Heat Shock Transcription Factor 1 (HSF1) is a Novel Supporter of NSCLC Anoikis Resistance Independent of Heat Shock Proteins

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DOCTOR OF PHILOSOPHY

By

Jack D. Carter

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ABSTRACT

Metastasis is the most lethal step in the progression of cancer, and the five-year survival rate for metastatic lung cancer patients is less than 5%. An essential step for metastasis is resistance to anoikis, a cell death program physiologically induced by detachment of cells from the extracellular matrix. Heat shock transcription factor 1 (HSF1) is the master regulator of heat shock proteins (HSP), and HSF1 and HSP promote cell survival and protein homeostasis during stress. In cancer, HSF1 dynamically controls a network of genes beyond HSP, is a mediator of malignant transformation, and promotes metastasis. HSF1 has been linked to anchorage-independent growth, but whether it exerts its effect by supporting anoikis resistance is largely unknown. Using NSCLC cells, we identified HSF1 as a novel supporter of anoikis resistance. Knockdown of HSF1 sensitizes NSCLC cells to anoikis, yet HSF1 expression or activation does not confer anoikis resistance to normal bronchial epithelial cells, suggesting parallel oncogenic pathways may be required to inhibit anoikis. Consistent with the ability of HSF1 to regulate HSP, HSF1 knockdown partially inhibited HSP72, HSP40, and HSP27. However, targeted inhibition of each HSP did not induce anoikis, suggesting the mechanism of HSF1 is unrelated to these HSP. Intriguingly, HSF1 activation markers were increased in response to cell detachment in H460 cells. Except for HSP60 in A549 cells, cell detachment did not induce HSP, further suggesting an alternative mechanism for HSF1. Interestingly, knockdown of HSP60 sensitized A549 cells to anoikis, despite HSF1 knockdown having no effect on HSP60. This work provides novel evidence that HSF1 and HSP60 can promote anchorage independence by supporting anoikis resistance, and may be valuable targets for future efforts to therapeutically suppress metastasis.

UNIVERSITY OF THE SCIENCES IN PHILADELPHIA

This is to Certify that the Dissertation Prepared by

Jack Carter

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Complies with the Policies of the Graduate Faculty of the University of the Sciences in Philadelphia and is Approved by the Research Advisory Committee as Fulfilling the Dissertation Requirements for the degree of

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_____	_____
Dr. Suzanne K. Murphy	Dr. Bela Peethambaran
Chairman, Research Advisory Committee	Member, Research Advisory Committee
_____	_____
Dr. Dana Pape-Zambito	Dr. Catherine Moore
Member, Research Advisory Committee	Member, Research Advisory Committee

_____
Dr. Bin Chen
Reviewer

DEDICATION

This dissertation is dedicated to my wife, whose unwavering and unconditional love and support is the most treasured aspect of my life, and without which I could never have completed this work. To my mother, I am forever grateful for your love and sacrifices that were integral in guiding me toward a path of success.

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BIOGRAPHICAL SKETCH

Jack Carter was raised in Pennsylvania. He began his college endeavors by taking courses at Northampton Community College during high school. He earned his Bachelor of Science (BS) degree in Biology from the University of the Sciences in Philadelphia in 2011. Jack officially entered the accelerated BS/MS program in Cell Biology and Biotechnology in 2011. In 2012, he transferred into the Ph.D. program in Cell and Molecular Biology. He served as a graduate student instructor for the Department of Biological Sciences from 2012-2016.

LIST OF ABBREVIATIONS AND SYMBOLS

HSF1 – heat shock transcription factor 1

pS326-HSF1 – HSF1 phosphorylated at serine 326 (S326)

siRNA – small interfering RNA

siHSF1 – siRNA targeting HSF1

EV – empty vector

hHSF1 – wild-type human HSF1 plasmid DNA

hHSF1ΔRDT – active human HSF1 plasmid DNA

HSE – heat shock element

HSP – heat shock protein

HSP90 – heat shock protein 90

HSP72 – heat shock protein 72

siHSP72 – siRNA targeting HSPA1A/HSPA1B

HSP60 – heat shock protein 60

siHSP60 – siRNA targeting HSPD1 (HSP60)

HSP40 – heat shock protein 40

siHSP40 – siRNA targeting DNAJB1 (HSP40)

HSP27 – heat shock protein 27

pS82-HSP27 – HSP27 phosphorylated at serine 82 (S82)

siHSP27 – siRNA targeting HSPB1 (HSP27)

siCon – non-targeting scrambled siRNA negative control

CC7 – cleaved caspase 7

PARP – poly-ADP ribose polymerase

c-PARP – cleaved PARP

NSCLC – non-small cell lung cancer

PI3K – phosphatidylinositol-3-kinase

AKT – protein kinase B

MAPK – mitogen-activated protein kinase

17AAG – 17-(Allylamino)-17-demethoxygeldanamycin

DMSO – dimethyl sulfoxide

ECM – extracellular matrix

RFU – relative fluorescence units

RLU – relative luminescence units

polyHEMA – poly-2-hydroxyethyl methacrylate

SD – standard deviation

MAPKAPK2 – MAPK-activated protein kinase 2

HSR – heat shock response

DBD – DNA binding domain

HR-A/B – oligomerization domain

HR-C – suppresses trimer formation

RD – regulatory domain

TAD – transactivation domain

LIST OF TABLES

LIST OF FIGURES

TABLE OF CONTENTS

1. INTRODUCTION

1.1 Introduction: Cancer and Metastasis

1.1.1 General Introduction

Advancing our understanding of tumor biology is of critical importance in furthering the development of much needed effective cancer therapies. While survival rates of cancer patients have continued to improve over time, in 2017 it is still projected that over 1.6 million new cancer cases and over 600,000 cancer deaths will occur in the U.S alone. One-third of patients diagnosed with cancer do not survive five years (Howlader et al., SEER Cancer Statistics Review 1975-2013; Siegel et al., 2017). Globally, cancer remains one of the leading causes of death (Ferlay et al., 2013). Tumor metastasis is a particularly devastating step during cancer progression, and dramatically lowers the survival rates of patients, especially in lung cancer (Table 1.1) (Howlader et al., SEER Cancer Statistics Review 1975-2013). Therefore, rigorous investigation into the physiological mechanisms of tumor initiation, progression, and metastasis, and the translation of this knowledge into therapeutic intervention, remains as important as ever. One such mechanism in metastatic cancers is resistance to anoikis, a physiological cell death program normally induced by detachment of cells from the extracellular matrix (ECM) and important in maintaining proper tissue development, homeostasis, and specificity (Buchheit et al., 2012; Frisch and Francis, 1994; Frisch and Screaton, 2001; Grossmann, 2002; Paoli et al., 2013; Sakamoto and Kyprianou, 2010; Taddei et al., 2012; Zhong and Rescorla, 2012). Presented in this dissertation is evidence of novel supporters

Table 1.1. Five-year survival rates for patients with common cancers in localized and metastatic cases.

Type	Localized	Metastatic
Lung	18.1%	4.5%
Breast	89.7%	26.9%
Prostate	98.6%	29.8%

of anoikis resistance in metastatic lung cancer, including the major cell stress and oncogenic signaling mediator, heat shock transcription factor 1 (HSF1).

Carcinogenesis is a process by which genetic mutations and altered cellular mechanisms transform normal cells into potentially malignant entities. Such changes represent a hijacking of normal cellular processes in a way that enables cancer cells to survive and flourish in a malignant fashion at the expense of an organism. Hanahan and Weinberg (2000, 2011) have described hallmark capabilities acquired by cells during tumor development, including the ability to maintain proliferative signaling, escape growth suppression, inhibit cell death, replicate in an unlimited fashion, induce angiogenesis, and initiate invasion and metastatic dissemination (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). Furthermore, altered energy metabolism (Hanahan and Weinberg, 2011; Phan et al., 2014), evasion of the immune system (Hanahan and Weinberg, 2011; Vinay et al., 2015), interactions with the tumor microenvironment (Hanahan and Weinberg, 2011; Quail and Joyce, 2013; Wang et al., 2017b), tumor-promoting inflammation (Grivennikov et al., 2010; Hanahan and Weinberg, 2011), the presence of cancer stem cells in tumors, and mechanisms regulating tumor dormancy and their switch toward malignancy (Giancotti, 2013; Patel and Chen, 2012; Plaks et al., 2015; Sosa et al., 2014) are also of great importance in the development and spread of

cancer. Collectively, these features form a network of mechanisms that tumor cells manipulate to their benefit at multiple stages of tumor initiation, progression, and metastasis.

One of these features, the onset of metastasis, is the most devastating in the progression of cancer, leading to poor prognosis and having been described as playing a role directly or indirectly in approximately 90% of cancer-related deaths (Gupta and Massagué, 2006; Lambert et al., 2017; Mehlen and Puisieux, 2006; Sakamoto and Kyprianou, 2010; Sleeman and Steeg, 2010; Spano et al., 2012; Valastyan and Weinberg, 2011). Tumor metastasis is a complex and multistep process by which cancer spreads to and colonizes at distant sites, often referred to as the invasion-metastasis cascade (Figure 1.1). A single or a cluster of cells from the primary tumor are disseminated by first invading surrounding tissues, followed by intravasation into the circulatory system. Upon basement membrane penetration, disconnection from the surrounding ECM, and subsequent circulatory system transit, cells must survive independent of anchorage (Lambert et al., 2017; Valastyan and Weinberg, 2011). This task is critically reliant on sustained aberrant signaling of multiple pathways, one crucial piece of which is the suppression of anoikis, or detachment-induced apoptosis (Frisch and Francis, 1994; Frisch and Screaton, 2001; Grossmann, 2002; Guadamillas et al., 2011; Kim et al., 2012; Paoli et al., 2013; Pongrakhananon et al., 2014; Sakamoto and Kyprianou, 2010; Taddei et al., 2012; Tan et al., 2013). Eventually, circulating tumor cells (CTC) will arrest and extravasate from the vessel, invading the surrounding tissue, which is then colonized consequent to the survival and proliferation of these cells in the new microenvironment, forming secondary tumors (Lambert et al., 2017; Valastyan and Weinberg, 2011). Other

processes, such as angiogenesis (Bielenberg and Zetter, 2015; Moserle and Casanovas, 2013) and epithelial-to-mesenchymal transition (EMT), appear to be supportive of metastatic progression, although much about these mechanisms have yet to be clearly understood and remain under debate (Chaffer et al., 2016; Lambert et al., 2017; Nieto et al., 2016).

1.1.2 Introduction to Lung Cancer

Lung cancer is globally the most common type of cancer and is the leading cause of cancer death in the world (nearly 20% of cancer deaths) (Ferlay et al., 2013). In the U.S., lung cancer was estimated to have the second highest incidence in 2017, and continues to have the highest mortality rate of all cancers, over three times higher than the mortality rate of the second deadliest cancer. Only an estimated 18.1% of lung cancer patients survive for five years (2007-2013), which drops significantly to a dismal 4.5% in patients with metastatic lung cancer, representative of the highly aggressive nature of their secondary tumors. Approximately 84% of all lung cancer cases are non-small cell lung cancers (NSCLC) (Howlader et al., SEER Cancer Statistics Review 1975-2014). NSCLC has variants of different histological subtypes including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, among others. Adenocarcinomas make up about 40% of all lung cancer cases, whereas large cell carcinomas make up a smaller proportion (10-15%), but are often tougher to treat due to their increased growth rates and rapid metastasis (American Cancer Society, 2016).

Identification of patient-specific tumor characteristics are helpful in providing alternative or supplemental therapeutic strategies that can be implemented in addition to

chemotherapy and other conventional treatments (Gerber and Minna, 2010; Ke and Wu, 2016). Mutation of a variety of oncogenes (e.g. *EGFR*, *ALK*, *KRAS,* etc.) (Gerber et al., 2014; Minna et al., 2002) or tumor suppressor genes (e.g. *p53, Rb, p16*, etc.) (Kohno and Yokota, 1999; Minna et al., 2002) occur in NSCLC, some of which are diagnostically tested for as a step in personalizing therapeutic approaches (Gerber and Minna, 2010; Ke and Wu, 2016). Three of the most common gene alterations studied in NSCLC are in epidermal growth factor receptor (*EGFR*), anaplastic lymphoma kinase (*ALK*), and V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*).

EGFR-mutated and *ALK*-rearranged NSCLC have been targeted in FDA-approved therapies (Gerber et al., 2014; Ke and Wu, 2016). About 10% of NSCLC patients have *EGFR*-mutations (Gerber and Minna, 2010; Ke and Wu, 2016) and about 5% have *ALK* rearrangements (American Cancer Society, 2016; Gerber and Minna, 2010). Additionally, EGFR protein is overexpressed in tumors of over 60% of metastatic NSCLC patients (Gazdar, 2009; Sharma et al., 2007). EGFR is a transmembrane receptor for epidermal growth factor ligands, and is a member of the ErbB (or HER) family of receptor tyrosine kinases that transduce signals regulating cell proliferation and apoptosis. In NSCLC, *EGFR* mutations typically involve the ATP-binding site (Gazdar, 2009; Gerber et al., 2014; Huang and Fu, 2015), resulting in sustained activation of downstream signaling cascades such as the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) (PI3K/AKT pathway), RAS/rapidly accelerated fibrosarcoma (RAF)/mitogen-activated protein kinase kinase (MEK)/extracellular-signal-regulated kinase (ERK) (MAPK pathway) (Gerber et al., 2014; Huang and Fu, 2015; Ke and Wu, 2016), and janus kinase (JAK)/signal transducer and activator of transcription (STAT)

pathways. Dysregulation of the other targeted kinase, *ALK*, occurs via rearrangement consequent of fusion to echinoderm microtubule-like protein 4 (Gerber et al., 2014; Gerber and Minna, 2010; Huang and Fu, 2015). Aberrant *ALK* signaling can also influence PI3K/AKT, MAPK, and STAT-3 pathways leading to cell survival (Gerber et al., 2014; Takezawa et al., 2011). Inhibition of EGFR or ALK with tyrosine kinase inhibitors have been effective, but acquired resistance to such therapies is a long-term limitation and mechanisms of resistance continue to be studied (Gerber et al., 2014; Gerber and Minna, 2010; Huang and Fu, 2015; Ke and Wu, 2016).

The RAS family of GTPases are molecular switches that influence cell proliferation and survival, among other functions, through RAF/MEK/ERK and PI3K/AKT signaling pathways (Gerber et al., 2014; Hobbs et al., 2016; Mendoza et al., 2011; Simanshu et al., 2017). Oncogenic mutation of *RAS* is observed in about 30% of all human cancers (Lim et al., 2014). *KRAS* is mutated in about 15-20% of NSCLC (Gerber et al., 2014; Lim et al., 2014), and is typically mutually exclusive of other activating mutations (Gerber et al., 2014; Gerber and Minna, 2010). Unfortunately, KRAS-targeted therapies have not been very effective and *RAS* mutations are predictive of poor response to therapies due to a limitation in effective *RAS*-targeting drugs. Some promising studies have identified small molecules that can bind and inactivate mutated KRAS (G12C), impairing its downstream function (Hobbs et al., 2016; Lim et al., 2014; Ostrem et al., 2013), but much work is yet to be done. Meanwhile, other investigations are underway focused on targeting signaling cascades downstream of KRAS (Gerber et al., 2014; Konstantinidou et al., 2009). The NSCLC cell lines used in this study both harbor *KRAS* mutations (ATCC, 2016).

1.2 Anoikis

1.2.1 General Introduction and Importance

In anchorage-dependent cells, detachment from, or improper attachment to, the substratum and surrounding ECM induces a cell death program referred to as anoikis (coined from the Greek meaning "homelessness"). Physiologically, anoikis aids in proper embryonic tissue development and maintenance of tissue homeostasis (Buchheit et al., 2012; Diaz-Mendoza et al., 2013; Frisch and Francis, 1994; Frisch and Screaton, 2001; Grossmann, 2002; Ioannides et al., 2010; Mailleux et al., 2008; Paoli et al., 2013; Sakamoto and Kyprianou, 2010; Taddei et al., 2012; Zhong and Rescorla, 2012). For example, in vertebrates, anoikis has been implicated in helping to sculpt digits and interdigital webbing patterns (Diaz-Mendoza et al., 2013), and aids in proper separation of tracheal and esophageal tubes during development (Ioannides et al., 2010). Anoikis is also important in mammary gland morphogenesis by aiding in luminal clearing (Mailleux et al., 2008; Mailleux et al., 2007). Critically, anoikis is important for maintaining tissue specificity by preventing reattachment and dysplastic growth of cells in inappropriate locations (Buchheit et al., 2012; Frisch and Francis, 1994; Frisch and Screaton, 2001; Grossmann, 2002; Paoli et al., 2013; Sakamoto and Kyprianou, 2010; Taddei et al., 2012; Zhong and Rescorla, 2012).

Physiological inhibition of anoikis occurs as a result of adhesion-mediated signals transmitted through cell-ECM connections. Connection to the ECM is largely mediated through integrins, which provide a link between the ECM and intracellular signal transduction machinery. Consequently, proper attachment stimulates major signal

transduction pathways that suppress anoikis and promote proliferation, whereas detachment severs this signaling and induces the anoikis program (Grossmann, 2002; Paoli et al., 2013; Taddei et al., 2012; Zhan et al., 2004; Zhong and Rescorla, 2012). Anti-anoikis signaling is also triggered by cell-cell contact in anchored and suspended cells (Grossmann, 2002; Rayavarapu et al., 2015). Furthermore, anoikis can be temporarily inhibited during cell migration (Paoli et al., 2013), and can be delayed through detachment-induced autophagy (Buchheit et al., 2012; Fung et al., 2008). Pathologically, excessive anoikis can lead to tissue degeneration and is suggested to be involved in cardiovascular diseases (e.g. smooth muscle cell loss contributing to aneurysms) (Michel, 2003; Taddei et al., 2012), diabetes (e.g. vascular complications and diabetic retinopathy) (Dobler et al., 2006; Taddei et al., 2012), congenital muscular dystrophy (Rahimov and Kunkel, 2013), and other tissue degenerative or remodeling dysfunctions. Relatedly, efforts to regenerate tissue through transplantation of mesenchymal stem cells have been limited in clinic, in part due to anoikis induction, and reversal of this could have therapeutic value for treating degenerative disease (Lee et al., 2015; Taddei et al., 2012).

Two essential steps in tumor progression are the evasion of apoptosis and onset of metastasis (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). The anoikis program is relevant to these two essential steps. For metastasis to occur, cellular detachment from the ECM is required, an action that would typically induce anoikis in normal cells. One way that metastatic tumor cells support anchorage independence and metastasis is through the suppression of anoikis (Figure 1.1). It is evident that anoikis resistance is a required trait of metastatic cancers, enabling malignant cancer cells to

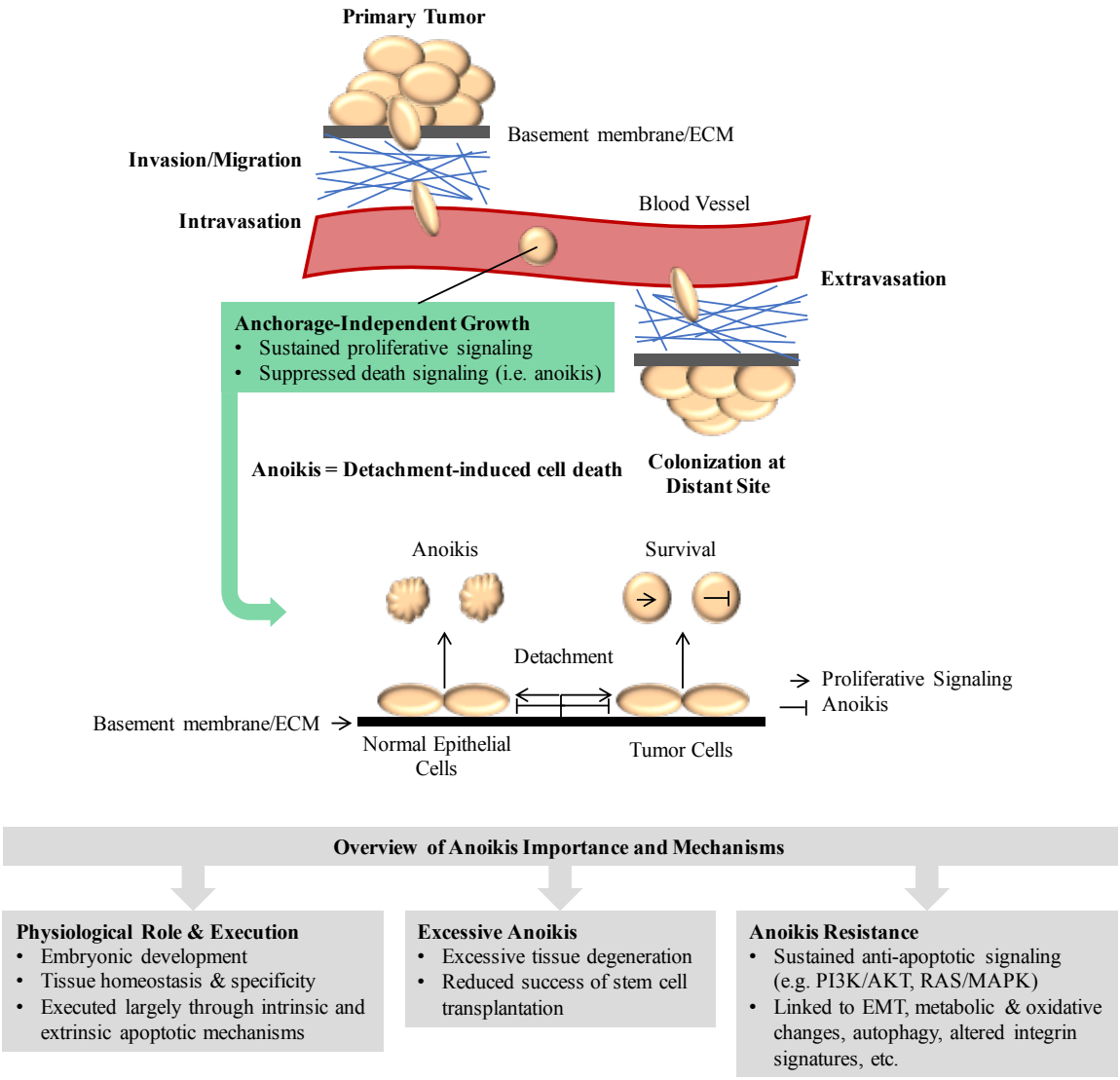

Figure 1.1. Simplified overview of metastasis, with a spotlight on anoikis.

leave the primary tumor and survive independent of anchorage during travel to distant sites (Buchheit et al., 2012; Grossmann, 2002; Paoli et al., 2013; Sakamoto and Kyprianou, 2010; Taddei et al., 2012; Zhong and Rescorla, 2012). Furthermore, it is likely that anoikis resistance is relevant not only during circulatory system transit when adhesion is completely lost, but at other steps as well (Buchheit et al., 2012; Horbinski et al., 2010; Paoli et al., 2013). Anoikis resistance could conceivably play a role during

early stages of tumor hyperproliferation, during which there is significant apoptosis, possibly due to physical displacement of cells from the ECM (Paoli et al., 2013). Changes to the cell-ECM connection during tissue invasion and cell migration, angiogenesis, as well as the presence of an unfamiliar ECM at metastatic sites, would seemingly also require cells to suppress anoikis (Buchheit et al., 2012; Dobler et al., 2006; Horbinski et al., 2010; Paoli et al., 2013; Taddei et al., 2012; Zhang et al., 2011b). Our understanding of the precise mechanisms by which anoikis resistance influences such processes is not well understood, and further study of this will be important. Ultimately, the identification and therapeutic targeting of specific anoikis mediators is an important area of research, with the potential to impede or limit metastatic dissemination.

1.2.2 Anoikis Mechanisms

With some exception, anoikis signaling is largely executed in similar ways compared to other apoptotic mechanisms. Anoikis signaling can take place through either intrinsic (mitochondrial) or extrinsic (death receptor) types of apoptotic mechanisms, both leading to caspase activation and the subsequent characteristic changes associated with apoptotic death (Frisch, 1999; Grossmann, 2002; Paoli et al., 2013; Sakamoto and Kyprianou, 2010; Taddei et al., 2012; Zhan et al., 2004; Zhong and Rescorla, 2012). Interestingly, some evidence of a caspase-independent alternate anoikis pathway has also been discovered (Jenning et al., 2013). Our understanding of anoikis mechanism is still relatively young and there exists some diversity in mechanisms between cell types, highlighting the need for further study to provide more targetable therapeutic options. Some anoikis signaling mechanisms and effectors are briefly discussed in these sections.

1.2.2.1 Anoikis via the Intrinsic Pathway

The intrinsic pathway of apoptosis involves manipulation of antagonistic members of the B-cell lymphoma 2 (BCL-2) family, and the balance of pro- and anti-apoptotic proteins of this family can decide the fate of the cell, causing perturbation of the mitochondria when leading to apoptosis (Beere, 2004; Elmore, 2007; Sreedhar and Csermely, 2004). Members of the BCL-2 family are categorized into anti-apoptotic and pro-apoptotic, and these functions often relate to their underlying structure (variation in BCL-2 homology (BH) domains). Key anti-apoptotic members include BCL-2, BCL-extra large (BCL-XL), and myeloid cell leukemia sequence 1 (MCL-1). BCL-2-associated X protein (BAX) and BCL-2 homologous antagonist killer (BAK) are well-studied multi-BH3 domain pro-apoptotic members, and BCL-2-like protein 11 (BIM), BH3 interacting-domain death agonist (BID), BCL-2-associated agonist of cell death (BAD), BCL-2 interacting killer (BIK), BCL-2 modifying factor (BMF), Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA), and p53 upregulated modulator of apoptosis (PUMA) are BH3-only pro-apoptotic members (Beere, 2004; Kuwana et al., 2005; Paoli et al., 2013; Taddei et al., 2012; Wazir et al., 2015). Briefly, changes in the expression, location, and binding partners of this family of proteins ultimately lead to the formation of BAX-BAK oligomers in the outer mitochondrial membrane (OMM), resulting in its permeabilization. Subsequently, cytochrome c (and SMAC/DIABLO) is released through the resulting channels, which aids in the formation of the apoptosome along with caspase-9 and apoptosis protease activating factor 1 (APAF-1). This event leads to activation of effector caspases (e.g. caspase-3, caspase-7), resulting in proteolysis of targets such as the DNA repair protein, poly-ADP ribose polymerase (PARP), and

leading to the implementation of apoptosis (Beere, 2004; Elmore, 2007; Kim et al., 2012; Oliver et al., 1998; Paoli et al., 2013; Sreedhar and Csermely, 2004; Taddei et al., 2012).

The anoikis activator, BIM, is a major modulator of anoikis occurring via the intrinsic pathway. Detachment of cells from the ECM leads to activation of BIM (Reginato et al., 2003; Woods et al., 2007). During appropriate integrin-ECM interaction, BIM is sequestered in dynein cytoskeletal complexes (Puthalakath et al., 1999). Cell detachment disrupts these interactions leading to the release of BIM, its translocation to the mitochondria, and ultimately apoptosis (Kim et al., 2012; Paoli et al., 2013; Taddei et al., 2012). In parallel, detachment-induced disruption of PI3K/AKT and ERK signaling prevents BIM from being targeted to the proteasome via phosphorylation (Luciano et al., 2003; Qi et al., 2006). Similarly, detachment induces the translocation of full-length BID (Valentijn and Gilmore, 2004), BMF (Puthalakath et al., 2001), and BAX (Valentijn et al., 2003) to the mitochondria. However, BIM and BID are activators that directly lead to the formation of BAX-BAK oligomers in the OMM to promote apoptosis, whereas other pro-apoptotic BCL-2 family members act as sensitizers that indirectly trigger mitochondrial perturbation by competitively antagonizing anti-apoptotic family members (Kuwana et al., 2005; Letai et al., 2002). PUMA has also been described as an activator of mitochondrial apoptosis (Bean et al., 2013; Woods et al., 2007), and is suggested to be involved in anoikis (Gilmore, 2005; Kim et al., 2012; Paoli et al., 2013), but its role is unclear (Woods et al., 2007). Sensitizers like BMF (Puthalakath et al., 2001), BAD (Idogawa et al., 2003), BIK, and NOXA are suggested to be involved in anoikis (Gilmore, 2005; Kim et al., 2012; Paoli et al., 2013). For example, similar to BIM, the detachment-induced release and mitochondrial translocation of BMF from myosin V

motors induces anoikis by binding anti-apoptotic BCL-2 proteins (Puthalakath et al., 2001). Also, BAD can be inactivated through phosphorylation, which is reduced upon detachment of cells, and overexpression of BAD enhances anoikis (Idogawa et al., 2003). Further, the expression of anti-apoptotic members BCL-XL (Frankel et al., 2001) and MCL-1 (Woods et al., 2007) are reduced when cells are detached, and this was important for anoikis induction. However, evidence suggests an activator is ultimately required to induce anoikis (Rosen et al., 2002; Woods et al., 2007). In summary, these studies highlight mechanisms by which anoikis can be executed through mitochondrial-mediated apoptosis. Much of this apoptotic machinery is known to be influenced by heat shock proteins (HSP), whose expression are driven by heat shock transcription factor 1 (HSF1), and as such we have studied whether HSF1 or HSP may be influential in anoikis resistance.

1.2.2.2 Anoikis via the Extrinsic or Caspase-Independent Pathways

The extrinsic, or death receptor, pathway is regulated through receptor-ligand signaling via receptors of FAS, tumor necrosis factor (TNF), and TNF-related apoptosis-inducing ligand (TRAIL) (Beere, 2004; Elmore, 2007; Paoli et al., 2013; Taddei et al., 2012; Wazir et al., 2015). Briefly, at the death-inducing signaling complex (DISC), "death domains" of the transmembrane receptors interact with adapter proteins like Fas-associated death domain (FADD) to ultimately activate initiator caspase-8. Subsequently, caspase-8 activates effector caspases-3 and -7 directly, or cleaves and activates BID into truncated BID (t-BID), a crosstalk mechanism that triggers mitochondrial apoptosis (Elmore, 2007; Paoli et al., 2013; Taddei et al., 2012; Wazir et al., 2015). Notably, an

alternative to FADD-dependent mechanisms exists, and a different adaptor molecule, death domain-associated protein 6 (DAXX), can independently drive Fas-mediated apoptosis (Beere, 2004; Charette et al., 2000). Extrinsic anoikis mechanisms are evident by the inhibition of anoikis upon expression of dominant-negative FADD (Frisch, 1999), and the anoikis-sensitizing detachment-induced upregulation of the FAS receptor and ligand (Aoudjit and Vuori, 2001; Rosen et al., 2002). Furthermore, cell detachment reduces expression of the caspase-8 antagonist, FLICE inhibitory protein (c-FLIP)(Aoudjit and Vuori, 2001), whose overexpression has been shown to inhibit anoikis (Aoudjit and Vuori, 2001; Marconi et al., 2004).

In addition to the caspase-dependent pathways that regulate anoikis, there is evidence of a caspase-independent anoikis mediator, the mitochondrial protein, BIT1 (Jan et al., 2004; Jenning et al., 2013; Yao et al., 2014). Detachment of cells induces the release of BIT1 from the mitochondria, enabling its complex with amino-terminal enhancer of split (AES) and initiating caspase-independent apoptosis. Furthermore, integrin-mediated attachment blocks BIT1-mediated anoikis (Jan et al., 2004; Jenning et al., 2013). Evidence of BIT1 influence on anoikis has been observed in various tumor models (Jenning et al., 2013; Yao et al., 2014). Collectively, an assortment of anoikis mechanisms have been observed, and these mechanisms can be hijacked in tumor cells. Such mechanisms can be influenced by aberrant signaling through integrin-mediated pathways, some of which are discussed in the following section.

1.2.3 Anoikis Resistance Mechanisms in Cancer

Anoikis resistance is an essential step in the process of metastasis, and is characteristic of metastatic cancers. Malignant tumor cells circumvent anoikis largely through aberrant sustained activation of anti-apoptotic signaling, manifested at multiple levels, including dysregulation of integrins or growth factor receptors, key signal transducers, or downstream anoikis effectors. Such pathways are stimulated often through mutation of relevant oncogenes and tumor suppressor genes, but also through phenomena observed in malignant cancers such as EMT, altered mechanisms for redox regulation and metabolism, and autophagy. These mechanisms are briefly discussed in this section.

Intrinsically, normal cells have the ability to suppress anoikis under anchored conditions due to signaling initiated by proper contacts to the ECM. Understanding which pathways are activated under these conditions is helpful because these mechanisms are largely taken advantage of without triggers from the ECM to support malignancy and metastatic dissemination. In anchored cells, integrin-mediated signaling plays a vital role in suppressing anoikis. Integrins (e.g. $\alpha v\beta 3$, $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$, etc.) promote survival of anchored cells through signaling pathways and proteins such as PI3K/AKT, MAPK, Jun-kinase (JNK), focal adhesion kinase (FAK), Src kinase, and integrin-linked kinase (ILK) (Gilmore, 2005; Grossmann, 2002; Paoli et al., 2013; Sakamoto and Kyprianou, 2010; Taddei et al., 2012; Zhan et al., 2004; Zhong and Rescorla, 2012). PI3K/AKT and MAPK signaling pathways play particularly significant roles in suppressing anoikis and are triggered by other key proteins. For instance, FAK is recruited into focal adhesions and activated in anchored cells, and in concert with Src, recruits key signaling molecules to these sites leading to PI3K/AKT and MAPK signaling

(Paoli et al., 2013; Sakamoto and Kyprianou, 2010; Taddei et al., 2012; Wozniak et al., 2004). ILK also activates signaling through AKT to suppress anoikis (Attwell et al., 2000). Caveolin-1 (CAV-1) promotes survival, in part, due to its ability to enhance β1-integrin signaling through Shc/MAPK (Wary et al., 1998). Furthermore, proper integrin-ECM interaction can lead to ligand-independent activation of growth factor receptors such as EGFR, platelet-derived growth factor receptor (PDGFR), and vascular endothelial growth factor receptor (VEGFR), activators of PI3K/AKT and MAPK signaling (Kim et al., 2012; Paoli et al., 2013; Taddei et al., 2012).

Convergence on PI3K/AKT or MAPK signaling can inhibit cell death in several ways, relevant to anchorage-induced signaling or constitutive activation in tumor cells. Such signaling can influence intrinsic apoptosis through transcriptional and posttranslational (proteasomal targeting) inhibition of anoikis activator BIM (Luciano et al., 2003; Qi et al., 2006), inhibitory phosphorylation of BAD (Datta et al., 1997; Idogawa et al., 2003), upregulation of multiple pro-survival BCL-2 family proteins (Paoli et al., 2013), and caspase inhibition (Cardone et al., 1998). Further, ERK signaling can influence extrinsic apoptosis through altered expression of c-FLIP (Aoudjit and Vuori, 2001). Even further, these pathways influence apoptosis, including anoikis, through modulation of transcription factors such as nuclear factor-κB (NF-κB)(Romashkova and Makarov, 1999; Toruner et al., 2006) and Fork-head transcription factors (Skurk et al., 2004; Taddei et al., 2012), which regulate expression of genes involved in cell death. In normal cells, disruption of integrin-ECM interaction severs these survival signals, contributing to anoikis sensitivity, whereas aberrant sustained signaling through these pathways in tumor cells is associated with anoikis resistance (Gilmore, 2005; Grossmann,

2002; Paoli et al., 2013; Sakamoto and Kyprianou, 2010; Taddei et al., 2012; Zhan et al., 2004; Zhong and Rescorla, 2012). Constitutive activation of the PI3K/AKT and MAPK signaling is a common result of carcinogenesis and tumor progression, and can be attained through activating mutations of RAS or deactivating mutations of the tumor suppressor phosphatase and tensin homolog (PTEN) (Edwards et al., 2005; Rytömaa et al., 2000; Tokunaga et al., 2008), both of which lead to increased anoikis resistance (Frisch and Francis, 1994; Vitolo et al., 2009). Additionally, sustained activation of FAK (Duxbury et al., 2004), Src (Frisch and Francis, 1994; Windham et al., 2002), or ILK (Attwell et al., 2000; Paoli et al., 2013; Sakamoto and Kyprianou, 2010) in tumor cells, can also trigger PI3K/AKT or MAPK signaling. Furthermore, sustained activation of growth factor receptors such as neurotrophic tyrosine kinase receptor B (TrkB)(Douma et al., 2004; Geiger and Peeper, 2007) and ErbB receptors EGFR and HER-2/Neu (ErbB2)(Grassian et al., 2011; Haenssen et al., 2010; Sharma et al., 2007) also significantly contribute to anoikis resistance through activation of PI3K/AKT, MAPK, and other pathways. Of note, the subject of our investigations, HSF1, which we found to support anoikis resistance, is a major mediator of oncogenic signaling through PI3K/AKT and MAPK pathways (see figure 1.2C)(Dai et al., 2007; Khaleque et al., 2005; Meng et al., 2010; O'Callaghan-Sunol and Sherman, 2006; Schulz et al., 2014).

Interestingly, an upstream phenomenon of relevance is the dysregulation of integrins themselves in malignant tumor cells. Different cell types have different integrin profiles, and the expression or signature of these can be altered as cells progress toward a metastatic phenotype. Some tumor cells switch integrin expression signatures compared to their normal counterparts in such a manner that favors signaling through integrins that

promote survival (Felding-Habermann et al., 2002; Gehlsen et al., 1992; Janes and Watt, 2004). As such, this is one mechanism that might enable sustained aberrant signaling in malignant tumor cells, and has been associated with anoikis resistance and the metastatic phenotype (Guadamillas et al., 2011; Paoli et al., 2013; Taddei et al., 2012). While not described here, altered expression or activation of various downstream apoptosis mediators through alternate signaling, transcriptional, and post-translational mechanisms as a result of tumorigenesis can certainly also promote anoikis resistance.

Other cellular processes can promote anoikis resistance in tumor cells, such as EMT and altered redox and metabolic mechanisms (Buchheit et al., 2012; Frisch et al., 2013; Giannoni et al., 2008; Guadamillas et al., 2011; Kim et al., 2012; Paoli et al., 2013; Taddei et al., 2012). EMT is a process leading to the phenotypic change of epithelial cells to a mesenchymal phenotype, and its induction is associated with increased cell motility, invasiveness, and acquisition of anoikis resistance. Intracellularly, EMT induction is associated with reduced expression of epithelial cell markers (e.g. E-cadherin), increased expression of mesenchymal cell markers (e.g. N-cadherin, vimentin, etc.), and altered regulation of apoptosis-related genes. In multiple tumor types EMT is regulated by several transcription factors (e.g. SNAIL, SLUG, TWIST, ZEB1/2, NF-κB, HIF1/2, etc.) that influence the expression of key targets that enable anoikis resistance. For instance, such mechanisms increase the expression of anti-apoptotic proteins (e.g. BCL-2, BCL-XL, XIAP) and reduce the expression of pro-apoptotic proteins (e.g. BIM, BID, BMF, BAX, BAD, PTEN, p21, NOXA, etc.). Multiple links between EMT, anoikis resistance, and FAK, ILK, PI3K/AKT, and MAPK-related signaling mechanisms are also known. One major mechanism involves neurotrophic tyrosine kinase receptor B (TrkB), a potent

anoikis suppressor that utilizes the PI3K/AKT pathway, and a promoter of EMT that involves MAPK signaling (Douma et al., 2004; Frisch et al., 2013; Guadamillas et al., 2011; Kim et al., 2012; Paoli et al., 2013; Taddei et al., 2012). Importantly, suppression of EMT might be a possible approach for reinstating the anoikis program in malignant tumor cells, and is therefore an important mechanism to continue elucidating. Of interest to the topic of this dissertation, it has been recently reported that EMT transcription factor genes *SLUG* (Carpenter et al., 2015), *SNAIL*, *ZEB*, and *TWIST1* (Powell et al., 2016) contain heat shock element (HSE) sequences, the binding site for our target of interest, HSF1, and HSF1 was shown to transcriptionally activate SLUG (Carpenter et al., 2015). We have shown here that HSF1 supports anoikis resistance, and have focused on its canonical mechanism, but a potential connection between HSF1, EMT, and anoikis resistance warrants investigation.

Detachment of cells causes oxidative stress and alterations in cell metabolism, which must be dealt with for continued cell survival and functioning. To do so, maintenance of appropriate redox balance and reprogramming of metabolic machinery is essential. Tumor cells have increased glucose uptake and preferentially use glycolysis for energy production, referred to as the Warburg effect (Kamarajugadda et al., 2012; Kim et al., 2012; Paoli et al., 2013; Taddei et al., 2012; Vander Heiden et al., 2009). Intriguingly, production of reactive oxygen species (ROS) in cancer is associated with both increased survival and metastatic progression, as well as apoptosis when in excess. As such, increased production of ROS can promote anoikis resistance, and antioxidant mechanisms are important to maintain this delicate balance to prevent excessive ROS from triggering apoptosis. ROS modulates integrin and growth factor receptor signaling

pathways (e.g. Src, PI3K/AKT, MAPK, etc.) and transcription factor activation (e.g. NF-κB, HIF1/2, Nrf2, etc.), which affect multiple layers of anoikis machinery (Buchheit et al., 2012; Giannoni et al., 2008; Guadamillas et al., 2011; Kim et al., 2012; Paoli et al., 2013; Taddei et al., 2012). For instance, sub-lethal hydrogen peroxide exposure increases lung carcinoma anoikis resistance through sustained expression of CAV-1 (Halim and Chanvorachote, 2012; Rungtabnapa et al., 2011). CAV-1 promotes anoikis resistance, in part, through sustained expression of MCL-1 by suppressing its proteasomal degradation, and through direct interaction (Chunhacha et al., 2012), possibly through PI3K/AKT signaling (Lloyd, 2012). Similarly, increased superoxide production also supports anoikis resistance through PI3K/AKT and MAPK signaling, through a mechanism that involves angiopoietin-like 4 mimicking of cell adhesion by binding to β1 and β5 integrins (Zhu et al., 2011). Yet, maintaining a non-lethal level of ROS is similarly important, for instance, detachment-induced activation of AMP-activated protein kinase (AMPK) promotes survival by preventing excessive ROS production through altered NADPH mechanisms (Jeon et al., 2012). Interestingly, among other mechanisms, tumor cells experiencing hypoxic conditions can adapt and increase anoikis resistance by inducing EMT (e.g. through HIF-1α) and activating pro-survival signaling (Kim et al., 2012; Paoli et al., 2013; Taddei et al., 2012; Whelan et al., 2010; Zhang et al., 2015). In normal cells, detachment also leads to reduced glucose uptake and energy-related cell stress. Consequent to oncogenic signaling mechanisms, tumor cells can restore glucose uptake and therefore reduce such stress (Mason et al., 2017). For example, overexpression of ErbB2 rescues suspended epithelial cells by reestablishing glucose uptake through an EGFR-PI3K/AKT and antioxidant-producing mechanism (Schafer et al., 2009). Also,

pyruvate dehydrogenase (PDH) kinase 4 (PDK4) was found to promote anoikis resistance in mammary epithelial cells by suppressing PDH, leading to decreased glucose oxidation and oxidative stress, adaptations that facilitate tumor survival and metastasis (Kamarajugadda et al., 2012). With respect to the topic of this dissertation, we identified HSF1 activity was capable of being triggered by cell detachment, without inducing HSP. It is notable that HSF1 is known to respond to oxidative stress (e.g. hydrogen peroxide) (Ahn and Thiele, 2003), and modulates glucose metabolism in tumor cells (Dai et al., 2007), and while not examined here, these are potential avenues for future research.

Another complex and developing area of research is the connection between autophagy and anoikis resistance. Briefly, autophagy is a stress-induced cellular process of self-digestion that involves lysosomal-mediated degradation of cell components, which can act both as a mechanism of cell death or cell survival (Yang et al., 2013). Although the mechanisms remain complex and unclear, ECM detachment induces autophagy and promotes anchorage-independent survival in the face of anoikis induction, and requires autophagy-related protein 7 (ATG7) and ATG5 (Fung et al., 2008). Many other mechanisms for autophagy have been described, and while not discussed here, have been reviewed by Yang *et al.* (2013) in the context of interplay with anoikis resistance (Yang et al., 2013). In this context, autophagy can promote improved cell homeostasis, allowing cells an opportunity to re-attach, and essentially providing a bypass to anoikis that can promote dissemination of malignant tumor cells. With respect to the findings presented in this dissertation, HSF1 was activated by cell detachment but did not trigger induction of HSP. It is notable that HSF1 has been reported to transcriptionally regulate ATG7 to

control autophagy (Desai et al., 2013), and it may be valuable to evaluate in future studies whether HSF1 might play a dual role in anoikis resistance and autophagy.

In summary, tumor cells acquire anoikis resistance by dysregulating signaling responsible for suppressing anoikis in anchored cells, through detachment-induced mechanisms, and by using to their benefit other altered biological mechanisms and environmental conditions they encounter (Fung et al., 2008; Guadamillas et al., 2011; Kim et al., 2012; Malagobadan and Nagoor, 2015; Paoli et al., 2013; Taddei et al., 2012). While the goals of this dissertation were focused on canonical HSF1 mechanisms, there is significant overlap between mechanisms of anoikis resistance and processes influenced by HSF1. Our findings, presented in this dissertation, leave open the possibility that HSF1 could promote anoikis resistance through one or more of the mechanisms discussed in this section. Many of these processes would be of interest to study in future work, in an effort to identify what non-canonical mechanisms for HSF1-mediated anoikis resistance might exist.

1.3 Heat Shock Transcription Factor 1 (HSF1)

1.3.1 General Introduction

Heat shock transcription factor 1 (HSF1) is a transcription factor classically known as a master regulator of heat shock protein (HSP) transcription (Calderwood et al., 2010; Ciocca et al., 2012; Inouye et al., 2003; Morimoto, 1998; Sarge et al., 1993; Stanhill et al., 2005). HSF1 is activated in response to a wide variety of biological and clinical stresses, leading to activation of the heat shock response (HSR), which can

temporarily help cells survive stress. Activation of the HSF1-mediated HSR is associated with response to heat shock, changes in pH, UV radiation, heavy metal exposure, and osmotic stress. Particularly relevant to cancer, HSF1 can also respond to changes in ROS production, hypoxic conditions, and various drugs (Åkerfelt et al., 2010; Caruccio et al., 1997; Dai et al., 2012; Dayalan Naidu and Dinkova-Kostova, 2017; Dayalan Naidu et al., 2016; Morimoto, 2011; Vydra et al., 2013; Vydra et al., 2014). In general, the HSF1-mediated HSR has the underlying role of responding to a disruption in protein homeostasis, and is a crucial process for maintaining the health and life span of cells and organisms. Central to the HSR is the upregulation of HSP, key molecular chaperones with a primary function of maintaining proper protein folding and conformation, preventing protein aggregation, and maintaining protein stability. Consequently, HSF1-mediated HSP regulation plays a critical role in promoting cell survival (Calderwood et al., 2010; Morimoto, 2011). Furthermore, HSP have a variety of mechanisms for directly preventing stress-induced apoptosis (Beere, 2004; Sreedhar and Csermely, 2004). The underlying biological mechanisms associated with HSP upregulation via HSF1 activation are relevant in disease etiology, having roles in aging, neurodegeneration, cardiovascular disease, and cancer (Calderwood et al., 2010; Dayalan Naidu et al., 2016). A complete understanding of the reasons for such involvement has not been fully attained, and dueling or opposing roles in these processes is a challenge that remains to be solved, yet is of great therapeutic value to pursue (Arneaud and Douglas, 2016; Calderwood et al., 2010).

Of particular interest is the role HSF1 plays in cancer, which appears to be an expansive role that includes, but extends far beyond, the regulation of HSP. HSF1 is

overexpressed and activated in multiple cancer models, and appears to be associated with highly malignant tumors (Chuma et al., 2014; Cui et al., 2015; Dai et al., 2007; Engerud et al., 2014; Fang et al., 2012; Li et al., 2014b; Mendillo et al., 2012; Santagata et al., 2011). While not an oncogene or tumor suppressor itself, HSF1 is a major mediator of oncogenic signaling, having been described as a "powerful multifaceted modifier of carcinogenesis" (Dai et al., 2007), and its nuclear localization correlates with poor prognosis in lung, breast, and colon cancer (Dayalan Naidu et al., 2016; Mendillo et al., 2012; Santagata et al., 2011). In malignant tumor cells, HSF1 controls a broad network of genes distinct from the stress-induced HSR, linking it to a wide array of cellular processes (Mendillo et al., 2012). In this context, HSF1 contributes to malignant transformation and metastasis, having roles in apoptosis suppression, invasiveness, migration, anchorage-independent growth, EMT, angiogenesis, glucose metabolism, redox regulation, autophagy, cell cycle regulation, protein translation, multi-drug resistance, and other processes (Ahn and Thiele, 2003; Cui et al., 2015; Dai et al., 2007; Desai et al., 2013; Fang et al., 2012; Gabai et al., 2012; Khaleque et al., 2005; Liu et al., 2016; Mendillo et al., 2012; O'Callaghan-Sunol and Sherman, 2006; Powell et al., 2016; Scott et al., 2011; Tchénio et al., 2006; Toma-Jonik et al., 2015; Wang et al., 2006a). Deciphering the precise mechanisms of HSF1 in these processes will further our understanding of this far-reaching protein and aid in the development of therapeutic strategies to target cancer and suppress metastasis.

1.3.2 Structure and Activation

In eukaryotes, HSF1 structure (Figure 1.2A) is highly conserved. Located at the N-terminus is a DNA-binding domain (DBD) (a helix-turn-helix), which binds to highly conserved cis-acting elements known as heat shock elements (HSE), composed of at least three inverted nGAAn pentamers (Fujimoto and Nakai, 2010; Vihervaara and Sistonen, 2014) and proximally located in target promoters (Fujimoto and Nakai, 2010). Adjacent to this domain is an oligomerization domain consisting of hydrophobic heptad repeats (HR-A/B) that, through coiled-coil interactions, enables the formation of the HSF1 trimers that actively bind HSE (Vihervaara and Sistonen, 2014). A leucine zipper-like motif containing a heptad repeat (HR-C) exists that can fold in such a way to contact the HR-A/B domain and prevent spontaneous trimer formation (Fujimoto et al., 2005; Vihervaara and Sistonen, 2014; Wang et al., 2003). As such, in non-stressed cells HSF1 is found as an inactive monomer (Calderwood et al., 2010; Fujimoto and Nakai, 2010). HSF1 also contains a transactivation domain (TAD) that is responsible for transcriptional activation of target genes (Fujimoto et al., 2005; Vihervaara and Sistonen, 2014; Wang et al., 2003). The TAD is normally masked by the regulatory domain (RD), located between HR-A/B and HR-C, and containing many sites for post-translational modifications (PTMs) that control HSF1 transactivational competence (Fujimoto et al., 2005).

Activation of HSF1 by stress is a complex and multi-step process (Figure 1.2B-C). In non-stressed cells, HSF1 is found as an inactive monomer distributed between the cytoplasm and nucleus (Morimoto, 1998; Sarge et al., 1993; Stanhill et al., 2005). HSF1 is kept in its inactive state through sequestration by HSP90, HSP70, and HSP40 (Morimoto, 2011). A driving theory for the stress-induced activation of monomeric HSF1

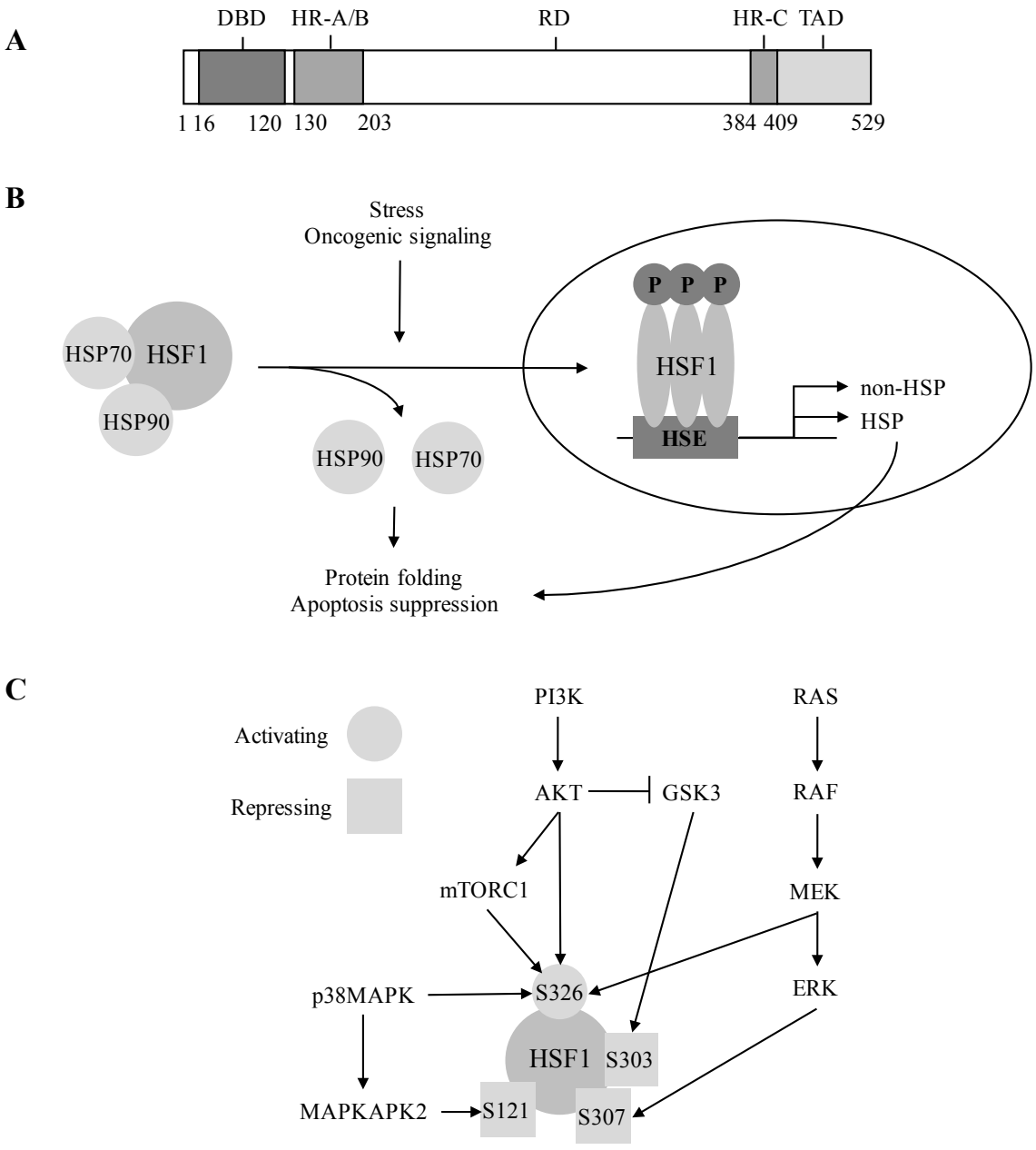

Figure 1.2. HSF1 structure and activation. (A) Schematic representing HSF1 structure (DBD = DNA binding domain, HR-A/B = oligomerization domain, RD = regulatory domain, HR-C = suppresses spontaneous trimer formation, TAD = transactivation domain). (B) Under normal conditions monomeric HSF1 is sequestered by a chaperone complex. Stress and other inducers of HSF1 activation trigger its release from this complex, trimerization, and increased nuclear localization, where it binds HSE within *HSP* and other genes that prevent protein aggregation and apoptosis. (C) Overview of common signaling pathways regulating HSF1 transactivation through post-translational modification.

is that an increase in misfolded proteins recruits HSP away from HSF1, thereby releasing it as the first step toward its activation. After being released from its chaperone complex, HSF1 becomes hyperphosphorylated (with some exception), forms trimers, and localizes into nuclear stress granules (Calderwood et al., 2010; Ciocca et al., 2012; Morimoto, 1998; Sarge et al., 1993; Stanhill et al., 2005). In the nucleus, HSF1 trimers bind the highly conserved HSE within the promoters of target genes to control gene expression. To achieve full transactivational competence, HSF1 is generally thought to be regulated through multiple PTMs on amino acids within the RD. Providing a negative regulatory circuit, upregulation of HSP expression can subsequently lead to inhibition of HSF1 (Calderwood et al., 2010; Ciocca et al., 2012; Guettouche et al., 2005; Morimoto, 1998; Sarge et al., 1993; Stanhill et al., 2005; Vihervaara and Sistonen, 2014).

A variety of HSF1 phosphorylation events primarily at serine, but also at threonine, residues has been observed in response to stress (Guettouche et al., 2005; Xu et al., 2012). Only three activating phosphorylation events have been identified, occurring at serine 326 (S326)(Guettouche et al., 2005), serine 230 (S230)(Holmberg et al., 2001), and serine 320 (S320)(Murshid et al., 2010), and are responsive to stress. Activation of HSF1 via S230 has been shown to depend on calcium/calmodulin-dependent protein kinase II (CaMKII) (Holmberg et al., 2001). Phosphorylation of S320 is dependent on protein kinase A (PKA)(Murshid et al., 2010). In response to stress, phosphorylation at S326 rapidly occurs and typically dissipates during the recovery phase while transcription and translation occurs (Guettouche et al., 2005). Phosphorylation of S326 is perhaps the most crucial modification for induction of the HSR, and is regarded as a well-established hallmark of HSF1 activity in response to stress and in highly malignant tumor cells

(Dayalan Naidu et al., 2016; Guettouche et al., 2005; Mendillo et al., 2012). Recently, an additional function for S326 phosphorylation in promoting HSF1 stability was observed in NSCLC cells (Yoon et al., 2014). Activators of HSF1 via S326 have been largely unknown, but a variety of recent work has identified some important mediators (Figure 1.2C). First, it was identified that in HeLa cells mTOR (mTORC1 complex) regulated the phosphorylation of HSF1 at S326 in response to stress (Chou et al., 2012). Interestingly, mTOR inhibition prevented HSP synthesis and inhibited nuclear accumulation of HSF1 in response to an HSP90 inhibitor (Acquaviva et al., 2014), which could suggest perhaps S326 phosphorylation might also play a role in the initial release of HSF1 from HSP90. It was later identified that in breast cancer cells, AKT can directly bind and phosphorylate HSF1 at S326, independent of mTOR (Carpenter et al., 2015). Furthermore, there is now evidence that MEK (Tang et al., 2015) and p38MAPK (Dayalan Naidu et al., 2016) also regulate HSF1 phosphorylation at S326. Phosphorylation events important for repressing HSF1, such as through serine 303 (S303)/serine 307 (S307) and serine 121 (S121), have also been identified. Repressive phosphorylation of HSF1 through S303/S307 is stimulated through MAPK/ERK1/glycogen-synthase kinase 3 (GSK3) signaling (Chu et al., 1998; Kline and Morimoto, 1997; Knauf et al., 1996). Meanwhile, MAPK-activated protein kinase 2 (MAPKAPK2) can repress HSF1 activity via S121 phosphorylation, while also promoting its binding to HSP90 (Wang et al., 2006b). Collectively, these findings highlight the complexity of HSF1 activation and link it to major pathways, such as PI3K/AKT and MAPK signaling, that regulate an array of processes, such as cell proliferation, apoptosis, and transformation.

While specific PTMs are important for regulating HSF1 transactivational competence, a precise role for hyperphosphorylation (i.e. mass phosphorylation of multiple sites in the RD) remains somewhat unclear. Hyperphosphorylation of HSF1, apparent through a clear shift in electrophoretic mobility, is a typical, but not always observed, response to stress. Recent evidence suggests that hyperphosphorylation may be dispensable for the stress-induced HSF1 activation (Budzyński et al., 2015), and functional HSF1-mediated increases in HSP have been observed without any apparent electrophoretic mobility shift (Khaleque et al., 2005). A possible uncoupling between HSF1 hyperphosphorylation and activation was demonstrated since an HSF1 mutant, where 15 phosphorylation sites within the RD were simultaneously mutated, was effectively activated by stress. Mutations included major sites known for both activating (e.g. S326) and repressing (e.g. S303/S307) HSF1, and it was suggested the discrepancy could represent differences in approach between single site and large scale mutagenesis (Budzyński et al., 2015). Perhaps the net balance of activating and repressive PTMs in the RD could possibly drive the outcome of HSF1 transactivational competence. Furthermore, it was suggested that RD PTMs may act as a signature, which might have some role in directing HSF1 to drive precise transcriptional programs in response to different situations (Budzyński et al., 2015). Indeed, this hypothesis would provide a mechanism for controlling the broad transcriptional repertoire HSF1 has in response to various stressors and in cancer.

1.3.3 Roles in Cancer and Metastasis

In a pioneering study, Dai *et al.* (2007) found that HSF1 was a strikingly "powerful multifaceted modifier of carcinogenesis" that orchestrates a broad regulatory network to promote multiple cellular processes supportive of malignant transformation and sustained oncogenic signaling. In this study, HSF1 was not found to act as an oncogene or tumor suppressor gene, but rather, as a crucial mediator of oncogene-induced proliferation and survival signaling, through RAS/MAPK and other pathways. HSF1 was key for maintaining the transformed phenotype and its loss in transformed cells reduced viability, most significantly in cells with advanced oncogenic states. Furthermore, roles for HSF1 in modulating translation machinery and glucose metabolism were identified (Dai et al., 2007). Now a decade later, a number of roles for HSF1 in cancer have been established (Figure 1.3), and elucidation of HSF1 mechanisms in cancer remains an important and popular area of research. A second study, conducted by Mendillo *et al.* (2012), found that not only is HSF1 a mediator of carcinogenesis, but its elevated expression and activation is associated with highly malignant tumors, metastasis, and poor patient prognosis in breast, lung, and colon cancers. Strikingly, it was identified in this study that HSF1 specifically supports highly malignant cancers through a transcriptional program distinct from the canonical HSF1-mediated HSR. In addition to regulation of HSP, HSF1 mediates malignant signaling by influencing expression of targets influential in apoptosis, energy metabolism, cell adhesion, cell-cycle regulation, development, ECM formation, transcription, translation, DNA repair, and chromatin remodeling (Mendillo et al., 2012)(Figure 1.3). Of particular relevance to the questions addressed in this dissertation, HSF1 could potentially control apoptotic

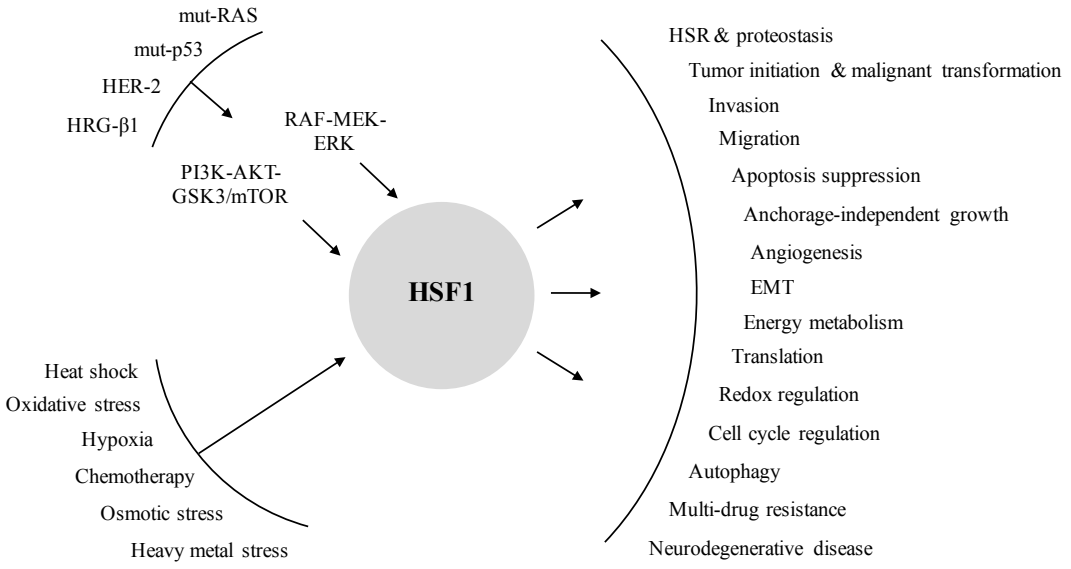

Figure 1.3. HSF1 is activated by and regulates a diverse set of cellular processes.

machinery important for anoikis resistance. Furthermore, these observations appear to be consistent with genome-wide analyses in other model organisms that indicate HSF1 has a far reaching transcriptional repertoire and may influence up to 3% of the genome (Brunquell et al., 2016; Hahn et al., 2004).

Multiple clinical and laboratory studies have demonstrated that HSF1 is overexpressed and activated in lung cancer (Cui et al., 2015; Mendillo et al., 2012). Furthermore, HSF1 overexpression and activation has been observed in other tumor types including breast cancer (Mendillo et al., 2012; Santagata et al., 2011), prostate cancer (Hoang et al., 2000), hepatocellular carcinoma (HCC)(Chuma et al., 2014; Fang et al., 2012; Li et al., 2014b), oral squamous cell carcinoma (Ishiwata et al., 2012), colon cancer (Cen et al., 2004; Mendillo et al., 2012), pancreatic cancer (Dudeja et al., 2011), and endometrial carcinoma (Engerud et al., 2014). In many of these circumstances, including lung cancer, HSF1 expression and activation was associated with the most aggressive, malignant, metastatic phenotypes and correlated with poor patient prognosis (Chuma et

al., 2014; Cui et al., 2015; Engerud et al., 2014; Fang et al., 2012; Li et al., 2014b; Mendillo et al., 2012; Santagata et al., 2011).

HSF1 is a key factor in oncogenic signaling through MAPK and PI3K/AKT pathways, heavily involved in promoting tumor progression and metastasis (Dai et al., 2007; Khaleque et al., 2005; Meng et al., 2010; O'Callaghan-Sunol and Sherman, 2006; Schulz et al., 2014). Multiple roles have been identified for HSF1 linking it to invasiveness and metastasis. In a genome-wide screen, HSF1 was found to be one of just six invasion-enhancing genes that drive metastasis in melanoma (Scott et al., 2011), and was also found to promote invasion in HCC (Fang et al., 2012). Furthermore, HSF1 promotes metastasis through enhanced cell migration (Fang et al., 2012; O'Callaghan-Sunol and Sherman, 2006; Toma-Jonik et al., 2015), induction of EMT (Powell et al., 2016; Xi et al., 2012), regulation of metabolism (Dai et al., 2007; Jin et al., 2011; Zhao et al., 2009), angiogenesis (Cui et al., 2015; Gabai et al., 2012), and interplay with the tumor stroma (Scherz-Shouval et al., 2014). A number of studies have provided evidence of the association of HSF1 and improved anchorage-independent growth on soft agar (Khaleque et al., 2005; Toma-Jonik et al., 2015). HSF1 interacts with, and alters the expression of the co-repressor protein, metastasis-associated protein 1 (MTA1), a component of the NuRD complex involved in chromatin remodeling. This interaction is induced by the heregulin ligand, which induces transformation, and leads to repression of estrogen-induced genes (Khaleque et al., 2008). HSF1 also interacts with, and alters the expression of, HIF-1, involved in angiogenesis, invasion, and survival under hypoxic conditions (Gabai et al., 2012).

One of the most important occurrences for tumor progression and metastasis is the suppression of apoptosis, and HSF1 can inhibit apoptosis in multiple ways (Kumar et al., 2013) (summarized in Figure 1.4). HSF1 is the master regulator of HSP, who have a multitude of direct interactions with key members of the intrinsic and extrinsic apoptotic pathways (see section 1.4.2) (Beere, 2004; Sreedhar and Csermely, 2004). Furthermore, HSF1 is also a known mediator of the co-chaperone, BAG3, whose expression can suppress apoptosis and has been linked to cancer and metastasis (Antonietti et al., 2017; Jacobs and Marnett, 2009), including in lung cancer (Chiappetta et al., 2014). Interestingly, endoplasmic reticulum stress induces apoptosis in part by upregulating BIM, and heat shock prevents the expression of BIM and apoptosis, independent of HSP70 (Kennedy et al., 2014). HSF1 can mediate oncogenic signaling through PI3K/AKT and MAPK pathways, and these pathways are known to play significant roles in suppressing apoptosis through multiple mechanisms (Khaleque et al., 2005; Li et al., 2014a; Meng et al., 2010; O'Callaghan-Sunol and Sherman, 2006; Schulz et al., 2014). Silencing of HSF1 induced apoptosis in RKO colon carcinoma cells via inhibition of BCL-XL expression and JNK activation (Jacobs and Marnett, 2007). Moreover, the expression of pro-apoptotic protein, XIAP-associated factor 1 (XAF1), an antagonist of XIAP, is inhibited through direct transcriptional repression by HSF1 (Wang et al., 2006a). Additionally, HSF1 can also directly repress *TNF-α* (Singh et al., 2002), *IL-1β* (Cahill et al., 1996), and *c-fos* (Xie et al., 2003), each of which can influence apoptosis in various contexts (Friedlander et al., 1996; Preston et al., 1996; Varfolomeev and Ashkenazi, 2004; Wajant et al., 2003). HSF1 has also been associated with altered activation of NFκB and activating protein-1 (AP-1)(Knowlton, 2006), which have

varying roles in apoptosis (Barkett and Gilmore, 1999; Khandelwal et al., 2011; Shaulian and Karin, 2001; Zhang et al., 2009). In summary, HSF1 is a key mediator in a multitude of cellular, tumorigenic, and metastatic processes. Continuing to elucidate such mechanisms is of great value to better understand how HSF1 or its targets might be therapeutically targeted to treat cancer and suppress metastasis.

1.4 Heat Shock Proteins (HSP)

1.4.1 General Introduction

As previously described, HSF1 transcriptionally regulates HSP by binding to HSE within *HSP* promoters to drive their expression, key to the induction of the conserved HSR. HSP are highly conserved molecular chaperones, are categorized by their molecular weight, and they play major roles in protein folding, regulation of homeostasis, and suppression of apoptosis (Ciocca et al., 2012; Richter et al., 2010; Sreedhar and Csermely, 2004). Response to proteostatic imbalances consequent to genetic mutation and environmental stress is critical to the overall health of an organism, is an evolutionary relevant mechanism, and there are continued efforts exploring how HSP (e.g. HSP90) influence evolutionary change and response to disease (Karras et al., 2017). The HSF1-HSP mechanism is ultimately responsive to a diverse set of biological and clinical stimuli. HSP influence a variety of other cellular processes such as maintenance of proper cell cycle function and proliferation. Five major classes of HSP families are induced by stress, including HSP70, HSP90, HSP60, small HSP (e.g. HSP27), and large HSP (e.g. HSP104)(Ciocca et al., 2012; Richter et al., 2010; Sreedhar and Csermely,

2004). Our understanding of HSF1 and HSP has expanded significantly, and we now know that they are relevant to the etiology of various diseases, influencing dysfunctions such as cardiovascular disease, neurodegeneration and aging, and cancer (Calderwood et al., 2010; Dayalan Naidu et al., 2016).

The HSP70 family of chaperones is one of the most conserved chaperone families. Under normal conditions, HSP70 functions in de novo protein folding. Cell stress can lead to an increase in unfolded or misfolded proteins, and HSP70 functions to prevent aggregation of, or correctly refold, such proteins. HSP70 activity occurs through an ATP-dependent process via its ATPase domain, and is reliant on co-chaperones (Calderwood and Gong, 2016; Richter et al., 2010). The most notable HSP70 co-chaperones are the HSP40/J-domain-containing proteins, which facilitate the delivery of misfolded proteins to the HSP70 protein binding domain, and stimulate its ATPase (Richter et al., 2010). Perhaps the most appreciated HSP associated with the stress response is a member of the HSP70 family, inducible HSP70 (HSP72)(Ciocca et al., 2012; Kampinga et al., 2009). HSP72 is encoded by two genes that produce nearly identical protein products, *HSPA1A* and *HSPA1B* (Kampinga et al., 2009). HSP72 is a potent inhibitor of apoptosis and is supportive of cancer cell survival and metastasis (Beere, 2004; Calderwood and Gong, 2016; Calderwood et al., 2006; Ciocca et al., 2012; Sherman and Gabai, 2015; Sreedhar and Csermely, 2004).

HSP90 is highly expressed under physiological conditions, and its expression is also induced upon exposure to stress. HSP90 is dependent on its ATPase function and relies on a variety of co-chaperones. In addition to HSP90 aiding in protein folding, it also binds and stabilizes native proteins (Ciocca et al., 2012; Richter et al., 2010). Loss of

HSP90 is highly detrimental to eukaryotes and often results in the degradation of its client proteins. HSP90 is highly relevant to cancer, in part, because HSP90 clients include major cancer-promoting cell signaling proteins such as AKT, an array of oncoproteins, and mutant p53 (Calderwood and Gong, 2016; Calderwood et al., 2006; Ciocca et al., 2012). Furthermore, HSP90 can directly inhibit apoptosis through a variety of mechanisms (Beere, 2004; Ciocca et al., 2012; Sreedhar and Csermely, 2004). More effective targeting of HSP90, alone or in combination with other drugs, remains an important focus in the development of cancer therapies (Ciocca et al., 2012).

HSP60 chaperones are most often associated with the mitochondria, although evidence of important roles for cytosolic and extracellular HSP60 is growing (Cappello et al., 2008). HSP60 utilizes an ATP-dependent mechanism to fold proteins, including correctly folding nascent proteins, often cooperating with another mitochondrial chaperone, HSP10 (Calderwood and Gong, 2016; Cappello et al., 2008; Ciocca et al., 2012; Ghosh et al., 2008). Additionally, HSP60 is thought to be important for transporting proteins into the mitochondria (Ciocca et al., 2012). HSP60 can influence apoptosis in both positive and negative fashions. In cancer, HSP60 has been shown to utilize multiple mechanisms that promote tumor cell survival (Cappello et al., 2008; Ciocca et al., 2012; Ghosh et al., 2008; Ghosh et al., 2010).

HSP27 is a small HSP that contains a conserved α-crystallin domain and has the ability to oligomerize. In contrast to other HSP, HSP27 is an ATP-independent chaperone, and is thought to function as a holdase, holding its unfolded protein targets until in the presence of other chaperones that can actively refold them (Ciocca et al., 2012; Richter et al., 2010). Oligomerization and phosphorylation of HSP27 are both

influential in its function (Charette et al., 2000; Garrido, 2002; Katsogiannou et al., 2014; Paul et al., 2010). Though not the only pathway, it is well-established that in response to stress, HSP27 is phosphorylated by MAPKAPK2, which is phosphorylated and activated by p38MAPK. Phosphorylation of HSP27 is widely associated with stress-induced cytoskeletal reorganization through its regulation of actin filament dynamics (Clarke and Mearow, 2013; Katsogiannou et al., 2014; Paul et al., 2010; Sreedhar and Csermely, 2004). HSP27 is also a potent inhibitor of apoptosis and is important in cancer progression and metastasis (Beere, 2004; Ciocca et al., 2012; Sreedhar and Csermely, 2004). Generally, multiple HSP appear to play roles in suppression of apoptosis, including in cancer, and the following section provides examples of such mechanisms.

1.4.2 HSP as Suppressors of Apoptosis

Overexpression of HSP in cancer is a frequent occurrence and is indicative of poor prognosis. There is strong evidence that HSP can influence multiple cellular processes that contribute to cancer and metastasis, although much about these mechanisms remains unknown. The underlying chaperoning nature of HSP provides one major route toward promoting tumor cell survival, proliferation, and metastasis by managing increased proteotoxic stress and stabilizing oncogenic proteins in malignant cells. In this way, malignant cells can commandeer the canonical adaptive mechanisms of HSP to survive and flourish. There is evidence that, in tumor cells, increased HSP can promote cell proliferation, potently suppress apoptosis, inhibit cell senescence, aid in angiogenesis, and promote invasion, EMT, and metastasis (Calderwood and Gong, 2016; Ciocca et al., 2012; Dai et al., 2012). For the purposes of this dissertation, we have

focused on HSP as suppressors of apoptosis. The remainder of this section provides some insight into apoptosis-suppressing mechanisms of HSP (summarized in Figure 1.4), which could be taken advantage of in tumor cells overexpressing HSP.

HSP can regulate the activation of a variety of signaling cascades that are important for initiation and modulation of apoptosis (Beere, 2004; Sreedhar and Csermely, 2004). For instance, HSP72 (Gabai et al., 2002; Mosser et al., 2000) and HSP27 (Stetler et al., 2008) can suppress JNK activation, and JNK-mediated phosphorylation of BCL-2 and BCL-XL antagonizes their anti-apoptotic function (Fan et al., 2000). Furthermore, JNK phosphorylates BIM and BMF leading to their release from dynein and myosin V motor complexes and BAX-dependent apoptosis (Lei and Davis, 2003). HSP90 and HSP27 help stabilize or activate AKT, which has a myriad of anti-apoptotic functions (Basso et al., 2002; Havasi et al., 2008; Nakagomi et al., 2003; Rane et al., 2003; Sato et al., 2000). For instance, AKT inhibits apoptosis by phosphorylating BAD (Datta et al., 1997), which can prevent it from neutralizing BCL-XL (Zha et al., 1996). Furthermore, degradation or inhibition of AKT by HSP90 or HSP27 depletion leads to BAX-dependent release of pro-apoptotic factors from the mitochondria (Havasi et al., 2008; Nimmanapalli et al., 2003). HSP90 has a wide range of influence and can also influence apoptosis through JNK, NF-κB, p53, Raf-1, and others (Lanneau et al., 2008)

Multiple HSP can also directly influence key factors for mitochondrial-mediated apoptosis (Beere, 2004; Sreedhar and Csermely, 2004). HSP72, in cooperation with HSP40, prevents apoptosis by inhibiting translocation of BAX to the mitochondria (Gotoh et al., 2004). HSP72 and HSP27 have been linked to suppression of BID-

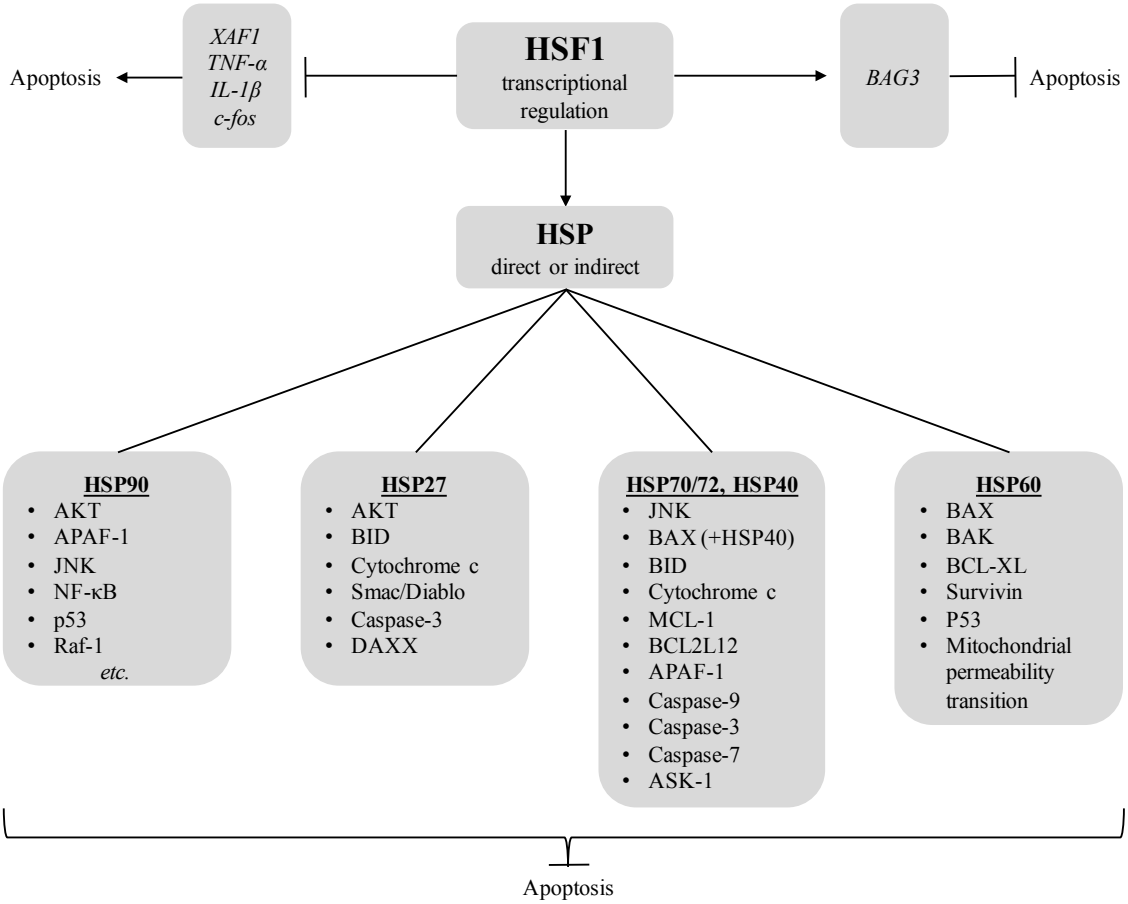

Figure 1.4. HSF1 and HSP are suppressors of apoptosis. A broad summary of potential direct or indirect links between HSF1, HSP, and apoptosis-related targets.

dependent apoptosis (a crosstalk pathway between extrinsic and intrinsic apoptosis) (Gabai et al., 2002; Paul et al., 2002). Furthermore, HSP72 and HSP27 are associated with the inhibition of pro-apoptotic factors cytochrome c (Bruey et al., 2000; Garrido, 2002; Mosser et al., 2000; Paul et al., 2002) and SMAC/DIABLO (Chauhan et al., 2003) that help to mediate apoptosome formation. A possible role for HSP, other than HSP70, in regulating BIM may exist, as heat shock was shown to prevent BIM-mediated apoptosis in response to endoplasmic reticulum stress (Kennedy et al., 2014). HSP70 stabilizes anti-apoptotic proteins MCL-1 (Stankiewicz et al., 2009) and BCL2L12 (a

caspase-3 and -7 inhibitor)(Yang et al., 2009), by preventing their proteasomal degradation. While both pro- and anti-apoptotic functions have been identified for HSP60 (Cappello et al., 2008; Sreedhar and Csermely, 2004), depletion of HSP60 triggers cytochrome c release, and cytosolic HSP60 sequesters BAX and BAK to prevent apoptosis (Kirchhoff et al., 2002). Furthermore, HSP60 expression can increase BCL-XL by inhibiting its ubiquitination (Shan et al., 2003). In tumor cells, HSP60 plays a pivotal role in stabilizing the mitochondrial pool of the inhibitor of apoptosis (IAP) survivin, and in restraining p53 to prevent BAX-dependent apoptosis (Ghosh et al., 2008). Moreover, in a multichaperone complex with HSP90 and TNF receptor-associated protein-1 (TRAP-1), HSP60 inhibits cyclophilin D-mediated mitochondrial permeability transition and subsequent apoptosis induction, specifically in tumor cells (Ghosh et al., 2010). Signaling downstream of the mitochondria is also influenced by multiple HSP. HSP72 and HSP90 are linked to disruption of the apoptosome through direct interaction with APAF-1, thereby preventing caspase-9 activation (Beere et al., 2000; Pandey et al., 2000b; Saleh et al., 2000). Expression of HSP72 has been associated with reduced processing of procaspase-9 (Mosser et al., 2000), and directly binds procaspase-3, and procaspase-7 (Komarova et al., 2004). Furthermore, HSP27 has been shown to associate with procaspase-3 to repress its activation by caspase-9 (Concannon et al., 2001; Pandey et al., 2000a).

HSP can also influence extrinsic apoptosis. For instance, HSP27 and HSP72 can prevent FAS-mediated apoptosis by binding to and inhibiting DAXX (Charette et al., 2000) or apoptosis signal-regulating kinase 1 (ASK1)(Park et al., 2002), respectively. In the case of HSP27, complex signatures of HSP27 structural organization with respect to

both phosphorylation and variations in the size of oligomers can generally lead to the favoring of one function over another, and can also enable it to adapt to different apoptosis-inducing stressors (Paul et al., 2010). For instance, phosphorylated HSP27 dimers, but not non-phosphorylated HSP27 oligomers, interact with DAXX to prevent FAS-mediated apoptosis (Charette et al., 2000). HSP27 negatively regulates BID-dependent apoptosis by stabilizing the F-actin network (Paul et al., 2002), and HSP27 regulation of actin filament dynamics is related to its phosphorylation status (Clarke and Mearow, 2013; Katsogiannou et al., 2014; Paul et al., 2010; Sreedhar and Csermely, 2004). Furthermore, large oligomers of HSP27 have been described as important for its chaperone activity and may also be important for some of its involvement with actors in the intrinsic apoptotic pathway (Garrido, 2002; Katsogiannou et al., 2014; Paul et al., 2010).

1.5 Summary and Hypothesis

In summary, HSF1 plays significant roles in mediating oncogenic signaling by regulating a broad transcriptional network that influences a diverse set of cellular processes to promote tumor progression and metastasis. One feature of metastatic cancers is increased anchorage-independent growth, which requires both increased growth signaling and suppression of anoikis. In support of its pro-metastatic function, HSF1 has been associated with increased anchorage-independent growth, but whether this reflects a role for HSF1 specifically in suppressing anoikis is unclear. An array of commonalities exists between processes and mechanisms influenced by HSF1 and those that promote anoikis resistance. Most notably, HSF1 and its canonical targets, HSP, play significant

roles in apoptosis suppression. Oncogenic signaling pathways such as PI3K/AKT and MAPK pathways are both mediated by HSF1 and are critical for anoikis resistance. Furthermore, processes such as EMT, altered energy metabolism and redox regulation, and autophagy, are examples of mechanisms linked to both HSF1 and anoikis resistance. We hypothesized that HSF1 might function mechanistically to suppress anoikis in tumor cells, and that this may happen, in part, through transcriptional regulation of HSP. We chose to evaluate this in a NSCLC model, the metastatic variants of which are associated with a dismal five-year survival rate of less than 5%. The broader goal of this area of research is to identify suppressors of anoikis in cancer, and to better understand their mechanisms so that they might be targeted therapeutically to suppress metastasis.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell Lines

Two human NSCLC cell lines, NCI-H460 (H460; ATCC HTB-177) and A549 (ATCC CCL-185), were obtained from American Type Culture Collection (ATCC, Manassas, VA). H460 cells are a large cell lung carcinoma subtype of NSCLC, and are lung epithelial cells derived from a pleural effusion site. A549 cells are an adenocarcinoma subtype of NSCLC, and are lung alveolar basal epithelial cells. Both tumor cell lines have mutant *KRAS*, and H460 cells also have mutant *PIK3CA* (ATCC, 2016). Both cell lines have previously been used to study anoikis resistance (Pongrakhananon et al., 2014; Yao et al., 2014). A normal, non-cancerous, human bronchial epithelial cell line (BEAS-2B; ATCC CRL-9609), that is sensitive to anoikis (Geissler et al., 2013), was also used. All cell lines have adherent growth properties.

2.1.2 Chemicals and Common Supplies

Dimethyl sulfoxide (DMSO), tris base, ponceau s, sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), glycine, cryovials, conical tubes, microcentrifuge tubes, cell scrapers, tissue culture dishes (35 mm, 60 mm, 100 mm, 12-well, 24-well), and 1X phosphate buffered saline (PBS) were purchased from Thermo Fisher Scientific (Waltham, MA); 200 proof absolute/anhydrous ethanol was purchased

from Pharmco-AAPER (Brookfield, CT); Tris-hydrochloride (Tris-HCl), glycerol, β-mercaptoethanol, protease inhibitor cocktail, methanol, acetic acid, agarose, ethidium bromide, sodium-dodecyl sulfate (SDS), and poly-2-hydroxyethyl methacrylate (polyHEMA) were purchased from Sigma-Aldrich (St. Louis, MO); Bromophenol blue, TEMED, and ammonium persulfate (APS) were purchased from Bio-Rad (Hercules, CA); phosphatase inhibitor cocktail set III was purchased from Calbiochem (Billerica, MA); LB agar and LB broth were purchased from BD (Franklin Lakes, NJ); RPMI 1640 (1X) + GlutaMAX-1, trypsin-EDTA, and ampicillin were purchased from Gibco (Carlsbad, CA); fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA).

2.1.3 Antibodies

HSF1 (#4356), HSP90 (#4874), HSP60 (#4870), HSP40 (#4868), phospho-HSP27 (S82) (#2401), caspase-7 (#9494), PARP (#9542), GAPDH (D16H11) XP (#5174), anti-Rb IgG HRP linked (#7074), and anti-Ms IgG HRP linked (#7076) antibodies were purchased from Cell Signaling Technology (Beverly, MA); HSF1 (phospho S326) [EP1713&] (#ab76076) antibody was purchased from Abcam (Cambridge, MA); HSP27 (#AHO1132) was purchased from Invitrogen (Carlsbad, CA); HSP72 (#ADI-SPA-810) antibody was purchased from Enzo Life Sciences (Farmingdale, NY); β-actin (#sc-1616-R) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); Chloramphenicol Acetyl Transferase antibody (#C9336) was purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Experimental Designs and Protocols

2.2.1 Cell Culture

All cells were maintained in complete medium, RPMI 1640 + GlutaMAX-1 supplemented with 10% fetal bovine serum (FBS), and kept in a 37°C/5% CO_2 incubator (Sanyo Scientific, Wood Dale, IL). Cells were passaged when approximately 80% confluency was reached. For passaging in 60 mm tissue culture dishes, in a sterile tissue culture hood, old medium was aspirated from the dish and cells were washed once with 5 mL 1X phosphate buffered saline (PBS). Cells were fully detached by incubating cells with 500 μL 1X Trypsin-EDTA for 2-5 min at room temperature. When necessary, cells were placed in a 37°C incubator to aid in detachment. Detached cells were brought up in fresh complete medium and diluted into new dishes at a total volume of 5 mL.

To freeze cells for storage in liquid nitrogen, cells were washed with 1X PBS and detached with 1X Trypsin-EDTA. Cells were then brought up in 1 mL freezing medium (complete medium + 5% DMSO) and placed in a cryovial. Cells were kept overnight at -80°C before being transferred to liquid nitrogen.

For thawing cells from liquid nitrogen, 5 mL complete medium was added to two 60 mm dishes and placed in a 37°C/5% CO_2 incubator for 5 min. Cryovials containing cells were warmed in a 37°C water bath for 1 min. In a sterile tissue culture hood, cells were then transferred from the cryovial into the warmed complete medium from dishes and allowed to adhere overnight. The next day cells were washed with 5 mL 1X PBS to remove the DMSO-containing freezing medium and fresh complete medium was added. Two passages were conducted prior to use for experiments.

2.2.2 Anoikis Assays

To sustain cells in suspension, tissue culture dishes were coated with a plastic substance, poly-2-hydroxyethyl methacrylate (polyHEMA), preventing cell-dish interaction. In a sterile tissue culture hood, a 20 mg/mL solution of polyHEMA was made in 95% non-denatured ethanol and kept in a sterile 50 mL Falcon tube. The solution was mixed overnight on a shaker (VWR Standard Analog Shaker 1000-STD, VWR, Radnor, PA) at room temperature to fully dissolve before plating. Coating was done in a sterile tissue culture hood and an appropriate amount of polyHEMA solution (e.g. 250 μL/well in a 12-well plate; 1.5 mL/60 mm dish) was pipetted into dishes, which were then allowed to dry overnight in the sterile environment. Plates were washed twice with 1X PBS before use. For suspension cultures, cells were seeded at a density of 1x10^5 cells/well in 12-well plates and scaled up or down appropriately for other dishes. Attached cells or cells collected at the time of seeding were included. Cultures were maintained in the same medium and incubator as described for attached cells. Dishes were not disturbed after seeding and cells were allowed to aggregate normally. Anoikis was measured using two established markers of apoptosis, cleavage of executioner caspase-7 (Shi, 2002) and PARP (Oliver et al., 1998). In some cases, anoikis was also measured with the CellTox Green Cytotoxicity Assay (Promega).

2.2.3 RNA Interference

All Silencer Select small-interfering RNAs (siRNAs) were purchased from Ambion (Waltham, MA). Silencer Select Negative Control No. 1 (#4390843) and Silencer Select Negative Control No. 2 (#4390846) non-targeting siRNAs were both

Table 2.1. siRNA reference sequences.

Product #	Target	Sequence (5' to 3')
4392420 ID: s6952	HSF1 (HSF1)	Sense: CUGGUGCAGUCAAACCGGAtt Antisense: UCCGGUUUGACUGCACCAGtg
4392420 ID: s6968	HSPA1B/HSPA1A (HSP72)	Sense: CGAUAUGUUCAUUAGAAUUtt Antisense: AAUUCUAAUGAACAUAUCGgt
4392420 ID: s7008	DNAJB1 (HSP40)	Sense: CAUUCGAAACGAAGACAAAtt Antisense: UUUGUCUUCGUUUCGAAUGct
4392420 ID: s7004	HSPD1 (HSP60)	Sense: CAAUGACCAUUGCUAAGAAtt Antisense: UUCUUAGCAAUGGUCAUUGct
4392420 ID: s194538	HSPB1 (HSP27)	Sense: GCUGCAAAAUCCGAUGAGAtt Antisense: UCUCAUCGGAUUUUGCAGCtt

tested as controls. All siRNAs are targeted against human. Sequence information for siRNAs are listed in Table 2.1. RNA interference was conducted using Lipofectamine RNAiMAX Reagent purchased from Invitrogen (Carlsbad, CA). Optimization of siRNAs were performed by testing a range of concentrations from 1-100 nM, a range of incubation times of 24-72 h, and multiple ratios of siRNA:RNAiMAX to achieve the most effective knockdown with the lowest toxicity.

One day prior to transfection, cells were seeded in complete medium such that they would be 40-70% confluent at the time of transfection (fast growing H460 cells required lower confluency to avoid reaching an overconfluent state by the end of the transfection incubation time). For transfection in 12-well plates, an appropriate amount of siRNA was diluted in 100 μL serum-free medium (SFM) in one tube. After gentle mixing, the appropriate amount of Lipofectamine RNAiMAX (0.1 μL/1 pmol siRNA) was added to 100 μL of SFM in a second tube. Each tube was mixed gently and incubated at room temperature for 5 min. Next, the Lipofectamine RNAiMAX was combined with the diluted siRNA and incubated for 15 min at room temperature, during which time 800 μL fresh complete medium was added to cells. Complexes were then

added to the cells, bringing the volume up to 1 mL, and achieving the desired final concentration of siRNA. Approximately 21 h post transfection, the media was gently changed to limit toxicity. Cells were allowed to continue incubating to a total transfection time of 48 h (H460) or 72 h (A549). Transfections in other plate formats were scaled appropriately.

2.2.4 Transformation and Bacterial Culture Preparation

MAX Efficiency DH5α Competent Cells were purchased from Invitrogen (Carlsbad, CA). Typical sterile technique for bacterial work was used to carry out procedures. Competent cells were thawed on ice, gently mixed, and 100 μL were added to chilled tubes. Plasmid DNA was diluted in sterile 1X TE buffer to 10 ng/μL and 1 μL was added to competent cells, which were gently mixed by tapping tubes. Cells were incubated on ice for 30 min, during which pre-prepared LB agar/Ampicillin (AMP) plates were allowed to reach room temperature. After incubation, cells were heat-shocked for precisely 45 sec in a 42°C water bath (IsoTemp 215, Thermo Fisher Scientific, Waltham, MA) and immediately after placed on ice for 2 min. Next, 900 μL room temperature S.O.C. medium was added to cells and they were allowed to shake for 1 h at 225 rpm and 37°C. Afterward, a mixture of 50 μL bacterial cells and 50 μL S.O.C. medium was spread on an LB agar plates containing 100 μg/mL Ampicillin and incubated overnight at 37°C. The next day, colonies were picked with a sterile tip and dropped into a tube containing 2 mL LB broth with 100 μg/mL AMP to make a starter culture that was incubated for 8 h at 250 rpm and 37°C. At this point, the starter culture was added to 148 mL LB broth with 100 μg/mL AMP and grown for 12-18 h at 200 rpm and 37°C on a 12400 Incubator

Shaker (New Brunswick Scientific, Hauppauge, NY). Optical density readings were taken on a Genesys20 4001/4 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) to ensure the proper density for mini or midi prep kits was reached. Glycerol stocks were prepared by diluting a small portion of cultures 1:1 with sterile 40% glycerol to obtain a 20% glycerol stock, which was stored at -80°C.

2.2.5 Plasmid DNA Extraction and Quantification

The GenElute HP Plasmid Miniprep Kit and GenElute HP Plasmid Midi Prep Kits (Sigma-Aldrich, St. Louis, MO) were used to extract plasmid DNA from bacterial cultures according to the manufacturer's spin method protocol. DNA was quantified using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and NanoDrop 1000 3.8.1 software. To clean the machine, 1 μL of distilled water was added and wiped off twice with a Kimwipe (Thermo Fisher Scientific, Waltham, MA). Next, a reading with 1 μL distilled water was done to initialize the machine. A 1 μL blank using the elution buffer (either kit "elution solution" or 1X Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)) was measured. Lastly, 1 μL of DNA samples were loaded and measured, cleaning the machine with distilled water between each reading. In addition to the concentration, the quality of plasmid DNA was confirmed by ensuring the 260/280 and 260/230 values were in normal ranges.

2.2.6 Plasmids

Wild-type HSF1 (hHSF1) and constitutively active HSF1 (hHSF1ΔRDT) plasmids were kindly provided by Dr. Akira Nakai (Dept. of Biochemistry and Molecular

Biology, Yamaguchi University School of Medicine, Ube, Japan). The constitutively activate mutant has had the RD deleted, and contains a leucine to glutamic acid substitution at amino acid 395 within the HR-C that prevents trimer suppression (Fujimoto et al., 2005). Plasmids were in a pcDNA3.1(+) vector backbone. The pcDNA3.1(+) empty vector backbone and pcDNA3.1/Chloramphenicol Acetyltransferase (CAT) were purchased from Invitrogen (Carlsbad, CA). Luciferase reporter plasmids, pGL4.41[*luc2P*/HSE/Hygro] and pGL4.73[*hRluc*/SV40] were purchased from Promega (Madison, WI). The pGL4.41[*luc2P*/HSE/Hygro] luciferase reporter is driven by four consensus HSE and the pGL4.73[*hRluc*/SV40] vector was co-transfected as an internal control.

All restriction enzymes were purchased from New England Biolabs (Ipswich, MA). Restriction digestions were conducted according to the enzyme-specific protocol recommended by the manufacturer. A sample reaction mixture included restriction enzyme(s), plasmid DNA, reaction buffer, and distilled water. Enzymes were always kept on ice and added to the mixture last. Reactions were incubated in a water bath at the recommended temperature and time. This was followed by a heat inactivation step according to the manufacturer protocols when appropriate for the enzyme used. Digested plasmid DNA (100ng, brought up to 10 μL with DNA loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF)) was run on 0.8-1.1% agarose gels containing 0.5 μg/mL Ethidium Bromide (EtBr) in 1X Tris-Acetate-EDTA (TAE) buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA, 0.5 μg/mL EtBr) using either a Mini-Horizontal Unit FB-SB-710 or Midi-Horizontal System FB-SB-1316 (Thermo Fisher Scientific, Waltham, MA). Gels were run at 80-115 volts until dye front nearly

reached the end of the gel. 1 Kb and 100 bp ladders (New England Biolabs, Ipswich, MA) were run as standards. Gels were visualized using the BioChemi System Epi Chemi II Darkroom (UVP BioImaging Systems, Upland, CA) with Ver. 4.6.00.0 of Lab Works Image Acquisition and Analysis Software. Additionally, plasmids were sent to GENEWIZ (South Plainfield, NJ) for sequencing, using their T7/BGHR universal primers to flank the multiple cloning site containing target sequence, and SV40-promoter/SV40pA-R universal primers to sequence the resistance gene. Sequences were aligned using Clustal Omega software.

2.2.7 Transfection

FuGENE HD Transfection Reagent was purchased from Promega (Madison, WI). Transfection protocols (12-well plates) were optimized with each plasmid by testing a range of DNA (31.25-2000 ng), FuGENE:DNA ratio (2.0:1-4.0:1), volume of complexes add (25-100 μL), length of complex incubation (5-15 min), and length of transfection (6-48 h). The pcDNA3.1/CAT vector was used as an additional aid to optimize transfection of vector backbone. For luciferase vectors, mass ratios of the two reporter plasmids was also optimized by testing a range of 10:1-100:1 HSE:SV40 (firefly:renilla). Furthermore, efficiency of co-transfection of the two luciferase vectors alone and with experimental plasmids was assessed. Transfection efficiency of each plasmid was assessed by comparing the protein expression of wild-type HSF1, truncated HSF1, and CAT between conditions after transfection with their respective plasmids. Additionally, we validated that the hHSF1ΔRDT expression vector successfully caused increased HSF1 binding to HSE by co-transfecting with the two luciferase vectors and measuring reporter output

using the Dual-Glo Luciferase Assay (Promega). The most efficient transfection conditions with the least toxicity observed were selected and the same conditions were used to transfect all plasmids being compared.

For transfections, cells were seeded one day prior to transfection such that cells would be approximately 60-80% confluent at the time of transfection. To transfect, DNA was diluted to the desired working concentration in SFM. An appropriate amount of FuGENE HD Transfection Reagent for desired FuGENE:DNA ratio was added, gently mixed, and incubated at room temperature for 10 min, during which fresh complete medium was added to cells. The FuGENE:DNA mixture was then added to cells at the desired volume to achieve appropriate final concentrations. After 24 h the medium was replaced to limit toxicity. Experiments were conducted 24-48 h post transfection.

2.2.8 Protein Extraction and Quantification

Laemmli Sample Buffer without bromophenol blue (62.5 mM Tris-HCl, 25% Glycerol, 2% sodium dodecyl sulfate (SDS)) was first prepared. Next, Laemmli Lysis Buffer (93% Laemmli Sample Buffer, 5% β-mercaptoethanol, 1% protease inhibitor, 1% phosphatase inhibitor) was prepared. The lysis procedure was conducted on ice. For protein extraction from attached cells, the cells were washed with 1X PBS, which was aspirated, and the desired amount of Laemmli Lysis Buffer was added (e.g. 40 μL for confluent well in a 12-well plate). A cell scraper was used to mechanically lyse cells and the viscous lysate was collected using a P200 with the end of the tip cut off. Samples were boiled 5-10 min depending on lysate volume, and kept at -20°C until use. For protein extraction from suspension cells, the cells were collected by centrifugation at 3 xg

for 7 min, using a Fisher Scientific accuSpin Micro 17 microcentrifuge (Thermo Fisher Scientific, Waltham, MA). The supernatant was decanted and cells were re-suspended in ice-cold 1X PBS before being pelleted again at 3 xg for 7 min. The supernatant was carefully removed by pipetting without disturbing the pellet. The desired amount of Laemmli Lysis Buffer was added depending on pellet size, and cells were mechanically lysed with a pipet tip and vigorous mixing until a clear homogenous lysate remained.

Protein was quantified using the Pierce 660 nm Protein Assay Reagent coupled with the Ionic Detergent Compatibility Reagent (IDCR; both reagents from Thermo Fisher Scientific, Waltham, MA). For quantification, 1 μL of protein sample was diluted in 9 μL of distilled water and the entire amount was transferred to a Costar clear 96-well flat bottom non-treated assay plate (Thermo Fisher Scientific, Waltham, MA), along with a range of pre-diluted bovine serum albumin (BSA) standard (Thermo Fisher Scientific, Waltham, MA) from 125 μg/mL to 2000 μg/mL, and a 10 μL of a blank control. All samples were quantitated in duplicate. The protein assay reagent was prepared by making a mixture of .05g IDCR/mL 660 nm reagent. Next, 150 μL of the prepared reagent was added to each well, placed on an Ortho multi-well plate Shaker Incubator (Ortho-Clinical Diagnostics, Raritan, NJ) at medium speed for 1 min, and then incubated at room temperature for 5 min. The absorbance was measured at 660 nm with a SpectraMax PLUS 384 spectrophotometer (Molecular Devices, Sunnyvale, CA) using the SoftMax Pro V 5.3 software. Protein concentrations were determined by averaging duplicates, plotting a trend line, and using the equation and absorbance of unknown protein samples to determine the diluted concentration. Final concentrations were determined by multiplying by the dilution factor.

2.2.9 Gel Casting

Glass plates and combs for casting polyacrylamide gels were purchased from Bio-Rad (Hercules, CA). Polyacrylamide gels were made with a 4% stacking portion and a resolving portion ranging from 6.5%-11% depending on molecular weight of targets. Glass plates were wiped with ethanol and assembled on a plate stand. Amount of Acrylamide 40% Solution (Acrylamide:Bis-Acrylamide, 37.5:1; Thermo Fisher Scientific, Waltham, MA) for 100 mL of resolving or tacking solutions was calculated as 2.5(X%) = mL 40% Acrylamide/Bis. Calculations were appropriately scaled for desired volumes. The resolving solution consisted of the calculated amount of Acrylamide/Bis, 25% 1.5 M Tris-HCl pH 8.8, 0.1% SDS, distilled water to reach calculated volume, 0.05% Ammonium Persulfate (APS), and 0.075% TEMED. The stacking solution consisted of 10% of the calculated amount of Acrylamide/Bis, 25% 0.5 M Tris-HCl pH 6.8, 0.1% SDS, distilled water to reach calculated volume, 0.05% APS, and 0.1% TEMED. Reagents were added in the listed order and mixed well. TEMED was not added until ready to set that portion of the gel. Resolving solution was pipetted between glass plates until approximately three-fourths filled. Distilled water was then added to completely fill the space between plates, and gels were allowed to solidify for at least 30 min. Next, the entire gel casting apparatus was overturned over a sink to drain the distilled water. TEMED was added to the stacking solution, mixed, and pipetted between plates on top of resolving gel until almost full, leaving only a thin space. Combs were placed between plates and gels were allowed to solidify for at least 20 min. When solidified, gel-containing plates were rinsed with distilled water and wrapped in wet paper towels, followed by wrapping in saran wrap and storage at 4°C.

2.2.10 SDS-PAGE and Western Blot

SDS-PAGE gels were rinsed with distilled water prior to use. Samples were thawed on ice. All sample volumes were equalized by bringing the volumes of each sample up to that of the highest sample volume required for desired μg of protein using Laemmli Sample Buffer containing 0.01% bromophenol blue (maximum of 30 μL). Depending on the protein targets, 15-30 μg of protein was loaded into prepared polyacrylamide gels. Approximately 5 μL of Precision Plus Protein Dual Color Standard (Bio-Rad, Hercules, CA) was loaded as a molecular weight reference. Empty wells were filled with Laemmli Sample Buffer at equivalent volume. Running buffer was diluted to 1X with distilled water from pre-prepared 10X running buffer (250 mM Tris Base, 1920 mM Glycine, 1% SDS, pH 8.3) and gel boxes were filled with buffer. Gels were run on a Mini-Trans-Blot Cell apparatus with a PowerPac HC from Bio-Rad (Hercules, CA) at 65 volts until dye front passed the stacking portion of the gel, and 115 volts the remaining time until dye front reached the bottom of the gel.

During this time 10X transfer buffer (250 mM Tris Base, 1920 mM Glycine, pH 8.3) was diluted to 1X transfer buffer (10% 10X transfer buffer, 20% methanol, 70% distilled water). Nitrocellulose membrane (0.45μm) purchased from Bio-Rad (Hercules, CA) were cut to the size of the gel. Foam pads (8 x 11 cm), filter paper (7.5 x 10 cm) (both from Bio-Rad, Hercules, CA), and nitrocellulose membrane were pre-soaked in the 1X transfer buffer. Plates were opened with a gel opening lever (Biorad, Hercules, CA) and a gel-membrane "sandwich" was prepared in a holder, and while fully submerged in 1X transfer buffer, as follows: BOTTOM (black side) – fiber pad – filter paper – gel – nitrocellulose membrane – filter paper – fiber pad – TOP (clear/red). The gel was always

placed appropriately to read lanes on final membrane from left to right. A roller (BioRad, Hercules, CA) was used to carefully roll out any air bubbles from the sandwich. The sandwich was placed in a box containing the transfer apparatus fully submerged in 1X transfer buffer. A stir bar was placed in the box, the entire box/apparatus was placed in an ice bin and surrounded to the top by an ice/ice water mixture, and samples were transferred at 90 volts for precisely 1 h and 15 min while stirring on medium speed. After transfer, the membrane was carefully removed from the sandwich with tweezers and placed "face-up" (side against the gel) in an appropriately sized western blotting box. To ensure effective transfer and equal loading, membranes were covered in Ponceau S Staining Solution (0.1% Ponceau S, 5% acetic acid) and placed on a rocker (Labnet International Inc, Edison, NJ) for 5 min. Afterward, excess stain was removed by washing membrane in the box in distilled water five times while gently manually shaking boxes. Images of the stained lanes/bands were scanned and saved. While submerged in distilled water, dots were made with a pencil between lanes at the marker location where membranes would be cut into pieces to separately probe for multiple targets of different molecular weights. This acted as a guide to cut appropriately after de-staining. To de-stain membranes, 10X Tris-Buffered Saline (TBS; 200 mM Tris Base, 1370 mM NaCl, pH 7.6) was diluted to 1X with distilled water and 0.1% Tween-20 (Bio-Rad, Hercules, CA) was added to make 1X TBS-T. The membranes were then de-stained with 1X TBS-T by performing three 5 min washes on a shaker. For all washes and incubations, membranes were covered in a sufficient volume to submerge, varying based on the size of the box. Next, membranes were blocked on a rocker at room temperature for 1 h in either 5% non-fat dry milk (Lab Scientific Inc, Highlands, NJ) or 5% BSA (Thermo

Fisher Scientific, Waltham, MA) solutions prepared in 1X TBS-T. BSA was only used for blocking when probing for phospho-proteins. After blocking, two quick washes (add buffer, drain), followed by three washes for 5 min on a shaker, were done in 1X TBS-T. Primary antibodies were prepared according to the manufacturer's protocol and at the pre-optimized dilution. A β-actin or GAPDH control was included for all western blots. Membranes were incubated in primary antibody overnight at 4°C on a rocker. The next day, antibodies were removed and two quick washes, followed by three washes for 5 min on a shaker, were done in 1X TBS-T. The appropriate species horseradish peroxidase-tagged secondary antibody for each primary antibody was prepared in 2.5% non-fat dry milk in 1X TBS-T at the pre-optimized dilution. Membranes were incubated in secondary antibody for 1 h at room temperature on a rocker, followed by two quick washes and three washes for 5 min on a shaker, done in 1X TBS-T. Membranes were never allowed to be in dry conditions.

For chemiluminescent detection, all work was done in a dark room. Equal volumes of Luminol Enhancer Solution and Peroxide Solution from the Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA) were mixed to make ECL solution. Membranes edges were dabbed on a paper towel to remove excess wash buffer (but not dried), then placed on a flat surface, and enough ECL was pipetted on top of membranes to evenly coat the entire piece of membrane. Membranes were incubated in the dark for 1 min at room temperature, gently dabbed on a paper towel to remove excess ECL, and then transferred to a cassette fitted with a cut sheet protector such that membranes were sandwiched between two sheets. Any air pockets between sheets were gently rolled out by passing over the closed sheets with a paper towel. In

complete dark, Blue Lite Autorad Film (BioExpress, Kaysville, UT) was placed on membranes for the desired exposure length and films were developed. Films were then labeled with a marker and scanned.

2.2.11 CellTox Green Cytotoxicity Assay

Cell death was also assessed using the CellTox Green Cytotoxicity Assay (Promega, Madison, WI). This assay measures changes in membrane integrity with a cyanine dye that is excluded from viable cells, but in dead cells will bind to DNA and enhance the dye's fluorescent properties. Costar black, clear-bottomed, tissue culture treated 96 well plates (Thermo Fisher Scientific, Waltham, MA) were coated with 75 μL of polyHEMA as previously described. Kit reagents were prepared according to the manufacturer's protocol and the linear range of the assay was determined. For simple detachment time courses, cells were prepared at a density of 1x10^5 cells/mL and 2 μL CellTox Green Dye/mL of cells was added. A total volume of 100 μL (1x10^4 cells) of cell/dye suspension was added to each well and samples were run in duplicate. A maximum death control was included, in which a Lysis Solution was added 15 min prior to readings. Media and media/dye controls in coated wells were included for background measurements. Fluorescence was measured initially (time 0) and 24 h, 48 h, and 72 h after incubation of suspension culture in a 37°C/5% CO_2. A Bio-Rad Biotek SYN2 96-well fluorometer/luminometer (Winooski, VT) with Gen5 1.10 software was used to take readings. After shaking for 1 min in the machine, readings were taken from the bottom at 485/20 excitation and 528/20 emission settings, in a dark room. After subtracting media/dye background, cell death was calculated as experimental RFU/positive lysis

control RFU*100. For siRNA experiments, RNA interference was performed as previously described and cells were seeded as described here post-transfection.

2.2.12 Dual-Glo Luciferase Assay

The Dual-Glo Luciferase Assay was purchased from Promega (Madison, WI). Reagents were prepared according to the manufacturer's protocol. Responsiveness of the HSE reporter was first validated by treating reporter-containing cells with 17-(Allylamino)-17-demethoxygeldanamycin (17AAG; Selleckchem, Houston, TX), which can induce HSF1 activation (Figure 2.1). Optimal assay conditions were tested by trying a range of cell numbers, treating cells for varying treatment times and with varying concentrations of 17AAG, and adjusting the luminometer sensitivity. Cells were transfected with pGL4.41[*luc2P*/HSE/Hygro] and pGL4.73[*hRluc*/SV40] as previously described. To measure HSE binding induced by transfection of HSF1 plasmids, an empty vector or each HSF1 plasmid was co-transfected with the luciferase plasmids. The assay was performed by detaching transfected cells, counting, and seeding 1x10^4 cells in 75 μL complete medium in white, clear-bottomed**,** tissue culture treated 96-well plates (Thermo Fisher Scientific, Waltham, MA). Next, 75 μL of Dual-Glo Luciferase Reagent was added to cells, covered with aluminum foil, and incubated at room temperature for 10 min. Firefly luciferase activity was measured after 30 sec shaking in the luminometer. Immediately after, 75 μL of Dual-Glo Stop & Glo Reagent was added and cells were incubated at room temperature for 10 min. This reagent acts to simultaneously quench the first reaction and act as the substrate for the second. *Renilla* luciferase activity was then measured after 30 sec shaking in the luminometer. Readings were taken top-down and

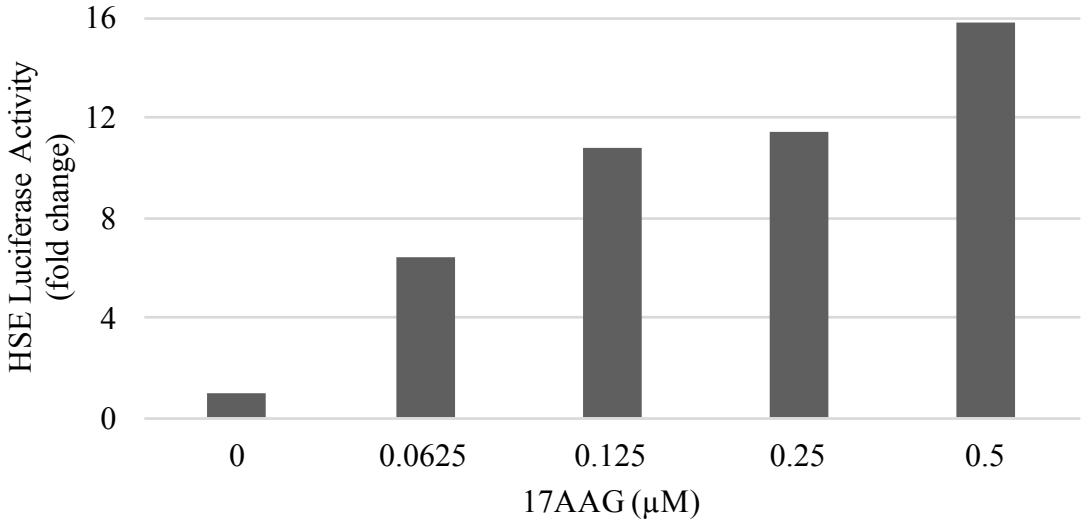

Figure 2.1. An HSE-driven reporter is responsive to 17AAG, known to induce HSF1 activity. BEAS-2B cells were transfected with pGL4.41[*luc2P*/HSE/Hygro] and pGL4.73[*hRluc*/SV40] followed by treatment with 0-0.5 μM 17AAG for 6 h and luminescence was measured.

assay sensitivity settings were consistent between biological replicates, and all experiments were run with at least two technical replicates that were averaged. To assess the results, relative luminescence units (RLU) of a background control containing non-transfected cells, media, and all appropriate reagents was first subtracted, followed by normalizing the HSE (firefly) data to the internal *Renilla* control. Experimental samples were then compared to mock-transfected controls, represented as fold change.

For taking luciferase measurements over time in suspension culture, the same protocol was employed with the following exceptions. After transfection in 12-well plates, 1x10^4 cells were seeded in polyHEMA-coated white 96-well plates containing 75 μL complete medium, and were then kept in a 37°C/5% CO_2 incubator for desired times. To prevent contamination, each time point was seeded in a separate plate because these plates must be read top-down and the lid must be removed for readings. At each

time point the plate was removed from the incubator 15 min prior to addition of the Dual-Glo Luciferase Reagent to equilibrate to room temperature. Lastly, to ensure that transfection of the reporter was successful, a positive control was treated with 17AAG 6 h prior to the initial seeding of suspension cultures, and read at the same time as the non-treated initial time point (time 0). Assay sensitivity was kept consistent for all readings within the time course (as well as between biological replicates). After background subtraction and *Renilla* normalization, suspension growth samples were compared to control cells measured at the time of seeding, represented as fold change.

2.2.13 Statistical Analysis

Statistical analysis was performed using PRISM software and Microsoft Excel. Data are presented as the mean +/- standard deviation of three independent replicates at minimum. Differences between paired samples were analyzed by applying two-sided Student's *t*-Tests. In multivariate cases, ANOVA with a Dunnett's post hoc analysis was also employed. Values of $p < 0.05$ were considered significant.

3. HEAT SHOCK TRANSCRIPTION FACTOR 1 (HSF1) IS A NOVEL SUPPORTER OF NSCLC ANOIKIS RESISTANCE

3.1 Rationale

Changes in the cellular environment or application of therapeutics often result in cell stress. The metastatic process itself contains a variety of significant environmental changes for tumor cells, beginning with their detachment and movement away from the primary tumor and surrounding extracellular matrix (ECM). Normally, loss of such connections alters the matrix-mediated and cell-cell signaling that critically regulate growth and survival of the cell (Kim et al., 2012; Paoli et al., 2013; Sakamoto and Kyprianou, 2010; Taddei et al., 2012). Yet, tumor cells acquire characteristics enabling them to regulate such signals autonomously, and through other processes, thus overcoming these significant environmental changes to continue to survive and proliferate (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). One such critical signaling pathway is anoikis, a programmed cell death signaling repertoire induced by detachment from the ECM, thereby preventing the abnormal survival of cells that would enable inappropriate relocation (Frisch and Francis, 1994; Frisch and Screaton, 2001; Paoli et al., 2013; Sakamoto and Kyprianou, 2010).

Detachment of cells from the ECM can lead to stressful intracellular changes (e.g ROS production, altered glucose regulation, etc.)(Guadamillas et al., 2011; Mason et al., 2017; Paoli et al., 2013). Similar to cells resisting other stressors or cell death processes, resistance to anoikis relies on manipulation of pro-survival and anti-death signaling molecules within a cell (Kim et al., 2012; Paoli et al., 2013; Taddei et al., 2012; Yao et

al., 2014). Heat Shock Transcription Factor 1 (HSF1) is known to be induced by a variety of biological and clinical stressors in a protective role to support survival of the cell. As a transcription factor often induced by stress, HSF1 is a master regulator of HSP (Calderwood et al., 2010; Ciocca et al., 2012; Kumar et al., 2013; Morimoto, 1998; Vydra et al., 2014), a well-known group of chaperones that have an array of clientele and typically function to promote cell survival (Beere, 2004; Ciocca et al., 2012; Kumar et al., 2013; Sreedhar and Csermely, 2004). Furthermore, HSF1 in lung, breast, and colon tumor cells influences expression of a broad array of genes, far beyond its canonical HSP regulatory role, influencing such processes as cell survival, proliferation, and metabolism (Mendillo et al., 2012). This is consistent with genome-wide analyses in *C. elegans* and yeast models that also indicate HSF1 has a far reaching transcriptional repertoire (Brunquell et al., 2016; Hahn et al., 2004). Tumor cells have been shown to overexpress and manipulate HSF1 and its targets to promote survival and resistance to multiple stressors (Calderwood and Gong, 2012; Calderwood et al., 2006; Ciocca and Calderwood, 2005; Dai et al., 2007; Jiang et al., 2015; Khaleque et al., 2005; Kumar et al., 2013; Mendillo et al., 2012; Stanhill et al., 2005; Vydra et al., 2014), and there exists some indirect evidence that it may influence anoikis resistance. HSF1 supports malignant transformation (Dai et al., 2007; Jiang et al., 2015; Khaleque et al., 2005; Kumar et al., 2013; Mendillo et al., 2012; Stanhill et al., 2005; Vydra et al., 2014), including RAS-mediated transformation (Dai et al., 2007; Stanhill et al., 2005), an oncogene whose activation promotes anoikis resistance (Rytömaa et al., 2000) through influence of the PI3K/AKT pathway (Taddei et al., 2012; Zhong and Rescorla, 2012). In U251 glioma cells, knockdown of BAG-3 (a transcriptional target of HSF1) reduced cell-matrix

adhesion on various matrices. While BAG-3 knockdown alone did not induce anoikis in suspended cells, it was suggested that the reduction in such cell-matrix interactions could prime cells to AT-101 (a pan BCL-2 inhibitor)-induced anoikis. In attached cells, similar death mechanisms were observed in AT-101 combination treatments with BAG-3 knockdown or BAG-3-depleting pharmacological inhibition of HSF1, implying targeting HSF1 might also reduce cell-matrix adhesion or be important for increased AT-101-induced anoikis (Antonietti et al., 2017). Further, HSF1 supports metastasis as a pro-invasion gene in melanoma (Scott et al., 2011) and by aiding in mouse embryonic fibroblast (MEF) cell migration (O'Callaghan-Sunol and Sherman, 2006). HSF1 supports anchorage-independent growth in heregulin β1 (HRGβ1)-stimulated MEF (Khaleque et al., 2005), B16-F10 mouse melanoma cells (Toma-Jonik et al., 2015), HEY and SKOV3 ovarian cancer cells (Powell et al., 2016), OE33 esophageal cancer cells (Asano et al., 2016), HeLa cervical cancer cells (Asano et al., 2016; Salamanca et al., 2014), and metastatic breast cancer cells (MDA-MB-231 and metastatic variants; also showed reduced anchorage-dependent growth)(Carpenter et al., 2017). In summary, HSF1 is overexpressed and activated to support malignant lung cancer, is connected to major anoikis signaling pathways, regulates apoptosis, and supports metastasis at multiple steps including anchorage-independent growth, and therefore could be a regulator of anoikis resistance.

Collectively, these roles and evidence certainly make HSF1 a suitable candidate to be a mediator of anoikis resistance. However, to date, whether HSF1 is directly involved in supporting anchorage-independent growth through the suppression of anoikis remains unknown. We therefore asked whether HSF1, with its pro-survival and pro-

metastasis capabilities, would specifically be important for anoikis resistance using a non-small cell lung cancer (NSCLC) cell model. Furthermore, we questioned whether cell detachment itself could act as a stressor capable of triggering changes in HSF1 associated with its activation, in anoikis-resistant cells.

3.2 Confirmation of Anoikis Resistance in NSCLC Cell Lines

H460 and A549 NSCLC cell lines have previously been used to study anoikis resistance (Yao et al., 2014). In order to confirm that our H460 and A549 cells did indeed have elevated anoikis resistance, sensitivity to anoikis in suspension cultures of each cell line was assessed and compared to anoikis-sensitive normal bronchial epithelial cells (BEAS-2B). Moreover, we assessed anoikis resistance in a selected cell line generated from parental H460 cells (H460-S) to study potential differences between cells that form large multicellular aggregates and those that grow alone or in small clusters. This cell line was selected through five sequential cycles of suspension and adherent culture. Cells were allowed to aggregate in suspension before being passed through a 40 μm cell strainer to select for single cells or small clusters that were then allowed to reattach and grow to confluency in normal tissue culture dishes.

Two intracellular markers of apoptosis, cleavage of effector caspase-7 and DNA repair protein, poly-ADP ribose polymerase (PARP), were measured by western blot. H460 and A549 cells grown in suspension for 24 h both displayed characteristics of anoikis resistance compared to suspended BEAS-2B cells, having significantly reduced cleaved caspase-7 (CC7) and cleaved PARP (c-PARP) (Figure 3.1A-C). Furthermore, in comparison to suspended BEAS-2B cells, suspended H460 and A549 cells had

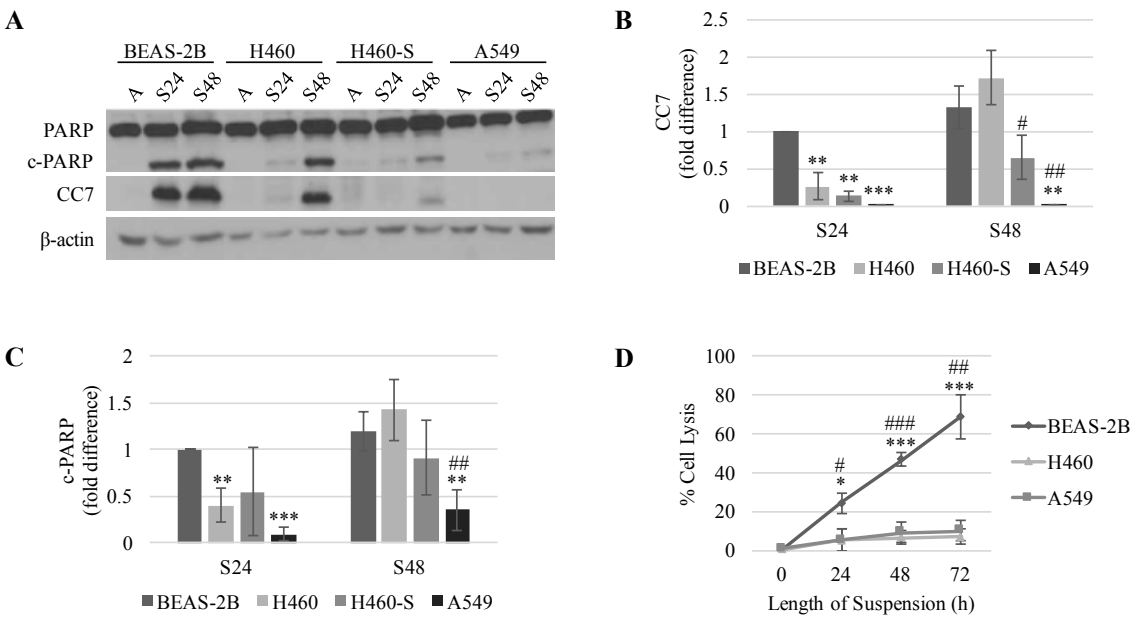

Figure 3.1. NSCLC cells have increased anoikis resistance. *(A)* BEAS-2B, H460, H460-S, and A549 cells were maintained under normal attached (A) conditions or suspended (S) on polyHEMA-coated dishes for 24-48 h. Western blots were performed to measure cleavage of caspase-7 (CC7) and PARP (c-PARP). β-actin was used as a loading control. CC7 immunoblot is from a separate gel, with similar β-actin as shown above (compiled graphs use gel-specific loading control) *(B/C)* Densitometric analysis of western blots of CC7 *(B)* and c-PARP *(C)* are represented as fold change from BEAS-2B cells 24 h post-detachment. Statistical significance from BEAS-2B S24 control cells was interpreted with a Student's *t*-Test (SD, * indicates significance from BEAS-2B cells and # indicates significance from H460 cells, at the same time point; #p<.05, **/##p < 0.01, ***p < 0.001, n=4). ANOVA analysis of the non-normalized data confirmed significance (Dunnett's post hoc test, all indicated differences at least p<.05 according to ANOVA). *(D)* BEAS-2B, H460, and A549 cells were suspended on polyHEMA-coated dishes with the CellTox Green binding dye for 0-72 h and fluorescence was measured every 24 h (kinetic measurement). After subtracting media/dye background, % cell lysis was calculated as Experimental RFU/Maximum Lysis Control RFU*100. Statistical significance was interpreted with a Student's *t*-Test (SD, * indicates significance between BEAS-2B and H460 cells and # indicates significance between BEAS-2B and A549 cells; */#p < 0.05, ##p < 0.01, ***/###p < 0.001, n=3).

significantly reduced anoikis, measured by the ability of a cyanine dye to penetrate cells and bind DNA (Figure 3.1D) (CellTox Green Cytotoxicity Assay). Comparing between tumor cell types, A549 cells exhibited very little cleavage of either marker, while H460 cells exhibited at least some level of spontaneous anoikis over time (Figure 3.1A-C), a general observation consistent with previous studies (Yao et al., 2014). H460-S cells appeared to have increased anoikis resistance over extended suspension culture, with significantly reduced CC7 (c-PARP tended to be less, but there was some variation) compared to parental H460 cells (Figure 3.1A-C). Moreover, the fact that these cells were not in large multicellular aggregates did not appear to be a random event, as there was a clear reduction in aggregate formation when these cells were suspended (data not shown). We noted that cell death measured with the CellTox Green Cytotoxicity Assay did not indicate a difference in H460 cells at later times (>48h) that may have been expected based on intracellular markers. Since this assay requires entry of dye into the cell, it more likely provides readout of cells in later stage apoptosis or secondary necrosis when there is disruption or leakiness of the plasma membrane, and may explain the delayed timing. Furthermore, all three cell lines aggregate in suspension, and perhaps there is some limitation in dye access to cells within anoikis-resistant cell aggregates. Perhaps increased proliferation of H460 cells may increase aggregate size and could physically block the smaller portion of dead cells from sloughing off, whereas increased anoikis sensitivity in BEAS-2B cells enables more rapid cell dissociation and easier measurement. While there may be some delayed timing with this assay, the findings generally agree with the intracellular measurements that these tumor cells have increased anoikis resistance, as expected.

3.3 Detachment of NSCLC Cells Induced Markers of HSF1 Activation

Detachment of cells from the ECM can induce changes in cellular functioning, and HSF1 is a downstream mediator of integrin-mediated signaling pathways that influence anoikis. Therefore, we investigated whether cell detachment could lead to modulation of HSF1, perhaps responding to a detachment-induced "stress". We first asked whether growth of cells in suspension would lead to changes in the expression of HSF1 in anoikis-sensitive or –resistant cells. BEAS-2B, H460, and A549 cells were grown in suspension for 24 h, and we observed no difference in HSF1 expression between attached and suspended cells (Figure 3.2A-B). Since functionality of HSF1 is typically associated with its activation, we asked whether cell detachment could act as a trigger for HSF1 activation in anoikis-resistant cells. Phosphorylation of HSF1 on S326 (pS326-HSF1) is a well-established marker of HSF1 activity (Chou et al., 2015; Guettouche et al., 2005; Mendillo et al., 2012). Therefore, we first investigated whether cell detachment could trigger induction of pS326-HSF1 in anoikis-resistant cells. Indeed, in contrast to BEAS-2B cells, detachment of H460 cells triggered a significant induction of pS326-HSF1 after 30 min of suspension culture (Figure 3.2C-D). Interestingly, we did not observe any consistent change in pS326-HSF1 in A549 cells, suggesting that HSF1 is either not being activated by detachment in these cells, or that it is occurring through an unknown mechanism. Consistent with stress induction such as heat shock, pS326-HSF1 was rapidly induced, and appeared to return toward baseline over time (Appendix Figure A.2.1). Thus, we identified that cell detachment induces a marker associated with HSF1 activation, pS326-HSF1, in anoikis-resistant H460 cells.

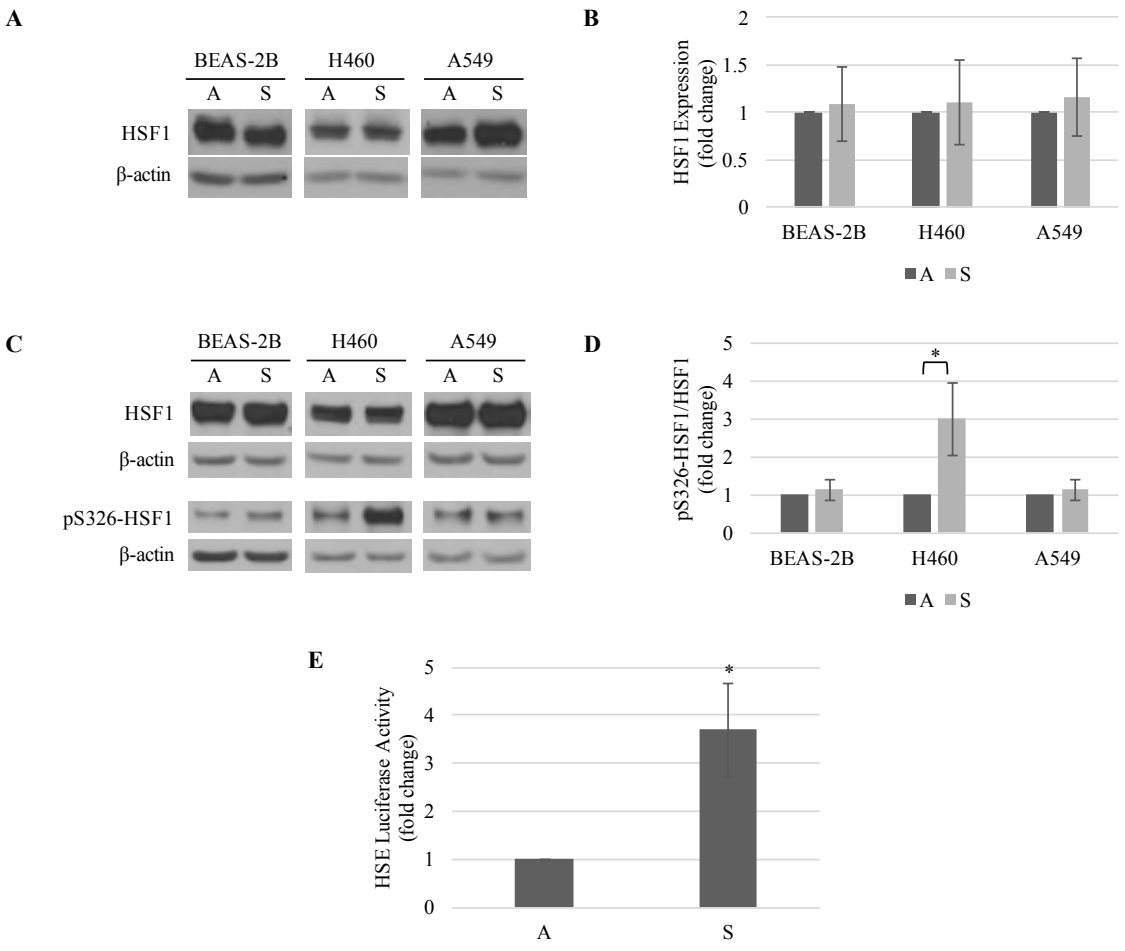

Figure 3.2. Detachment of NSCLC cells induced markers of HSF1 activation. *(A)* BEAS-2B, H460, and A549 cells were detached and suspended on polyHEMA-coated culture dishes for 24 h. HSF1 expression in suspended (S) or attached (A) control cells was measured by western blot. β-actin was used as a loading control. *(B)* Densitometric analysis of HSF1 expression (SD, n=6 BEAS-2B/A549, n=7 H460). *(C)* BEAS-2B, H460, and A549 cells were detached and suspended on polyHEMA-coated culture dishes for 30 min. Expression of HSF1 and pS326-HSF1 was measured by western blot. β-actin was used as a loading control. *(D)* Densitometric analysis of pS326-HSF1 expression, normalized to total HSF1 (SD, *$p < 0.05$, n=3). *(E)* H460 cells were transfected with pGL4.41[*luc2P*/HSE/Hygro] and pGL4.73[*hRluc*/SV40] for 24 h in 12-well plates. Cells were seeded on polyHEMA-coated 96-well plates for 24 h. Luminescence from HSE reporter activation was measured at the time of seeding and after suspension culture (SD, *$p < 0.05$, n=3). In all cases, statistical significance was interpreted with a Student's *t*-Test. All data are represented as fold change from attached control for each cell-type.

Activation of HSF1 is also often measured using luciferase reporters driven by promoters of well-known HSF1 targets, such as the genes for HSP72 (Chou et al., 2012) or HSP70B (Khaleque et al., 2005; Stanhill et al., 2005), or driven by the conserved HSE sequences themselves (Stanhill et al., 2005; Yoon et al., 2014). Therefore, to further investigate whether HSF1 may be activated in H460 cells upon growth in suspension, we employed a luciferase reporter driven by copies of the consensus HSE sequence. Indeed, H460 cells grown in suspension for 24 h showed increased stimulation of the HSE-driven reporter (Figure 3.2E). Therefore, detachment and suspension culture of H460 cells led to increases in two established markers of HSF1 activity, pS326-HSF1 and binding to HSE. Collectively, this evidence suggests that loss of cell anchorage can act as a trigger for HSF1 activation in these cells.

3.4 HSF1 Knockdown Increases Sensitivity of NSCLC Cells to Anoikis

We next asked whether HSF1 plays a direct role in anoikis resistance of NSCLC cells. To determine whether HSF1 is necessary for H460 and A549 cell anoikis resistance, cells were transfected with siRNA specifically targeting HSF1 (siHSF1) or a scrambled siRNA non-targeting control (siCon) prior to cell detachment. The expression of HSF1 was significantly inhibited in cells transfected with siHSF1 (Figure 3.3A-B). Transfected cells were subsequently suspended on polyHEMA-coated plates and induction of anoikis was evaluated by measuring caspase-7 and PARP cleavage. H460 cells transfected with siHSF1 and grown in suspension for 6 h had significantly elevated CC7 and c-PARP, compared to siRNA control cells (Figure 3.3A, C-D). Changes in apoptosis markers in H460 cells were observed over time and as early as 1.5 h post-suspension (Appendix Figure A.2.2). Similarly, suspended A549 cells transfected with

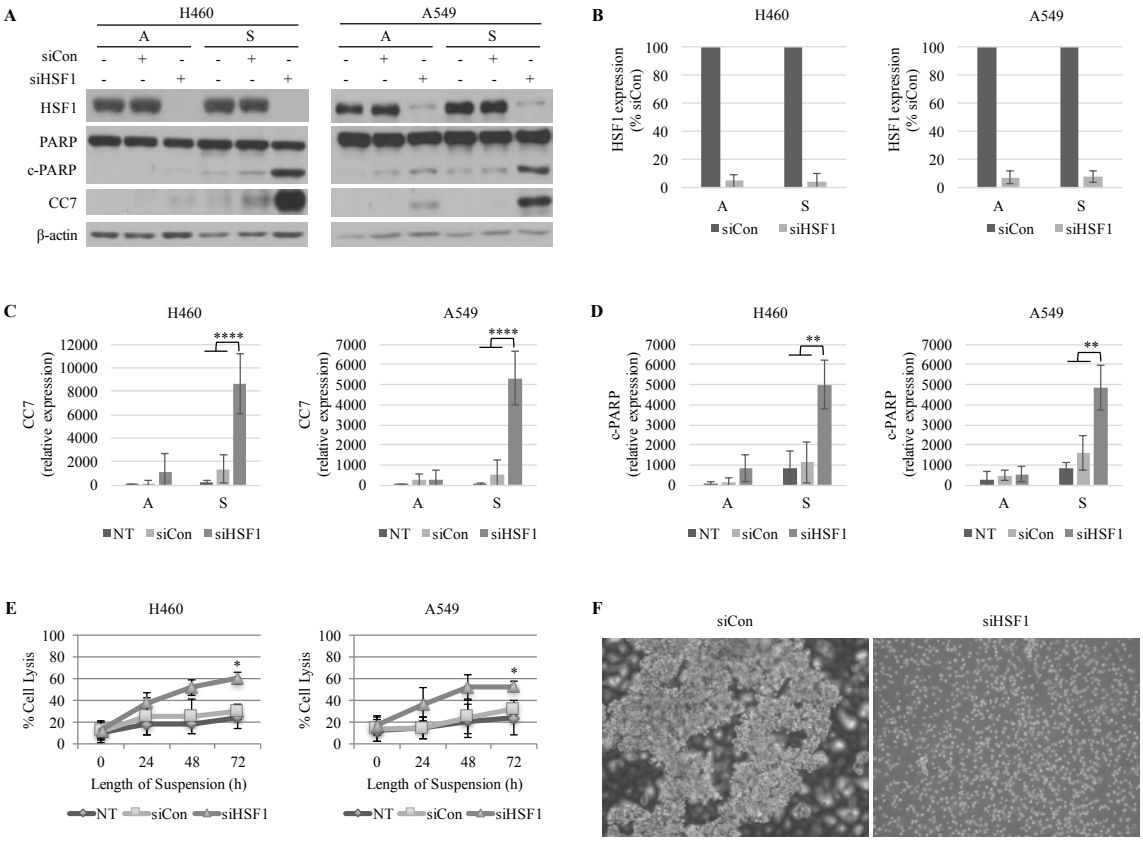

Figure 3.3. Knockdown of HSF1 potentiates anoikis in NSCLC cells. *(A)* H460 and A549 cells were transfected with HSF1 (siHSF1) or a scrambled control (siCon) siRNA for 48 h (H460) or 72 h (A549) prior to suspension. Cells were then suspended for 6 h (H460) or 24 h (A549). Suspended (S) and attached control (A) cells were harvested and western blots were performed to measure changes in HSF1 and cleavage of caspase-7 (CC7) and PARP (c-PARP). β-actin was used as a loading control. HSF1 (H460) and PARP (A549) immunoblots are from separate gels, with similar β-actin as shown above (compiled graphs use gel-specific loading control). *(B/C/D)* Densitometric analysis of HSF1 knockdown efficiency *(B)*, CC7 *(C)*, and c-PARP *(D)* (SD, **p < 0.01, ****p < 0.0001, n=11 (H460, CC7), n=5 (A549, CC7), n=4 (H460/A549, PARP)). *(E)* H460 and A549 cells transfected with siCon or siHSF1 were suspended with the CellTox Green binding dye for 0-72 h and fluorescence was measured kinetically (SE, *p < 0.05 compared to siCon, n=3; HSF1 knockdown similar to panel *B*). *(F)* Phase contrast images (100X) of H460 cells transfected with siCon or siHSF1 and suspended for 24 h. Statistical significance between control and siHSF1 samples were interpreted with a Student's *t*-Test.

siHSF1 had significantly elevated CC7 and c-PARP after 24 h in suspension, compared to siRNA control cells. Comparatively, minimal changes in CC7 or c-PARP were observed in HSF1-depleted H460 or A549 attached controls, indicating that loss of HSF1 sensitizes cells grown under anchorage-independent conditions to anoikis.

In concurrence, when measuring cell death with the CellTox Green Cytotoxicity Assay, we observed a significant increase in anoikis in suspended HSF1-knockdown cells, with no effect of HSF1 depletion at the time of detachment (Figure 3.3E). Furthermore, we observed that, in contrast to control cells that normally form multicellular aggregates when grown in suspension, siHSF1-treated cells were more dispersed (H460 cells shown in Figure 3.3F) and clearly displayed morphological changes consistent with apoptosis, such as membrane blebbing and cell shrinkage. Therefore, we have identified that HSF1 is necessary for anoikis resistance in two NSCLC cell lines.

3.5 Expression of Wild-Type or Constitutively Active HSF1 in Non-Tumorigenic Bronchial Epithelial Cells is Not Sufficient to Confer Anoikis Resistance

We next asked whether expression of HSF1, or its constitutive activation, was sufficient to confer anoikis resistance to BEAS-2B cells. To evaluate whether HSF1 expression or activity could promote anoikis resistance in BEAS-2B cells, wild-type human HSF1 (hHSF1) or constitutively active human HSF1 (hHSF1ΔRDT) expression vectors (kindly provided by Dr. Akira Nakai) were transiently transfected into BEAS-2B cells prior to cell detachment. This hHSF1ΔRDT expression vector has been used and

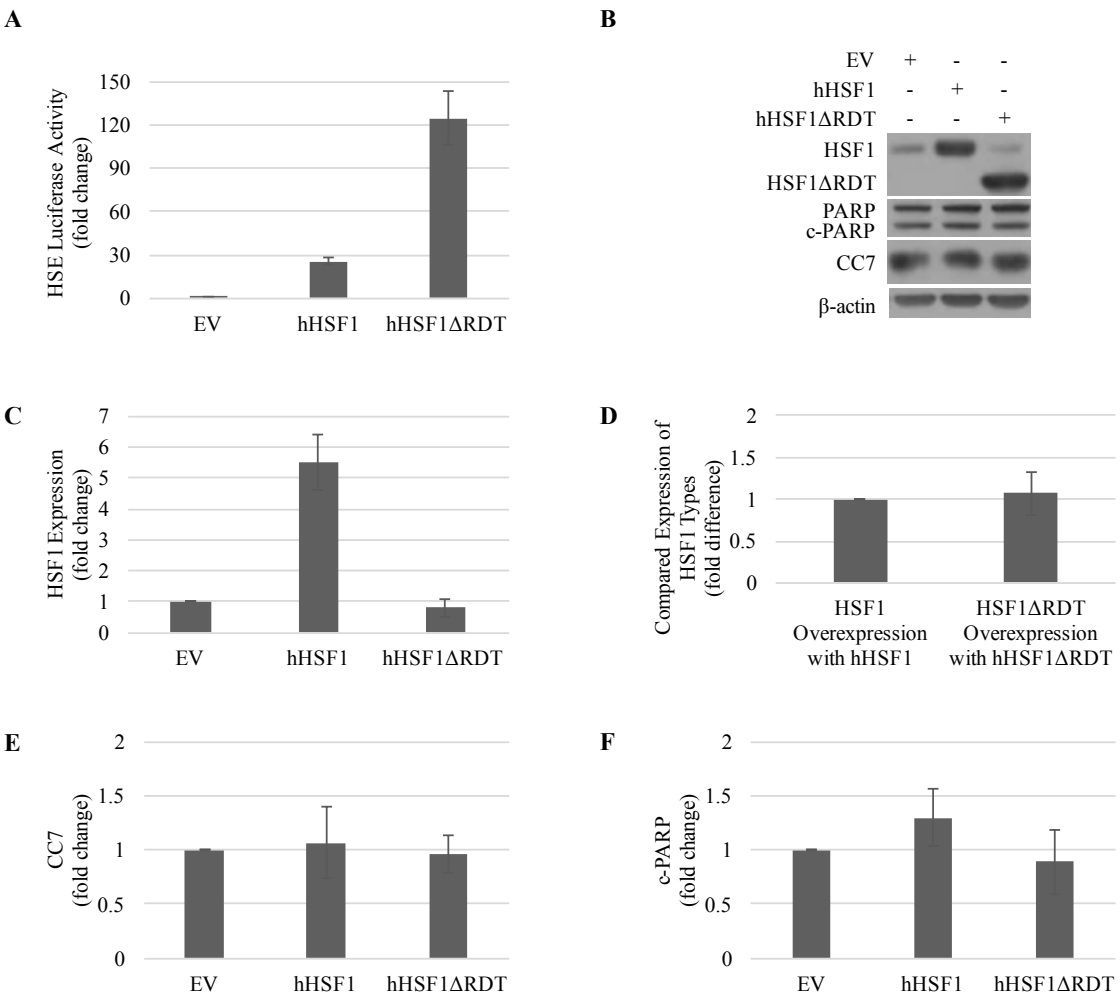

Figure 3.4. Expression of wild-type or active HSF1 did not confer anoikis resistance to normal bronchial epithelial cells. *(A)* Activation of exogenously expressed HSF1 was confirmed by co-transfecting BEAS-2B cells with an HSE-containing luciferase vector (and *Renilla* control) and wild-type HSF1 (hHSF1), active HSF1 (hHSF1ΔRDT), or an empty vector (EV). Luciferase activity was measured 24 h post-transfection. *(B)* BEAS-2B cells transfected with HSF1 expression vectors were suspended on polyHEMA-coated dishes for 24 h, which resulted in anoikis induction in EV cells, as expected. Western blots were performed to measure changes in both forms of HSF1 and cleavage of caspase-7 (CC7) and PARP (c-PARP). β-actin was used as a loading control. *(C-D)* Densitometric analysis of full length HSF1 *(C)* and comparison showing similar total overexpression of full length and truncated (active) forms of HSF1 *(D)*. *(E-F)* Densitometric analysis of CC7 *(D)* and c-PARP *(E)*. Statistical significance from EV control was interpreted with Student's *t*-Tests (SD, n=3).

demonstrated in previous studies to express functionally active HSF1 that successfully binds HSE and drives transcription of targets without requiring stress induction (Fujimoto et al., 2005). The HSE-driven luciferase reporter was co-transfected with each HSF1 plasmid and we confirmed that expression of hHSF1ΔRDT resulted in increased binding to HSE (Figure 3.4A). As anticipated, expression of hHSF1 also caused some increase in reporter stimulation, though significantly less than observed with hHSF1ΔRDT expression, reflective of basal activity occurring in the cells. Transfection of BEAS-2B cells with hHSF1 yielded elevated wild-type HSF1 expression compared to the empty vector control (Figure 3.4B-C). Transfection of BEAS-2B cells with hHSF1ΔRDT yielded elevated expression of truncated HSF1 (HSF1ΔRDT), and the level of overexpression was comparable to that observed for wild-type HSF1 (Figure 3.4B, D).

To investigate the effect on anoikis induction, control, hHSF1, or hHSF1ΔRDT-transfected cells were suspended on polyHEMA-coated plates and anoikis was evaluated by measuring caspase-7 and PARP cleavage. Anoikis was induced in BEAS-2B cells transfected with an empty vector and suspended for 24 h (Figure 3.4B), compared to attached controls, similar to previous experiments (attached samples not shown, reference Figure 3.1A-C). Interestingly, no significant protection from anoikis was observed in suspended BEAS-2B cells expressing hHSF1 or hHSF1ΔRDT, evident by the similar induction of CC7 and c-PARP compared to suspended cells transfected with the empty vector control (Figure 3.4B, E-F). Additionally, there were no changes in anoikis induction when directly comparing between suspended cells expressing hHSF1 or hHSF1ΔRDT. No significant toxicity was observed in attached cells transfected with any of the plasmids, measured by caspase-7 and PARP cleavage (data not shown). In possible

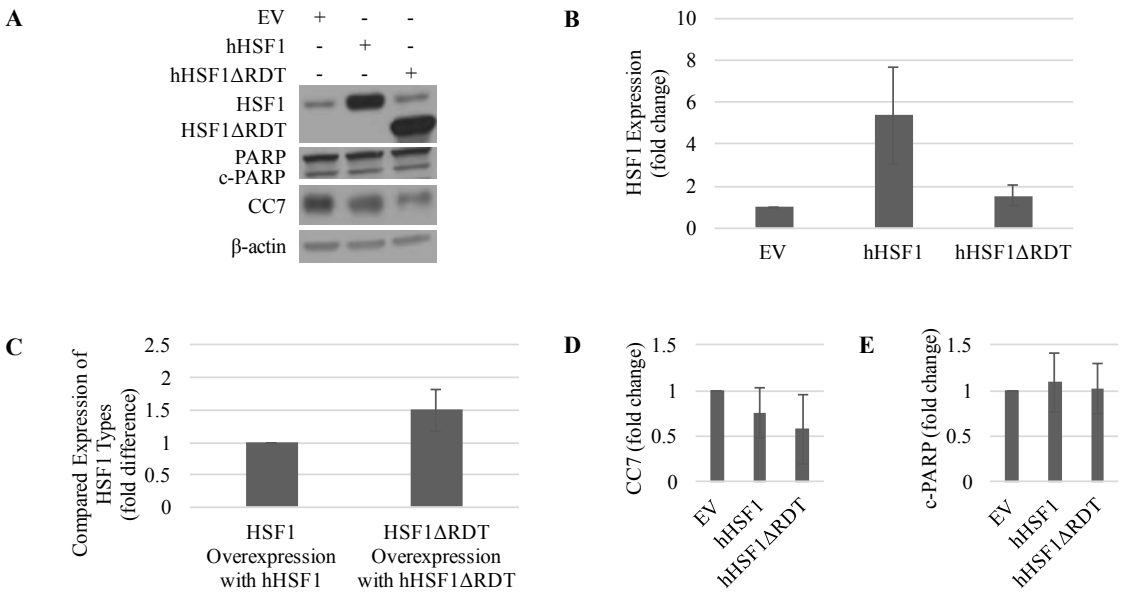

Figure 3.5. Expression or activation of HSF1 had variable effects on prolonged H460 cell survival in suspension. *(A)* H460 cells were transfected with wild-type HSF1 (hHSF1), active HSF1 (hHSF1ΔRDT), or an empty vector (EV) for 24 h and were suspended on polyHEMA-coated dishes for 48 h, which resulted in some spontaneous anoikis induction in EV cells, as expected. To reduce transfection-related toxicity observed in these cells, luciferase vectors were excluded from these experiments. Previous experiments validated that transfection of each HSF1 vector led to a similar HSE reporter activation in H460 cells as shown in other cell types. Western blots were performed to measure changes in both forms of HSF1 and cleavage of caspase-7 (CC7) and PARP (c-PARP). β-actin was used as a loading control. *(B-C)* Densitometric analysis of full length HSF1 *(B)* and comparison of total overexpression of full length and truncated (active) forms of HSF1 *(C)*. *(D-E)* Densitometric analysis of CC7 *(D)* and c-PARP *(E)*. Statistical significance from EV control was interpreted with Student's *t*-Tests (SD, n=4).

support of these findings, preliminary experiments indicated that exposing BEAS-2B cells to heat shock prior to detachment also had no effect on anoikis induction (Appendix Figure A.2.3). Therefore, we observed that neither the expression nor the activation of

HSF1 was alone sufficient to confer anoikis resistance to these normal bronchial epithelial cells.

Because we observed increases in apoptosis markers with extended (>48 h) suspension culture of H460 cells (Figure 3.1A-C), we performed a complementary experiment to examine whether we could prolong survival by overexpressing or overactivating HSF1 in these cells. We reasoned that, while loss of HSF1 leads to rapid induction of anoikis and is likely important early after detachment, further enhanced HSF1 activation might also prevent the eventual partial spontaneous anoikis induction observed in these cells. H460 cells were transfected with an empty vector, hHSF1, or hHSF1ΔRDT, and grown in suspension for 48 h. On average, transfection of H460 cells with active HSF1 appeared to reduce caspase-7 activation after extended suspension culture; however, due to variability this finding did not reach statistical significance. Furthermore, we did not observe a consistent change in PARP cleavage at this time (Figure 3.5). Currently, we are unable to conclude that increased HSF1 activity in these cells can extend their anchorage-independent survival for prolonged times. If not a consequence of experimental variation, we suggest this could be reflective of alternate and separate anoikis, or other apoptotic mechanisms, being triggered at these extended times.

3.6 Discussion

The experiments presented here provide direct and novel evidence that HSF1 is important for NSCLC cell anoikis resistance. To the best of our knowledge, this is the first study to explicitly demonstrate that HSF1 exerts its support of anchorage-

independence through suppression of anoikis. Furthermore, we provide novel evidence that suggests HSF1 activity can be triggered by cell detachment. It has been demonstrated that phosphorylation of serine 326 (S326) is specifically (through site-directed mutagenesis) and critically important for the transactivational competence of HSF1 in response to heat stress (Guettouche et al., 2005), and is a well-established marker of HSF1 activation (Chou et al., 2015; Guettouche et al., 2005; Mendillo et al., 2012). While our experiments did not indicate any changes in the total expression of HSF1, we observed that cell detachment rapidly stimulated pS326-HSF1 in anoikis-resistant H460 cells, but not anoikis-sensitive BEAS-2B cells (Figure 3.2A-B). Perhaps reflecting differences between heat shock and cancer HSF1 mechanisms, there was no major shift in electrophoretic mobility of HSF1, which results from heat shock-induced hyperphosphorylation, though its precise function is unclear (Budzyński et al., 2015; Sarge et al., 1993; Xia and Voellmy, 1997). Recently, it was demonstrated that HSF1 transcriptional activity could be uncoupled from its hyperphosphorylation. Budzyński and colleagues (2015) showed that multisite mutagenesis of 15 phosphorylation sites in the HSF1 regulatory domain (which included S326) did not prevent (and actually increased) heat shock-induced transcriptional activation of HSP genes, and cautioned against using phosphorylation signatures as sole markers for HSF1 activation (Budzyński et al., 2015).

Activation of HSF1 is also frequently assessed by measuring changes in HSP protein expression, activity at the promoters of well-known HSP targets, such as the genes for HSP72 (Chou et al., 2012) or HSP70B (Khaleque et al., 2005; Stanhill et al., 2005), or binding at the conserved HSE sequences themselves (Stanhill et al., 2005; Yoon et al., 2014). A potential limitation to solely assessing HSF1 activity by HSP expression

or HSP promoter activity is the reliance on changes in a specific target. This is particularly relevant in cancer, where HSF1 can control HSP and non-HSP targets, and changes in different HSP are variable and not always correlative with changes in HSF1 (Whitesell and Lindquist, 2009). Thus, we measured changes in binding to conserved HSE sequences to provide broader insight into HSF1 activity, independent of any singular target. In agreement with our phosphorylation data, detachment of H460 cells led to increased binding of HSE (Figure 3.2D), further suggesting that detachment and growth of H460 cells in suspension can trigger changes in HSF1 associated with its activation. Collectively, this data indicated a potential role for HSF1 in supporting anchorage independence in these cells, while also providing novel evidence that cell detachment can modulate HSF1.

HSF1 has been linked to anchorage-independence before. For instance, when grown on soft agar, B16-F10 mouse melanoma cells expressing active HSF1 (RD delete) grew faster and generated more colonies, although silencing HSF1 notably did not prevent such growth (Toma-Jonik et al., 2015). Khaleque et al. (2005) demonstrated that while HRGβ1 stimulated soft agar growth of MEF *HSF1*+/+, MEF *HSF1-/-* were unable to form colonies with or without HRGβ1 treatment. HRGβ1 increased both HSF1 expression and activity leading to increased HSP expression (perhaps notably, there was similarly no shift in HSF1 electrophoretic mobility). HSF1 was important for HRGβ1-mediated protection from apoptosis stimulators such as *cis*-platinum and heat shock in MEF, leading the authors to suggest the possibility that HSF1 might influence anchorage independence through increased cell survival (Khaleque et al., 2005). Other instances of HSF1 influencing the ability of cells to grow independent of anchorage have also been

observed (Asano et al., 2016; Carpenter et al., 2017; Powell et al., 2016; Salamanca et al., 2014). Identifying what type of pathways (proliferative, anti-anoikis, etc.) anchorage independence-promoting targets influence is important to fully understand the underlying mechanisms. There have been instances where the inability of cells to grow on soft agar was specifically consequent to deficient proliferative signaling, despite cells being resistant to anoikis (Fiucci et al., 2002). We postulated that HSF1 might influence anchorage independence through increased cell survival, and sought to directly test this hypothesis by specifically evaluating whether HSF1 is important for anoikis resistance using our NSCLC cell model. Indeed, we identified that siRNA-mediated knockdown of HSF1 potentiated anoikis in both H460 and A549 lung carcinoma cells, while comparatively having minimal effects on attached cells (Figure 3.3). Thus, using our NSCLC cell model, we have provided novel evidence that HSF1 supports anchorage independence specifically through the suppression of anoikis.

Tumor cells frequently have increased expression and activation of HSF1 (Calderwood and Gong, 2012; Calderwood et al., 2006; Ciocca and Calderwood, 2005; Dai et al., 2007; Mendillo et al., 2012), and our experiments indicated detachment of cells could modulate changes in HSF1 associated with its activation in at least one tumor type. Thus, we further asked whether expression or activation of HSF1 would alone be sufficient to drive anoikis resistance in non-tumorigenic bronchial epithelial cells. However, we found that neither HSF1 expression or activation in BEAS-2B cells protected cells from undergoing anoikis (Figure 3.4). It is notable that HSF1 has not been considered to act as an oncogene, but rather by controlling a broad transcriptional repertoire and promoting tumor progression and metastasis by mediating oncogenic

signals (Dai et al., 2007; Mendillo et al., 2012). These findings suggest that increasing HSF1 expression or activation alone is not sufficient to overcome anoikis signaling in non-tumorigenic bronchial epithelial cells. While HSF1 is needed for anoikis resistance in lung carcinoma cells, additional oncogenic signaling, present in lung cancer cells, but not in BEAS-2B cells, appears to be required for successful anoikis inhibition. While active HSF1 was shown to increase colony formation in B16-F10 mouse melanoma cells (Toma-Jonik et al., 2015), perhaps this difference relates to the tumorigenic status of the model, if not simply differing species and tumor type. Thus, while HSF1 may be necessary for anoikis resistance in lung cancer cells, expressing or activating it in non-tumorigenic cells may be insufficient to block anoikis without simultaneously providing additional factors. Because they undergo some spontaneous anoikis (comparably to BEAS-2B; >48h measured by caspase-7 and PARP cleavage), we additionally experimented in H460 cells to see whether overexpressing or activating HSF1 might prevent this delayed (>48 h) onset of anoikis. While our present analysis was variable, and ultimately inconclusive, there is some suggestive (but not statistically significant) evidence of this by measure of caspase-7 cleavage, and we suggest it warrants further analysis. However, if this is not the case, it may reflect separate anoikis signaling pathways are executed by this time, independent of HSF1. With respect to our gain-of-function experiments, the time frame during which these experiments were conducted should be considered. We have measured the effect of HSF1 expression or activation on BEAS-2B anoikis at 24 h (48 h for H460), the time at which we observed anoikis in these cells. From an alternative perspective, perhaps increased HSF1 expression or activation could have some anti-anoikis effect early, but by 24 h (48 h for H460), anoikis is still

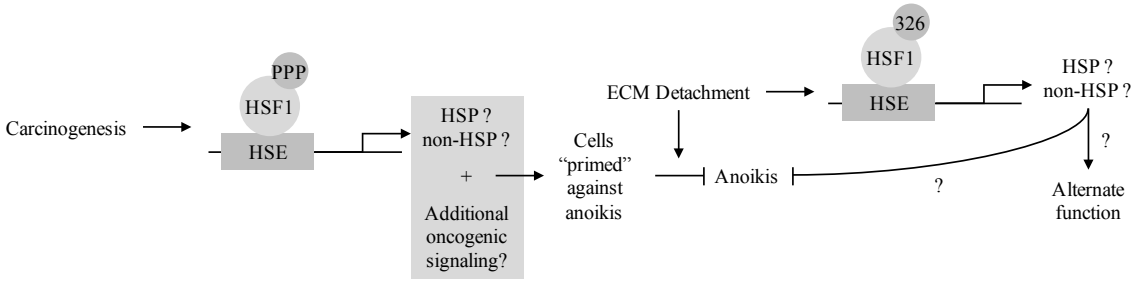

Figure 3.6. Hypothesis for HSF1-mediated suppression of anoikis in NSCLC cells. Schematic representing hypothesis that HSF1 promotes anoikis resistance by controlling basal and/or detachment-induced expression of anoikis effectors in NSCLC cells (post-translational modifications (PPP), pS326-HSF1 (S326)).

executed, or an increased surviving population of cells could be masked by the larger population of cells undergoing anoikis at these times. Conducting expanded time course studies and applying a broader measure of viability would be beneficial for future studies.

Based on our findings and the known functions of HSF1, we suggest that HSF1 regulates the expression of one or more anoikis effectors in NSCLC cells, and that this may or may not involve detachment-induced HSF1 activation (Figure 3.6). We have yet to identify whether detachment-induced changes in HSF1 activation markers are specifically critical for anoikis suppression. One argument against this would be the lack of detachment-induced pS326-HSF1 in A549 cells, despite their anoikis sensitivity in response to HSF1 depletion. This could represent a cell-type specific response, or alternatively suggest S326 phosphorylation specifically is not required for driving HSF1-mediated anoikis resistance in these cells, possibly reflective of an uncoupling, as recently described (Budzyński et al., 2015). We note that we were unable to assess changes in HSE binding in A549 cells, and therefore cannot rule out that detachment still induces HSF1 activation, which warrants further analysis. In either case, it seems likely

that this response in H460 cells plays some important role. The rapid and transient nature of the response is reminiscent of cells responding to a stress, suggesting loss of adhesion may trigger a stress-like response in these cells. Identifying downstream changes occurring in response to this will be important, and determining the time frame during which they occur will provide relevant insight into its role with respect to anoikis resistance. Notably, depletion of HSF1 led to a rapid induction of anoikis, perhaps suggestive that detachment-induced transcriptional changes might not happen quick enough to explain its anoikis-suppressing function in these cells. Given that HSF1 can control basal expression of transcriptional targets, we hypothesize that HSF1 in cancer cells may drive the increased basal expression of key anti-anoikis effectors, essentially priming these cells to resist anoikis prior to ECM disengagement. Thus, depletion of HSF1 prior to cell detachment may downregulate anti-anoikis effectors, thereby permitting unimpeded activation of anoikis machinery rapidly after detachment. Further, HSF1 has been shown to transcriptionally repress pro-apoptotic genes (Wang et al., 2006a), and thus could also act in a repressive capacity toward anoikis activators. Interestingly, one gene that HSF1 represses is *XAF1* (Wang et al., 2006a), which has been implicated in complex mechanisms that regulate autophagy and anoikis in A549 cells (Cai et al., 2016). In parallel, perhaps detachment-induced HSF1 activation might represent a separate or redundant mechanism of anoikis suppression. Moreover, HSF1 may also differentially regulate targets in suspended cells to prepare them to deal with detachment-induced changes, such as increased production of ROS or changes in glucose metabolism. Thus, identification of downstream targets of HSF1 in suspended cells will be important regardless of its specific effect on anoikis resistance.

Our findings keep in line with, and add to, the ability of HSF1 to influence apoptosis in a variety of contexts (e.g. cell stress, chemotherapy, regulation of apoptosis related genes and signaling pathways, etc.) (Dai et al., 2007; Franceschelli et al., 2008; Jiang et al., 2015; Khaleque et al., 2005; Mendillo et al., 2012; Vydra et al., 2014; Wang et al., 2006a). Tumor cells overexpress an array of proteins that support tumor progression (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011), and increased HSF1 expression and activity, and HSP overexpression, are common occurrences in cancer cells (Calderwood and Gong, 2012; Calderwood et al., 2006; Ciocca and Calderwood, 2005; Dai et al., 2007; Mendillo et al., 2012). Importantly, there is significant commonality between cellular processes and proteins that lead to anoikis resistance and those that HSF1 has been associated with, suggesting there are a number of mechanisms by which HSF1 might promote anoikis resistance. For instance, HSF1 has been linked to apoptosis regulation, energy metabolism, response to oxidative stress and hypoxia, EMT, autophagy, and is a mediator of signaling through PI3K/AKT, MAPK, and other overactive pathways in anoikis resistant cells (Ahn and Thiele, 2003; Dai et al., 2007; Desai et al., 2013; Gabai et al., 2012; Khaleque et al., 2005; Liu et al., 2016; Mendillo et al., 2012; O'Callaghan-Sunol and Sherman, 2006; Powell et al., 2016; Wang et al., 2006a). While not explicitly shown to regulate anoikis, candidates for such anoikis effectors might include HSP, the most well-known targets of HSF1, and known suppressors of apoptosis. Some indirect evidence also suggests HSP may be involved in anoikis resistance (Sreedhar and Csermely, 2004), although given the diverse transcriptional repertoire of HSF1 in cancer (Dai et al., 2007; Mendillo et al., 2012), non-HSP candidates are also possible.

Some future directions of this work include generation of siRNA-resistant HSF1 expression constructs for wild-type, active, DNA-binding, and S326 phosphorylation mutant variants. This will enable the use of early time-frame rescue experiments in lung carcinoma cells that will help to identify what role each of these steps plays in enabling HSF1-mediated anoikis resistance in NSCLC. Regardless of the yet unexplored mechanism, it is clearly evident that HSF1 is important for NSCLC cell anoikis resistance. Given the broad array of HSF1 targets in cancer, evidence of such a role in anoikis signaling mechanisms could have a profound impact by broadening the scope of potential anoikis effectors. It would be of great interest in the future to broadly compare and analyze changes in gene expression in attached and suspended cells with or without HSF1 using, for instance, a microarray analysis. Beginning to elucidate the mechanism by which HSF1 influences anoikis resistance will help in understanding how it, or its downstream targets, might be taken advantage of therapeutically to suppress metastasis. Our work, presented in the next chapter, begins to investigate HSF1 anoikis mechanism by focusing on its most well-known targets for apoptosis suppression, HSP.

4. Heat Shock Transcription Factor 1 (HSF1) Supports NSCLC Anoikis Resistance Independent of Canonical Heat Shock Protein Regulation

4.1 Rationale

We previously reported that HSF1 plays a role in maintaining anoikis resistance in NSCLC cells. Furthermore, we observed that cell detachment led to increases in indicators of HSF1 activity. Presently, it is evident that HSF1 controls a vast array of genes with a variety of functions in cancer (Dai et al., 2007; Mendillo et al., 2012), in addition to its classical role as a master regulator of HSP transcription (Calderwood et al., 2010; Ciocca et al., 2012; Inouye et al., 2003; Morimoto, 1998; Sarge et al., 1993; Stanhill et al., 2005). Such genes include those involved in apoptosis suppression (Ciocca et al., 2012; Kumar et al., 2013; Mendillo et al., 2012), affording multiple potential targets for HSF1 in its regulation of anoikis resistance. Yet, perhaps the most likely targets for HSF1 control of anoikis resistance may still be HSP, as they both act as chaperones for important apoptosis mediators and are themselves capable of inhibiting apoptosis. Therefore, we questioned whether detachment-induced activation of HSF1 may occur to induce a canonical HSP stress response, or whether overexpression of HSP in tumor cells may be regulated by HSF1 to essentially "prime" cells to resist anoikis before matrix detachment.

HSP suppress apoptosis through a variety of mechanisms both in response to stress and in tumor cells. In addition to their chaperoning function, the ability of HSP to promote cell survival is also related to their ability to directly influence both the intrinsic

and extrinsic pathways of apoptosis at multiple steps (Beere, 2004; Sreedhar and Csermely, 2004). HSP can regulate the activation or stability of key members of signaling cascades, such as JNK (Gabai et al., 2002; Mosser et al., 2000; Stetler et al., 2008) and PI3K/AKT (Basso et al., 2002; Havasi et al., 2008; Nakagomi et al., 2003; Rane et al., 2003; Sato et al., 2000), important for initiation and modulation of apoptosis (Beere et al., 2000; Datta et al., 1997; Fan et al., 2000; Havasi et al., 2008; Lei and Davis, 2003; Nimmanapalli et al., 2003; Zha et al., 1996). The intrinsic, or mitochondrial, pathway of apoptosis relies heavily on the antagonistic relationship of opposing members of the BCL-2 family, the balance of which can decide the fate of the cell (Beere, 2004; Sreedhar and Csermely, 2004). HSP can directly influence key BCL-2 family members (Gabai et al., 2002; Gotoh et al., 2004; Kirchhoff et al., 2002; Paul et al., 2002; Shan et al., 2003; Stankiewicz et al., 2009; Yang et al., 2009), as well as inhibitors of apoptosis (IAP), p53 (Ghosh et al., 2008), and the mitochondrial permeability transition (Ghosh et al., 2010). A possible role for HSP in regulating the anoikis activator BIM might exist, since heat shock prevented BIM-mediated apoptosis in response to endoplasmic reticulum stress (Kennedy et al., 2014). Furthermore, HSP can also inhibit pro-apoptotic factors released from the mitochondria, cytochrome c (Bruey et al., 2000; Garrido, 2002; Mosser et al., 2000; Paul et al., 2002) and SMAC/DIABLO (Chauhan et al., 2003), that help to mediate apoptosome formation. Signaling downstream of the mitochondria is influenced by multiple HSP that disrupt apoptosome formation by associating with targets such as APAF-1, thereby preventing caspase-9 activation (Beere et al., 2000; Pandey et al., 2000b; Saleh et al., 2000). HSP can bind and influence processing of procaspases, and consequentially their activation and cleavage of targets (Concannon et

al., 2001; Komarova et al., 2004; Mosser et al., 2000; Pandey et al., 2000a). HSP can also influence extrinsic apoptosis through, for instance, binding to and inhibiting DAXX or ASK1 to suppress FAS-mediated apoptosis (Charette et al., 2000; Park et al., 2002). Notably, HSP27 in particular is frequently dependent on its phosphorylation status, important with respect to its function as a regulator of actin filament dynamics and apoptosis (Charette et al., 2000; Clarke and Mearow, 2013; Garrido, 2002; Katsogiannou et al., 2014; Paul et al., 2002; Paul et al., 2010; Sreedhar and Csermely, 2004).

Anoikis induction involves intrinsic and extrinsic pathways of apoptosis, and resistance to anoikis is achieved through stimulation of a variety of signaling pathways and manipulation of key apoptosis effectors (Gilmore, 2005; Grossmann, 2002; Paoli et al., 2013; Taddei et al., 2012), some of which overlap with known anti-apoptotic functions of HSP. Furthermore, in addition to our finding that HSF1 plays a key role in NSCLC cell anoikis suppression, there is other indirect evidence of HSP involvement in anchorage-independence, and perhaps anoikis resistance (Sreedhar and Csermely, 2004). FAK is an important integrin signaling molecule that can suppress anoikis. FAK is a client of HSP90, and treatment of mice harboring SiHa cervical xenografts with the HSP90 inhibitor, 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), reduced FAK signaling, tumor growth, and metastasis. Transfection of dominant-negative FAK into SiHa cells reduced colony formation on soft agar, implicating the FAK-stabilizing HSP90 in this process (Schwock et al., 2009). BAG-3, a co-chaperone of HSP70 and transcriptional target for HSF1, contains a BAG domain important for its interaction with HSP70 and HSC70 (Franceschelli et al., 2008; Frisch, 1999; Jacobs and Marnett, 2009; Rosati et al., 2011). The BAG domain was found to be

required for silencer of death domains (SODD)-mediated protection of MDCK cells from anoikis (Frisch, 1999). In U251 glioma cells, knockdown of BAG-3 reduced cell-matrix adhesion on various matrices, and pharmacological inhibition of HSF1 reduced constitutive BAG-3 and HSP70 expression. While BAG-3 knockdown alone did not induce anoikis in suspended cells, it was suggested that the reduction in such cell-matrix interactions could prime cells to AT-101 (a pan BCL-2 inhibitor)-induced anoikis. In attached cells, similar death mechanisms were observed in AT-101 combination treatments with BAG-3 knockdown or preventing HSP70-BAG-3 interaction, implying targeting HSP70 might also reduce cell-matrix adhesion or be important for increased AT-101-induced anoikis. Interestingly, knockdown of BAG-3 in suspended glioma cells also suppressed detachment-induced FAK phosphorylation (Antonietti et al., 2017). In H460 cells, photo-activated aloe-emodin caused cell death (described as anoikis) leading not only to multiple alterations in apoptotic machinery, but also reduced p38MAPK and HSP27 expression, and alterations to the actin cytoskeleton (Chang et al., 2012; Lee et al., 2010).

Relationships between integrins and HSP have also been found. Overexpression of HSP27 surprisingly inhibited the ability of melanoma cells to form colonies on soft agar, and HSP27-transfected cells had a marked reduction in the integrin αvβ3 (Aldrian et al., 2002). Using metastatic breast cancer cells it was demonstrated that exogenously added HSP60 can bind and activate integrin α3β1, which mediates attachment of breast cancer cells to metastatic sites such as lymph nodes and bone, in addition to other roles in tumor cell proliferation, metastasis, invasion, motility, and angiogenesis (Barazi et al., 2002). Additionally, adhesion of platelets to collagen through the $\alpha_2\beta_1$ integrin resulted in

dissociation of a protein phosphatase 1 (PP1) complex with HSC70/HSP90 and their subsequent dephosphorylation, and the phosphorylation of HSP27 was linked to platelet aggregation (Polanowska-Grabowska and Gear, 2000). Collectively, there are numerous roles for HSF1 and HSP in the suppression of apoptosis and a variety of indirect evidence that suggests HSP could be involved in anoikis. Moreover, our previous finding that HSF1 directly influences anoikis resistance and can be activated upon cell detachment provides a solid rationale for study of HSP involvement in HSF1-mediated anoikis resistance. Our work here aims to begin investigating whether cell detachment triggers HSF1 activation to induce HSP expression, and whether various HSP are important for HSF1-mediated anoikis resistance in NSCLC cells.

4.2 Detachment of NSCLC Cells Does Not Induce a Canonical Stress-Induced HSP Response

We previously reported that cell detachment could induce changes in markers of HSF1 activity in tumor cells that can be sensitized to anoikis through HSF1 depletion, but the significance of this was unclear. Here, we asked whether detachment-induced HSF1 activation might lead to a canonical upregulation of HSP similar to a stress response, to influence anoikis resistance or perform some other function. To examine this, H460 and A549 NSCLC cells were suspended on polyHEMA-coated dishes for 24 h and the expression of multiple HSP including HSP72, HSP40, HSP27, HSP60, and HSP90 were measured. Compared to attached controls, growth in suspension did not result in any significant changes in the expression of HSP72, HSP40, HSP27, or HSP90 in either cell line. Suspension of cells for shorter or longer times yielded similar results (data not

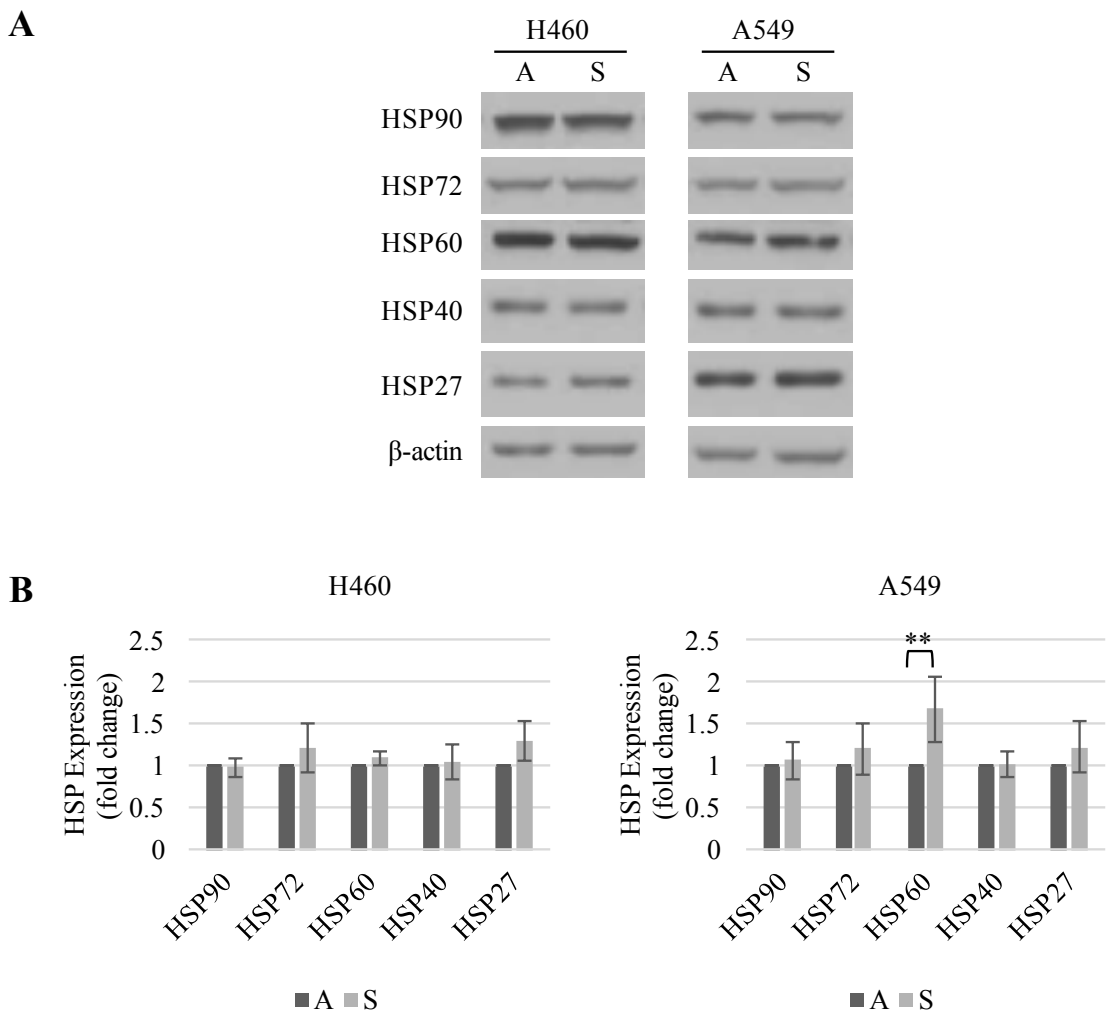

Figure 4.1. Cell detachment does not induce a canonical HSP-mediated stress response. *(A)* H460 and A549 cells were maintained under normal attached (A) conditions or suspended (S) on polyHEMA-coated dishes for 24 h. Western blots were performed to measure expression of various HSP. β-actin was used as a loading control. *(B)* Densitometric analysis of HSP expression, represented as fold change from attached control. Statistical significance was interpreted with Student's *t*-Tests (SD, **p < 0.01, n=7 (HSP90, HSP72, HSP27, H460 – HSP60), n=6 (A549 – HSP60), n=4 (HSP40)).

shown). Interestingly, we did observe an increase in HSP60 expression in suspended A549 cells, but not H460 cells (Figure 4.1). This was particularly intriguing considering we did not observe increased pS326-HSF1 in suspended A549 cells, although we did not

rule out the possibility that HSF1 is activated through an alternate mechanism in these cells. Taken in entirety, we found no evidence that would generally support the hypothesis that cell detachment triggers a canonical HSF1-HSP-mediated stress response, but perhaps HSP60 plays a specific role in suspended A549 cells. Notably, none of the examined HSP were altered in suspended H460 cells in which we had previously identified increases in pS326-HSF1 and HSE binding, suggesting cell detachment may trigger HSF1 to regulate a target not associated with the canonical stress response. These findings add to the recent and expanding literature reflecting non-canonical roles for HSF1 in cancer, unrelated to HSP.

4.3 HSF1 Partially Controls Expression of Multiple HSP in NSCLC

While there are distinct mechanisms for HSF1 with regard to the HSR compared to in cancer (Mendillo et al., 2012), HSF1 certainly can still regulate expression of HSP in tumor cells. Basal overexpression of HSP in tumor cells has been observed (Calderwood and Gong, 2012; Calderwood et al., 2006; Ciocca and Calderwood, 2005; Dai et al., 2007; Mendillo et al., 2012) and such HSP have a variety of anti-apoptotic functions (Beere, 2004; Ciocca et al., 2012). Yet, not all increases in HSP in cancer are correlative with HSF1 activation, specific HSP changes can be tumor-specific, and HSF1 depletion does not always abrogate overexpressed HSP in tumor cells back to normal levels (Whitesell and Lindquist, 2009). Since anoikis was induced quickly in HSF1-depleted tumor cells, we hypothesized that increased HSF1 activity in these cells may lead to altered basal regulation of anoikis effectors to essentially prime cells to resist anoikis prior to ECM disengagement. While detachment-induced HSF1 activation does

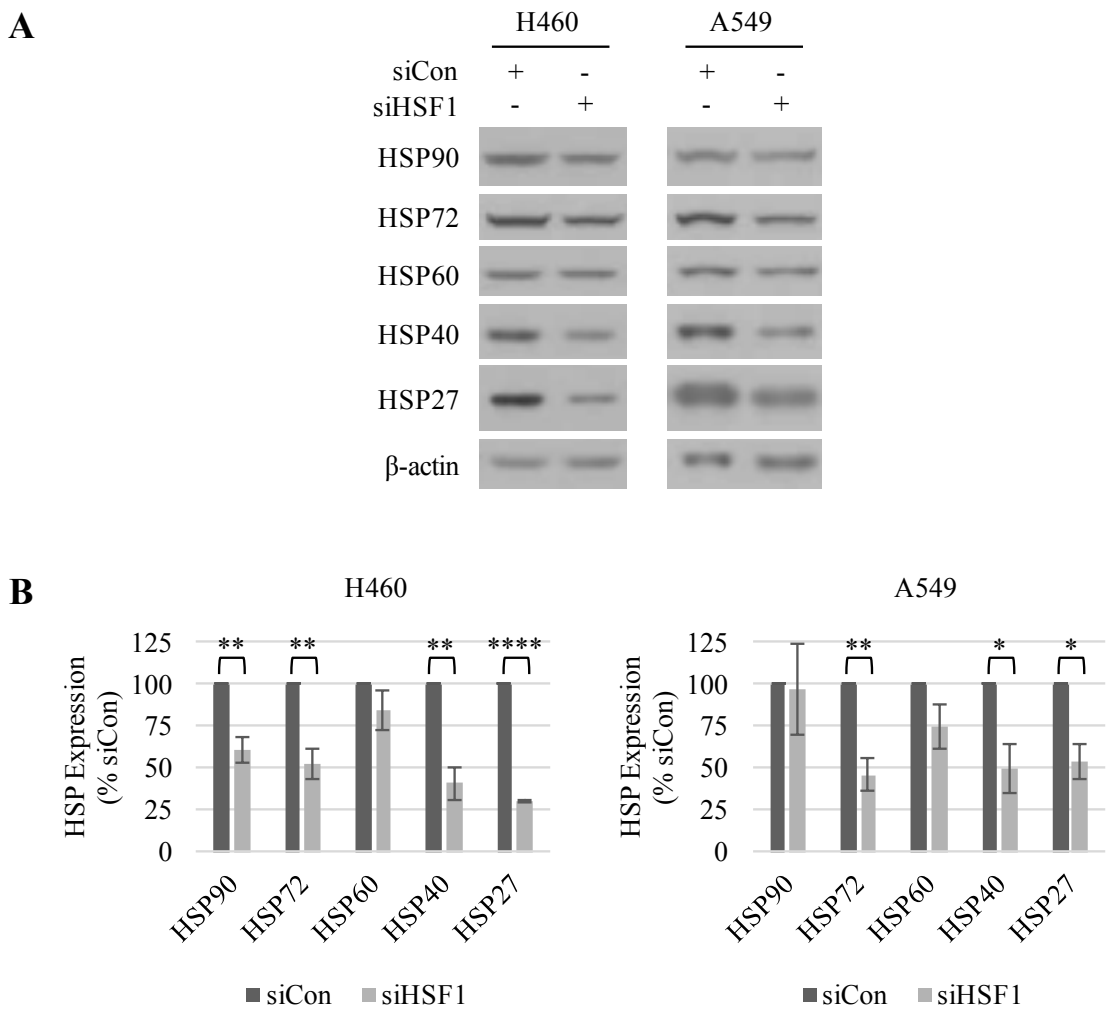

Figure 4.2. Knockdown of HSF1 causes partial inhibition of HSP expression in NSCLC cells. *(A)* H460 and A549 cells were transfected with siRNA targeting HSF1 (siHSF1) or a scrambled control (siCon) for 48 h (H460) or 72 h (A549). Western blots were performed to measure expression of various HSP. β-actin was used as a loading control. HSP72 (H460) and HSP90/HSP60/HSP40 (A549) immunoblots are from a separate gel, with similar β-actin as shown above (compiled graphs use gel-specific loading control) *(B)* Densitometric analysis of HSP expression, represented as fold change from siCon. Statistical significance was interpreted with Student's *t*-Tests (SD, *p<.05, **p < 0.01, ****p<.0001, n=4 (H460), n=3 (A549)).

not lead to a broad upregulation of HSP, HSF1 depletion in attached cells may lead to multiple changes in its transcriptional targets, including HSP. Therefore, we asked whether knockdown of HSF1 inhibited the basal expression of HSP72, HSP40, HSP27, HSP60, or HSP90 in lung carcinoma cells. Indeed, in both H460 and A549 cells, knockdown of HSF1 partially reduced expression of HSP72, HSP40, and HSP27. Additionally, HSF1 depletion caused partial inhibition of HSP90 in H460 cells, but not A549 cells (Figure 4.2). HSF1 knockdown had minimal effect on HSP60 expression in either cell type, particularly notable in A549 cells, in which we observed a detachment-induced upregulation of HSP60, and suggesting that, at least basally, HSF1 does not regulate HSP60. Notably, knockdown of HSF1 resulted in only partial inhibition of these HSP, indicating there are other factors involved in maintaining their basal expression. Furthermore, our observations also add to evidence of tumor-type specificity sometimes seen with regards to HSF1-HSP regulation in cancer. A summary of HSF1 and HSP changes observed in each tumor cell line is presented in Appendix Table A.2.1. However, collectively we observed a reduction in expression of multiple anti-apoptotic HSP as a consequence of HSF1 depletion, which we hypothesized, could be of relevance in the re-sensitization of anoikis in these cells.

4.4 Cell Detachment Induces Phosphorylation of HSP27 on Serine 82

Of the measured HSP, the regulation of HSP27 function in particular is also strongly regulated by its phosphorylation status, and occurs in a rapid fashion. Changes in HSP27 phosphorylation can lead to differential functions, including influence of

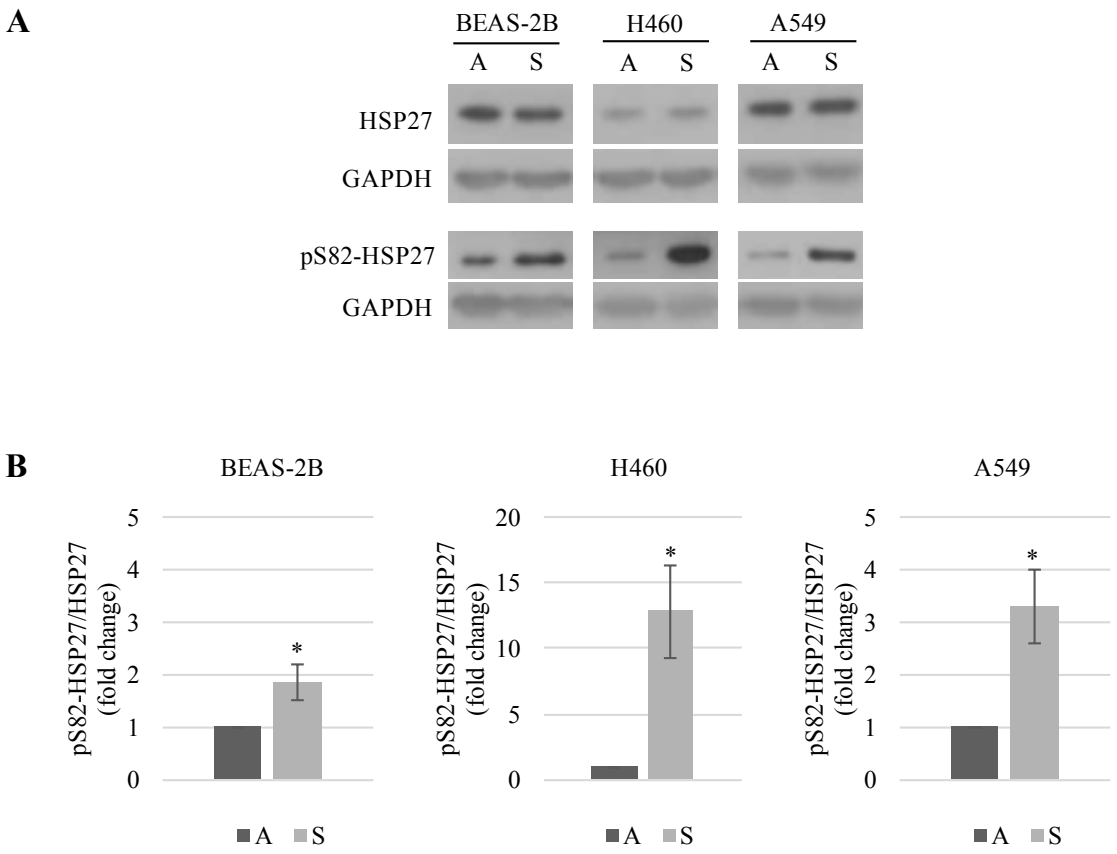

Figure 4.3. Cell detachment induces phosphorylation of HSP27 at serine 82. *(A)* BEAS-2B, H460 and A549 cells were maintained under normal attached (A) conditions or suspended (S) on polyHEMA-coated dishes for 24 h. Western blots were performed to measure expression of HSP27 and pS82-HSP27. GAPDH was used as a loading control. *(B)* Densitometric analysis of pS82-HSP27 expression, normalized to total HSP27 expression, represented as fold change from attached control. Statistical significance was interpreted with Student's *t*-Tests (SD, *p < 0.05, n=3).

apoptosis and regulation of actin filament dynamics (Beere, 2004; Clarke and Mearow, 2013; Katsogiannou et al., 2014; Paul et al., 2010; Sreedhar and Csermely, 2004), both relevant to changes in cell adhesion. While there were no changes in total HSP27 expression induced by cell detachment, HSF1 knockdown resulted in reduced HSP27 expression. We hypothesized that cell detachment might trigger HSP27 phosphorylation, leading to a response that would be influential in the suppression of anoikis, an event that

would be reduced consequent to HSP27 downregulation in anoikis-sensitized HSF1-knockdown cells. Indeed, we observed that HSP27 was phosphorylated on serine 82 (pS82-HSP27) in both NSCLC cell lines grown in suspension for 24 h compared to attached controls, and was most robustly elevated in H460 cells (Figure 4.3). Preliminary experiments indicated this event may occur as early as 1.5 h post-detachment in tumor cells, and be sustained (data not shown). However, to a lesser extent we did also observe an increase in pS82-HSP27 in anoikis-sensitive BEAS-2B cells, which could be indicative of a more generic role for this event upon loss of adhesiveness. Nevertheless, the difference in robustness in the response was notable, and perhaps this could reflect some additional importance in suspended tumor cells. Taken with our observation that anoikis-potentiating HSF1 knockdown inhibited HSP27 expression (and other HSP), this provides further rationale to evaluate the possibility that HSP27 (related or unrelated to phosphorylation status) or other inhibited HSP could be pertinent to HSF1-mediated anoikis resistance.

4.5 Knockdown of HSP72, HSP40, HSP27, or HSP60 Did Not Sensitize H460 NSCLC Cells to Anoikis

Thus far, we have presented evidence that anoikis-inducing HSF1 depletion also inhibits HSP expression, including that of HSP27 which was robustly phosphorylated in suspended H460 cells. With that in mind, and since these HSP individually have not yet been directly evaluated for potential roles in anoikis resistance, we took an siRNA-based approach to determine whether inhibition of individual HSP would potentiate anoikis similar to that seen after HSF1 depletion in H460 cells. To determine whether HSP72,

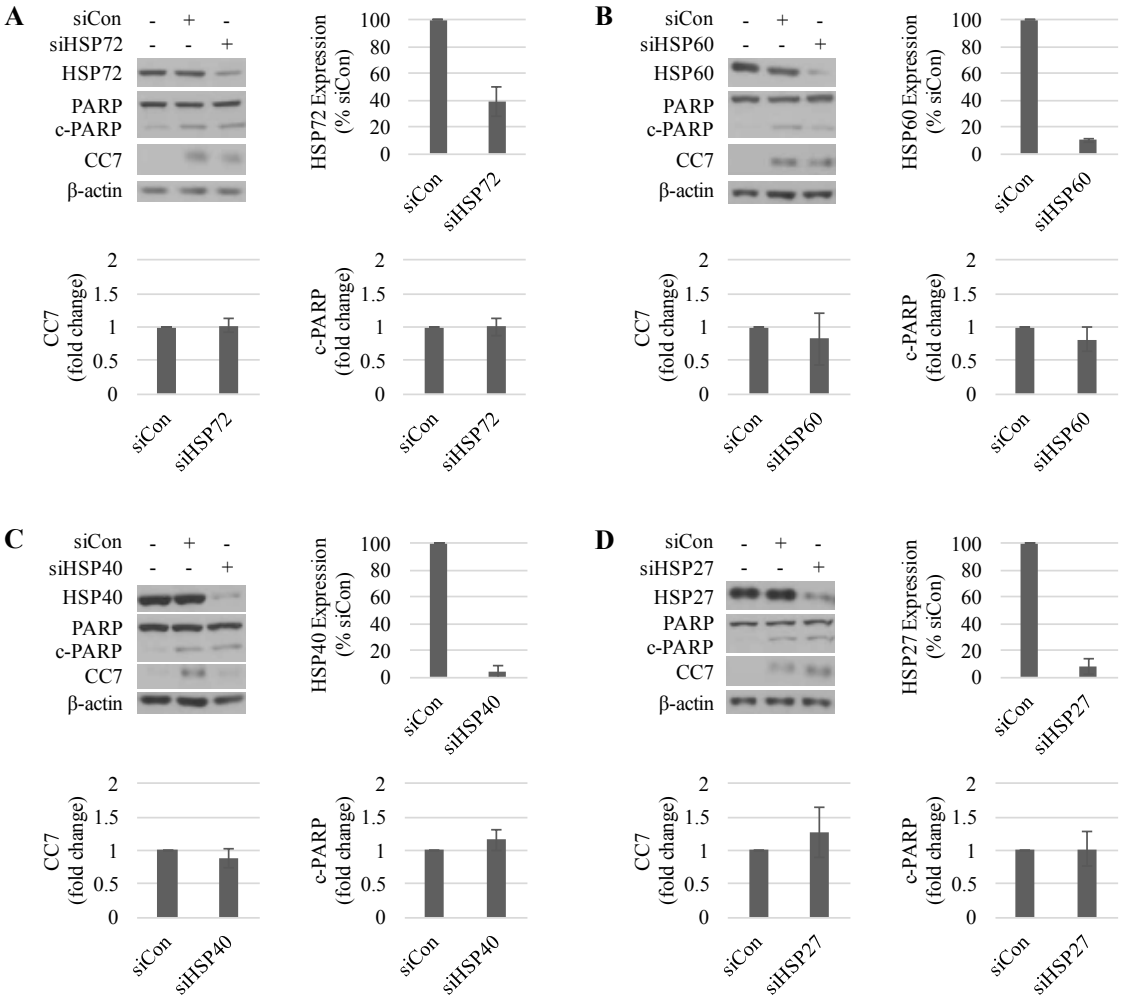

Figure 4.4. Knockdown of HSP72, HSP40, HSP27, or HSP60 did not induce anoikis similar to HSF1 knockdown in H460 cells. H460 cells were transfected for 48 h with siRNA targeting HSP72 *(A)*, HSP60 *(B)*, HSP40 *(C)*, or HSP27 *(D)*. An siRNA scrambled control (siCon) was included in each experiment. Cells were subsequently suspended on polyHEMA-coated dishes for 6 h. Western blots were performed to measure cleavage of caspase-7 (CC7) and PARP (c-PARP), and the knockdown efficiency of HSP72 *(A)*, HSP60 *(B)*, HSP40 *(C)*, and HSP27 *(D)*. β-actin was used as a loading control. CC7 (panels *A*-*C*) and HSP40 immunoblots are from a separate gel, with similar β-actin as shown above (compiled graphs use gel-specific loading control). Graphs show the densitometric analysis of western blots. Statistical significance was interpreted with Student's *t*-Tests (SD, n=4 (panels *A*, *C*, and *D*), n=3 (panel *B*)).

HSP40, HSP27, or HSP60 are necessary for H460 cell anoikis resistance, cells were transfected with a scrambled siRNA non-targeting control (siCon) or siRNA specifically targeting HSP72 (siHSP72), HSP40 (siHSP40), HSP27 (siHSP27), or HSP60 (siHSP60) prior to cell detachment. We previously showed that, in response to HSF1 knockdown in H460 cells, there was a reduction in HSP72 (47% reduction), HSP40 (59% reduction), and HSP27 (70% reduction) (Figure 4.2). Our siRNA-mediated knockdown of each these individual HSP (HSP72 – 61% reduction, HSP40 – 96% reduction, HSP27 – 92% reduction) was greater than that observed as a consequence of anoikis-potentiating HSF1 knockdown (Figure 4.4). As was done for HSF1 knockdown experiments, transfected cells were suspended on polyHEMA-coated plates for 6 h and induction of anoikis was evaluated by cleavage of caspase-7 (CC7) and PARP (c-PARP). Interestingly, suspension of H460 cells transfected with siHSP72, siHSP40, siHSP27, or siHSP60 had no significant induction of CC7 and c-PARP, compared to suspended cells transfected with the control siRNA (Figure 4.4). Preliminary experiments suggested increasing the suspension time to 24 h also did not increase anoikis after treatment with any siRNA (data not shown). No dissociation of aggregates or morphological characteristics of apoptosis were observed after treatment with any siRNA. No noticeable toxicity, assessed by caspase-7 and PARP cleavage, was observed in attached cells transfected with siHSP72, siHSP40, or siHSP60. It appeared that anchored, HSP27-depleted, cells grew at a slower rate (observational), and we observed some CC7 and c-PARP in these cells (data not shown). Perhaps HSP27 might promote proliferation or survival in attached H460 cells, but further work is needed to confirm this. Nevertheless, we clearly observed no difference in anoikis between suspended cells treated with control or anti-HSP27 siRNA.

In summary, the robust caspase-7 and PARP cleavage observed in H460 HSF1-depleted suspension cells was not mimicked by inhibition of HSP72, HSP40, HSP27, or HSP60 (a comparison to A549 cells – see section 4.6) to equivalent or lower levels than caused by HSF1 knockdown. Therefore, our data suggests that anoikis induced in H460 cells by HSF1 depletion is not a consequence of reduced expression of the individual HSP analyzed. Moreover, our evidence does not appear to point to HSP72, HSP40, HSP27, or HSP60 as being required for anoikis resistance in H460 lung carcinoma cells.

4.6 HSP60 Knockdown Increases Sensitivity of A549 NSCLC Cells to Anoikis

Similar to that observed in H460 cells, preliminary experiments suggested that knockdown of HSP40 or HSP27 did not lead to induction of anoikis in suspended A549 cells (Appendix Figure A.2.4). Previously, we observed only in the A549 cell line a slight increase in HSP60 expression in suspended cells (Figure 4.1). We also observed no significant effect on HSP60 expression upon HSF1 knockdown (Figure 4.2), indicating some other factor is primarily responsible for controlling its basal expression in tumor cells. Since HSP60 plays a role in the suppression of tumor cell apoptosis (Ghosh et al., 2008; Ghosh et al., 2010), we questioned whether HSP60 generally may play some role in anoikis resistance still, perhaps independent of HSF1. Indeed, we found that knockdown of HSP60 increased the anoikis sensitivity of A549 cells suspended for 24 h, evident by increased caspase-7 and PARP cleavage in these cells compared to the siRNA control (Figure 4.5). Suspended cells transfected with siHSP60 were also more dispersed than those transfected with siCon, and an increased number of cells displayed

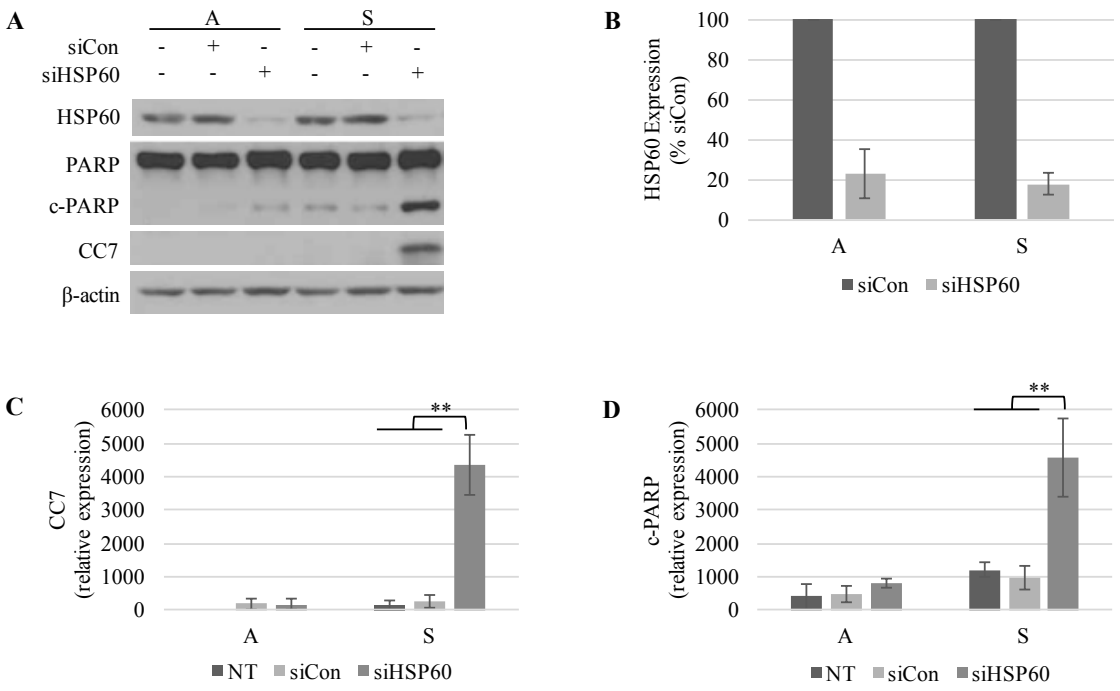

Figure 4.5. Knockdown of HSP60 increases the sensitivity of A549 cells to anoikis. *(A)* A549 cells were transfected with siRNA targeting HSP60 (siHSP60) or a scrambled control (siCon) for 72 h prior to suspension. Cells were subsequently suspended on polyHEMA-coated dishes for 24 h (A549). Lysates of suspended (S) cells and attached control (A) cells were harvested and western blots were performed to measure changes in HSP60 and cleavage of caspase-7 (CC7) and PARP (c-PARP). β-actin was used as a loading control. HSP60 and c-PARP immunoblots are from a separate gel, with similar β-actin as shown above (compiled graphs use gel-specific loading control). *(B/C/D)* Densitometric analysis of HSP60 knockdown efficiency *(B)*, CC7 *(C)*, and c-PARP *(D)*. Statistical significance was interpreted with Student's *t*-Tests (SD, ** $p<.01$, n=3).

morphological changes consistent with apoptosis. Minimal toxicity was observed in attached cells transfected with siHSP60. Therefore, we have identified for the first time that HSP60 supports increased anoikis resistance, as shown in A549 NSCLC cells, although the mechanism of this is unclear, and regrettably an expanded analysis of HSP60 mechanism could not be done for this dissertation.

4.7 Discussion

HSP are the most well-known targets of HSF1 transcriptional regulation and are known apoptosis suppressors. Therefore, we began investigating the mechanism of HSF1-mediated anoikis resistance by examining what role, if any, a variety of HSP might play in it. The experiments presented here provide new insight into the mechanism of HSF1-mediated anoikis resistance as it pertains to its canonical function as a master regulator of HSP. We previously identified that loss of cell adhesion was capable of triggering changes in HSF1 associated with its activation, and hypothesized this might be to regulate a stress-like HSP response to influence anoikis resistance or play some alternate role. Furthermore, we hypothesized that increased activation of HSF1 in malignant tumor cells might drive increased expression of targets that may prime cells to resist anoikis prior to ECM detachment.

We found that, despite increased markers of HSF1 activity in suspended H460 cells, the expression of five HSP canonically influenced by stress (i.e. HSP72, HSP40, HSP27, HSP60, and HSP90) were unaffected by suspension culture. Four of these HSP were similarly unaffected in suspended A549 cells, with the lone exception of HSP60, which was slightly upregulated (Figure 4.1). This suggests cell detachment does not induce a canonical HSP response, especially in H460 cells, but that HSP60 may have some role in anchorage-independence in A549 cells. Furthermore, it suggests that detachment-induced modulation of HSF1 may be important in the transcriptional regulation of some non-HSP target, in line with growing evidence that HSF1 runs a broader transcriptional program in cancer cells, beyond HSP (Mendillo et al., 2012). The precise reason for detachment-induced HSF1 activity in H460 cells remains elusive, and

it is unclear whether basal levels of HSF1 activation in these cells might be sufficient to promote anoikis resistance. We posit that detachment-induced changes in HSF1 activation may be a redundant mechanism of anoikis suppression, perhaps to respond to increased ROS production or altered glucose metabolism, which can be induced by cell detachment (Buchheit et al., 2012; Giannoni et al., 2008; Guadamillas et al., 2011; Kim et al., 2012; Paoli et al., 2013; Taddei et al., 2012).

A mechanism by which increased expression or activation of HSF1 in malignant tumor cells drives the expression of key anoikis effectors, which prime cells to resist anoikis prior to ECM disengagement, is supported by our evidence that HSF1 depletion appears to induce anoikis rapidly after detachment. Since HSP are frequently overexpressed in cancer (Calderwood and Gong, 2012; Calderwood et al., 2006; Ciocca and Calderwood, 2005; Dai et al., 2007; Mendillo et al., 2012), and HSF1 can, but does not always completely, drive HSP overexpression in cancer (Whitesell and Lindquist, 2009), we asked whether HSF1 knockdown would inhibit various apoptosis-modulating HSP. Indeed, HSF1 depletion significantly inhibited the expression of HSP72, HSP40, and HSP27 in both tumor cell lines (Figure 4.2). We observed a cell-type specific decrease in HSP90 in H460 cells, adding to evidence HSF1-HSP mechanisms in cancer can be specific to tumor type. Intriguingly, we observed minimal effect on HSP60 in either cell line, despite its detachment-induced upregulation in A549 cells, although this does not rule out that HSF1 might specifically control its detachment-induced changes. The partial inhibition of downregulated HSP indicates other transcription factors may be also be influencing their expression. One transcription factor previously identified as capable of regulating HSP is Signal Transducer and Activator of Transcription-1 (STAT-

1), which itself has been implicated in cancer (Stephanou et al., 1999; Stephanou and Latchman, 2011).

We furthermore observed that the phosphorylation-regulated, apoptosis- and cytoskeleton-modulating protein, HSP27, was robustly phosphorylated in suspended tumor cells, although the importance was unclear, as it was also moderately increased in BEAS-2B cells (Figure 4.3). Thus, both this event, and the downregulation of multiple anti-apoptotic HSP in HSF1-depleted cells suggested that the depletion of HSP might be important in the mechanism of HSF1-mediated anoikis resistance. In contrast to this, inhibition of HSP72, HSP40, or HSP27 to lower levels than observed in HSF1-depleted H460 cells did not similarly potentiate anoikis (Figure 4.4), and was supported by preliminary findings in A549 cells (Appendix Figure A.2.4). We observed no evidence indicating a compensatory mechanism, whereby knockdown of one HSP might affect the expression of another (data not shown). Ultimately, this data supports a model in which HSF1-mediated suppression of anoikis is independent of its role as a major regulator of HSP, and suggests HSF1 may regulate non-HSP targets to promote anoikis resistance in NSCLC (Figure 4.6). Furthermore, to the best of our knowledge, this is the first study to directly test whether inhibition of HSP can induce anoikis. Our data further implies that HSP72, HSP40, and HSP27 are not critical for suppressing anoikis in H460 cells in an HSF1-dependent or -independent fashion. While we suggest such an effect might be evident earlier, our preliminary results by observation and through apoptosis markers also indicated that extended suspension culture (>24 h) of HSP-depleted cells did not induce anoikis (data not shown). Nevertheless, we do not entirely rule out an HSF1-independent role for these HSP in H460 cell anoikis resistance, if we did not capture a more delayed

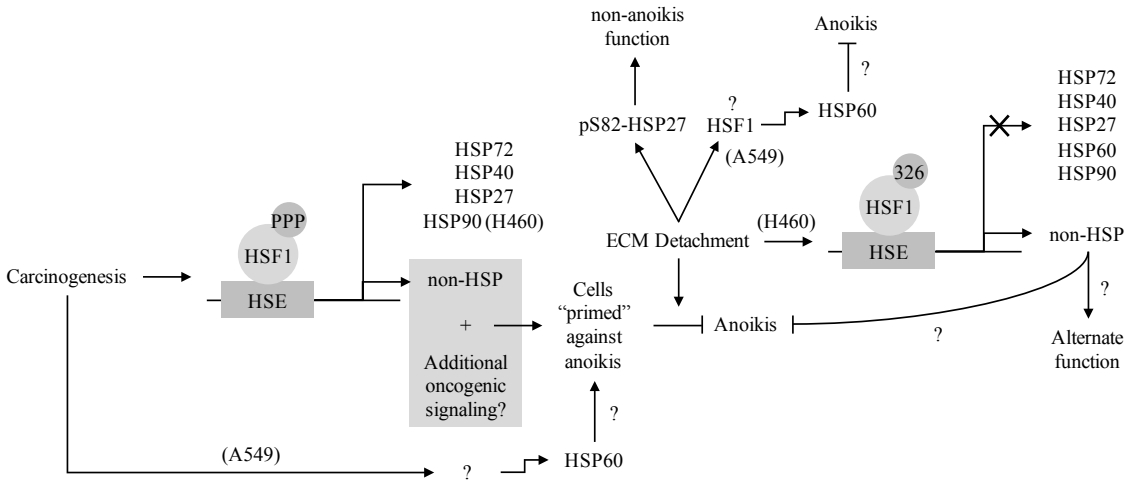

Figure 4.6. Hypothesis for HSP-independent HSF1-mediated suppression of anoikis in NSCLC. Schematic representing hypothesis that HSF1 promotes anoikis resistance by controlling basal and/or detachment-induced expression of non-HSP anoikis effectors, and summary of changes associated with cell detachment (post-translational modifications (PPP), pS326-HSF1 (S326)).

effect. However, our data does not support this, and suggests otherwise. In future studies, we propose complementing this work with additional toxicity assays over a longer time frame. We also note that our HSP72 knockdown was limited to approximately 60% inhibition, despite troubleshooting with multiple siRNA and targeting of both HSPA1A and HSPA1B, which produce nearly identical HSP72 protein. We find it unlikely given the lack of response, but caution that if the remaining intracellular HSP72 levels were sufficient to inhibit anoikis, the effect of HSP72-depletion could be masked. It would be ideal to identify an approach with stronger effects, either through siRNA or through genetic knockouts (i.e. CRISPR/CAS9). Importantly though, with respect to whether HSP72 is responsible for anoikis induction caused by HSF1 depletion, it was evident this was not the case, as our targeted inhibition of HSP72 was greater than that observed via HSF1 knockdown and did not induce anoikis. Furthermore, if a small pool of remaining

HSP72 could suppress anoikis, it would have been insufficient to overcome anoikis induced by HSF1 depletion, which only partially inhibited HSP72.

Our finding that HSP27 knockdown did not induce anoikis in tumor cells suggests its phosphorylation is an unrelated event. Perhaps phosphorylation of HSP27 reflects a role in the cytoskeletal changes taking place upon loss of matrix adhesion. It is well-established that HSP27 phosphorylation is modulated in response to stress via the p38MAPK-MAPKAPK2 pathway, and regulates actin filament dynamics (Clarke and Mearow, 2013; Katsogiannou et al., 2014; Paul et al., 2010; Sreedhar and Csermely, 2004). Indeed, our preliminary experiments suggest p38MAPK and MAPKAPK2 are also phosphorylated rapidly in tumor cells after cell detachment (data not shown). HSP27 function has been associated with the cytoskeleton specifically in NSCLC cells in response to various drugs (Chang et al., 2012; Kausar et al., 2013; Lee et al., 2010; Wang et al., 2017a). In H460 cells, photo-activated aloe-emodin caused cell death leading to multiple alterations in apoptotic machinery, reduced p38MAPK and HSP27 expression, and alterations to the actin cytoskeleton. Based on the cytoskeletal changes seen in attached cells, it was suggested this treatment induced anoikis (Chang et al., 2012; Lee et al., 2010), although whether HSP27 is specifically required for this was unclear. Our finding might suggest its role is either unrelated to apoptosis or is different in a drug-induced model. Stress-induced phosphorylation of HSP27 via P38MAPK pathway activation has been linked to cell adhesion and detachment (de Graauw et al., 2005; Du et al., 2010), but in what capacity appears to be unclear or different depending on the model. Notably, our “stressor” is cell detachment itself, and our experiments indicate cell detachment influences HSP27, as opposed to a stressor inducing changes in cell adhesion.

We cannot fully rule out that treating cells with trypsin to detach them might influence HSP27, as there is some evidence that trypsin can induce p38MAPK phosphorylation (Zhang et al., 2012). However, while our preliminary work indicated that pS82-HSP27 may be induced as early as 1.5 h (data not shown), we observed robust increases after 24 h in suspension, suggesting this is sustained, and likely not a transient effect due to trypsin. Interestingly, other reports have shown that HSP27 did not influence cell adhesion or detachment, but instead increased tumor cell invasion and proliferation (Voll et al., 2014). Perhaps the increased robustness of pS82-HSP27 in suspended tumor cells could foreshadow a different mechanism unrelated to anoikis, such as promoting invasiveness or proliferation during altered growth conditions. Notably, knockdown of HSP27 appeared to reduce proliferation in attached H460 cells (observational), which should be followed up on in future studies.

While both H460 and A549 cells are classified as NSCLC, they are also derived from different histological subtypes (H460 – large cell carcinoma, A549 – adenocarcinoma). While HSF1 was important for anoikis resistance in both cell types, we observed some cell-type specific differences, most notably regarding HSP60 in A549 cells. HSP60 expression was elevated in suspended A549 cells (Figure 4.1). Moreover, knockdown of HSP60 increased the sensitivity of A549 cells (Figure 4.5), but not H460 cells (Figure 4.4), to anoikis. Furthermore, HSF1 did not control the constitutive expression of HSP60 in either cell type (Figure 4.2). Whether HSF1 might be specifically involved in the detachment-induced regulation of HSP60 remains unclear. Therefore, if detachment-induced HSP60 upregulation is responsible for increased anoikis resistance in A549 cells, we cannot rule out HSF1 might be influential in this mechanism. However,

we have yet to establish whether the detachment-induced change is specifically required for anoikis resistance, or whether elevated expression of HSP60 in A549 cells is sufficient for this. In the latter case, the mechanism of action would appear to be independent of HSF1 (Figure 4.6). In future work, we propose measuring HSP60 expression in suspended HSF1 knockdown cells to determine whether detachment-induced upregulation is inhibited. If so, we further suggest identifying whether exogenous expression of HSP60 might prevent anoikis sensitivity due to HSF1 depletion. If not, it would be of interest to identify the regulator of constitutive HSP60 expression in these tumor cells, and the detachment-induced changes might represent a redundant or alternate function, as we have proposed for detachment-induced activation of HSF1.

HSP60 has been described as primarily a mitochondrial HSP, but functions for cytosolic HSP60 have surfaced, and both fractions can influence apoptosis (Cappello et al., 2008; Ghosh et al., 2008; Sreedhar and Csermely, 2004). For instance, depletion of HSP60 leads to cytochrome c release and cytosolic HSP60 sequesters pro-apoptotic BAX and BAK to prevent apoptosis (Kirchhoff et al., 2002). It would be interesting to examine the localization of HSP60 in attached and suspended cells, and which fractions are of importance for anoikis resistance. HSP60 expression also increases BCL-XL by inhibiting its proteasomal degradation (Shan et al., 2003), and plays a pivotal role in tumor cells by stabilizing the mitochondrial pool of survivin, and in restraining p53 to prevent BAX-dependent apoptosis (Ghosh et al., 2008). Furthermore, HSP60 complexes with HSP90 and TRAP-1 to inhibit cyclophilin D-mediated mitochondrial permeability transition and subsequent apoptosis induction in tumor cells (Ghosh et al., 2010). Therefore, there is past evidence to support a role for HSP60 in suppressing apoptosis in

tumor cells, and perhaps these may be potential mechanisms of action for HSP60 in A549 cell anoikis resistance.

In summary, we have provided novel insights into the mechanism of HSF1-mediated anoikis suppression in NSCLC cells by demonstrating its effect is largely independent of its canonical role as a master regulator of HSP. We also identified for the first time that HSP60 is beneficial for A549 NSCLC anoikis resistance, seemingly independent of HSF1. To the best of our knowledge, this is also the first study to directly examine whether HSP72, HSP40, HSP27, or HSP60 are required for cells to resist anoikis. Furthermore, we observed changes in HSP27 phosphorylation in detached cells, which appears to be unrelated to anoikis resistance. While the precise mechanism of HSF1 in protecting NSCLC cells from anoikis remains unknown, we have provided a solid start in elucidating this mechanism by examining the possible involvement of HSP in this process. Implementation of a large-scale analysis of gene expression influenced by HSF1 in attached and suspended NSCLC cells would be valuable in identifying possible non-HSP targets of it for this mechanism. Continuing to learn about HSF1- and HSP60-mediated mechanisms of anoikis resistance will help in understanding how they, or their targets, might be therapeutically manipulated for the purposes of metastasis suppression.

5. GENERAL DISCUSSION AND FUTURE DIRECTIONS

5.1 General Discussion

Anoikis resistance in malignant cancers is an attractive area of research that is yet in its early stages. An assortment of signaling pathways and effector molecules have been found to be important for acquisition and maintenance of anoikis resistance. The variety of pathways involved and cell-type specificity that has been observed with regard to anoikis resistance mechanisms presents a current challenge for therapeutic intervention. Yet, with the possible imprint of anoikis resistance on multiple stages of the metastatic process, the potential value of targeting these mechanisms to suppress metastatic dissemination is immense. Therefore, it is critical to identify anoikis mediators, evaluate their mechanisms, and search for commonalities in those mechanisms to provide better therapeutic avenues for the re-initiation of anoikis in metastatic tumor cells. Importantly, our work was conducted in NSCLC cells, a tumor type with which metastasis is associated with a less than 5% five-year survival rate in patients (Howlader et al.), highlighting the need to find new potential avenues for the therapeutic suppression of metastasis. Here, we identified HSF1 as a novel supporter of anoikis resistance in multiple NSCLC cell types and provided evidence to suggest its influence is not exerted through canonical HSR mechanisms or by driving basal overexpression of specific HSP in these tumor cells. Furthermore, we identified a new potential trigger for HSF1 activation in NSCLC, matrix detachment, which also largely reflected an HSP-independent response. Additionally, we show for the first time that HSP60 depletion can increase the sensitivity of A549 lung adenocarcinoma cells to anoikis, despite minimal

influence of HSF1 levels on its basal expression. These findings suggest that HSF1, and perhaps HSP60, are promising targets for anti-metastasis therapy, provide new leads to the field for possible anoikis effectors, and add to building evidence of HSP-independent HSF1 functions in cancer.

HSF1 was originally identified as a major stress-responsive transcriptional regulator of HSP, which promotes improved proteostasis and increased cell survival (Calderwood et al., 2010; Morimoto, 2011). Response to proteostatic imbalances due to genetic mutation or environmental stress is critical to the health of organisms, is an evolutionary relevant mechanism, and efforts continue to explore how HSP (e.g. HSP90) shapes evolutionary change and response to disease (Karras et al., 2017). Our understanding of HSF1 and HSP has also evolved dramatically, and we now know that it is relevant to the etiology of multiple diseases, having roles in cardiovascular disease, neurodegeneration and aging, and cancer (Calderwood et al., 2010; Dayalan Naidu et al., 2016). Unfortunately, HSF1 functions with striking duality when contrasting its influence in such diseases. With respect to neurodegeneration, aging, and cardiovascular disease, HSF1 plays a protective role. As a driver of chaperone proteins, HSF1 plays a major role in protecting against neurodegenerative diseases associated with aggregation of misfolded proteins, such as Alzheimer's, Parkinson's, Huntington's, and amyotrophic lateral sclerosis (ALS) diseases (Neef et al., 2011). With respect to aging, there is evidence that HSF1 can promote longevity (Hsu et al., 2003; Seo et al., 2013). HSF1 also plays a role in protecting cardiomyocytes from ischemia and protects against myocardial infarction (Zhang et al., 2011a; Zou et al., 2003). Therapeutically, HSF1-mediated heat shock improves the viability of transplanted stem cells for the purposes of intervention in

cardiac dysfunctions (Feng et al., 2014). Notably, the viability of transplanted stem cells is linked to anoikis induction (Lee et al., 2015; Taddei et al., 2012), and a potential area of future study may be evaluating whether HSF1-activating heat shock might also improve stem cell transplantation through anoikis suppression. Unfortunately, despite such positive roles for HSF1 in normal cellular functioning and fighting against various diseases, in cancer, these functions are hijacked and are utilized to the extreme detriment of the organism (Dai et al., 2007).

HSF1 is overexpressed and activated in multiple tumor types, is associated with the most aggressive malignant stages of cancer, is a major mediator of oncogenic signaling, and has influence on multiple key processes important for metastatic dissemination (Cui et al., 2015; Dai et al., 2007; Li et al., 2014b; Mendillo et al., 2012; Santagata et al., 2011). In some organisms, HSF1 can control up to 3% of the genome (Hahn et al., 2004), and we have learned that HSF1 regulates a wide variety of genes extending beyond chaperones in malignant cancers, distinct from canonical heat-shock mechanisms and related to an array of cellular processes (Mendillo et al., 2012). Previous work has demonstrated a link between HSF1 and anchorage-independent growth (Asano et al., 2016; Carpenter et al., 2017; Khaleque et al., 2005; Powell et al., 2016; Salamanca et al., 2014; Toma-Jonik et al., 2015), yet whether HSF1 exerts its effects through the modulation of anoikis resistance remains unclear. Our findings demonstrate a role for HSF1 in supporting NSCLC anoikis resistance, suggesting this is one mechanism by which HSF1 can promote anchorage-independence. This role is consistent with the ability of HSF1 to promote survival in tumor cells exposed to a variety of biological and clinical stresses (Jiang et al., 2015; Khaleque et al., 2005; Vydra et al., 2014). Further supporting

this, there is considerable overlap between HSF1-driven cellular processes in cancer and those that are manipulated by metastatic cancer cells to circumvent anoikis. In addition to apoptosis suppression, HSF1 can influence energy metabolism, EMT, autophagy, respond to oxidative stress and hypoxia, and mediate sustained oncogenic signaling through PI3K/AKT, MAPK, and other pathways (Ahn and Thiele, 2003; Dai et al., 2007; Desai et al., 2013; Gabai et al., 2012; Khaleque et al., 2005; Liu et al., 2016; Mendillo et al., 2012; O'Callaghan-Sunol and Sherman, 2006; Powell et al., 2016; Wang et al., 2006a). Many of these processes, and the sustained activation of signaling molecules that drive them, are also important for anoikis resistance (Buchheit et al., 2012; Guadamillas et al., 2011; Kim et al., 2012; Paoli et al., 2013).

Our findings suggest that HSF1 is needed early after cells detach from the ECM, as HSF1 knockdown induced robust induction of late-stage apoptotic markers as early 1.5-6 h post-detachment (Figure 3.3A, C, D; Figure A.2.2). Our experiments indicated the downregulation of individual HSP, whose basal expression were partially controlled by HSF1 in tumor cells sensitized to anoikis by HSF1 knockdown, were not responsible for the induction of anoikis (Figures 4.2 and 4.4). In at least one cell-type, H460 cells, we also observed detachment-induced modulation in pS326-HSF1 and HSE binding, two established markers of HSF1 activation (Figure 3.2C, D). HSF1 phosphorylation was rapid (within 30 min) (Figure 3.2C, D) and transient (Figure A.2.1A), reminiscent of a HSR (Figure A.2.1B), yet in these cells we found no corresponding changes in expression of five major HSP (i.e. HSP90, HSP72, HSP40, HSP60, and HSP27) (Figure 4.1). However, it is currently unclear whether detachment-induced changes leading to a transcriptional response could happen quick enough to blunt anoikis induction during the

time frame we observed in HSF1-depleted cells. Based on our findings, we propose that in malignant cells, HSF1 regulates the constitutive expression of a yet unknown anoikis effector, which may essentially prime cells to resist anoikis prior to ECM disengagement. Furthermore, detachment-induced HSF1 activation may act to stimulate redundant or parallel pathways to inhibit anoikis. Through either carcinogenesis-induced (i.e. either initiation, or progression toward a malignant phenotype) or detachment-induced mechanisms, our data suggests HSF1 acts to regulate targets in a manner largely independent of individual HSP (Figure 4.6 and Figure 5.1). HSF1 likely works in parallel with other oncogenic signals, as its expression and constitutive activation did not confer anoikis resistance to non-tumorigenic bronchial epithelial cells (Figure 3.4). Thus, while HSF1 was a necessary component for anoikis resistance in NSCLC cells, it may require cooperation with other proteins or coordination with parallel signaling pathways that are not active in normal cells. Since cell-type specificity is a frequent observation with respect to anoikis mechanisms, perhaps it is alternatively possible that different anoikis-sensitive cells have differing levels of dependency on HSF1.

We had sought to identify what targets HSF1 might regulate to influence anoikis resistance, and focused on understanding whether HSF1 may act in a canonical manner to regulate HSP, whose clientele include key pieces of apoptotic machinery utilized by anoikis, at multiple levels. Further, there are several pieces of evidence that also indirectly suggested the role of HSP in anoikis resistance (Beere, 2004; Sreedhar and Csermely, 2004). Further yet, despite the distinct transcriptional program of HSF1 in malignant cells (Mendillo et al., 2012), HSF1 can still regulate HSP, whose overexpression in cancer is well-appreciated (Ciocca et al., 2012). Indeed, we observed

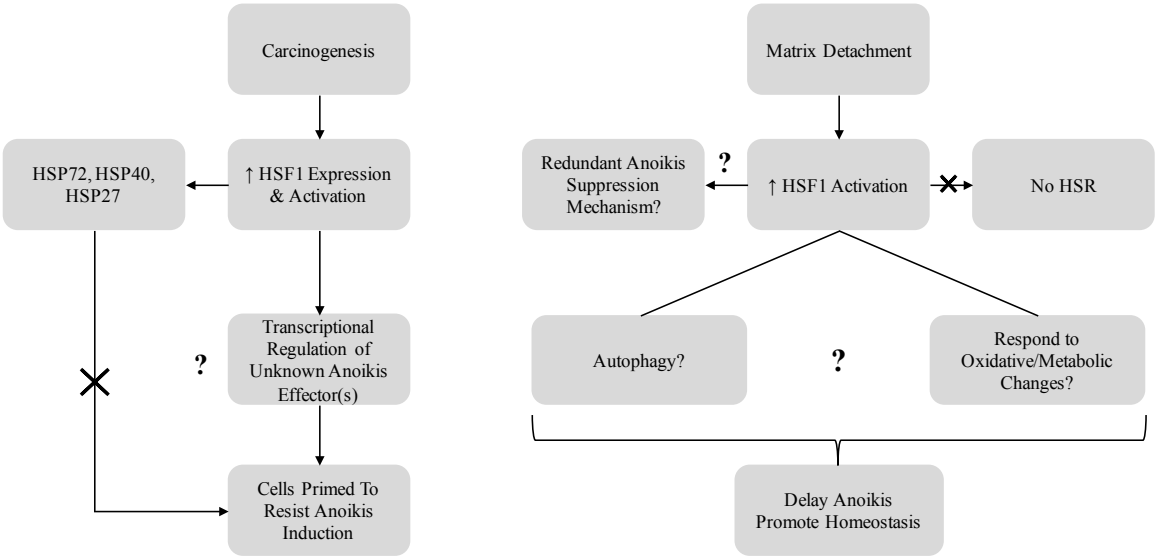

Figure 5.1. Hypothesis for HSF1-mediated anoikis resistance in NSCLC, highlighting some potential questions raised for future studies. HSF1 is overexpressed and activated in highly malignant lung cancer. Given the rapid induction of anoikis in HSF1-depleted cells, we hypothesize altered HSF1 activity in lung cancer cells leads to altered transcriptional regulation of a yet unknown anoikis effector to prime cells to resist anoikis prior to ECM disengagement. As depletion of individual HSP controlled by HSF1 in these cells did not induce anoikis, we posit HSF1 works through non-canonical mechanisms to support anoikis resistance. Furthermore, matrix detachment can modulate HSF1 in H460 cells, but does not induce a canonical HSR. Since HSF1 appears to be needed soon after matrix detachment, it remains unclear whether a detachment-induced transcriptional response could blunt anoikis during this time frame. However, there may be redundant or parallel mechanisms by which these cells resist anoikis. Autophagy and oxidative and metabolic changes are associated with anoikis resistance and are linked to HSF1 through various mechanisms. We pose the questions for future research, might HSF1 be modulated to respond to detachment-induced oxidative or metabolic changes, or to promote autophagy, in an effort to improve cell survival and homeostasis when under anchorage-independent conditions?

partial inhibition of HSP72, HSP40, and HSP27 in both tumor cell lines sensitized to anoikis upon HSF1 knockdown (Figure 4.2). Consistent with HSP expression in cancer not always correlating with HSF1 expression or activation or being HSP- and tumor-specific (Whitesell and Lindquist, 2009), we observed cell-type specific changes (HSP90), and HSP60 was largely unaffected by HSF1 knockdown. Ultimately, as we have discussed, none of our data indicated the involvement of the evaluated HSP in HSF1-mediated anoikis resistance. Therefore, our data suggests that HSF1 regulates either a chaperone protein we have not evaluated or a non-HSP target to support anoikis resistance.

HSF1 can regulate targets in both an activating and repressive fashion (Mendillo et al., 2012; Wang et al., 2006a). We suspect HSF1 may drive the upregulation of anti-anoikis, or repression of pro-anoikis, effectors in a manner that may prime cells for anoikis resistance before ECM disengagement, and lead to their sustained expression when cells are detached. Therefore, depletion of HSF1 may result in loss of anti-anoikis, or upregulation of pro-anoikis effectors, permitting rapid and unimpeded activation of anoikis when cells are detached. HSF1 is a major mediator of oncogenic signaling through PI3K/AKT and MAPK pathways (Dai et al., 2007; Khaleque et al., 2005; Li et al., 2014a; Meng et al., 2010; O'Callaghan-Sunol and Sherman, 2006; Schulz et al., 2014), which are highly involved in anoikis resistance (Grossmann, 2002; Paoli et al., 2013; Taddei et al., 2012), and it could exert its effects by mediating these pathways. In terms of potential downstream targets for HSF1, one possibility could be XAF1, a repressor of the inhibitor of apoptosis XIAP. XAF1 is transcriptionally repressed by HSF1 (Wang et al., 2006a), and has been implicated in complex mechanisms regulating

autophagy and anoikis in A549 cells (Cai et al., 2016). HSF1 is also known to transcriptionally repress TNFα (Singh et al., 2002), IL-1β (Cahill et al., 1996), and c-fos (Xie et al., 2003), which have been implicated previously in apoptosis mechanisms (Friedlander et al., 1996; Preston et al., 1996; Varfolomeev and Ashkenazi, 2004; Wajant et al., 2003). HSF1 positively regulates the transcription of BAG3, an apoptosis suppressor linked to cancer, metastasis, cell-matrix adhesion, and priming of AT-101-treated glioma cells to anoikis (Antonietti et al., 2017; Chiappetta et al., 2014; Jacobs and Marnett, 2009). Through ChIP-Seq and microarray analyses, an array of non-HSP HSF1 targets were identified, including some involved in regulation of apoptosis, adhesion and the ECM, energy metabolism, and other processes (Mendillo et al., 2012). Alternatively, perhaps HSF1 could influence other apoptotic targets, which have not yet been identified as regulated by HSF1. For example, BIM-mediated apoptosis is induced in response to endoplasmic reticulum stress, which was blunted by heat shock independent of HSP70 (Kennedy et al., 2014), and it would be of interest to evaluate whether HSF1 might influence the expression of BIM, a major anoikis activator. In summary, our data indicating that HSF1 supports NSCLC anoikis resistance through non-canonical mechanisms offers a wealth of opportunities for future study into possible targets for HSF1 in this mechanism.

Detachment-induced changes can delay and control the timing of anoikis, and in tumor cells, help tip the balance of pro- and anti-apoptotic signaling toward cell survival (Liu et al., 2006). We have yet to identify whether the detachment-induced changes in HSF1 phosphorylation and HSE binding are specifically involved in anoikis resistance in H460 cells. Presently, our experimentation potently depleted HSF1, rather than inhibiting

only these detachment-induced changes. Regrettably, we could not assess changes in detachment-induced HSE binding in A549 cells, but did not observe changes in pS326-HSF1. An uncoupling between HSF1 phosphorylation and its stress-induced activation was recently described, and it was suggested that PTMs might represent a signature to "fine-tune" transcriptionally competent HSF1 and possibly direct what transcriptional program is enacted (Budzyński et al., 2015). In this context, we cannot entirely rule out that S326 is unnecessary for HSF1 activation in detached A549 cells, and that detachment-induced activation is a common feature in both of our NSCLC cell lines. Future work should be done to measure HSE binding in these cells. In either case, at least in H460 cells, it is possible that HSF1 modulation by detachment reflects a parallel or redundant mechanism to respond to detachment-induced changes or mechanistic changes that support anoikis resistance (Figure 5.1). For instance, cell detachment can lead to changes in ROS levels, glucose metabolism, and the induction of autophagy, all of which are linked to anoikis resistance, (Buchheit et al., 2012; Fung et al., 2008; Giannoni et al., 2008; Guadamillas et al., 2011; Kim et al., 2012; Mason et al., 2017; Paoli et al., 2013; Taddei et al., 2012; Yang et al., 2013). HSF1 is known to respond to oxidative stress (e.g. hydrogen peroxide)(Ahn and Thiele, 2003) and to modulate glucose metabolism in tumor cells (Dai et al., 2007). HSF1 is also known to transcriptionally regulate the autophagy mediator, ATG7, shown to be important for bypassing anoikis (Desai et al., 2013; Fung et al., 2008). Some evidence also suggests that cell detachment might also further promote EMT in lung (Chunhacha et al., 2013) and breast cancer cells (Ryu et al., 2011), and EMT is associated with increased anoikis resistance (Frisch et al., 2013; Paoli et al., 2013). HSF1 has been shown to transcriptionally regulate the EMT regulator SLUG

(Carpenter et al., 2015), and HSE have been located in the promoters of *SNAIL*, *ZEB*, and *TWIST1* EMT regulators (Powell et al., 2016). Therefore, detachment-induced HSF1 activation could perhaps be related to at least four major processes involved in the acquisition of anoikis resistance, and our findings provide many new opportunities for the study of HSF1 mechanisms with respect to anoikis resistance.

Interestingly, we observed a slight, cell-type specific, increase in HSP60 expression in suspended A549 cells (Figure 4.1). Basal HSP60 expression was not significantly influenced by HSF1 knockdown (Figure 4.2). Intriguingly, our evidence suggests for the first time that loss of HSP60 can increase anoikis sensitivity, in A549 lung adenocarcinoma cells, leading to increases in apoptosis markers. As reduced HSF1 levels do not significantly influence HSP60 expression, this is not likely to be a mechanism by which HSF1 influences anoikis resistance. Furthermore, knockdown of HSP60 in H460 cells did not lead to induction of anoikis. Nonetheless, we cannot rule out a mechanism by which HSP60 induction is controlled by HSF1 specifically in suspended cells. It is presently unclear whether detachment-induced changes, as opposed to basal overexpression of HSP60, plays a role in this mechanism. We noted individual replicates with less clear HSP60 induction had comparable increases in apoptotic markers when HSP60 was knocked down, perhaps indicating basal HSP60 levels are sufficient to aid in anoikis resistance. Furthermore, other transcription factors could be involved in this mechanism by maintaining the expression of HSP60 in these cells. Regrettably, we were unable to expand on this analysis during the completion of this dissertation. However, in either case, HSP60 has been previously shown to inhibit mitochondrial-mediated apoptosis in tumor cells by stabilizing mitochondrial survivin, restraining p53, and

inhibiting mitochondrial permeability transition (Ghosh et al., 2008; Ghosh et al., 2010), and such mechanisms could perhaps be at play here.

Notably, we also observed the detachment-induced phosphorylation of HSP27 in both tumor cell lines, as well as BEAS-2B cells to a lesser extent. Since HSP27 depletion did not induce anoikis, this event likely does not influence anoikis resistance. HSP27 phosphorylation regulates its function, often through p38MAPK-MAPKAPK2 signaling, including its influence of actin filament dynamics (Clarke and Mearow, 2013; Katsogiannou et al., 2014; Paul et al., 2010; Sreedhar and Csermely, 2004). Our preliminary work suggests p38MAPK and MAPKAPK2 are also phosphorylated in suspended NSCLC cells (data not shown), perhaps implicating this pathway. Indeed, links between cell adhesion, cell detachment, and HSP27 phosphorylation have been made, although the mechanisms may differ in various models (de Graauw et al., 2005; Du et al., 2010). Interestingly, our work suggests cell detachment is itself capable of triggering such a change, in contrast to reports studying the reverse. It is therefore possible that phosphorylation of HSP27, through the canonical p38MAPK-MAPKAPK2 pathway, is involved in the cytoskeletal rearrangements that would inevitably happen as cells round upon detachment. Notably, other literature indicates that HSP27 can promote invasiveness and proliferation, while not affecting cell adhesion or detachment (Voll et al., 2014). Our experiments do indicate that phosphorylation of HSP27 remains elevated after 24 h in suspension, and perhaps the increased robustness of this effect in suspended tumor cells might also foreshadow its role in those processes, or in cell migration.

5.2 Future Directions

Based on our findings, we propose a number of future studies. It would be of tremendous value to conduct a microarray analysis to identify what changes in gene expression HSF1 may be driving in attached or suspended cells. We have begun conducting some preliminary studies to evaluate the expression of common BCL-2 family members (e.g. BIM) in response to HSF1 knockdown, and continued work in this area would be of value to better understand HSF1 mechanism in these cells. Evaluation of transcript and protein levels of prospective HSF1 regulatory targets such as XAF1 and BAG3, in HSF1 knockdown cells, could provide further mechanistic insight. Furthermore, in an effort to understand whether HSF1 may be a common regulator of anoikis across multiple cell and tumor types, we propose expanding our analysis to other types of metastatic lung cancer cells, and multiple other tumor types. Moreover, using additional anoikis-sensitive cells to conduct our HSF1 gain of function analyses might be useful. Additional models, such as the application of a *RAS*-transformed wild-type and knockout *HSF1* MEF model, or CRISPR/CAS-9 knockout models, could also be valuable tools to improve our analysis.

We also propose some specific future experiments that could expand upon and improve our analysis. Through site-directed mutagenesis, we propose generating siRNA-resistant expression constructs for wild-type, active, DNA-binding, and S326 phosphorylation (alanine and phosphomimetic) mutant variants. Such constructs will help identify what role each of these HSF1 activation events plays in HSF1-mediated anoikis resistance by enabling us to perform early time-frame rescue experiments in tumor cells with depleted endogenous HSF1. While there was some indication that HSF1 activation

might prolong extended anoikis resistance (>48 h), this was inconclusive. Further, our approach may have been limited to a variable fraction of cells undergoing anoikis, and possibly skewed by alternate apoptotic mechanisms being activated by these times. The proposed approach will provide a more direct understanding of HSF1 mechanisms during the time frame that HSF1 appears to be most important. Understanding whether detachment-induced changes are specifically important for anoikis resistance in our model will also be useful. Perhaps titrating dominant-negative S326A or DNA-binding mutants in cells that still have endogenous HSF1 might reduce the proportion of HSF1 phosphorylated at S326 or bound to HSE in response to detachment by competing with endogenous HSF1, providing further insight into their importance.

Future evaluation of mechanisms associated with detachment-induced HSF1 would be highly beneficial in understanding the relevance of these changes with respect to anchorage-independence. As we have outlined, there is considerable overlap between detachment-induced intracellular changes and what changes HSF1 responds to. We propose evaluating whether HSF1 is activated in response to detachment-induced ROS production or changes in glucose regulation. Furthermore, we propose evaluating whether cell detachment might lead to HSF1 regulation of autophagy effectors, such as ATG7, to induce autophagy and provide a parallel mechanism to bypass anoikis. Lastly, measuring detachment induced changes in EMT-related transcription factors that can be regulated by HSF1 (SLUG), or contain HSE (*SNAIL*, *ZEB*, *TWIST1*), could reveal new mechanisms by which cell detachment could lead to the enhancement of EMT, and consequently, anoikis resistance.

Some studies have selected for highly anoikis-resistant subpopulations through varying protocols, enabling a comparison of these and parental cells (Godin-Heymann et al., 2016; Halim et al., 2012). While studying the importance of cell aggregation in suspended cells, we too generated such a population (H460-S) (Figure 3.1A), and preliminary work indicated they were similarly affected by HSF1 depletion (data not shown). Future comparison between these and parental H460 cells might provide some added analytical benefit. Notably, we selected for single or small clusters of cells by passing them through a cell strainer after each successive cycle of attached and suspension culture, and these cells aggregate considerably less in suspension (data not shown). Generating a second cell line without straining the cells could also be useful to assure we have not introduced another variable into the comparison. With respect to this topic, anoikis resistance baselines between studies can be variable, likely reflecting variation in assay selection, whether they solely measure cell death, at what stage, and if anoikis-sensitive cell lines are used for comparison. Multiple reports generally agree with our baseline assessment (Wei et al., 2001; Wei et al., 2004; Yao et al., 2014; Zhang et al., 2010).

With respect to our HSP60 findings, future studies should be done to measure the expression of HSP60 in suspended HSF1 knockdown cells, enabling us to identify whether HSF1 is responsible for detachment-induced expression of HSP60. If so, expressing HSP60 in HSF1-depleted A549 cells would be a useful experiment to determine whether HSF1 promotes anoikis resistance in these cells through this mechanism. Whether dependent or independent of HSF1, further evaluation of the mechanism of HSP60-mediated anoikis resistance is warranted. Previous reports show

that HSP60 primarily influences apoptosis through modulation of the mitochondria (Ghosh et al., 2008; Ghosh et al., 2010; Kirchhoff et al., 2002). We propose future studies to interpret changes in BCL-2 family targets such as BAX or BAK, survivin, and general evaluation of apoptosis-related mitochondrial changes, as a starting point for the elucidation of the HSP60 anoikis-suppressing mechanism.

Future studies into the role of HSP27 phosphorylation in suspended cells should also be conducted. Our preliminary evidence implicates the p38MAPK-MAPKAPK2 pathway. This work should be confirmed and elaborated on using inhibitors of these targets to determine whether this pathway leads to the phosphorylation event we observed. Evaluating changes to the actin cytoskeleton through fluorescent microscopy would be of interest to better understand how HSP27 could be influencing cytoskeletal rearrangement consequent to cell detachment. Studies could also be conducted to determine whether HSP27 promotes migration or invasiveness in these cells.

We also suggest using alternate viability or toxicity assays moving forward, such as the Cell Death Detection ELISA Assay (Sigma-Aldrich). The dye-based assay we used in some instances, relied on entry of the compound into cells, and there was some undesirable variability and possibly some reduced assay sensitivity. Multicellular aggregation resulting from suspension culture provides a challenge for use of common cytotoxicity (or proliferation/viability) assays, as variation or limitation in access to cell permeable, or impermeable, dyes occurs. Adding centrifugation and cell dissociation steps prior to time course readings can lead to variability, particularly if cells are stressed or in later stages of cell death. The Cell Death Detection ELISA quantitates DNA

fragmentation and the release of histones from the nucleus to the cytoplasm during apoptosis, and use of this could also limit such variables.

Cell aggregation can promote increased proliferation and anoikis resistance (Rayavarapu et al., 2015; Zhang et al., 2010), and cluster formation is physiologically relevant to metastatic dissemination, but such characteristics are not always discussed in anoikis-related literature. CTC can travel both as single cells or in cell clusters, and CTC clusters may be more efficient at seeding metastatic colonies, in part, due to increased apoptosis resistance (Lambert et al., 2017). Interestingly, the comparative size or nature of multicellular aggregates formed by different lung cancer cell lines did not correlate to their anoikis sensitivity (Zhang et al., 2010), yet the underlying cell-cell contact seems to increase anoikis resistance. An H460 subpopulation selected by growing cells to overconfluency and collecting and passaging only floating cells, appears to grow primarily as single cells or in small clusters (Godin-Heymann et al., 2016), unlike parental H460 cells based on our, and others (Zhang et al., 2010), observations. Our observations that our selected H460-S cell line tended to have comparatively higher anoikis resistance (Figure 3.1A), and markedly reduced aggregation when suspended (observational, data not shown), is generally supportive of this concept. We suggest increased focus should be placed on whether cell aggregation is influential in various anoikis resistance models, and that this should be carefully considered when selecting viability or toxicity assays. Furthermore, signaling mechanisms associated with aggregate formation will be important to better understand how this event influences anoikis resistance. During this doctoral work, we have begun evaluating such mechanisms with respect to our model, and this work would be of benefit to continue moving forward.

In summary, we have proposed future studies that aim to ascertain whether HSF1 may be a mediator of anoikis resistance in multiple tumor types, to evaluate what transcriptional targets it may be regulating under attached or suspended conditions, to identify what steps are important for its detachment-induced activation, and to examine whether HSF1 might regulate anoikis resistance in a multifaceted way to respond to detachment-induced intracellular changes. Furthermore, we have proposed further evaluation of HSP60 anoikis resistance mechanisms based on its known anti-apoptotic functions related to mitochondrial-mediated apoptosis. Elucidation of HSF1- and HSP60-related mechanisms that can contribute to anoikis resistance will be of importance in learning how these targets might be manipulated therapeutically to suppress metastasis.

5.3 Therapeutic Relevance

It is becoming increasingly evident that inhibiting HSF1 as an anti-cancer and anti-metastasis strategy may have significant potential. HSF1 is dispensable for the survival and growth of non-malignant cells during non-stressed conditions (Dai et al., 2007; de Thonel et al., 2011; Heimberger et al., 2013), and while *HSF1*(-/-) mice have some developmental deficiencies, they live to adulthood without adverse outcome under normal living conditions (Dai et al., 2007; Xiao et al., 1999). In primary human cells, knockdown of HSF1 has little effect on cell survival and proliferation, yet is highly detrimental to multiple premalignant and malignant human cancer cell lines, through similar mechanisms and despite genetic diversity (Dai et al., 2007). HSF1 influences cancer at multiple stages, enabling tumor initiation, maintaining tumor growth and survival, being associated with advanced malignant states, and supporting metastasis at

multiple steps of the cascade (Dai et al., 2007; Khaleque et al., 2005; Mendillo et al., 2012; O'Callaghan-Sunol and Sherman, 2006; Scott et al., 2011). Based on our findings, this also includes supporting anoikis resistance, one mechanism that enables anchorage-independence, allowing for successful transit during metastatic dissemination. Furthermore, a number of studies have provided evidence that HSF1 may also increase the effectiveness of other therapeutics, such as cisplatin or HSP90 inhibitors (de Thonel et al., 2011; Whitesell and Lindquist, 2009). Therefore, targeting HSF1 has the potential to provide significant advantages such as having minimal effects on the growth and survival of normal cells, simultaneously effecting multiple steps in the onset, progression, and spread of cancer, coordinating similar programs across diverse genetic backgrounds, and improving efficacy of drugs that might be used in combinatorial therapy. Identifying that HSF1 exerts its effect on anchorage-independence, at least in part, by supporting anoikis resistance, provides new and valuable information about how targeting HSF1 may lead to cancer cell death and the suppression of metastasis. Furthermore, it will be valuable to develop a better understanding of whether the anoikis program might need to be bypassed during the stages of metastasis flanking circulatory system transit, during which transient or improper connection to the ECM is likely to occur.

Despite the clear potential value of targeting HSF1 as an anti-cancer and anti-metastasis therapy, significant consideration will need to be placed on understanding the potential unintended negative impacts. HSF1 plays significant roles in protecting against neurodegenerative diseases such as ALS, Parkinson's, Huntington's, and Alzheimer's disease by preventing protein aggregation as a consequence of a buildup of misfolded proteins (Neef et al., 2011). Furthermore, HSF1 has been linked to mechanisms that

support longevity (Hsu et al., 2003; Seo et al., 2013) and protect against cardiac injury related to ischemia (Zhang et al., 2011a; Zou et al., 2003). Identification of HSF1 as a supporter of anoikis resistance could also spur research efforts to identify whether HSF1 activation can improve viability of human embryonic or mesenchymal stem cells during cell transplantation through anoikis suppression (Lee et al., 2015; Taddei et al., 2012). Indeed, some evidence indicates HSF1-mediated heat shock improves the viability of transplanted stem cells used to intervene in cardiac dysfunctions (Feng et al., 2014), although whether this could be, in part, due to anoikis resistance, has not been established. Furthermore, whether such treatment would increase the incidence of cancer is unknown, and will be critical to determine. It will be critical to unravel mechanisms associated with how HSF1 controls each process, so that we might better devise strategies to target HSF1 for the purpose of anti-cancer and anti-metastasis therapy, without simultaneously increasing risk of neurodegenerative or cardiac diseases, or reduced aging/life span. Adjusting the treatment length or approach, or designing HSF1 inhibitors that do not cross the blood-brain barrier, may be methods to try and limit certain negative effects (de Thonel et al., 2011; Whitesell and Lindquist, 2009). Furthermore, as HSF1 inhibition as an anti-cancer strategy progresses, how treatment affects such mechanisms *in vivo*, and particularly in clinic, will be of critical importance to closely evaluate and report.

Therapeutic targeting of HSF1 for these purposes is still in early stages of evaluation, but there is an expanding number of HSF1-modulating compounds being identified. This is perhaps less a reflection of a lack of possibility, but more so of the relatively recent and rapid increase in our understanding of HSF1 importance in cancer,

and a need to dedicate more resources to this effort. Currently, a variety of compounds have shown some ability to inhibit HSF1 or HSP induction (e.g. quercetin, KNK437, etc.); however, questions remain about their specificity (de Thonel et al., 2011; Whitesell and Lindquist, 2009). The small molecule inhibitor, triptolide, is a potent HSF1 inhibitor that prevents HSF1 transactivation through unknown, possibly non-specific, mechanisms (de Thonel et al., 2011; Heimberger et al., 2013; Whitesell and Lindquist, 2009). Many questions remain regarding the complex mechanisms of HSF1 activation, as well as how HSF1 regulates such a diverse program in malignant cells, but small molecule inhibitors continue to be evaluated for their possible use to target HSF1.

HSP60 has also been increasingly implicated in tumor cell survival, including by our findings, and is becoming an appealing target for possible anti-cancer therapy (Cappello et al., 2008; Ghosh et al., 2008). Our work indicating depletion of HSP60 can sensitize certain tumor cells to anoikis, possibly through an HSF1-independent mechanism, adds to this evidence, and warrants further study. To date, however, not much information regarding specific HSP60-targeted anti-cancer therapies appears to be available, aside from general inhibitors of HSP synthesis. Identification of specific HSP60 inhibitors and continued evaluation of HSP60 mechanisms in tumor cell apoptosis will be important for its potential advancement as an anti-cancer or anti-metastatic therapeutic option.

In conclusion, revealing mechanisms associated with NSCLC metastasis is important, as patients with NSCLC have a less than 5% five-year survival rate. Our work builds on evidence that HSF1 supports metastasis by identifying that it supports anoikis resistance in NSCLC, a key enabling feature for anchorage-independence and metastatic

dissemination. Furthermore, we have identified matrix detachment as a potential trigger for HSF1 activation in NSCLC. Both of these mechanisms appear to be largely independent of HSP. Furthermore, we revealed new information indicating HSP60 can support anoikis resistance in A549 lung adenocarcinoma cells, perhaps independent of HSF1. These findings suggest that HSF1, and perhaps HSP60, are promising targets to evaluate for the purposes of suppressing metastasis. Furthermore, our work provides significant new leads to pursue for the study of anoikis resistance, consequent to the large transcriptional program HSF1 orchestrates, and adds to building evidence of HSP-independent HSF1 functions in cancer.

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APPENDIX

A.1 Investigation of HSP90 Inhibition and Dual Targeting of HSP90 and HSF1 in Attached and Suspended H460 Cells

Our previous experiments indicated that HSP90 expression is partially reduced by HSF1 depletion in H460, but not A549, cells. While we suspect that HSF1 exerts its influence on anoikis by regulating a common target in both cell types, we cannot rule out that the partial reduction in HSP90 might contribute to increased anoikis sensitivity in HSF1-depleted H460 cells. Evaluating this response can be challenging due to the relationship of HSP90 and HSF1 under normal, non-stressed conditions. A variety of HSP90 inhibitors including the geldanamycin-derivative, 17AAG, have been evaluated in clinical trials (Usmani et al., 2009). As we and others have observed, because HSP90 sequesters HSF1 in an inactive state, HSP90 inhibition often leads to activation of HSF1 (Kim et al., 2015). Simultaneously, HSP90 inhibition results in the degradation of multiple HSP90 clientele, including a variety of oncogenic proteins (Ciocca et al., 2012). Here, we have conducted some preliminary experiments to begin to understand whether inhibition of HSP90 might contribute to anoikis potentiation in HSF1-depleted cells.

To inhibit HSP90 functionality, we optimized treatment of H460 cells with 17AAG over a range of times and concentrations by evaluating the expression of AKT, a major client that is stabilized by HSP90 (data not shown). Treatment of H460 cells with 0.25 μM 17AAG for 24 h significantly reduced AKT expression, and there were signs of increased HSF1 activation evident by elevated expression of HSP90 (Figure A.1.1A-B) and apparent increases in HSP72 (Figure A.1.1C, E). Furthermore, we had previously

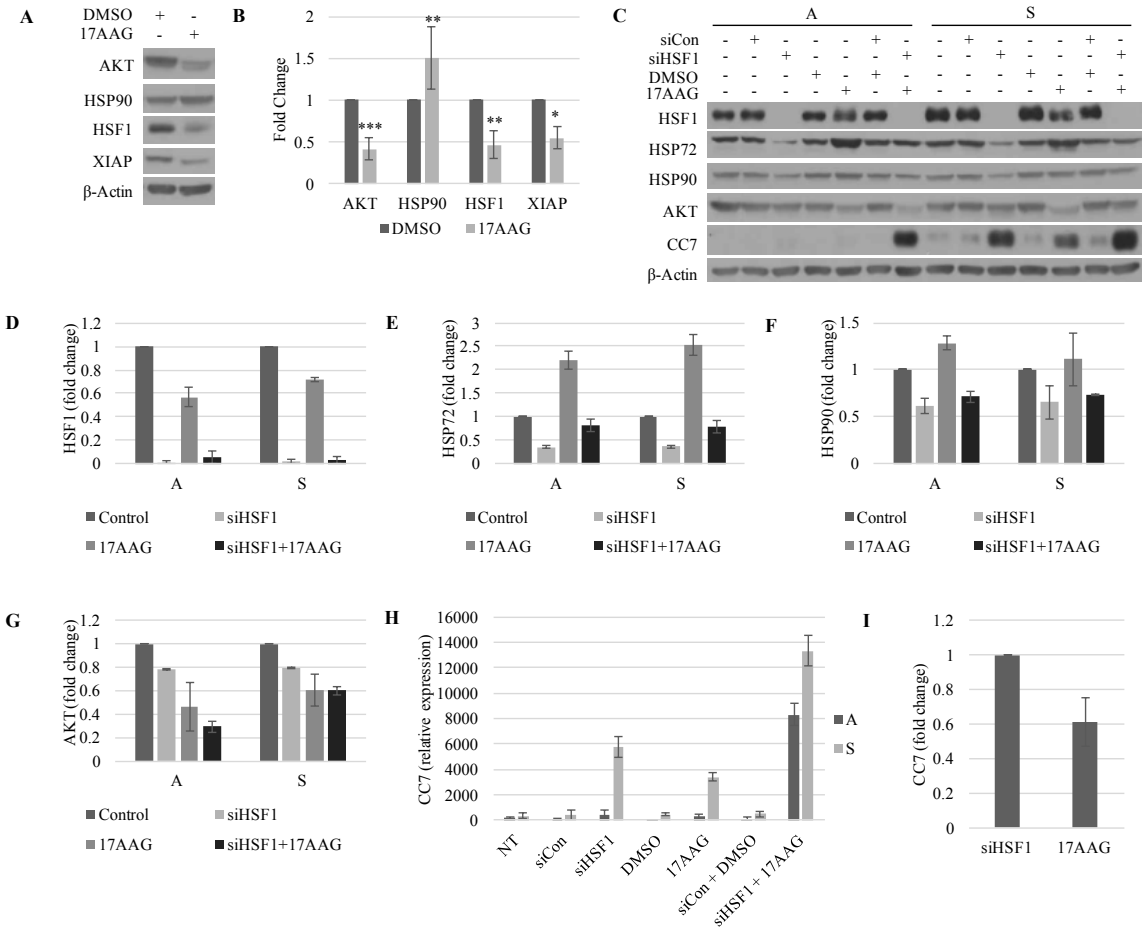

Figure A.1.1. Investigation of HSP90 inhibition and dual targeting of HSP90 and HSF1 in attached and suspended H460 Cells. (A) H460 cells were treated with 0.25μM 17AAG or DMSO (0.1%) for 24 h. Western blots were performed to measure expression of pan-AKT, HSP90, HSF1, and XIAP. (B) Densitometric analysis of western blots in panel A. Statistical significance was interpreted with Student's *t*-Tests (SD, n=10 (AKT/HSP90), n=5 (HSF1), n=3 (XIAP), *p<.05, **p<.01, ***p<.001). (C) H460 cells were transfected with a control siRNA (siCon) or siRNA targeting HSF1 (siHSF1) for 48 h. After 24 h transfection with siRNA, indicated samples were treated with 0.25μM 17AAG or DMSO (0.1%) for 24 h. Cells were then suspended on polyHEMA-coated culture dishes for 6 h. Western blots were performed to measure expression of HSF1, AKT, HSP72, HSP90, and cleaved caspase-7 (CC7). Blots are representative of two independent experiments. (D-I) Densitometric analysis of western blots in panel A for HSF1 (D), HSP72 (E), HSP90 (F), AKT (G), and CC7 (H – relative expression; I – direct comparison of siHSF1 and 17AAG). Error bars are the range of the two replicates.

used 17AAG as a positive control to induce binding of an HSE-driven reporter. Dual targeting of cells with an HSF1 siRNA and 17AAG appeared to offset changes in HSP72, further indicating HSF1 can mediate 17AAG-induced apparent upregulation of HSP72 (Figure A.1.1C, E). Furthermore, treatment of 17AAG led to a significant reduction in XIAP (Figure A.1.1A-B), a member of the IAP family that inhibits apoptosis, and that is a downstream target of AKT (Dan et al., 2004). Surprisingly, we also observed a significant reduction of intracellular HSF1 expression in response to 17AAG (Figure A.1.1A-B). Initially, we suspected that perhaps we failed to detect a higher molecular weight band for HSF1, which we might observe in cells in which HSF1 has been activated by a stress. However, we have previously observed such a shift successfully in heat shocked controls and, to the best of our present experimentation, we did not observe this shift, suggesting that intracellular HSF1 levels may indeed be reduced. Because this reduction is partial, increased activation of the remaining HSF1 protein could still account for increased HSP expression. Furthermore, this unexpected decrease in total HSF1 expression was also observed in breast cancer cells treated with 17AAG, similarly with no electrophoretic mobility shift (Pimienta et al., 2011), providing support for our findings.

We next began evaluating whether 17AAG could increase sensitivity of H460 cells to anoikis, and compared this to the effects observed in HSF1 knockdown cells. Our preliminary work is suggestive that functional inhibition of HSP90 by 17AAG may increase anoikis sensitivity, but to a lesser extent (Figure A.1.1C, H, I), and over a longer time frame, than HSF1 depletion (data not shown). Cells treated with either 17AAG or transfected with siHSF1 were suspended on polyHEMA-coated dishes for 6 h. As

previously shown, HSF1 knockdown induced anoikis. Comparatively, 17AAG treatment appears to result in less activation of caspase-7 (Figure A.1C, H-I). While this finding might initially appear to suggest that reduction of HSP90 by HSF1 depletion might partially contribute to anoikis induction, a number of variables complicate this assessment. Unfortunately, in addition to increasing our sample size, a more thorough analysis than we could provide during this study would need to be done to effectively draw a conclusion.

We propose taking added steps to ensure that we are not misidentifying an undetected shift in HSF1 molecular weight as reduced intracellular expression. While we have previously observed these shifts, including a heat-shocked control directly on these western blots would provide a solid comparison. Furthermore, perhaps an electrophoretic mobility shift assay could be employed for more thorough confirmation. The importance of this in assessing this data is high. Presently, we cannot rule out that the lesser amount of CC7 in suspended 17AAG-treated cells is actually reflective of the partial reduction in HSF1 expression, which subsequently induces anoikis. Indeed, comparatively siHSF1 potently inhibits HSF1 and 17AAG only partially inhibits it, and this may be relatable to the differences in CC7. However, considering that AKT, XIAP, and other HSP90 clients are inhibited by treatment with 17AAG, and that many of these are relevant to anoikis, it remains possible that this is a separate mechanism (Liu et al., 2006; Paoli et al., 2013; Simpson et al., 2008).

One limitation is that we cannot directly compare the expression of HSP90 resulting from HSF1 knockdown and that by 17AAG. However, 17AAG does appear to have a stronger effect on HSP90 clientele (i.e. AKT) (Figure A.1.1C, G). If the partial

inhibition of HSP90 by HSF1 knockdown was responsible for anoikis induction, we would expect that a more robust inhibition of HSP90 would lead to a more robust caspase response, which we did not observe. Importantly, HSF1 depletion in A549 cells also induced anoikis, but did not affect the expression of HSP90, in general support of HSP90 inhibition not being the primary cause of anoikis potentiation in HSF1-depleted cells. We suggest a better direct comparison would be to knockdown HSP90 with siRNA so we can titrate HSP90 levels to be directly comparable to that caused by HSF1 depletion. Furthermore, we could complement this work by rescuing HSP90 to endogenous levels in HSF1-depleted cells to determine whether this could rescue cells from anoikis, although whether this might inhibit HSF1 activity would be a pointed question. Notably, when targeting both HSF1 and HSP90 in attached H460 cells, we observed a robust increase in CC7 (Figure A.1.1C, H, I). Other instances of increased susceptibility of cells to 17AAG treatment upon HSF1 depletion have been previously shown (Kim et al., 2015). Lastly, we noted some variation in response by attached controls to 17AAG, and this requires further analysis to ensure general induction of apoptosis does not significantly influence our analysis.

While much work is required to elucidate this mechanism and we cannot presently draw a definitive conclusion from these experiments, we have developed a working hypothesis based on our current findings, to be investigated in future studies. Recently, it was identified that AKT can directly bind and phosphorylate HSF1 at S326 (Carpenter et al., 2015). In H460 cells, a possible role for S326 phosphorylation in preventing HSF1 degradation was presented, as an inhibitor of HSF1 dephosphorylated S326 and induced HSF1 degradation (Yoon et al., 2014). Future work is needed to

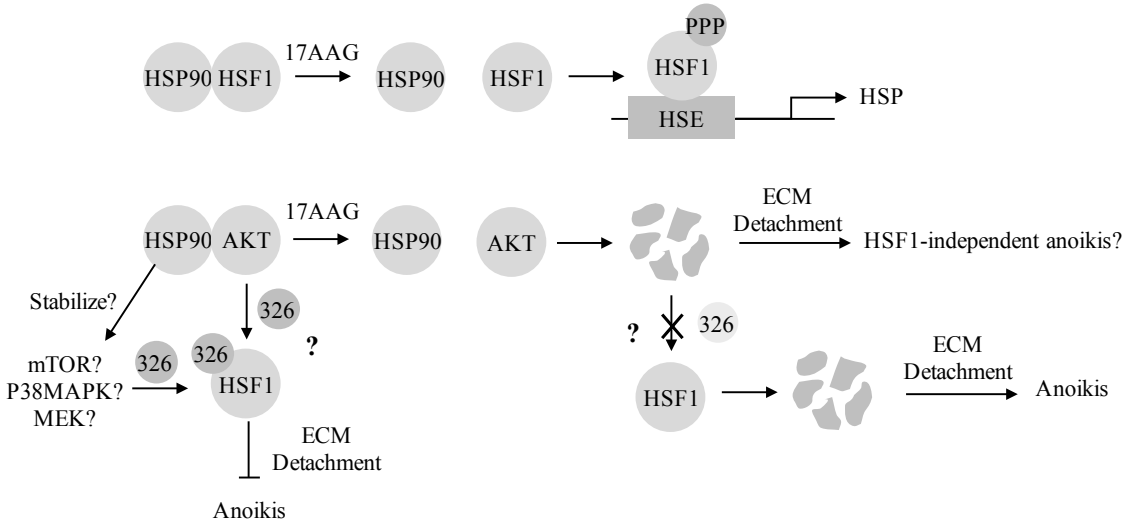

Figure A.1.2. Possible mechanisms of 17AAG effect on suspended H460 cells. Schematic summarizing observations and potential mechanisms of 17AAG effects on downstream targets and how that might relate to anoikis induction in H460 cells (post-translational modifications (PPP), pS326-HSF1 (S326)).

determine whether this effect might be specific to this compound, but it represents one possible mechanism for the degradation of HSF1. We suggest it may be worth investigating whether degradation of AKT, consequent to HSP90 inhibition, might inadvertently lead to HSF1 degradation by reducing AKT-mediated S326 phosphorylation. Subsequently, partial HSF1 degradation could be responsible for any increases in anoikis, through alternate mechanisms (Figure A.1.2). Notably, we found that AKT, XIAP, and HSF1 are all significantly reduced to comparable levels by 17AAG (Figure A.1.1A-B), and XIAP has been shown to be stabilized by AKT to prevent its degradation, a mechanism that could be similar to what we have suggested. Alternatively, other proteins such as mTOR (Chou et al., 2012), P38MAPK (Dayalan Naidu et al., 2016), and MEK (Tang et al., 2015) can phosphorylate HSF1 at S326; however, we have not evaluated these targets in our experiments. Certainly, one question that presents itself

is the compatibility between 17AAG-induced HSF1 activation, classically associated with S326 phosphorylation, and de-stabilization through S326 de-phosphorylation. Interestingly, a previous study indicated that 17AAG, despite its HSF1-activating capabilities, did not increase S326 phosphorylation (Chou et al., 2012), and thus HSF1 activation and reduced S326 phosphorylation might be compatible. Furthermore, recent evidence suggests uncoupling between stress-induced HSF1 activation and phosphorylation is possible, and PTMs might act as mechanism that help direct specific transcriptional programs (Budzyński et al., 2015). Ultimately, future experiments investigating the effects of HSP90 inhibition on both HSF1 and anoikis are warranted.

A.2 Supporting Figures

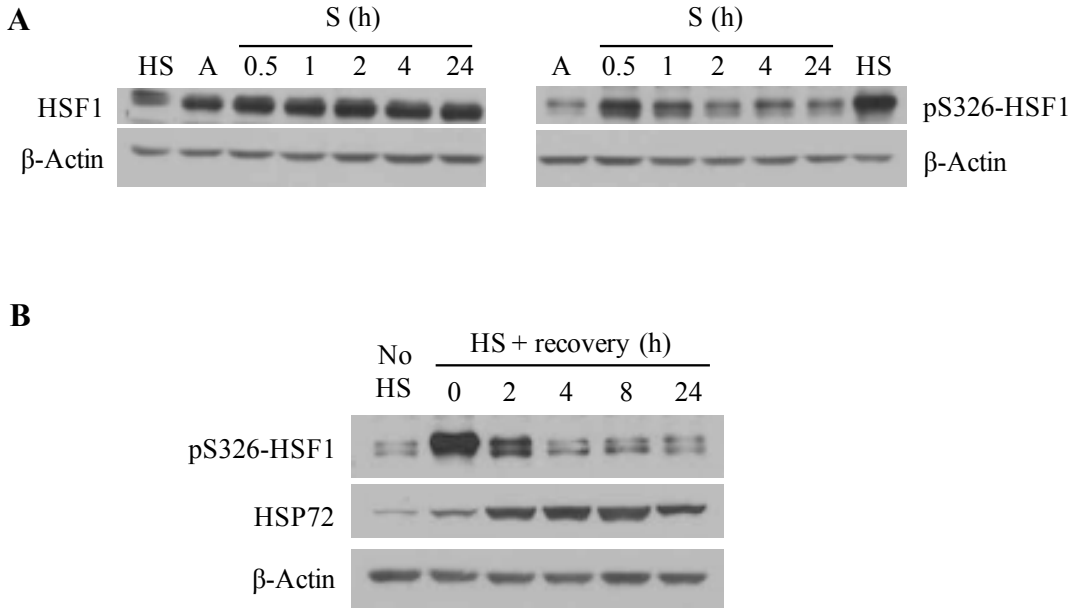

Figure A.2.1. Detachment-induced phosphorylation of HSF1 at S326 in H460 cells is transient, similar to that observed after heat shock. (A) H460 cells were detached and suspended on polyHEMA-coated culture dishes for the indicated times. Expression of HSF1 and pS326-HSF1 in suspended (S) or attached (A) control cells was measured by western blot. For reference, a heat-shocked control was included (HS). β-actin was used as a loading control. Blots are representative of at least three independent experiments. (B) For reference, BEAS-2B cells were heat-shocked at 43°C for 1 h and allowed to recover for the indicated lengths of time. Expression of pS326-HSF1 and HSP72 were measured by western blot. β-actin was used as a loading control.

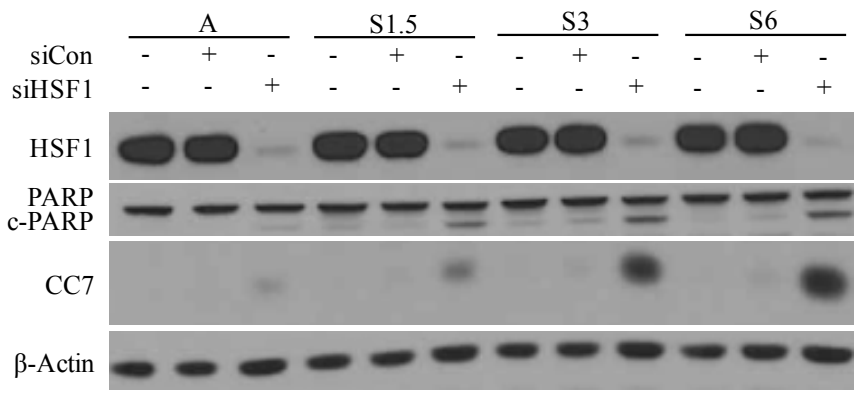

Figure A.2.2. Anoikis is triggered quickly after detachment in HSF1-depleted H460 cells. H460 cells were transfected with siRNA targeting HSF1 (siHSF1) or a scrambled control (siCon) for 48 h (H460) prior to suspension. Cells were subsequently suspended on polyHEMA-coated dishes for the indicated times up to 6 h. Lysates of suspended (S) cells and attached control (A) cells were harvested and western blots were performed to measure changes in HSF1 and cleavage of caspase-7 (CC7) and PARP (c-PARP). β-actin was used as a loading control. Early time points on blots are representative of at least two independent experiments.

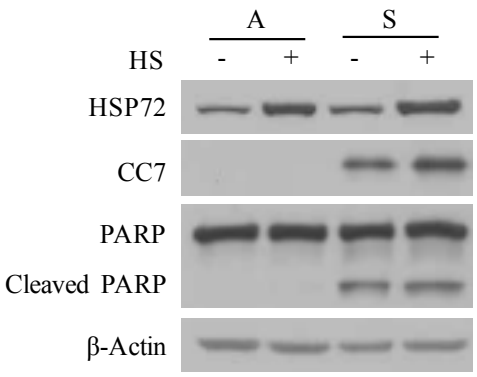

Figure A.2.3. Supporting preliminary data indicating heat shock does not rescue BEAS-2B cells from anoikis. BEAS-2B cells were heat-shocked at 43°C for 1 h and immediately detached and seeded on polyHEMA-coated dishes for 24 h to induce anoikis. Expression of HSP72 and cleavage of caspase-7 (CC7) and PARP (c-PARP) were measured by western blot. β-actin was used as a loading control.

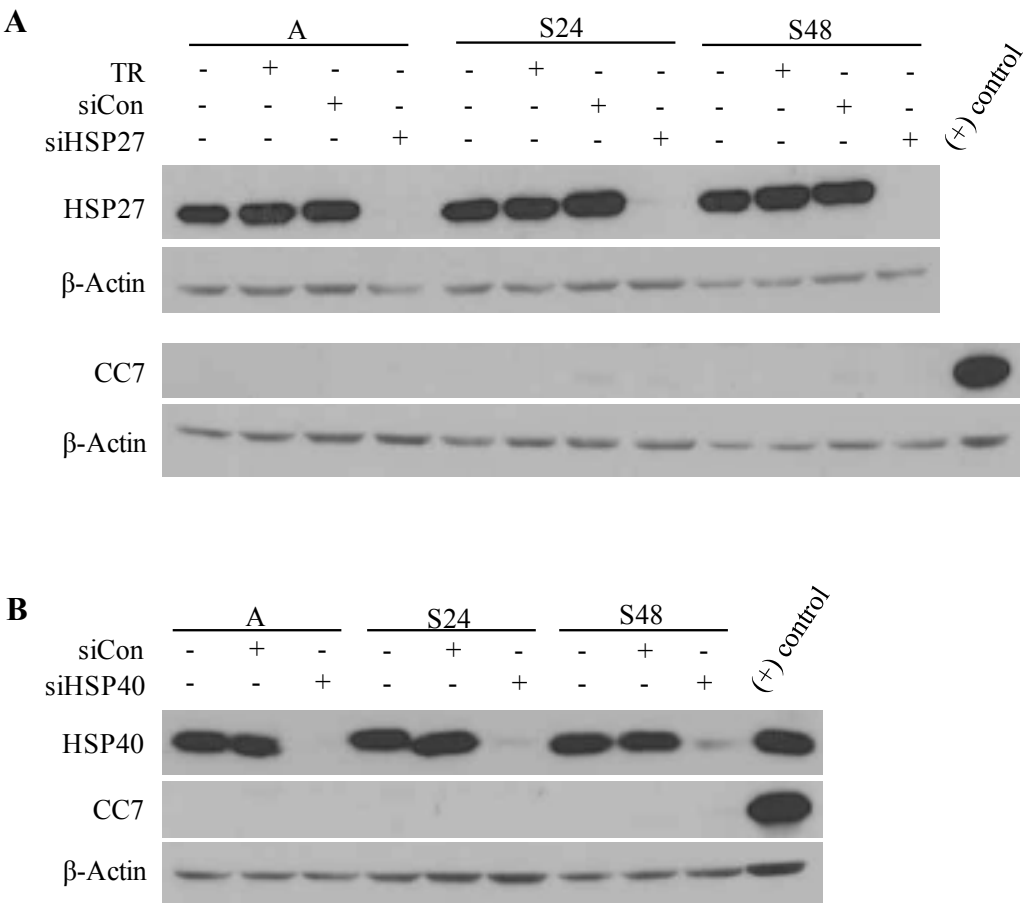

Figure A.2.4. Preliminary data indicating knockdown of HSP27 or HSP40 in A549 cells does not appear to influence anoikis. A549 cells were transfected with siRNA targeting HSP27 (siHSP27) (A) or HSP40 (siHSP40) (B) or a scrambled control (siCon) for 72 h prior to suspension. Panel A includes a transfection reagent (TR) control. Cells were subsequently suspended on polyHEMA-coated dishes for varying times up to 48 h. Lysates of suspended (S) cells and attached control (A) cells were harvested and western blots were performed to measure changes in HSF1 and cleavage of caspase-7 (CC7). β-actin was used as a loading control.

Table A.2.1. HSF1 and HSP changes observed in each tumor type.

Treatment	Target	H460	A549
Detachment	HSF1	-	-
	pS326-HSF1	↑	-
	HSE binding	↑	?
	HSP90	-	-
	HSP72	-	-
	HSP60	-	↑
	HSP40	-	-
	HSP27	-	-
	pS82-HSP27	↑	↑
HSF1 Knockdown	HSP90	↓	-
	HSP72	↓	↓
	HSP60	-	-
	HSP40	↓	↓
	HSP27	↓	↓