The Role of HSP70/HSP90 Organizing Protein (Hop) in the Heat Shock Factor 1 (HSF1)-mediated Stress Response

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ABSTRACT

Molecular chaperones regulate cellular proteostasis. They control protein conformation and prevent misfolding and aggregation under both normal and stressful environments, ultimately resulting in cell survival. The project aimed to understand the role of the HSP70 – HSP90 Organizing Protein (Hop/STIP1) in the survival of stressed cells and the function of the stress-responsive transcription factor, Heat Shock Factor 1 (HSF1). HSF1 protein levels were significantly reduced in Hop-depleted HEK293T cells compared to controls by ELISA, western blot, and mass spectrometry. HSF1 transcriptional activity at the HSP70 promoter, and binding of a biotinylated HSE oligonucleotide under basal conditions were significantly reduced, consistent with the reduced levels of HSF1. In response to heat shock, HSF1 levels in Hop-depleted cells increased to that of controls, but there was still significantly lowerHSF1 transcriptional activity and HSE binding. Hop-depleted HEK293T cells were more sensitive than controls to the HSF1 inhibitor KRIBB11 and showed reduced short-term and long-term proliferation. Unlike the HSP90 inhibitor 17-DMAG, which had no effect, the HSP70 inhibitor JG98, further decreased the levels of HSF1 in Hop-depleted cells, suggesting a role for HSP70 in the Hop-mediated effects. There was punctate nuclear staining for HSF1 in Hop-depleted cells under both basal and heat shock conditions, as well as reduced nuclear localization and increased cytoplasmic accumulation of HSF1 in response to heat shock. Hop and HSF1 colocalized in cells, and HSF1 could be isolated in complex with Hop and HSP70. Loss of Hop reduced HSF1 in HSP70complexes but did not affect HSF1 abundance in HSP90 complexes. Hop-depleted cells showed reduced short-term and long-term survival compared to controls, an effect that was potentiated by the JG98 HSP70 inhibitor. Taken together, these data suggest that Hop regulation of HSF1activity is via a mechanism involving reductions in HSP70 interaction, as well as reduced nuclear localization, and DNA binding, and is consistent with reduced cellular fitness under basal and stress conditions.

DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy in Biochemistry of Rhodes University. It has not been submitted before for any degree or examination at any other university.

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Grahamstown

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LIST OF ABBREVIATIONS

ATP	Adenosine Triphosphate
Aha1	Activator of Heat Shock Protein 90
ANOVA	Analysis of Variance
AR	Androgen Receptor
BSA	Bovine Serum Albumin
Cdc37	Cell division cycle 37
Cdc42	Cell division cycle 42
CHIP	Carboxyl terminus of HSC70-interacting Protein
C-terminal	Carboxyl terminal
Dox	Doxycycline
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DTSSP	3,3'-Dithiobis(sulfosuccinimidyl propionate)
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EEVD	Glutamate-glutamate-valine-aspartate motif
FBS	Foetal Bovine Serum
Fkbp51/52	FK506 binding protein-51/52
HEK293T	Human Embryonic Kidney 293T cell line
Hip	HSC70-interacting protein
Hop	HSP70/HSP90 Organizing Protein
HRP	Horseradish Peroxidase
HSF1	Heat Shock Factor 1
HSP	Heat Shock Protein
NEAA	Non-essential amino acids
N-terminal	Amino-terminal
PBS	Phosphate Buffered Saline

PSA	Penicillin-Streptomycin-Amphotericin
PIC	Phosphate Inhibitory Cocktail
Puro	Puromycin
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA
TBS	TRIS Buffered Saline
TBST	TBS-Tween
TMB	3,3’,5,5’-Tetramethylbenzidiene
TPR	Tetratricopeptide Repeat

LIST OF SYMBOLS

α	Alpha
β	Beta
°C	Degree Centigrade
%	Percentage
V	Volts
xg	Relative Centrifugal Force to Gravity
M	Molar
mM	Millimolar
µM	Micromolar
nM	Nanomolar
g	Grams
mg	Milligrams
µg	Micrograms
ng	Nanograms
L	Litre
mL	Millilitre
µg	Micrograms
bp	Base pairs
kDa	Kilodaltons
min	Minutes
v/v	Volume/Volume
w/v	Weight per Volume

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OUTPUTS

Publications

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International:

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CHAPTER 1: Introduction

The rate of protein synthesis is rapid in living cells, which sometimes leads to misfolding of proteins. Molecular chaperones prevent this aggregation by providing support for correct folding, conformational change, maturation, and degradation of numerous signaling proteins and transcription factors (Lindquist, 1986a; Hartl, 1996). Chaperones are ubiquitous and highly conserved proteins (Csermely *et al.*, 1997). These are essential for cell survival after exposure to environmental stress that causes protein damage. Stressors like heat shock, proteasomal stress, or any entity that causes a sudden change in the cell environment stimulates the synthesis of a variety of chaperones (Blagosklonny, 2001). Hence many molecular chaperones are members of the heat shock protein (HSP) families (Welch & Brown, 1996; Snyder *et al.*, 1998) and include the major chaperones heat shock protein 70 kDa (HSP70) and heat shock protein 90 kDa (HSP90). However, stress can also cause adaptive responses and aging in cells (Labbadia & Morimoto, 2015). Stress regulation occurs at transcriptional, translational, and post-translational levels (Söti& Csermely, 2007).

1.1 Heat Shock Proteins (HSPs)

Many molecular chaperones are members of the Heat Shock Proteins (HSPs) superfamily and are traditionally classified based on their molecular weight. The various families of HSPs include HSPH (HSP100), HSPC (HSP90), HSPA (HSP70), DnaJ (HSP40), HSPD/E (HSP60/10), CCT (TRiC) and HSPB (small Hsps). Two of the major molecular chaperone families are the 70kDa and 90 kDa heat shock proteins, HSP70 and HSP90. Most HSPs are diverse in structure and size, and the majority but not all isoforms are dependent on ATP (Edkins & Boshoff, 2014; Baindur-Hudson *et al.*, 2015)

1.2 Heat Shock Protein 70(HSP70)

There are 13 types of HSP70 isoform in human cells with distribution in almost every subcellular compartment (Kampinga *et al.*, 2009). HSP70 has a wide array of functions, including protein folding, holdase, and re-foldase activity, translocation, aggregation, and

targeted proteasomal degradation (Edkins & Boshoff, 2014).The isoform of the HSP70 family responsible for basal proteostasis is heat shock cognate 70 (HSC70), whereas HSP70 is considered the stress-inducible isoform responsive for HSR or PSR (Young, 2016).During apoptosis, HSP70 inhibits both caspase-dependent and caspase-independent pathways. Overexpression of HSP70 provides resistance against apoptosis inducers like TNFα, staurosporine, doxorubicin. Cancer cells have increased levels of HSP70, which correlate with the risk of malignancy and reduced sensitivity towards therapy (Jäättelä *et al.*, 1998; Jaattela, 1999). HSP70 has significance in pathological conditions, including cancer and neurodegenerative disorders, and hence is a putative drug target (Patury *et al.*, 2009; Evans *et al.*, 2010; Young *et al.*, 2016).

Structurally the N-terminal domain of HSP70 is the ATPase domain or nucleotide-binding domain, which is conserved across the isoforms. The C-terminal domain has a β sandwich structure, and the substrate-binding domain varies in the different isoforms (Edkins & Boshoff, 2014). HSP70 is dependent on several co-chaperones and co-factors for appropriate function, especially HSP40 isoforms that trigger the low basal ATPase activity of HSP70 in addition to ensuring substrate specificity.HSP40 co-chaperones (DnaJ in prokaryotes) have a canonical J domain consisting of four α helices. Between the first and third helix there is a highly conserved HPD motif (Histidine-Proline-Aspartic Acid), which is the catalytic residues required for inducing ATPase activity of HSP70. There are 49 different isoforms that differ structurally and functionally, although the primary function is to act as a co-chaperone of HSP70 and regulate ATP dependent client binding (Sterrenberg *et al.*, 2011; Edkins & Boshoff, 2014). Nucleotide exchange factors(NEFs) like HSP110 and BAG allow the release of the folded client and re-binding of ATP to initiate a new chaperone cycle (Johnson & Craig, 2001; Andreasson *et al.*, 2008) (Figure 1).

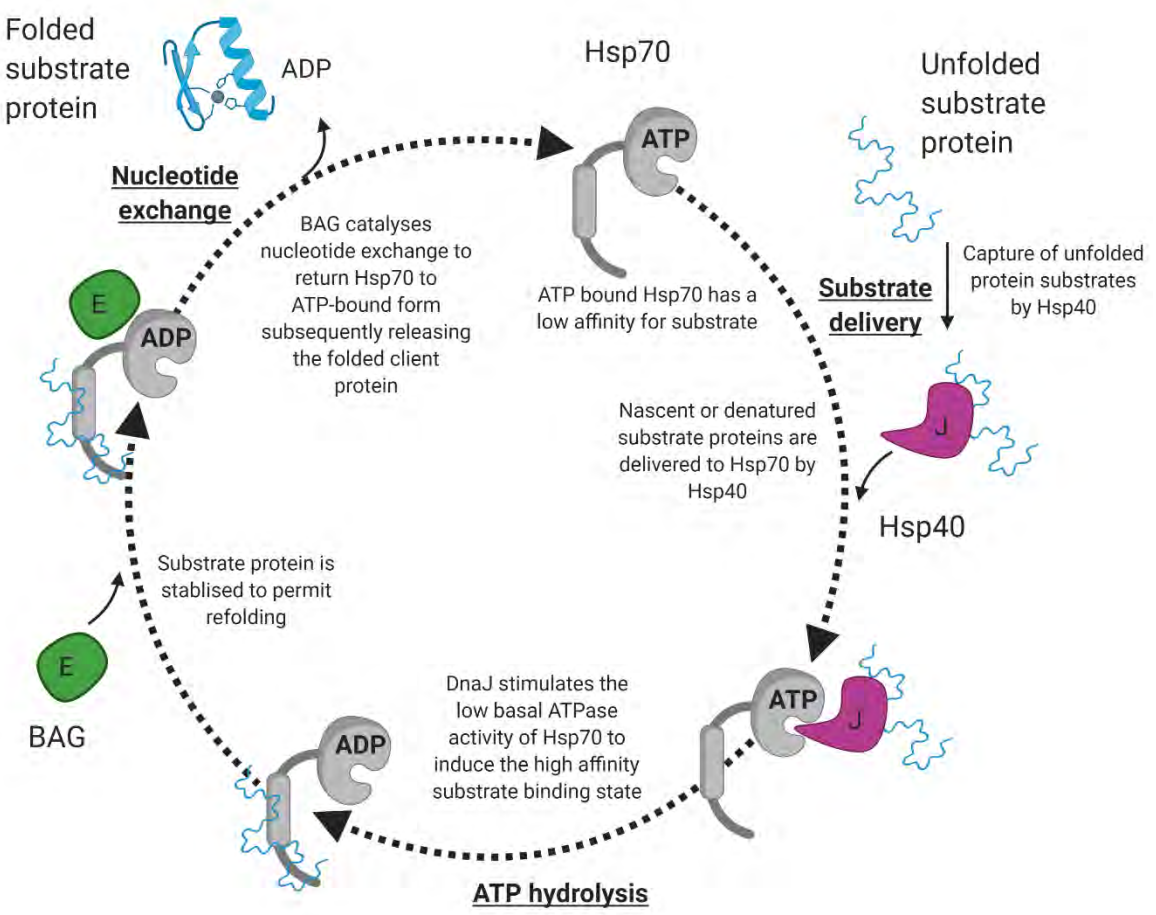


Figure 1: HSP70 chaperone cycle
Misfolded client proteins are captured by HSP40 and delivered to HSP70 in an ATP bound form. HSP40 stimulates the ATPase activity of HSP70 to convert HSP70 to an ADP-bound form with increased affinity for substrate and promotes client binding. The replacement of ADP with ATP by nucleotide exchange is catalyzed by BAG isoforms and results in client protein release.

The cytoplasmic eukaryotic HSP70 contains an EEVD motif at the C-terminal end, which is the binding site for tetratricopeptide repeat (TPR) containing co-chaperones such as CHIP, Hop, and Hip (Ballinger et al., 1999; Hohfeld, 1995; Chen & Smith, 1998). During a chaperone cycle, HSP40 stimulates the ATPase activity of HSP70, thereby catalyzing the binding of Hop to HSP70. Hip interacts with the ATPase domain of HSC70 after HSP40 has induced HSP70 to hydrolyze ATP, leading to a stable HSP70-ADP conformation. The ATPase cycle is hindered by the CHIP-HSP70 interaction to ensure reduced protein folding. CHIP also competes with Hop in terms of binding to the C-terminus of both HSC70/HSP70 and HSP90 (Stankiewicz *et al.*, 2010; Edkins & Boshoff, 2014; Baindur-Hudson *et al.*, 2015).

In eukaryotes, the most abundant chaperone is HSP90 comprising about 1-2% of the cytosolic fraction under basal conditions. HSP90βhas an indispensable role in mammalian cells, and *Hsp90ab1* knockout is embryonic lethal in the mouse (Csermely *et al.*, 1998; Voss *et al.*, 2000; Edkins & Boshoff, 2014). Although the two HSP90 isoforms show 86% sequence identity, *Hsp90aa1* knockout mice are viable (Grad *et al.*, 2010). HSP90 also has extracellular and membrane-bound isoforms, in addition to intracellular and organelle ones, namely, mitochondrial HSP90 (TRAP-1/HSP75/HSPC5) and endoplasmic reticulum HSP90 (Grp94/Grp96/HSPC4). HSP90 in prokaryotes, like *E.coli,*is known as HtpG. The localization and function of HSP90 is influenced by PTM like phosphorylation, acetylation, *s*-nitrolysation. Significant linkage between HSP90 and multiple signaling cascades has led to the design various inhibitors (many derived from or informed by the ATPase inhibitory natural product geldanamycin, which can also inhibit Grp94 and TRAP1 (Lawson *et al.*, 1998; Felts *et al.*, 2000; Soti *et al.*, 2005; Edkins & Boshoff, 2014).

Structurally HSP90 has a dimeric conformation consisting of three conserved domains per monomer, namely N domain (25kDa; amino-terminal), M domain (35kDa; middle domain), and C domain (12kDa; carboxyl-terminal). The N and M domains are linked by a charged linker, which is species and isoform-specific (Jahn *et al.*, 2014). The ATPase activity is triggered by the interaction of the co-chaperone Aha1 at the N and M domain, while the HSP90 C-terminal end has a dimerization domain and terminates in an EEVD motif to bind TPR domain-containing co-chaperones. There is a second nucleotide-binding site that displays preferential binding for GTP/UTP and gets exposed only when the N-terminal domain is ATP bound. Novobiocin, a C-terminal inhibitor binds at a site which overlaps with the dimerization domain (amino acid 538-728) and competes with ATP binding, as well as disrupting co-chaperone interaction (Prodromou *et al.*, 1997; Meyer *et al.*, 2003; Soti *et al.*, 2003; Terasawa *et al.*, 2005). HSP90 has over 300 clients that depend on it for proper folding and functionality, with steroid receptors being one of the classical HSP90 clients. HSP90 also has roles in pathological conditions like Alzheimer's disease, and cancer (Luo *et al.*, 2010; Salminen *et al.*, 2011; Drecoll *et al.*, 2014; Edkins & Boshoff, 2014; Baindur-Hudson *et al.*, 2015; Jaeger & Whitesell,

2019).The mitochondrial HSP90 or TRAP1 (TNF associated receptor protein 1) has six isoforms, exists as a dimer, with ATPase activity induced by heat shock. TRAP1 is inhibited by geldanamycin but has more affinity for ATP than cytosolicHSP90 and lacks the EEVD motif for TPR containing co-chaperone binding. The N-terminal domain of TRAP1 has a mitochondrial targeting sequence (Neckers *et al.*, 2007; Leskovar *et al.*, 2008).Grp94 also exits as a dimer, is inhibited by geldanamycin, and has a conserved C-terminal end with the KDEL motif required for retention of proteins within the ER. The N-terminal region has an ER signal peptide (Munro & Pelham, 1987; Marzec *et al.*, 2012; Edkins & Boshoff, 2014).

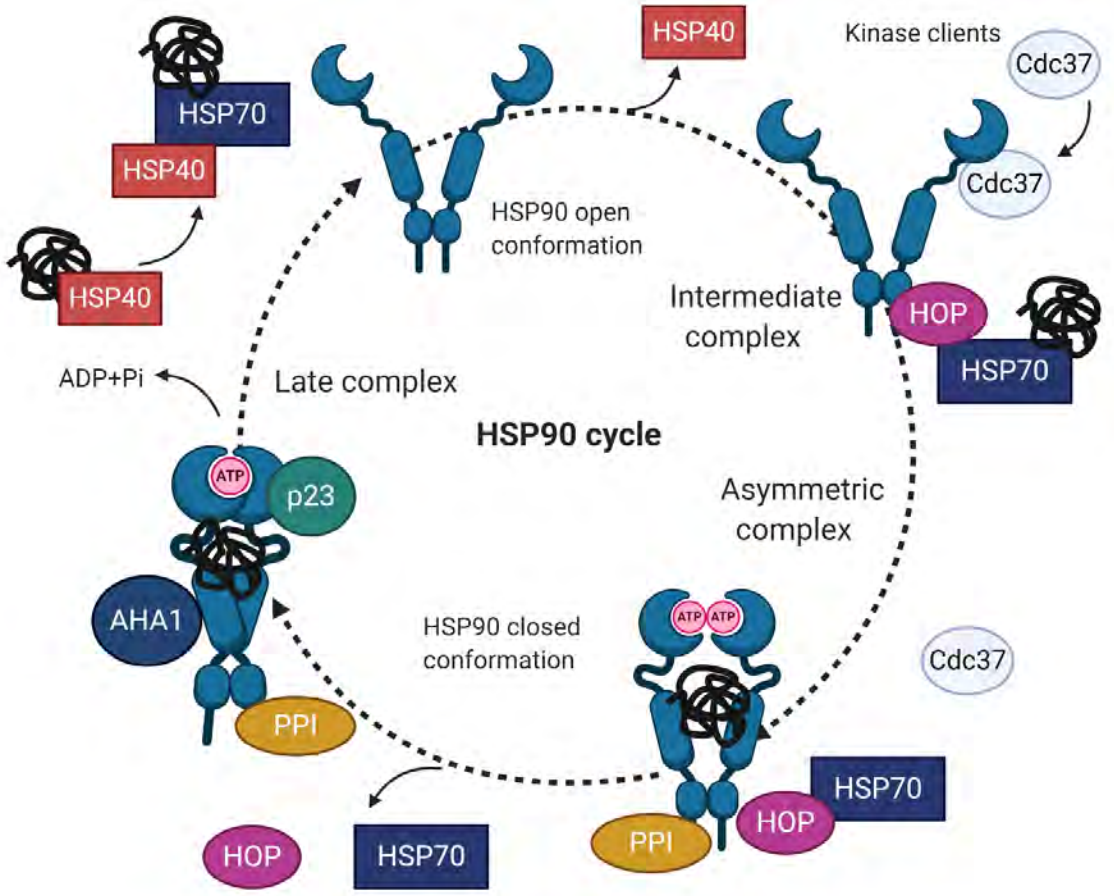


Figure 2: HSP90 chaperone cycle
Misfolded client proteins captured by the HSP70-HSP40 chaperone complex are transferred to the open HSP90 client via Hop and Cdc37. HSP90 binds ATP and undergoes N-terminal dimerization and transfer of the client protein. HSP90 undergoes further conformational changes, trapping the client protein and acquiring co-chaperones, including PPI and p23. Client release is promoted by ATP hydrolysis, which is stimulated by the co-chaperone AHA1.

The chaperoning of clients by HSP90 is due to a series of conformational changes. HSP90 is constitutively dimerized via the C-terminal dimerization domain, and when inactive, the N-terminal domains are separate, ATP is not bound, and HSP90 has an “open” conformation. Association of client proteins, some co-chaperones, and ATP binding leads to the closure of the clamp at the N-terminal domain leading to HSP90 being in a “closed” conformation. ATP hydrolysis results in reversion to the open state and release of the client. This HSP90 cycle is under the regulation of numerous co-chaperones which alter enzymatic activity, HSP90 conformation, or client binding (Li *et al.*, 2012a)(Figure2).

1.3 Chaperone-assisted protein folding by theHSP70-HSP90 complex

The HSP70 and HSP90 chaperones co-operate during chaperone-mediated folding (Figure 1 and 2). The folding process by this network is best understood for the steroid hormone receptor (SHR) clients. During the initial steps, HSP70, along with a co-chaperone isoform, HSP40, binds the client protein to form the early complex. Then in the presence of the adaptor co-chaperone HSP70-HSP90 organizing protein (Hop, also known as Sti1, STIP1, or p60), this complex is transferred from HSP70 to HSP90. A single molecule of Hopcan sufficiently stabilize the open conformation of HSP90. At the same time, the other TPR-acceptor arm of HSP90 binds to a PPIase (FKBP51 or FKBP52 members of PPIase family),forming an asymmetric intermediate complex(Pirkl & Buchner, 2001; Riggs *et al.*, 2007). The TPR-containing PPIase regulates the interconversion of the *cis-trans* isomerization of peptide bonds at proline residues (Pirkl & Buchner, 2001; Kramer *et al.*, 2004). The association of ATP with the complex converts the open conformation of HSP90 to the closed conformation, while the co-chaperone p23binds and promotes stability of the closed state of HSP90 (Johnson *et al.*, 1998). This does not favour the binding of Hop, thus stimulating it to dissociate from the complex. In the later stages, other PPIases may associate with the HSP90 and p23 complex. The hydrolysis of ATP, stimulated by Aha1, causes the release of p23 along with the client protein from HSP90 (Young & Hartl, 2000). In the case of newly synthesized kinases, after interaction with HSP70 and HSP40, the protein kinases are transferred to HSP90 by

the presence of Hop and the kinase-specific co-chaperone Cdc37, both of which contribute towards stabilization of the HSP90/kinase complex. Hop exits from the complex in the presence of protein phosphatase PP5 and the ATPase activator Aha1. In the later stages, Aha1 releases Cdc37 along with nucleotides from HSP90 (Li *et al.*, 2012a; Xu *et al.*, 2012).

1.4 HSP70-HSP90 organizing protein (Hop)

The HSP70/HSP90 organizing protein (Hop) or stress-inducible phosphoprotein 1 (STIP1) is a co-chaperone that binds to and interacts with the molecular chaperones, HSP70 and HSP90 (Wegele *et al.*, 2004; Onuoha *et al.*, 2008; Röhl *et al.*, 2015; Karam *et al.*, 2017). Hop functions as an adaptor protein whereby it facilitates the folding and transfer of client proteins between the molecular chaperones HSP70 and HSP90 (Chen & Smith, 1998; Wegele *et al.*, 2004; Baindur-Hudson *et al.*, 2015). Originally Hop was thought to be required for HSP90 and HSP70 interactions in eukaryotes. It has now been shown in yeast that HSP90 (HSP82) and HSP70 (Ssa1) interact directly, independent of the Hop homolog (Sti1), via a region of middle domain of yeast HSP90 (Kravats *et al.*, 2018). The folding of luciferase by HSP70 was stimulated in the presence of Hop and further enhanced when HSP90 was introduced (Johnson *et al.*, 1998). Hop is also able to modulate several signal transduction pathways, including those which can lead to cancer (Walsh *et al.*, 2009, 2011). Prion protein, PrPc, can also bind to Hop (Lopes *et al.*, 2005). Hop serves as a receptor or ligand to regulate signaling pathways through interactions with both chaperones and PrPc, and Hop has been shown to have ATPase activity (Yamamoto *et al.*, 2014). Hop is essential in the mouse and has a pivotal role to play for the proper development of embryos (Beraldo *et al.*, 2013).

Hop is a universal co-chaperone protein, first identified in yeast (Onuoha *et al.*, 2008; Johnson & Brown, 2009; Röhl *et al.*, 2015; Karam *et al.*, 2017). Hop is also present in the genomes of model organisms used in genetic studies like nematode, fruit fly, zebrafish, mouse and even in Coelacanth (*Latimeria* sp), a living fossil (Woods *et al.*, 2005; Song *et al.*, 2009; Amemiya *et al.*, 2013). Hop is a phylogenetically conserved protein, but its

structural and functional variations are species-specific, for example, the *Caenorhabditis elegans* Hop lacks TPR1 and consequently only has 56% similarity with human Hop (Chang *et al.*, 1997).

Even though Hop is mostly cytoplasmic, it has been detected in the nucleus, Golgi complex, extracellular matrix (ECM), and cell membrane (Honore *et al.*, 1992; Longshaw *et al.*, 2004; Hajj *et al.*, 2013). Dogma suggests that these forms of Hop derive from different localization of the same isoform. The mammalian Hop contains a bipartite nuclear localization signal (NLS) that regulates the transportation of the molecule to the nucleus when the cell is in a stressed condition (Longshaw *et al.*, 2004). The movement of Hop from the cytoplasm to the nucleus occurs at the G1/S phase of the cell cycle by phosphorylation of Hop by casein kinase II. Hop was restricted to the cytoplasm if phosphorylation happened by cell division cycle 2 (cdc2) kinase (Longshaw *et al.*, 2004; Daniel *et al.*, 2008). In an astrocyte cell line study, PIAS (protein inhibitor of activated STAT1) had a role in retaining Hop in the nucleus (Soares *et al.*, 2013).

Structurally, Hop contains repeating units of two domains (Scheufler *et al.*, 2000; Odunuga *et al.*, 2004; Onuoha *et al.*, 2008). The first is the TPR domain. There are 3 TPR domains (TPR1, TPR2A, and TPR2B) in Hop, each containing three TPR motifs. This domain comprises tandem repeats of anti-parallel alpha-helices, making an amphipathic groove, the site of protein-protein interaction with HSP70 and HSP90 (Kenneth Allan & Ratajczak, 2011). Hop can differentiate between HSP70 and HSP90 C-terminal EEVD motifs due to the presence of specific TPR residues. The residues of TPR1 and TPR2A facilitate domain-specific binding to HSP70 and HSP90, respectively, via the C-terminal motifs of the chaperones (GPTIEEVD for HSP70 and MEEVD for HSP90) (Odunuga *et al.*, 2003). In mammals, phosphorylation of serine and threonine residues near the C-terminal EEVD motifs of the HSP70 or HSP90 regulates Hop binding (Carrigan *et al.*, 2004; Odunuga *et al.*, 2004; Muller *et al.*, 2012) and controls Hop-mediated protein folding. The length between the C-terminal dimerization domain of HSP90 and the MEEVD motif determines the rate of binding of Hop-HSP90 (Lee *et al.*, 2012; Schmid *et al.*, 2012).

The second type of domain in Hop is the DP domain. These are alpha-helical structures, rich in aspartate and proline amino acids. There are two DP domains in Hop. DP1 is located between TPR1 and TPR2A, while DP2 is in between the C terminal and TPR2B of Hop. Although their role remains mostly elusive, DP2 mutants showed reduced binding to HSP70 and were essential for protein activation in an *in vivo* system (Muller *et al.*, 2012; Schmid *et al.*, 2012). The TPR1-DP1-TPR2AB-DP2 modular structure is conserved for humans, mouse, and yeast, but variations are seen in *Drosophila* (DP1 domain absent) and C. *elegans* (absence of TPR1 domain and the linker region with DP1 domain preceding the TPR2A domain) (Carrigan *et al.*, 2005; Baindur-Hudson *et al.*, 2015)

1.5 Role of Hop in Cancer

Hop is a factor involved in the entry of a diverse range of client proteins to the HSP90 complex. Client proteins may be kinases, transcription factors, steroid receptors or signaling intermediates that have a critical role in aberrant pathways in the context of disease, like cancer (Muller et al., 2012a; Schmid et al., 2012). HSPs have a significant role in pathological states, as their levels rise in cancer, promoting tumorigenesis, while the levels drop, causing protein aggregation in the case of Alzheimer's disease (Santagata *et al.*, 2011; Calderwood & Gong, 2012). Hop is overexpressed in several human cancers, such as hepatocellular, pancreatic, colon, ovarian, and thyroid cancer (Walsh *et al.*, 2011). Using proteomic analysis, increased levels of Hop were found in a highly invasive human pancreatic cancer cell line compared to a less invasive cell line from the same individual (Walsh *et al.*, 2009). Hop expression in cell lines is induced through promoter activation by loss of p53 function and Ras gain of function mutations, which makes it part of the cancer gene signature. In Hs578T, HEK293T, SV40-transformed MEF1 cell lines showing expression of mutant or loss of p53, an increase in the Hop promoter activity was observed, while when wild type p53 was expressed, there was a reduction in the Hop promoter activity (Mattison *et al.*, 2016). Recent studies done in HEK293T, MCF7 and MDA-MB-231 cell lines report downregulation of emerin, a nuclear structural protein, upon Hop depletion or overexpression due to proteasomal or lysosomal degradation. Immunoprecipitation results validated that emerin and Hop were

in a shared complex with HSP70 but not HSP90, and overexpression of emerin could rescue the altered nuclear morphology seen with Hop depletion (Kituyi & Edkins, 2018).

Additionally, knockdown of Hop (using siRNA) in the highly invasive human pancreatic cell line resulted in a decrease in cell invasion and proliferation (Walsh *et al.*, 2011). Hop knockdown (using siRNA) in endothelial HUVEC cells showed a reduction in cell migration. Hop co-localized with microtubules, interacted with tubulin, and was involved in regulating tube formation and angiogenesis (Li *et al.*, 2012b). Silencing Hop in epithelial ovarian cancer (EOC) cells led to an inhibition of cell proliferation and invasion (Cho *et al.*, 2014). Decreasing the levels of Hop in MDA-MB-231 and Hs578T breast cancer cell lines (using a siRNA Hop knockdown system) led to a decrease in cell migration. Hop was enriched predominantly in the pseudopodia of Hs578T cells, where Hop co-localized with actin, a cytoskeletal protein. Knockdown of Hop in these cells resulted in a reduction in pseudopodia formation, reduced cell migration and loss of RhoC (Willmer *et al.*, 2013).

Hop has some seemingly chaperone independent functions. For example, the Hop-PrPc complex contributes to stimulate many cellular functions like neural development, memory, and cognition, in addition to preventing apoptosis in nerves (Zanata *et al.*, 2002; Coltinho *et al.*, 2007). A study using *PrPc* null mice has revealed the role of Hop on neural stem cells, which includes proliferation, differentiation, and response to ischemic stress. Other functions, like retinal proliferation, is a PrPc independent function of Hop (Zanata *et al.*, 2002). Extracellular Hop can act as a cytokine to transduce some signaling cascades, including SMAD, ERK, and PKA pathway (Baindur-Hudson et al., 2015; Chao et al., 2013). In a proteomic assay, Hop was identified as a granzyme-B substrate that can be cleaved in *in vitro* conditions making the cells undergo granzyme-B-induced apoptosis. Murine embryonic fibroblasts isolated from *Hop* null embryos showed increased levels of caspase3, leading to cell death and a lower proliferative potential of the cells(Beraldo *et al.*, 2013).

1.6 Heat shock factor 1 (HSF1)

The expression of most HSP, some co-chaperones (including Hop), and a range of other cellular proteins are regulated at the level of transcription by Heat Shock Factors (HSF) (Hartl *et al.*, 2011; Ruckova *et al.*, 2012). HSF1 is the best characterized of the HSF isoform family. This includes HSF2 (which is involved mainly in development and has tumor suppressor activity), HSF4 (a constitutively active protein required for eye development), HSF5 and HSF3 (identified only in the mouse). HSFX and HSFY have only been partially characterized (Anckar & Sistonen, 2007a, 2011; Åkerfelt *et al.*, 2010a, 2010b; Dai & Sampson, 2016). HSF1 is most closely associated with the response to cellular stress, and regulation of expression of molecular chaperones required to support proteostasis (Kline & Morimoto, 1997; Shi *et al.*, 1998; Anckar & Sistonen, 2011; Dai & Sampson, 2016; Solís *et al.*, 2016; Su *et al.*, 2016). HSF1 also regulates the expression of proteins related to autophagy, ribosome biogenesis, and chromatin remodeling (Kovács *et al.*, 2019). HSF1 binds as either a homotrimer or heterotrimer along with HSF2, to the extended sequence in the major groove of the DNA helix referred to as heat shock elements (HSE). HSEs are comprised of inverted repeats of the sequence GAAn, and at least three repeats are preferred for HSF1 binding (Amin *et al.*, 1988; Xiao & Lis, 1988; Anckar & Sistonen, 2011). Mice lacking the *hsf1* gene failed to express elevated levels of HSPs when exposed to thermal shock and also had a reduced survival (Xiao *et al.*, 1999). In addition, *hsf1-/-* murine fibroblasts did not show stress-induced transcription of HSP genes and succumbed to heat-induced apoptosis, suggesting a crucial role for HSF1 to combat stress in mammals. Cells with a disrupted *HSF2* gene were still able to trigger a distinct heat shock response resulting in a different expression pattern which indicates that HSF2 may be a modulator of some HSP promoters (McMillan *et al.*, 1998; Östling *et al.*, 2007)

1.7 HSF1 in Cancer

HSF1 aids malignant cells to proliferate by promoting mitosis, anabolism, invasion, and metastasis by blocking apoptotic pathways (Dudeja *et al.*, 2011; Santagata *et al.*, 2014). This pro-survival activity is ribosome dependent and under strict regulation of translational processes (Santagata *et al.*, 2014). This is also validated from the findings of the LINCS database, with animal models showing that the ribosome-HSF1 interaction is a putative target to disable the cytoprotective action of HSF1 in malignant cells (Santagata *et al.*, 2014). Immunohistochemistry studies conducted in patients with breast cancer revealed the highest expression of nuclear-activated HSF1 in invasive breast cancer and in *in situ* analysis. Kaplan-Meier analysis indicated a lower survival in breast cancer patients with elevated HSF1 mRNA levels, which contributes to more extensive tumor formation. Increased HSF1 mRNA levels were correlated with high-grade ER (Estrogen Receptor) positive breast cancer cells (Santagata *et al.*, 2011). In experiments using *hsf1* null mice, skin cancer induced by carcinogen treatments was suppressed, suggesting a role of hsf1 in tumor formation. *In vitro* cell culture studies showed that HSF1-driven malignancy changed cell signaling pathways for glucose uptake, and protein synthesis, and that these modulations result in cancer cell survival by altered metabolic processes (Whitesell *et al.*, 2007). Suppression of the proteotoxic stress response was seen in cells when they were treated with the anti-diabetic drug metformin, which is also a metabolic stressor. Metformin inactivated HSF1 via AMPK, which phosphorylated HSF1 at Ser121. This inactivation of HSF1 resulted in the downregulation of two classical stress response genes, HSP72 and HSP25, both at the transcriptional and protein level under heat shock. These data link the metabolic stress sensor AMPK to proteotoxic stress-induced by HSF1 (Dai *et al.*, 2015). mTOR, a proteotoxic stress sensor, shares a reciprocal relationship with HSF1 where suppression of mTOR is followed by an elevation of HSF1 levels, which in turn suppresses JNK to stabilize mTOR integrity (Chou *et al.*, 2012; Su *et al.*, 2016). The link between JNK and mTOR is altered in the Proteotoxic Stress Response (PSR) whereby mTOR and RAPTOR are phosphorylated by JNK at Ser863 and Ser567, respectively, resulting in decay of mTOR and blocking of translation. Hence the molecular cross-talk between HSF1, JNK, and mTOR regulate stress resistance and determine

growth rates in cells and organs (Su *et al.*, 2016). In *Nf-/-*(neurofibromatosis type I) MEFs, the HSR was triggered by elevated HSF1, supported by a dysregulated MAPK pathway, resulting in PSR tolerance. In animal model studies using *hsf1-/-*mice, blockage of the RAS/MAPK signal cascade blocked Nf1 associated oncogenesis, while in *Nf-/-* human malignant peripheral nerve sheath tumors (MPNSTs) cells, overexpression of active phosphorylated nuclear HSF1 was seen. This suggests that although Nf1 helps in cell survival, eventually, it is also responsible for the death of the organism due to oncogenesis (Dai *et al.*, 2012).

Experiments conducted in Cancer-Associated Fibroblasts (CAFs) identified active HSF1. HSF1 aids in the survival of malignant cells, but the transcriptional machinery is different in the surrounding cells compared to CAFs. It has been observed that HSF1 is increased in fibroblasts in co-culture with cancer cells, which results in the survival of malignant cells via a mechanism involving the TGFβ and SDF1 signaling pathway. Bioinformatics showed that the SDF1 promoter possesses HSE for direct HSF1 binding in stromal cells, and there exists a correlation between high levels of HSF1 with poor survival of lung and breast cancer patients even from an early stage of detection (Scherz-Shouval *et al.*, 2014). In CAF cells, the HSF1 effector molecule, Dickkopf-3 (DKK3), was identified as a promoter of tumor survival both *in vitro* and *in vivo*, as well as contributing to increased aggressiveness in breast, ovarian and colorectal cancer. Also in CAFs, there is an established link between HSF1, the Wnt pathway, and YAP/TAZ signaling that results in tumor survival (Ferrari *et al.*, 2019). In Hela and COS-1 cells treated with the anti-cancer chemical, sulforaphane (SFN), HSP27 was upregulated, along with the induction of proteasomal activity and HSF1 activation. SFN was able to induce the heat shock response (HSR), but the phosphorylation of HSP27 was independent of SFN induced proteasomal activity (Gan *et al.*, 2010). Studies involving the highly malignant BPLER cell line and lesser malignant HMLER cells showed the presence of active, phosphorylated HSF1 at Ser326. CHIP-Seq experiments in the same cell lines were done at 37°C and 42°C and showed distinct genome occupancy by HSF1 in heat shock versus cancer conditions. The genome occupancy by HSF1 was more or less conserved across a broad

range of carcinomas (Mendillo *et al.*, 2012) and suggests that HSF1 supports a cancer phenotype through its transcriptome.

1.8 Structure of HSF1

The HSF family has a domain structure that is mostly conserved across isoforms and is linked closely to HSF function. The N-terminal DNA-binding domain (DBD) is highly conserved and composed of a looped helix-turn-helix. The loop stabilizes the DNA-bound HSF trimer by protein-protein interactions (Vuister *et al.*, 1994; Anckar & Sistonen, 2011). Residues in the DBD form disulfide bonds between the adjacent HSF1 monomers to stabilize the HSF1trimer (Lu *et al.*, 2009), and mutation of any of these residues inhibits HSF1 DNA-binding ability, hindering the heat shock response (Ahn & Thiele, 2003).

Inactive HSF1 is a monomer that undergoes trimerization upon activation. Trimerization of HSF1 increases the affinity for the HSE by several-fold and is regulated by the α-helix rich oligomerization domain, located next to DBD (Perisic *et al.*, 1989). This oligomerization domain has two subdomains, each having an amphiphilic helix with hydrophobic heptad repeats HR-A and HR-B (Peteranderl & Nelson, 1992; Peteranderl *et al.*, 1999). In the HSF trimer, both HR-A and HR-B make intermolecular hydrophobic interactions resulting in the formation of triple-stranded coil-coiled interactions. An additional hydrophobic heptad repeat, HR-C, located between the regulatory and the *trans*-activation domains (TADs), folds back and suppresses the trimerization of HSF1 through the interaction with the HR-A/B. Additionally, the linker region connecting the DBD to the HR-A/B also modulates the process of trimerization (Rabindran *et al.*, 1993; Liu & Thiele, 1999)

The C-terminal part of HSF1, which stimulates the transcriptional activation of target genes, is composed of 150 amino acids containing two TADs, TAD1, and TAD2 (Newton *et al.*, 1996). The α-helical hydrophobic amino acid-rich TAD1 is located between residues 401-420 and interacts with TAF-9 (TATA box binding protein [TBP]-associated factor), whereas TAD2 is non-helical in structure and rich in hydrophobic amino acids and proline residues, and is localized between amino acids 431-529 (Newton *et al.*, 1996). Both TAD1

and TAD2 can regulate transcriptional elongation and initiation, but the TAD1 domain precisely controls transcriptional initiation (Newton *et al.*, 1996). Elongation is regulated by hydrophobic residues that stimulate the entry of BRG1, which is the subunit of the chromatin remodeler SWI/SNF (switch/sucrose non-fermentable) complex responsible for heat-induced chromatin remodeling of HSP genes (Sullivan *et al.*, 2001; Corey *et al.*, 2003).In a stress-free environment, the regulatory domain (RD) between the HR-A/B and HR-C domains restrains the HSF1 TAD(Green *et al.*, 1995). The RD also has a negative regulation over TAD and blocks HSF1 activation when there is no protein damage (Green *et al.*, 1995). The HSF1 RD can sense heat stress and confers heat inducibility when fused with TAD of the herpes simplex virus protein VP16 (Newton *et al.*, 1996).

1.9 HSF1 and the stress response

Stress acts as a triggering factor for the transcription of molecular chaperones, which ultimately supports the refolding of denatured proteins or the degradation of proteins damaged beyond repair via proteasomal or lysosomal degradation. HSF1 is the main transcription factor that regulates HSP expression in response to stress. Most cell types and tissues express HSF1 constitutively. In mammalian cells, heat shock stress increases RNA II promoter-proximal pausing that leads to transcriptional repression of thousands of genes(Mahat *et al.*, 2016). Often considered the ‘master regulator of the stress response,’ recent studies show that HSF1 instead controls the expression of a restricted number of predominantly chaperone genes during stress, and other transcription factors including serum response factor (SRF) in mammalian cells and Msn2/4 in yeast are important for the rest of the response (Mahat *et al.*, 2016; Solís *et al.*, 2016).Upon stress, the extent of condensation and phase transition of HSF1 acts as a determinant of cell survival or death where it not only acts as a driving force for chaperone transcription but localizes in nucleus as stress foci (Lindquist, 1986b; Vihervaara & Sistonen, 2014).Using techniques like multiplexed tissue imaging, HSF1 foci have also been located in tumors although these are inversely proportional to the level of chaperone expression. Data from live cell imaging and single cell microscopy experiments show a link between dissolution and formation of HSF1 foci which aids in cell survival. Chronic stress exposure resulted

in insoluble gel-like HSF1 followed by decreased chaperone transcription ultimately culminating into apoptotic cells. As such, HSF1 foci can act as a sensory switch to turn on cytoprotection in an irreversible manner (Gaglia *et al.*, 2020). In mammalian cells, the ability of HSF1 to induce expression of these genes was related to a release of RNA pol II from promotor proximal pause (Mahat *et al.*, 2016).

Under non-stressed conditions, HSF1 is mostly in an inert monomeric state, bound to chaperone complexes containing at least HSP70, HSP40, HSP90, and members of the TRiC/CCT chaperonins (Shi *et al.*, 1998; Zou *et al.*, 1998; Mosser & Morimoto, 2004; Kijima *et al.*, 2018). HSF1 is distributed diffusely in the nucleus due to the presence of a strong nuclear localization (NLS) signal (Vujanac *et al.*, 2005), although HSF1 can also shuttle between nucleoplasm and cytoplasm. Upon introduction of stress, HSF1 is quickly converted from the monomer to a transcriptionally active, trimeric form with competence for DNA-binding activity and nuclear accumulation, with an increase in trans-activating capacity and phosphorylation (Biamonti, 2004; Anckar & Sistonen, 2007a, 2007b). Upon exposure to thermal stress, the movement of HSF1 to the cytoplasm is inhibited, and it accumulates in the nucleus (Vujanac *et al.*, 2005). Nuclear localization of HSF1 is critical for cell survival under stress, as forced export led to cell death due to global protein aggregation (Solís *et al.*, 2016). In human cells, the accumulation of the activated trimeric HSF1 occurs in particular sub-nuclear structure called nuclear stress bodies (nSBs) (Biamonti, 2004). HSF1 transcriptional programs and regulation are distinct in developmental, tumorigenic, and heat shock response pathways (Calderwood & Gong, 2012; Mendillo *et al.*, 2012; Li *et al.*, 2016). *In vitro* studies of the activation of HSF1 suggest that the protein has an inherent ability to sense proteotoxic stress and purified HSF1 protein can be trimerized in presence of a range of stress-inducing conditions, including heat shock, increased calcium concentration, hydrogen peroxide and low pH (Mosser *et al.*, 1990; Farkas *et al.*, 1998; Zhong *et al.*, 1998; Ahn & Thiele, 2003; Xie *et al.*, 2003).

1.10 Regulation of HSF1 function

The HSF1 activation cycle involves a concerted series of stages, including oligomerization, nuclear accumulation, and DNA binding, which subsequently lead to transcriptional activation (Figure 3). This response must be carefully regulated, and hence there is a process of attenuation and HSF1 degradation, which follows heat shock (Wu, 1995; Kline & Morimoto, 1997; Guo *et al.*, 2001). There are two main mechanisms by which HSF1 is regulated, namely through post-translational modifications (PTLM) and regulation by selected HSP and chaperones. The other factors that contribute to HSF1 regulation are protein degradation and trafficking (Boyault *et al.*, 2007; Raychaudhuri *et al.*, 2014), co-activator/suppressor of transcription and alternate isoforms of Heat Shock Factor (e.g. HSF2) (Whitesell & Lindquist, 2009), normal cellular transcriptional machinery (Calderwood., S. K., Neckers., 2016), and rearrangement of chromatin (Fritah *et al.*, 2009; Neckers *et al.*, 2018).

1.11 Regulation of HSF1 function by post-translational modification

Regulation of HSF1 by PTLM in general, and phosphorylation in particular, has long been considered critical for HSF1 activity and/or expression levels. As the field has developed, it is now understood that the PTLM of HSF1 by phosphorylation, ubiquitination, acetylation, and SUMOylation fine-tunes HSF1 function, and links the transcription factor to cellular signaling pathways, particularly those regulated by kinases. PTLMs regulate numerous aspects of HSF1 function, including activation, repression, expression levels, localization, and protein-protein interactions (Table 1). The heptad repeats (HR)-A/B and regulatory domain (RD) are extensively regulated by phosphorylation, acetylation and binding of SUMO (small ubiquitin-like modifier), which is phosphate-dependent and mediated predominantly by kinases (Chu *et al.*, 1996, 1998; Knauf *et al.*, 1996; Kline & Morimoto, 1997; Xia *et al.*, 1998; Guettouche *et al.*, 2005; Wang *et al.*, 2006; Anckar & Sistonen, 2011; Dai & Sampson, 2016).

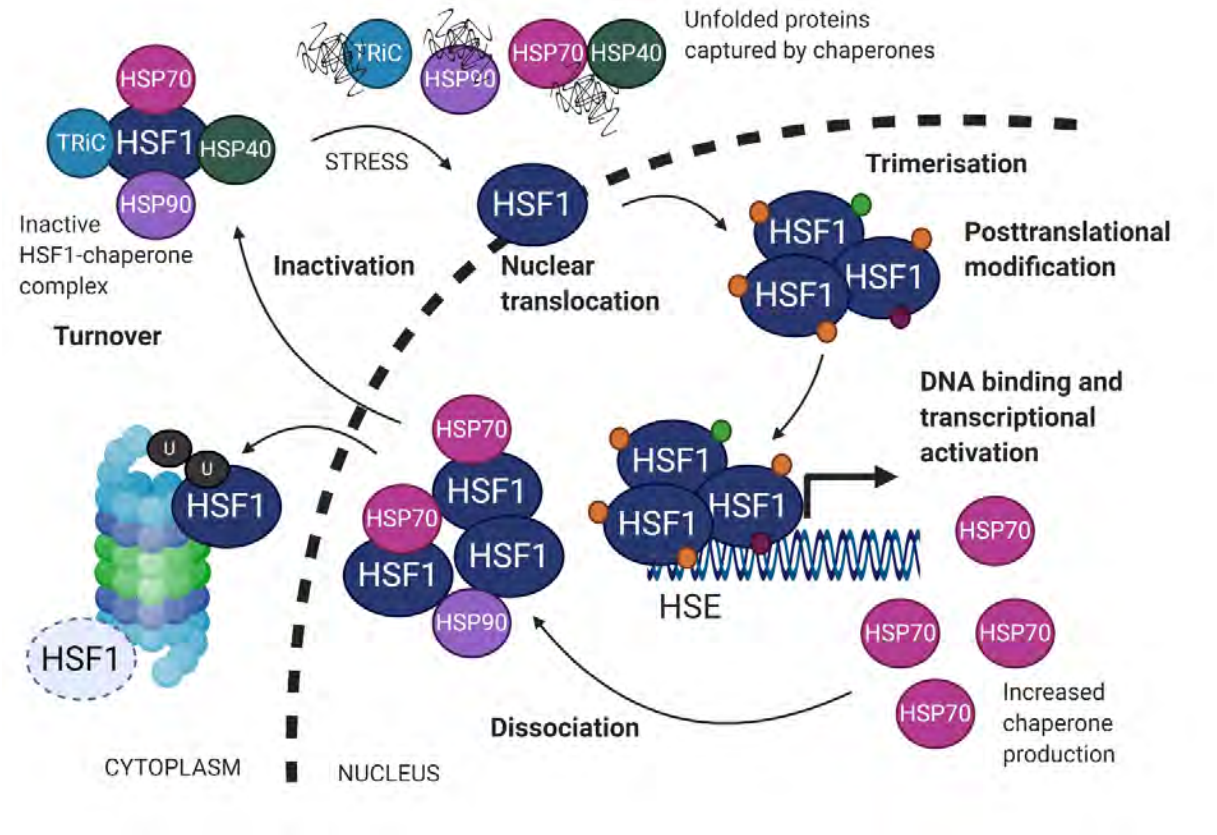


Figure 3: HSF1 cycle in response to stress
HSF1 is held in an inactive monomeric state in the cytoplasm by molecular chaperones. Stress induces protein misfolding, which titrates chaperones away and releases HSF1. HSF1 undergoes nuclear translocation, trimerization, and posttranslational modifications becoming competent to bind DNA and promote transcription of mainly chaperone genes. As chaperone levels increase, isoforms like HSP70 and HSP90 associate with HSF1 to remove it from the DNA and promote attenuation and recycling to the inactive form, and/or turnover via the proteasome.

The acetylation of HSF1 is linked to the stability and activity of the protein. HSF1 is acetylated at Lys80, Lys208, and Lys298 by the histone acetylase p300 and deacetylated by the protein sirtuin (SIRT1) (Raychaudhuri *et al.*, 2014; Zelin & Freeman, 2015). Acetylation at Lys80 blocks HSF1 interaction with DNA and therefore blocks transcriptional activity. The acetylation of Lys208 and Lys298 increases the stability of the HSF1 protein and prevents proteasomal degradation (Raychaudhuri *et al.*, 2014). The regulation of acetylation by p300 is linked to HSF1 levels under basal conditions and during recovery from heat stress. Interestingly, changes in the levels of acetylases and deacetylases are considered a contributing factor to changes in HSF1 levels during aging (Morley & Morimoto, 2004; Zelin *et al.*, 2012).

The major site for SUMOylation of HSF1 is at Lys298 in the RD, although this is dependent on phosphorylation of the adjacent Ser303 (Table 1). The SUMOylation of Lys298 was induced by stress, but not required for transcriptional activation of HSF1. Instead, this PTLM appears to be involved in the deactivation of HSF1 during the attenuation phase after the stress response (Hietakangas et al., 2006; Anckar & Sistonen, 2007; Anckar & Sistonen, 2011).

A total of 73 of the 153 Ser/Thr residues in HSF1 can be phosphorylated (Zheng *et al.*, 2016), and hyperphosphorylation of HSF1 is induced by heat shock. Some of the phosphorylated HSF1 residues, the kinases responsible, and the effect on HSF1 function are summarized in Table 1. For example, phosphorylation associated with deactivation of HSF1 includes Ser121 (which is related to metabolic stress sensors), Ser303, Ser307, and Ser363 (Kline & Morimoto, 1997; Wang *et al.*, 2003, 2006; Dai *et al.*, 2018). Phosphorylation of HSF1 at Ser230 by CaMKII (calcium/calmodulin-dependent protein kinase II), Ser320 by PKA (protein kinase A), Thr142 by CKII (casein kinase II), Ser410 by PLKII (polo-like kinase II) and Ser326 by mTOR and RAS/MAPK, activate HSF1 and are associated with stress-induced HSF1 activity (Holmberg *et al.*, 2001; Soncin *et al.*, 2003; Guettouche *et al.*, 2005; Zhang *et al.*, 2011; Chou *et al.*, 2012; Tang *et al.*, 2015; Dai & Sampson, 2016). The correlation between HSF1 hyperphosphorylation and transcriptional activity meant that for many years, phosphorylation was considered essential for transcriptional activity and a marker for active HSF1. However, mutational studies have demonstrated that blocking phosphorylation does not inhibit HSF1 transcriptional activation, although differential phosphorylation has been linked with cell-to-cell variation in responses and leads to variation in HSP90 levels (Zheng *et al.*, 2018). Consequently, phosphorylation is now considered a mechanism by which to modulate or fine-tune HSF1 activity, rather than an absolute requirement for activity. Also, phosphorylation is a mechanism by which to prolong HSF1 activity during heat stress (Zheng *et al.*, 2016).

Table 1: List of selected post-translational modifications in HSF1 and the effect on HSF1 activity

Mode	Position	Domain	Agent	Function	Reference
Phosphorylation	Ser307	RD	ERK1/2	Basal constitutive suppression	(Wang *et al.*, 2003)
Phosphorylation	Ser326	RD	RAS/MAPK, MEK, p38, mTOR	Activator of HSR under HS	(Guettouche *et al.*, 2005; Chou *et al.*, 2012; Dayalan Naidu *et al.*, 2016; Tang *et al.,* 2015)
Phosphorylation	Ser121	DBD	AMPK	Inhibition of nuclear shuttling and stabilization	(Dai *et al.*, 2015)
Phosphorylation	Ser303	RD	GSK3	Negative regulator of HSF1, aids in nuclear export of HSF1 and inhibitor of transactivation due to SUMOylation	(Hietakangas *et al.*, 2003; Wang *et al.*, 2003; Huang *et al.*, 2018)
Phosphorylation	Ser363	RD	JNK/SAPK	Inhibitor of HSF1 activation	(Dai *et al.*, 2000; Su *et al.*, 2017)
Phosphorylation	Ser419 Ser216	TAD RD	PLK PLK	Activator for the nuclear shuttling in HS Blocks of HSF1 degradation by CDC20 in mitosis	(Kim *et al.*, 2005, 2009)
Phosphorylation	Ser320	RD	PKA	Aids nuclear localization and activation	(Li *et al.*, 2000; Murshid *et al.*, 2010)
Phosphorylation	Thr142	HR-A/B	CK2	In HS, DNA binding and HSP transcriptional response	(Soncin *et al.*, 2003; Trembley *et al.*, 2009)
Phosphorylation	Thr 120	DBD	PIM2	Promoter of tumorigenesis, proteostasis and breast tumor survival	(Yang *et al.*, 2019)
SUMOylation	Lys298 (major)	RD	Ubc9 Phosphorylation at S303 required	Negative regulation of HSF1 transactivation	(Hietakangas *et al.*, 2003, 2006)
Acetylation	Lys80	DBD	P300, GCN5 (acetylation) SIRT1, HDAC7, HDAC9 (deacetylation)	Deacetylase activity to maintain HSF1 in a DNA-bound state	(Westerheide*et al.*, 2009)
Acetylation	Lys208	DBD	P300	Acetylation stabilizes HSF1 protein by blocking proteasomal degradation	(Raychaudhuri *et al.*, 2014)

1.12 Regulation of HSF1 by molecular chaperones

In non-stressed environments, HSF1 monomers are held in a folded but inactive conformation due to interactions with the HSP90, HSP70, HSP40, and the TRiC/CCT chaperonin complex (Shi *et al.*, 1998; Hu & Mivechi, 2003; Neef *et al.*, 2014). The long-standing dogma is that chaperones are subsequently liberated from HSF1 due to an increase in protein misfolding when the cells are exposed to proteotoxic or heat shock. This is referred to as the chaperone titration mechanism of HSF1 regulation (Voellmy & Boellmann, 2007; Zheng *et al.*, 2016).

Most studies have focused on the role of HSP90 and HSP70 in the regulation of HSF1 function. Dogma suggested that HSP90 was responsible for maintaining the inactive state of HSF1 (Zou *et al.*, 1998; Guo *et al.*, 2001; Boyault *et al.*, 2007) When HSP90 was pharmacologically inhibited, HSF1 was converted to the trimeric conformation with spontaneous DNA-binding activity (Ali *et al.*, 1998; Bharadwaj *et al.*, 1999). Although monomeric HSF1 binds to HSP90, the RD of the HSF1 trimer forms a complex with HSP90-FKBP52-p23 in cells exposed to heat shock (Ali *et al.*, 1998; Bharadwaj *et al.*, 1999; Guo *et al.*, 2001). If this complex is disturbed, there is a delay in HSF1 DNA-binding activity, suggesting an essential role of HSP90 complex for inhibition of the trimeric HSF1 (Ali *et al.*, 1998; Bharadwaj *et al.*, 1999). More recent studies suggest that the HSP90-HSF1 interaction is transient, and strong binding is only observed between HSF1 and the closed conformation (which is not readily observed in cells). These interaction studies were done with ATP dependent “closed” conformational mutants of HSP90, HSP90αE47A, and HSP90βE42A, and showed robust co-precipitation along with full-length HSF1. The stress-responsive HSP90α isoform had a higher affinity for HSF1, and overexpression of HSP90α downregulated HSF1 expression, establishing the concept of a negative feedback loop to repress the HSR (Kijima *et al.*, 2018). Flag-tagged HSF1 truncations and internal deletion mutants along with HSP90αE47A and HSP90βE42A were used to map the HSP70 and HSP90 binding sites and revealed strong binding with the “closed” HSP90 at the HR-A/B trimerization domain (part of the RD). The C-terminal half of HR-A/B (amino acids 183-214) could bind HSP90, and the interaction was

increased in HSF1 HR-C and TAD deletion mutants. Treatment with the HSP90 inhibitor 17-AAG led to increased transcriptional activity of an Hsp70 promoter-reporter above basal levels, and heat shock without 17-AAG, suggesting that HSP90 inhibition increases or activates the HSR. HSF1-ChIP data showed that a combination of HSP90 inhibition and heat shock resulted in a significant fold enrichment of HSF1 bound to the HSP70 promoter compared to only heat shock. Upon treatment with N-terminal inhibitors, HSF1 was seen to dissociate even from the closed conformational mutants of HSP90. N-terminal inhibition also increased HSF1 transcriptional activity culminating in an increase of HSP70 mRNA and prolonged the time span of HSP70 mRNA synthesis by HSF1 post heat shock. The authors interpret these data to suggest that HSP90 is involved in attenuation of the HSR in an ATP dependent manner, and that this is inhibited by N-terminal inhibitors (Kijima *et al.*, 2018).

HSP70 and HSF1 work through a negative feedback loop to ensure the coordinated function of HSF1 according to the environment or the expression of target genes (Zheng *et al.*, 2016, 2018; Krakowiak *et al.*, 2018). The transcription of molecular chaperones slows down post-stress exposure, and during this recovery phase, levels return to basal conditions. During this attenuation cycle, HSF1 also reverts from its active phosphorylated trimers to the inactive monomeric conformation, and this event co-occurs with upregulated levels of chaperones, particularly HSP70 (Abravaya, Klara, Myers MP, Murphy SP & Biology, 1992). It has been reported that HSP70 and the co-chaperone, Hdj1 (DNAJB1), interact directly with the transactivation domain (TAD) of HSF1, which results in the suppression of the HSF1 transcriptional machinery. Repression of the transcriptional potential of the GAL4-HSF1 activation domain fusion protein and endogenous HSF1 occurred when either HSP70 or Hdj1 were overexpressed in cells, although neither the inducible phosphorylation nor the DNA binding capacity of HSF1 was affected. Thus, while attenuation proceeds, there is suppression of the transcription of HSP genes, which takes place due to the interaction of HSP70 and the TAD domain of HSF1, validating this as an autoregulatory loop of the HSR (Shi *et al.*, 1998). Endogenous HSP70 interaction with the isolated RD and other domains of HSF1 has been observed, along with weak binding to the isolated HR-A/B domain of HSF1 (Kijima *et al.*, 2018;

Peffer *et al.*, 2019). This interaction with HSF1 is thought to occur via the HSP70 substrate-binding domain (Masser *et al.*, 2019; Peffer *et al.*, 2019) and involve HSP70 recognition sequences in the N and C termini of HSF1 (Krakowiak *et al.*, 2018; Peffer *et al.*, 2019). Mutation of these motifs abrogated the interaction with HSP70 and activated HSF1 transcriptional activity, suggesting that the HSP70-HSF1 interaction inactivates HSF1 (Krakowiak *et al.*, 2018; Peffer *et al.*, 2019). Indeed, deactivation of HSF1 after stress required transcriptional induction of HSP70 (Krakowiak *et al.*, 2018).The HSF1-HSP70 interaction occurred under basal conditions and was dissociated and re-associated during and after heat shock, suggesting that HSP70 serves as an “ON/OFF” switch for the HSR. Overexpression of HSF1 impeded growth in yeast; however, the phenotype was rescued by elevated levels of HSP70 and HSP40. Recent reports suggest that HSP70 may also regulate the ability of HSF1 to bind DNA. HSP70-HSF1 complexes can be classified as active complexes when HSE DNA binding is possible, or latent complexes unable to bind DNA. Increasing HSP70 levels increased the proportion of latent complexes, which would then reduce DNA binding by HSF1 (Masser *et al.*, 2019). Taken together, these data suggest that HSP70 may have an important role in both constitutive as well as stress induced HSR via regulation of HSF1, although the mechanism and its implications are not fully understood in humans (Zheng *et al.*, 2016, 2018; Krakowiak *et al.*, 2018). In addition, the role of many co-chaperones in the regulation of HSF1 has not been studied.

1.13 Motivation for the current study

Hop is an important cellular protein that regulates the characteristics of cancer cells. In particular, Hop levels are increased in cancers compared to healthy cells, and loss of Hop reverses malignant characteristics. Therefore, Hop is considered a possible drug target for cancer therapy, and therefore it is crucial to understand its biological functions fully. The Hop promoter contains HSE sequences and is regulated by HSF1 in response to stress. We identified changes in the level of HSF1 in a global proteomics analysis of Hop-depleted cells, suggesting that Hop may be a regulator of the stress-responsive transcription factor HSF1. The HSP70 and HSP90 chaperones, both of which interact with Hop, have been shown to regulate HSF1 function. However, a role for Hop in these processes has not been studied in detail. Herein, we study the possible relationship between Hop and HSF1 levels and activation to determine if this can be linked to stress resilience in Hop-depleted cell lines.

1.14 Hypothesis

Hop regulates HSF1 activity to influence the survival of cells under stress.

1.15 Aims

1. Analyze the effect of Hop depletion on HSF1 levels, activity and cell survival under basal and heat shock conditions.
2. Determine whether Hop regulation of HSF1 involves HSP70 or HSP90 chaperones.

CHAPTER 2: Materials and Methods

2.1 Plasmids used

The pGL3-Hsp70pro-Luc plasmid, which contains the HSP70 stress-inducible promoter regulating luciferase expression, was a kind gift from Stuart Calderwood (Olst *et al.*, 2012). The pLV-eGFP plasmid encoding GFP for mammalian expression was a gift from Pantelis Tsoulfas (Addgene#36083). The HA-HSP90αE47A plasmid was a kind gift from Len Neckers (NIH) (Kijima *et al.*, 2018). The pcDNA3-HA-Hop plasmid was designed in house and synthesized by Genscript (Mattison *et al.*, 2016).

2.2 Cell line maintenance and induction of shRNA expression

HEK293T cells (a gift from Sharon Prince, University of Cape Town) were stably transfected with TRIPZ plasmids encoding doxycycline-inducible shRNA against Hop (referred to henceforth as HEK-shHOP or KD) or a control, non-targeting shRNA (referred to as HEK-shNT or NT). HEK-shNT and HEK-shHOP cells were grown in DMEM with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, streptomycin and amphotericin (PSA), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids (NEAA), 500 µg/ml G418, and 2µg/ml puromycin under 9% CO_2 at 37°C. The induction of expression of Hop-specific and control shRNA was done for specified periods by daily addition of 1 µg/ml doxycycline to the complete growth media. Assays were conducted with and without doxycycline treatment in both the HEK-shNT and HEK-shHOP cells.

2.3 Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

Cell lysates were made in one of four lysis buffers (Table 1) containing protease inhibitor cocktail, PIC (Sigma-Aldrich,USA), and protein concentration was quantified using absorbance at 280 nm on a Nanodrop spectrophotometer. The proteins in the lysates were separated by SDS-PAGE following the standard modifications of the protocol of

Laemmli (1970), and the expression levels of various proteins were determined by western blot analysis according to the established methods (Towbin and Gordon, 1979). For SDS-PAGE, a 4% (v/v) stacking gel and 10% (v/v) resolving gel was used. The membranes were blocked with 1% (w/v) BLOTTO in 1XTBS (50mM Tris pH 7.5, 0.15M NaCl) for 1hr at room temperature, and incubated with primary antibody at 4°C overnight with primary antibodies Histone, tubulin, and HRP-Conjugated GAPDH antibodies were used as loading controls. The membranes were washed five times for 5-7 mins each wash with TBS-T [0.1% (v/v) Tween-20 (Sigma-Aldrich, USA) in 1XTBS] and incubated at room temperature with species-specific HRP-conjugated secondary antibody in 1XTBS-BLOTTO. The membranes were washed using TBS-T and visualized using Clarity luminescence substrate with the ChemiDocXRS+ system (Bio-rad).

Table 2:Lysis buffers used

Lysis Buffer	Composition
RIPA	50 mM Tris-HCl pH 8.0, 0.15 M NaCl, 1% (v/v) NP40, 0.1% (w/v) SDS, 0.5%(w/v) deoxycholic acid.
NP40	1% (v/v) NP40 in 1XPBS
SDS	1% (w/v) SDS in 50mMTris-HCl pH8.0, 10mMEDTA
CelLytic	Commercial product from Sigma Aldrich [C29780]

2.4 Mass Spectrometry Analysis

Biognosys, Switzerland conducted the SWATH mass spectrometry. Samples were shipped frozen in Biognosys' proprietary cell lysis buffer containing 8 M urea and 0.1 M ammonium bicarbonate. A total of 50 µg protein per sample was reduced using 5 mM Tris(2-carboxyethyl)phosphine (TCEP), alkylated using 10 mM iodoacetamide, and digested overnight with sequencing grade modified trypsin (Promega) at a protein:protease ratio of 50:1. C18 cleanup for mass spectrometry was carried out using MICROSpin columns (The Nest Group) according to the manufacturer's instructions. Peptides were dried down to complete dryness using a SpeedVac system and

redissolved in LC solvent A [1% (v/v) acetonitrile in water with 0.1% formic acid (FA)] containing Biognosys' iRT-peptide mix for retention time calibration. Peptides (calculated amount 1 µg per sample) were injected to an in-house packed C18 column (Magic AQ, 3µm particle size, 200 Å pore size, Michrom; 75 µm inner diameter, New Objective) on a Thermo Scientific Easy nLC nano-liquid chromatography system for all mass spectrometric analysis. LC solvents were A: 1% (v/v/) acetonitrile in water with 0.1% (v/v) FA; B: 3% (v/v) water in acetonitrile with 0.1% (v/v) FA.

The LC gradient for shotgun analysis was 0-72% (v/v) solvent B in 120 mins (non-linear) followed by 72-100% B in 2 mins and 100% B for 8 mins (total gradient length was 130 mins). The column length was 30 cm. LC-MS/MS shotgun runs for peptide discovery were carried out on a Thermo Scientific Q Exactive mass spectrometer equipped with a standard nano-electrospray source. Full MS covered the *m/z* range of 400-1200 with a resolution of 70'000 (AGC target value was $1e^6$) and was followed by 12 data-dependent MS2 scans with a resolution of 17'500 (AGC target value was $5e^5$). MS2 acquisition precursor isolation width was 2 *m/z*, while normalized collision energy was centered at 25 (10% stepped collision energy), and the default charge state was 2+. In HRM-MS™ mode, full MS covered the *m/z* range of 400-1220, and all-ion fragmentation (AIF) scan range was 200-1800 m/z.

LC-MS/MS datasets were analyzed using the MaxQuant software package v 1.3.0.5, and searches were performed against the UniProt HUMAN database. HRM-MS™ maps were analyzed with Spectronaut™ software using the library generated from MaxQuant searches of shotgun runs. The applied false discovery rate cutoff was 0.01. A total of 1061 protein groups with a p-value of pairwise comparisons ≤ 0.05 were identified, and the networks of these analyzed.

2.5 Survival and expression analysis in normal and tumor samples

Publically available data on survival and expression levels of HSF1 and Hop in normal and cancer patient samples from The Cancer Genome Atlas Network (TCGA;

http://cancergenome.nih.gov/) and Genotype-Tissue Expression project (GTex;https://gtexportal.org/home/) (GTex Consortium, 2013) were analyzed using the Gene Expression Portal Interactive Analysis (GEPIA) portal (http://gepia.cancer-pku.cn/) (Tang *et al.*, 2017).

2.6 Heat shock and inhibitor treatment

Heat shock was performed at 42°C for 1 hr without recovery, unless otherwise indicated. The Hsp90 inhibitor 17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) (Sigma-Aldrich, USA) was used at a concentration of 1 μM, and untreated cells served as a control. The HSF1 inhibitor KRIBB11 (Sigma-Aldrich, USA) and the HSP70 inhibitor JG98 (a gift from Dr.Jason Gestwicki, UCSF, USA) were used at a concentration of 5 μM, and DMSO treatment served as a control.

2.7 Long-term clonogenic survival assay

HEK-shNT and HEK-shHOP cells remained untreated or were treated with doxycycline for 72 hrs before seeding. Cells were seeded at 1000 cells/ml with/without doxycycline in a 6-well plate, allowed to sit overnight and treated with heat shock or inhibitor treatment, and allowed to grow for 8-12 days with the addition of fresh complete media every third day. When colonies reached approximately 50 cells, they were fixed using a mixture of 3:1 methanol:acetic acid for 2 mins and washed with 1ml of PBS. The colonies were stained using 0.5% (w/v) crystal violet in methanol, washed in distilled water, and air-dried. Images of wells were captured, and then crystal violet dye solubilized in equal volumes of 1% (v/v) acetic acid and absorbance read at 595nm.Results were normalized to untreated, basal HEK-shNT sample. Images are representative of biological triplicate experiments.

2.8 Luciferase reporter assay

HEK-shNT and HEK-shHOP cells (either untreated or treated with doxycycline to induce shRNA for 72 hrs) were seeded at a density of $1X10^5$ cells/ml and transfected with pGL3-

Hsp70pro-Luc plasmid and pLV-eGFP plasmid (as transfection efficiency control) in a ratio of 1:1 (µg DNA to µl reagent) with X-tremeGENEHP transfection reagent (Roche). The transfection mixture was kept at 22°C for 15-20 mins before addition to the cells. Total transfection time was 48 hrs. Cells were heat-shocked for 1 hr, followed by a recovery time of 8 hrs and then lysed with 10% (v/v) Triton-X 100. Luciferase activity was quantified after addition of FLAR buffer (20mM Tricine pH 7.4, 100µM EDTA, 2.67mM$MgSO_4$,17mM DTT, 250 µMATP, 250 µM D-luciferin) (Olst *et al.*, 2012). The fluorescence due to EGFP was detected at an excitation of 485 nm and emission at 525 nm. The reporter activity was determined as the ratio of the luminescence to the EGFP signal for the corresponding treatment.

2.9 Nuclear-cytoplasmic fractionation

HEK-shNT and HEK-shHOP cells remained untreated or were treated with doxycycline for 96 hrs to induce shRNA expression. Nuclear-cytoplasmic fractionation was done with the NE-PER kit as per the manufacturer's instructions (Thermo Fischer Scientific) and the levels of proteins in fractions determined by SDS-PAGE and western blot analysis.

2.10 Immunofluorescence and confocal microscopy

HEK-shNT and HEK-shHOP cells remained untreated or were treated with doxycycline for 72 hrs to induce expression of the relevant shRNA and then seeded in complete growth media on glass coverslips coated with 0.1 mg/ml poly-L-lysine (Sigma Aldrich, USA). Cells were permeabilized with 0.1% (v/v) PBS-T for 15 mins at RT and blocked with 1% (w/v) BSA-PBS-T for 1 hr at RT. Primary anti-HSF1 antibody, anti-Hop, and anti-pSer326:HSF1 were added at a dilution of 1:100 at 4°C overnight, and the coverslips washed for 5 mins three times in 0.1%(w/v) BSA/TBS-T. Secondary species-specific antibody, anti-rabbit, and anti-mouse was diluted in 0.1%(w/v) BSA/TBS-T in a ratio of 1:500, followed by incubation at RT in thedark for 1hr. All the primary antibodies were validated for specificity by the supplier or in the literature in terms of specificity. Experiments validating the low non-specificity of secondary antibodies were routinely

conducted in the laboratory (data not shown). Coverslips were washed three times for 5 mins each with 0.1%(w/v) BSA/TBS-T. Finally, a wash was done with sterile water containing 1 µg/ml Hoechst 33342 in order to stain the nucleus. DAKO was used as the mounting medium. Samples were analyzed using the Zeiss LSM780 Confocal Microscope, and quantification was done using Image J. Colocalization analysis was performed using the ICA plugin (Manders, Stap, Brakenhoff, Driel, & Aten, 1992; Li et al., 2004).

2.11 Transfection and HA immunoprecipitation

Transient transfection was performed in HEK-shNT and HEK-shHOP cells (with or without prior doxycycline treatment for 72 hrs) with 1 µg of HA-HSP90AαE47A plasmid with1 µl of XtremeGeneHP transfection reagent (Roche) (1:1 ratio) in 100 µl of OptiMEM in accordance to the manufacturer's instructions. Untransfected cells served as a negative control. Co-immunoprecipitation was done using the Anti-HA Co-IP kit (Sigma-Aldrich) following the manufacturer's instructions, and isolated complexes analyzed by SDS-PAGE and western blot analysis. The whole-cell lysate was used for input. Primary antibodies were used to probe for overnight at 4°C, and species-specific secondary antibodies were used at RT for 1 hr.

2.12 Endogenous HSP70 immunoprecipitation (IP)

HEK-shNT and HEK-shHOP (untreated or treated with doxycycline for 72 hrs) were seeded at 80% confluency in 10 cm dishes, washed twice with PBS at RT and incubated for 5 mins at RT with 10ml PBS containing the cross-linker 2mM DTSSP (Sigma-Aldrich,USA). The DTSSP cross-linker was quenched with the addition of 10 mM glycine, and the cells washed once with PBS. Cells were harvested and lysed on ice using RIPA buffer (Table 1) with 1% (v/v) PIC for 15 mins followed by centrifugation at 16000*xg* for 10 mins, and protein concentration was determined using absorbance at 280 nm on the Nanodrop2000 spectrophotometer (Thermo Scientific). A total of 800 µg of protein was used for IP. Lysates were incubated at 4°C overnight with 5 µg of HSP70/HSC70 antibody

(W27, Santa Cruz Biotechnology, SCBSC-24) and then 20 µl Agarose-IgA/G beads were added (Santa Cruz Biotechnology, SC-2003). Beads alone were used as a negative control. IP samples were collected by centrifugation at 16000*xg* at 4°C for 2 mins and washed four times with PBS. The washed samples were eluted in SDS-PAGE sample buffer containing β-mercaptoethanol, boiled for 5 mins at 95°C and analyzed by SDS-PAGE followed by western blot.

2.13 Resazurin cytotoxicity and cell proliferation assay

HEK-shNT and HEK-shHOP cells treated for 72hrs with 1µg/ml and without doxycycline were seeded at a density of $1X10^5$cells/ml in 96 well plates. For cytotoxicity, cells were treated with a range of doubling dilutions of KRIBB11, starting from the concentration of 200µM for 72 hrs. For proliferation assay, cells were incubated at 37°C for 72 hrs. In both cases, after the 72-hr incubation, 0.54 mM resazurin solution was added and incubated for 4 hrs. The plate was read at an excitation of 560 nm and an emission of 590 nm. The half-maximal inhibitory concentration (IC_{50}) was calculated by non-linear regression using GraphPad Prism 4.0.

2.14 Enzyme-linked immunosorbent assay (ELISA) for HSF1 levels

A total of 1 mg of whole cell lysate in 100 ul PBS or known amounts of purified human HSF1 (Abcam, ab204184) were coated on to wells of a high-binding 96-well plate (Greiner Bio-one, 655001) overnight at 4°C. Wells were blocked with 200µl of 3% (w/v) BSA in PBS for 1 hr at room temperature. Primary anti-HSF1 antibody (Abcam, ab2923) was used at 1:2500 dilution in 1% (w/v) BSA-PBS for overnight, washed 3X in PBS and species-specific HRP-conjugated secondary antibody in 1% (w/v) BSA-PBS used at 1:10,000 for 2 hrs at room temperature, followed by three washes. For HRP detection, HRP Buffer [25.7 mM Citric Acid, pH 5.0, 48.6mM Na_2HPO_4, 1 mg/ml TMB in DMSO, 0.001%(v/v) H_2O_2] was added, and the reaction stopped with the addition of 1 M H_2SO_4, and absorbance read at 450 nm. The concentration of HSF1 per mg of lysate was determined from the standard curve.

2.15 Protein-DNA binding assay

A microtitre based assay to measure HSF1 DNA binding was modified from published protocols to detect DNA-protein interactions (Underwood *et al.*, 2013). Cell lysates were prepared from untreated or doxycycline-treated HEK-shNT and HEK-shHOP cells under basal or heat shock conditions (42°C for 1 hr). A total of 500 µg of whole cell lysate in PBS per well was incubated at 4°C in a 96-well high binding plate overnight, followed by blocking in 3% (w/v) BSA-PBS for 1 hr at room temperature. A stock concentration of 10 mM biotin-labelled or unlabeled competitor oligonucleotides containing an HSE target sequence (Yoon *et al.*, 2011)(Table 3) were annealed in acetate buffer (1 M potassium acetate, 300 mM HEPES, pH 7.5) by heating to 94°C and cooling at a rate of 1°C/minute according to the manufacturer's instructions (IDT).

Table 3: Oligonucleotide sequences used to generate double-stranded DNA probes to detect HSF1 binding

Name	Sequence	Label
HSE oligonucleotide F	GATCTAGAACGTTCTAGAACGTTCTAGAACGT TCTA	5'-biotin
HSE oligonucleotide R	CTAGATCTTGCAAGATCTTGCAAGATCTTGCA AGAT	5'-biotin
HSE competitor F	GATCTAGAACGTTCTAGAACGTTCTAGAACGT TCTA	None
HSE competitor R	CTAGATCTTGCAAGATCTTGCAAGATCTTGCA AGAT	None

The annealed double-stranded oligonucleotides (10 nM) with or without unlabeled competitor (100 nM) were added to the wells containing the cell lysates and incubated in Kingston buffer [24mM HEPES, pH7.9, 120mMKCl, 4mMMgCl_2, 0.24mM EDTA, 0.6mMPMSF, 0.6mMDTT, 24% (v/v) glycerol] (Baler *et al.*, 1993) at RT with shaking for 2 hrs followed by 2 washes in PBS. Streptavidin-HRP (Thermo Scientific) was incubated

at 1:1000 in PBS at room temperature for 2 hrs, followed by two washes in PBS. The HRP activity was developed using HRP buffer and read at 450 nm.

2.16 Statistical Analysis

All data shown are representative of at least 3 independent experiments unless otherwise stated. Statistical analysis was performed by either t-test or ANOVA in GraphPad Prism 4.0, and values below 0.05 were taken as significant.

CHAPTER 3: Results:

3.1 Global proteomics indicated that Hop depletion in HEK293Tcellsresulted in HSF1 depletion

To study the effect of Hop depletion on cell biology, we conducted a global proteomics analysis by SWATH-MS to identify the levels of cellular proteins in triplicate HEK293T whole cell lysates expressing either control or Hop-specific shRNA. Those proteins showing significant differences in levels between Hop-depleted and control lysates were analyzed using Cytoscape. A total of 1061 proteins were significantly different between the two groups ($p \leq 0.05$). Hop (STIP1 in Table 1, $\log_2$FC of -2.548, p-value 0.0000) was the most significantly downregulated protein, which suggested successful depletion in shHOP-expressing cells compared to control cells expressing shNT. Of the significantly deregulated proteins, our analysis identified changes in the levels of the stress-responsive transcription factor, HSF1, as well as several of its reported target genes (Table 2). In HEK293Tcells expressing shRNA against Hop, HSF1 was significantly downregulated ($\log_2$FC of -0.414, p-value 0.0484), while putative HSF1 target genes were both downregulated (Table 2, blue) and upregulated (Table 2, red). These data suggested that Hop and HSF1 expression might be linked.

Table 4: Changes in protein levels in HEK293T cells upon Hop depletion

UNIPROT	P VALUE	LOG_2FC HOP KD vs. NT	FULL NAME
STIP1_HUMAN	0.0000	-2.548	Stress-induced-phosphoprotein 1
AGAL_HUMAN	0.0000	-1.125	Alpha-galactosidase A
E41L1_HUMAN	0.0236	-0.832	Band 4.1-like protein 1
A4_HUMAN	0.0000	-0.831	Amyloid-beta precursor protein
APOO_HUMAN	0.0062	-0.602	Apolipoprotein O/MICOS complex subunit MIC26
PCKGM_HUMAN	0.0023	-0.583	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial
HMOX1_HUMAN	0.0000	-0.545	Heme oxygenase 1
VATG1_HUMAN	0.0021	-0.483	V-type proton ATPase subunit G 1
TPP1_HUMAN	0.0003	-0.482	Tripeptidyl-peptidase 1
RPOM_HUMAN	0.0101	-0.462	DNA-directed RNA polymerase, mitochondrial
SPSY_HUMAN	0.0040	-0.437	Spermine synthase
NDRG1_HUMAN	0.0000	-0.415	Protein NDRG1
HSF1_HUMAN	**0.0484**	**-0.414**	**Heat shock factor protein 1**
AMRP_HUMAN	0.0001	-0.406	Alpha-2-macroglobulin receptor-associated protein
RL26_HUMAN	0.0069	-0.406	60S ribosomal protein L26
RL1D1_HUMAN	0.0004	-0.391	Ribosomal L1 domain-containing protein 1
4F2_HUMAN	0.0000	-0.391	4F2 cell-surface antigen heavy chain
IRS4_HUMAN	0.0039	-0.384	Insulin receptor substrate 4
ERP29_HUMAN	0.0000	-0.373	Endoplasmic reticulum resident protein 29
ROA2_HUMAN	0.0016	-0.373	Heterogeneous nuclear ribonucleoproteins A2/B1
MOC2B_HUMAN	0.0002	-0.370	Molybdopterin synthase catalytic subunit
MLP3B_HUMAN	0.0493	-0.366	Microtubule-associated proteins 1A/1B light chain 3B
DPOA2_HUMAN	0.0234	-0.350	DNA polymerase alpha subunit B
LAP2A_HUMAN	0.0046	-0.343	Lamina-associated polypeptide 2, isoform alpha
CY1_HUMAN	0.0000	-0.341	Cytochrome c1, heme protein, mitochondrial
SQSTM_HUMAN	0.0227	-0.336	Sequestosome-1
PAPOA_HUMAN	0.0046	0.267	Poly(A) polymerase alpha
CDC73_HUMAN	0.0000	0.268	Cell division cycle protein 73
IF2B2_HUMAN	0.0000	0.269	Insulin-like growth factor 2 mRNA-binding protein 2
IF5_HUMAN	0.0029	0.269	Eukaryotic translation initiation factor 5
GLO2_HUMAN	0.0217	0.278	Hydroxyacylglutathione hydrolase, mitochondrial

FTO_HUMAN	0.0004	0.285	Alpha-ketoglutarate-dependent dioxygenase FTO
GMPPB_HUMAN	0.0078	0.299	Mannose-1-phosphate guanyltransferase beta
IQGA1_HUMAN	0.0000	0.305	Ras GTPase-activating-like protein IQGAP1
UBE2O_HUMAN	0.0000	0.305	E3-independent E2 ubiquitin-conjugating enzyme
AMPB_HUMAN	0.0000	0.305	Aminopeptidase B
TCTP_HUMAN	0.0016	0.316	Translationally-controlled tumor protein
SRP14_HUMAN	0.0000	0.325	Signal recognition particle 14 kDa protein
ODO1_HUMAN	0.0361	0.336	2-oxoglutarate dehydrogenase, mitochondrial
SUGT1_HUMAN	0.0136	0.341	Protein SGT1 homolog
USP9X_HUMAN	0.0169	0.344	Probable ubiquitin carboxyl-terminal hydrolase FAF-X
CPNE1_HUMAN	0.0000	0.351	Copine-1
FXR1_HUMAN	0.0000	0.359	Fragile X mental retardation syndrome-related protein 1
HSP71_HUMAN	0.0000	0.359	Heat shock 70 kDa protein 1A
FAAA_HUMAN	0.0000	0.361	Fumarylacetoacetase
PSMG4_HUMAN	0.0156	0.362	Proteasome assembly chaperone 4
IMP4_HUMAN	0.0040	0.362	U3 small nucleolar ribonucleoprotein protein IMP4
FA98B_HUMAN	0.0000	0.369	Protein FAM98B
WDR11_HUMAN	0.0301	0.373	WD repeat-containing protein 11
FERM2_HUMAN	0.0000	0.378	Fermitin family homolog 2
SBP1_HUMAN	0.0232	0.383	Methanethiol oxidase
MAAI_HUMAN	0.0142	0.402	Maleylacetoacetate isomerase
ARL2_HUMAN	0.0106	0.433	ADP-ribosylation factor-like protein 2
TBCE_HUMAN	0.0001	0.452	Tubulin-specific chaperone E
IPO4_HUMAN	0.0104	0.453	Importin-4
ASSY_HUMAN	0.0013	0.496	Argininosuccinate synthase
IF4A2_HUMAN	0.0015	0.508	Eukaryotic initiation factor 4A-II
NEST_HUMAN	0.0000	0.516	Nestin
NBN_HUMAN	0.0422	0.527	Nibrin
DPOD2_HUMAN	0.0092	0.586	DNA polymerase delta subunit 2
TIA1_HUMAN	0.0035	0.608	Nucleolysin TIA-1 isoform p40
FANCI_HUMAN	0.0153	0.683	Fanconi anemia group I protein
RO60_HUMAN	0.0051	0.716	60 kDa SS-A/Ro ribonucleoprotein
KTHY_HUMAN	0.0429	0.742	Thymidylate kinase
EPIPL_HUMAN	0.0000	0.746	Epiplakin

3.2 Hop and HSF1 levels are correlated in tumors

Our global proteomics was conducted in the HEK293T cell line. To determine if there was any relationship between Hop and HSF1 expression in tumor samples, we analyzed publicly available data from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression project (GTex) (Figure 4).

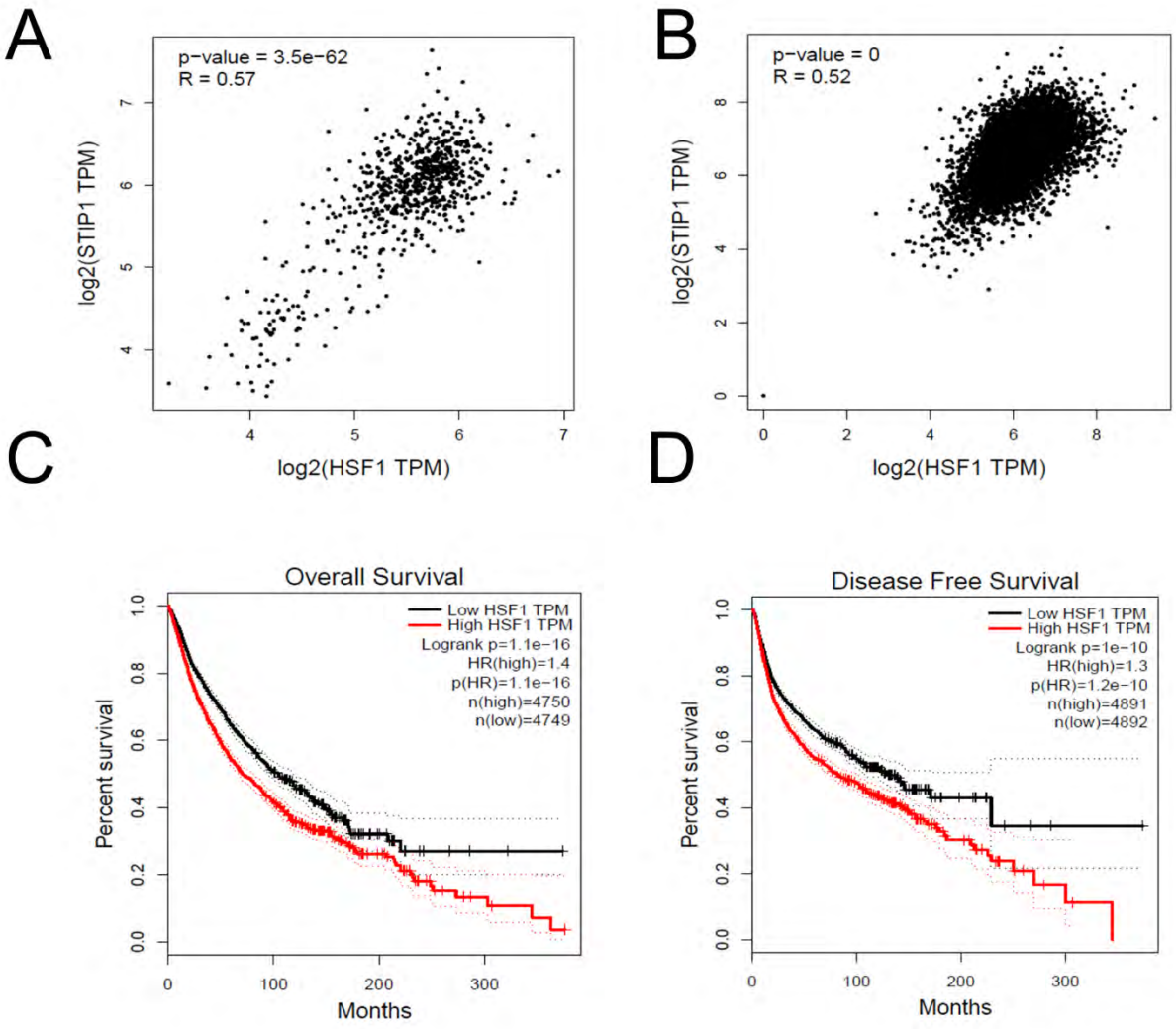


Figure 4: Correlation between Hop and HSF1 expression in cell lines and tumor samples.
Correlation of mRNA levels for Hop and HSF1 in (A) normal tissues from GTex data and (B) tumor samples from The Cancer Genome Atlas (TCGA). TCGA tumor data showing the relationship between HSF1 levels and (C) overall and (D) disease-free survival.

Analysis of the data from TCGA and GTex consortium showed a significant positive correlation between HSF1 and Hop mRNA levels in both normal (Figure 4A) and tumor samples (Figure 4B). In addition, the survival data from the TGCA tumor database showed an increase in survival with a lower expression of HSF1 in terms of both overall and disease-free survival (Figure. 4C and D). Taken together, these data suggested a relationship between Hop and HSF1 expression, which may lead to changes in HSF1 transcriptional activity. Furthermore, if high HSF1 levels are prognostic for poor tumor outcomes (Santagata *et al.*, 2011), then the regulation of HSF1 by Hop may be relevant in cancer.

3.3 Validation of reduction of HSF1 protein levels by western blot analysis

Global proteomics may give false results, and therefore it is necessary to validate the observations using alternative methods. To validate the results which suggested a downregulation of HSF1 following the silencing of Hop using shRNA against Hop or the shNT control, we analyzed the level of HSF1 protein by western blot analysis (Figure 5). Since the shRNA expression in our study was induced with doxycycline, untreated HEK-shNT and HEK-shHOP cells were included as an additional control. We used four different lysis buffers, including RIPA (Figure 5A), 1% (w/v) SDS lysis buffer (Figure 5B), 1% (v/v) NP40 lysis buffer (Figure 5C) and the commercial lysis buffer CelLytic M (Figure 5D). The specific details of the buffers can be found in the methods section. Lysates prepared in all buffers showed the same trend of reduced levels of HSF1 upon Hop depletion in the HEK-shHOP cells treated with doxycycline to induce the Hop-specific shRNA. In contrast, the lysates from HEK-shNT cells, both with and without doxycycline treatment and the HEK-shHOP cells without doxycycline, showed higher and similar levels of HSF1 protein. We observed multiple bands for HSF1 in the western blot which is consistent with different modifications of HSF1 reported in the literature (Zou *et al.*, 1998).

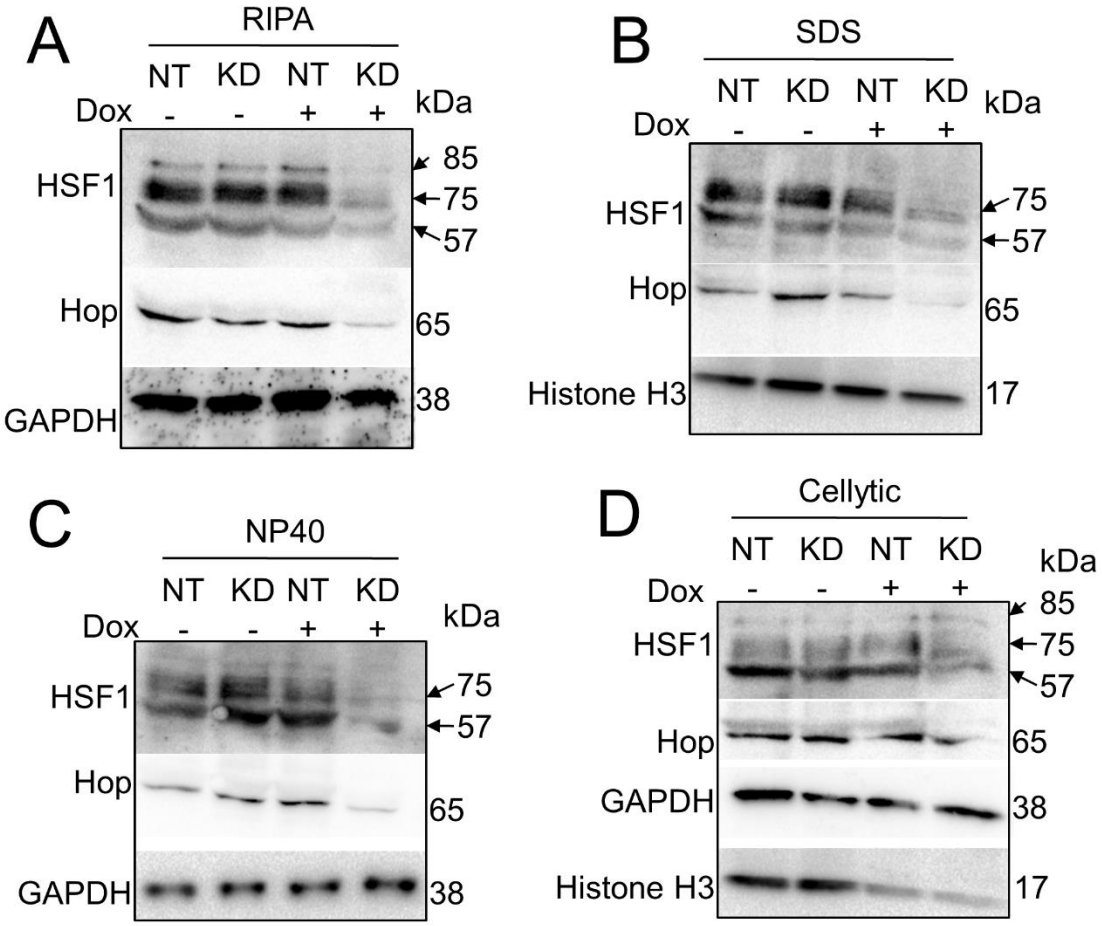


Figure 5: Hop depletion reduced HSF1 protein levels in cell lysates.

Western blot analysis of levels of Hop and HSF1 in lysates of HEK-shNT and HEK-shHOP cells with and without doxycycline treatment prepared using cell lysates prepared in (A) RIPA [50 mM Tris-HCl pH 8.0, 0.15 M NaCl,1%(v/v) NP40,0.1% (w/v) SDS, 0.5 % (w/v) deoxycholic acid] (B) 1% SDS [1% (w/v) SDS in 50 mMTris-HCl pH8.0, 10mMEDTA] (C) 1% NP40 [1% (v/v) NP40 in 1XPBS] (D) CelLytic M lysis buffers. GAPDH and Histone H3 served as loading controls.

To further validate the depletion of HSF1 upon Hop depletion, we conducted a doxycycline washout assay (Figure 6). Lysates were prepared for western blot from HEK-shHOP and HEK-shNT cells after 72 hrs of treatment with or without doxycycline, and then at 24-hr time periods after subsequent culture in doxycycline free medium for a further 72 hrs (Figure 6A). Western blot analysis showed stable levels of HSF1 and Hop in the HEK-shNT lysates with or without doxycycline, and in the HEK-shHOP lysates without doxycycline (Figure 6B). In contrast, there were reduced levels of Hop and HSF1

in HEK-shHOP lysates after 72 hrs of doxycycline treatment, and these levels increased from 24 hrs post doxycycline removal. This shows reversal of the reduction of HSF1 occurs upon removal of doxycycline in HEK-shHOP cells. Taken together, these data suggested that HSF1 levels are reduced in HEK-shHOP cells in response to the depletion of Hop levels by shRNA.

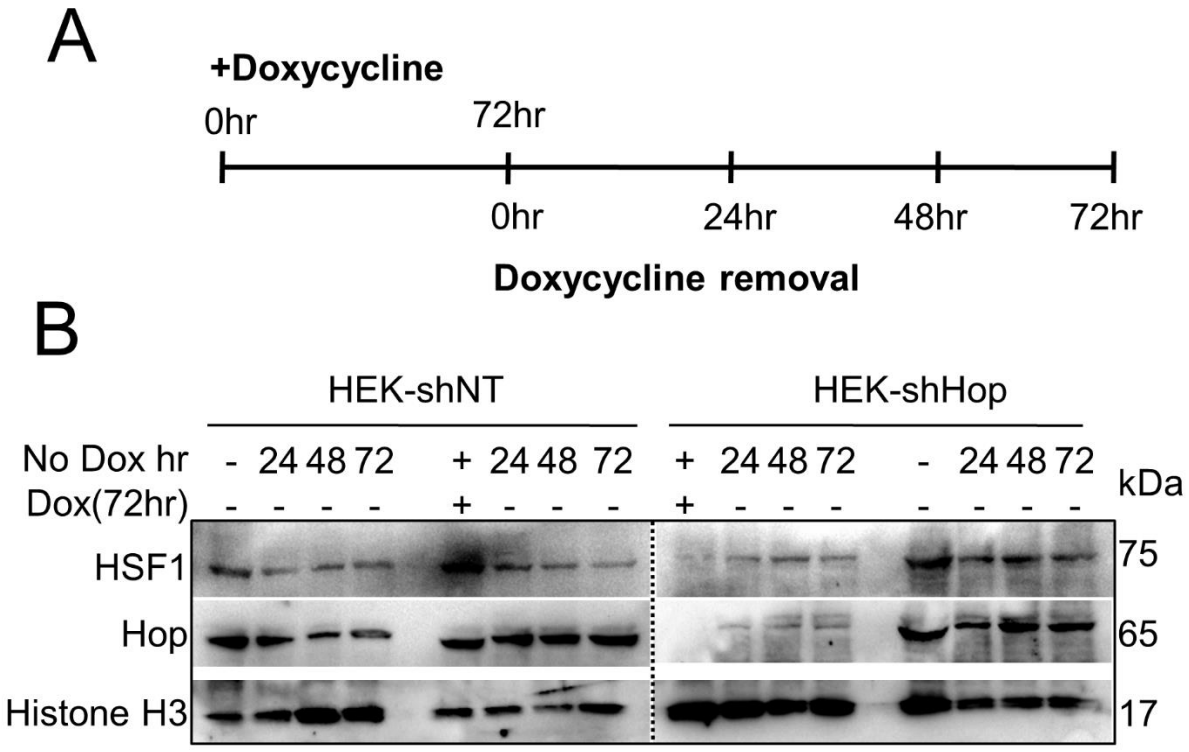


Figure 6: Recovery of HSF1 protein levels upon doxycycline removal.

(A) Schematic diagram showing the experimental design of time course of effect of doxycycline (Dox) removal on levels of Hop and HSF1. Samples were collected for western blot analysis for the indicated time points (hrs). (B) Western blot analysis showing levels of HSF1 and Hop in HEK-shNT and HEK-shHOP cells with (+) and without (-) doxycycline treatment and/or doxycycline withdrawal throughout the experiment. Histone H3 served as a loading control.

3.4 Basal reductions in HSF1 protein levels upon Hop depletion are reversed in response to heat shock

Western blot analysis is only semi-quantitative, and hence we used enzyme-linked immunosorbent assay (ELISA) to quantify the levels of HSF1 in HEK-shNT and HEK-shHOP lysates with and without doxycycline treatment under basal and heat shock conditions (Figure 7).

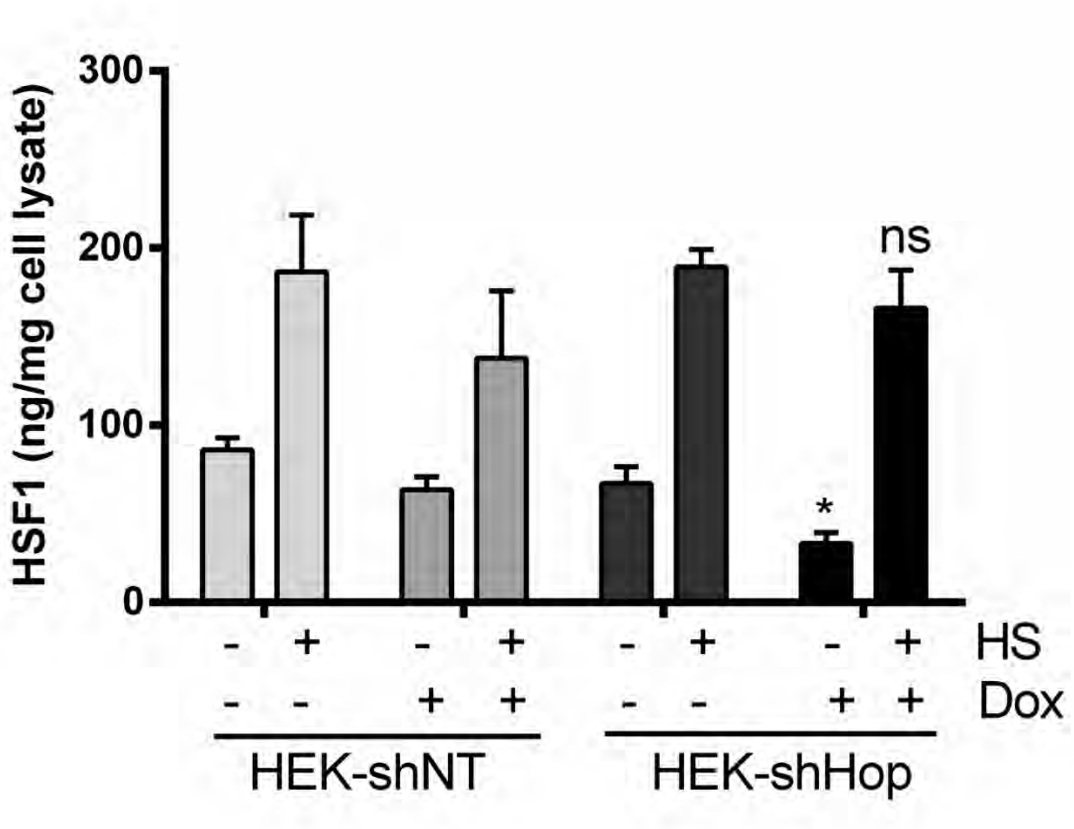


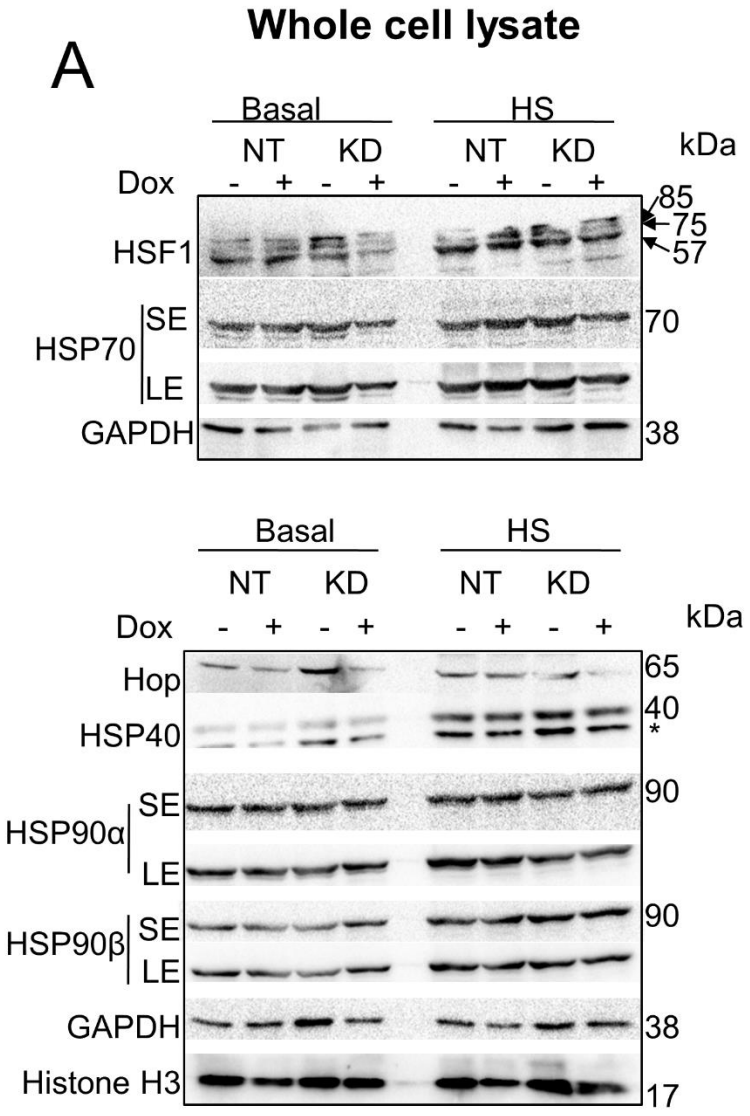
Figure 7: Quantification of changes in HSF1 levels under basal and heat shock conditions.

ELISA to quantify average levels of HSF1 per mg of whole cell lysate (±SD, n=3) from samples prepared in RIPA buffer from HEK-shNT and HEK-shHOP cells with (+) and without (-) doxycycline (Dox) treated under basal and heat shock (HS) conditions. Statistical analysis was done by unpaired t-test comparing doxycycline-treated HEK-shHOP cells with other treatments (*$p<0.05$, ns not significant)

HSF1 levels were not significantly different in the HEK-shNT lysates with and without doxycycline treatment (86.1±11.5ng HSF1/mg cell lysate compared to 63.6±12.6 ng HSF1/mg cell lysate, respectively) or in the HEK-shHOP lysates without doxycycline (67.2±16.4 ng HSF1/mg cell lysate). In contrast, the HEK-shHOP lysates treated with doxycycline showed a significant reduction in HSF1 levels to approximately 50% of the untreated HEK-shHOP cells (33.3±10.3 ng HSF1/mg cell lysate, $p<0.05$). Upon heat shock, the levels of HSF1 were significantly increased compared to basal levels for all lysates, with there being no significant difference in the levels between HEK-shHOP lysates with and without doxycycline (189.5±16.8ng HSF1/mg cell lysate compared to 166.1±37.2 ng HSF1/mg cell lysate, respectively). These data suggest that HSF1 levels are reduced under basal conditions with Hop depletion, but Hop depletion does not prevent heat shock-induced increases in HSF1 levels.

3.5 HSF1 subcellular distribution is altered in Hop-depleted cells

HSF1 is known to shuttle between the nucleus and the cytoplasm. Therefore, we analyzed if Hop depletion resulted in alterations in the subcellular localization of HSF1. We conducted biochemical fractionation of the cytoplasmic and nuclear fractions from HEK-shNT and HEK-shHOP cells with and without doxycycline under basal and heat shock conditions (Figure 8).



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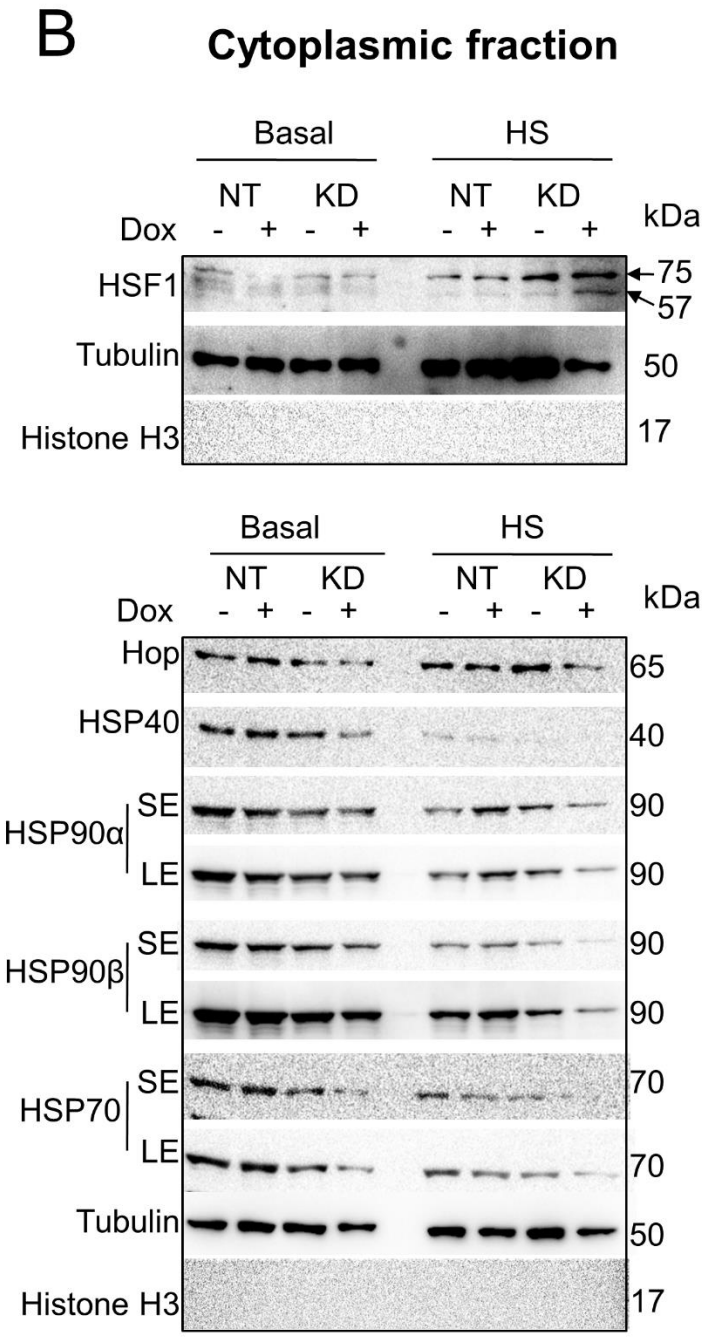

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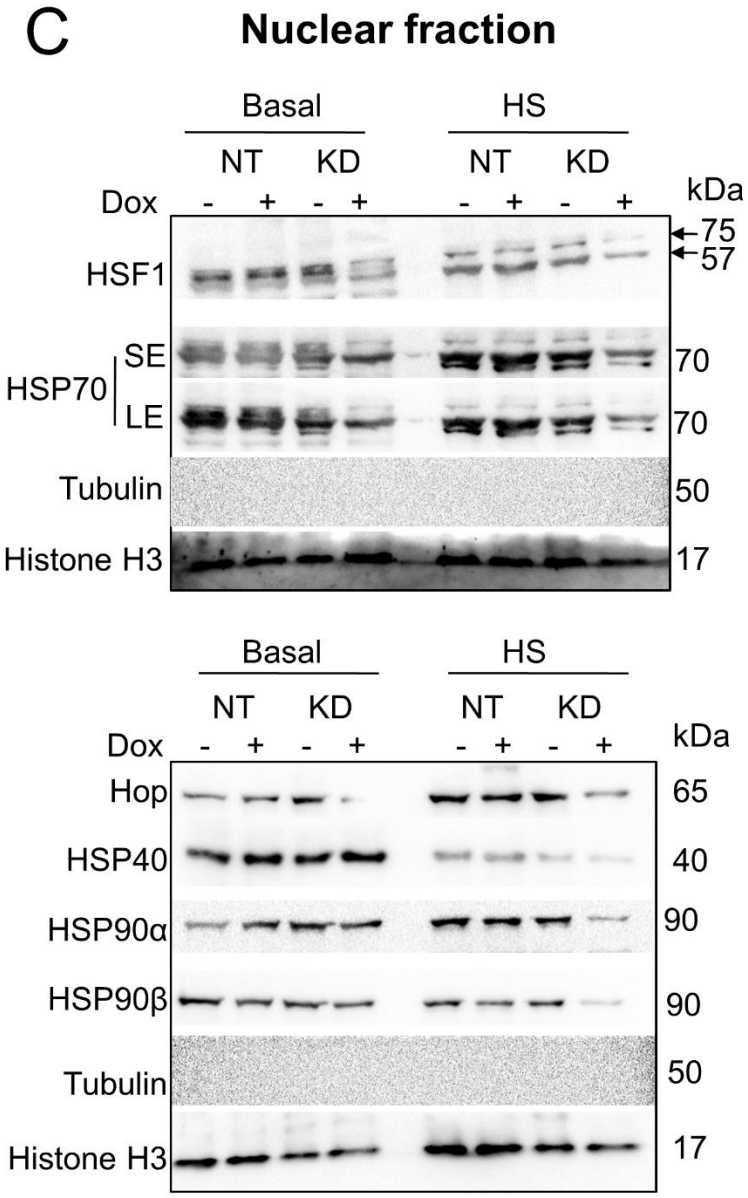


Figure 8: HSF1 accumulation in the cytoplasm with Hop depletion under heat shock.

HEK-shNT and HEK-shHOP lysates treated with (+) and without (-) doxycycline under basal and heat shock (HS) conditions (42 °C for 1 hr without recovery) were fractionated and protein levels in(A) whole cell lysate, (B) cytoplasmic fraction and (C) nuclear fractions analyzed by western blot analysis. GAPDH, tubulin and histone H3 served as loading controls and markers for successful fractionation of cytoplasm and nucleus. SE: short exposure, LE: long exposure. Images are representative of independent duplicate experiments.

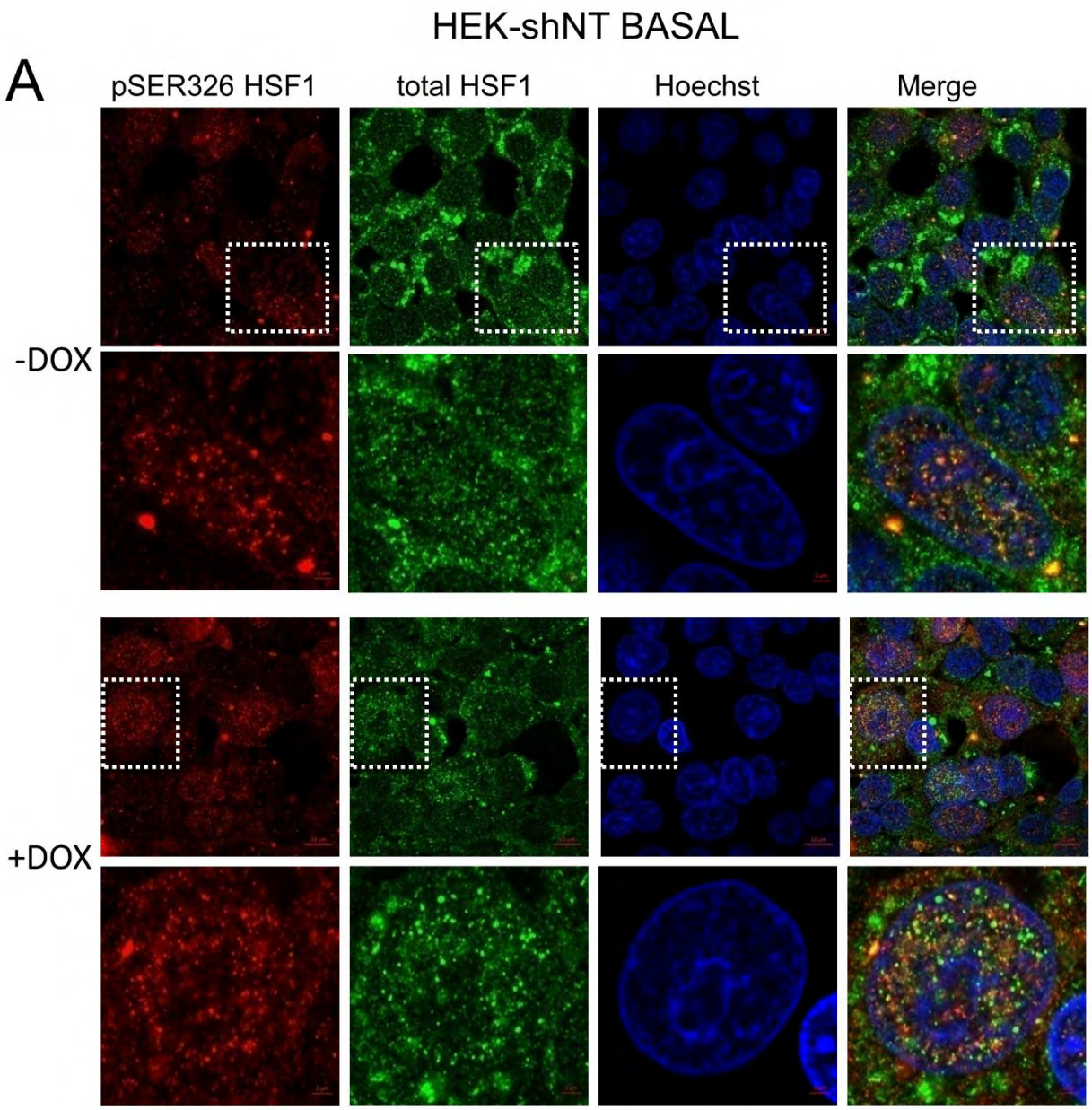
Western blot analysis in the whole cell lysate under basal conditions indicated that HSF1 levels were reduced in HEK-shHOP cells treated with doxycycline, compared to the controls of HEK-shHOP without doxycycline and HEK-shNT with and without doxycycline (Figure 8A). The levels of HSF1 were increased in both HEK-shNT and HEK-shHOP cells irrespective of doxycycline treatment upon heat shock (Figure 8A), which was consistent with the ELISA data (Figure 7). HSP70 levels were slightly reduced in the Hop-depleted cells compared to the controls under both basal and heat shock conditions, but no major changes were seen in levels of HSP90α and HSP90β. HSP40 levels were increased in response to heat shock irrespective of Hop depletion (Figure 8A).

In the cytoplasmic fractions, western blot showed similar levels of HSF1 in all lysates under basal conditions. However, an accumulation of HSF1 levels in the cytoplasmic fraction was seen upon heat shock only in the HEK-shHOP cells treated with doxycycline to induce Hop depletion (Figure 8B). Reduced protein expression was observed in the cytoplasmic fraction of the HEK-shHOP lysate with doxycycline and heat shock for HSP40, HSP90α, HSP90β, and HSP70. Tubulin served as a positive loading control for cytoplasm and histone as a negative control to confirm the successful isolation of the cytoplasmic fraction (Figure 8B).

In the nuclear fractions, western blot analysis showed reduced HSF1 levels for the HEK-shHOP cells with doxycycline compared to the controls under both basal and heat shock conditions (Figure 8C). HSP70 levels were also lower in the Hop-depleted lysates compared to controls in both basal and heat shock conditions, whereas HSP40 levels were low in all cell lines upon heat shock. HSP90α and HSP90β levels in Hop-depleted cells were reduced in the nucleus upon heat shock in Hop-depleted lysates compared to controls but were not different under basal conditions (Figure 8C).

The biochemical fractionation studies suggested that Hop depletion resulted in an altered nuclear to cytoplasmic ratio for HSF1 upon heat shock. To support these data, we conducted confocal microscopy on HEK-shNT and HEK-shHOP with and without doxycycline treatment, under basal and heat shock conditions (Figure 9). We analyzed

the localization and distribution of total HSF1 (in green) and pSER326:HSF1 (in red), which is a classical site of HSF1 phosphorylation in response to heat shock (Boellmann *et al.*, 2004; Guettouche *et al.*, 2005). Under basal conditions, the HEK-shNT with and without doxycycline showed a diffuse staining pattern across the cytoplasm and nucleus for both the total HSF1 and pSER326:HSF1 (Figure 9A). Heat shock treatment increased the proportion of total and pSER326:HSF1 signal in the nucleus (Figure 9B). For the HEK-shNT without doxycycline, a similar diffuse HSF1 staining pattern to the HEK-shNT cells was observed under basal conditions (Figure 9C). In contrast, in the HEK-shHOP cells with doxycycline treatment, the staining pattern of HSF1 and pSER326:HSF1 was punctate and predominantly in the nucleus (Figure 9C). Upon heat shock, the HEK-shHOP cells without doxycycline showed an increase in total HSF1 and pSER326:HSF1 in the nucleus, similar to the HEK-shNT cells. In the HEK-shHOP cells treated with doxycycline, the punctate nuclear staining for total HSF1 and pSER326:HSF1 was observed, as was an apparent accumulation of total but not pSER326:HSF1 in the cytoplasm. (Figure 9D).



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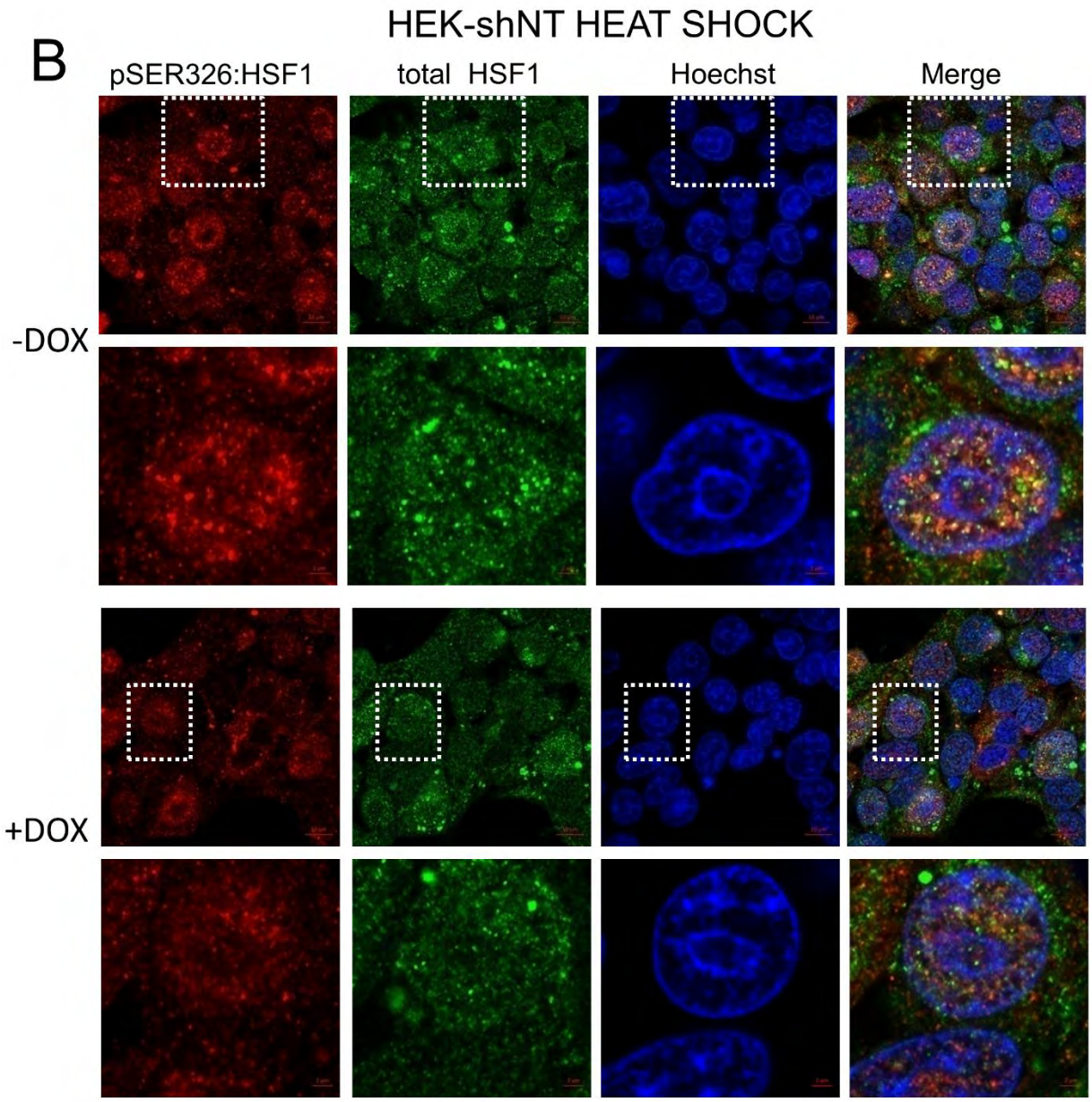

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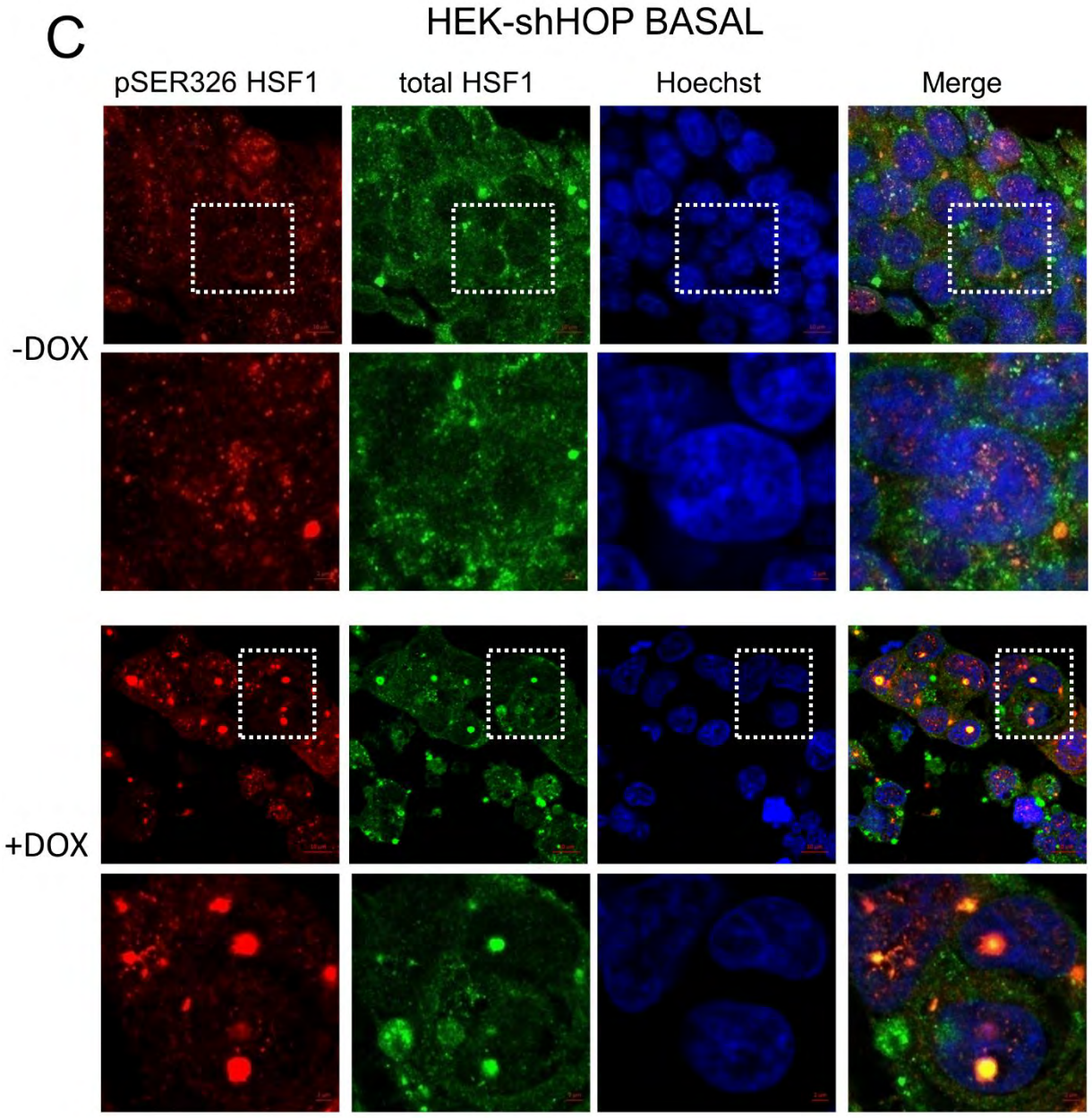

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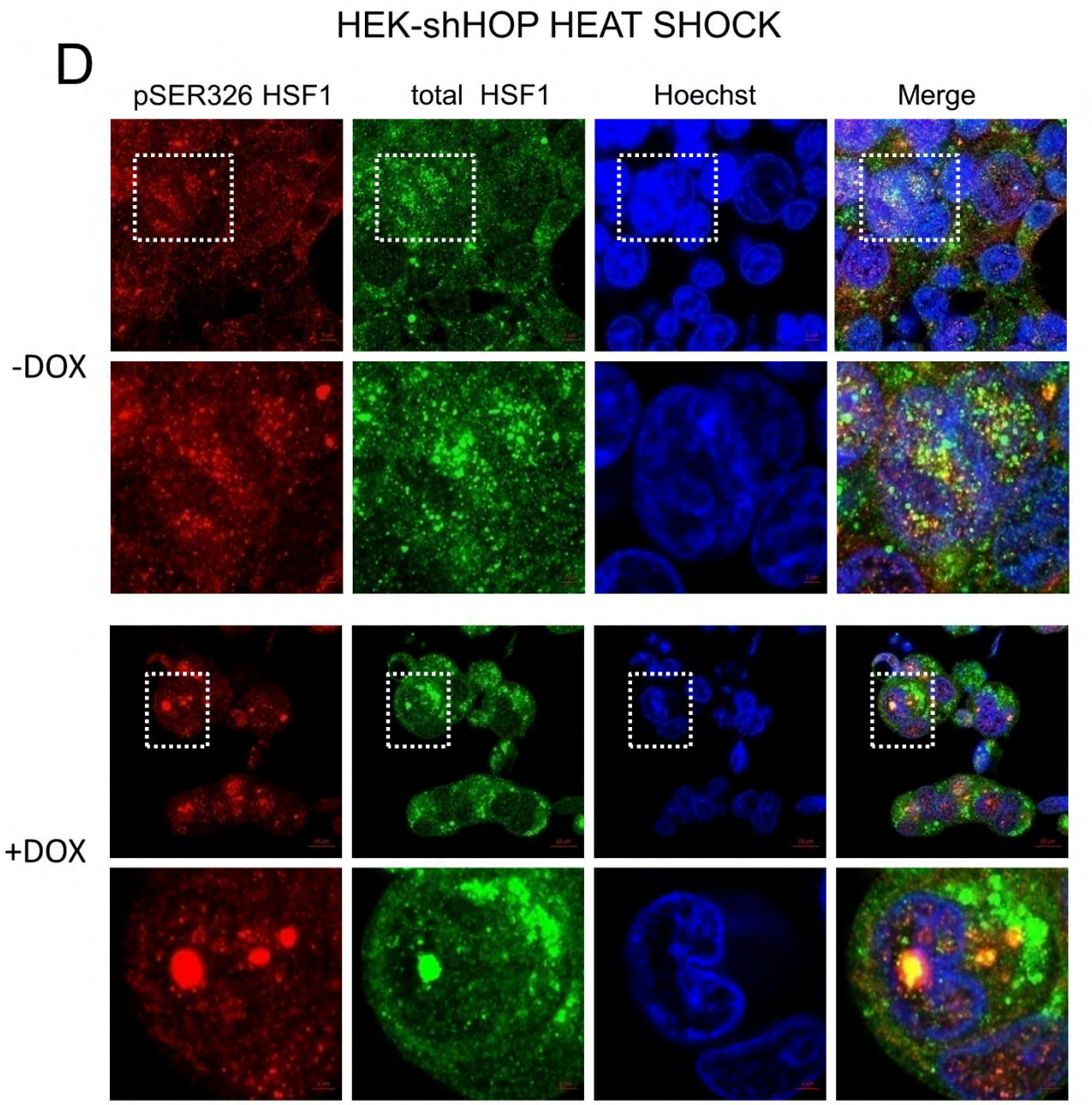


Figure 9: Changes in subcellular localization of HSF1 upon Hop depletion.

Confocal microscopy to detect total HSF1 (green) or HSF1 phosphorylated on Ser326 (red) in HEK-shNT with and without doxycycline under (A) basal and (B) heat shock conditions (42 °C for 1 hr without recovery), and in HEK-shHOP with and without doxycycline treatment under (C) basal and (D) heat shock conditions. Hoechst 33324 was used to stain the nucleus (blue). Upper panels in each grouping show cells captured at 63x magnification. The lower panels show magnified images of the areas represented in white boxes.

3.6 HSF1-DNA binding and transcriptional activity altered in Hop-depleted HEK293T cells

Having shown changes in the HSF1 protein levels and subcellular localization, we next analyzed whether these changes would culminate in a change in HSF1 activity (Figure 10). First, we assessed the ability of HSF1 in cell lysates to bind a biotinylated oligonucleotide containing a canonical HSE under basal and heat shock conditions (Underwood *et al.*, 2013) (Figure 10A). The binding of the HSE was inferred from the absorbance after detection with streptavidin-HRP conjugate. The data shown are from equivalent amounts of cell lysate, have had background binding from a control peptide containing a mutant HSE subtracted, and have been normalized to the HEK-shNT cells without doxycycline under basal conditions (Figure 10A). There was equivalent binding of the HSE containing peptide above background under basal conditions in the HEK-shNT cell lysates with or without doxycycline, and the HEK-shHOP cell lysates without doxycycline. However, HEK-shHOP cells treated with doxycycline led to a reduction in the amount of HSE containing peptide bound. Upon heat shock, there was a significant increase relative to basal conditions in the binding of the HSE containing peptide in the HEK-shNT irrespective of doxycycline treatment and in the HEK-shHOP lysates lacking doxycycline. However, while there was a minor increase in HSE peptide binding in the heat-shocked HEK-shHOP lysates with doxycycline treatment, this was not significantly different from the basal conditions. The reduction in HSE peptide binding in the HEK-shHOP with doxycycline in response to heat shock was, however, significantly different from the heat shock response detected in other cell lysates

In addition, we analyzed the transcriptional activity from an HSF1 regulated reporter based on the HSP70 promoter under basal and heat shock conditions. HSF1 transcriptional activity was determined as the amount of luciferase activity relative to the EGFP transcription control. Equivalent levels of transcriptional activity were detected in the untreated and doxycycline-treated HEK-shNT, cells as well as the HEK-shHOP without doxycycline under basal conditions. However, basal levels of transcriptional activity were reduced in HEK-shHOP cells with doxycycline. Heat shock-induced a

significant increase in transcriptional activity in the HEK-shNT cells irrespective of doxycycline treatment, and in the HEK-shHOP cells without doxycycline. In contrast, there were significantly reduced levels of transcriptional activity in the heat-shocked HEK-shHOP cells with doxycycline compared to the other cells (Figure 10B). Taken together, these data suggest that HSF1 HSE binding and transcriptional activity are reduced in Hop-depleted cells.

3.7 Hop-depleted cells are more sensitive to HSF1 inhibition

Given the reduced levels and activity of HSF1 in the Hop-depleted cells, we tested whether Hop depletion affected the sensitivity of cells to an inhibitor of HSF1, known as KRIBB11. KRIBB11 inhibits HSF1 transcription by impeding the recruitment of p-TEFb in transcriptional complex (Yoon *et al.*, 2011). The analysis showed that HEK-shHOP with doxycycline treatment had an IC_{50} value for KRIBB11 (2.8±1.2 μM) that was approximately ten-fold lower than the respective control cell lines (HEK-shNT with and without doxycycline (36.3±1.1 and 37.1±1.0 μM, respectively) and HEK-shHOP without doxycycline (43.0±1.2 μM) (Table 5). This suggested that Hop-depleted cells are more sensitive to HSF1 inhibition which would correlate with reduced basal levels of HSF1 upon Hop depletion.

Table 5: Cytotoxicity of KRIBB11 against Hop-expressing or depleted cell lines.

Cell line and treatment*	KRIBB11 IC_{50}±SEM (μM)
HEK-shNT-Dox	36.3± 1.1
HEK-shNT+Dox	37.1±1.0
HEK-shHOP-Dox	43.0±1.2
HEK-shHOP+Dox	2.8±1.2

*Dox: doxycycline; +: with; -: without.

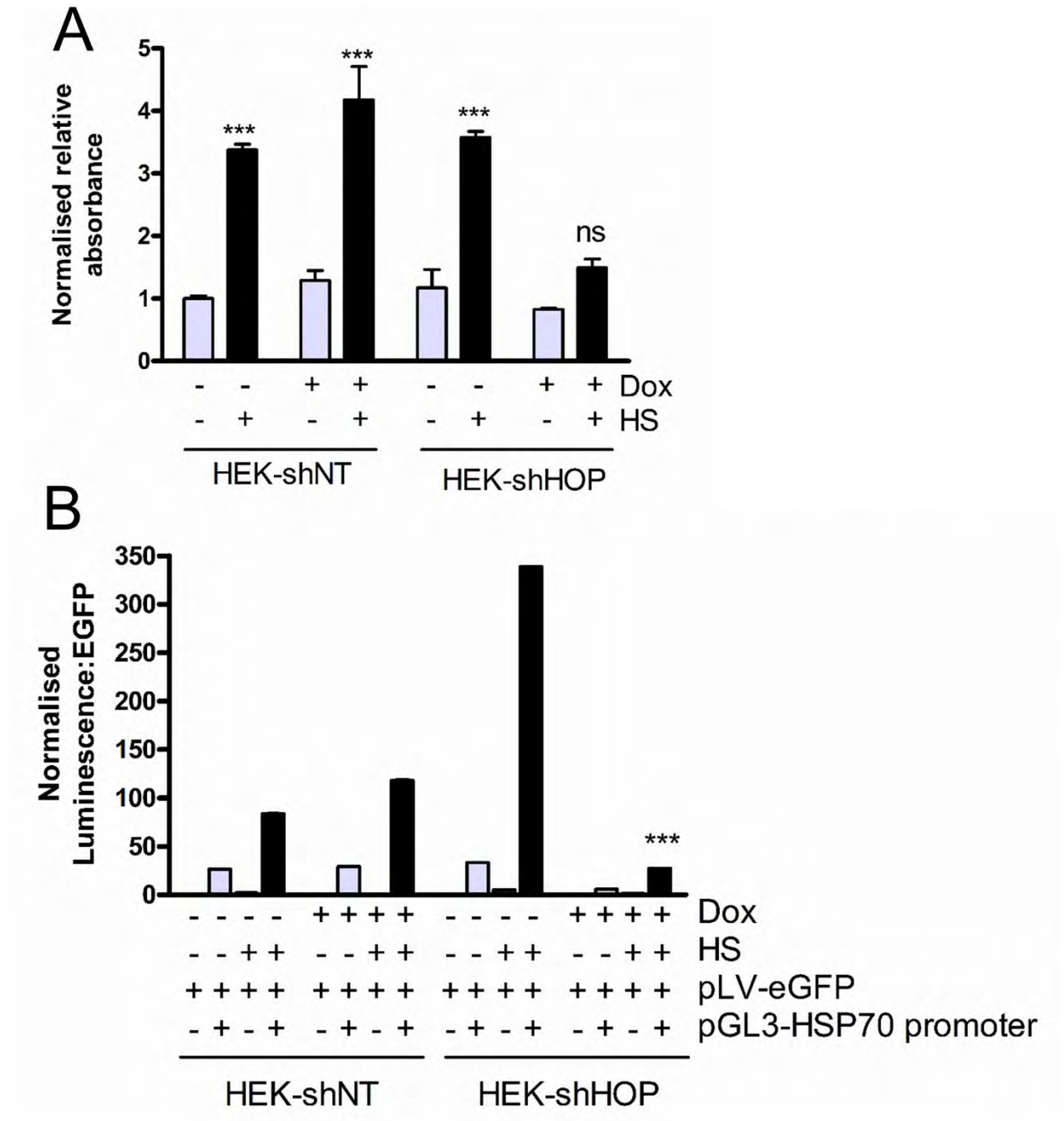


Figure 10: HSF1 activity is reduced in Hop-depleted cells.

Analysis of HSF1 activity in HEK-shNT and HEK-shHOP cells with and without doxycycline treatment under basal and heat shock conditions using (A) binding of DNA probe containing a canonical HSE sequence and (B) transcriptional activity from an HSF1 responsive reporter plasmid measured as luciferase activity normalized to GFP fluorescence from the pLV-eGFP plasmid used as a transfection efficiency control. In (A), the data have undergone subtraction of background data from the binding of a competitor HSE probe and then were normalized to the basal HEK-shNT sample without doxycycline. Error bars represent ±SD (n=3). Statistical analysis was done by two-way ANOVA with Bonferroni post-test (***p<0.0001).

3.8 Interaction of HSF1 and Hop in cell lines

Our analysis had suggested that HSF1 levels and activity were altered in Hop-depleted cells. We, therefore, attempt to understand how this occurred in more detail. We first tested for possible interactions between HSF1 and Hop in cell lines (Figure 11). Confocal microscopy showed that HSF1 and Hop colocalized in both wild type and HEK-shNT cells, as well as the colon cancer cell line HCT116 and cervical cancer cell line HeLa (Figure 11A). In the wild type HEK293T and the HEK-shNT cells, both HSF1 and Hop were predominantly located in the cytoplasm. In the HCT116 and the HeLa cell lines, both HSF1 and Hop staining was detected in the nucleus and cytoplasm. Quantitative colocalization analysis was conducted in Image J to calculate the degree of pixel-on-pixel colocalization using Pearson's correlation coefficients (R). R values of 1 represent perfect correlation, while R values of -1 represent complete exclusion. In all cell lines analyses, there was a significant colocalization of Hop and HSF1 with average R values (±SD) above 0.7 (Figure 11A). To test if HSF1 and Hop could be isolated in complex, we performed immunoprecipitations from HEK-shNT cells transfected with a plasmid expressing HA-Hop or an untransfected lysate as a control followed by western blot analysis. The whole cell lysate was used as input to validate successful transfection and show the presence of endogenous HSP70 and HSF1. The HA-IP detected the presence of both HA-Hop and HSF1 in the IP fractions, but not the control. In addition, the Hop interacting chaperone HSP70 was also detected in the complex. Taken together, these data demonstrate the colocalization of HSF1 and Hop in cells and indicate that HSF1 and Hop can be isolated in a joint complex that can accommodate HSP70 (Figure 11B).

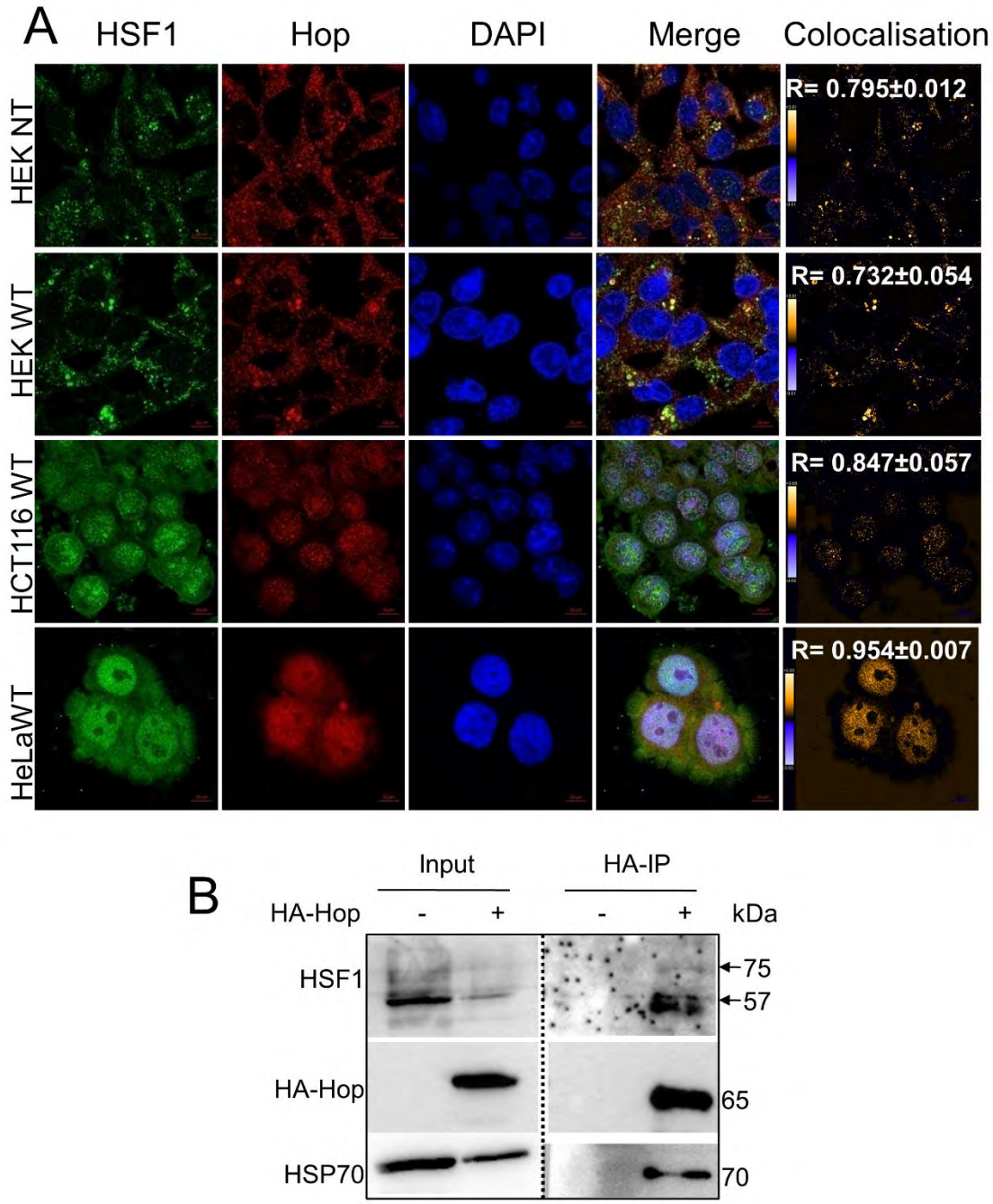


Figure 11: Interaction of HSF1 and Hop in cell lines.

(A) Confocal microscopy for HSF1 (green) and Hop (red) in HEK-shNT, HEK293 WT, HCT116 WT (wild type), and HeLa WT cell lines. Nuclei were stained using Hoechst 33324 (blue).The R value shown in white writing on the merged image represents the average Pearson correlation coefficient (±SD, from a minimum of 5 different frames). (B) Detection of proteins in complex with HA-Hop by western blot analysis after HA-Hop immunoprecipitation from transfected HEK-shNT cell lysates.

3.9 Effect of Hop depletion on interaction ofHSF1 with HSP70 and HSP90

HSF1 is known to interact with HSP70 and HSP90 chaperones, both of which interact with and are regulated by Hop. Therefore, we assessed whether Hop depletion would alter the interaction of HSF1 with these chaperones. First, we used immunoprecipitation of endogenous HSP70 and analyzed complexes by western blot from HEK-shNT and HEK-shHOP cells with and without doxycycline treatment (Figure 12).

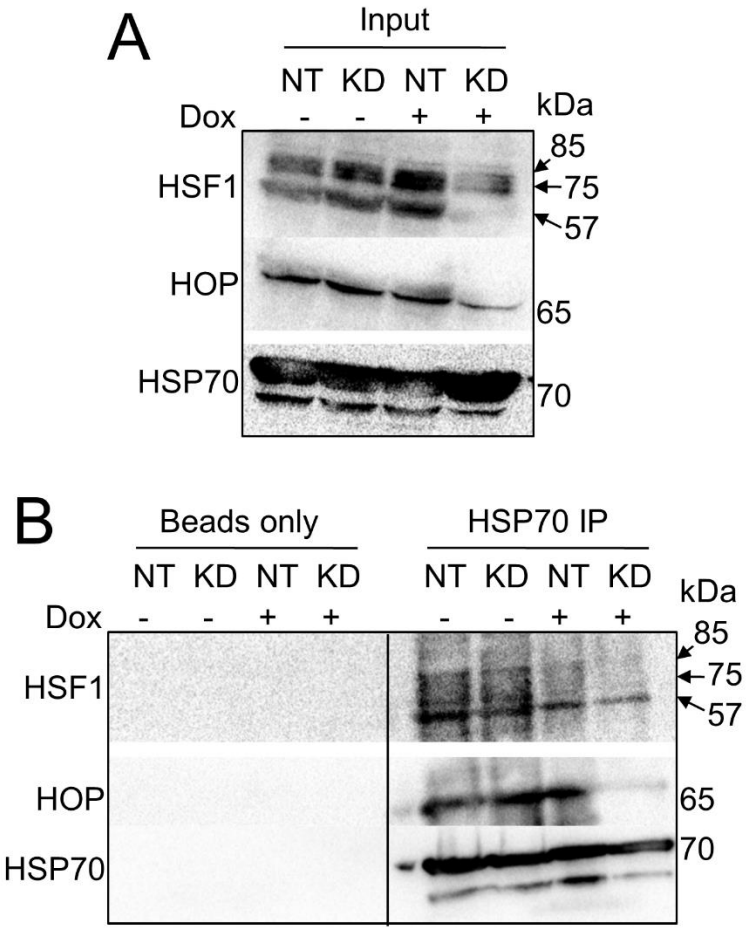


Figure 12: Hop depletion reduces HSF1 in HSP70 complexes.

HSP70 was isolated from HEK-shNT and HEK-shHOP lysates with and without doxycycline treatment (A) and (B) associating proteins in complexes analyzed by western blot analysis in control (beads only) and HSP70 immunoprecipitations.

The input showed reduced HSF1 levels in the Hop-depleted cells compared to controls (Figure 12A). Equivalent levels of HSP70 were isolated in the immunoprecipitations for all treatments. HSF1 was detected in all the HSP70 complexes, but the amount of HSF1 isolated with HSP70 complexes in Hop-depleted cells was lower than the other conditions (Figure 12B).

Next, we tested the effect of Hop depletion on HSF1 interaction with HSP90. The endogenous HSF1-HSP90 interaction is known to be transient and difficult to isolate since HSF1 binds preferentially to the N-terminal dimerized HSP90 conformation, which is rarely detected in cells (Zou *et al.*, 1998; Kijima *et al.*, 2018). Therefore, we made use of an HSP90αE47A mutant that traps the chaperone in a closed conformation to study the HSF1-HSP90 interaction (Kijima *et al.*, 2018). The HSP90α isoform was used as this showed higher binding to HSF1 than HSP90β (Kijima *et al.*, 2018). HEK-shNT and HEK-shHOP cells with and without doxycycline treatment were transfected with the HA-HSP90αE47A plasmid, followed by HA immunoprecipitation and western blot analysis. Untransfected cell lysates were used as a control for the IP. HSF1 levels were reduced only in lysates with depleted Hop levels (Figure 13A). HA-HSP90αE47A was successfully isolated in complex with HSF1. In this case, there was no change in the amount of HSF1 isolated when Hop was depleted. Interestingly, however, there was a reduction in the amount of HSP70 detected in the complexes with Hop depletion (Figure 13B). Future experiments conducting reciprocal IPs using anti-HSF1 antibodies and detection of HSP90, HSP70 and Hop in associating complexes would increase confidence in these data.

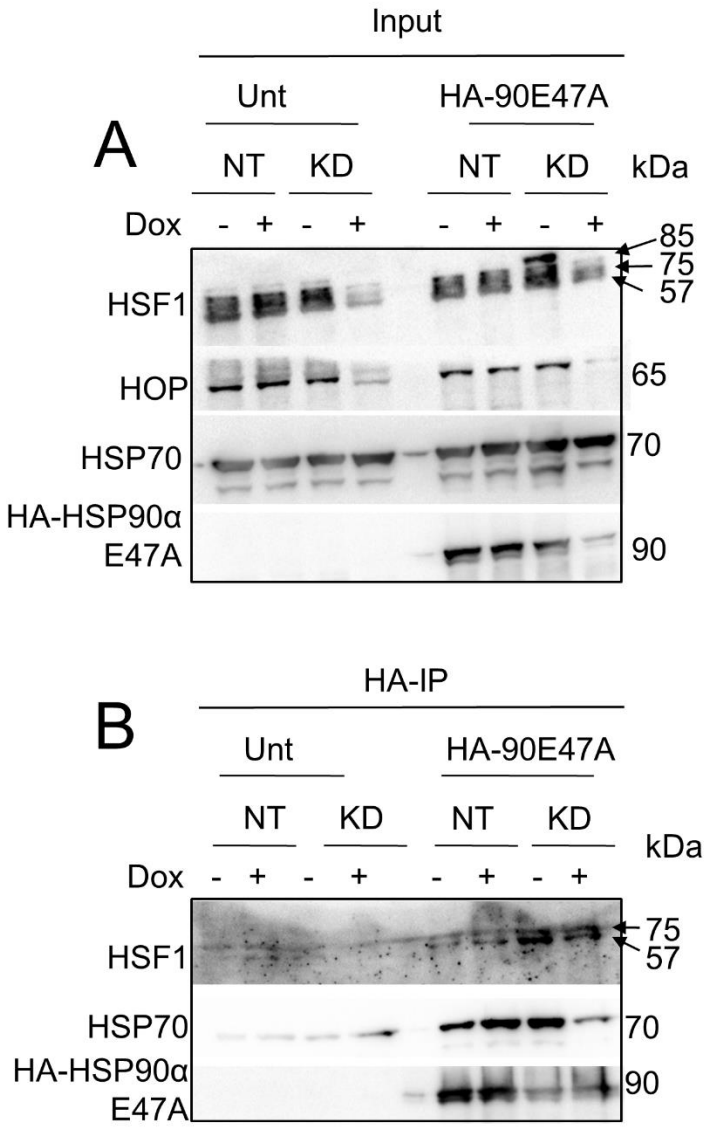


Figure 13: Hop depletion does not affect HSF1 association with the closed conformation of HSP90α.

The conformationally closed HA-HSP90αE47A mutant was isolated from transfected HEK-shNT, and HEK-shHOP lysates with and without doxycycline treatment (A) and (B) associating protein complexes analyzed by western blot analysis in HA immunoprecipitations from untransfected lysates (control) and transfected lysates.

3.10 Effect of HSP70 and HSP90 inhibition on HSF1 levels in Hop-depleted cells

We next aimed to understand the effect of inhibition of HSP90 on HSF1 levels in Hop-depleted cells. HEK-shNT and HEK-shHOP cells with and without doxycycline treatment under basal and heat shock conditions were untreated or treated with 1 μM of the HSP90 inhibitor 17-DMAG for 16 hrs and analyzed by western blot (Figure 14). Under basal conditions, 17-DMAG did not alter the HSF1 levels in the HEK-shNT cells irrespective of doxycycline treatment. However, heat shock-induced an accumulation in the 57 kDa band of HSF1 in response to 17-DMAG treatment in HEK-shNT with or without doxycycline. Hop levels were also reduced in 17-DMAG treated HEK-shNT cells only when combined with heat shock and irrespective of doxycycline treatment (Figure 14A). Depletion in the levels of the known HSP90 client Cdk4 (Smith *et al.*, 2005) under both basal and heat shock conditions in the 17-DMAG treatment served as a positive control for HSP90 inhibition.

In the HEK-shHOP cells, there was a reduction in HSF1 levels under basal conditions with and without 17-DMAG treatment only in Hop-depleted lysates treated with doxycycline (Figure 14B). Heat shock resulted in increased levels of HSF1 in both the untreated and 17-DMAG treated HEK-shHOP lysates in the absence of doxycycline. In the HEK-shHOP lysates with doxycycline treatment, there was an increase in HSF1 levels in the heat-shocked lysates compared to the equivalent sample under basal conditions in the absence but not the presence of 17-DMAG (Figure 14B). Under basal conditions, Cdk4 levels were reduced in Hop-depleted lysates to an equivalent level to 17-DMAG treatment while combined Hop depletion and 17-DMAG reduced levels further. The reduction in Cdk4 upon Hop depletion was greater than 17-DMAG in the heat-shocked samples, with the combination of Hop depletion and 17-DMAG further reducing levels. This suggested that Hop depletion may inhibit HSP90 function and that the combination of Hop depletion and HSP90 inhibition reduced the recovery of HSF1 in response to heat shock.

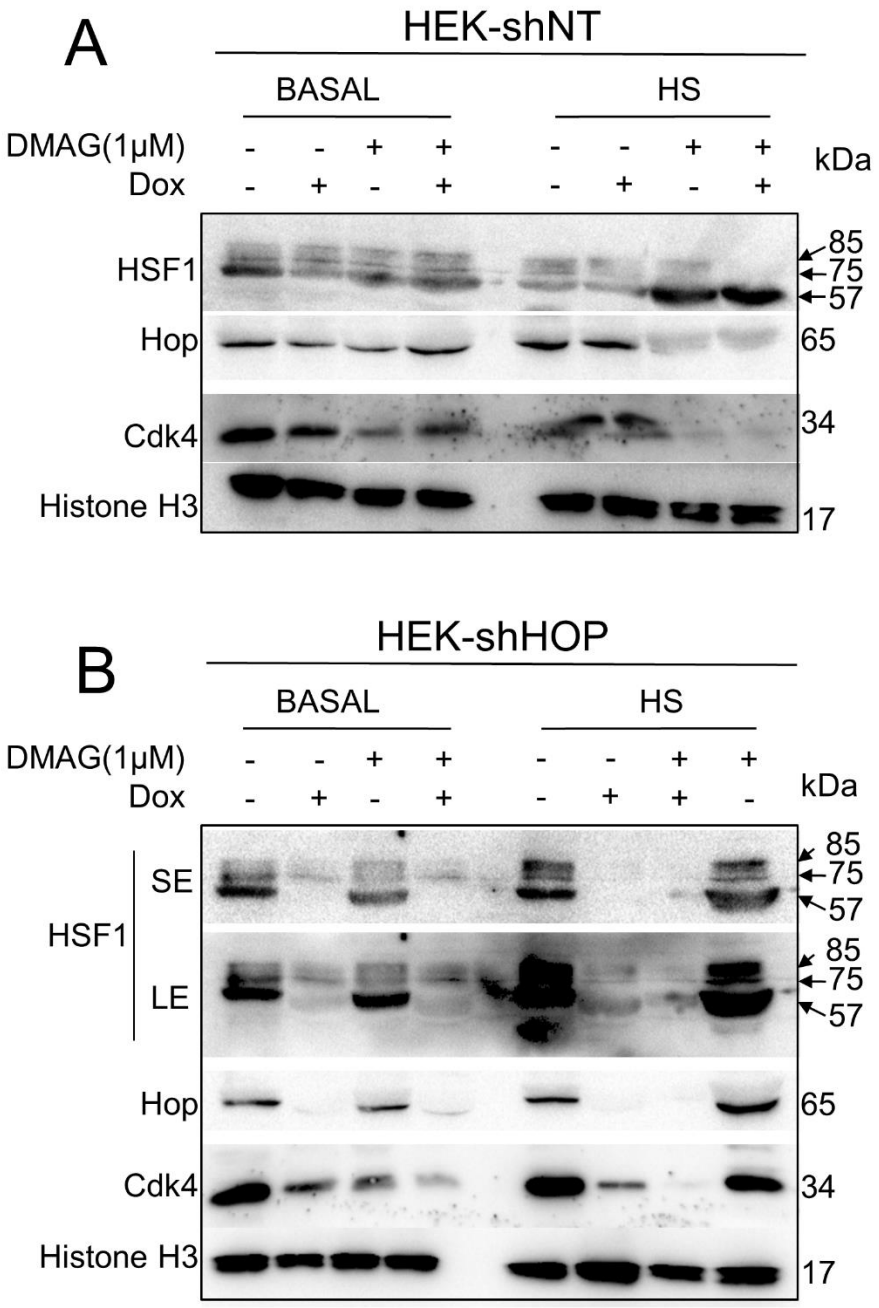
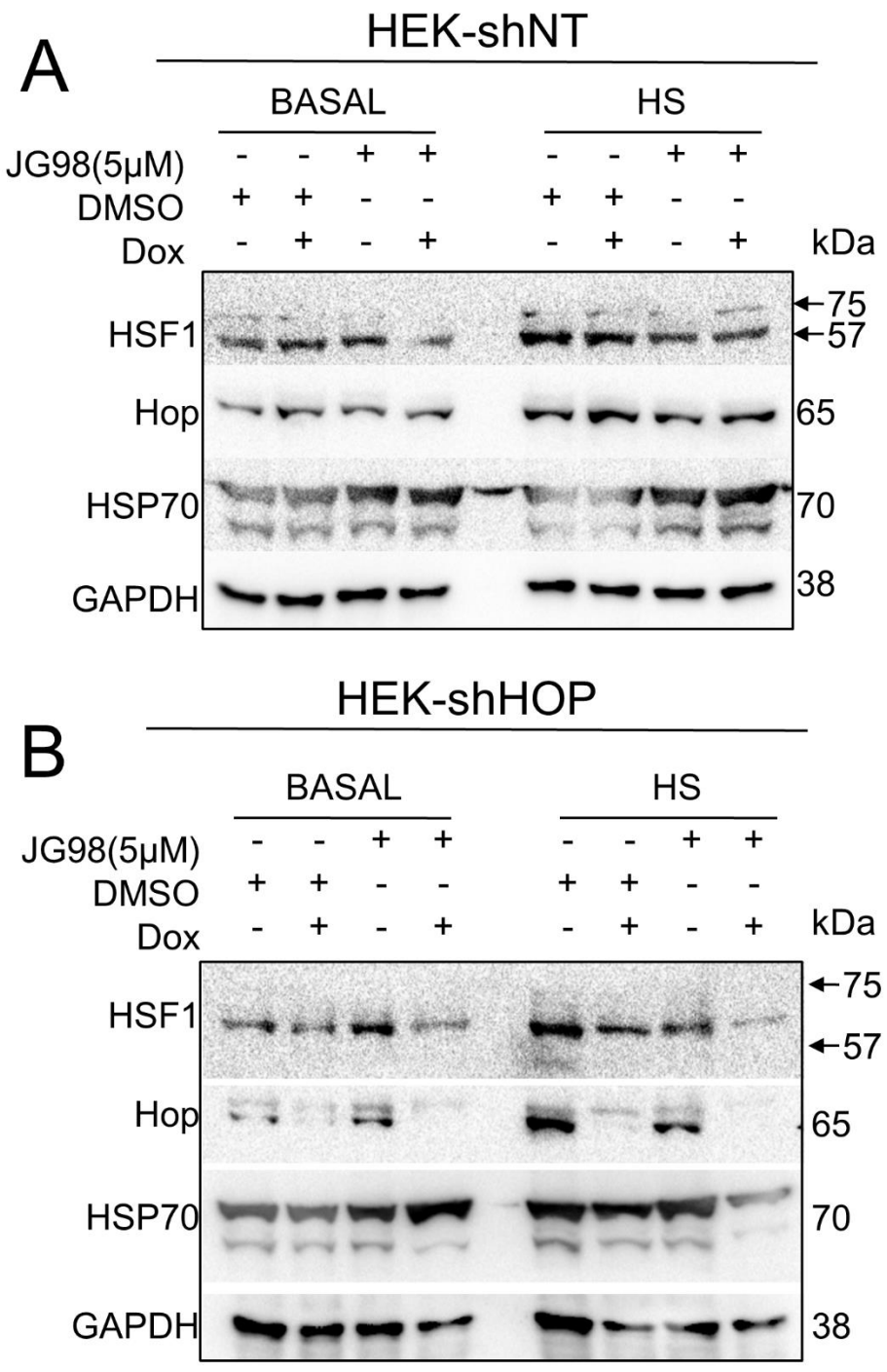


Figure 14: Effect of HSP90 inhibition on HSF1 upon Hop depletion under basal and heat shock conditions.

Western blot analysis of levels of HSF1, Hop, and Cdk4 (positive control for HSP90 inhibition) in response to 17-DMAG treatment under basal and heat shock conditions in (A) HEK-shNT and (B) HEK-shHOP with and without doxycycline treatment.

We next tested the effect of HSP70 inhibition on HSF1 levels in Hop-expressing and depleted lysates using the JG98 inhibitor (Yaglom *et al.*, 2018)(Figure 15). HEK-shNT and HEK-shHOP cells with and without doxycycline were treated with 5 μM JG98 or DMSO control for 16 hrs and protein levels in cell lysates analyzed by western blot. JG98 treatment under basal conditions in HEK-shNT with or without doxycycline did not substantially alter HSF1 levels. However, under heat shock, there was a minor reduction in HSF1 levels in the JG98 treated lysates (irrespective of doxycycline treatment) (Figure 15A).

In the HEK-shHOP cells under basal conditions, HSF1 was lower in DMSO treated cells with doxycycline compared to without doxycycline and reduced further in JG98 treated lysates lacking Hop. Under heat shock conditions, there was a recovery in HSF1 levels in Hop-depleted lysates compared to the DMSO treated, but JG98 treatment reduced the HSF1 and HSP70 levels in the Hop-depleted HEK-shHOP lysates compared to the controls and compared to the equivalent treatment under basal conditions (Figure 15B). HEK-shNT and HEK-shHOP cells with and without doxycycline were subsequently treated under basal conditions with DMSO, or a dose-response of the HSP70 inhibitor JG98 for 16hrs. Cell lysates were analyzed by western blot, which showed that JG98 induced a loss inHSF1 protein levels in the HEK-shNT with and without doxycycline, and the HEK-shHOP cells without doxycycline treatment (Figure 15C). However, the combination of Hop depletion and JG98 led to a loss of detectable HSF1 levels at concentrations of 5 and 10 μM in HEK-shHOP cells treated with doxycycline (Figure 15C). However, future experiments should confirm the effect of JG98 using a validated marker of HSP70 inhibition.



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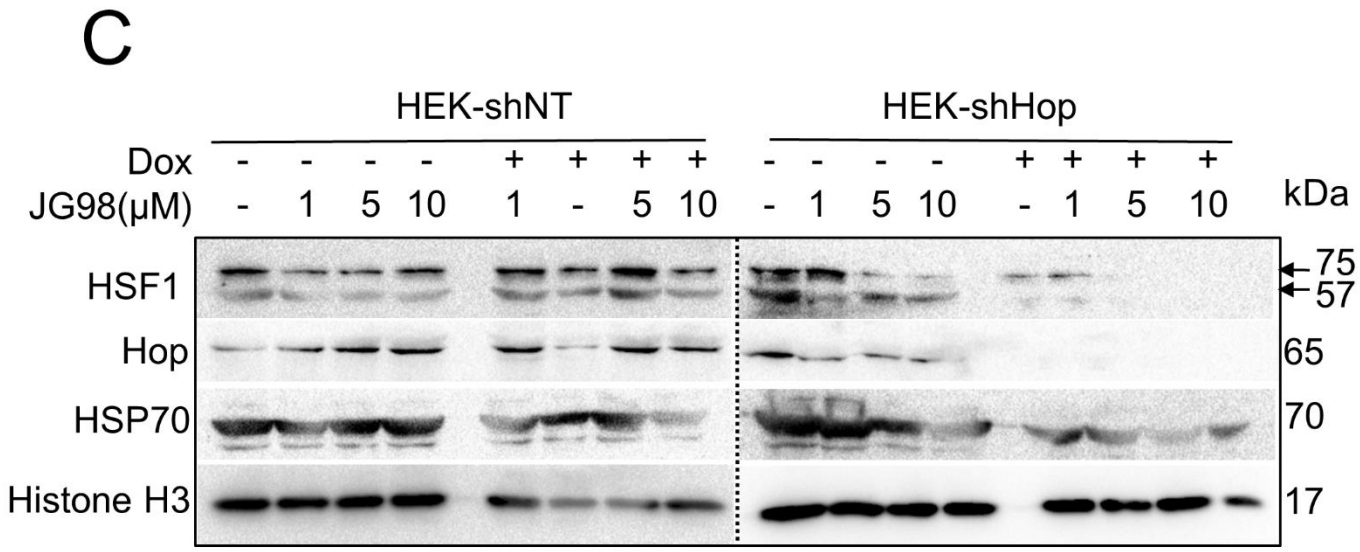

Figure 15: Effect of HSP70 inhibition on HSF1 levels in Hop-depleted cells.

Western blot analysis of levels of HSF1, Hop, and HSP70 in response to JG98 treatment under basal and heat shock conditions in (A) HEK-shNT and (B) HEK-shHOP with and without doxycycline treatment. (C) effect of a range of JG98 doses in HEK-shNT and HEK-shHOP with and without doxycycline treatment under basal conditions.

3.11 Analysis of the viability of Hop-depleted cells in response to HSP70 and HSP90 inhibition and heat shock

To determine if Hop depletion resulted in changes in cell viability, we analyzed the proliferation of HEK-shNT and HEK-shHOP cells with and without doxycycline-treated using a resazurin based microtitre based assay. Cell viability after 72 hrs was normalized to the HEK-shNT cells without doxycycline, which was taken as 100%. There was a significant reduction in average viability of the HEK-shHOP cells treated with doxycycline (18.9±3.6) compared to the HEK-shNT with or without doxycycline and the HEK-shHOP without doxycycline (96.7±5.0, 100±3.6 and 94.4±12.1, respectively) (Figure 16).

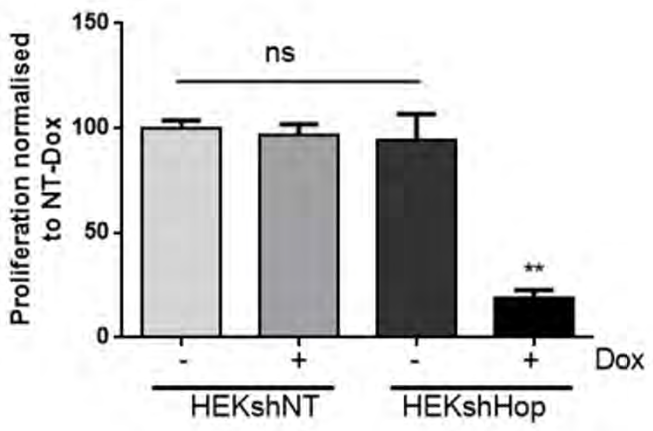

Figure 16: Hop depletion reduces cell viability

Viability of HEK-shNT and HEK-shHOP cells with and without doxycycline treatment was assessed by resazurin assay after 72 hrs in culture. Average absorbance (±SD, n=3) was normalized to HEK-shNT without doxycycline, which was taken as 100% viability. Statistical analysis was done using two-way ANOVA with Bonferroni post-test (**p<0.01).

We next used clonogenic assays to measure the long-term survival of cells under basal and heat shock conditions and in response to HSP90 inhibition (Figure 17). Images were captured of wells (Figure 17A) and average cell survival quantified by solubilization of crystal violet dye retained by cells and normalized to the HEK-shNT without doxycycline under basal conditions (Figure 17B). HEK-shNT cells (irrespective of doxycycline treatment) and HEK-shHOP cells without doxycycline treatment showed a significantly higher cell survival compared to HEK-shHOP cells treated with doxycycline under both basal and heat shock conditions in the presence and absence of 17-DMAG. However, while the HEK-shHOP cells treated with doxycycline had significantly reduced survival compared to other cells under all conditions, heat shock and/or 17-DMAG treatment did not significantly alter the average long term survival of the (already slow growing) Hop-depleted cells compared to basal conditions or lack of inhibitor (Figure 17).

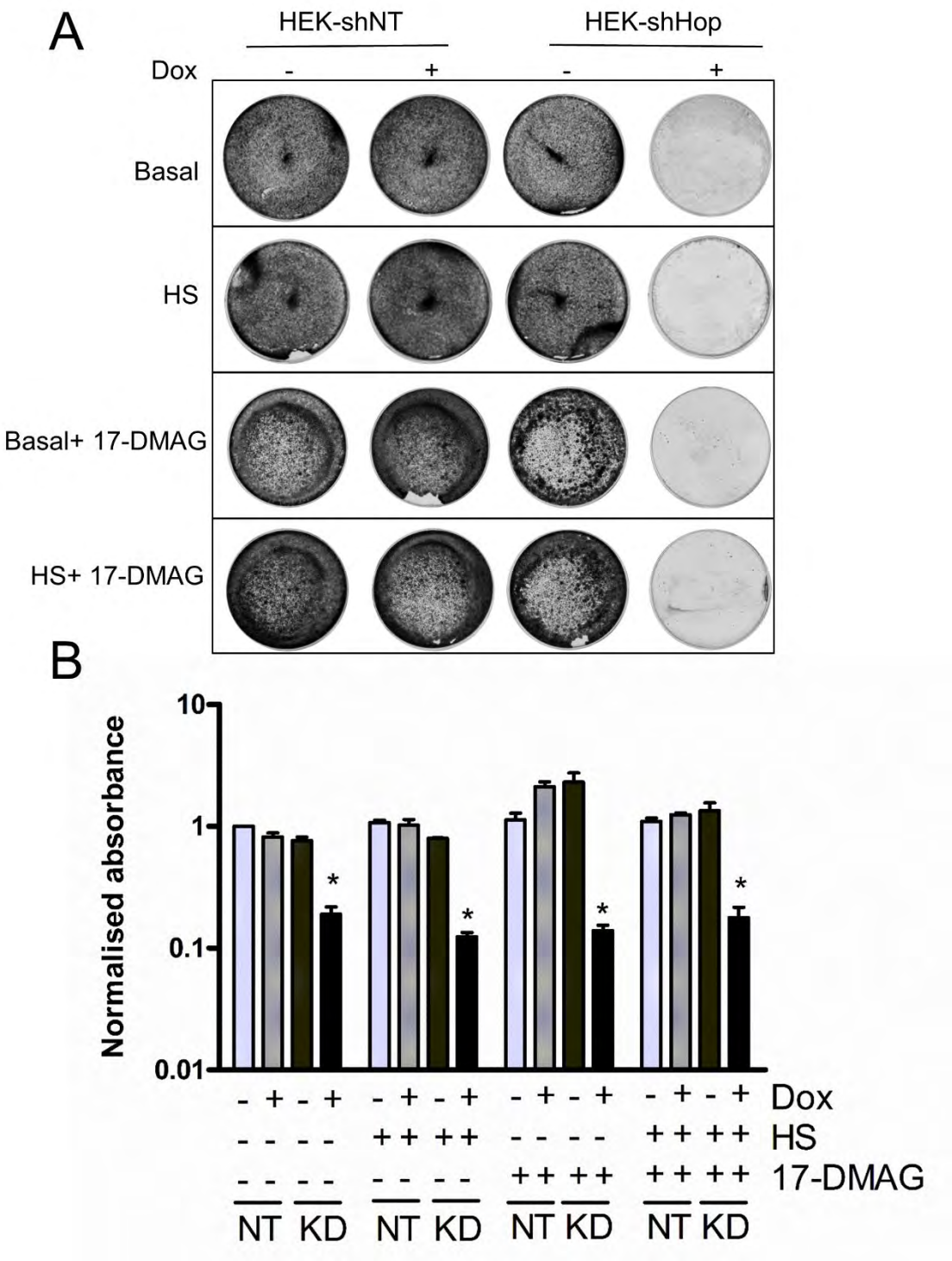

Figure 17: Effect of HSP90 inhibition on the survival of Hop-depleted cells.

Long-term cell survival determined by a clonogenic assay in HEK-shHOP and HEK-shNT cells, with and without doxycycline treatment under basal and heat shock conditions and in the presence of HSP90 inhibitor 17-DMAG. (A) Representative images of wells after crystal violet staining, (B) average absorbance (±SD, n=3) of solubilized crystal violet dye normalized to the HEK-shNT cells without doxycycline treatment. Statistical analysis was done by two-way ANOVA with Bonferroni post-test (*p<0.05).

We next tested the effect of long-term survival of Hop-depleted and expressing cells in response to HSP70 inhibition with JG98 under basal and heat shock conditions (Figure 18). JG98 did not significantly alter the ability of the control cells to survive in the clonogenic assay under basal and heat shock conditions. Similar to the previous analysis (Figure 17), the HEK-shHOP cells with doxycycline treatment showed significantly reduced long-term survival compared to the controls under all conditions. However, in contrast to the 17-DMAG treatments, HSP70 inhibition with JG98 resulted in further reductions in long term survival under both basal and heat shock conditions. Taken together, these data suggest that Hop depletion reduces cell survival and proliferation, which is increased under stress conditions in response to HSP70 but not HSP90 inhibition.

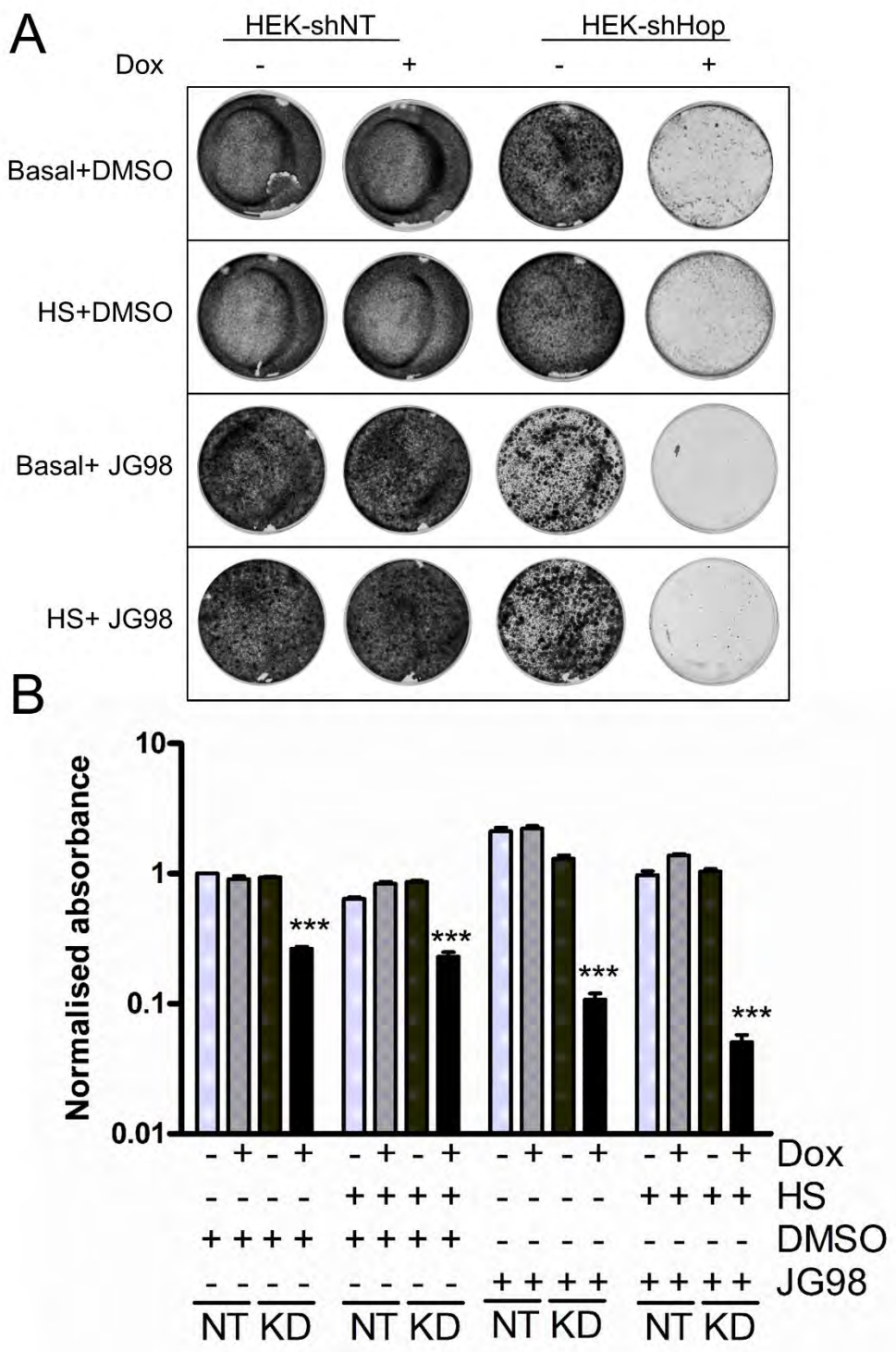

Figure 18: Effect of HSP70 inhibition on the survival of Hop-depleted cells.

Long-term cell survival determined by a clonogenic assay in HEK-shHOP and HEK-shNT cells, with and without doxycycline treatment under basal and heat shock conditions and in the presence of HSP70 inhibitorJG98. (A) Representative images of wells after crystal violet staining, (B) average absorbance (±SD, n=3) of solubilized crystal violet dye normalized to the HEK-shNT cells without doxycycline treatment. Statistical analysis was done by two-way ANOVA with Bonferroni post-test (***p<0.001).

CHAPTER 4: Discussion

HSF1 is a transcription factor that regulates the expression of a selected number of chaperone genes in response to cellular stress (Anckar & Sistonen, 2011). While HSF1 may only regulate a restricted number of stress-responsive genes, its function is critical for cell survival after stress, as the genes it regulates are required to respond to the disruption of protein homeostasis caused by stress (Solís *et al.*, 2016). In addition, HSF1 mediates a distinct transcriptional program in oncogenesis (Mendillo *et al.*, 2012). Inactive HSF1 held in the cytoplasm in a complex with chaperones undergoes activation when stress-induced misfolding competes for chaperone binding (Zheng *et al.*, 2016). HSF1 undergoes nuclear translocation, trimerization, and posttranslational modifications to bind DNA and become transcriptionally active. HSF1 is subsequently deactivated through posttranslational modifications, chaperone binding, and/or degradation (Hietakangas *et al.*, 2006; Anckar & Sistonen, 2011; Vihervaara & Sistonen, 2014; Gomez-Pastor *et al.*, 2018; Joutsen & Sistonen, 2019). Phosphorylation is a mechanism to fine-tune HSF1 function, including the duration of the heat shock response (Zheng *et al.*, 2016). In particular, the interaction of HSF1 with HSP70 and stress-related HSF1-induced HSP70 transcription, are linked to attenuation or inhibition of the heat shock response (Krakowiak *et al.*, 2018). Therefore, any stimulus which perturbs these processes will likely have consequences for HSF1 levels and activity.

While the role of HSP70 and HSP90 in HSF1 activation and stabilization has been studied in detail (Mosser *et al.*, 1993; Rabindran *et al.*, 1994; Nunes & Calderwood, 1995; Shi *et al.*, 1998; Zheng *et al.*, 2016; Kijima *et al.*, 2018; Krakowiak *et al.*, 2018), there have been limited reports on the role of the HSP70-HSP90 co-chaperone Hop. Here, we report in mammalian cell lines that Hop and HSF1 colocalized in cell lines and could be isolated in a common complex together with HSP70. HSF1 levels were reduced in Hop-depleted cells under basal conditions, although HSF1 levels increased to levels equivalent to the controls upon heat shock. There was a decrease in the nuclear localization of HSF1, HSP70, HSP90, and HSP40 with a concomitant increase in cytoplasmic levels upon Hop depletion when combined with heat shock. In addition, HSF1 and HSF1 phosphorylated

at Ser326 was punctate in the nucleus upon Hop depletion under basal conditions. While heat-shocked Hop-depleted cells retained the nuclear HSF1 puncta and showed no increase in nuclear HSF1, there was also an increase in perinuclear cytoplasmic HSF1 staining, which was consistent with the biochemical fractionation. Hop depletion led to a reduction in HSE binding and transcriptional activity from an HSF1 reporter plasmid under basal and heat shock conditions. Given the increase of HSF1 levels in Hop-depleted cells upon cell stress, these data suggested that the reduced binding and transcriptional activation in response to heat shock were not solely due to reduced HSF1 levels. Hop-depleted cells were more sensitive to the HSF1 inhibitor KRIBB11. Loss of Hop did not affect the interaction of HSF1 with the closed conformation of HSP90 but did reduce the interaction with HSP70. Treatment of cells with the HSP70 inhibitor JG98, which blocks the association between HSP70 and BAG proteins (Li *et al.*, 2015), did not alter HSF1 levels in control cells but led to further reductions in HSF1 protein levels in Hop-depleted cells. Furthermore, heat shock reduced cell survival in Hop-depleted cells, which was further reduced by HSP70 inhibition with JG98, but not HSP90 inhibition with 17-DMAG. Taken together, these data suggest that Hop is involved in stabilization of HSF1 protein under basal conditions and that it regulates the levels and activity of HSF1 through mechanisms that may involve reduced HSP70 interaction together with reduced nuclear localization, reduced DNA binding, and transcriptional activity.

Hop was originally identified in yeast (termed STI1 for stress-inducible gene 1 since its expression was increased tenfold by heat shock) (Nicolet & Craig, 1989). STI1 was found in a screen for proteins regulating the expression of the yeast HSP70 SSA4 (homologous to HSPA1A in humans). Yeast lacking STI1 showed normal growth under basal conditions but reduced growth at elevated or lowered temperatures suggesting a role in stress adaption. Also, STI1 overexpression led to the transactivation of the SSA4 promoter, suggesting a role of STI1 in the stress response, which we now know is regulated in part by HSF1 (Nicolet & Craig, 1989). HSP70 isoforms are classical targets for HSF1 (Trinklein *et al.*, 2004). Ruckova and colleagues showed that in cell lines, siRNA against Hop reduced HSF1 protein levels, while Hop overexpression had no significant effect on HSF1 protein levels (Ruckova *et al.*, 2012). Hop was itself shown to be stress-inducible and

regulated by HSF1 binding to two HSEs (GAANNTTCNNGAA) in the Hop promoter sequence between positions -260 and -680 base pairs from the transcription start site. This suggested a reciprocal relationship between HSF1 and Hop levels (Ruckova *et al.*, 2012).These two studies are consistent with and support our finding that Hop depletion reduces HSF1 levels and activity and suggests that the response is conserved between mammalian and yeast cells. However, the recent finding in yeast that deletion of Hop resulted in a remarkable decrease in HSP90 availability, and a strong HSR activation is not consistent with our study (Alford & Brandman, 2018).

Mathematical modeling showed that the total concentration of HSF1 is a determining factor for the stress response under both basal and stress conditions, irrespective of the presence of excess HSF1 compared to HSE sequences. Even a 25% decrease in HSF1 from the basal level at 37°C translates to an equivalent decrease in transcriptional response and *vice versa* (Rieger *et al.*, 2005). This would suggest that the reduced HSF1 activity and HSE binding under basal conditions upon Hop depletion was due to the 50% reduction in HSF1 protein.

However, the mechanism by which HSF1 levels are reduced in Hop-depleted cells remains undefined. There are limited studies on the stability of HSF1 protein levels, and those which are available suggest that acetylation, particularly at residues K208 and K298, may stabilize HSF1 and prevent turnover via the proteasome (Raychaudhuri *et al.*, 2014). The effect of Hop depletion on HSF1 acetylation could be studied in the future to address this question. Also, the HS70/HSP90 co-chaperone CHIP, which mediates the degradation of chaperone clients via the proteasome, competes with Hop for binding to chaperones (Muller *et al.*, 2012). Therefore, in the absence of Hop, there may be more CHIP associated with the HSP90 or HSP70 chaperones, increasing proteasomal degradation. However, our proteomic analyses suggest that there is not a global turnover of proteins upon Hop depletion, and hence this effect would need to be client-specific were it to explain the basal reductions in HSF1. In addition, increased CHIP expression is involved in nuclear translocation and activation of HSF1, an effect that was abrogated by mutation of a critical residue required for HSP70 interaction in the CHIP TPR domain

(Dai *et al.*, 2003). This suggests that the CHIP-HSP70 interaction is required for the CHIP-mediated activation of HSF1, and in this way, increasing the CHIP-HSP70 interaction upon Hop depletion would not be expected to culminate in our observed HSF1 inhibition. Interestingly, the levels of HSF1 in Hop-depleted cells were returned to levels equivalent to the controls in response to heat shock, which suggested that either Hop does not regulate stress-induced expression of HSF1 (and assumes stability of HSF1 under normal and stress conditions is different), or that HSF1 is somehow stabilized by changes associated with heat shock. However, Hop is clearly required for active HSF1 during heat shock, since there was still reduced HSE binding and transcriptional activation compared to controls despite the increased protein levels.

Besides its role as a receptor for the prion protein, the predominant role for Hop in the cell has been to function as an essential mediator of the interaction between HSP70 and HSP90 (Patricia Hernández *et al.*, 2002; Carrigan *et al.*, 2004; Beraldo *et al.*, 2013; Baindur-Hudson *et al.*, 2015). Hop is absent from prokaryotes, where HSP90 and HSP70 interact directly (Genest *et al.*, 2015). Recent studies have also shown that HSP90 and HSP70 can both interact and chaperone clients in eukaryotic cells (Kravats *et al.*, 2018). This then indicates that Hop is not essential for the HSP70-HSP90 interaction and begs the question of the exact biological function of Hop. It is possible that Hop is not required for the HSP70-HSP90 chaperone machine to function, but rather that it serves a regulatory role, mediating the rate of chaperone folding and perhaps governing the entry of selected (groups) of client proteins. This is relevant in the context of this study, given the central role of HSP70 and HSP90 in the regulation of HSF1 function. The HSP70-HSF1 interaction is stronger than the HSF1-HSP90 interaction, suggesting that HSP70 has a constitutive stress repressive role in HSR in mammalian cells (Kijima *et al.*, 2018). Recently HSP90 has been shown to be involved in HSF1 deactivation rather than keeping in HSF1 in a monomeric form before activation (Kijima *et al.*, 2018). Hop stabilizes the “open” conformation of HSP90 and promotes transfer of the client protein from the HSP70 complex to HSP90 (Chang *et al.*, 1997; Lee *et al.*, 2012; Baindur-Hudson *et al.*, 2015). This suggests that in turn loss of Hop should promote the closed HSP90 conformation, which should lead to increased association between HSP90 and HSF1. This was not

observed in our study but could be because it is difficult to quantify due to the transient nature of the wild type HSP90-HSF1 interaction (Kijima *et al.*, 2018).

The HSP70-HSF1 interaction is a major determinant of HSF1 regulation (Shi *et al.*, 1998; Zheng *et al.*, 2016; Krakowiak *et al.*, 2018; Peffer *et al.*, 2019). Our data show that HSF1 colocalized with and could be isolated in Hop complexes containing HSP70. The depletion of Hop resulted in a reduction in the amount of HSF1 isolated in HSP70 complexes but did not affect the interaction of HSF1 with the closed conformation of HSP90. This HSP70 phenotype is consistent with the role of Hop as a co-chaperone involved in mediating substrate association with chaperones. However, Hop has been predominantly viewed as a co-chaperone of HSP90 rather than HSP70, and so it is interesting that the HSP70 interaction and not the HSP90 interaction with HSF1 was perturbed. These data support a role for Hop as a regulator of HSP70 activity independently of HSP90.

The effect of Hop depletion on HSF1 function may be due to reduced HSF1-HSP70 interaction. The consensus from several studies by different groups is that HSP70 is a negative regulator of HSF1 function and is important in attenuation of the heat shock response. HSF1 forms bipartite interactions with the HSP70 substrate binding site (Peffer *et al.*, 2019). Disruption of the HSP70-HSF1 interaction has been shown to result in HSF1 activation, induction of the heat shock response, and delayed attenuation (Zheng *et al.*, 2016, 2018; Krakowiak *et al.*, 2018). This contrasts with our data, where we see a reduction in HSF1-HSP70 interaction in Hop-depleted cells coinciding with altered HSF1 nuclear staining patterns under basal and heat shock conditions, as well as reduced HSF1 nuclear localization, HSE binding and transcriptional activity at an HSF1-responsive promoter in response to heat shock. These contradictory data could be interpreted to mean either that Hop is required for HSF1 activity upon release from HSP70 repression, or that the reduced association of HSF1 from HSP70 is not what induces the loss in HSF1 activity in Hop-depleted cells.

Hop depletion led to a profound reduction in HSF1 levels when combined with JG98, the allosteric HSP70 inhibitor which blocks the BAG-HSP70 interaction and destabilizes client proteins (Jin *et al.*, 2015; Yaglom *et al.*, 2018). BAG3 is thought to have an analogous scaffolding function for HSP70 chaperone complexes to Hop (Rauch & Gestwicki, 2014), and BAG3 interacts with HSF1 (Jin *et al.*, 2015). The disruption of the BAG-HSP70 complex would be expected to prevent nucleotide exchange linked to substrate release by HSP70 and, consequently, may be expected to extend the HSF1-HSP70 interaction. HSF1 is bound at the HSP70 substrate binding site, and these blocked HSP70 complexes would then lead to degradation of HSF1, similar to that seen for other HSP70 clients like Raf1 and Akt (Li *et al.*, 2013). Hop and BAG3 interact with distinct sites of HSP70, and steric hindrance between the different sites influences binding (Gebauer *et al.*, 1998). Therefore, the proportion of BAG3 in HSP70 complexes may be altered in Hop-depleted cells. Given the role of BAG3 in promoting the release of HSP70 substrates, it could be speculated that increased BAG3 binding in the absence of Hop promotes the release of HSF1 from HSP70. However, again this would not account for lack of transcriptional activation since releasing HSF1 from HSP70 by overexpression of a yeast NEF has been shown to promote HSF1 activation (Masser *et al.*, 2019). Similarly, it is not clear if BAG-HSP70 complexes were to be increased upon Hop depletion, why JG98 (which disrupts BAG-HSP70 interactions) would potentiate the loss of HSF1 in Hop-depleted cells rather than stabilize them.

HSF1 shuttles between the nucleus and cytoplasm under basal conditions, but nuclear accumulation is observed under stress due to reduced export and is essential for HSF1 activity (Vujanac *et al.*, 2005). Accumulated nuclear HSF1 is usually considered to be activated and in a trimerized form, which is capable of binding the conserved upstream HSE sequences of HSF1 target genes like HSPA1A to initiate the stress response (Trinklein *et al.*, 2004; Anckar & Sistonen, 2011). Our data showed that in Hop-depleted cells, HSF1 distribution was not significantly different to controls under basal conditions. However, HSF1 failed to translocate to the nucleus in response to heat stress and accumulated in the cytoplasm, which would explain the loss in HSF1 DNA binding and transcriptional activity in Hop-depleted cells upon heat shock. HSP40 has been shown to

colocalize to the nucleus with HSP70, followed by a return to the cytoplasm during attenuation (Hattori *et al.*, 1993). However, there was also a reduction in nuclear translocation in response to heat shock of selected chaperones in Hop-depleted cells. In general, cell stress results in the collapse of the Ran gradient causing nuclear accumulation of α-importin and blocking the classical nuclear import of NLS-containing proteins(Stochaj *et al.*, 2000; Miyamoto *et al.*, 2004). Under stress conditions, HSP70 is translocated to the nucleus by a novel stress-related import mechanism mediated by the protein known as Hikeshi (Imamoto & Kose, 2012). Hop is predominantly a cytoplasmic protein but possesses a bipartite NLS motif enabling the protein to move in and out of the nucleus during stages of the cell cycle. This shuttling is controlled by phosphorylation by cdc2 kinase at the cdc2-kinase-NLS motif at amino acids 180-239 of Hop (Longshaw *et al.*, 2004). However, Hop nuclear translocation under stress is not dependent on this sequence (Daniel *et al.*, 2008), although if Hikeshi regulates stress-related nuclear translocation of other chaperones is not known. These data suggest that Hop or Hop-mediated chaperone complexes regulate nuclear localization of HSF1 during heat shock. Whether the reduced nuclear localization is due to decreased import or increased export of HSF1 is currently unknown. Interestingly, the co-chaperone BAG3, which binds HSF1 and competes with Hop for binding to HSP70 (Gebauer *et al.*, 1998), translocated to the nucleus and increased export of HSF1 during attenuation (Franceschelli *et al.*, 2008).

Under both basal and heat shock conditions, a defect in HSE binding and punctate nuclear HSF1 staining was observed in Hop-depleted cells. The morphology of the HSF1 nuclear staining in Hop-depleted cells resembled that seen with nuclear stress bodies (nSB) (Raychaudhuri *et al.*, 2014). These nSBs are associated with transcriptionally active HSF1 complexes (Alastalo *et al.*, 2003). However, this is not necessarily consistent with our observations of reduced HSF1 activity in Hop-depleted cells, which suggests that the puncta likely do not represent functional nSB. However, taken together, these data do suggest a change in HSF1 DNA interactions in Hop-depleted cells. It is unlikely that this phenotype is related to the defect in nuclear translocation in response to stress since the phenotype was conserved in Hop-depleted cells under both basal and stress conditions. Whether this is related to the changes in HSP70-HSF1 interaction remains to

be determined. A study in yeast triggering the release of HSF1 from HSP70 by forced nuclear translocation of the NEF Sse1 led to transcriptional activation of HSF1 (Masser *et al.*, 2019). This would be consistent with the formation of nuclear puncta, but not with a lack of transcriptional activity.

The reduced HSF1 levels and activity in Hop-depleted cells corresponded to reduced short-term proliferation under basal conditions and reduced long-term survival under basal and heat shock conditions. In *sti1* null yeast cells, there was reduced growth at both 30°C and 37°C (Nicolet & Craig, 1989). HSP70 inhibition (with JG98), not HSP90 inhibition (with 17-DMAG), potentiated the low cell survival of Hop-depleted cells, and JG98 was associated with further decreases in HSF1 protein levels. Hop-depleted cells were also more sensitive to the HSF1 inhibitor KRIBB11 than controls. KRIBB11 treatment and HSF1 depletion by RNA have been shown to produce the same phenotype (Fok *et al.*, 2018), and hence it is likely that Hop depletion and KRIBB11 combine in inhibiting HSF1. Together, these data suggest that reduced cellular fitness in Hop-depleted cells is linked to HSP70-dependent reductions in HSF1.

In conclusion, our data demonstrate a role for Hop in the regulation of the levels and activity of the stress-responsive transcription factor HSF1. Depletion of Hop impaired HSF1 function. The most plausible explanation for this based on our data and those of others in the field is that Hop depletion restricts the nuclear localization of HSF1 under heat shock, which reduces its ability to bind HSE and become transcriptionally active, culminating in reductions in cell survival. Our data also suggest a role for regulation of HSP70, but not HSP90, by Hop in these processes, which substantiates that Hop is not only involved as a co-chaperone in mediating HSP70-HSP90 complexes. Work into understanding the mechanism in more detail is ongoing, including extending analysis to other cell lines to determine the significance and broaden the generalization of the findings.

CHAPTER 5: Appendix I

Table 6: List of primary antibodies used and their respective dilutions for western blot analysis

Name	Source	Catalog no	Dilution
HSF1	Abcam	ab2923	1:1000
HSF1	Enzo Life Sciences	ADA-SPA-901	1:1000
Hop	Abcam	ab126724	1:5000
Histone	Abcam	ab1791	1:5000
HSP40	Abcam		1:5000
Tubulin	Abcam	ab7291	1:5000
HA	Abcam	ab9110	1:10000
HSP90αβ	Santa cruz biotechnology	sc-13119	1:5000
HSP90α	Enzo life sciences	adi-spa-840	1:10000
HSP90β	Abcam	ab1198333	1:5000
HSP70	Santa cruz biotechnology	sc-24	1:5000
GAPDH-HRP Conjugated	Abcam	ab185059	1:10000
pSer326:HSF1	Abcam	ab76076	1:1000
Cdk4	Abcam	ab108357	1:5000
HSP40	Abcam	ab69402	1:5000

Table 7: List of secondary antibodies used and their respective dilutions for western blot analysis

Name	Source	Catalog no	Dilution
Anti-Rabbit	Abcam	ab97064	1:10000
Anti-Mouse	Abcam	ab97023	1:5000
Anti-Rat	Abcam	ab97057	1:10000

Table 8: List of primary antibodies used and their respective dilutions for confocal microscopy

Name	Source	Catalog no	Dilution
HSF1	Abcam	ab2923	1:100
HSF1	Santa cruz biotechnology	sc-13156	1:100
Hop	Abcam	ab126724	1:100
Hop	Santa cruz biotechnology	sc-390206	1:100
pSer326:HSF1	Abcam	ab76076	1:100

Table 9: List of secondary antibodies used and their respective dilutions for confocal microscopy

Name	Source	Catalog no	Dilution
Alexa 488 anti-Rabbit	Abcam	ab150073	1:500
Alexa 555 anti-Rabbit	Abcam	ab150074	1:500
Alexa 488 anti-Mouse	Abcam	ab150106	1:500

CHAPTER 6: References

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