye to digest and load, leaving an empty well in-between each sample.
3. Use a 1kb ladder and run for approximately 45 minutes, photograph gel.

Purify Plasmid Backbone by Gel Extraction Using a Qiagen Gel Extraction Kit

1. Extract desired bands under UV light using a clean blade for each excised band.
2. Place excised gel bands into to pre-weighed 1.5 ml tube and reweigh to determine mass of gel.

3. Add 3 volumes buffer QG. 0.100 g = 100 µl e.g. gel weighs 0.32 g, add 960 µl buffer QG
4. Incubate at 50ºC for 10 minutes. Mix sample every 2 minutes. All gel should be dissolved when finished.
5. Add 1 volume isopropanol. e.g. gel weighs 0.32 g add 320 µl isopropanol.
6. Add to column and spin at 13,000 rpm for 1 minute; discard the flow through.
7. Add 500 µl buffer QG, spin, discard flow through.
8. Add 750 µl buffer PE, spin, discard flow through.
9. Spin, move column to clean 1.5 ml tube.
10. Add 30 µl buffer EB, wait 1 minute.
11. Spin, label and save the eluent. Note: Yield cannot be determined by UV spectrophotometry due to carryover from QG buffer.

Ligate Plasmid and shRNA Sequence

1. In a 1.5 ml tube, add 2 µl 10X T4 ligase buffer, 8 µl shRNA insert, 4 µl backbone plasmid, 1 µl ligase and nuclease free H2O.
2. Incubate at 15ºC for 1 hour. Note: plasmid may be ligated overnight.

Transform NEB5α Cells with Ligation Product

1. Thaw NEB5 alpha cells on ice.
2. Add 8 µl of ligated product.
3. Incubate on ice for 15 minutes.
4. Heat shock cells at 42ºC for 35 seconds.
5. Incubate on ice for 10 minutes.
6. Add 750 µl room temp SOC medium.
7. Incubate at 37ºC for 1 hour with 250rpm shaking.

8. Spread on appropriate LB agar plates.
    i. Spread 20 µl, 200 µl, and the remainder across three plates.
9. Incubate overnight at 37ºC.
    i. Colonies should appear by morning, store at 4ºC after 16 - 18 hours incubation.

Purify Plasmid DNA Using Qiagen Miniprep

1. Prepare 15 ml conical tubes by adding 10 ml LB broth and appropriate antibiotic.
2. Inoculate each broth with a single colony using a flame sterilized inoculating wand.
3. Incubate overnight at 37ºC with 250rpm shaking.
4. Pellet cells by spinning at 5,000rpm for 15 minutes at 4ºC.
5. Aspirate media.
6. Re-suspend cells in 250 µl buffer P1 and transfer to a new 1.5 ml tube.
7. Add 250 µl buffer P2 and invert 4 - 6 times. Solution will be completely blue when done.
8. Add 350 µl buffer N3 and invert 4 - 6 times. Blue color will be gone when done inverting.
9. Spin for 10 minutes at 13,000rpm.
10. Transfer supernatant to column.
11. Spin at 13,000rpm for 1 minute; discard flow through.
12. Add 500 µl buffer PB, spin, discard flow through.
13. Add 750 µl buffer PE, spin, discard flow through.
14. Spin at 13,000rpm and move column to new tube.
15. Add 30 µl buffer EB, wait 1 minute then spin again.
16. Determine DNA concentration of eluent, label and save.

Generation of Stable Cell Lines

The pTripZ doxycycline inducible targeting short hairpin RNA (shRNA) works with a second generation lentiviral system. This requires the use of pCGP packaging and pVSVG envelope plasmids. Retroviral plasmids may require different packaging and envelop plasmids.

1. Culture HEK293T cells in the cell medium the cell line that is to be infected uses. A single 10 cm dish will generate enough viral supernatant for multiple rounds of infection.
2. Transfect HEK293T cells with Polyfect Transfection Reagent (Qiagen) using a 1:1:1 ratio of pTripZ, pCGP, and pVSVG.
    i. Culture cells to approximately 60% confluence
    ii. Add 4 µg total plasmid DNA to 400 µl serum and antibiotic free medium. Use a 1:1:1 ratio of plasmids.
    iii. Add 25 µl polyfect, vortex, spin and incubate at room temperature for 5 minutes.
    iv. During incubation, aspirate medium from cells and replace with 8 ml complete medium.
    v. Add 1 ml complete medium to polyfect mixture mix by pipetting and add to cells. Rock plates to mix.
3. 12 - 24 hours post-transfection, remove medium with transfection reagent and replace with fresh medium.
4. 24 hours later harvest medium containing viral stock.
5. Filter using a 0.45 micron PVDF filter with Leur lock attached to a syringe.
6. Viral stock may be frozen back at -80ºC if it will not be used for 48 hours.
7. Add polybrene to a final concentration of 34.6 ug/ml. Save as 1.5 ml aliquots; when added to 5 ml medium this will yield a final concentration of 8µg/ml polybrene.
8. Infect actively dividing cells at approximately 30% confluency with 1.5 ml of viral supernatant per 5ml cell medium.

9. 12 – 24 hours later, replace cell medium and treat with 0.5 – 5 µg/ml puromycin. The amount needed depends on cell type and cell density.
10. Replace medium as cells die off. Passage cells as needed.

Freezing Cells

It is imperative that cells are healthy and actively dividing to obtain viable cell stocks.

1. Remove medium from cells, wash, and release cells.
2. Once cells are detached add 5 ml warm medium, and transfer to centrifuge tube. Use 15 ml sterile centrifuge tubes and combine cells of same type.
3. Lightly pellet cells by spinning at 1,000 rpm for 5 minutes and remove medium. Up to 1,500rpm may be needed to pellet some cell lines.
4. Resuspend cells in enough freezing medium to create a cell suspension of 1 – 2 x 10^6 cells per ml.
5. Pipette up and down gently to ensure an even mixture. Aliquot approximately 1 ml into cryo-vials. This will provide 1 – 2 x 10^6 cells per cryo-vial.
6. Place cryo-vials into Mr. Frosty™ freezing container and store in -80ºC freezer overnight.
7. Cells may be transferred to liquid nitrogen the next day. If a dedicated 1ºC per hour freezing container is not available, place cells in a Styrofoam cooler to achieve a similar controlled freezing.

Clonogenecity Assay

1. Culture cells to 70% confluence. It is critical that the cells are healthy and actively dividing.
2. Release cells with trypsin and break up cell clumps. Be thorough to ensure a single cell mixture is obtained.
3. Count cells using a hemocytometer. To ensure accuracy count cells twice.

4. Plate 500 cells per well in 6 well plates. Plating from the same cell pool to ensure all wells receive the same number of cells. Treat with doxycycline as needed.
5. Retreat doxycycline samples on day 4. Doxycycline has a 48 hour half-life.
6. Remove plates after 8 days or when discreet substantial colonies form.
7. Aspirate medium and stain cells with 1% crystal violet (w/v) in methanol for 10 minutes.
8. Gently rinse 3 times by submerging plates in clean deionized water.
9. Photograph and count colonies. Lyse stained colonies using 200 µl per well of 1% SDS and placing plates on rocker for 30 minutes.
10. Measure absorbance from each well's lysate in triplicate at 590 nm using Biotek® plate reader.

Migration Assay

1. Treat with doxycycline 48 hours before start of assay.
2. 24 hours before the assay cells should be approximately 75% confluent and serum starved by using serum free medium.
3. Thoroughly release cells with trypsin and pipette to ensure no cell aggregates are in solution.
4. To ensure accuracy count cells twice.
5. Dilute cells to 1 X 10^5 cells/ml in serum free medium. Add 250 µl to the upper chamber insert. Perform in triplicate.
6. Add 400 µl of medium containing serum as chemoattractant to bottom of wells. Wells are filled second to avoid floating inserts.
7. After 16 hours, remove cells which did not migrate using a cotton swab. Do not let inserts and cells dry out.

8. Stain cells using 1% (w/v) crystal violet in methanol for 10 minutes. Wash 3 times by submerging in deionized water.
9. Cut out membrane from insert using scalpel and mount on glass slides; a cover slip is not needed.
10. Count and average 10 random field of views for each insert.

Viability Assay for Testing Drug Sensitivity

1. Cells to be treated with doxycycline should be done 48 hours before plating. One 60% confluent 10 cm plate is needed for each 96 well plate.
2. Release with trypsin and count cells
3. Dilute to 1.5 x 10^5 cell/ml in a multichannel boat. 10 ml of cell solution is needed per 96 well plate.
4. Using a multichannel pipette, add 100 µl per well to 96 well black side clear bottom plates. Be sure to mix cell solution in boat to maintain homogeneous solution.
5. Incubate for 6 hours to allow cells to adhere. Treat with drugs or vehicle control in triplicate. Dilutions should be created fresh.
6. After a 16 hour treatment, wash cells 3 times with PBS. Note: More washes may be needed as doxorubicin and curcumin can affect PrestoBlue® assay due to strong color.
7. Add warm 50 µl RPMI medium to each well followed by 5 µl of PrestoBlue® Cell Viability reagent.
8. Incubate for 45 minute at 37ºC. Incubation time is critical and may need to be adjusted for some treatments.
9. Measure fluorescence using excitation at 570 nm and emission at 600 nm using a microplate reader (BioTek).

10. Calculate percent viability, subtract dead cell control value from all wells and divide each sample reading by the vehicle control.
11. Kill curves can be calculated using variable slope linear regression analysis with GraphPad Prism® 7 software.

Wound Healing Assay

This procedure can be performed with standard or fibronectin coated plates. It makes little difference in cell morphology and migration when using SKOV-3 and HEY cell lines. It is imperative that the cells are on the cusp of full confluence, and that the level of confluence is equal between treated and untreated pairs.

1. Plate cells at 3 x 10^5 cells per ml in each well of a 6 well plate. Treat knockdown samples with 1 µg/ml doxycycline.
2. After 36 – 48 hours use a sterile 2 µl pipette tip to scrape 2 horizontal and 2 vertical lines, creating a "#" pattern. Be sure to use even pressure so the wounds are consistent.
3. Thoroughly aspirate and wash twice with PBS solution. Wash more if detached cells are not all removed from medium.
4. Aspirate PBS and replace with warm serum-free medium.
5. Photograph each of the intersections created by scraping; keeping track of each of the 4 individual scrape intersections.
6. Repeat photograph after 12 hours. None of the scrape intersections should be completely healed, but some should be close. The time interval may be adjusted as needed.
7. Analyze wound closure using TScratch software [365]. Be sure to use identical parameters for image analysis across the entire experiment.

BCA Protein Assay

The bicinchoninic acid (BCA) assay is tolerant of ionic detergents and has a wide range of detection from 20 – 2000 µg/ml. This protocol calls for a clear 96 well plate and sample dilutions which serve to save sample and ensure the concentration is within range of the assay.

1. Prepare RIPA lysis buffer within a 1.5 ml tube, using 250 µl per cell pellet. Add appropriate amount of 100x protease inhibitors and 100x phosphatase inhibitors.
2. Add 250 µl RIPA buffer containing inhibitors to each pellet.
3. Place in 4ºC mixer set at 1,000rpm for 10 minutes.
4. Pellet cell debris by spinning at 13,000rpm for 12 minutes at 4ºC.
5. Transfer supernatant to new tubes. This step can be omitted if there is no debris.
6. Prepare working reagent by mixing BCA reagents A and B at a 50:1 ratio. Determine total amount needed as follows: number of samples X replicates X 200 µl + 100 µl.
7. Mix well. Working reagent may be stored for 5 days in a sealed container at room temperature.
8. Prepare a 2:3 dilution by the addition of 10 µl sample and 15 µl PBS followed by 200 µl working reagent.
9. Incubate at 37ºC for 30 minutes and cover to prevent evaporation.
10. Read plate absorbance at 562 nm using BioTek microplate reader.
11. If available, use a quadratic fit curve to generate standard curve. Use curve to calculate concentrations. Correct for dilution factor as needed.

Western Blots

For consistency, 10% SDS-PAGE gels should be used unless other percentages are absolutely required. Gels should be run at 125 volts until the dye front nears the bottom. A total of 25 µg protein should be run per well. If the lysate is not concentrated enough a lower amount

of total protein may be used. When possible, a positive control cell lysate sample should be run on the gel to serve as a reference point for signal intensity. Decaying signal intensity of the control sample indicates the antibody is going bad, or the western procedure was not performed optimally.

Antibodies should be aliquoted in glycerol and stored at -20ºC. This will ensure the longest shelf life possible and prevent contamination. While antibodies containing sodium azide are said to last a year or more at 4ºC they often lose potency and become contaminated over time. Storing aliquots at -20ºC ensures there is another source of a given antibody to use if a current aliquot is thought to be bad. Used antibodies in TBST may be saved for 1 – 2 weeks if sodium azide is added to a final concentration of 0.05% (w/v). It is important to save antibodies in 1% (w/v) non-fat milk TBST instead of 5% milk because the milk component readily aggregates and falls out of solution at 5%. This precipitation can be further avoided by using BSA in lieu of non-fat milk. An additional consideration is that sodium azide irreversibly inhibits conjugated horseradish peroxidase secondary antibodies. Three washes between incubation with sodium azide solutions and the secondary horseradish peroxidase antibody is sufficient to remove residual sodium azide.

1. Cut a 0.2 µm pore polyvinylidene difluoride (PVDF) membrane to the exact size of gel to be transferred, or slightly larger.
2. Briefly soak PVDF membrane in methanol. Ethanol and isopropyl alcohol will also work if methanol is not available.
3. Transfer PVDF membrane to Towbin transfer buffer and allow to soak until membrane sinks. This takes about 3 minutes. Towbin buffer consists of 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3. If probing for proteins larger than 100 kDa add 0.1% (m/v) sodium dodecyl sulphate.
4. Create a transfer sandwich top to bottom as follows: extra thick blotting paper, trimmed SDS-PAGE gel, PVDF membrane, extra thick blotting paper. Double layers of standard

thickness blotting paper may substituted if needed. Assemble the blot sandwich in transfer buffer, being sure to remove any air bubbles by massaging the transfer sandwich.

5. Move blot sandwich to semi dry transfer apparatus, being sure the layers of the blot have not shifted and do not hang over. Blots with hang over will allow the current to go around the membrane instead of through it, resulting in decreased transfer of proteins.
6. Transfer for 45 - 60 minutes at 150 mA. Double the current with each blot being transferred.
7. Rinse membrane for 2 minutes in TBS (20 mM Tris, 150 mM NaCl, pH 7.6). Repeat for a total of 3 times.
8. Briefly stain in Ponceau Red for 5 minutes (0.1% w/v ponceau, 5% v/v acetic acid, in diH_2O).
9. Rinse 3 times in TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.6).
10. Block in TBST 1% (w/v) non-fat milk for 30 minutes at room temperature.
11. Replace TBST milk and add antibody. Incubate as directed (Table S.3).
12. After incubation period wash 3 times with TBST milk.
13. Add appropriate secondary antibody and incubate for 2 hours at room temperature with agitation on a plate rocker.
14. Rinse 5 times, in 5 minutes intervals with TBST milk. It is imperative to remove any free secondary antibody.
15. Prepare 200 µl developing solution by mixing 100 µl ECL Prime working solution (50 µl reagent A and 50 µl reagent B) with 100 µl PBS. Further dilution of ECL Prime may be used if the signal is too intense, or there is substantial background signal.
16. Blot edge of membrane on a kimwipe to remove excess fluid.
17. Evenly distribute developing solution where protein bands are expected and cover membrane with plastic wrap.

18. Tape down covered membrane in the top left corner of developing cassette and expose. Use 15 seconds, 30 seconds, 1 minute, and 5 minute exposures. If developing 1 or 2 blots, use each corner of the x-ray film individually. This ensures that 4 exposures can be obtained from a single sheet by flipping the x-ray film over.

Stripping Western Blot PVDF Membranes

Western blots using PVDF membranes may be striped of antibodies multiple times. This procedure will not work with nitrocellulose membranes. It is imperative to wash stripping solution off thoroughly to avoid denatured antibodies when re-probing.

1. Rinse blot for 5 minutes with 5 ml TBST.
2. Decant TBST and add 5 ml stripping solution (6M GnHCl, 0.2% Nonidet P-40, 0.1M β-mercaptoethanol, 20mM Tris-HCl,). β-mercaptoethanol should be added when it is fresh. Stripping solution may be warmed to help the GnHCl dissolve.
3. Place on a rocker or shaker for 10 minutes.
4. Decant stripping solution and briefly rinse with TBST.
5. Wash for 5 minutes with TBST. Repeat twice.
6. Re-block membrane with appropriate blocking solution.

RNA Extraction with Tri Reagent

Samples should be prepared directly after harvest. If using frozen samples the time in storage should be minimized. The Tri reagent can be added directly to the frozen samples to thaw. The procedure should be performed in 1.5 ml tubes.

1. For monolayer cells, homogenize the cells by pipetting 1 ml Tri reagent per 10 cm plate. For spheroids add 1 ml Tri reagent and homogenize using a Dounce homogenizer in 1.5 ml tubes. After lysing spheroids, spin at 12,000g for 10 minutes at 4ºC to pellet debris.

2. Incubate for 5 minutes at room temperature on plate rocker.
3. Add 0.2 ml chloroform and shake vigorously for 15 seconds. Allow to stand for 10 minutes.
4. Spin at 12,000g for 15 minutes at 4ºC.
5. Transfer the top aqueous phase to a new tube. Avoid the white interphase as it contains DNA.
6. Add 0.5 ml of isopropanol. Mix and let stand 8 minutes at room temperature.
7. Centrifuge at 12,000g for 10 minutes at 4ºC.
8. Remove supernatant and wash pellet with 1 ml cold 75% ethanol.
9. Vortex briefly and spin at 7,500g for 5 minutes at 4ºC. If pellets floats, perform at 12,000g for 5 minutes. Samples can be saved after this step in ethanol for 4 days at 4ºC, or 3 months at -20ºC.
10. Aspirate ethanol wash with a micropipette and air dry for 20 minutes.
11. Dissolve remaining pellet in 20 µl DNase/RNase free water.

When assessing quality and yield, A260/A280 should be greater than 1.7. Yield from epithelial cells should be between 8 - 15 µg/1 x 10^6 cells. If the extracted RNA is resolved on an agarose gel, strong crisp bands should be observed at 2kb and 5kb if the sample is of good quality. These bands result from the large and small ribosomal RNAs.

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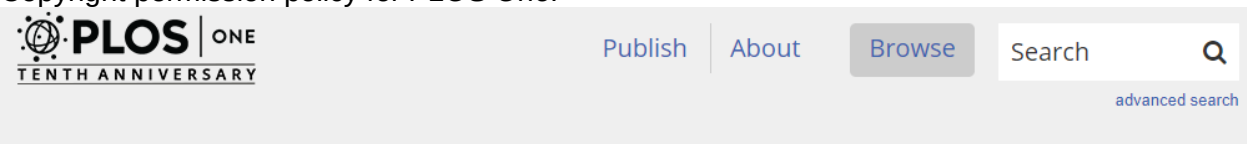

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