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SIRT1 Regulation of the Heat Shock Response in an HSF1-Dependent Manner and the Impact of Caloric Restriction

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SIRT1 Regulation of the Heat Shock Response in an HSF1-Dependent Manner

and the Impact of Caloric Restriction

by

Rachel Raynes

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
Department of Cell Biology, Microbiology, and Molecular Biology
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Keywords: Caloric Restriction; Hormesis; Cytoprotection; Heat Shock Proteins; AROS; DBC1; NAD+/NADH ratio; Celastrol; C. elegans

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DEDICATION

This manuscript is dedicated to my parents, Matthew L. Blake and Becky R. Blake, for their unwavering understanding, love, and support, whose guidance led me to be the individual that I am today. Likewise, I must thank my mentor, Sandy D. Westerheide, for molding me into the scientist that I am today.
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Abstract

Introduction

Methods

Cell culture and heat shock
Quantitative RT-PCR
Immunoblotting Analysis
Chromatin Immunoprecipitation

Results

RPS19 mRNA and protein levels increase upon HS
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<tbody>
<tr>
<td>AL</td>
<td><em>ad libitum</em></td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>AROS</td>
<td>Active Regulator of SIRT1</td>
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<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
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<td>CR</td>
<td>Caloric Restriction</td>
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<td>DBC1</td>
<td>Deleted in Breast Cancer 1</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>HSE</td>
<td>Heat Shock Element</td>
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<td>IR</td>
<td>Ischemia Reperfusion</td>
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<td>M-PER</td>
<td>Mammalian Protein Extraction Reagent</td>
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<tr>
<td>NAD⁺, NADH</td>
<td>Nicotinamide Adenine Dinucleotide</td>
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<td>NAM</td>
<td>Nicotinamide</td>
</tr>
<tr>
<td>NAMPT</td>
<td>Nicotinamide Phosphoribosyltransferase</td>
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<tr>
<td>ON</td>
<td>Overnight</td>
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<tr>
<td>OSR</td>
<td>Oxidative Stress Response</td>
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<tr>
<td>PAGE</td>
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<td>Transcription and Translation</td>
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<td>Western Blot</td>
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ABSTRACT

The heat shock response (HSR) is the cell’s molecular reaction to protein damaging stress and is critical in the management of denatured proteins. Activation of HSF1, the master transcriptional regulator of the HSR, results in the induction of molecular chaperones called heat shock proteins (HSPs). Transcription of hsp genes is promoted by the hyperphosphorylation of HSF1, while the attenuation of the HSR is regulated by a dual mechanism involving negative feedback inhibition from HSPs and acetylation at a critical lysine residue within the DNA binding domain of HSF1, which results in a loss of affinity for DNA. SIRT1 is a NAD⁺-dependent histone deacetylase that has been reported to deacetylate HSF1, thus promoting stress-induced HSF1 DNA binding ability and increasing HSP expression (Westerheide, Anckar et al. 2009). While an abundance of research is aimed to investigate SIRT1 substrate regulation, the mechanism in which SIRT1 itself is regulated is less understood (Haigis and Sinclair 2010). Positive and negative modulators of SIRT1 include AROS and DBC1, respectively, and have yet to be investigated in relation to SIRT1-dependent regulation of the HSR. In addition, metabolic stress such as caloric restriction has been shown to modulate SIRT1 activity in yeast (Rahat, Maoz et al. 2011), but the effect of caloric restriction on the HSR is unknown.

Using cell-based assays, we have investigated how the HSR may be controlled by factors influencing SIRT1 activity. We found that heat shock results in an increase in the cellular NAD⁺/NADH ratio and an increase in recruitment of SIRT1 to the hsp70 promoter. Furthermore, we found that the SIRT1 modulators, AROS and DBC1, impact hsp70 transcription, HSF1 acetylation status, and HSF1 recruitment to the hsp70 promoter. The nematode Caenorhabditis elegans is a useful model organism for testing the relationship between the HSR and metabolism, as these animals can easily be
calorically-restricted via bacterial limitation and possess the mammalian SIRT1 homolog, Sir2.1. Using *C. elegans*, we demonstrate that caloric restriction and heat shock have a synergistic effect on the HSR in a *sir2.1*-dependent manner. We show that caloric restriction increases the ability of heat shock to promote thermotolerance and fitness in wild-type animals and to preserve movement in a polyglutamine toxicity neurodegenerative disease model and that this effect is dependent on *sir2.1*. These studies provide insight into SIRT1-dependent regulation of the HSR and the impact of metabolism on this response. We highlight the SIRT1 modulators AROS and DBC1 as two new targets available for therapeutic regulation of the HSR and add caloric restriction as another HSR activator that can synergize with heat shock.
CHAPTER 1. INTRODUCTION

The Discovery of the Heat Shock Response

The heat shock response (HSR) was discovered serendipitously in the 1960’s by an Italian geneticist named Ferruccio Ritossa. While working at the Genetic Institute in Pavia, Ritossa designed a project to study the nucleic acids that were synthesized in the chromosomal puffs observed in Drosophila salivary glands at active sites of transcription. Sometime throughout his research, one of Ritossa’s colleagues increased the temperature of his incubator and consequently changed the puffing pattern he observed. After determining the optimal temperature for this shift, Ritossa observed new RNA synthesis within 2-3 minutes of thermal stress (Ritossa 1962; Ritossa 1996). He observed the rapid induction of new RNA across different tissues, developmental times, and species of Drosophila and eventually published his work in Experientia in 1962 (Ritossa 1962).

The work was not well received and it wasn’t until several years later that the heat shock response became an established field. In the late 1970’s it was discovered that an elevation in temperature resulted in a change of the total protein profile for the cell. Pulse radiolabeling of mRNA and proteins indicated that production of the aptly named heat shock proteins (HSPs) increased upon thermal stress, while the production of other constitutively expressed proteins decreased (Tissiere.A, Mitchell et al. 1974; Lewis, Helmsing et al. 1975; Spradling, Pardue et al. 1977). The cloning of a rapidly inducible HSP known as hsp70 and the subsequent analysis of the hsp70 promoter lead to the identification of an evolutionarily conserved heat shock element (HSE) that is found upstream of HSP genes. Through the use of affinity chromatography with HSE-oligonucleotide-sepharose beads, the biochemical purification and characterization of the HSE-binding protein resulted in the identification of the Heat Shock Factor 1 (HSF1) transcription factor for S. cerevisiae, Drosophila, and human cell culture (Parker...
These studies lead to the establishment of the HSR field that is now recognized to have a vital role in the regulation of protein homeostasis.

**HSF1 Regulation of the Heat Shock Response**

The HSR is an ancient inducible state designed to allow a cell to manage denaturing stress and promote organismal survival. Stressors that perturb the HSR include, but are not limited to, heat, heavy metals, oxygen-free radicals, cancer, aging, infection, protein misfolding diseases, and the presence of various growth factors (Morimoto, Westerheide et al. 2009). Initiation of the HSR results in transcriptional upregulation of molecular chaperones known as heat shock proteins (HSPs). The elevated level of HSPs allows a cell to manage stress and obtain protein homeostasis. Once a stressor triggers the HSR, HSF1 trimerizes and translocates to the nucleus (Figure 1.1).

**Figure 1.1. HSF1 regulation of the heat shock response (HSR).**

HSF1 exists as an inactive monomer in the cytoplasm. Upon induction of the HSR, it trimerizes and translocates to the nucleus where it binds to heat shock elements (HSEs) found in the promoter region of HSP genes. Transcription is induced when HSF1 is
hyperphosphorylated. Upon acetylation, HSF1 loses affinity for the DNA. SIRT1 is a deacetylase that promotes HSF1 DNA binding.

Upon translocation into the nucleus, HSF1 binds conserved regulatory upstream promoter elements known as heat shock elements (HSEs). The HSEs consists of several inverted repeats of the pentamer sequence nGAAn (Sorger and Pelham 1987; Amin, Ananthan et al. 1988). Each DNA-binding foot of the HSF1 trimer is capable of binding to an inverted repeat of the HSE, therefore the transcription binding site is typically a region of contiguous repeats with the sequence TTCnnGAAAnTTC (Amin, Ananthan et al. 1988; Xiao and Lis 1988; Perisic, Xiao et al. 1989). Many HSP genes have several regions of HSE repeats resulting in the binding of multiple HSF1 transcription factors. This binding occurs in a cooperative manner in which the binding of one HSF1 facilitates the binding of the next one (Xiao, Perisic et al. 1991). This cooperativity may account for the differential expression of heat shock genes, whereby the number of HSEs impacts the level of gene induction. Interestingly, the C. elegans hsp70 family member, F44E5.5, possesses four regions of HSE clusters (Figure 1.2A), while C12C8.1 possesses one region of HSE clusters (Figure 1.2B). The integrity or stringency of an HSE may also vary as demonstrated by the mouse hsp70.1 gene, which contains two regions of HSEs, each containing typical and atypical groupings of the nGAAn inverted pentamer (Christians, Michel et al. 1997) (Figure 1.2C).

While trimerization is all that is necessary for HSF1 to bind to DNA, activation of transcription only occurs upon HSF1 hyperphosphorylation. As an example, the anti-inflammatory agent sodium salicylate is capable of inducing HSF1 to bind to the DNA, but does not cause the phosphorylation required for initiation of transcription which would result in upregulation of HSPs (Jurivich, Sistonen et al. 1992). Phosphorylation of HSF1 is promiscuous as several protein kinase families including MAPK, PKC, and CAMKII are reported to phosphorylate HSF1 at various residues (Westerheide, Raynes et al. 2012).

Attenuation of the HSR is regulated by a dual mechanism involving negative feedback inhibition from HSPs (Shi, Mosser et al. 1998) and acetylation at a critical lysine residue
within the DNA binding domain of HSF1 causing loss of affinity for DNA (Westerheide, Anckar et al. 2009). It is not clear if multiple histone acetyl transferases (HATs) are involved in the HSR, but p300 has been shown to acetylate HSF1 when overexpressed (Westerheide, Anckar et al. 2009) and to interact with HSF1 in vivo (Zhang, Murshid et al. 2011). SIRT1 is a NAD⁺-dependent histone deacetylase that deacetylates HSF1, thus promoting stress-induced HSF1 DNA binding ability and increased HSP expression (Westerheide, Anckar et al. 2009). The end result of the HSR is the upregulation of HSPs, thus promoting the cell’s ability to cope with denaturing stress.

Figure 1.2. Representation of the HSE elements found in hsp70 family genes for C. elegans and M. musculus.

The hsp70 gene family is highly inducible and is frequently used as a marker for initiation of the HSR. Two hsp70 family genes in C. elegans, (A) F44E5.5 and (B) C12C8.1, vary in the number of HSEs found in the promoter region of each gene. (C) HSE sequences may be atypical as demonstrated by the M. musculus hsp70.1 gene. HSEs that differ from the standard nGAAAn sequence appear underlined. The numbers
Nuclear Stress Bodies

In addition to canonical HSEs being found in all eukaryotic heat shock-responsive genes, degenerate HSEs also compose structures in the nucleus called nuclear stress bodies (nSBs) (Sarge, Murphy et al. 1993; Biamonti and Vourc'h 2010). Several nuclear factors that are distributed throughout the nucleoplasm can accumulate in nuclear subcompartments such as nucleoli, nuclear speckles, Cajal bodies, promyelocytic leukaemia (PML) bodies, and the perinucleolar compartment (PNC) [reviewed by (Spector 2001; Lamond and Spector 2003)]. nSBs are transient subnuclear structures that are distinct from other nuclear bodies, such as PML and Cajal bodies (Cotto, Fox et al. 1997; Chiodi, Biggiogera et al. 2000). Very little is known regarding the function of nSB’s since their discovery in the late 1980’s and their subsequent association with the HSR.

In 1989, it was first demonstrated that HS resulted in the recruitment of heterogeneous ribonucleoprotein particles (hnRNPs) to distinct perichromatin granules (PGs), now known as nSBs (Mahl, Lutz et al. 1989). Subsequently, immunofluorescence indicated that HSF1 also translocates to these nuclear bodies upon HS stress (Sarge, Murphy et al. 1993) (Figure 1.3). The kinetics of nSB formation is dependent on the nature and duration of the stressor, but is consistent with HSF1 activation of HSP transcription. However, classical heat-induced HSPs do not colocalize to the nSB structures (Leroy, Perrin et al. 1997). Interestingly, the cycling of nSB assembly/disassembly upon successive rounds of short HS treatments results in their formation at the same positions on the chromatin (Jolly, Usson et al. 1999). Investigation of these nuclear positions led to the finding that upon HS, HSF1 binds to the pericentric heterochromatic 9qh region of the human chromosome (Jolly, Konecny et al. 2002), which is a block of heterochromatin composed of satellite III repeats (Jones, Prosser et al. 1973). The transcriptional activation of these sites results in the production of non-coding RNA molecules that remain fixed at the transcription site and are composed of multiple repeats of the
sequence \((\text{GGAAT})_n\text{CAACCGAGT}\) (Valgardsdottir, Chiodi et al. 2005). Therefore, while this sequence is not a canonical HSE, this degenerate nGAAn motif may be responsible for recruiting HSF1 specifically to the genomic satellite III fragments, as other satellite sequences such as Sat II and \(\alpha\)-satellite do not recruit HSF1 (Grady, Ratliff et al. 1992; Jolly, Metz et al. 2004).

Figure 1.3. HSF1 localizes to nuclear stress bodies upon HS.
HeLa cells were fixed in formaldehyde and blocked in FBS. Immunocytochemistry was performed using an anti-HSF1 primary antibody (Stressgen cat#SPA-901) detected by an RFP secondary antibody (Invitrogen Alexa-Fluor #555) and the cells were Dapi stained. A 42°C HS results in localization of HSF1 (RFP) to nuclear stress bodies (nSBs).

While the function of nSBs is unknown, the accumulation of satellite III transcripts results in the recruitment of several RNA-processing proteins and splicing factors [reviewed by (Sandqvist and Sistonen 2004)]. This sequestration of RNA maturation factors may hypothetically aid in the global decrease in translation observed at the onset of the HSR. Conversely, translation of HSPs is maximized upon HS activation of HSF1. The rapid increase in HSPs may be facilitated by HSF1 forming a protein interaction with symplekin, a protein subunit found in a CstF/CPSF polyadenylation factor complex responsible for RNA maturation (Xing, Mayhew et al. 2004). Thus HSF1 may directly enhance the production of mature HSP transcripts by acting as a polyadenylation stimulatory factor.

Interestingly, nSBs are only found in human and monkey cell lines and have not been discovered in any other eukaryote, including rodents (Denegri, Moralli et al. 2002).
However, recent characterization of a transgenic *C. elegans* strain expressing tagged HSF-1::GFP at physiological levels has demonstrated localization of HSF-1 to subnuclear compartments that are reminiscent of primate nSBs (Morton and Lamitina 2012). Like nSBs, the induced *C. elegans* stress granules are transient and recruit HSF1 with rapid kinetics and reformation of these bodies upon subsequent HS occurs in approximately the same nuclear positions. Further research is necessary to determine if nSBs are an evolutionarily conserved mechanism of HSR regulation of proteostasis.

**Molecular Chaperones and Heat Shock Proteins**

The unifying characteristic of molecular chaperones is the ability to bind to denatured proteins. Molecular chaperones are proteins that non-covalently bind and form stable complexes with non-native proteins. Disassociation of a chaperone and its substrate most often requires the binding and subsequent hydrolysis of adenine triphosphate (ATP) (Fink 1999). Chaperones are ubiquitous and assist in the folding, assembly, transport, and degradation of proteins (Saibil, Zheng et al. 1993). Although their assistance is critical in these processes, chaperones do not contribute to the final protein conformation or activity.

As a nascent polypeptide chain emerges from the ribosome, highly interactive hydrophobic regions of the chain will tend to self-aggregate. Protein aggregation is an immediate impediment to successful folding and the achievement of a native state. It is not clear what percentage of proteins can successfully self-assemble into an active conformation. *In vitro* experiments indicate that small proteins have the ability to assemble in a few tens of milliseconds without ever achieving an intermediate folding conformation (Hartl 1996; Fink 1999). But for most proteins, the binding of chaperones to these interactive regions is imperative to successful folding.

In addition to assisting in the prevention of nascent protein aggregation, chaperones may act as holdsases for some intermediate stages of protein folding (Hoffmann, Linke et al. 2004). This role contributes to the correct folding pathway of the protein, but also acts as a method to prevent inappropriate folding. Macromolecular crowding in the cellular
environment often leads to the restriction of available free water. Cytoskeletal elements, organelles, membranes, and a high concentration of other proteins all contribute to the crowded environment (Ellis 2001). Intracellular crowding leads to a general increase in reaction efficiencies. Without the mechanism of chaperone control, proteins may attain conformation prematurely, which may interfere with transcriptional regulation, cell signaling, and translocation of the protein (Zhou, Rivas et al. 2008).

Premature folding is specifically problematic in protein recruitment to cellular membranes and translocation. In order for translocation to occur, a protein must pass through intermembrane channels. Native proteins may be too large or bulky to pass through such narrow conduits. Thus, a chaperone may be necessary to act as a holdase to regulate protein folding and the subsequent transport of the protein to the appropriate membrane for translocation. Once past the membrane, additional chaperones may contribute to the folding of the protein into its active conformation (Ambjornsson and Metzler 2004).

In addition to binding to newly formed polypeptides, molecular chaperones are also responsible for managing proteins that have undergone denaturing stress. Denaturation may occur due to exposure of the protein to extreme pH, heat, organic solvents, high concentrations of inorganic salts, and other factors not conducive to the cellular environment (Hartl 1996). Upon encountering a denatured protein, molecular chaperones perform triage, either directing the protein into the correct folding pathway or shuttling the damaged protein to the proteasome for destruction (Roelofs, Park et al. 2009). Many molecular chaperones that are important in protein folding have also been shown to have roles in maintaining protein homeostasis such as the HSR. Molecular chaperones that are inducible in this manner are called heat shock proteins (HSPs) and make up the majority of the classes of chaperones. Progressively as research contributes to what is known about the HSR, several chaperones reported to assist in protein folding have been reclassified as HSPs.

While chaperone families are not stringently classified and often overlap, there are five families of HSPs commonly discussed in the literature: small HSPs, HSP40, HSP70,
HSP90, and HSP100. Several HSP chaperone families have members that are constitutive and do not have a role in the HSR. For instance, HSP70 is a highly inducible molecular chaperone that is often used as a sensor of the HSR, but the constitutive Hsc70 is maintained at a constant level in the cell and is not induced by stress (Takayama, Xie et al. 1999). The unifying characteristic of the HSP family of proteins is their inducibility by the master transcriptional regulator, HSF1.

Domains of HSF1

Similar to many eukaryotic transcription factors, HSF1 possesses a complex modular structure with several functional domains that have been mapped by mutational analyses (Wiederrecht, Seto et al. 1988; Sorger and Nelson 1989; Green, Schuetz et al. 1995; Newton, Knauf et al. 1996). Approximately 90% of eukaryotic transcription factors possess long regions of intrinsic disorder, which may contribute to their flexibility and allow protein interaction with multiple cellular partners (Liu, Perumal et al. 2006). More than half of the HSF1 protein is predicted to be disordered (Westerheide, Raynes et al. 2012). In fact, only two of the HSF1 functional domains, the DNA-binding domain (DBD, residues 15-120) and the hydrophobic repeat HR-A/B domain (residues 130-203), are predicted to be ordered. The HR region C, which is located at the beginning of the transactivation domain (TAD, residues 384-409), is predicted to be partially ordered. The DBD and HR-A/B domains are connected by a flexible linker and the remainder of the protein, including two important functional domains, the regulatory domain (RD, residues 221-310) and the TAD (residues 371-529) and their linker, is mostly disordered.

DNA binding domain (DBD)

The DBD is the most conserved domain of HSF1 from C. elegans to humans (Figure 1.4). Both the crystal structure of the DBD for Kluyveromyces lactis HSF and the solution structure of the DBDs for K. lactis and D. melanogaster HSF1 show that the DBD is a helix-turn-helix motif (Damberger, Pelton et al. 1994; Harrison, Bohm et al.

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1 Portions of this section have been previously published by the author (Westerheide, Raynes, et al 2012) and is utilized with permission of the publisher. See Appendix E.
The helix-turn-helix structure contains 3 alpha helices, 4 beta strands, and an exposed loop. Upon trimerization, the DBD of each HSF1 monomer recognizes an inverted nGAAAn repeat of the HSE (Fernandes, Xiao et al. 1994). The domains make contact with the major groove and with the DNA phosphate backbone by helix 3 (Vuister, Kim et al. 1994; Littlefield and Nelson 1999). Helix 3 is a region rich in positive charge and is the most conserved region of the DBD. Unlike other helix-turn-helix proteins, the loop in the HSF1 DBD does not bind DNA. Instead, the loop forms an interaction with the surface between adjacent HSF monomers (Littlefield and Nelson 1999).

**Trimerization domains**

The HSF1 trimerization domain is composed of three arrays of hydrophobic heptad repeats (HR-A/B) (Sorger and Nelson 1989). HSF1 trimerization is unusual in that when the monomers come together, they form a leucine zipper, an artifact typically seen in dimerization. Trimerization is constitutive and is actively suppressed by the hydrophobic heptad repeat HR-C (Rabindran, Haroun et al. 1993; Zuo, Baler et al. 1994). When a mutation is introduced into the HR-C domain, trimerization and binding to the DNA is able to persist. *S. cerevisiae* and *K. lactis* HSFs have constitutive DNA-binding activity. In these HSFs, the HR-C is poorly conserved and does not actively suppress trimerization. Mutating the HR-C does not affect the oligomerization status of HSF in these organisms (Chen, Barlev et al. 1993).

**Transactivation domains (TAD)**

Transcriptional elongation of *hsp* genes is regulated by the transactivation domains (TAD) of HSF1. In the absence of HSF1, RNA polymerase pauses at 46-49 bases downstream from the transcription start site even in the presence of excess free nucleotides (Brown, Imbalzano et al. 1996). The HSF1 transactivation domain can be divided into two distinct regions rich in hydrophobic and acidic residues: activation domain I and activation domain II, spanning amino acids 371-430 and 431-505, respectively (Green, Schuetz et al. 1995; Shi, Kroeger et al. 1995; Zuo, Rungger et al.
1995). These domains have been shown to equivalently stimulate both initiation and elongation of transcription (Brown, Weirich et al. 1998). This redundancy may be in effect due to the importance of HSPs in maintaining protein homeostasis.

**Regulatory domain (RD)**

The regulatory activity of HSF1 is located between amino acids 221 and 310 and is functionally conserved between mammalian *hsf1* genes, but not between other members of the HSF family (Green, Schuetz et al. 1995). Upon non-stress conditions in which protein homeostasis is preserved, the transactivator activity of TAD I and TAD II is repressed by the regulatory domain and an exposure to stress leads to a reversal of this repression (Green, Schuetz et al. 1995; Newton, Knauf et al. 1996). The regulatory domain of HSF1 contains several serine residues that are responsive to protein kinases and lead to repression of the TADs upon phosphorylation. Phosphorylation by the MAP kinase ERK is responsible for this transactivator repression and mutation of these phosphorylation sites abolishes the suppression of the transactivation domains even in the absence of stress (Chu, Soncin et al. 1996).

**Post-translational Modification Regulation of HSF1**

HSF1 is extensively modified by post-translational modifications including phosphorylation, sumoylation, and acetylation (Figure 1.5). This extensive modification may form a code to allow fine-tuning of the activity of HSF1 to the precise needs of the cell.

**Phosphorylation**

HSF is phosphorylated at several residues in order to promote or inhibit transactivation. HSF was first reported to display a reduction in electrophoretic mobility as a result of

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Figure 1.4. Amino acid sequence alignment of HSFs (with highlighted DBD) from different organisms using BLAST analysis.
Commonly discussed post-translational modification sites for HSF1.

In addition to these sites, Guettouche, et al. (2005) have indicated phosphorylation sites on serine residues 97, 230, 292, 314, 319, 344, and 363. While K80 is a critical lysine residue that when acetylated will cause a loss of affinity for DNA, other uncharacterized sites for acetylation include lysine residues 116, 118, 126, 148, 157, 208, 224, 298, 208, and 224 (Westerheide, Anckar et al. 2009).

Phosphorylation in *S. cerevisiae* (Sorger and Pelham 1988). Mammalian HSF1 also exhibits a similar electrophoretic shift due to hyperphosphorylation upon activation of the heat shock response. Phosphorylation regulates transcriptional activity and not DNA binding, as some inducers of HS, such as the anti-inflammatory agent sodium salicylate, are capable of inducing HSF1 to bind to the DNA, but do not cause hyperphosphorylation (Jurivich, Sistonen et al. 1992). Another example of the distinction between these two activities is observed in yeast. In *S. cerevisiae*, HSF exists as a trimer with constitutive DNA-binding activity. However, transcriptional activation only occurs in the presence of increased phosphorylation (Sorger, Lewis et al. 1987; Sorger and Pelham 1988; Sorger 1990).

Through phosphoamino acid analyses and mass spectrometry, several phosphorylation sites within HSF1 have been mapped. While phosphorylation has been primarily mapped to serine residues, a low level of threonine phosphorylation has also been detected (Chu, Soncin et al. 1996; Cotto, Kline et al. 1996; Kline and Morimoto 1997; Xia and Voellmy 1997; Holmberg, Hietakangas et al. 2001). Of the first phosphorylation sites to be
characterized are serine residues S303, S307, and S363 located within the C-terminal region of the regulatory domain. These sites are proline-directed and typically experience phosphorylation in the absence of stress. Upon stress, the repressive activity of these residues is superseded by the activation of HSF1 (Chu, Soncin et al. 1996; Knauf, Newton et al. 1996; Kline and Morimoto 1997). An HSF1 phosphorylation site that leads to transcriptional activation is serine residue 230, located within the N-terminal region of the regulatory domain (Holmberg, Hietakangas et al. 2001). Many sites within HSF1 can be phosphorylated, and a mass spectrometry analysis of HSF1 activated by heat shock found phosphorylation on Ser121, Ser230, Ser292, Ser303, Ser307, Ser314, Ser319, Ser326, Ser344, Ser363, Ser419, and Ser444 (Guettouche, Boellmann et al. 2005). Phosphorylation of Ser326, but none of the other serine residues, was found to contribute significantly to activation of HSF1 upon stress in this study (Guettouche, Boellmann et al. 2005). While the implications of HSF1 phosphorylation have yet to be fully characterized, it is clear that dynamic phosphorylation modulates HSF1 activity in both a positive and negative manner.

HSF1 phosphorylation is regulated by a number of kinases with several of the residues suspected to undergo modification by more than one kinase. MAP kinases have been shown to negatively regulate HSF1 activity and the HSF1 Serine residues S303, 307 and 363 have been found to be MAP kinase targets. The MAP kinase ERK1 has been shown to phosphorylate S307, after which S303 is receptive to phosphorylation by glycogen synthase-3 kinase (GSK-3) (Chu, Soncin et al. 1996; Knauf, Newton et al. 1996; Kline and Morimoto 1997). The MAP kinase p38 has also been reported to phosphorylate S303 and/or S307, resulting in basal repression of HSF1 (Knauf, Newton et al. 1996). JNK, another MAP kinase family member, and the PKC isoforms alpha and zeta have been suggested to phosphorylate S363, thereby contributing to the maintenance of HSF1 in an inert state (Chu, Zhong et al. 1998; Dai, Frejtag et al. 2000). Inhibition has also been shown to occur through the phosphorylation of S121 by the proinflammatory protein kinase MK2, which may lead to an increase in affinity for Hsp90 (Wang, Khaleque et al. 2006). Various kinases suspected of activating HSF1 have also been discovered. For instance, the Ca2+/calmodulin-dependent kinase II (CaMK II) phosphorylates S230 and promotes transcriptional activity (Holmberg, Hietakangas et al. 2001).
2001). Given the broad range of stimuli that induce the HSR and the vast number of HSF1 phosphorylation sites, it may be that different kinases regulate HSF1 depending on the nature of the stress. As an example, in yeast, Snf1 protein kinase has been shown to regulate HSF1 in low glucose conditions, but does not phosphorylate HSF upon temperature heat shock (Hahn and Thiele 2004).

**Sumoylation**

Upon stress, HSF1 is modified by SUMO-1 at the well-conserved lysine residue 298 within the N-terminal region of the regulatory domain (Hietakangas, Ahlskog et al. 2003). In order for K298 to become sumoylated, S303 must first undergo phosphorylation, which has a repressive effect on HSF1 activation (Hietakangas, Ahlskog et al. 2003). There may be a regulatory interplay of modifications at this residue as residue K298 is also an acetylation site (Westerheide, Anckar et al. 2009).

**Acetylation**

While the kinetics of phosphorylation and sumoylation are brought on rapidly upon stress, acetylation is delayed and coincides with a reduction of HSF1 DNA-binding activity at the decline of the HSR (Westerheide, Anckar et al. 2009). Acetylation of HSF1 by the histone acetyltransferase p300 occurs to diminish HSF1 binding to the DNA. The deacetylation of HSF1 is regulated by the NAD⁺-dependent deacetylase SIRT1. When SIRT1 is inhibited by siRNA, not only are the levels of heat shock-induced hsp70 mRNA significantly decreased, but the ability of HSF1 to bind to the hsp70 promoter region is also greatly reduced (Westerheide, Anckar et al. 2009).

**Other Heat Shock Transcription Factors**

The mammalian HSF family is composed of HSF1, 2, 3, 4, 5, Y and X (Fujimoto and Nakai 2010). While there is some redundancy, the HSF family members maintain

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different functionalities. Mammalian HSF1 is the ortholog of the single, essential HSF that is present in *S. cerevisiae*, *C. elegans*, and *D. melanogaster*. HSF1 is the best-characterized member of the HSF family and the factor responsible for the HSR. HSF2 and HSF4 have various roles in development and differentiation (Pirkkala, Nykanen et al. 2001). Mammalian HSF3, 5, Y and X have not yet been well characterized.

**HSF2**

HSF2 has been shown to recruit to satellite III repeats in nuclear stress bodies (NSBs) along with HSF1 (Alastalo, Hellesuo et al. 2003). At this colocalization site, HSF2 has been indicated to play a role in the HSR by modulating HSF1 in a heterocomplex formation (Ostling, Bjork et al. 2007). HSF2 was originally discovered in tandem with HSF1 and similarly was shown to stimulate HSE-dependent transcription *in vitro* (Sarge, Zimarino et al. 1991; Schuetz, Gallo et al. 1991). While HSF2 is capable of inducing the transcription of HSPs, it is not activated by heat and therefore has not been associated with the induction of the HSR (Sarge, Zimarino et al. 1991; Sistonen, Sarge et al. 1992). However, HSF2 has been shown to be involved in development and cellular differentiation (Sistonen, Sarge et al. 1992; Mezger, Rallu et al. 1994; Murphy, Gorzowski et al. 1994; Sarge, Park-Sarge et al. 1994; Rallu, Loones et al. 1997).

Despite low amino acid sequence homology between HSF1 and HSF2, the DNA binding domain, as well as the N-terminal and C-terminal trimerization domains, are conserved. Similar to human HSF1, more than half of HSF2 is predicted to be disordered. In addition, HSF2 possesses several ordered functional domains including a DBD (residues 7-112), a HR-A/B domain (residues 119-192), and a HR-C domain (residues 360-385). The remainder of HSF2 is mostly disordered. However, the manner of DNA-binding differs between the two proteins. HSF2 appears to regulate a different set of target genes compared to HSF1 and also experiences variable expression patterns in different tissues and cell types (Sistonen, Sarge et al. 1992; Rallu, Loones et al. 1997). The DNA binding-specificity of HSF1 is determined by the loop within the DNA binding domain. Chimeric HSF2 containing the HSF1 DBD loop is capable of binding to HSF1 target genes upon HS stress (Ahn, Liu et al. 2001). In addition, the transactivation activity of
HSF2 is considerably weaker than HSF1, possibly as a result of a dispersed AD (Yoshima, Yura et al. 1998). In addition to the role of HSF2 in development, evidence has indicated that HSF2 may act as a modulator of HSF1 transcription. While HSF2 has not been shown to directly regulate the HSR, HSF2 has been shown to interact with HSF1, is recruited to nuclear stress bodies along with HSF1, and has been shown to stimulate HSF1-mediated transcription upon heat shock stress (He, Soncin et al. 2003).

**HSF4**

HSF4 exhibits limited sequence homology to the other HSF family members, but regions of similarity correspond to the DNA binding domain and the N-terminal hydrophobic repeats (HR-A/B). Like other HSF family members, HSF4 is able to bind to HSEs. However, when HSF4 is co-transfected into Cos7 cells along with a transcriptional HSE-reporter construct, HSF4 is unable to induce transcription and is therefore not a typical activator for the transcription of *hsp* genes (Nakai, Tanabe et al. 1997). In addition, HSF4 lacks a *cis*-regulatory domain that represses HSF1 under non-stress conditions (Green, Schuetz et al. 1995).

HSF4 has also been shown to exhibit crosstalk with HSF1. Together, HSF1 and HSF4 are involved in the maintenance of sensory organs and are critical during lens development (Fujimoto, Oshima et al. 2008). HSF4 expression is specific to the brain and lungs and has been indicated to play a role in protein quality control (Tanabe, Sasai et al. 1999; Fujimoto, Izu et al. 2004). Mutations of HSF4 have been shown to lead to cataractogenesis and the breakdown of the lens microarchitecture (Bu, Jin et al. 2002; Smaoui, Beltaief et al. 2004). Two HSF4 missense mutations have been identified by screening age-related cataract patients (Shi, Shi et al. 2008). These mutations appear to have an effect on HSF4 DNA-binding to HSEs resulting in an under-expression of heat shock proteins in the lens, which consequently lead to an increase in protein aggregates that cause cataracts.
HSF3 was first identified in chicken, where it is the HSF that is essential for activation of the HSR in this species (Nakai and Morimoto 1993). Mammalian HSF3 has only recently been identified in mouse, and is capable of activating non-classical heat inducible genes during HS by binding to the PDZ domain-containing 3 (Pdzk3) promoter (Sistonen, Sarge et al. 1992). Although sequences related to HSF3 have also been found in the orthologous region of the human genome, this sequence is thought to be a pseudogene as no transcripts corresponding to this gene have been found (Fujimoto and Nakai 2010).

HSF5 has recently been discovered as part of a large gene characterization project (Ahn, Liu et al. 2001) and has been identified as a potential transcription factor within the HSF family, but has not undergone characterization. HSFY and HSFX are the only members of the HSF family found on the sex chromosomes. HSFY is present on the human Y chromosome as well as murine chromosome 2. It is predominantly expressed in the testes and may potentially have a role in spermatogenesis (Shinka, Sato et al. 2004; Tessari, Salata et al. 2004; Bhowmick, Takahata et al. 2006). Even less has been characterized regarding HSFX, but both HSFs have been found to exist as two identical copies (Bhowmick, Takahata et al. 2006).

Sensory Regulation of HSF1 and the Heat Shock Response

Due to the importance of HSF1 as a regulator of protein homeostasis, the activation and disengagement of transcriptional activity is a finely tuned process. While many studies have focused on the function of HSF1 as the master regulator of cytoprotective genes, little work has focused on the manner in which HSF1 senses proteotoxic stress and mounts or attenuates a response. The triggering of HSF1 by temperature sensitive RNAs and negative feedback by HSPs are two mechanisms of HSF1 control.
Activation of HSF1 by Temperature Sensitive RNA

In *E. coli*, the HSR is regulated by an alternative sigma factor known as $\sigma^{32}$, which recruits RNA polymerase to transcribe HSPs (Grossman, Erickson et al. 1984; Taylor, Straus et al. 1984). Upon thermal stress, HSP transcription is rapidly induced, then declines as the cells adapt to the stress, and finally reaches an equilibrium that is typically observed in high temperature bacteria (Straus, Walter et al. 1987). Once the cells reach homeostatic conditions, excess HSPs are diluted out of the cells as they divide. An upshift in temperature results in the translation of $\sigma^{32}$, which is encoded by the *rpoH* gene. One of the hallmarks of translational control of $\sigma^{32}$ is the ability of the *rpoH* mRNA to react to changes in temperature. At $30^\circ C$, *rpoH* mRNA undergoes a structural transition that results in base-pairing that occludes the Shine-Dalgarno sequence (Figure 1.6), thus inhibiting translation. However, at high temperatures the *rpoH* RNA secondary structure is resolved and translation of $\sigma^{32}$ is no longer inhibited (Morita, Kanemori et al. 1999; Morita, Tanaka et al. 1999). Therefore, *rpoH* mRNA acts as a thermosensor to regulate the abundance of the HSR sigma factor, $\sigma^{32}$, and thus it’s ability to mount and attenuate a response to denaturing stress.

In eukaryotes, temperature-sensing RNA has also been indicated to play a regulatory role in the HSR. In a study to identify putative HSF1 interacting partners, fractionation of lysate from HS-treated HeLa or hamster BHK-21 cells on an HSF1-Sepharose column and subsequent mass spectrometry analysis resulted in the identification of an aminoacyl-tRNA-binding translation elongation factor known as eEF1A (Shamovsky, Ivannikov et al. 2006). Co-immunoprecipitation of eEF1A from BHK-21 cells indicated an increase in eEF1A-bound HSF1 upon HS, but exogenous eEF1A was unable to activate HSF1 *in vitro* (Shamovsky, Ivannikov et al. 2006). Further analysis indicated that the presence of a specific non-coding RNA (ncRNA), subsequently named Heat Shock RNA-1 (HSR-1), was required to mediate eEF1A conversion of HSF1 into its trimeric DNA-bound state (Shamovsky and Nudler 2009). While little is know regarding HSR-1 RNA, it is a proposed thermosensor that may undergo structural changes resembling the mechanism of translational control in the bacterial *rpoH* RNA thermometer.
Figure 1.6. Secondary RNA structure for the bacterial rpoH mRNA.
The rpoH RNA undergoes a secondary structure upon homeostatic conditions that blocks translational access of the ribosomal subunits to the Shine-Dalgarno sequence. Induction of thermal stress results in the melting of the rpoH RNA structure and allows the ribosome to translate sigma factor 32 (Johansson and Cossart 2003).

Negative feedback of HSF1 by Heat Shock Proteins

During the recovery phase of the HSR, an accumulation of HSPs corresponds to a reduction of hsp transcription, therefore attenuating the response. Perturbation of HSP translation or functionality results in a prolonged HSR in which hsp transcription does not abate, indicating a self-regulating mechanism for transcriptional control of HSF1 (Didomenico, Bugaisky et al. 1982). HSP regulation of HSF1 is present in homeostatic conditions, whereby HSP90 sequesters the inactive HSF1 monomer in the cytoplasm; upon activation of HSF1 by denaturing stress, the HSP90-HSF1 interaction is greatly diminished (Zou, Guo et al. 1998). Furthermore, inhibition of HSP90 by geldanamycin in Xenopus oocytes results in the conversion of HSF1 to an active trimer (Ali, Bharadwaj
et al. 1998). In addition to releasing the monomeric HSF1 for trimeric activation in the cytoplasm, HSP90 may play a role in inactivation of HSF1 in the nucleus. An HSP90-FKBP52-p23 complex binds to the HSF1 regulatory domain (RD) and disruption of this complex promotes HSF1 DNA-binding at *hsp* promoters (Ali, Bharadwaj et al. 1998; Bharadwaj, Ali et al. 1999; Guo, Guettouche et al. 2001).

In addition to HSP90 regulation, the robust increase of HSP70 levels that accrue throughout the HSR also play a role in the negative feedback of HSF1. HSP70, along with co-chaperone HSP40, bind to the HSF1 activation domain (AD) and inhibit HSF1 transactivation (Shi, Mosser et al. 1998). This model is a method to sequester monomeric HSF1 in the cytoplasm during unstressed conditions. HSP70/HSP40 is then titrated away from HSF1 upon the presence of misfolded proteins, thus allowing the transactivation of HSF1. Moreover, accumulation of HSP chaperones and the unavailability of denatured proteins causes HSP70/HSP40 to disassociate HSF1 trimers (Morimoto, Sarge et al. 1992; Shi, Mosser et al. 1998). However, the robust molar excess of HSP70 in heat shocked cells would presumably make fine-tuning of HSF1 activity problematic (Shamovsky and Nudler 2008). Therefore, negative feedback by the HSP70/HSP40 complex may be a passive mode of control compared to HSP90 regulation. Additionally, post-translational acetylation of the HSF1 DBD is a more rapid measure to repress HSF1, with SIRT1 acting as an inhibitor of HSF1 repression.

**An Introduction to Sirtuins**

The sirtuin family is evolutionarily conserved from prokaryotes to eukaryotes (Brachmann, Sherman et al. 1995). Sir2 (silent information regulator 2) is the original sirtuin and was first observed in yeast during an extensive screen for silencing factors (Rine, Strathern et al. 1979). Sir2 is a Class III NAD⁺-dependent histone deacetylase that possesses transcriptional silencing activity due to the removal of histone acetyl groups resulting in the tighter packaging of chromatin (Fritze, Verschueren et al. 1997). The mechanism of sirtuin deacetylation is unique, in which the SIRT protein binds to a NAD⁺ molecule, thus cleaving nicotinamide and transferring the acetyl group from a substrate protein onto the NAD⁺ backbone. The result is a 1:1:1 ratio of nicotinamide, *O*-acyethyl-
ADP-ribose, and the deacetylated substrate protein as measured by HPLC quantification. In the absence of enzyme or acetylation of the substrate protein, no ADP-ribose or nicotinamide is generated from available NAD\(^+\), thus establishing the necessity of NAD\(^+\) hydrolysis in the catalysis of SIRT1 deacetylation activity (Landry, Slama et al. 2000). Additionally, some sirtuin family members, such as SIRT4 and SIRT6, possess ADP-ribosyltransferase activity capable of transferring the O-acetyl-ADP-ribose onto acceptor protein residues (Haigis and Sinclair 2010). ADP-ribosyltransferase activity occurs most often in the presence of proteins containing acetyl-lysines and is not specific to sirtuin deacetylation of threonine and serine residues (Landry, Sutton et al. 2000).

Interest in sirtuins has grown with their association with longevity. It was found that an extra copy of Sir2 transformed into yeast resulted in a reduction in recombination of ribosomal DNA and a subsequent 30% increase in lifespan of the yeast mother cell (Kaeberlein, McVey et al. 2001). The Sir2 homolog Sir2.1 was later discovered to extend lifespan in *C. elegans*, suggesting that Sir2 homologs and the NAD\(^+\)/NADH ratio may also be responsible for extending lifespan in higher eukaryotes, such as mammals (Tissenbaum and Guarente 2001). While several studies have supported the role of Sir2 in longevity, recently the impact of sirtuins in fly and worm lifespan extension has been questioned on the basis that several aging studies did not utilize the appropriate controls. By outcrossing Sir2 overexpression transgenic animals to wild-type animals, the intensity observed in previous lifespan studies was diminished, thus bringing to light that differing genetic backgrounds related to transgene insertion must be a measure controlled in experimental design (Burnett, Valentini et al. 2011). Recent experiments to address this issue in *C. elegans* with identical genetic backgrounds now show that while the original effect on lifespan by Sir2 overexpression was overestimated, there is still a 10–14% lifespan extension (Viswanathan and Guarente 2011).

**Modulators of SIRT1**

While evidence has emerged that the NAD\(^+\)/NADH ratio plays a critical role in SIRT1 regulation, until recently little investigation had been done concerning proteins that may play a role in regulating the function of SIRT1. Two potentially significant modulators
of SIRT1 activity include the positive and negative regulators AROS and DBC1, respectively. AROS, an alias for Active Regulator of SIRT1, is a nuclear protein also referred to as ribosomal protein S19 binding protein 1 (RPS19bp1). Mutations in Ribosomal protein S19 (RPS19) are found in patients diagnosed with a type of aplastic iron deficiency known as Diamond-Blackfan anemia (DBA) (Draptchinskaia, Gustavsson et al. 1999) and RPS19bp1 was discovered as a result of a study investigating the role of RPS19 in erythropoiesis (Maeda, Toku et al. 2006). AROS has been shown to enhance SIRT1-mediated deacetylation of p53, thus inhibiting p53-mediated transcriptional activity and promoting cell survival. In addition, AROS activity is abrogated by SIRT1 inhibitors and SIRT1 siRNA (Kim, Kho et al. 2007).

DBC1, an alias for Deleted in Breast Cancer 1, was initially cloned from a region (8p21) homozygously deleted in 3.5% of breast cancers (Hamaguchi, Meth et al. 2002). DBC1 has been found to directly interact with SIRT1 and inhibit deacetylase activity both in vivo and in vitro (Kim, Chen et al. 2008). DBC-1 mediated down-regulation of SIRT1 has an effect on the transcriptional activity of other sirtuin substrates, such as p53. The repression of SIRT1 by DBC1 leads to an increase in p53 acetylation, and therefore an upregulation of p53 apoptotic activity. Likewise, the RNA interference of DBC1 results in an increase of SIRT1-mediated deacetylation thus inhibiting p53-dependent apoptosis (Zhao, Kruse et al. 2008). In a similar manner, DBC1 has also been shown to regulate the acetylation status of the nuclear receptor PPARγ (Santagata, Hu et al. 2011). Both AROS and DBC-1 have yet to be investigated in relation to SIRT1 and the HSR.

**Molecular function of SIRT1**

The deacetylase activity of SIRT1 has been well documented in the regulation of several stress-induced transcription factors including p53 (Luo, Nikolaev et al. 2001; Vaziri, Dessain et al. 2001), NF-κB (Yeung, Hoberg et al. 2004; Salminen, Huuskonen et al. 2008; Jung, Lee et al. 2009), PGC-1α (Rodgers, Lerin et al. 2005; Rodgers, Lerin et al. 2008), HIV Tat (Chen, Zhou et al. 2005; Pagans, Pedal et al. 2005), and the FOXO family of transcription factors (Brunet, Sweeney et al. 2004; Motta, Divecha et al. 2004; Viswanathan, Kim et al. 2005). Research has highlighted SIRT1 as a potential oncogene
based on its ability to deacetylate p53, thus resulting in cell survival (Vaziri, Dessain et al. 2001). SIRT1 deacetylation of transcription factors may result in their activation or inactivation. For instance, SIRT1 deacetylation of the FOXO family of transcription factors activates genes that are important for gluconeogenesis (Brunet, Sweeney et al. 2004; Giannakou and Partridge 2004), while deacetylation of NF-κB results in the suppression of inflammatory responses (Yang, Zhang et al. 2012). In addition, SIRT1 deacetylation of Tat, the viral trans-activating regulatory protein, has been shown to increase HIV expression (Pagans, Pedal et al. 2005). Most recently, SIRT1 has been shown to be an HSF1 deacetylase, indicating SIRT1 as a regulator of protein homeostasis (Westerheide, Anckar et al. 2009). The activation of SIRT1 upon stress may be an evolutionarily conserved role to drive cellular homeostasis by invoking a variety of stress response pathways.

**SIRT1 and Metabolic Stress**

Caloric restriction (CR) is a 30-40% decrease in dietary intake with maintained nutrition that has been shown to increase longevity and protect against age-related disease (Bishop and Guarente 2007). The life extension effects of CR were first established as early as 1935 using calorically-restricted rats (McCay, Crowell et al. 1989) and have now been extended to yeast (Fabrizio, Pozza et al. 2001; Kaeberlein, Powers et al. 2005), nematodes (Johnson 2008; Greer and Brunet 2009), flies (Giannakou, Goss et al. 2008; Min, Yamamoto et al. 2008), and mice (Anderson, Shanmuganayagam et al. 2009). Sirtuins are linked to metabolism based on their unique ability to breakdown NAD$^+$ during protein deacetylation, resulting in the formation of nicotinamide and O-acetyl-ADP-ribose (Denu 2003). Nicotinamide phosphoribosyltransferase (NAMPT) is an important component in the NAD$^+$ synthesis pathway that converts nicotinamide to NAD$^+$ and has been shown to regulate the transcriptional function of Sir2α in mammalian cells (Revollo, Grimm et al. 2004). In fasted mice, an increase in NAMPT activity correlated with an increase in NAD$^+$ levels and enhanced SIRT1 transcriptional activity (Hayashida, Arimoto et al. 2010) (Figure 1.7). Interestingly, it has been reported that a 10-fold change in cellular NAD$^+$ concentration is required to affect Sir2 activity.
(Anderson, Latorre-Esteves et al. 2003). However, CR studies in yeast have shown that Sir2 activity is also regulated by a reduction of NADH, a competitive inhibitor of Sir2 (Lin, Ford et al. 2004; Rahat, Maoz et al. 2011). CR was found to increase the replicative lifespan of budding yeast by Sir2 activation due to decreasing the NADH levels resulting in an increase in the NAD$^+/\text{NADH}$ ratio (Lin, Ford et al. 2004). In both calorically restricted rats and human cell culture treated with serum from these animals, CR results in an increase of SIRT1 expression (Cohen, Miller et al. 2004).

**Figure 1.7. Model of sirtuin deacetylation.**

Sirtuins bind to NAD$^+$ and an acetylated substrate protein in order to catalyze the transfer of the acetyl group to the NAD$^+$-backbone, thus resulting in the deacetylated substrate protein, nicotinamide, and $O$-acyetyl-ADP-ribose in a 1:1:1 ratio. Nicotinamide, a SIRT inhibitor, is converted back to NAD$^+$ upon CR induction of the NAD$^+$ biogenesis pathway through NAMPT.

Furthermore, while SIRT1 is regulated in part by metabolism, SIRT1 is also required for the CR phenotype. In yeast, the deletion of Sir2 blocks the beneficial effects of CR on yeast lifespan (Lin, Kaeberlein et al. 2002). In mammals, CR produces complex behavioral changes, including increased physical activity and an increase in distance
coverage, which is most likely associated with foraging behavior (Weed, Lane et al. 1997). Moreover, wild-type mice and SIRT1 knockout (KO) mice demonstrate vastly different phenotypes upon CR, with the SIRT1 KO mice exhibiting traits very similar to wild-type control mice that are not on a restricted diet (Chen, Steele et al. 2005).

**Metabolism and the Heat Shock Response**

Overnutrition is a major health concern in developed countries and is the result of an unbalanced lifestyle in which energy consumption surpasses energy expenditure. This imbalance can lead to the development of a condition known as metabolic syndrome that is characterized by insulin resistance, hypertension, obesity, and type 2 diabetes (Zimmet, Magliano et al. 2005). The metabolic dysfunction characterized by diabetes can lead to a loss of protein homeostasis that impacts a number of biological systems. For example, post-translational glycation of the small HSP α-crystallin in the lens leads to a decrease in chaperone activity that contributes to the formation of cataracts (Kumar, Kumar et al. 2007). Diabetic patients exhibit decreased expression of HSP70 in skeletal muscle (Kurucz, Morva et al. 2002) and diabetic monkeys display a significant decline of both HSP70 and HSP90 in the blood and liver (Kavanagh, Zhang et al. 2009). While an impaired HSR is a hallmark of diabetic disease, induction of the HSR can rescue metabolic distress. For instance, physical exercise has been shown to induce the HSR resulting in the upregulation of HSPs (Smolka, Zoppi et al. 2000; McArdle, Pattwell et al. 2001; Milne and Noble 2002). Furthermore, streptozotocin-induced diabetic rats have impaired HSP72 expression in the heart, liver, and muscles, but 8 weeks of endurance training increased HSP72 expression and compensated for some of the deleterious effects associated with diabetes (Atalay, Oksala et al. 2004; Lappalainen, Lappalainen et al. 2010). Interestingly, rats on a high-fat diet that were treated weekly with a 20 min HS at 41° C for 12 weeks have improved glucose tolerance, increased insulin signaling, and increased expression of HSP72 and HSP25 (Gupte, Bomhoff et al. 2009).

Due to the fact that overnutrition leads to proteotoxic stress, it stands to reason that caloric restriction has a positive impact on protein homeostasis. CR reverses the age-related decline of HSP70 in rat hepatocytes (Heydari, Wu et al. 1993). Moreover,
CR promotes cytoprotection to thermal stress allowing for 100% survival compared to 50% survival of old rats on an *ad libitum* diet (Hall, Oberley et al. 2000). Other forms of diet restriction including glucose restriction with 2-deoxy-D-glucose (DOG), a nonmetabolizable glucose analog, have been shown to increase HSP70 levels in the striatum, hippocampus, and synaptic terminals of rat brains (Duan and Mattson 1999; Lee, Bruce-Keller et al. 1999; Yu and Mattson 1999; Guo and Mattson 2000). DOG-treated animals experienced cytoprotection from oxidative damage and mitochondrial dysfunction upon exposure to amyloid beta-peptide (Guo and Mattson 2000). However, the cytoprotective nature of a restricted diet appears to be due to a number of protective mechanisms that overlap with the HSR. In *C. elegans*, HSF-1 is required for longevity (Morley and Morimoto 2004), but glucose restriction via DOG-treatment results in lifespan extension that is independent of the HSF-1 regulator, Sir2.1 (Schulz, Zarse et al. 2007). There are several methods to induce dietary restriction that differentially invoke a number of cytoprotective pathways including the forkhead transcription factor FOXO, the AMP-dependent protein kinase AMPK, and the oxidative stress inducible Nrf2, in addition to HSF1 [reviewed in (Dancso, Spiro et al. 2010)]. Regardless of the crosstalk between these various pathways, it is evident that the HSR and metabolism are linked and dynamically impact organismal health.

**SIRT1 Regulation of HSF1**

SIRT1 regulates the HSR in an HSF1-dependent manner. In cell culture, SIRT1 siRNA inhibition results in a significant reduction of HSF1 recruitment to the *hsp70* promoter and a drastic decline of *hsp70* mRNA levels upon HS (Westerheide, Anckar et al. 2009). Moreover, only wild-type SIRT1, and not a catalytic mutant, is capable of deacetylating HSF1 in an *in vivo* acetylation assay (Westerheide, Anckar et al. 2009). Recently, SIRT1 has also been shown to deacetylate HSF1 in neurons stressed by mutated α-synuclein. SIRT1 activated the HSR in an HSF1-dependent manner, thereby increasing *hsp70* transcription resulting in a reduction of α-synuclein aggregation and conferring neuroprotection (Donmez, Arun et al. 2012).
Since the initial finding that SIRT1 regulates the HSR in an HSF1-dependent manner, little work has been done to understand the role of SIRT1 on the HSR. This dissertation aims to elucidate the role of SIRT1 on the heat shock response and the impact of metabolism.

**Specific Aims**

The objective of this study is to investigate the mechanism by which SIRT1 regulates HSF1 activity and the impact of caloric restriction. **The hypothesis of this proposal is that SIRT1 undergoes a change in activity upon stress, including heat and caloric restriction, resulting in the modulation of the heat shock response.** The following specific aims are designed to test this hypothesis.

*Investigate the Mechanism of SIRT1 regulation of the Heat Shock Response (Chapter 2)*

**Aim 1:** Determine the mechanism of SIRT1 activity regulation upon initiation of the heat shock response.

1.1: Determine whether sirt1 mRNA or protein levels change upon initiation of the HSR.
1.2: Investigate how heat shock influences the cellular NAD$^+/$/NADH ratio.
1.3: Determine if initiation of the HSR alters SIRT1 recruitment to the hsp70 promoter.
1.4: Investigate whether AROS and DBC1 impact SIRT1 deacetylation of HSF1.

**Aim 2:** Investigate the role of AROS and DBC1 in SIRT1 regulation of the HSR in an HSF1 dependent manner.

2.1: Determine if AROS and DBC1 mRNA or protein levels vary throughout a heat shock time course.
2.2: Determine if AROS and DBC1 have a positive and negative impact, respectively, on the HSR.
2.3: Determine if AROS and DBC1 alter HSF1 recruitment to and activity at the hsp70 promoter.
Aim 1: Investigate the effect of metabolism on Sir2.1-regulation of the heat shock response.

1.1: Determine if heat shock and metabolism have the same effect on the HSR using a strain of *C. elegans* possessing GFP integrated with a heat inducible promoter.
1.2: Determine whether a synergistic effect between elevated temperature and caloric restriction is observed at the transcriptional level for multiple members of the *hsp70* family.
1.3: Investigate whether a synergistic effect between elevated temperature and caloric restriction is dependent on Sir2.1, specifically.

Aim 2: Investigate the functional effects of synergy between elevated temperature and caloric restriction.

2.1: Investigate the biological significance of synergy between elevated temperature and caloric restriction by evaluating organismal cytoprotection.
2.2: Investigate the impact of synergy between elevated temperature and caloric restriction using a neurodegenerative disease model.

**Studies**

The first two chapters consist of published manuscripts designed to address the Specific Aims (pages 28-29). Chapter 1 evaluates the roles of AROS and DBC1 in regulation of the heat shock response. Chapter 2 evaluates the capacity for caloric restriction and temperature heat shock to synergize to promote the induction of the heat shock response and cytoprotection in the *Caenorhabditis elegans* model organism. Chapter 3 consists of unpublished data supporting the hypothesis that RPS19, an AROS-interacting protein, is a transcriptional target of HSF1.
CHAPTER 2: THE SIRT1 MODULATORS AROS AND DBC1 REGULATE HSF1 ACTIVITY AND THE HEAT SHOCK RESPONSE

Authored by Rachel Raynes, Kathleen M. Pombier, Kevin Nguyen, Jessica Brunquell, Jamie E. Mendez, and Sandy D. Westerheide.


Experiments were designed, performed, and analyzed by R. Raynes or performed under the direction of R. Raynes. K. Pombier performed overexpression transfection in Figure 2.4B,C. K. Nguyen performed qPCR replicates for Figures 2.5C,D. J. Brunquell and R. Raynes measured cell viability in Figure 2.5B and 2.6C. J. Mendez and R. Raynes measured luciferase activity in Figure 2.4C.

All data analysis and figures were generated by R. Raynes. The manuscript was written by R. Raynes and S.D. Westerheide. See Appendix E for copyright permission.

Abstract

The heat shock response, the cellular response to protein damaging stress, is critical in maintaining proteostasis. The heat shock response is regulated by the transcription factor HSF1, which is activated upon heat shock and other stresses to induce the expression of molecular chaperones. SIRT1 has previously been shown to activate HSF1 by deacetylating it, leading to increased DNA binding ability. We have investigated how the heat shock response may be controlled by factors influencing SIRT1 activity. We found that heat shock results in an increase in the cellular NAD\(^+\)/NADH ratio and an increase in recruitment of SIRT1 to the *hsp70* promoter. Furthermore, we found that the SIRT1 modulators AROS and DBC1 have an impact on *hsp70* transcription, HSF1 acetylation
status, and HSF1 recruitment to the hsp70 promoter. Therefore, AROS and DBC1 are now two new targets available for therapeutic regulation of the heat shock response.

**Introduction**

The heat shock response (HSR) is a cellular response to diverse stressors that results in the induction of genes encoding molecular chaperones essential for protection and recovery from cellular damage (Anckar and Sistonen 2011). The stressors that activate transcription of these heat shock genes include elevated temperature, heavy metals, chemical toxicants, and oxidative stress. Heat shock proteins function to guide protein folding and protect cells against a wide array of stress conditions (Morimoto, Westerheide et al. 2009). The HSR is regulated at the transcriptional level by the heat shock transcription factor 1 (HSF1).

SIRT1 is a NAD\(^+\)-dependent deacetylase that regulates a number of target proteins, including p53 (Luo, Nikolaev et al. 2001; Vaziri, Dessain et al. 2001), PGC-1\(\alpha\) (Rodgers, Lerin et al. 2005; Rodgers, Lerin et al. 2008), HIV Tat (Chen, Zhou et al. 2005; Pagans, Pedal et al. 2005), NF-\(\kappa\)B (Yeung, Hoberg et al. 2004; Salminen, Huuskonen et al. 2008; Jung, Lee et al. 2009), and the FOXO family of transcription factors (Brunet, Sweeney et al. 2004; Motta, Divecha et al. 2004; Viswanathan, Kim et al. 2005). We have previously shown that SIRT1 also impacts the HSR by deacetylation of the transcription factor HSF1 (Westerheide, Anckar et al. 2009). However, the mechanism by which heat shock (HS) may regulate SIRT1 is still unknown.

SIRT1 has been shown to be regulated by diverse mechanisms. SIRT1 mRNA levels are stabilized by HuR, a ubiquitously expressed RNA binding protein, and oxidative stress has been reported to dissociate the *HuR-sirt1* mRNA complex allowing for *sirt1* mRNA decay (Abdelmohsen, Pullmann et al. 2007). SIRT1 enzymatic activity can be regulated by the cellular NAD\(^+\)/NADH ratio, as the hydrolysis of NAD\(^+\) is necessary to drive SIRT1 enzymatic activity (Borra, Langer et al. 2004). Caloric restriction (CR) was shown to increase the replicative lifespan of yeast by activating Sir2, the yeast SIRT1 homolog, due to decreasing NADH levels (Lin, Ford et al. 2004). Interestingly, CR
induces the expression of NAMPT, which triggers the NAD$^+$ salvage pathway converting nicotinamide (NAM) to NAD$^+$ (Menssen, Hydbring et al. 2012).

Recently, DBC1 and AROS have been indicated to have a role in SIRT1 regulation. DBC1, identified as a SIRT1 interactor via co-purification, was found to inhibit the deacetylase activity of SIRT1, resulting in increased p53 acetylation and the upregulation of p53 function and p21 gene expression (Kim, Chen et al. 2008; Zhao, Kruse et al. 2008). Conversely, AROS, identified as a SIRT1 interactor via a yeast two-hybrid screen, was found to activate SIRT1, promoting the deacetylation of p53 and leading to the downregulation of p53 function (Kim, Kho et al. 2007). The impact of SIRT1 expression levels, changes in the cellular NAD$^+$/NADH ratio, and the SIRT1 modulators DBC1 and AROS have yet to be investigated as they relate to the regulation of the HSR.

Here we further investigate the mechanism of SIRT1 regulation of the HSR. Previously, HSF1 has been shown to interact with SIRT1 via co-immunoprecipitation, indicating that HSF1 and SIRT1 interact, but not indicating whether or not the interaction is direct (Westerheide, Anckar et al. 2009; Donmez, Arun et al. 2012). We now show with a GST pull-down assay that the HSF1-SIRT1 interaction is direct. We report that induction of the HSR correlates with an increase in the cellular NAD$^+$/NADH ratio and with an increase in recruitment of SIRT1 to the hsp70 promoter. Additionally, we find that the SIRT1 modulators AROS and DBC1 have an impact on hsp70 transcription, HSF1 acetylation status, and HSF1 recruitment to the hsp70 promoter.

**Methods**

*Cell culture and heat shock*

HeLa, HEK293, and HeLa hsp70.1 promoter-luciferase reporter cells (Westerheide, Bosman et al. 2004) were utilized in this study. All cell lines were cultured in DMEM media (CellGro cat#15-017-CV) supplemented with 10% fetal bovine serum (GIBCO cat#10437-028) and 1% Pen-Strep-Glutamine (CellGro cat#30-0090Cl) at 37° C with 5% CO$_2$. HS was induced as previously described (Westerheide, Anckar et al. 2009).
Briefly, plates were wrapped in parafilm and submerged in a water bath set to 42°C for the designated times prior to collection with 1X PBS.

**Plasmids**

Plasmids used in this study include expression vectors for SIRT-MYC (Langley, Pearson et al. 2002), p300 (Eckner, Ewen et al. 1994), DBC1-HA (Kim, Chen et al. 2008), AROS (Kim, Kho et al. 2007), Flag-HSF1, and Myc-HSF1 (Cotto, Fox et al. 1997; Holmberg, Hietakangas et al. 2001).

**NAD⁺/NADH quantification**

HeLa cells were exposed to control conditions or a 2 hour HS in a 42°C water bath. The cells were collected in 1X PBS and NAD⁺/NADH extracted using the FluoroNAD™ Fluorescent NAD/NADH Detection Kit by Cell Technology, Inc (cat#FLNADH 100-2), according to the manufacturer’s instructions. The concentration readings were applied to a NADH standard curve and the NAD⁺/NADH ratio was determined.

**Chromatin immunoprecipitation**

HEK293 cells were fixed in 1% formaldehyde and neutralized in 125 mM glycine. The cells were collected in 1X PBS, lysed in Cell Lysis Buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP40) with the addition of Halt™ Protease Inhibitors (Thermo Scientific cat#78430), and then centrifuged, with the resulting pellet resuspended in Nuclei Lysis Buffer (50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS) with the addition of Halt™ Protease Inhibitors. Chromatin shearing was preformed for 45 minutes, cycling on and off for 30 seconds each using the Diagenode Bioruptor 300. Immunoselection and immunoprecipitation were performed essentially as previously described (Beresford and Boss 2001) using 5 µL of HSF1 (Enzo cat#ADI-SPA-901), SIRT1 (Millipore cat #07-131), or IgG (Cell Signaling Technology cat#2729S) antibody. The DNA was purified using phenol/chloroform/isoamyl alcohol (25:24:1) by standard procedure. qPCR was performed on the ChIP DNA with ABI’s Step One Plus Real-time PCR
system using BioRad’s iTaq™ Fast SYBR® Green Supermix with ROX (cat#172-5101) according to manufacturer’s protocol using primers to the hsp70 promoter-proximal HSE site, an upstream hsp70 promoter site, and the gapdh promoter (Table 1). Statistical data analysis and determination of relative fold increase from control samples without HS was performed according to standard protocol (Bookout and Mangelsdorf 2003).

Transient transfection and siRNA knockdown

Transfections were performed with Polyfect® Transfection Reagent (Qiagen cat#301107) according to the manufacturer’s protocol. HeLa hsp70.1 promoter-luciferase reporter and HEK293 cells were transfected with DharmaFECT transfection reagent (Thermo) according to the manufacturer’s protocol, using 50 nM of Dharmacon SmartPool DBC1, AROS, HSF1, or non-targeting (NT) control siRNA. RNA was isolated using Trizol 48 hours after transfection. Knockdown was confirmed via qRT-PCR.

Cell viability assay

Cell viability was measured 48 hours after treatment with siRNA or expression plasmids using PrestoBlue (Life Technologies Ltd. cat#A13262) according to the manufacturer’s instructions. Briefly, HEK293 cells were seeded in a 96 well plate and transfected as described above. After 48 hours of treatment, PrestoBlue was added to each well at a final concentration of 10%. After a 10 minute incubation at 37°C, total fluorescence was measured at excitation 525+/20 nm and emission 590+/35 nm.

HSF1 acetylation assay

HEK293 cells were transfected with expression plasmids Flag-HSF1 and p300, and either SIRT1-MYC, DBC1-HA, or AROS. Cell lysates were subjected to immunoprecipitation with Flag beads, and acetylated HSF1 was detected by Western blotting with an antibody that recognizes acetylated lysines (Cell Signaling Technology cat#9441).
Luciferase assay

HeLa hsp70.1 promoter-luciferase reporter cells were seeded in a 96 well plate at a density of 7.5 x 10^3 cells per well. All transfections were performed 24 hours after plating. 24 hours post-transfection cells were treated with or without 5 µM celastrol. Luciferase assays were performed 24 hours post-treatment with Promega’s Bright-Glo Luciferase Assay (cat#E2620) according to manufacturer’s instructions and as previously described (Westerheide, Kawahara et al. 2006).

Quantitative RT-PCR

qRT-PCR was performed to quantify mRNA levels for aros, dbc1, hsp27, hsp70, hsp90, sirt1, and gapdh using gene-specific primers (Table 1). Cells were collected in PBS and RNA was extracted using Trizol per standard protocol. RNA was reverse transcribed using Applied Biosystem’s High Capacity cDNA Reverse Transcription Kit (cat#4368814) according to the manufacturer’s protocol. The samples were diluted to 50 ng/µL and used as a template for qRT-PCR. qRT-PCR was performed with Applied Biosystem’s Step One Plus Real-time PCR system using BioRad’s iTaq™ Fast SYBR® Green Supermix with ROX (cat#172-5101) according to manufacturer’s protocol. Statistical data analysis and determination of relative fold increase from control samples was performed according to standard calculations (Bookout and Mangelsdorf 2003).

Immunoblotting analysis

The cells were collected in 1X PBS and then extracted using M-PER (Thermo Scientific cat#78503) with the addition of Halt™ Protease Inhibitors (Thermo Scientific cat#78430). Protein was quantified with Biorad Protein Assay (cat#500-0006) and 20 µg of protein was run on 10% SDS-PAGE gels. Gels were stained with 1% Ponceau in 10% glacial acetic acid for visualization of normalized protein levels before being blotted to nitrocellulose and immunostained with primary antibody in 5% milk. Primary antibodies used include ACTIN (Santa Cruz cat#sc-1616-r), AROS (Santa Cruz cat#sc-86210), β-tubulin (Cell Technology cat#2128), DBC1 (Abcam cat#ab70242), HA (Convance
cat#MMS-101P), c-MYC (Sigma cat#M4439), HSF1 (Assay Design cat#SPA-950), SIRT1 (Abcam cat#ab 32441), Acetylated-Lysine (Cell Signaling Technology cat#9441). HRP-conjugated secondary antibodies were from Millipore (cat#12-349 and 12-348) and Jackson ImmunoResearch (cat#112-035-062). ECL Plus Western Blotting Detection System (Amersham™ cat#RPN2132) was used to incubate blots prior to film exposure (Kodak ClinicSelect Blue X-ray film cat#604-1768).

Results

*SIRT1 is not regulated by a change in expression upon HS*

We sought to determine if HS might regulate the activity of SIRT1 through changes in SIRT1 expression levels. HeLa cells were treated with a 42°C HS from 0 to 6 hours and, as expected, *hsp70* mRNA levels increased substantially by 2 hours of HS and then began to decline by 6 hours of treatment correlating with the attenuation phase of the HSR (Figure 2.1A). The mRNA levels for *hsp27* and *hsp90* were also quantified, demonstrating that HS induces the transcription of multiple HSPs (Figure 2.1B,C). Conversely, *sirt1* mRNA levels were not altered during the initial 2 hours of HS treatment (Figure 2.1D). While *sirt1* mRNA levels did begin to decline by 4 hours of HS treatment, Western blot analysis shows that SIRT1 protein levels remained constant throughout the 6 hour HS time course, while HSP70 and HSP27 protein levels were increased at the 4 and 6 hour timepoints as expected (Figure 2.1E,F). HSP90 protein levels did not show a significant increase over this time course, likely due to the abundance and stability of this protein (data not shown) (Ethridge, Hellmich et al. 1998). Therefore, we conclude that the regulation of SIRT1 abundance is likely not a major mechanism by which HS controls SIRT1 activity, at least during the initial phases of the HSR.

*Heat shock induces an increase in the cellular NAD⁺/NADH ratio*

We next investigated whether HS could induce a change in the cellular NAD⁺/NADH ratio, as SIRT1 is well-documented as an NAD⁺-dependent deacetylase (Blander and
Guarente 2004; Baur, Pearson et al. 2006; Lavu, Boss et al. 2008; Zschoernig and Mahlknecht 2008). The cellular NAD$^+$ and NADH levels from whole cell extracts were quantified for HEK293 cells that were treated with HS at 42°C for 2 hours and the levels compared to unstressed cells. HS did not induce a significant increase in the cellular NAD$^+$ levels (Figure 2.2A), but did significantly decrease the cellular NADH levels by approximately 2-fold (Figure 2.2B). Overall, HS resulted in an increase of the NAD$^+$/NADH ratio from 2.5 to 6.5 (Figure 2.2C). HS may therefore have a positive impact on SIRT1 activity due to an increase in available NAD$^+$ and a reduction of NADH. We note that as SIRT1 is a nuclear protein, a more relevant measurement may be of the nuclear (instead of the whole cell) NAD$^+$/NADH ratio. Future studies will be required to determine if the nuclear NAD$^+$/NADH ratio is altered upon HS.

**SIRT1 recruitment to the hsp70 promoter increases upon heat shock**

We then tested SIRT1 recruitment to the hsp70 promoter over a HS time course to see if SIRT1 may be recruited to DNA with the same kinetics as HSF1. HEK293 cells were treated with HS at 42°C for 0, 2, 4, and 6 hours followed by chromatin immunoprecipitation (ChIP) using an antibody to SIRT1. We found that upon a 2 hour HS, there was a 4-fold increase of SIRT1 recruitment to the hsp70 promoter (Figure 2.3B). After 6 hours of HS, recruitment of SIRT1 to the hsp70 promoter was reduced to a 2-fold increase as compared to unstressed cells. These kinetics correspond to what has previously been reported for HSF1 DNA recruitment (Westerheide, Anckar et al. 2009) and also correlates with our HSF1 recruitment results in Figure 2.6D. Therefore, it is likely that SIRT1 has the ability to regulate HSF1 while it is on the promoter. Recruitment was also evaluated at an upstream non-specific site of the hsp70 promoter and at the gapdh promoter. We found that SIRT1 recruitment to these sites do not increase upon HS (Figure 2.3C,D). Therefore, increased recruitment of SIRT1 is specific to the HSE-binding site within the hsp70 promoter.
AROS and DBC1 expression have an impact on the HSR

As SIRT1 activity is positively regulated by AROS and negatively regulated by DBC1, we next sought to determine the impact of these proteins on the HSR. While aros and dbc1 mRNA levels do not change upon 0-3 hours of HS and show only minimal change between 3-6 hours of HS (Figure 2.4A), AROS and DBC1 overexpression have significant effects on hsp70 transcription (Figure 2.4B,C). AROS, DBC1, or empty vector was overexpressed in HEK293 cells and the induction of the HSR was assessed using the transcription of hsp70 as a marker throughout a 6 hour HS time course. We found that AROS overexpression resulted in a striking increase in HS-induced hsp70 mRNA levels (Figure 2.4B). Conversely, DBC1 overexpression resulted in a decrease in HS-induced hsp70 mRNA levels (Figure 2.4C).

Next, we investigated the impact of AROS and DBC1 siRNA knockdown on the HSR. We transfected HEK293 cells with AROS, DBC1, or a non-targeting (NT) siRNA and treated the cells with either DMSO (mock) or celastrol (5 μM), a potent inducer of the HSR (Westerheide, Bosman et al. 2004). Transfection with siRNA resulted in significant knockdown of aros and dbc1, which did not impact cell viability compared to the NT control (Figure 2.5A,B). Cells transfected with AROS siRNA displayed a decrease in hsp70 induction by celastrol (Figure 2.5C), while conversely cells transfected with DBC1 siRNA displayed an increase in hsp70 induction by celastrol (Figure 2.5D). Therefore, we conclude from these experiments that AROS and DBC1 may be significant regulators of the HSR, with AROS having a positive effect on the HSR and DBC1 having a negative effect.

AROS and DBC1 affect HSF1 acetylation

We next examined the impact of AROS and DBC1 on HSF1 acetylation status using an in vivo acetylation assay. Cells were transfected with Flag-HSF1 and p300 together with SIRT1, DBC1, AROS, or empty vector expression plasmids prior to treatment with or without HS (Figure 2.6A). HS led to HSF1 acetylation (lane 2), while the overexpression of SIRT1 inhibited HS-induced HSF1 acetylation (lane 4), consistent with previous
results (Westerheide, Anckar et al. 2009). Interestingly, the overexpression of DBC1, the SIRT1 inhibitor, enhanced HSF1 acetylation under both non-stress and stress conditions (lanes 5 and 6), while the overexpression of the SIRT1 activator AROS reduced HS-induced HSF1 acetylation to a similar degree as SIRT1 itself (compare lanes 4 and 8). Western blot analysis was used to verify the overexpression of SIRT1, DBC1 and AROS (Figure 2.6B), and cell viability results show that the overexpression of these factors does not affect overall cellular fitness (Figure 2.6C). We therefore conclude that AROS and DBC1 can modulate the acetylation status of HSF1. We would like to point out that while it is likely that the effects of AROS and DBC1 on HSF1 deacetylation are occurring through SIRT1, we have not demonstrated this here and it could be possible that other deacetylases are involved.

**AROS and DBC1 impact HSF1 DNA binding**

As HSF1 acetylation status has previously been shown to affect DNA binding ability (Westerheide, Anckar et al. 2009), we investigated the effect of AROS and DBC1 expression on HSF1 binding to the hsp70 promoter. HEK293 cells were transfected with AROS, DBC1, or empty vector and then treated with HS over a 6 hour time course followed by ChIP using an antibody to HSF1. As expected, a 2 hour HS resulted in recruitment of HSF1 to the hsp70 promoter, and this binding decreased by 6 hours of continuous HS (Figure 2.6D). AROS overexpression enhanced HS-induced recruitment of HSF1 to the hsp70 promoter. Conversely, DBC1 overexpression led to the inhibition of HS-induced HSF1 recruitment. Thus, AROS and DBC1 can modulate both HSF1 acetylation status as well as HS-induced binding of HSF1 to target DNA sites.

We next evaluated the effect of AROS and DBC1 knockdown on the activation of an hsp promoter reporter. HeLa cells expressing a stable hsp70.1 promoter-luciferase reporter were transfected with siRNA to HSF1, AROS, DBC1, or a non-targeting (NT) siRNA control. Cells were treated with either DMSO (mock) or celastrol (5 µM) 24 hours post-transfection and luciferase activity was measured 24 hours post-treatment. We found that knockdown of AROS resulted in a 2-fold reduction of celastrol-induced luciferase activity compared to the NT siRNA control (Figure 2.6E). This result is similar to the
result produced with HSF1 siRNA treatment. Conversely, DBC1 knockdown resulted in an approximate 2.3-fold increase in celastrol-induced luciferase activity compared to the non-targeting siRNA control (Figure 2.6E). This data provides further support that AROS and DBC1 positively and negatively impact expression from the hsp70 promoter, respectively.

Discussion

HSF1, the master regulator of genes involved in protein quality control, is highly post-translationally modified (Anckar and Sistonen 2011). These modifications are hypothesized to allow fine-tuning of the transcriptional activity of HSF1 in order to respond to the precise needs of the cell. We have previously shown that HSF1 is acetylated and SIRT1 activates HSF1 through deacetylation at a key lysine residue within the DNA binding domain, thereby promoting DNA binding (Westerheide, Anckar et al. 2009). With this study, we have expanded our analysis of the regulation of HSF1 by SIRT1 and find that AROS and DBC1, two SIRT1 modulators, can regulate HSF1’s acetylation status and thus control the HSR.

SIRT1 and the HSR

Prior work by us and others has established a connection between SIRT1 and the HSR in mammalian tissue culture cells, mice, and C. elegans (Westerheide, Anckar et al. 2009; Donmez, Arun et al. 2012; Raynes, Leckey et al. 2012). We have shown that SIRT1 prolongs HSF1 DNA binding, thus serving to enhance the HSR by increasing HS-induced chaperone expression (Westerheide, Anckar et al. 2009). The connection between SIRT1 and the HSR has been further established with an α-synuclein mouse model (Donmez, Arun et al. 2012). In these mice, SIRT1 was shown to deacetylate HSF1 and increase HSP70 levels in the brain, leading to α-synuclein aggregate suppression and an overall greater survival (Donmez, Arun et al. 2012). We have also recently shown that the C. elegans SIRT1 homolog Sir2.1 is linked to the HSR (Raynes, Leckey et al. 2012). Caloric restriction was found to synergize with HS to induce hsp70 gene expression in the worm, and this effect was dependent on Sir2.1 (Raynes, Leckey et
Our finding here that DBC1 and AROS, two modulators of SIRT1 activity, can regulate the HSR provides yet further support for a role of SIRT1 in the HSR.

The regulation of SIRT1 during heat shock

We sought to discover how HS may regulate SIRT1 activity. We first tested whether SIRT1 may be regulated at the transcription or translation level during HS. However, we did not find major changes in SIRT1 abundance throughout a HS time course. SIRT1 is a NAD\(^+\) binding protein and has been shown to be activated by an increase in the cellular NAD\(^+\)/NADH ratio (Borra, Langer et al. 2004). Previous studies have indicated that a 10-fold change in NAD\(^+\) concentration was required to affect Sir2 activity (Anderson, Latorre-Esteves et al. 2003). However, a decrease in NADH, a competitive inhibitor of SIRT1 that is present at much lower levels in the cell compared to NAD\(^+\), may have a greater impact on SIRT1 activity. Caloric restriction studies in yeast have shown that Sir2 activity is regulated by a reduction of NADH resulting in an increase in the NAD\(^+\)/NADH ratio (Lin, Ford et al. 2004; Rahat, Maoz et al. 2011). We demonstrate in this study that cellular NADH levels decrease upon HS resulting in an increase in the NAD\(^+\)/NADH ratio and therefore this may be one mechanism by which HS regulates HSF1 activity. These results suggest that the HS-induced increase in the NAD\(^+\)/NADH ratio may be an important factor in SIRT1 regulation of the HSR.

While SIRT1 is not a DNA binding protein, it can be recruited to promoters through association with other DNA binding proteins. For instance, BCL11A, a zinc finger transcription factor that functions as a myeloid and B-cell proto-oncogene, has been shown to recruit SIRT1 to a promoter template (Senawong, Peterson et al. 2005). Furthermore, SIRT1 converges to the same DNA regulatory elements as its transcription factor substrate CLOCK, indicating that these proteins operate on circadian promoters in a chromatin regulatory complex (Nakahata, Kaluzova et al. 2008). We sought to investigate whether SIRT1 was recruited to the hsp70 promoter with similar kinetics as HSF1 upon HS and found that it is. Therefore, SIRT1 may be in a chromatin-bound complex together with HSF1 on target promoters, leading to enhanced HSF1 activity.
AROS and DBC1 regulate HSF1

AROS and DBC1 are two regulators of SIRT1 activity. DBC1 has been shown to form a stable complex with SIRT1 by binding to its catalytic domain and inhibiting its activity by blocking substrate access (Kim, Chen et al. 2008; Zhao, Kruse et al. 2008). While AROS regulation of SIRT1 has been comparatively understudied, it is a SIRT1 binding partner that positively regulates SIRT1 deacetylase activity (Kim, Kho et al. 2007). AROS and DBC1 were first characterized to affect SIRT1 regulation of p53-mediated apoptosis through alteration of the p53 acetylation status. DBC1 has also recently been characterized to regulate the acetylation status of the nuclear receptor PPARγ (Santagata, Hu et al. 2011).

We show here that the overexpression and knockdown of AROS and DBC1 have a striking impact on hsp70 transcription. Overexpression of DBC1 results in a decrease in hsp70 transcription, while knockdown with DBC1 siRNA results in an increase in HSR induction. Conversely, overexpression of AROS results in an increase in hsp70 transcription, while knockdown with AROS siRNA results in a decrease in HSR induction. In an acetylation assay, AROS is able to completely deacetylate HSF1 in a manner similar to SIRT1, while DBC1 increases HSF1 acetylation. Given that AROS and DBC1 were found to regulate HSF1 acetylation, and HSF1 acetylation can affect DNA binding ability, we sought to investigate whether AROS and DBC1 expression could affect HSF1 DNA binding. As expected, we found that overexpression of AROS led to an increase in HSF1 recruitment to the hsp70 promoter and that, conversely, overexpression of DBC1 led to a decrease in HSF1 recruitment to the promoter. Thus, we have now added HSF1 to the list of SIRT1 target proteins that are modulated at the acetylation status level by AROS and DBC1. This work expands our knowledge of the control of the HSR and provides AROS and DBC1 as new therapeutic targets for modulating this response.
Table 2.1. List of qRT-PCR primers used in this study.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence*</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
</table>
| aros                    | F: 5’- GAAGGCAATTCAGGCCAGAAAACCT- 3’  
R: 5’- TCGTCCTGGTCAGAAACTTCAGGT- 3’ | 131           |
| dbc1                    | Primer mix from GeneCopoeia, Inc.  
Cat # HQP015872 (KIAA1967)      | Not provided  |
| gapdh                   | F: 5’- CCACTCCTCCACTTTTGAC - 3’  
R: 5’- ACCCTGTTGTGTAGCCA - 3’ | 102           |
| gapdh promoter¹         | F: 5’- TACTAGCGGTTTTACGGGCG - 3’  
R: 5’- TCGAACAGAGGAGCAGAGGCA - 3’ | 166           |
| hsp27²                  | F: 5’- CAAGTTTCTCTCTCCTTGT - 3’  
R: 5’- GCCAGTCTCTCATGGATTTTG - 3’ | 156           |
| hsp70³                  | F: 5’- AGAGCGGAGCAGACAGAG - 3’  
R: 5’- CACCTTGCCGTGTTGGAAC - 3’ | 110           |
| hsp70 promoter 1³ (HSE site) | F: 5’- GGCAGAAAACCTGGAGATATTCCCGA - 3’  
F: 5’- AGCCTTGGAACACGGGAG - 3’ | 191           |
| hsp70 promoter 2³ (NS site) | F: 5’- CCTCCCGAGGAGCTGGGACT - 3’  
R: 5’- CGAGGGCGGGCCGTGACT - 3’ | 134           |
| hsp90⁴                  | F: 5’- GGCAGTCAAGCAGCTTTCTCTGTA - 3’  
R: 5’- GTCACCACACCAAGATAA - 3’ | 199           |
| sirt1⁵                  | F: 5’- TCCTGGACAAATTCGAGGCCATCTCCT - 3’  
R: 5’- TTTCCAGCGTGCATGTTTCTGAGGT - 3’ | 103           |

*F: forward, R: reverse
³Primers from Westerheide et al. Science 2009 323, 1063.
⁴Primers from McLean et al. Biochemical and Biophysical Research Communications 2006 351:3.
Figure 2.1. SIRT1 is not regulated by a change in expression upon HS.

(A-C) *hsp70*, *hsp27*, and *hsp90* mRNA expression levels are induced during a HS time course. HeLa cells were exposed to a 42°C HS from 0 to 6 hours and mRNA levels were determined by qRT-PCR. (D) *sirt1* mRNA expression levels are not altered in the early stages of HS. HeLa cells were exposed to a 42°C HS from 0 to 6 hours and mRNA levels were determined qRT-PCR. Results for A-D are in technical triplicates and are representative of biological duplicates. Statistical significance was measured by Student’s t test as compared to 0 hr HS (*P<0.05; **P<0.01). (E) SIRT1 protein levels are not altered during a HS time course. HeLa cells were treated with a HS time course and SIRT1, HSP70, HSP27, and β-tubulin protein levels were determined by Western analysis. (F) Protein levels from E were quantified and plotted using ImageJ.
Figure 2.2. HS induces an increase in the NAD$^+/NADH$ ratio.
(A) NAD$^+$ and (B) NADH concentrations were determined for HeLa cells exposed to either control conditions or a 42°C HS for 2 hours. (C) HS induces an increase in the NAD$^+/NADH$ ratio as a result of decreased NADH levels. Mean values of the NAD$^+/NADH$ ratios from (A) and (B) are plotted. Results in A-C are in technical triplicates and are representative of biological duplicates. Statistical significance was measured by Student’s t test as compared to -HS (*P<0.05; **P<0.01; ***P<0.001).
Figure 2.3. HS results in an increase in SIRT1 recruitment that is specific to the HSE within the hsp70 promoter.

(A) Schematic representation of primers designed to amplify the HSE site and a nonspecific (NS) site at the hsp70 promoter. (B) SIRT1 is recruited to the hsp70 promoter HSE site during HS. HEK293 cells were exposed to a 42°C HS from 0 to 6 hours prior to chromatin immunoprecipitation (ChIP) with a SIRT1 antibody. SIRT1 is not recruited upon HS to a non-specific (NS) upstream hsp70 promoter site (C) or to the gapdh promoter (D). Results in B-D are in technical triplicates and are representative of biological duplicates. Statistical significance was measured by Student’s t test as compared to - HS (*P<0.05; ** P<0.01; ***P<0.001).
Figure 2.4. AROS and DBC1 overexpression impacts the transcription of hsp70 and initiation of the HSR.

(A) aros and dbc1 mRNA expression levels do not undergo a significant change upon 0-3 hours of HS, while small changes in levels are observed between 3-6 hours of HS. HEK293 cells were exposed to a 42°C HS from 0 to 6 hours and mRNA levels were determined by qRT-PCR. (B) AROS overexpression enhances HS induction of hsp70 mRNA, while (C) DBC1 overexpression inhibits HS induction of hsp70 mRNA. For B and C, HEK293 cells were transfected with empty vector (EV), AROS, or DBC1 and then exposed to a 42°C HS from 0 to 6 hours. The mRNA levels were determined for hsp70 by qRT-PCR. Results in A-C are in technical triplicates and are representative of biological duplicates. Statistical significance was measured by Student’s t test as compared to 0 hr HS (*P<0.05; **P<0.01; ***P<0.001).
Figure 2.5. AROS and DBC1 knockdown impact the transcription of hsp70 and initiation of the HSR.

(A) AROS and DBC1 siRNA is able to knockdown gene expression. HEK293 cells were transfected with siRNA for DBC1, AROS, or a non-targeting (NT) control and aros and dbc1 gene expression was assessed by qRT-PCR. (B) AROS, DBC1, and HSF1 siRNA do not significantly impact cell viability compared to the NT siRNA control as measured by a PrestoBlue assay. Cell viability assay was performed in biological and technical triplicates and statistical significance was measured by Student’s t test compared to NT siRNA control. (C) AROS knockdown decreases the induction of hsp70 by celastrol. HEK293 cells were transfected with AROS or NT control siRNA and treated with and without celastrol (5 µM). The mRNA levels were determined for hsp70 by qRT-PCR. (D) DBC1 knockdown increases the induction of hsp70 by celastrol. HEK293 cells were transfected with DBC1 or NT control siRNA and treated with and without celastrol.
(5 µM). The mRNA levels were determined for hsp70 by qRT-PCR. Results in A, C, and D are in technical triplicates and are representative of biological duplicates. Statistical significance was measured by Student’s t test as compared to control (*P<0.05; **P<0.01; ***P<0.001).
Figure 2.6. AROS and DBC1 impact HSF1 acetylation, DNA recruitment, and activity at the hsp70 promoter.

(A) AROS and DBC1 overexpression impact HSF1 acetylation. HEK293 cells were transfected with p300 and FLAG-HSF1 as well as empty vector, SIRT1-MYC, DBC1-HA, or AROS as indicated and exposed to 0 or 2 hours of HS at 42°C. A FLAG immunoprecipitation was run on an SDS-PAGE gel and the acetylation status of HSF1 was determined using an acetyl-lysine antibody. Results are representative of biological triplicates. (B) The expression of transfected SIRT1-MYC, DBC1-HA, and AROS was verified by Western analysis. (C) Overexpression of HSF1, DBC1, and AROS do not significantly impact cell viability compared to the empty vector (EV) control as measured by PrestoBlue. The cell viability assay was performed in biological and technical triplicates and statistical significance was measured by Student’s t test compared to EV. (D) AROS and DBC1 overexpression impact HSF1 recruitment to the hsp70 promoter. AROS, DBC1, or empty vector (EV) was overexpressed in HEK293 cells prior to HS for 0, 2, or 6 hours and chromatin immunoprecipitation (ChIP) was performed with an HSF1 antibody. The purified DNA was then analyzed by qPCR and DNA levels were determined for the hsp70 promoter. The qPCR results are in technical triplicates and statistical significance was measured by Student’s t test as compared to EV at 0 hr HS (*P<0.05; ** P<0.01). ChIP was performed in biological duplicates. (E) AROS and
DBC1 knockdown impact *hsp70.1* promoter-luciferase reporter activity. HeLa *hsp70.1* promoter-luciferase reporter cells were transfected with 50 nM of Dharmacon SmartPool DBC1, AROS, HSF1, or non-targeting (NT) control siRNA and HS was induced with celastrol (5 µM). Luciferase activity was measured and compared to that of the NT control. Luciferase assays were performed in biological triplicate. Statistical significance was measured by Student’s t test as compared to the NT siRNA control with celastrol (5 µM) treatment (*P<0.05*).
CHAPTER 3: HEAT SHOCK AND CALORIC RESTRICTION HAVE A SYNERGISTIC EFFECT ON THE HEAT SHOCK RESPONSE IN A SIR2.1-DEPENDENT MANNER IN CAENORHABDITIS ELEGANS

Authored by Rachel Raynes, Bruce D. Leckey Jr., Kevin Nguyen, and Sandy D. Westerheide.


Experiments were designed, performed, and analyzed by R. Raynes or performed under the direction of R. Raynes. B.D. Leckey and R. Raynes performed phenotypic replicates for Figures 3.5 and 3.6C. K. Nguyen and R. Raynes performed qPCR replicates for Figures 3.3-4.

All data analysis and figures were generated by R. Raynes. The manuscript was written by R. Raynes and S.D. Westerheide. See Appendix E for copyright permission.

Summary

The heat shock response (HSR) is responsible for maintaining cellular and organismal health through the regulation of proteostasis. Recent data demonstrating that the mammalian HSR is regulated by SIRT1 suggests that this response may be under metabolic control. To test this hypothesis, we have determined the effect of caloric restriction in C. elegans on the activation of the HSR and have found a synergistic effect on the induction of hsp70 gene expression. The homolog of mammalian SIRT1 in C. elegans is Sir2.1. Using a mutated C. elegans strain with a sir2.1 deletion, we show that heat shock and caloric restriction cooperate to promote increased survivability and fitness in a sir2.1-dependent manner. Finally, we show that caloric restriction increases
the ability of heat shock to preserve movement in a polyglutamine toxicity neurodegenerative disease model and that this effect is dependent on sir2.1.

**Introduction**

The heat shock response (HSR) is a cytoprotective response that increases longevity and protects against diseases of aging in model organisms (Akerfelt, Morimoto et al. 2010; Anckar and Sistonen 2011). This response enables an organism to manage protein damaging stress through the activation of the transcription factor HSF1. HSF1 is responsible for transcriptionally upregulating the heat shock protein genes (HSPs). In mammalian cells, flies, nematodes, and plants, this transcription factor exists as an inactive monomer, however in response to denaturing stress will trimerize and bind to heat shock elements (HSEs) located in the promoter regions of the hsp genes (Sarge, Murphy et al. 1993; Yao, Munson et al. 2006; von Koskull-Doring, Scharf et al. 2007; Chiang, Ching et al. 2012). Increased expression of HSPs, such as HSP70 family members, results in cellular protection from a variety of stressors including elevated temperatures, oxidative stress, heavy metals, proteasome inhibitors, and infection (Westerheide and Morimoto 2005).

A characteristic of many HSR activators is the ability to elicit hormesis, a biological process that occurs when a low level stress is applied that promotes cytoprotection against a subsequent exposure to a more severe stress (Cypser and Johnson 2002). For instance, exposure to a moderate heat shock (HS) can protect against exposure to a subsequent high-temperature HS in Caenorhabditis elegans (Lithgow, White et al. 1995). Hormesis occurs, in part, through the upregulation of molecular chaperones during the first mild stress treatment, which then protect cells from subsequent acute protein damage.

Caloric restriction (CR), a 30-40% decrease in dietary intake, increases longevity and protects against diseases of aging (Bishop and Guarente 2007). An association between CR and longevity was established as early as 1935 through studies with calorically-restricted rats (McCay, Crowell et al. 1989). CR has now been shown to
increase longevity in many other models including *S. cerevisiae* (Fabrizio, Pozza et al. 2001; Kaeberlein, Powers et al. 2005), *C. elegans* (Johnson 2008; Greer and Brunet 2009), *D. melanogaster* (Giannakou, Goss et al. 2008; Min, Yamamoto et al. 2008), and *M. musculus* (Anderson, Shanmuganayagam et al. 2009). In addition to increasing longevity, CR slows the progression of many age-related diseases including neurodegenerative diseases (Bishop and Guarente 2007). For instance, in various *C. elegans* models of protein aggregation diseases, CR has been shown to protect against age-associated paralysis (Steinkraus, Smith et al. 2008). Genetic experiments in model organisms have implicated that CR mediates its effects through a number of genes, including the sirtuins (Donmez and Guarente 2010).

The sirtuins are a family of NAD+-dependent deacetylases that have been characterized to play a role in a number of biological processes. The first family member identified was the yeast Sir2α, based on its responsibility for establishing transcriptional silencing of mating-type loci (Laurenson and Rine 1992). The mammalian homolog to yeast Sir2α, SIRT1 deacetylates histones and many stress-inducible transcription factors including p53, FOXO and NF-kB (Luo, Nikolaev et al. 2001; Brunet, Sweeney et al. 2004; Yeung, Hoberg et al. 2004). Recent findings have indicated that SIRT1 also plays a critical role in the HSR by directly deacetylating HSF1 within its DNA binding domain to promote HSF1 occupancy at heat shock promoters (Westerheide, Anckar et al. 2009). As SIRT1 is an important metabolic regulator and because HSF1 and SIRT1 function together to protect cells from stress, we were interested in examining a direct link between the HSR and metabolism.

*C. elegans* is a useful model organism for testing the relationship between the HSR and metabolism, as these animals can easily be calorically-restricted via bacterial limitation. Here, we show that CR and HS synergize to induce the HSR and that this effect depends on the *C. elegans* SIRT1 homolog Sir2.1.
Methods

C. elegans strains and maintenance

The following C. elegans strains were utilized in this study: wild-type N2, C12C8.1::GFP reporter fusion (Morley and Morimoto 2004), sir2.1 (ok434), sir2.3 (ok444), Q0-YFP, Q24-YFP, and Q35-YFP (Morley, Brignull et al. 2002). Wild-type (N2), C12C8.1::GFP, Q0-YFP, and Q35-YFP animals were obtained from Dr. R.I. Morimoto. All other strains were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). All strains were handled with standard procedures (Brenner 1974) and grown on standard NGM agar plates seeded with OP50 E. coli and incubated at 23°C. The Q24-YFP; sir2.1 (ok434) double mutant strain was constructed by mating Q24-YFP males with sir2.1 (ok434) hermaphrodites. F1 cross progeny that displayed the YFP reporter were picked to individual plates and allowed to have F2 progeny. Approximately twenty F2 progeny were picked to individual plates and allowed to self fertilize. Once F3 progeny were obtained, the F2 parents were tested for the sir2.1 deletion by PCR using primers flanking the deletion (5’-CAGCGACGCTGTCTCAAAAA, 3’-GATCAATGAGCATTCGGCT) (Wang and Tissenbaum 2006).

Bleach synchronization

Animals were bleach synchronized in order to obtain the same developmental time point prior to any experimental conditions as previously described (Lewis and Fleming 1995). Briefly, a petri dish of C. elegans was washed with M9 buffer to dislodge the animals for transfer into a 15mL conical for centrifugation. The pellet was washed with 20% alkaline hypochlorite solution to kill all animals except for the eggs, which are resistant to the bleach. These were then centrifuged and washed three times with M9 and the final pellet was resuspended in 7 mL of M9 and incubated at room temperature at 220 rpm to allow the eggs to reach the L1 larva stage. Synchronized L1 larvae were used for all experimental conditions.
Caloric Restriction and Heat Shock Conditions

Synchronized L1 larvae were cultured in S-basal media with OP50 *E. coli* in either *ad libitum* (AL) or CR conditions, 1.9 x 10^{10} cells/mL or 2.6 x 10^{9} cells per mL of bacteria, respectively (Hansen, Chandra et al. 2008). Cultures were incubated at 23°C at 220 rpm. The life cycle progression of the animals was monitored daily under a dissecting microscope until they reached adulthood. Once the animals reached adulthood, each AL and CR flask was equally divided into two flasks (one for control and one for HS). HS was performed for 15 minutes or 1 hour in a 33°C circulating water bath and worms were allowed to recover at 23°C for 30 minutes or 6 hours as indicated.

Quantitative RT-PCR

qRT-PCR was performed to quantify mRNA levels for the *hsp70* family members C12C8.1, F44e5.4 and F44e5.5 as well as *sir2.1* using gene-specific primers. Animals were collected via centrifugation and RNA extracted using TRIzol per standard protocol. RNA was reverse transcribed using Applied Biosystem’s High Capacity cDNA Reverse Transcription Kit (cat#4368814) according to the manufacturer’s protocol. The samples were diluted to 50 ng/µL and used as a template for qRT-PCR. qRT-PCR was performed with Applied Biosystem’s Step One Plus Real-time PCR system using BioRad’s iTaq™ Fast SYBR® Green Supermix with ROX (cat#172-5101) according to manufacturer’s protocol. Statistical data analysis and determination of relative fold increase from control samples was performed according to standard calculations (Bookout and Mangelsdorf 2003).

Fluorescence Microscopy

Animals were analyzed for GFP expression using an EVOS fluorescence microscope. Animals were photographed individually by pipetting onto a layer of 1% agarose on top of a glass slide. Animals were paralyzed prior to microscopy by adding 1-2 drops of 1mM Levamisole onto the slide.
**Immunoblotting Analysis**

For each treatment condition, cultured *C. elegans* were centrifuged at 5,000 rpm for 5 minutes and the resulting pellet was repeatedly washed in phosphate buffered saline to clear bacteria from the extraction. Animals were lysed in Buffer C (20mM HEPES pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl2, and 0.2 mM EDT) with the addition of Halt™ Protease Inhibitors (Thermo Scientific cat#78430) and sonicated for 15 minutes, cycling on and off for 30 second intervals, using the Diagenode Bioruptor 300. Protein extracts were quantified and run on a 10% SDS-PAGE gel. Blots were probed with anti-GFP (Santa Cruz, cat#SC-9996) at a 1:1000 dilution and anti-Actin (Amersham, cat#JLA20-C) at a 1:750 dilution.

**Thermotolerance Assay**

Animals grown in AL or CR conditions were either left untreated or heat shocked for 1 hour at 33°C in liquid culture in a circulating water bath and allowed to recover overnight at 23°C. The next day, 100 animals from each of the 4 liquid cultures were plated to separate NGM agar plates without bacteria. These were then exposed to a lethal heat shock at 36°C in a circulating water bath for 45 minutes with a 3 hour recovery. Survivability of the animals was then manually quantified using a dissecting microscope. Animals were scored as alive if they responded to stimuli by poking with a platinum wire.

**Thrashing Assay**

After animals were scored in the thermotolerance assay, lethal HS survivors were individually transferred to a drop of M9 buffer on a glass slide. The animals were allowed to recover from the transfer for 1 minute. Thrashing was judged as a mid-body bend. The number of thrashes was counted for 1 minute after recovery from the transfer.
Paralysis Assay

Q24 animals grown in AL or CR conditions were either left untreated or heat shocked for 1 hour at 33°C in a circulating water bath and allowed to recover overnight at 23°C. At day 4, paralysis was determined by transferring individual animals to a seeded NGM plate. Animals that did not move through the bacterial lawn and create a trail within 5 minutes were scored as paralyzed. Animals were scored as alive if they responded to stimuli by poking with a platinum wire.

Polyglutamine Protein Aggregation Assay

Q35-YFP animals were cultured as described in the paralysis assay. At day 3, animals were visualized to analyze GFP expression. Animals were photographed as described previously and the number of protein aggregates was scored.

Results

Caloric Restriction and Heat Shock Synergize to Activate an hsp70 Promoter Reporter

CR has previously been reported to induce hsp70 expression (Heydari, Wu et al. 1993; Ehrenfried, Evers et al. 1996). To verify that our CR conditions induce a HSR in C. elegans, we visualized green fluorescent protein expression (GFP) which is under the regulatory control of the hsp70 promoter (C12C8.1::GFP) by microscopy (Morley and Morimoto 2004). Animals were maintained under ad libitum (AL) or CR conditions with either 1.9 x 10^{10} or 2.6 x 10^{9} bacterial cells per mL, respectively. As expected, HS treated animals showed induction of HS-responsive GFP expression, as compared to untreated animals (Figure 3.1A). CR also resulted in an increase in expression of the HS-responsive reporter, however GFP levels were not as robust as in animals receiving HS treatment. Interestingly, animals subjected to both CR and HS displayed an increase in reporter activation as compared to either treatment condition alone. The increase in GFP expression observed by fluorescence microscopy was corroborated by quantification.
of GFP levels (Figure 3.1B) and by Western analysis using an anti-GFP antibody (Figure 3.1C). While immunoblotting was not sensitive enough to detect GFP expression in the animals treated with CR alone, quantitation of GFP fluorescence confirmed that animals subjected to CR and HS induced a 2-fold increase in GFP expression over animals treated with HS alone.

**Caloric Restriction and Heat Shock Synergize to Activate Endogenous Heat Shock Gene Expression**

To get a more accurate quantification of the synergy between CR and HS, endogenous heat shock gene expression was measured in the N2 C. elegans strain. Wild-type N2 animals cultured in either AL or CR conditions were exposed to a 33°C heat shock for 15 minutes with a 30 minute recovery. Induction of C12C8.1 (hsp70) transcripts was quantitated by qRT-PCR (Figure 3.2). As expected, animals maintained under AL conditions showed a 40-fold increase in C12C8.1 mRNA levels upon HS (Figure 3.2A). Consistent with results shown in Figure 3.1A, N2 animals cultured under CR conditions also showed a 20-fold increase in C12C8.1 expression. Strikingly, animals cultured in CR conditions and HS together exhibited a 4,000-fold increase in C12C8.1 mRNA levels, indicating a synergistic effect between HS and CR. To determine whether similar results were observed for multiple members of the hsp70 family, we also performed qRT-PCR using primers against F44e5.4 and F44e5.5. Similar to C12C8.1, F44e5.4 and F44e5.5 expression significantly increased in response to CR and HS treatment, as compared to either treatment alone (Figure 3.2B,C).

**sir2.1 is Required for the Synergy between HS and CR on hsp70 Induction**

We have previously established that mammalian cells required the NAD\(^+\)-dependent deacetylase SIRT1 for full transcriptional activity of HSF1 (Westerheide, Anckar et al. 2009). As CR stimulates SIRT1 activity by changing the NAD\(^+\) to NADH ratio, we tested whether the synergistic effect observed between HS and CR was dependent on *sir2.1*. The *sir2.1* (ok434) strain possesses a homozygous *sir2.1* partial gene deletion that encodes for a non-functional protein. To initiate experiments, animals were treated with
HS, CR or both as previously described in Figure 3.2 and qRT-PCR was performed to evaluate mRNA expression for the hsp70 family members C12C8.1, F44E5.4, and F44E5.5 (Figure 3.3). For reasons that are not clear, the hsp70 genes are induced to a higher level upon HS treatment in the sir2.1 (ok434) strain than in the N2 strain under AL conditions. Interestingly, though, the sir2.1 (ok434) strain was no longer able to upregulate C12C8.1 in response to CR (Figure 3.3A). Furthermore, the ability of CR and HS treatments to synergistically upregulate C12C8.1, F44E5.4, and F44E5.5 gene expression was lost upon deletion of sir2.1 (Figure 3.3A-C).

To determine whether the synergistic upregulation of the hsp70 gene family was dependent on sir2.1 and not some other sirtuin family member, the sir2.3 (ok444) knockout strain was treated with CR, HS, or both CR and HS and C12C8.1, F44e5.4 and F44e5.5 expression was measured by qRT-PCR. As shown in Figure 3.4, animals treated with both CR and HS showed a synergistic increase in the expression of the hsp70 gene family, indicating the specificity of the response to sir2.1. Thus, expression of the sir2.1 gene product, but not sir2.3, is required to synergistically upregulate hsp70 genes in response to HS and CR in C. elegans.

**CR enhances the ability of HS to induce thermotolerance**

As a prior induction of hsp genes upon mild stress can lead to increased thermotolerance upon subsequent exposure to a lethal HS in a variety of organisms (Lithgow, White et al. 1995; Cypser and Johnson 2002), we investigated whether CR and HS could heighten thermotolerance in C. elegans above levels produced with HS alone. N2 animals were exposed to a 33°C conditioning HS with an overnight recovery prior to receiving a 36°C lethal heat shock. After a 3 hour recovery, the animals were scored for survivability. As expected, preconditioning with a mild HS provided thermotolerance to lethal HS treatment with a 25% increase in survival rate, compared to animals without HS preconditioning (Figure 3.5A). CR preconditioning prior to lethal HS treatment also provided modest thermotolerance, with a 14% increase in survival. Similar to what we observed with hsp70 gene induction, animals preconditioned with both CR and HS
displayed heightened thermotolerance. Animals increased from 48% survival with no preconditioning to 94% survival upon preconditioning with both CR and HS together. Both sir2.1 (ok434) and sir2.3 (ok444) deletion strains were examined for responses to thermotolerance. Consistent with our other data, sir2.1, but not sir2.3, was required for thermotolerance. Therefore, as for the induction of hsp70 genes, the ability of CR to enhance HS-induced thermotolerance requires sir2.1.

**CR enhances the ability of HS Preconditioning to Increase the Fitness of Lethal HS Survivors**

To measure whether HS and CR preconditioning increased overall fitness after lethal HS exposure, thrashing assays were performed immediately after scoring for thermotolerance by transporting individual animals into a drop of M9 buffer. After a 1 minute recovery from the transfer, the number of thrashes, or mid-body bends, was scored for 1 minute. Similar to survival assays, preconditioning with either HS or CR alone prior to exposure to lethal HS increased the number of thrashes per minute (Figure 3.5B). Moreover, animals preconditioned with both CR and HS displayed heightened fitness as compared to either treatment alone, in a manner dependent on sir2.1 (Figure 3.5B-E). Collectively, our data indicate that CR works in conjunction with HS to effectively provide increased thermotolerance, survival and fitness, a process specifically requiring sir2.1.

**CR increases the ability of HS to preserve movement in a C. elegans model of polyglutamine diseases**

*C. elegans* strains genetically engineered to express a polyglutamine (polyQ) repeat tract fused to YFP in the body wall muscle serve as models of polyQ disease where the expression of polyQ expansions in body wall muscle cells causes animal paralysis that develops in a polyQ length- and age-dependent manner (Morley, Brignull et al. 2002). In polyQ models in a variety of organisms, induction of the HSR protects animals from polyQ aggregation and aggregation-induced toxicity (Waza, Adachi et al. 2005; Fujikake, Nagai et al. 2008; Calamini, Silva et al. 2011). We were interested in determining whether CR would enhance the ability of HS to protect from polyQ aggregation and
cytotoxicity. Q35-YFP worms produce aggregates that can be easily visualized by fluorescence microscopy by day 3 of adulthood (Morimoto 2008). Upon testing the effect of HS and CR on the Q35-YFP strain, we found a statistically significant decrease in the number of aggregates with either CR or HS pretreatment (Figure 3.6A, B). There was no increased effect observed upon treatment with HS and CR together, which could perhaps be due to the strong propensity of Q35-YFP to aggregate. To test whether CR could enhance the cytoprotective effect of HS on a more sensitive polyQ strain, we assayed polyQ-induced paralysis in Q24-YFP animals. While this strain does not produce visual aggregates, it does result in age-dependent paralysis that is evident at day 4 of adulthood. CR was observed to increase the ability of HS to alleviate paralysis in this strain (Figure 3.6C). A Q24-YFP; sir2.1 (ok434) strain was created to determine if the ability of CR to enhance HS-induced cytoprotection is dependent upon sir2.1. The Q24-YFP; sir2.1 (ok434) double mutant displayed a higher degree of paralysis and eliminated the ability of CR to enhance HS-induced movement preservation (Figure 3.6C). Therefore, we conclude that CR can increase the ability of HS to preserve movement ability in this polyQ model in a sir2.1-dependent manner.

The transcriptional regulation of the HSR was further evaluated for the Q24-YFP mutant to determine if the cytotoxicity of the polyglutamine repeat altered the HS and CR synergism observed for the hsp70 genes in N2 animals. Q24-YFP animals cultured in either AL or CR conditions were exposed to a 33°C heat shock for 15 minutes with a 30 minute recovery. Induction of hsp70 family transcripts was quantitated by qRT-PCR (Figure 3.7A-C). We found that the Q24 repeats did not impact the synergy between HS and CR, although the overall inducibility of the hsp70 genes was reduced. We tested to see if the expression of Q24-YFP influenced sir2.1 expression and found it was decreased by 43% (Figure 3.7D). Thus, we conclude that while Q24-YFP expression dampens sir2.1 mRNA expression and the inducibility of the HSR, the ability of CR to synergize with HS is maintained.

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Discussion

Prior work on mammalian HSF1 has implicated that it may be regulated by metabolism due to control by the NAD$^+$-dependent sirtuin SIRT1 (Westerheide, Anckar et al. 2009; Donmez, Arun et al. 2012). SIRT1 activity promotes the HSR, so we therefore reasoned that CR, by changing the NAD$^+$ to NADH ratio, would also promote the HSR. We have tested this hypothesis in *C. elegans*, an organism that is easily amenable to growth in CR conditions by limiting the bacterial food source. The studies presented here demonstrate that CR conditions do indeed enhance the HSR as observed in a C12C8.1(*hsp70*)::GFP reporter strain. Additionally, striking synergy occurs in the HS-induced levels of *hsp70* mRNA in the presence of CR. This effect is dependent on *sir2.1*, as evidenced by experiments using *sir2.1*-deletion animals. The synergy that we observe between CR and HS is biologically relevant, as CR can enhance the ability of HS to cause thermotolerance and increase thrashing fitness after a lethal HS in a *sir2.1*-dependent manner. In addition, CR and HS can work together to preserve movement in a *C. elegans* polyQ cytotoxicity model. To ensure the survival of the organism, it makes sense that stress induced by CR would enhance the cytoprotective effects of HS-induced stress.

HSP70 is the chaperone that is highly induced by the HSR and protects cells from cytotoxic stress (Riabowol, Mizzen et al. 1988; Angelidis, Lazaridis et al. 1991; Li, Li et al. 1991). There is an age-related decline in HSP70 levels during aging in many model organisms (Deguchi, Negoro et al. 1988; Faassen, O'Leary et al. 1989; Liu, Lin et al. 1989; Fargnoli, Kunisada et al. 1990; Blake, Fargnoli et al. 1991; Campanini, Petronini et al. 1992; Pahlavani, Harris et al. 1995), which correlates with a decline in the ability to respond to stress. Interestingly, CR has been shown to restore the ability of cells to mount a HSR in aged rat hepatocytes (Heydari, You et al. 1996). In these cells, CR was found to function not by increasing HSF1 levels, but by increasing the ability of HSF1 to bind to DNA upon HS. It was proposed that HSF1 may be post-translationally altered by CR to allow it to be more active. Our studies support a positive influence of CR upon induction of the HSR in a second model organism, *C. elegans*. The synergy that we observe between CR and HS requires *sir2.1*.
A direct interaction between the insulin/IGF-1-like signaling pathway and HSR in *C. elegans* has recently been described. Upon increased insulin/IGF-1-like signaling, an inhibitory complex containing the *C. elegans* proteins DDL-1, DDL-2, and HSB-1 was found to associate with HSF-1 and sequester it (Chiang, Ching et al. 2012). CR conditions, by decreasing insulin/IGF-1-like signaling, may thus enhance the HSR via two mechanisms: 1) an increased NAD$^+$ to NADH ratio activates Sir2.1 and thus the HSR and 2) the release of HSF-1 from the DDL-1/DDL-2/HSB-1 inhibitory complex leads to an increase in free HSF-1 capable of being activated. Homologs of the DDL-1, DDL-2 and HSB-1 proteins are also present in mammals, indicating a possible preservation of this pathway.

A characteristic of various activators of the HSR is that they can often function synergistically with each other. For example, the non-steroidal anti-inflammatory drug indomethacin can cooperate with a mild heat stress to induce increased levels of chaperone gene expression over treatment with just heat alone (Lee, Chen et al. 1995). Other small molecules shown to work synergistically with HS include the inflammatory pathway intermediate arachidonic acid, the hydroxylamine derivative bimoclomol, and the triterpenoid celastrol (Jurivich, Sistonen et al. 1994; Torok, Tsvetkova et al. 2003; Westerheide, Bosman et al. 2004). We now add CR as another HSR activator that can synergize with HS.
Figure 3.1. Caloric restriction synergizes with heat shock to activate the C12C8.1(hsp70) promoter.

A, Epifluorescence (E) and phase contrast (P) images are shown of a C12C8.1(hsp70)::GFP reporter strain of C. elegans treated with or without HS and CR, as indicated. B, GFP levels observed by fluorescence microscopy in the worms in (A) were quantified by Image J and plotted as fluorescence intensity in arbitrary units. Statistical significance was measured by Student’s t test as compared to AL (*P<0.05, ** P<0.01, ***P<0.001). C, GFP protein levels were determined by Western analysis using protein extracts from C12C8.1(hsp70)::GFP reporter animals treated as in (A). The relative intensities of the GFP bands were quantitated by Image J and the fold inductions, relative to the untreated animals, are shown. Results are representative of three or more experiments.
Figure 3.2. Caloric restriction synergizes with heat shock to induce the endogenous \textit{hsp70} genes C12C8.1, F44e5.4 and F44e5.5.

A, N2 animals were treated with or without HS and CR, as indicated, and mRNA levels were determined for C12C8.1 by qRT-PCR. B, N2 animals were treated as in (A) and mRNA levels were determined for F44e5.4. C, N2 animals were treated as in (A) and mRNA levels were determined for F44e5.5. Results are in technical triplicates and are representative of biological duplicates. Statistical significance was measured by Student’s t test as compared to AL (*P<0.05, ** P<0.01, ***P<0.001).
Figure 3.3. The synergistic effect between heat shock and caloric restriction on hsp70 gene induction is dependent upon sir2.1.

A, sir2.1 deletion animals (ok434) were treated with or without HS and CR, as indicated, and mRNA levels were determined for C12C8.1 by qRT-PCR. B, sir2.1 (ok434) animals were treated as in (A) and mRNA levels were determined for F44E5.4. C, sir2.1 (ok434) animals were treated as in (A) and mRNA levels were determined for F44E5.5. Results are in technical triplicates and are representative of biological duplicates. Statistical significance was measured by Student’s t test as compared to AL (*P<0.05, ** P<0.01, ***P<0.001).
Figure 3.4. *sir2.3* deletion does not alter the synergistic effect between heat shock and caloric restriction on *hsp70* gene induction.

A, *sir2.3* deletion animals (ok444) were treated with or without HS and CR, as indicated, and mRNA levels were determined for C12C8.1 by qRT-PCR. B, *sir2.3* (ok444) animals were treated as in (A) and mRNA levels were determined for F44E5.4. C, *sir2.3* (ok444) animals were treated as in (A) and mRNA levels were determined for F44E5.5. Results are in technical triplicates and are representative of biological duplicates. Statistical significance was measured by Student’s t test as compared to AL (*P*<0.05, **P*<0.01, ***P*<0.001).
Figure 3.5. Caloric restriction enhances the ability of heat shock to induce thermotolerance and thrashing fitness in a sir2.1-dependent manner.

A, The indicated *C. elegans* strains were treated with or without HS and CR, as indicated, prior to a lethal HS treatment of 36°C for 45 minutes followed by a 3 hour recovery. Percent survival was quantified. *sir2.1* was required for the observed synergistic thermotolerance effect, while *sir2.3* was not. B and C, The fitness of the surviving animals from (A) was tested by a thrashing assay by quantitating the number of mid-body bends per minute. B, N2 animals exposed to both HS and CR exhibited synergy in thrashing activity. C, *sir2.1* deletion animals did not show a synergistic effect between HS and CR in thrashing fitness. Results for thermotolerance are in biological triplicates. Fifty animals were scored for each variable in the thrashing assay, with results representative of two experiments. Statistical significance was measured by Student’s t test as compared to AL (*P<0.05, **P<0.01, ***P<0.001).
Figure 3.6. Caloric restriction and heat shock reduce aggregate numbers and preserve movement in a C. elegans polyglutamine cytotoxicity model.

A) Q35-YFP animals show a reduction in aggregate formation when pretreated with HS, CR, or HS and CR together. The numbers refer to the amount of aggregates observed for the animal pictured in the panel. B) Quantitation of the aggregate numbers from (A). At least 50 animals were scored for each variable. C) Q24-YFP animals show a preservation of movement when pretreated with HS or CR, and CR increases the ability of HS to preserve movement in a manner dependent on sir2.1. Q24-YFP or Q24-YFP;sir2.1 deletion animals were treated with HS, CR, or CR+HS as indicated. At least 100 animals were scored for each variable. Results for the paralysis assay are in biological triplicates. Statistical significance for B-C was measured by Student’s t test as compared to AL (*P<0.05, **P<0.01, ***P<0.001).
Figure 3.7. PolyQ cytotoxicity does not alter the synergistic effect between heat shock and caloric restriction on hsp70 gene induction.

A, Q24-YFP animals were treated with or without HS and CR, as indicated, and mRNA levels were determined for C12C8.1 by qRT-PCR. B, Q24-YFP animals were treated as in (A) and mRNA levels were determined for F44e5.4. C, Q24-YFP animals were treated as in (A) and mRNA levels were determined for F44e5.5. D, N2 and Q24-YFP animals were analyzed for sir2.1 mRNA levels by qRT-PCR. Results are in technical triplicates and are representative of biological duplicates. Statistical significance was measured by Student’s t test as compared to AL (*P<0.05, ** P<0.01, ***P<0.001).
CHAPTER 4: RPS19, A KNOWN AROS-INTERACTING PROTEIN, IS A NEW TRANSCRIPTIONAL TARGET OF HSF1

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Unpublished data.

Experiments were designed, performed, and analyzed by R. Raynes or performed under the direction of R. Raynes. K. Nguyen performed a qPCR replicate for Figure 4.1.

All data analysis and figures were generated by R. Raynes. The manuscript was written by R. Raynes.

Abstract

HSF1 is the master regulator of the heat shock response (HSR) and consequently up-regulates heat shock (HS) inducible genes upon stress. HSF1 activity is determined by a number of post-translational modifications (PTMs). Because acetylation causes HSF1 to lose affinity for the DNA, the deacetylase SIRT1 plays a significant role in the propagation of heat shock proteins (HSPs). Recently, SIRT1 regulators DBC1 and AROS have also been shown to impact HSF1 activity and regulation of the HSR. Here, we investigate a ribosomal protein, RPS19, which binds to AROS and may play a role in the HSR. We found that RPS19 expression increases upon HS and that HSF1 is recruited to the rps19 promoter. We conclude that rps19 is a new transcriptional target of HSF1.
Introduction

The ribosome is a ribonucleoprotein assembly consisting of rRNA strands and multiple smaller protein subunits. Eukaryotes possess a small 40S and a large 60S subunit, as measured by the Svedberg rate of sedimentation unit. The 40S subunit is composed of 18S rRNA and 33 proteins while the 60S subunit is composed of a 5S, 28S, and 5.8S rRNA and approximately 49 proteins (Klinge, Voigts-Hoffmann et al. 2011; Rabl, Leibundgut et al. 2011). Ribosomal proteins (RP) are named according to molecular weight on a 2D gel and their formation with the small or large subunits, RPS or RPL, respectively.

Some ribosomal proteins, for instance the S. cerevisiae RPL26, have roles in optimizing pre-rRNA maturation and are non-essential to the translation of proteins. An rpl26aΔ;rpl26bΔ mutant undergoes identical growth and translation accuracy as compared to the wild-type strain, though does result in a slight deficit of 60S r-subunits and a delay in pre-rRNA processing (Babiano, Gamalinda et al. 2012). However, most RPs are essential and result in disease or lethality when defective. Mutations in the proteins RPS17, RPS19, RPS24, RPL5, RPL11, and RPL35a are associated with Diamond Blackfan Anemia (DBA). DBA is characterized by decreased erythroid progenitors in the bone marrow resulting in anemia that develops in infancy. Nearly half of the individuals diagnosed with DBA also possess congenital abnormalities including cardiac defects, craniofacial malformations, limb abnormalities, and cleft palate (Freedman 2000; Willig, Gazda et al. 2000). Approximately 25% of DBA cases have been linked to mutations in RPS19 (Draptchinskaia, Gustavsson et al. 1999; Willig, Draptchinskaia et al. 1999). This has led to the hypothesis that a defect in the ribosomal machinery may be the underlying cause of DBA.

In an effort to better understand the cause of DBA, a yeast two-hybrid screen aimed to identify novel proteins that associate with RPS19 led to the discovery of a small 15kDa protein that localizes to the nucleoli and was subsequently named RPS19 binding protein 1 (RPS19bp1) (Maeda, Toku et al. 2006). RPS19bp1 was later discovered in another yeast two-hybrid screen aimed to investigate direct regulators of SIRT1 (Kim,
Kho et al. 2007). It was found that RPS19bp1 binds directly to SIRT1 in order to suppress p53 transcriptional activity, thus leading the cell down a non-apoptotic pathway and earning the new name Active Regulator of SIRT1 (AROS) (Kim, Kho et al. 2007). In addition to regulating p53-mediated apoptosis, SIRT1 is also a known regulator of the HSR. SIRT1 deacetylates HSF1 thus increasing its affinity for DNA and prolonging the transcription of hsp genes (Westerheide, Anckar et al. 2009).

Recently, our laboratory has shown that the SIRT1 interactor AROS regulates HSF1 activity and the HSR. We found that AROS has an impact on hsp70 transcription, HSF1 acetylation status, and HSF1 recruitment to hsp promoters (Raynes, Pombier et al. 2013). Given that AROS has been shown to directly interact with RPS19 and because RPS19 has a critical role in protein translation, we sought to investigate whether RPS19 might play a role in the HSR. Here we show that RPS19 is upregulated upon initiation of the HSR and is a new HSF1 target for transcription.

**Methods**

**Cell culture and heat shock**

HeLa and HEK293 cells were utilized in this study. All cell lines were cultured and heat-shocked as previously described (Raynes, Pombier et al. 2013) (Studies, Chapter 1). Briefly, plates were wrapped in parafilm and submerged in a water bath set to 42°C for the designated times prior to collection with 1X PBS.

**Quantitative RT-PCR**

qRT-PCR was performed to quantify mRNA levels for rps19 and gapdh using gene-specific primers (Appendix B). qRT-PCR was performed as previously described (Raynes, Pombier et al. 2013) (Studies, Chapter 1). Briefly, RNA was extracted using Trizol and was reverse transcribed using Applied Biosystem’s High Capacity cDNA Reverse Transcription Kit (cat#4368814) according to the manufacturer’s protocol. The samples were diluted to 50 ng/µL and used as a template for qRT-PCR. Statistical data
analysis and determination of relative fold increase from control samples was performed according to standard calculations (Bookout and Mangelsdorf 2003).

**Immunoblotting Analysis**

HeLa cells were collected in 1X PBS and then extracted using M-PER (Thermo Scientific cat#78503) with the addition of Halt™ Protease Inhibitors (Thermo Scientific cat#78430). 20 µg of protein was run on a 10% SDS-PAGE gel and immunoblotting was performed as previously described (Raynes, Pombier et al. 2013) (Studies, Chapter 1). Primary antibodies used include ACTIN (Santa Cruz cat#sc-1616-r) and RPS19 (Sigma cat#SAB1404336). HRP-conjugated secondary antibodies were from Millipore (cat#12-349 and 12-348). Detection was performed using ECL Plus Western Blotting Detection System (Amersham™ cat#RPN2132).

**Chromatin Immunoprecipitation**

Chromatin Immunoprecipitation (ChIP) was performed as previously described (Raynes, Pombier et al. 2013) (Studies, Chapter 1). Immunoselection and immunoprecipitation were performed using 5 µL of HSF1 (Enzo cat#ADI-SPA-901) or IgG (Cell Signaling Technology cat#2729S) antibody. Purified DNA was used for qRT-PCR using primers to the rps19 promoter surrounding the proximal HSE (Appendix B). Statistical data analysis and determination of relative fold increase from control samples without heat shock was performed according to standard protocol (Bookout and Mangelsdorf 2003).

**Results**

*RPS19 mRNA and protein levels increase upon HS*

We first sought to investigate whether *rps19* transcription levels were affected by a HS time course. HeLa cells were treated with a 42°C HS from 0 to 6 hours and qRT-PCR was performed using primers to *rps19*. It is well established, and we have previously shown, that *hsp70* mRNA levels increase substantially by 2 hours of HS and then begin
to decline by 6 hours of HS due to the attenuation of the HSR (Figure 2.1B) (Raynes, Pombier et al. 2013). Similarly, \( rps19 \) mRNA levels increased after 30 min of HS and began to decline by 6 hours of continuous HS treatment (Figure 4.1). Western blot analysis showed that RPS19 protein is elevated upon 2 hours of HS and remained constant at 6 hours of treatment (Figure 4.2). We therefore conclude that RPS19 expression is induced by the HSR.

**The \( rps19 \) promoter possesses conserved HSF1 binding sites**

We next sought to investigate whether \( rps19 \) is a transcriptional target of HSF1 as suggested by Figures 4.1-2. The \( rps19 \) open reading is highly conserved from yeast to humans (Figure 4.3). The HSF1 targeted promoters possess evolutionarily conserved inverted repeats of heat shock elements (HSEs) composed of the pentamer sequence nGAAn (Sorger and Pelham 1987; Amin, Ananthan et al. 1988). Typically found in triplicate, each element is a sequence for one of the three DNA-binding domains of the HSF1 trimer to bind to the DNA. Sequence analysis of the \( C. \) elegans \( rps19 \) 5′UTR revealed a putative HSE 367 bps upstream of the ATG start codon (Figure 4.4). Sequence analysis of the \( H. \) sapiens \( rps19 \) 5′UTR revealed a putative HSE 150 bps upstream of the ATG start codon (Figure 4.5). This HSE is conserved in \( S. \) cerevisiae, \( C. \) elegans, \( D. \) melanogaster, and \( M. \) musculus (Figure 4.6).

**HSF1 is recruited to the \( rps19 \) promoter**

In order to verify that \( rps19 \) is an HSF1 target, we performed chromatin immunoprecipitation (ChIP). HEK293 cells were treated with HS over a 6 hour time course followed by ChIP using an antibody to HSF1. A 2 hour HS resulted in recruitment of HSF1 to the \( rps19 \) promoter (Figure 4.7). Unlike the kinetics observed for HSF1 recruitment to \( hsp \) targets (Figure 2.4B) (Raynes, Pombier et al. 2013), HSF1 recruitment to the \( rps19 \) promoter did not attenuate at 6 hours of HS treatment (Figure 4.7). Therefore, we conclude that \( rps19 \) is a transcriptional target of HSF1.
**Discussion**

Our results indicate that RPS19 is a new transcriptional target of HSF1. We provide evidence that RPS19 is upregulated upon HS and that HSF1 is recruited to the rps19 promoter. Upon initiation of the HSR, transcription and translation of most genes is reduced in favor of the upregulation of HSPs (Theodorakis and Morimoto 1987). It is therefore interesting that RPS19 interacts with AROS (Maeda, Toku et al. 2006), a positive SIRT1 modulator, and that RPS19 is also up-regulated upon HS. We feel that there are two hypotheses that may explain this phenomenon: 1) RPS19 may be a component in a SIRT1-AROS complex that positively regulates the HSR or 2) the expression of RPS19 is increased during the HSR to manage the elevation of HSP translation induced by HS.

**Future Directions**

It is well established that RPS19 interacts directly with AROS and that AROS interacts directly with SIRT1 (Maeda, Toku et al. 2006; Kim, Kho et al. 2007). In order to test the hypothesis that RPS19 forms a complex with SIRT1 and AROS, a co-immunoprecipitation pulling down SIRT1 should be performed under HS conditions and the presence of RPS19 can be confirmed via Western blot analysis. Additionally, the precipitation of RPS19 with HSF1 would provide further evidence of its involvement with HSR regulation. Association with SIRT1-AROS might provide a mechanism to recruit translational machinery to hsp promoters in order to rapidly transcribe nascent hsp RNA.

While knockdown studies would traditionally elucidate the role of a protein in the HSR, knockdown of RPS19 may be uninformative due to the fact that it is an essential ribosomal protein. Recently, depletion of the proteins RPS6, RPS7, RPS15, RPS16, RPS25, RPS28, RPL7, RPL14, and RPL26, in addition to the previously reported DBA-associated proteins including RPS19, demonstrated that they are essential for translation. Knockdown of individual RPs with siRNA resulted in a decreased production of other RPs within the same subunit and a decline in the production of mature
ribosomes, thus negatively affecting translation (Robledo, Idol et al. 2008). Similarly, overexpression of RPS19 may merely cause the increased expression of other proteins in the small ribosomal subunit and result in a general increase in HSPs, independent of RPS19 interaction with a SIRT1-AROS complex.

However, because the HSE found within the rps19 promoter is conserved in S. cerevisiae, the importance of HSF1 induction of RPS19 can be tested via site-directed mutagenesis in the yeast model system. Guanine and cytosine are the most conserved nucleotides of the HSE and may abolish HSF1 recruitment if mutated to purines (Trinklein, Murray et al. 2004). Upon mutation of the rps19 HSE, a decline in HS-stress resistance compared to the wild-type strain would indicate that the increased expression of RPS19 is critical for HSR regulation.

Additionally, the importance of RPS19 in HSR-regulated stress resistance can be measured in a D. melanogaster strain possessing a heterozygous deletion mutation for rps19b<sup>EY00801</sup> (Bloomington Drosophila Stock Center, Stock#15043). Loss of one copy of the rps19b allele may lead to reduced HS-stress resistance. Furthermore, a cross between an rps19b<sup>EY00801</sup> mutant and a polyQ-GFP reporter mutant may result in an increase in age-dependent aggregation. This would demonstrate the importance of rps19 in HSR regulation in a multicellular organism. While this can also be shown in C. elegans using RNAi to rps19, complete knockdown of rps19 may be detrimental to protein translation as a whole, thus resulting in misleading phenotypic interpretations.

In addition to the hypothesis that RPS19 is a member in an HSF1 regulatory complex, it would be interesting to investigate whether RPS19 may be a sensor of the HSR in regards to protein translation. Stress sensing mechanisms are critical for regulation of the HSR and are evident for the trimerization of HSF1. Inactive HSF1 monomers are bound by HSP90 and sequestered to the cytoplasm. Upon denaturing stress, HSP90 releases HSF1 in order to manage misfolded proteins, allowing HSF1 to trimerize and translocate to the nucleus (Ali, Bharadwaj et al. 1998). HSF1 trimerization is also aided by the eukaryotic elongation factor eEF1A and Heat Shock RNA-1 (HSR-1), which are hypothesized to recruit translational machinery to the nascent hsp mRNA (Shamovsky, Ivannikov et al. 1998).
HSR-1 is thermosensitive RNA that may act in a similar manner as the prokaryotic _rpoH_, which forms an inhibitory secondary RNA structure that can only be translated upon melting by denaturing stress (Morita, Kanemori et al. 1999; Morita, Tanaka et al. 1999). Likewise, _rps19_ may be sensitive to proteotoxic stress in order to promote the HSR by interacting with SIRT1-AROS or to prepare for the increased translation of HSPs. Interestingly, _in vitro_ studies have shown that RPS19 binds the 5′UTR region of its own RNA in a potential feedback mechanism (Schuster, Frojmark et al. 2010). Because RPS19 has RNA binding capabilities and is upregulated upon HS, its potential involvement with HSR-1 and eEF1A should be investigated.

In order to test the independent hypothesis that RPS19 expression is increased during the HSR as a global consequence to necessitate the elevation of HSP translation induced by HS, the expression levels of other ribosomal proteins upon HS should be evaluated. Moreover, evidence of HSEs within the promoter regions of other ribosomal genes would indicate that the increased expression of RPs during the HSR is a global reaction to increased translation. However, given that translation of constitutive genes decreases upon HS, it may be that the rate of general protein translation does not increase in order to compensate for increased HSP expression. Further confounding the matter is the hypothesis that nSB’s sequester protein translational machinery as a method to down-regulate translation of non-stress genes (Sandqvist and Sistonen 2004). Titrating away translation factors may not negatively impact HSP protein synthesis if they are specifically recruited with HSF1.

Further research is needed to determine if RPS19 upregulation by HSF1 is a hallmark of protein translation or a component of the SIRT1-AROS regulatory complex. This current work expands our knowledge of genes that are transcriptional targets of HSF1 and we add RPS19 as a protein that is inducible by HS stress.
Figure 4.1. *rps19* mRNA levels increase upon HS.

HeLa cells were exposed to a 42°C HS from 0 to 6 hours and qRT-PCR was performed using primers to *rps19*. The kinetics of *rps19* transcription is rapid, but does not change throughout the initiation of the HSR. However, similarly to *hsp70* induction (Figure 2.1A), *rps19* levels begin to decrease at 6 hours at 42°C. The qPCR results are in technical triplicates and statistical significance was measured by Student’s t test as compared to 0 hr HS (*P<0.05, **P<0.01, ***P<0.001).
Figure 4.2. RPS19 protein levels increase upon HS.
HeLa cells were exposed to a 42°C HS from 0 to 6 hours and RPS19 and ACTIN protein levels were determined by Western blot analysis. RPS19 protein levels begin to increase at 2 hours of HS. Results representative of biological duplicates.
Figure 4.3. The sequence alignment of the rps19 open reading frame is evolutionarily conserved.
Figure 4.4. Sequence of a putative HSE within the promoter region of the *C. elegans* rps19 homolog, T05F1.3.

A putative HSE was found 367 bps upstream of the *C. elegans* rps19 homolog. The orange bar indicates the ORF. Analysis was performed using Geneious® DNA sequence analysis software.
Figure 4.5. Sequence of a putative HSE within the promoter region of the *H. sapiens rps19* gene.

HSF1 binds to HSEs, consisting of variations of the inverted pentamer sequence nGAAn, within the promoter region of HS responsive genes. A HSE is 150 bps upstream of the *rps19* gene. The orange bar indicates the open reading frame (ORF). Analysis was performed using Geneious® DNA sequence analysis software.
Figure 4.6. Sequence alignment of the rps19 5’ UTR for S. cerevisiae, C. elegans, D. melanogaster, M. musculus, and H. sapiens.

A) Conserved nGAAAn pentamer. B) Conserved nTTCn pentamer. Other elements of the core promoter have not yet been identified. Sequence alignment was performed using Geneious® DNA sequence analysis software.

[Nucleotide notations: G= G, guanine; A= A, adenine; T = T, thymine; C= C, cytosine; R= G or A, purine; Y= T or C, pyrimidine; M= A or C, amino; K= G or T, keto; S= G or C, strong interaction (3 H bonds); W= A or T, weak interaction (2 H bonds); H= A or C or T, not a guanine; B= G or T or C, not an adenine; V= G or C or A, not a thymine; D= G or A or T, not a cytosine; N= any nucleotide.]
Figure 4.7. HSF1 is recruited to the \textit{rps19} promoter and undergoes an increase in recruitment upon HS.

Hek293 cells were exposed to HS for 0, 2, or 6 hours and chromatin immunoprecipitation (ChIP) was performed with an HSF1 antibody. The purified DNA was then analyzed by qPCR and DNA levels were determined for the \textit{rps19} promoter. The qPCR results are in technical triplicates and statistical significance was measured by Student’s t test as compared to 0 hr HS (*$P<0.05$, ** $P<0.01$). ChIP was performed in biological duplicates.
CHAPTER 5. IMPLICATIONS AND FUTURE DIRECTIONS

Implications for AROS and DBC1 Studies

HSF1 and Cancer

Heat shock proteins (HSPs) are elevated in a number of cells and tissues from a variety of cancers including prostate (Tang, Khaleque et al. 2005), lung (Zimmermann, Nickl et al. 2012), pancreas (Gress, Muller-Pillasch et al. 1994), bladder (Syrigos, Harrington et al. 2003), and breast (Cheng, Chang et al. 2012). Elevated HSP expression is often used as a biomarker for tumor progression and prognosis, though not all cancers (i.e. renal cell carcinoma) present this correlation (Ramp, Mahotka et al. 2007). Tumors take advantage of the HSR survival mechanism, which allows the cancer cells to proliferate. Overexpression of HSP27 in rat colon adenocarcinoma cells injected into syngenic animals increased tumorigenicity resulting in increased tumor size and a delay in tumor regression (Garrido, Fromentin et al. 1998). Furthermore, overexpression of HSP70 in primary cell cultures can lead to reversible oncogenic transformation (Volloch and Sherman 1999).

The manner in which increased expression of HSPs promote cancer is not fully understood. In cancer cells, the dysregulation of cell growth is the result of a shift toward cell proliferation over cell death. Therefore, HSPs may have a role in cytoprotection against apoptosis and interfere with apoptotic signaling. In tumor cells, knockdown of HSP70 with antisense oligomers led to the induction of apoptosis and the inhibition of tumor cell proliferation (Wei, Zhao et al. 1995). In addition, HSP70 has been shown to interfere with the assembly of the apoptosome by preventing the recruitment of procaspases 9 and 3 (Beere, Wolf et al. 2000). The mechanism by which HSP70 is cytoprotective against apoptosis may also be through inhibitory binding and sequestration.
of proapoptotic proteins (Jolly and Morimoto 2000). For instance, HSP70 has been shown to stabilize p53 in transformed cell culture (Pinhasi-Kimhi, Michalovitz et al. 1986). In addition to HSP70, other HSPs have been found to promote cancer cell proliferation, in a chaperone-specific manner that may not be redundant (Richards, Hickey et al. 1996).

Modulation of HSF1 as a Therapeutic Strategy

As expected, the upregulation of HSPs in cancer correlates with elevated expression of HSF1. Expression of HSF1 is increased in the more malignant human prostate carcinoma cells resulting in HSP induction (Tang, Khaleque et al. 2005). High levels of HSF1 may potentially act as a prognostic biomarker for tumor progression. A Nurses’ Health Study initiated in 1976 revealed that an increased level of HSF1 in mammary tumors is indicative of a poor prognosis (Santagata, Hu et al. 2011).

Due to the role of HSF1 in some cancers, the ability to regulate the HSR is an attractive avenue for cancer treatment. Our studies add to the current knowledge base of HSR regulation in the cell. We found that AROS and DBC1 are SIRT1 regulators that impact hsp70 transcription (Figure 2.4-5), HSF1 acetylation status (Figure 2.6A), and HSF1 recruitment to the hsp70 promoter (Figure 2.6D) (Raynes, Pombier et al. 2013). Specifically, DBC1 is shown to have a negative impact on hsp induction. Interestingly, through inhibitory binding to SIRT1, DBC1 also stimulates p53 by blocking its deacetylation (Kim, Chen et al. 2008). Acetylation of p53 results in its transactivation and the upregulation of p53-mediated function, thus leading cells down an apoptotic pathway. Consequently, a decrease in DBC1 inhibition of SIRT1 may promote cancer cell survival. Contrary to this hypothesis, DBC1 is overexpressed in a number of cancers and is associated with a shorter overall survival (Cha, Noh et al. 2009; Lee, Kim et al. 2011). However, while both SIRT1 and DBC1 expression is higher in breast cancer, their protein interaction is weaker in tumor tissue compared to normal tissue (Sung, Kim et al. 2010). This may be due to inhibitory post-translation modification to residues within the binding domains that allow for the SIRT1-DBC1 interaction. Furthermore, the
aberrant regulation of SIRT1 by DBC1 may contribute to cell proliferation in cancer as mediated by p53, as well as HSF1.

Recently, it was shown that DBC1 is phosphorylated at T454 upon genotoxic stress and mutation of that residue abolishes the SIRT1-DBC1 interaction (Yuan, Luo et al. 2012). DBC1 binds to the SIRT1 catalytic domain via its leucine zipper motif (amino acids 243-264) (Kim, Chen et al. 2008), but phosphorylation of DBC1 upon DNA damage creates a second binding site for SIRT1. Consequently, T454 phosphorylation of DBC1 resulted in a stronger DBC1-SIRT1 interaction and enhanced p53 acetylation (Yuan, Luo et al. 2012). The manner in which DBC1 senses DNA damage and becomes phosphorylated may involve recruitment of the MRN complex to modified histones at double-stranded DNA break sites, ultimately resulting in the activation of the ATM/ATR kinase pathway [reviewed in (Aguilera and Gomez-Gonzalez 2008)]. Therapeutic strategies aimed to phosphorylate DBC1 may have a dual function in treating cancer by 1) repressing p53 deacetylation thereby activating apoptosis and by 2) repressing HSF1 deacetylation resulting in down-regulation of HSPs.

**Future Studies for DBC1**

We have shown that the SIRT1 modulator DBC1 is able to negatively impact the HSR and hsp transcription. DBC1 is a homolog to cell cycle and apoptosis regulator 1 (CCAR1) in mammalian cells. While *C. elegans* does not have a DBC1 homolog, there is a CCAR1 ortholog known as LST-3. Due to the relevance of DBC1 in SIRT1 regulation and its ability to impact the HSR, current work in our laboratory is aimed to investigate the evolutionary significance of lst-3 in sir2.1 regulation of the HSR in *C. elegans*. Our laboratory has recently found that RNAi knockdown of *lst-3* results in an increase in fluorescence of an *Phsp70::GFP* reporter *C. elegans* strain (Figure S8,9) and the positive impact that *lst-3* knockdown has on the HSR is dependent upon the SIRT1 ortholog, sir2.1 (unpublished data). We hypothesize that DBC1 regulation of SIRT1 is an evolutionarily conserved mechanism that impacts the HSR and protein homeostasis. We propose to test this hypothesis using aging and age-related disease models in *C. elegans*.
to investigate the effect that lst-3 has on sir2.1 regulation of the HSR in *C. elegans* with the following aims:

**Aim 1:** Investigate the effect that lst-3 has on sir2.1 regulation of the HSR in *C. elegans.*

1.1: Determine the impact that RNAi knockdown of *lst*-3 has on the HSR as measured by transcription of *hsp70* family members and whether this is dependent on sir2.1.

1.2: Examine whether *lst*-3 RNAi impacts the acetylation status of HSF-1 by utilizing an HSF-1::GFP *C. elegans* strain, immunoprecipitating HSF-1::GFP, and analyzing acetylation of HSF-1 via Western blot.

1.3: Perform chromatin immunoprecipitation using an HSF-1::GFP *C. elegans* strain and a GFP antibody to determine the impact the *lst*-3 knockdown has on HSF-1 recruitment to *hsp* promoters via qRT-PCR.

**Aim 2:** Determine if RNAi knockdown of *lst*-3 is cytoprotective by evaluating thermotolerance and rescue of the deleterious phenotype of a polyglutamine *C. elegans* disease model.

2.1: Evaluate whether *lst*-3 knockdown results in thermotolerance and increased fitness for N2 (wild-type) animals exposed to lethal heat shock and whether this effect is reliant on sir2.1.

2.2: Examine the impact and sir2.1-dependency that *lst*-3 RNAi knockdown has on an age-dependent polyglutamine::YFP *C. elegans* model by assaying for YFP aggregation and paralysis.

**Aim 3:** Investigate whether RNAi knockdown of *lst*-3 leads to an increase in *C. elegans* lifespan in a sir2.1-dependent manner.

3.1: Evaluate whether *lst*-3 RNAi knockdown impacts lifespan due to increased stress adaptation caused by salvaging the age-related decline of *hsp* transcription.

3.2: Determine if sir2.1 plays a role in *lst*-3 modulation of lifespan using a sir2.1 (ok434) deletion mutant, sirtuin inhibitors/activators, and RNAi against sir2.1.

Together, these proposed aims will provide a basis for demonstrating the evolutionary conservation of HSF-1 regulation of the HSR, *lst*-3 involvement in protein aggregation in
a polyglutamine neurodegenerative disease model, and the impact of lst-3 regulation of HSF-1 on lifespan. This work will provide further support of DBC1 as an HSR regulator that may be a potential therapeutic target in HSR modulation.

In addition to the lst-3 studies in C. elegans evaluating the conservation of the impact of DBC1 on the HSR, our laboratory has recently found that DBC1 is degraded upon a HS time course (Figure S2). Future studies should evaluate the nature of DBC1 regulation and how it becomes degraded throughout induction of the HSR. The rate of DBC1 degradation can be determined by standard $[^{35}\text{S}]$-methionine pulse chase radiolabelling followed by immunoprecipitation of DBC1 and SDS-PAGE analysis by autoradiography. Moreover, inhibition of the proteasome with MG132 may impact the rate of DBC1 degradation. Additionally, it would be interesting to determine whether degradation of DBC1 upon HS is mediated by chaperone-mediated autophagy, which may be a more readily available pathway compared to ubiquitylation and proteolysis by the 26S proteasome.

Furthermore, DBC1 may also be regulated at the mRNA level as indicated by the slight decrease of $dbc1$ transcription observed upon prolonged HS (Figure 2.4A). The stability of the $dbc1$ mRNA can be evaluated by performing a nuclear run-on assay using HEK293 cells in the absence and presence of HS. In short, transcription is halted in the cells with actinomycin D treatment and RNA is collected over a 24-hour time course followed by qRT-PCR using primers to $dbc1$. Determining the method in which DBC1 is regulated will provide insight in how to modulate the HSR for therapeutic purposes.

**Future Studies for AROS**

Our studies regarding SIRT1 regulation of the HSR also indicated that the SIRT1 regulator AROS has a positive impact on hsp induction. However, compared to DBC1, AROS has been relatively understudied. In part, this may be due to the fact that there is currently no available commercial antibody for endogenous detection of AROS protein\(^4\).

\(^4\) We have successfully detected overexpressed AROS in transfected cell lysate using an antibody from Santa Cruz (cat#SC-86210).
Because AROS positively regulates SIRT1 deacetylation activity, it may have a role in fostering cell survival by 1) promoting deacetylation of p53 thereby leading cells away from a pro-apoptotic pathway and by 2) promoting the deacetylation of HSF1 resulting in upregulation of HSPs. Unlike DBC1, AROS does not possess an ortholog in invertebrate models and is therefore an evolutionarily recent mechanism for regulating SIRT1. It would be interesting to investigate whether AROS may play a role in cancer or if its activation could relieve polyQ aggregation toxicity in a cell culture model. In addition, the involvement of AROS in HSR regulation should be further investigated. It is tempting to suggest that there may be a dynamic role for AROS and DBC1 as competitors for SIRT1 binding and, consequently, HSF1 regulation. The endogenous activity of AROS and DBC1 upon stress would elucidate whether such an antagonistic relationship is a hallmark of SIRT1 regulation.

Final thoughts

The HSR is an ancient adaptive mechanism that has evolved to help organisms cope with stressful insults. Regulation of the HSR is complex involving redundant mechanisms and a number of feedback controls (Figure 5.1). While HSF1 has been shown to promote cytoprotection and cell survival, it has also been demonstrated to have a lethal role in cancer proliferation. In addition, a loss of protein homeostasis is a hallmark of many other diseases including heart disease, neurodegenerative diseases, and diabetes. Therefore it is of great significance to further understand the intricacies controlling this complex survival response.
Figure 5.1. A model for the regulation of the eukaryotic HSR.

Upon denaturing stress, misfolded proteins compete with HSF1 for binding to HSPs, thereby releasing HSF1 to trimerize and translocate to the nucleus where it binds HSEs within the promoter of hsp genes. Trimerization is aided in part by elongation factor eEF1A and the open confirmation of HSR-1 RNA, a complex which also serves to recruit translational machinery to the nascent hsp mRNA. Transactivation of HSF1 is aided in part by phosphorylation of the AD by a number of protein kinases (PK), while DNA binding of HSF1 is regulated by acetylation of the DBD by a histone acetyltransferase (HAT). HSF1 DNA-binding affinity is promoted via deacetylation by SIRT1, which is positively regulated by AROS and negatively regulated by DBC1. During recovery of cellular homeostasis, an excess of HSPs disassociates HSF1 trimers resulting in attenuation of the HSR.
Implications for Caloric Restriction and Heat Shock Synergy Studies

*Caloric Restriction as a Therapeutic Strategy*

Activation of the HSR and the up-regulation of HSPs are currently under investigation as a potential therapeutic to combat aging and protein aggregate diseases, including Alzheimer’s and Parkinson’s disease. Several small molecule activators have been found to activate HSF1 and are generally categorized as translation inhibitors, chaperone inhibitors, proteasome and protease inhibitors, amino acid analogs, and thiol reactive molecules (Westerheide and Morimoto 2005; Neef, Jaeger et al. 2011). Pharmacological activation of HSF1 is primarily caused by targeting and dysregulating protein homeostasis. By perturbing protein synthesis, folding, and degradation, an imbalance in protein homeostasis occurs, which leads to an increase in misfolded proteins that have the potential to oligomerize and aggregate, and thereby necessitate induction of the HSR (Salomons, Menendez-Benito et al. 2009; Wilde, Brack et al. 2011).

Several pharmacological HSR activators have proven effective against phenotypes related to neurodegenerative disease models. Celastrol, a plant-derived triterpene, has been shown to ameliorate cytotoxicity and inflammation in a rodent Alzheimer’s disease model (Allison, Cacabelos et al. 2001). HSF1A, a benzyl pyrazole derivative, elevates HSP expression, reduces protein misfolding and apoptosis in polyQ-expressing neuronal precursor cells, and promotes cytoprotection in a polyQ-mediated neurodegenerative fly model (Neef, Turski et al. 2010). Despite several studies that indicate the potential of using HSF1 activators therapeutically, the disadvantages of these compounds are too severe to apply to clinical trials. Many of the known HSR activators show hepatotoxicity in animal models at the higher concentrations that would be required in human studies. Additionally, there is a low degree of specificity regarding target activation, therefore signaling pathways independent of the HSR may be activated. Furthermore, while activation of the HSR is desirable in the treatment of protein aggregate diseases and to combat the decline of HSPs associated with aging, many cancers exhibit overexpression of HSPs and it is unknown if HSR activators could lead to the onset of cancerogenesis.
later in life (Santagata, Hu et al. 2011). In order for pharmacological activators of the HSR to ever be used in therapeutics, compounds must be engineered to have a higher degree of specificity and a lower degree of cellular toxicity.

Alternatively, caloric restriction (CR) is a relatively innocuous treatment to impose on an organism and appears to have no known negative side effects. CR has been shown to increase an organism’s resistance to a number of acute stressors (Sinclair 2005; Brown-Borg 2006). While the benefits of CR in model organisms have been demonstrated extensively, there have been relatively few studies to investigate the clinical application of CR in humans. The clinical studies that have been done indicate that humans on CR show increased metabolic fitness similar to laboratory mammals. In humans, CR has been shown to lower blood pressure, promote glucose homeostasis, and improve blood lipid profiles (Heilbronn, de Jonge et al. 2006; Weiss, Racette et al. 2006; Fontana and Klein 2007). The void of clinical CR studies is likely due to the impression that CR only has long-term benefits and that long-term self-imposed food restriction in humans is notoriously difficult.

Indeed, several studies indicate that long-term CR is required for the maximal effects of increased lifespan. In fruit flies, the effects of CR on longevity, as measured by daily mortality rate, depends on their current nutritional status with no apparent effect imparted by past nutrition (Mair, Goymer et al. 2003). While the greatest effects on longevity are due to long-term CR, short-term restriction has been shown to impart resistance to acute stressors. Mice that are pretreated with 5 and 8 months of CR show increased survivability over AL animals when injected with paraquat, a free radical-inducing agent (Sun, Muthukumar et al. 2001). The short-term benefits of CR have also been demonstrated using the surgical stress of ischemia reperfusion (IR) injury. The damage that is accrued during IR injury is caused, first, by the lack of blood flow (ischemia) that inflicts the targeted organs with oxygen deprivation, ATP depletion, and cellular toxicity resulting from the buildup of toxic byproducts; and secondly, by the restoration of blood flow (reperfusion) that inappropriately activates cellular oxidases and inflammation pathways in response to the initial tissue damage (Friedewald and Rabb 2004). In rats,
preconditioning CR for 12 months reduced myocardial oxidative stress and protected animals from the post-ischemic inflammatory response related to IR injury in comparison to AL fed control animals (Chandrasekar, Nelson et al. 2001). Interestingly, the length of time for the kinetic onset of CR benefits is shorter than previously anticipated. Both CR for 2-4 weeks and fasting for 3 days were similarly effective in protecting mice from renal IR injury when compared to mice on an AL diet (Mitchell, Verweij et al. 2010). In addition to exhibiting benefits as measured by kidney function biomarkers, short-term CR and fasted mice demonstrated a striking 60% increase in post-surgical survival.

The nutritional basis in which CR imparts biologically relevant benefits to mammals is poorly characterized. One hypothesis for the mechanism in which CR confers longevity, stress resistance, and hormesis is that the phenomenon is dependent on a reduction of amino acids (Segall and Timiras 1976; Ooka, Segall et al. 1988; Orentreich, Matias et al. 1993; Miller, Buehner et al. 2005; Troen, French et al. 2007; Grandison, Piper et al. 2009). Recently, it was found that when mice were pretreated with a protein-free diet for 1-2 weeks prior to IR injury, kidney function was protected preserving their ability to clear waste products from the blood (Peng, Robertson et al. 2012). Specifically, a tryptophan-deficient diet fed AL significantly protected kidney function compared to its pair-fed control. Additionally, at 35% dietary restriction, a protein- and tryptophan-free diet were significantly better than a complete diet in promoting hormesis, as measured by kidney function (Peng, Robertson et al. 2012).

Amino acid depletion is sensed by GCN2 (general control nonderepressible 2) kinase, which binds uncharged transfer RNAs (tRNAs) and consequently phosphorylates the eukaryotic translation initiation factor 2α (eIF2α) (Wek, Zhu et al. 1995; Dong, Qiu et al. 2000; Wek, Jiang et al. 2006). Phosphorylation of eIF2α inhibits the formation of the 43S pre-initiation complex and represses the onset of translation. Because Gcn2 is effectively an essential (EAA) and non-essential (NEAA) amino acid sensor, a Gcn2−/− mouse was used to test whether it is necessary for protection against IR injury. The deletion of Gcn2 abrogated the hormesis imparted by the tryptophan-free diet, while treatment with halofuginone, an activator of the Gcn2 pathway, was able to reproduce the
protective effect of amino acid deprivation (Peng, Robertson et al. 2012). While these studies have yet to be demonstrated in humans, the prospect of benefiting from a short-term tryptophan-restricted diet eaten \textit{ad libitum} versus a traditional CR diet may be a feasible additive therapeutic for surgical patients.

Our \textit{C. elegans} studies demonstrate that the hormeric effect of CR to protect against a lethal HS stressor occurred only in the presence of sir2.1 (Figure 3.5) (Raynes, Leckey et al. 2012). In addition, the ability of CR to protect against the age-dependent paralysis resulting from proteotoxicity of a polyglutamine model also required sir2.1 (Figure 3.6) (Raynes, Leckey et al. 2012). As expected, we have also shown that Q24-YFP animals have a 40\% decrease in \textit{sir}2.1 mRNA levels (Figure 3.7) (Raynes, Leckey et al. 2012). This is consistent with Western blot analysis of tissue samples from Huntington Disease patients, for which the polyglutamine repeats are a model (Pallas, Pizarro et al. 2008). Taken together, our results indicate that CR hormesis is dependent upon its ability to induce the HSR in the presence of stressors that perturb protein homeostasis.

It has been previously shown that SIRT1 protein levels increase upon CR in rats (Cohen, Miller et al. 2004). Likewise, recent evidence in our laboratory has shown that CR significantly increases \textit{sir}2.1 mRNA levels in \textit{C. elegans} by 1.6 fold (Figure S7). Interestingly, CR in a Q24-YFP background causes a 4.8 fold increase in \textit{sir}2.1 mRNA. While this result seems contradictory, a study of polyglutamine mutants in yeast has shown that protein oxidation and catalase activity increases in relation to the length of the polyQ tract only in glucose limiting conditions (Sorolla, Rodriguez-Colman et al. 2011). The oxidative stress resulting from the polyQ cytotoxicity results in an increase in Sir2 and subsequent reduction in protein aggregation. This has led us to hypothesize that there may be crosstalk between the HSR and the oxidative stress response (OSR).

\textit{The Oxidative Stress Response}

Under homeostatic conditions, cells are able to balance the production of oxidants and antioxidants resulting in redox equilibrium. Oxidative stress occurs when cells are subjected to excess levels of reactive oxygen species (ROS) or as a result of antioxidant
depletion, resulting in an imbalance of ROS and antioxidants. Common ROS include superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals (OH), and reactive nitrogen species (NO) (Rutkowski, Panciewicz et al. 2007). While normal cells require low level concentrations of these compounds for signal transduction, increased levels are lethal resulting in DNA base modifications, DNA strand breaks, lipid peroxidation, and protein aggregation (Burney, Niles et al. 1999; Klaunig, Kamendulis et al. 2010). Nuclear Factor Erythroid-derived 2-Related Factor 2 (NRF2) is the major transcriptional regulator of the OSR, which results in the induction of detoxification and antioxidant proteins. NRF2 is highly conserved and is known as SKN-1 in *C. elegans* (Bishop and Guarente 2007; Onken and Driscoll 2010) and CncC in *D. melanogaster* (Sykiotis and Bohmann 2008). Upon induction of the OSR, NRF2 binds to antioxidant response elements (AREs) within the promoter regions of target genes. AREs consist of the motif (T/C) TGCTGA (C/G) TCA (T/C), which are bond by the basic leucine zipper (bZIP) domain within NRF2 (Andrews, Kotkow et al. 1993). Modulation of the NRF2-mediated OSR involves the cytoplasmic protein Keap1, which sequesters NRF2 within the cytoplasm in basal conditions (Itoh, Wakabayashi et al. 1999). Upon oxidative stress, NRF2 disassociates from Keap1 and localizes to the nucleus where it forms heterodimers with small transcriptional activator Maf proteins and binds to the ARE (Sykiotis and Bohmann 2010).

Several studies have demonstrated that the generation of ROS is linked to hormesis (Ristow and Schmeisser 2011). Administration of antioxidants Vitamin C and E prevent the health-promoting effects of exercise in humans (Ristow, Zarse et al. 2009). A study in *C. elegans* has shown that glucose restriction causes increased ROS formation, thus promoting mitochondrial metabolism and resulting in an increase in longevity (Schulz, Zarse et al. 2007). A minor oxidative stress results in the upregulation of antioxidant genes on the cellular level that may increase the survival response of the organism, which is then better prepared to manage potentially lethal oxidative stressors. Similarly, induction of the HSR is cytoprotective against subsequent lethal HS stressors. Crosstalk between adaptive stress responses may provide organisms with increased survival and would, therefore, be evolutionarily advantageous.
Crosstalk between the HSR and the OSR

Adaptive stress responses are highly conserved in eukaryotes and enable an organism to manage a variety of stressors. In addition to thermal stress, HSF1 is activated by free radicals and oxidative damage, which also induce the NRF2-mediated OSR (Westerheide and Morimoto 2005). In addition, SIRT1 deacetylates a number of other transcription factors regulated by stress, including NRF2. In a K562 myelogenous leukemia cell line, overexpression of SIRT1 results in NRF2 deacetylation rendering antioxidant gene transcription inactive (Kawai, Garduno et al. 2011). Therefore, the NRF2-mediated OSR and the HSF1-mediated HSR are both stress-induced cytoprotective systems regulated in part by SIRT1 (Kawai, Garduno et al. 2011; Zhang, Ahn et al. 2011). Certain sulfhydryl-reactive small molecule activators have been found to induce both HSF1 and NRF2 transcriptional targets including celastrol and nitro-oleic acid (Westerheide and Morimoto 2005; Trott, West et al. 2008; Kansanen, Jyrkkkanen et al. 2009). Sulfoxythiocarbamate inducers were recently demonstrated to act as dual activators of NRF2 and HSF1, though much higher concentrations were required to induce hsp70 transcription (Zhang, Ahn et al. 2011). This differential induction of both stress responses may indicate that upon oxidative stress, the more rapid kinetics of NRF2 activation allow cells to respond to immediate stress, while the slower kinetics of HSF1 activation allow cells to manage the resulting proteomic stress. Interestingly, SIRT1 has been shown to deacetylate NRF2 resulting in a loss of affinity for the ARE element in target promoters and a decrease in NRF2-dependent gene transcription (Kawai, Garduno et al. 2011). The role of SIRT1 in the activation and regulation of stress response mechanisms may represent a vital evolutionarily conserved function in cellular homeostasis.

Future studies

Glutathione S-transferase 4 (gst-4) is a transcriptional target for skn-1, the Nrf2 C. elegans homolog, and can be used as a sensor for OSR induction. In order to evaluate the interplay between the HSR and the OSR, a Pgst-4::RFP strain of C. elegans, in which
the *gst-4* promoter is fused to RFP, can be utilized to report transactivation of *skn-1*. A
*Pgst-4::RFP;Phsp70::GFP* strain could be used in a compound screen for dual inducers
of the transcription factors *skn-1* and *hsf-1*. The C12C8.1::GFP strain used in Studies
Chapter 2 would be a suitable reporter for HSR induction. However construction of a
GFP reporter activated by the F44E5.5 promoter, an *hsp70* family member, would likely
give a more robust fluorescence due to the increased number of HSEs present in the
sequence (Figure 1.2), which results in increased transcriptional activity (Figure 3.3)
(Raynes, Leckey et al. 2012).

A number of cytoprotective assays using combinations of oxidative and thermal stressors
to precondition against lethal oxidative or thermal damage would elucidate crosstalk
between the HSR and OSR. For instance, a minor HS preconditioning promotes
thermotolerance against a lethal HS, but can it also protect against lethal oxidative
damage by juglone, H$_2$O$_2$, or copper? Likewise, can a preconditioning treatment with
ROS reagents protect against lethal HS? Furthermore, does a minor oxidative stress act
in an additive or synergistic manner with HS or CR to maximize cytoprotection? In
addition, it would be interesting to investigate the sir2.1-dependency of these
mechanisms with RNAi.

*Final thoughts*

A loss of protein homeostasis is a hallmark of many diseases associated with aging. As
lifespan increases in Western civilizations, so does the need to manage the imbalance
between proteotoxic stress and cytoprotective genes. By understanding adaptive stress
responses and how they can be exploited in order to promote hormesis, the treatment of
age-related disease may be directed toward the broad approach of extending healthspan.
REFERENCES


APPENDICES

Appendix A. Supplementary Figures

**Figure S1. SIRT1 can interact directly with HSF1.**

SIRT1 interacts directly with HSF1. A TNT-synthesized HSF1 prey protein precipitates with SIRT1-GST expressed by *E. coli* in a GST pull-down assay.

**Methods:** SIRT1-GST bait protein was generated using the Gateway Cloning system to shuttle full-length SIRT1, lacking a stop codon, in the ORF Express Gateway Plus Shuttle clone (Genecopoeia cat#GC-U1443-CF) into the Gateway® pDEST™24 GST Destination Vector (Invitrogen cat#12216-016). A GST pull-down assay was performed using Promega’s MagneGST™ Pull-Down System (cat#V8870). The HSF1 prey protein was synthesized using the TNT® T7 Master Mix from the full-length Myc-HSF1 plasmid (Holmberg, Hietakangas et al. 2001) described above. SIRT1-GST was transformed into BL21 (DE3) competent *E. coli* cells (NEB cat#C2527) and expression was induced with 1mM IPTG for 6 hours. The cells were lysed with MagneGST™ Cell Lysis Reagent and the SIRT1-GST fusion protein was immobilized with equilibrated MagneGST™ Particles that had been blocked with 10% BSA according to manufacturer’s instructions. The HSF1 prey protein was then captured by the immobilized SIRT1-GST bait protein and washed with 400 µL of MagneGST™ Binding/Washing buffer. The MagneGST™ Particles were eluted with SDS sample buffer and bound polypeptides were detected by Western blot analysis.
Figure S2. DBC1 protein expression decreases over a heat shock time course.
Protein collected from HeLa cells exposed to 42°C from 0 to 6 hours. Immunoblotting was performed using an antibody against DBC1 and Actin. The relative intensities of the DBC1 bands were quantitated using Image J and the fold intensities compared to the background is shown.
Figure S3. SIRT1 does not co-localize with HSF1 at nuclear stress bodies upon HS.

Immunofluorescence of HSF1 and SIRT1 nuclear localizations in HeLa cells. HSF1 localizes to the nucleus specifically to nuclear stress bodies, as previously described (Westerheide, Anckar et al. 2009). SIRT1 localizes to PML nuclear bodies prior to heat shock, also as previously described (Campagna, Herranz et al. 2010). Upon heat shock, SIRT1 does not appear to experience a change in localization.

Methods: HeLa cells were cultured on glass coverslips in 30 mm plates at a 1:10 dilution in DMEM media (CellGro cat#15-017-CV) supplemented with 10% fetal bovine serum (GIBCO cat#10437-028) and 1% Pen-Strep-Glutamine (CellGro cat#30-0090CI). Parafilmed plates were heat shocked in a 42°C water bath for 30 minutes with no time for recovery. At 75-90% confluency, cells were fixed in a 4% formaldehyde solution in PBS. The cells were then washed in PBS and blocked in blocking buffer (5% FBS and 0.3% Triton in 1X PBS) for 1 hour. After blocking, the cells were incubated overnight at 4°C with the primary antibodies, both anti-HSF1 (Stressgen cat#SPA-901) and anti-SIRT1 (Millipore cat#04-1557). Next, the cells were washed in PBS and incubated with the secondary antibodies for 1-2 hours, both Alexa-Fluor #555 and #488 (Invitrogen). The cells were then washed with PBS and the coverslips were placed on a slide with a drop of Vectorshield® with Dapi (Vector Laboratories). The coverslips were sealed with clear nail polish and observed on the EVOS scope.
Figure S4. Knockdown with AROS siRNA reduces hsp70 promoter activity upon HSR induction at 42°C.

HeLa hsp70.1 promoter-luciferase reporter cells were transfected with 50 nM of Dharmacon SmartPool DBC1, AROS, HSF1, or non-targeting control siRNA and HS was induced via 2 hours at 42°C. Luciferase activity was measured and compared to that of the non-targeting control. Luciferase assays were performed in biological triplicate. Statistical significance was measured by Student’s t test as compared to the non-targeting siRNA control with celastrol (5 μM) treatment (*P<0.05, ** P<0.01, ***P<0.001).
**Figure S5. Generation of the Q24-YFP; sir2.1 (ok434) strain.**

A) The Q24-YFP; sir2.1 (ok434) double mutant strain was constructed by mating Q24-YFP males with sir2.1 (ok434) hermaphrodites. F1 cross progeny that displayed the YFP reporter were picked to individual plates and allowed to have F2 progeny. Approximately twenty F2 progeny were picked to individual plates and allowed to self-fertilize. B) Once F3 progeny were obtained, the F2 parents were tested for the sir2.1 deletion by PCR using primers flanking the deletion (5′-CAGCGACGCTGTCTCAAAAA, 3′-GATCAAATGAGCGATTGGCT) (Wang and Tissenbaum 2006).
PCR was performed on genomic DNA from the F2 parents using \textit{sir2.1} deletion primers flanking the deletion. The deletion is confirmed by PCR amplification of a 300 bp amplicon.
Figure S7. *sir2.1* mRNA levels for N2 and Q24-YFP *C. elegans* strains. N2 and Q24-YFP animals were treated with or without HS and CR, as indicated, and mRNA levels were determined for *sir2.1* by qRT-PCR. Results are in technical triplicates and are representative of biological duplicates. Statistical significance was measured by Student’s t test as compared to AL (*\(P<0.05\), **\(P<0.01\)).
Figure S8. *hsp70* promoter reporter used in Studies, Chapter 2.
A green fluorescent protein (GFP) reporter driven by the promoter of the HSF-1 target gene *hsp-70* (*C12C8.1*) provides a fluorescence-based method for measuring HSF-1 transcriptional activity in *C. elegans*. The expression of GFP is highly inducible with heat.
Figure S9. *hsp-70* promoter induction is significantly increased upon HS in *C. elegans* when fed *lst-3* RNAi.

Bacteria (HT115) containing plasmid constructs made to produce double-stranded RNA were grown in LB and ampicillin and then spread onto fresh NGM agar plates containing 100µg/ml ampicillin and 1mM IPTG (Isopropyl β-D-1-thiogalactopyranoside). Bacterial lawns were induced overnight at room temperature. A C12C8.1::GFP strain was bleach synchronized and placed onto plates with either empty vector (pL4440), *hsf-1*, or *lst-3* RNAi. Upon adulthood, animals were heat-shocked at 33°C for 15 minutes. (Experimental designed by R. Raynes and J. Brunquell and performed by J. Brunquell.)
Appendix B. Supplementary Tables

Table S1. List of primer sets used for mammalian cell culture samples.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence*</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aros</td>
<td>F: 5’- GAAGGCAATTCAGGCCCAGAAACT- 3’ R: 5’- TCGTTCTGGTCAGAAACTTCAGGT- 3’</td>
<td>131</td>
</tr>
<tr>
<td>dbc1</td>
<td>Primer mix from GeneCopoeia, Inc. Cat # HP015872 (KIAA1967)</td>
<td>Not provided</td>
</tr>
<tr>
<td>gapdh</td>
<td>F: 5’- CCACCTCCTCCACTTTGAC - 3’ R: 5’- ACCTGTTGCTGTGACCA - 3’</td>
<td>102</td>
</tr>
<tr>
<td>gapdh promoter¹</td>
<td>F: 5’- TACTAGCGGTTTTACGGGCG - 3’ R: 5’- TCGAACAGGAGAGGAGGAGCGA - 3’</td>
<td>166</td>
</tr>
<tr>
<td>hsp27²</td>
<td>F: 5’- CAAGTTTCCTCCTCCTCTGT - 3’ R: 5’- GGCAAGTTCTCACTCGGATTTG - 3’</td>
<td>156</td>
</tr>
<tr>
<td>hsp70³</td>
<td>F: 5’- AGAGCGGAGGCCGAGAAGAG - 3’ R: 5’- CACCTTTCGCTGTTGAAAC - 3’</td>
<td>110</td>
</tr>
<tr>
<td>hsp70 promoter 1³</td>
<td>F: 5’- GGCGAAACCCCTGGAATAATCCTGGCAGA - 3’</td>
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<tr>
<td>hsp70 promoter 2 (HSE site)</td>
<td>F: 5’- CTCCTCCCGAGGAGCTGGGACT - 3’ R: 5’- CGAGCCGAGCGGATCTTTA - 3’</td>
<td>134</td>
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<tr>
<td>hsp90⁴</td>
<td>F: 5’- GGCAGTCAAGCATTTCCTGATGAG - 3’ R: 5’- GTCAACCACACCCAGGATAAA - 3’</td>
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<td>rps19</td>
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<td>111</td>
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<td>rps19 promoter</td>
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<td>sirt1¹</td>
<td>F: 5’- TCTCGGACGACATTCGAGCATTCTT - 3’ R: 5’- TTTCCAGCCTGTCTATGTCTGGG - 3’</td>
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</table>

*F: forward, R: reverse
³Primers from Westerheide et al. Science 2009 323, 1063.
⁴Primers from McLean et al. Biochemical and Biophysical Research Communications 2006 351:3.
Table S2. List of primer sets used for *C. elegans* samples.

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<td>C12C8.1</td>
<td>F: 5' - CCCGTTGGTTGAGGTTGAAGT - 3' R: 5' - CAGCTTCAGCCGTTTCTTTCT - 3'</td>
<td>100</td>
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<tr>
<td>gapdh</td>
<td>F: 5' - GGAACGTGTTACCTACGATGGAGA - 3' R: 5' - AAACTCCAGTAGACTGACAACG - 3'</td>
<td>148</td>
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<tr>
<td>F44E5.4</td>
<td>F: 5' - CTCGGTACTACGTACTCGTGTTTGTT - 3' R: 5' - GAATGCCACGTATGAGTGGAGT - 3'</td>
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<tr>
<td>F44E5.5</td>
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<td>120</td>
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<tr>
<td>sir2.1</td>
<td>F: 5' - GGGAATGAGATTCGTGAGGA - 3' R: 5' - TCCGAGATCCCTCTCAGGAGTA - 3'</td>
<td>105</td>
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<tr>
<td>sir2.1 deletion</td>
<td>F: 5' - CAGCGACGCTGTCTCAGGATTTAGG - 3' R: 5' - GATCAAATGAGCATTGGCT - 3'</td>
<td>304/1072^</td>
</tr>
</tbody>
</table>

*F: forward, R: reverse

^ The amplicon is 304 bp if a deletion occurs within the *sir2.1* gene and 1072 bp if one does not occur.
Table S3. Antibodies for Western blot (WB) analysis, immunoprecipitation (IP), and immunocytochemistry (ICC).

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<th>Application</th>
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<td>Acetyl-K</td>
<td>Cell Signaling Technology</td>
<td>9441</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>ACTIN</td>
<td>Santa Cruz</td>
<td>SC-1616-R</td>
<td>1:1000-1:5000</td>
<td>WB</td>
</tr>
<tr>
<td>ACTIN (C. elegans)</td>
<td>Amersham</td>
<td>JLA20-C</td>
<td>1:750</td>
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<tr>
<td>Alexa-Fluor #488</td>
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<td>A11008</td>
<td>2 µg/0.5 mL</td>
<td>ICC</td>
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<td>Alexa-Fluor #588</td>
<td>Invitrogen</td>
<td>A21422</td>
<td>2 µg/0.5 mL</td>
<td>ICC</td>
</tr>
<tr>
<td>AROS (S19bp)</td>
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<td>SC-86210</td>
<td>1:200</td>
<td>WB</td>
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<tr>
<td>BETA-TUBULIN</td>
<td>Cell Signaling Technology</td>
<td>2128</td>
<td>1:1000</td>
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<tr>
<td>DBC1</td>
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<td>AB 70242</td>
<td>1:2500-1:5000</td>
<td>WB</td>
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<td>GFP</td>
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<td>1:1000</td>
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<td>Sigma</td>
<td>F3165</td>
<td>1:500</td>
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<td>HA</td>
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<td>HRP-Mouse</td>
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<td>HRP-Rabbit</td>
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<td>HRP-Rat</td>
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<td>112-035-062</td>
<td>1:10,000</td>
<td>WB</td>
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<td>HSF1</td>
<td>Assay Design</td>
<td>SPA-950</td>
<td>1:750</td>
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<tr>
<td>HSF1</td>
<td>Stressgen</td>
<td>SPA-901</td>
<td>5 µL/0.5 mg, 10 µL/1 mL</td>
<td>IP, ICC</td>
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<td>Stressgen</td>
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<tr>
<td>Normal Rabbit IgG</td>
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<td>2729</td>
<td>5 µL/0.5 mg</td>
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Table S3 continued from previous page.

<table>
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<th>Protein A-HRP</th>
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<td>SIRT1</td>
<td>Abcam</td>
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<td>Millipore</td>
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</tbody>
</table>

HSF Transcription Factor Family, Heat Shock Response, and Protein Intrinsic Disorder.

Sandy D. Westerheide, Rachel Raynes, Chase Powell, Bin Xue and Vladimir N. Uversky

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HSF Transcription Factor Family, Heat Shock Response, and Protein Intrinsic Disorder

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Abstract: Intrinsically disordered proteins are highly abundant in all kingdoms of life, and several protein functional classes, such as transcription factors, transcriptional regulators, hub and scaffold proteins, signaling proteins, and chaperones are especially enriched in intrinsic disorder. One of the unique cellular reactions to protein damaging stress is the so-called heat shock response that results in the upregulation of heat shock proteins including molecular chaperones. This molecular protective mechanism is conserved from prokaryotes to eukaryotes and allows an organism to respond to various proteotoxic stressors, such as heat shock, oxidative stress, exposure to heavy metals, and drugs. The heat shock response-related proteins can be expressed during normal conditions (e.g., during the cell growth and development) or can be induced by various pathological conditions, such as infection, inflammation, and protein conformation diseases. The initiation of the heat shock response is manifested by the activation of the heat shock transcription factors HSF1, part of a family of related HSF transcription factors. This review analyzes the abundance and functional roles of intrinsic disorder in various heat shock transcription factors and clearly shows that the heat shock response requires HSF flexibility to be more efficient.

Keywords: Intrinsic disorder, heat shock response, heat shock factors, chaperones.
INTRINSICALLY DISORDERED PROTEINS: GENERAL OVERVIEW

Research over the last decade or so made it absolutely clear that in addition to well-folded and highly structured transmembrane, globular and fibrous proteins, the protein universe includes intrinsically disordered proteins (IDPs) and proteins with intrinsically disordered regions (IDRs). These IDPs and IDRs are biologically active and yet fail to form specific 3D structure; existing instead as collapsed or extended dynamically mobile conformational ensembles [1-7]. These floppy proteins and regions are known as pliable, rheomorphic [8], flexible [9], mobile [10], partially folded [11], natively denatured [12], natively unfolded [3, 13], natively disordered [6], intrinsically unstructured [2, 5], intrinsically denatured, [12] intrinsically unfolded [13], intrinsically disordered [14], vulnerable [15], chameleon [16], malleable [17], 4D [18], protein clouds [19], and dancing proteins [20], among several other terms. The variability of terms used to describe such proteins and regions is a simple reflection of their highly dynamic nature and the lack of unique 3-D structure. None of these terms or their combinations is completely appropriate, as the majority of them have been borrowed from fields such as protein folding or crystallography, which are not directly related to the biologically active proteins that normally exist as structural ensembles.

Since these proteins are highly abundant in any given proteome [21], the role of disorder in determining protein functionality in organisms can no longer be ignored. Native biologically active proteins were conceptualized as parts of the “protein trinity” [4] or the “protein quartet” [22] models, where functional proteins might exist in one of several conformations – ordered, collapsed-disordered (molten globule-like), partially collapsed-disordered (pre-molten globule-like) or extended-disordered (coil-like), and protein function might be derived from any one of these states and/or from the transitions between them. Disordered proteins are typically involved in regulation, cell signaling and control pathways [23-25], which complement the functional repertoire of ordered proteins, which have evolved mainly to carry out efficient catalysis [26]. It is also important to remember that sites of posttranslational modifications (acylation, hydroxylation, ubiquitination, methylation, phosphorylation, and others) and sites of regulatory proteolytic attack are frequently associated with regions of intrinsic disorder [14].

Because of the fact that IDPs play crucial roles in numerous biological processes, it was not too surprising to find that many of them are involved in human diseases [27]. Originally, this hypothesis was based on numerous case studies where a particular IDP was shown to be associated with a particular disease. For example, the presence of disorder has been directly observed in several cancer-associated proteins, including p53 [28], p53kip2 [29], Bcl-XL and Bcl-2 [30], c-Fos [31], thyroid cancer associated protein, TC-1 [32], and many others. Some other maladies associated with IDPs includes Alzheimer’s disease (deposition of amyloid, tau-protein, α-synuclein fragment NAC [33-36]), Niemann-Pick disease type C, subacute sclerosing panencephalitis, argyrophilic grain disease, myotonic dystrophy, and motor neuron disease with neurofibrillary tangles (accumulation of tau-protein in the form of neurofibrillary tangles [35]); Down’s syndrome (nonfilamentous amyloid-deposits [37]); Parkinson’s disease, dementia with Lewy body, diffuse Lewy body disease,
Lewy body variant of Alzheimer’s disease, multiple system atrophy and Hallervorden-Spatz disease (deposition of α-synuclein in a form of Lewy body, or Lewy neuritis [38]); prion diseases (deposition of PrPSc [39]); and a family of polyQ diseases, a group of neurodegenerative disorders caused by expansion of CAG trinucleotide repeats coding for polyQ in the gene products [40].

Three computational and bioinformatics approaches have been elaborated to estimate the abundance of IDRPs in various pathological conditions. The first approach was based on the assembly of specific datasets of proteins associated with a given disease and the computational analysis of these datasets using a number of disorder predictors [23, 27, 41-44]. In essence, this was an analysis of individual proteins extended to a set of proteins related to a given disease. Using this approach, a prevalence of intrinsic disorder was detected in proteins associated with cancer [23], cardiovascular disease [42], neurodegenerative diseases [43, 45], various amyloidoses [46] and diabetes [27]. A second approach utilized the diseasome, a network of genetic diseases where the related proteins are interlinked within one disease and between different diseases [47]. A third approach was based on the evaluation of the association between a particular protein function (including the disease-specific functional keywords) with the level of intrinsic disorder in a set of proteins known to carry out this function [48-50]. Based on the fact that IDRPs and proteins with long IDRIs were commonly found in various diseases, the “disorder in disorders” or D2 concept was introduced to summarize work in this area [27] and the concepts of the disease-related unfoldome and unfoldomics were developed [51].

INTRINSIC DISORDER AND TRANSCRIPTION REGULATION

Several protein functional classes (e.g., transcription factors [19, 52-54], transcriptional regulators [17, 55, 56], hub proteins [24, 56-61], scaffold proteins [62-65], signaling proteins [23], chaperones [66-73], etc.) were shown to be enriched in intrinsic disorder. Recent studies suggested that eukaryotic proteomes are highly enriched in IDRPs relative to bacterial and archaeal proteomes [74-76], which may reflect the greater need for signaling and transcriptional regulation in nucleated cells [14, 23, 77]. Transcription factors (TFs) act through the recognition of specific DNA sequences and recruitment and assembly of the transcription machinery. Therefore, both protein-DNA and protein-protein recognition are central processes in TF function. It has been reported that protein-protein and protein-DNA interaction are often accompanied by a local folding in a protein molecule [78]. One of the important biological implications of this coupled binding and folding scenario is that protein backbone mobility may play an important role in the early stages of a binding event [79], where the specific signal from the complex of protein with its binding partner emerges only after appropriate conformational changes take place [80].

Comprehensive computational analysis of several transcription factor datasets revealed that about 90% of TFs possess long regions of intrinsic disorder, and ≥70% proteins in the TF datasets were predicted to be wholly disordered [52]. Furthermore, the degree of disorder in eukaryotic TFs was shown to be significantly higher than in prokaryotic proteins. Eukaryotes have a well-developed
gene transcription system, which probably requires a great deal of flexibility and plasticity. The intrinsically disordered TFs or partially unstructured regions can offer significant advantages in response to different molecular targets, allowing one protein to interact with multiple cellular partners and allowing fine control over binding affinity [52]. This analysis also revealed the existence of two distinct classes of DNA-binding domains (DBDs) in TFs. In one class, the DBDs are well-structured and specifically recognize DNA using the molecular surfaces they present to the environment in another class, various DBDs such as basic domains and AT-hooks, are likely to be highly unstructured in isolation, and presumably undergo a disorder-to-order transition upon binding to specific DNA sequence [52]. The high prevalence of intrinsic disorder in TFs suggested that it may play a critical role in the primary functions of TFs, which are molecular recognition, DNA binding, and transcriptional regulation.

This hypothesis is in good agreement with several recent findings showing that eukaryotic TFs contain a variety of structural motifs that interact with specific DNA sequences [81] and are involved in activating transcription. For example, based on the analysis of binding of multiple zinc fingers to cognate DNA, a ‘snap-lock’ model has been recently introduced [82, 83]. According to this model, C2H2-type zinc finger domains consist of well-folded modules connected by highly conserved linker sequences that are mobile and unstructured in the absence of the cognate DNA. NMR analysis revealed that upon binding to the correct DNA sequence, the linker becomes highly structured and locks adjacent fingers in the correct orientations in the major groove [82, 83]. Furthermore, it has been shown that many alterations of this linker disrupt the conformation of the bound linker, increase its flexibility, and impair DNA binding, thereby altering both the biological function and sub-nuclear localization of the protein [82, 83]. This model illuminates the sophisticated relationship between the function of a TF, its domain structure, and intrinsic disorder.

It is known that two totally disordered types of DBDs, AT-hook and basic domain, act as a versatile minor groove tether to anchor TFs to particular DNA sites. In addition to having sequence-specific DNA-binding activity, many TFs contain a region involved in activating the transcription of the gene whose promoters or enhancers they have bound. Usually, this trans-activating region enables the TF to interact with a protein involved in binding RNA polymerase. Many trans-activating domains were predicted either unstructured or partly structured [52]. This was in agreement with the accumulated experimental data that many trans-activating domains are significantly disordered in an unbound form and their interactions with their targets involve coupled folding and binding events [23, 75, 77, 84]. Recently, the kinase-inducible activation domain of CREB (cAMP response element binding protein) [85], the trans-activation domain of p53 [86], and the acidic activation domain of herpes simplex virus VP16 [87] were comprehensively examined. These studies revealed that the activation domains remained mostly unstructured in their normal unbound states, and form a helix or helices upon binding to the target proteins.

In another elegant computational study, the abundance and functional roles of intrinsic disorder in human TFs were investigated [53]. The authors emphasized that eukaryotic TFs, especially so-
called *trans-acting* factors such as activators, repressors or enhancer-binding factors that specifically bind DNA *cis* elements, were noticeably larger than prokaryotic TFs. In fact, the average sequence of human TFs was more than twice as long as length of the TFs in prokaryotes. Furthermore, the fractions of sequence aligned to domains of known structure were 31% and 72% in human and bacterial TFs, respectively [53]. Therefore, as a rule, human TFs were long and poorly annotated. Analysis revealed that as high as 49% of the entire sequence of human TFs was occupied by IDR, and that more than half of the human TFs consisted of a small DBD and a set of long IDRs. In general, IDRs were shown to occupy a high fraction of TFs from eukaryotes, but not prokaryotes [53].

**AN OVERVIEW OF INTRINSIC DISORDER IN CHAPERONES**

Generally, a polypeptide chain of a protein contains all the information required to achieve functional conformation [88, 89]. Although this principle is generally correct for many proteins, the information contained in some potentially foldable proteins is not complete enough to guarantee the formation of a functionally active 3D structure. Many such potentially foldable proteins cannot fold spontaneously and require the help of molecular chaperones; i.e., cellular proteins which act to ensure that the folding of certain polypeptide chains and their assembly into oligomeric structures occurs correctly [90]. Chaperones are an important part of the cellular quality control system, maintaining an intricate balance between protein synthesis and degradation and protecting cells from the devastating consequences of uncontrolled protein aggregation. In addition to chaperones, this system includes the ubiquitin-proteasome system and the autophagy-lysosome system. Molecular chaperones protect cells from apoptosis induced by toxic oligomers. There are several mechanisms by which chaperones fight devastating consequences of misfolding and aggregation. These mechanisms can be grouped into three major classes of action: prevention, reversal and elimination. At the prevention stage, chaperones bind to unfolded stretches in proteins and keep them in a folding-competent state while preventing aggregation. In the reversal mechanism, chaperones act as disaggregating and unfolding machines, which help dissolve aggregates and give a misfolded protein a second chance for folding correctly. At the elimination step, chaperones target misfolded proteins for degradation by the ubiquitin-proteasome system and/or the autophagy-lysosome system.

The principal heat shock proteins that have chaperone activity belong to five conserved classes: Hsp33, Hsp60, Hsp70, Hsp90, Hsp100, and the small heat shock proteins. Molecular chaperones have been divided into three functional subclasses based on their mechanism of action. "Folding" chaperones (e.g., DnaK and GroEL in prokaryotes, and Hsp60 and Hsp70 in eukaryotes) rely on ATP-dependent conformational changes to mediate the net refolding/unfolding of their substrates. "Holding" chaperones (e.g., Hsp33 and Hsp31, or the HspB group of Hsp including Hsp27 and HspB1 in eukaryotes) bind partially folded proteins and maintain these substrates on their surface to await the availability of "folding" chaperones. "Disaggregating" chaperones (e.g., ClpB in
prokaryotes and Hsp104 in eukaryotes) promote the solubilization of proteins that have become aggregated as a result of stress.

Molecular chaperones are classified as either inducible or constitutively expressed according to their expression mechanisms. Both types of chaperones act by the selective binding of solvent-exposed hydrophobic segments of non-folded polypeptides, and, through multiple binding-release cycles, bring about the folding, transport, and assembly of the target polypeptides [91-93]. Some chaperones are ATPases; i.e., they use free-energy from ATP binding and/or hydrolysis to perform work on their substrates.

The concentration of inducible chaperones, also known as heat shock proteins (Hsp), increases as a response to stress conditions. These molecular chaperones prevent and reverse the misfolding and aggregation of proteins that occur as a consequence of stress [94, 95]. On the other hand, constitutively expressed chaperones, also known as heat shock cognate proteins (Hscs), facilitate protein translation, help newly synthesized proteins fold, promote the assembly of proteins into functional complexes, and assist the translocation of proteins into cellular compartments such as mitochondria and chloroplasts [92, 96]. In the Hsp70 family of proteins, in addition to the inducible Hsp70 form, there is a constitutively expressed form, the heat shock cognate protein 70 (Hsc70), which has 85% identity with human Hsp70 and binds to nascent polypeptides to facilitate correct folding.

Molecular chaperones have evolved to protect proteins from misfolding and aggregation regardless of their classification as inducible or constitutively expressed. One important feature of chaperones is that, although they assist the non-covalent folding/unfolding and the assembly/disassembly of other macromolecular structures, they do not occur in these structures when the latter are performing their normal biological functions. Generally, molecular chaperones have no effect on a protein’s folding rate. Of course, apparent folding and assembly rates can be increased by the elimination of non-productive oligomeraggregate formation. Furthermore, by binding to partially folded species and preventing their aggregation, chaperones increase the yield of functional folded/assembled proteins. However, these actions do not affect intramolecular folding rates. On the other hand, there is a last class of protein-helpers, which assist protein folding and are not present in the final folded/assembled functional form of a protein-substrate. Therefore, these helpers, known as foldases, belong to the family of chaperones. Contrary to the typical chaperones considered so far, foldases have evolved to catalyze the folding process by directly accelerating the protein folding rate-limiting steps. Well-known foldases include eukaryotic protein disulfide isomerase [97-99], peptidyl-prolyl cis/trans isomerase [99, 100], and lipase-specific foldases, Lifs, found in the periplasm of Gram-negative bacteria [99, 101].

Earlier, the prevalence of functional regions without a well-defined 3-D structure in RNA and protein chaperones was emphasized [73]. The analysis revealed a high proportion of predicted IDRss in chaperones, with 54% of residues of RNA chaperones falling into IDRs and with 40% of their
residues being located within the disordered regions longer than 30 consecutive residues. Intrinsic disorder was also found to be abundant in protein chaperones, for which corresponding values were 37% and 15%, respectively [73]. Based on the analysis of several individual cases it has been concluded that the IDR's in chaperones might function as molecular recognition elements solubilizing or locally loosening the structure of the kinetically trapped folding intermediate via transient binding to facilitate its conformational search. An "entropy transfer" model was proposed to account for the mechanistic role of structural disorder in chaperone function. According to this model, the binding of the disordered chaperone to the misfolded substrate was proposed to be accompanied by ordering of the chaperone with a concomitant unfolding of the substrate [73].

Recently, it was confirmed that intrinsic disorder is highly abundant in protein chaperones and plays a number of important roles in the action of these intricate machines. IDR's determine the promiscuity of chaperones, act as pliable molecular recognition elements, wrap misfolded chains and participate in disaggregation and local unfolding of the aggregated and misfolded species. The functions of chaperones, co-chaperones, and decorating proteins are precisely orchestrated. These proteins often act as large chaperone machines. They communicate with each other and form sophisticated chaperone networks. Protein intrinsic disorder plays a crucial role in the coordination and regulation of these chaperone machines and networks, thus helping to form a flexible net of malleable guardians [66].

THE HEAT SHOCK RESPONSE

The heat shock response is the cell's molecular reaction to protein damaging stress and results in the upregulation of heat shock proteins including molecular chaperones (reviewed in [102]). This response is conserved from prokaryotes to eukaryotes and allows an organism to respond to proteotoxic stressors, including heat shock, oxidative stress, heavy metals, and drugs, as well as cell growth and developmental conditions [103]. Pathology, such as inflammation or infection, may also induce the heat shock response, in addition to protein conformational diseases, such as Alzheimer's disease [103]. Upon initiation of the heat shock response, heat shock transcription factors (HSF's) are activated through trimerization and post-translational modifications [102]. A single, essential HSF1 gene exists in Saccharomyces cerevisiae and in Drosophila melanogaster, where this gene is responsible for both constitutive and heat-inducible heat shock gene expression [104-106]. Vertebrate species express a family of HSF proteins with diverse cellular roles.

HSF1 - THE MASTER HEAT SHOCK RESPONSE REGULATOR

HSF1 is the HSF family member responsible for stress-induced expression of heat shock proteins. Once the heat shock response is induced, HSF1 trimerizes, accumulates in the nucleus, and binds the heat shock elements (HSE's) located in the promoter regions of hsp genes Fig. (1). The HSEs consists of several adjacent and inverted repeats of the pentamer sequence nGAAn [107]. Activation of transcription occurs upon HSF1 hyperphosphorylation [108].
Attenuation of the heat shock response is important because the overexpression of HSPs is deleterious to the cell. Attenuation is regulated by a dual mechanism involving negative feedback inhibition from HSPs [109] and acetylation at a critical lysine residue within the DNA binding domain of HSF1 causing loss of affinity for DNA [110]. SIRT1 is a NAD-dependent histone deacetylase (i.e., the deacetylase, which splits NAD during each deacetylation cycle) that deacetylates HSF1, thus promoting stress-induced HSF1 DNA binding ability and increased HSP expression [110]. The end result of the heat shock response is the upregulation of HSPs, thus promoting the cell’s ability to cope with denaturing stress.

![Diagram of HSF1 activity cycle](image)

**Fig. 1.** The HSF1 activity cycle. HSF1 in unstressed cells is a monomer in both the cytoplasm and nucleus. Following heat shock, HSF1 accumulates in the nucleus in a trimeric form and is capable of binding to heat shock elements (HSE). Transcriptional activity requires hyperphosphorylation of HSF1 by various kinases. Attenuation of the cycle involves negative feedback by chaperones as well as acetylation of a conserved lysine within the DNA binding domain.

**DOMAINS OF HSF1**

Similar to many eukaryotic TFs, HSF1 possesses a complex modular structure with several functional domains shown schematically in Fig. (2a). These functional domains of HSF1 have been mapped by mutational analyses [106, 111-113]. Fig. (2b) represents the distribution of intrinsic disorder propensity within the human HSF1 sequence as estimated by two established disorder...
predictors, PONDR® VLXT (red line) and PONDR-FIT (black line). According to this analysis, more than half of the protein is predicted to be disordered. In fact, only two of the HSF1 functional do-mains, the DNA-binding domain (DBD, residues 15-120) and the hydrophobic repeat HR-A/B domain (residues 130-203) are predicted to be ordered. Fig. (1) also shows that the HR region C (which is located at the beginning of the transactivation domain (TAD), residues 384-409) is predicted to be partially ordered. These two domains are connected by a flexible linker, and the remainder of the protein, including two important functional domains, the regulatory domain (RD, residues 221-310) and TAD (residues 371-529) and their linker, is mostly disordered.

DNA Binding Domain (DBD)

The DBD is the most conserved domain of HSF1 from Caenorhabditis elegans to humans as shown in Fig. (3). Both the crystal structure of the DBD for Kluyveromyces lactis HSF and the solution structure of the DBDs for K. lactis and D. melanogaster HSF1 show that the DBD is a helix-turn-helix motif [114-116].

The helix-turn-helix structure contains 3 alpha helices, 4 beta strands, and an exposed loop. Upon trimerization, the DBD of each HSF1 monomer recognizes an inverted nGAAn repeat of the HSE [117]. The domains make contact with the major groove and with the DNA phosphate back-bone by helix 3 of domain [118, 119]. Helix 3 is a region rich in positive charge and is the most conserved region of the DBD. Unlike other helix-turn-helix proteins, the loop in the HSF1 DBD does not bind DNA. Instead, the loop forms an interaction with the surface between adjacent HSF monomers [119].

The characteristic helix-turn-helix structure of the HSF1 DBD is shown in Figs. (4b, 4c, and 4e) representing both solution and crystal structures of DBDs from non-mammalian HSFs. Comparison of the crystal and NMR structures of K. lactis HSF (cf. Fig. (4b and 4c)) revealed that DBD preserved the overall topology in solution. In fact, both structures contained a 4-stranded antiparallel beta-sheet and a 3-helix bundle [114, 115]. Seeing in the crystal structure an irregular segment defined as an α-helical bulge in helix H2 was consistent with the NMR results. Furthermore, in the solution structure, the ill-defined loop connecting strands B3 and B4 was consistent with the results of the X-ray model, which showed larger B-factors (also known as the temperature factors or the Debye-Waller factors, which are used to describe the attenuation of X-ray scattering caused by thermal motion and therefore reflect the fluctuation of atoms about their average positions, providing important information about protein dynamics) in this region than in the rest of the structure, and the absence of electron density for residues 76-79 [114, 115]. These data are also consistent with the result of disorder prediction for the K. lactis HSF. In fact, Fig. (4a) clearly shows that the C-terminal part of the DBD is predicted to have significant amount of intrinsically disordered residues. Comparison of Fig. (4b, 4c and 4e) also shows that the overall structure of the DBD is conserved between K. lactis and D. melanogaster, but the DBD of the D. melanogaster HSF is a bit less flexible in solution in comparison with the domain from K. lactis.
Once again, this is consistent with the results of disorder predictions for these two domains, since according to Fig. (4a and 4d), *D. melanogaster*’s DBD is expected to have less disorder propensity than its homologue from the yeast.

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

**Trimerization Domains**

The HSF1 trimerization domain is composed of three arrays of hydrophobic heptad repeats (HR-A/B) [113]. HSF1 trimerization is unusual in that when the monomers come together, they form a leucine zipper, an artifact typically seen in dimerization. Trimerization is constitutive and is actively suppressed by the hydrophobic heptad repeat HR-C [120, 121]. When a mutation is introduced into the HR-C domain, trimerization and binding to the DNA is able to persist. *S. cerevisiae* and *K. lactis* HSFs have constitutive DNA-binding activity. In these HSFs, the HR-C is poorly conserved and does not actively suppress trimerization. Mutating the HR-C does not affect the oligomerization status of HSF in these organisms [122].
Transactivation Domains (TAD)

Transcriptional elongation of *hsp* genes is regulated by the transactivation domains (TAD) of HSF1. In the absence of HSF1, RNA polymerase pauses at 46-49 bases downstream from the transcription start site even in the presence of excess free nucleotides [123]. The HSF1 transactivation domain can be divided into two distinct regions rich in hydrophobic and acidic residues: activation domain I and activation domain II, spanning amino acids 401-420 and 431-529, respectively [111, 124, 125]. These domains have been shown to equivalently stimulate both initiation and elongation of transcription [126]. This redundancy may be in effect due to the importance of heat shock proteins in maintaining protein homeostasis.
Fig. (4). Structural characterization of the DBDs from *Kluyveromyces lactis* (plots (a), (b) and (c)) and from *Drosophila melanogaster* (plots (d) and (e)). (a) and (d) Intrinsic disorder propensity evaluated by PONDR® VLXT (red lines) [190, 191] and PONDR-FIT (blue lines) [192]. Cyan shadow represents standard errors of disorder prediction by PONDR-FIT. DBDs are shown as shaded gray areas. (b) and (c) Crystal and solution structures of DBD of the *K. lactis* HSF, respectively. (e) Solution structure of the *D. melanogaster* HSF1 DBD.

**Regulatory Domain (RD)**

The regulatory activity of HSF1 is located between amino acids 221 and 310 and is functionally conserved between mammalian *hsf1* genes, but not between other members of the HSF family [111]. Upon non-stress conditions in which protein homeostasis is preserved, the transactivator activity of TAD I and TAD II is repressed by the regulatory domain and an exposure to stress leads to a reversal of this repression [111, 112]. The regulatory domain of HSF1 contains several serine residues that are responsive to protein kinases and lead to repression of the TADs upon phosphorylation. Phosphorylation by the MAP kinase ERK is responsible for this transactivator repression and mutation of these phosphorylation sites abolishes the suppression of the transactivation domains even in the absence of stress [127].

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CONSERVATION OF HSF ACROSS DIFFERENT SPECIES

Fig. (3) represents alignment of amino acid sequences of HSF from different species and clearly shows that the DBD and HR-A/B domains are highly conserved during evolution, whereas other functional domains of HSFs are much less conserved. Interestingly, two non-vertebrate HSFs possess long and poorly conserved N-terminal extensions. Fig. (5) shows the conservation of the distribution of intrinsic disorder propensity in the HSF family. In agreement with sequence alignment data, Fig. (5) shows that there is a great conservation of order propensity in the N-terminal parts of HSFs; i.e., in their DBDs and HR-A/B domains, with evolutionarily more advanced organisms typically showing very similar disorder patterns in this region as shown in Fig. (5a) and Fig. (5b). On the contrary, the C-terminal parts of these proteins generally do not show noticeable conservation except for the fact that all of them are predicted to be highly disordered. On the other hand, Fig. (5c) shows that the C-terminal fragments of these HSFs possessed relatively similar amino acid compositions, and also emphasizes that these regions, in general, have some features of typical disordered proteins, being depleted in major order-promoting residues and showing enrichment in major disorder-promoting residues.

ALTERNATIVE SPLICING OF HSF1

In mammalian cells, there are two distinct HSF1 mRNA isoforms that are produced via alternative splicing of the HSF1 pre-mRNA [128]. For example, in mouse, the two HSF1 mRNA isoforms differ by a single 66 bp exon of the HSF1 gene, which is present in the HSF1-α mRNA isoform but is skipped in the HSF1-β mRNA isoform. The extra 22 amino acid sequence in the HSF1-α isoform is inserted immediately adjacent to a C-terminal leucine zipper motif [128]. The levels of the two HSF1 isoforms were shown to be regulated in a tissue dependent manner, with testis expressing higher levels of the HSF1-β isoform, and with heart and brain expressing higher levels of the HSF1-α isoform [128]. In human, the shorter alternatively spliced isoform of HSF1 is different from the canonical isoform by lacking the last 40 residues and by changing the canonical residues GKQLVHYTAQFLDLPGSVDTG5NGLG6LP462-489 to the alternative sequence AGALHSAAAVPGPPLRQRH5EQRPAAGA562-489. Earlier, it has been pointed out that alternative splicing occurs mostly in regions of RNA that code for the disordered protein regions [129]. In agreement with these earlier findings, Fig. (6) clearly shows that in both mouse and human HSF1 proteins, the regions affected by alternative splicing are predicted to be mostly disordered.

POST-TRANSLATIONAL MODIFICATIONS REGULATE HSF1 TRANSCRIPTIONAL ACTIVITY

HSF1 is extensively modified by a range of post-translational modifications including phosphorylation, sumoylation and acetylation Figs. (1c and 7). This extensive modification may form a code to allow fine-tuning of the activity of HSF1 to the precise needs of the cell.

Phosphorylation
HSF is phosphorylated at several residues in order to promote or inhibit transactivation. HSF was first reported to display a reduction in electrophoretic mobility as a result of phosphorylation in S. cerevisiae [105]. Mammalian HSF1 also exhibits a similar electrophoretic shift due to hyper-phosphorylation upon activation of the heat shock response. Phosphorylation regulates transcriptional activity and not DNA binding, as some inducers of heat shock, such as the anti-inflammatory agent sodium salicylate, are capable of inducing HSF1 to bind to the DNA, but do not cause hyper-phosphorylation [130]. Another example of the distinction between these two activities is observed in yeast. In S. cerevisiae, HSF exists as a trimer with constitutive DNA-binding activity. However, transcriptional activation only occurs in the presence of increased phosphorylation [105, 131, 132].

Through phosphoamino acid analyses and mass spectrometry, several phosphorylation sites within HSF1 have been mapped. While phosphorylation has been primarily mapped to serine residues, a low level of threonine phosphorylation has also been detected [108, 127, 133-135]. Of the first phosphorylation sites to be characterized are serine residues S303, S307, and S363 located within the C-terminal region of the regulatory domain.

These sites are proline-directed and typically experience phosphorylation in the absence of stress. Upon stress, the repressive activity of these residues is superseded by the activation of HSF1 [108, 127, 136]. An HSF1 phosphorylation site that leads to transcriptional activation is serine residue 230, located within the N-terminal region of the regulatory domain [135]. Many sites within HSF1 can be phosphorylated, and a mass spectrometry analysis of HSF1 activated by heat shock found phosphorylation on Ser121, Ser230, Ser292, Ser303, Ser307, Ser314, Ser319, Ser326, Ser344, Ser363, Ser419, and Ser444 [137]. Phosphorylation of Ser326, but none of the other serine residues, was found to contribute significantly to activation of HSF1 upon stress in this study [137]. While the implications of HSF1 phosphorylation have yet to be fully characterized, it is clear that dynamic phosphorylation modulates HSF1 activity in both a positive and negative manner.

HSF1 phosphorylation is regulated by a number of kinases with several of the residues suspected to undergo modification by more than one kinase. MAP kinases have been shown to negatively regulate HSF1 activity and the HSF1 serine residues S303, 307 and 363 have been found to be MAP kinase targets. The MAP kinase ERK1 has been shown to phosphorylate S307, after which S303 is receptive to phosphorylation by glycogen synthase-3 kinase (GSK-3) [108, 127, 136]. The MAP kinase p38 has also been reported to phosphorylate S303 and/or S307, resulting in basal repression of HSF1 [138]. JNK, another MAP kinase family member, and the PKC isoforms alpha and zeta have been suggested to phosphorylate S363, thereby contributing to the maintenance of HSF1 in an inert state [139, 140]. Inhibition has also been shown to occur through the phosphorylation of S121 by the proinflammatory protein kinase MK2, which may lead to an increase in affinity for Hap90 [141].
Fig. (5). Conservation of intrinsic disorder in HSFs from different species. (a) PONDR-FIT plots for the evolutionary more-advanced organisms. (b) PONDR-FIT profiles of the evolutionary less-advanced organisms. (c) Compositional profiling of the C-terminal fragments of HSFs. The bar for a given amino acid represents the fractional difference in composition between a given protein and a set of ordered proteins. The fractional difference is calculated as $(C_X - C_{ordered})/C_{ordered}$, where $C_X$ is the content of a given amino acid in a given protein, and $C_{ordered}$ is the corresponding content in a set of ordered reference proteins and plotted for each amino acid [14, 193]. The amino acid residues are arranged according to the abundance of residues in a set of well-characterized IDPs from the DistProt database [194, 195]. Negative values indicate residues that a given protein has less than the reference set, positive values correspond to residues that are more abundant in a given protein in comparison with the reference set.
Fig. (6). Effect of alternative splicing on disorder profiles of the C-terminal regions of mouse (a) and human (b) HSF1 proteins. Propensity for intrinsic disorder was evaluated by PONDR-FIT for canonical (red) and short (blue) isoforms of these proteins. Standard errors of disorder predictions for long and short forms are shown as red and cyan shadows, respectively.

Various kinases suspected of activating HSF1 have also been discovered. For instance, the Ca2+/calmodulin-dependent kinase II (CaMK II) phosphorylates S230 and promotes transcriptional activity [135]. Given the broad range of stimuli that induce the heat shock response and the vast number of HSF1 phosphorylation sites, it may be that different kinases regulate HSF1 depending on the nature of the stress. As an example, in yeast Snf1 protein kinase has been shown to regulate HSF1 in low glucose conditions, but does not phosphorylate HSF upon heat shock [142].

SUMOylation

Upon stress, HSF1 is modified by SUMO-1 at the well-conserved lysine residue 298 within the N-terminal region of the regulatory domain [143]. In order for K298 to become sumoylated, S303 must first undergo phosphorylation, which has a repressive effect on HSF1 activation [143].
may be a regulatory interplay of modifications at this residue as residue K298 is also an acetylation site [110].

**Fig. (7).** Post-translational modification sites for HSF1 are indicated. In addition to these sites, additional phosphorylation sites include serine residues 97, 230, 292, 314, 319, 344, and 363 [137]. While K80 is a critical lysine residue that when acetylated will cause a loss of affinity for DNA, other uncharacterized sites for acetylation include lysine residues 116, 118, 126, 148, 157, 208, 224, and 298 [110].

**Acetylation**

While the kinetics of phosphorylation and sumoylation are brought on rapidly upon stress, acetylation is delayed and coincides with a reduction of HSF1 DNA-binding activity at the decline of the heat shock response [110]. Acetylation of HSF1 by the histone acetyltransferase p300 occurs to attenuate HSF1 off the DNA. The deacetylation of HSF1 is regulated by the NAD-dependent deacetylase SIRT1. When SIRT1 is inhibited by siRNA, not only are the levels of heat shock-induced hsp70 mRNA significantly decreased, but the ability of HSF1 to bind to the hsp70 promoter region is also greatly reduced [110].

**A Correlation between Intrinsic Disorder and PTMs**

In the past, a correlation between intrinsic disorder and PTM sites was found. In fact, in a study of the functions associated with more than 100 long disordered regions, many were found to contain sites of protein posttranslational modifications (PTMs) [144, 145]. These PTMs included phosphorylation, acetylation, fatty acid acylation, methylation, glycosylation, ubiquitination, and ADP-ribosylation suggesting the possibility that protein modifications commonly occur in regions of disorder. A particular advantage of disorder for regulatory and signaling regions is that changes, such as protein modification, lead to large-scale disorder-to-order structural transitions: such large-
scale structural changes are not subtle and so could be an advantage for signaling and regulation as compared to the much smaller changes that would be expected from the decoration of an ordered protein structure.

Protein phosphorylation and dephosphorylation are crucial for signaling. Indeed, about one-third of eukaryotic proteins are phosphorylated [146]. Many sites of protein phosphorylation were found to be in regions structurally characterized as intrinsically disordered [144, 145]. This conclusion was based on several lines of evidence, such as: very small number of PDB structures for both the unphosphorylated and phosphorylated forms of the same protein [147, 148]; the fact that the residues of the phosphorylation site often have extended, irregular conformation consistent with disordered structure [148]; the fact that the segments containing phosphorylation site not only lack secondary structure but are held in place by side chain burial and also by backbone hydrogen bonds to the surrounding kinase side chains [149-154]; the fact that regions flanking the sites of phosphorylation are enriched in the disorder-promoting amino acids [148]; the fact that the sequence complexity distribution of the residues flanking phosphorylation sites matches almost exactly the complexity distribution obtained for IDPs [148]; and the fact there is a high correspondence between the prediction of disorder and the occurrence of phosphorylation [148].

Ubiquitination, the reversible modification of proteins by the covalent attachment of ubiquitin, is implicated in the regulation of a variety of cellular processes and is involved in many diseases. Recently, 141 new ubiquitination sites were identified using a combination of liquid chromatography, mass spectrometry, and mutant yeast strains [155]. The detailed analysis of the sequence biases and structural preferences around known ubiquitination sites indicated that the properties of these sites were similar to those of IDPRs. In agreement with this computational study, structural information about the ubiquitination sites is sparse. In fact, despite the large size of PDB, only 7% of currently known ubiquitination sites in yeast could be confidently mapped to protein structures. The analysis of 3D structures of 32 homologous protein chains (with 15 of them being 100% identical with query proteins) containing 28 ubiquitination sites revealed that 10 ubiquitination sites were in crystal or interchain/intrachain contacts, and therefore the assignment of these sites to a specific structural element should be made with caution. Of the 18 sites that could be confidently assigned to ordered regions, 11 were located within coils (two of which were close to the observed disordered regions), four within helices, and three within strands. The majority of the sites within coils and helices were surface exposed and had high B-factor values indicating high flexibility [155]. The authors also pointed out that along with the lack of structural information for the majority of experimentally detected ubiquitination sites, there were several examples of ubiquitination sites located in the experimentally confirmed disordered regions [155]. Based on these observations it has been concluded that the involvement of flexible and disordered protein regions into various aspects of ubiquitination process provides a strong support for the functional importance of such regions. In addition to protease digestion, ubiquitination, and phosphorylation, several other types of PTMs, such as acetylation, fatty acid acylation, and methylation, have also been observed to occur in regions of intrinsic disorder [50, 145].
In agreement with all these earlier findings, Fig. (1c) shows that human HSF1 is not an exception and most of its experimentally verified PTM sites are located in the regions predicted to be disordered. From these findings, it is tempting to suggest that sites of protein modification in eukaryotic cells in general, and in HSF in particular, universally or at least very commonly exhibit a preference for intrinsically disordered regions. The likely explanation for this behavior is the fact that the modifying enzyme has to bind to and modify similar sites in a wide variety of proteins. If all the regions flanking these sites are disordered before binding to the modifying enzyme, it is easy to understand how a single enzyme could bind to and modify a wide variety of protein targets.

**HSF1 AS AN INTERACTION HUB**

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

During the resting state, chaperones including Hsp70 and Hsp90 contribute towards keeping HSF1 in an inactive state by preventing trimerization [109, 156-158]. Stress-induced activation of HSF1 may occur in part due to chaperones being titrated away from HSF1 by accumulating unfolded proteins, thus relieving the negative inhibition and allowing HSF1 to trimerize. In addition, heat actively promotes the trimerization of HSF1 through a heat-sensitive RNA called HSFR1, which has been proposed to act as a thermosensor [159]. Heat changes the conformation of HSFR1, and together with the translation elongation factor eEF1A, HSFR1 promotes the trimerization of HSF1 [159].

In order for HSF1 to be transcriptionally active, HSF1 must be hyperphosphorylated through transient interactions with the kinases described in the previous section. Hyper-phosphorylation may allow recruitment of basal transcription factors and coactivators required for transcription. HSF1 target promoters frequently contain a paused DNA polymerase that is poised to activate transcription rapidly upon stress. *D. melanogaster* HSF1 recruits elongation factors including pTEFb that allow release of the paused polymerase [160]. In addition, chromatin remodeling factors are recruited to target promoters by HSF1. The SWI/SNF chromatin-remodeling complex interacts with the activation domain of human HSF1, mediating the chromatin architecture changes required for proper Pol II elongation [161, 162]. *D. melanogaster* HSF1 also recruits the Mediator complex and the FACT complex to facilitate chromatin remodeling and promote transcription [163, 164]. Human HSF1 has been shown to interact with coactivators such as activating signal co-integrator 2 (ASC-2) to activate transcription on specific target promoters [165]. Interestingly, HSF1 is also linked to the recruitment of mRNA processing factors as evidenced by the binding of symplekin to human HSF1 [166]. Inhibition of this interaction inhibits hsp70 mRNA polyadenylation, suggesting that HSF1 is involved in cotranscriptional mRNA processing.
During the attenuation phase, HSF1 is under negative control by its own targets including Hsp70 and Hsp90. Hsp70 and its cochaperone Hdj1/Hsp40 bind to the activation domain of HSF1 trimers and interfere with its function [109, 156, 167]. HSF1 trimers also interact with the Hsp90-immunophilin (FKBP52)-p23 complex, resulting in a loss of transcriptional activity [168]. Additionally, acetylation of HSF1 at a conserved lysine residue within the DNA binding domain promotes this phase [110]. HSF1 can be acetylated by p300 and deacetylated by SIRT1 [110], so interactions with these factors have important implications in the regulation of the heat shock response. The unstructured regions of HSF1 likely facilitate the multitude of interactions and modifications that HSF1 participates in.

OTHER HEAT SHOCK TRANSCRIPTION FAMILY MEMBERS

The mammalian HSF family is composed of HSF1, 2, 3, 4, 5, Y and X [169]. While there is some redundancy, the HSF family members maintain different functionalities. Mammalian HSF1 is the ortholog of the single, essential HSF that is present in *S. cerevisiae* and *D. melanogaster*. HSF1 is the best-characterized member of the HSF family and the factor responsible for the heat shock response. HSF2 and HSF4, on the other hand, have various roles in development and differentiation [170]. Mammalian HSF3, 5, Y and X have not yet been well-characterized.

HSF2

HSF2 has been shown to recruit to satellite III repeats in nuclear stress bodies (NSBs) along with HSF1 [171]. At this colocalization site, HSF2 has been indicated to play a role in the heat shock response by modulating HSF1 in a hetero-complex formation [172]. HSF2 was originally discovered in tandem with HSF1 and similarly was shown to stimulate HSE-dependent transcription *in vitro* [173, 174]. While HSF2 is capable of inducing the transcription of heat shock proteins, it is not activated by heat and therefore has not been associated with the induction of the heat shock response [174, 175]. However, HSF2 has been shown to be involved in development and cellular differentiation [175-179].

Despite low amino acid sequence homology between HSF1 and HSF2, the DNA binding domain, as well as the N-terminal and C-terminal trimerization domains, are conserved. Fig. (8a) represents the distribution of disorder propensity within the human HSF2 sequence evaluated by PONDR® VLXT (red line) and PONDR-FIT (black line). Similar to human HSF1, more than a half of HSF2 is predicted to be disordered. Similar to HSF1, HSF2 possesses several ordered functional domains, DBD (residues 7-112), HR-A/B domain (residues 119-192), and HR-C (residues 360-385). The remainder of HSF2 is mostly disordered. Fig. (8b) compares PONDR® VLXT predictions for HSF2 (red line) and HSF1 (blue line) and shows that despite relatively low sequence homology, these two proteins possess remarkably similar disorder profiles. However, the manner of DNA-binding differs between the two proteins. HSF2 appears to regulate a different set of target genes compared to HSF1 and also experiences variable expression patterns in different tissues and cell types [175, 179]. The DNA binding-specificity of HSF1 is determined by the loop within the DNA binding
domain. Chimeric HSF2 containing the HSF1 DBD loop is capable of binding to HSF1 target genes upon heat shock stress [180]. In addition, the transactivation activity of HSF2 is considerably weaker than HSF1, possibly as a result of a dispersed activation domain [181]. As mentioned previously, in addition to the role of HSF2 in development, evidence has indicated that HSF2 may act as a modulator of HSF1 transcription. While HSF2 has not been shown to directly regulate the heat shock response, HSF2 has been shown to interact with HSF1, is recruited to nuclear stress bodies along with HSF1, and has been shown to stimulate HSF1-mediated transcription upon heat shock stress [182].

![Graphs showing PONDR score changes](image)

**Fig. (8).** Evaluating the intrinsic disorder propensity of human HSF2. (a) Per residue intrinsic disorder propensities evaluated by PONDR® VXT (red line) and PONDR-FIT (black line). Gray shadow represents standard errors of disorder prediction by PONDR-FIT. Thick bars on the top of this plot represent localization of the major domains. (b) Conservation of intrinsic disorder in human HSF2 (red line) and HSF1 (blue line) as evaluated by PONDR® VXT.

**HSF4**

HSF4 exhibits limited sequence homology to the other HSF family members, but regions of similarity correspond to the DNA binding domain and the N-terminal hydrophobic repeats (HR-A/B). Like other HSF family members, HSF4 is able to bind to HSEs. However, when HSF4 is co-
transfected into Cos7 cells along with a transcriptional HSE-reporter construct, HSF4 is unable to induce transcription and is therefore not a typical activator for the transcription of ksp genes [183]. In addition, HSF4 lacks the cis-regulatory domain that represses HSF1 under non-stress conditions [111].

HSF4 has also been shown to be involved in the crosstalk with HSF1. Together, HSF1 and HSF4 are involved in the maintenance of sensory organs and are critical during lens development [179]. HSF4 expression is specific to the brain and lungs and has been indicated to play a role in lens development and quality control [184, 185]. Mutations of HSF4 have been shown to lead to cataractogenesis and the breakdown of the lens microarchitecture [186, 187]. Two HSF4 missense mutations have been identified by screening age-related cataract patients [188]. These mutations appear to have an effect on HSF4 DNA-binding to HSEs resulting in an under-expression of heat shock proteins in the lens, which consequently lead to an increase in protein aggregates that cause cataracts.

![Graph](image)

**Fig. (9).** Evaluating disorder propensity distribution in human HSF4 by PONDR-FIT for canonical (red) and alternatively spliced isoforms (blue). Standard errors of disorder predictions for long and short forms are shown as red and cyan shadows, respectively.

**Fig. (9)** represents disorder propensity distribution in human HSF4 evaluated by PONDR-FIT for both canonical and alternatively spliced isoforms. Similar to HSF1 and HSF2, HSF4 has two ordered functional domains, DBD (residues 17-122) and HR-A/B domain (residues 129-203). Contrarily to HSF1 and HSF2, the functional HR-C domain of HSF4 (residues 364-389) is predicted to be disordered. The C-terminal half of human HSF4 is predicted to be mostly disordered. Intriguingly, the 245-322 region of HSF4 known to be involved in functional interactions with DUSP26, MAPK1, and MAPK2 contains important phosphorylation site (S298)
and is predicted to be mostly disordered. Fig. (9) also shows that this functional intrinsically disordered region is affected by alternative splicing.

**HSF3, 5, X and Y**

HSF3 was first identified in chicken, where it is the HSF that is essential for activation of the heat shock response in this species [189]. Mammalian HSF3 has only recently been identified in mouse, and is capable of activating non-classical heat shock genes during heatshock by binding to the PDZ domain-containing 3 (Pdzk3) promoter [175]. Although sequences related to HSF3 have also been found in the orthologous region of the human genome, this sequence is thought to be a pseudogene as no transcripts corresponding to this gene have been found [169].

**Fig. (10).** Evaluating the intrinsic disorder propensity of human HSF3 (a), HSF5 (b), HSFY (c) and HSFX (d). Propensity for intrinsic disorder was evaluated by PONDRT-FIT for canonical (red) and short (blue) isoforms of these proteins. Alternatively spliced isoforms of HSFX were not described as of yet, whereas HSFY has two short alternatively spliced isoforms (shown by blue and green lines in plot (c)). Standard errors of disorder predictions for long and short forms are shown as red and cyan (and light green) shadows, respectively.
HSF5 has recently been discovered as part of a large gene characterization project [180] and has been identified as a potential transcription factor within the HSF family, but has not undergone characterization. HSFY and HSFX are the only members of the HSF family found on the sex chromosomes. HSFY is present on the Y chromosome as well as murine chromosome 2. It is predominantly expressed in the testes and may potentially have a role in spermatogenesis [181-183]. Even less has been characterized regarding HSFX, but both HSFs have been found to exist as two identical copies [183]. Fig. (10) shows disorder propensity distributions in sequences of HSF3, HSF5, HSF, Y, and HSFX Figs. (10a, 10b, 10c and 10d), respectively) evaluated by PONDR-FIT for both canonical and short alternatively spliced isoforms.

All four proteins are predicted to possess a significant number of long disordered regions. Of particular interest is the fact that HSFY and HSFX have rather similar disorder profiles, in both of which a long disordered N-terminal region (50-80 residues) is followed by an ordered domain (100-120 residues), a long disordered domain (100-120 residues), another ordered domain (40-60 residues), and a disordered C-terminal region. HSF3, HSF5, and HSFY are known to have alternatively spliced isoforms. Similar mouse and human HSF1 proteins, the regions of HSF3, HSF5, and HSFY affected by alternative splicing are predicted to be mostly disordered.

CONCLUDING REMARKS

The modern literature on structural properties and functions of heat shock transcription factors has been systematically analyzed with a major focus on potential roles of intrinsic disorder in structural and functional peculiarities of these proteins. Published data clearly show that all HSF proteins contain substantial amount of intrinsic disorder and that intrinsic disorder has a very broad range of functional implementations. In fact, all HSFs have biologically important disordered regions. Sites of post-translational modifications and domains affected by alternative splicing are located within the intrinsically disordered regions.

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Stress-inducibility of SIRT1 and its role in cytoprotection and cancer.

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Written by R. Raynes and S.D. Westerheide, with contribution from J. Brunquell. All figures were created by R. Raynes. See Appendix E for copyright permission. Much of the text has been formatted from the Introduction and Implications of this dissertation.
Abstract

Cells must continuously respond to stressful insults via the upregulation of cytoprotective pathways. The longevity factor and deacetylase SIRT1 plays a critical role in coordinating this cellular response to stress. SIRT1 activity and levels are regulated by cellular stressors, including metabolic, genotoxic, oxidative, and proteotoxic stress. As a stress sensor, SIRT1 impacts cell survival by deacetylating substrate proteins to drive the cell towards a cytoprotective pathway. Extreme stress conditions, however, can cause SIRT1 to lead cells down an apoptotic pathway instead. SIRT1 is frequently dysregulated in cancer cells and has been characterized to have a dual role as both an oncogene and a tumor suppressor, likely due to its pivotal function in regulating cytoprotection. Recently, the ability of SIRT1 to regulate HSF1-dependent induction of the heat shock response has highlighted another pathway through which SIRT1 can modulate cytoprotection. Activation of HSF1 results in the production of cytoprotective chaperones that can facilitate the transformed phenotype of cancer cells. In this review, we discuss the stress-dependent regulation of SIRT1. We highlight the role of SIRT1 in stress management and cytoprotection and emphasize SIRT1-dependent activation of HSF1 as a potential mechanism for cancer promotion.

An Introduction to Sirtuins

The sirtuin family is evolutionarily conserved from prokaryotes to eukaryotes (1). Sir2 (silent information regulator 2), the original sirtuin to be characterized, was first observed in yeast during an extensive screen for silencing factors (2). Sir2 is a Class III NAD$^+$-dependent histone deacetylase that causes transcriptional silencing due to the removal of histone acetyl groups resulting in the tighter packaging of chromatin (3). Mammalian cells contain seven sirtuin family members, SIRT1-7 [reviewed in (4, 5)]. The mechanism of sirtuin deacetylation is unique, in which the SIRT protein binds to a NAD$^+$ molecule, thus cleaving nicotinamide and transferring the acetyl group from a substrate protein onto the NAD$^+$ backbone (6). The result is a 1:1:1 ratio of nicotinamide, O-acetyl-ADP-ribose, and the deacetylated substrate protein as measured by HPLC quantification (6). In the absence of enzyme or acetylation of the substrate
protein, no ADP-ribose or nicotinamide is generated from available NAD\(^+\), thus establishing the necessity of NAD\(^+\) hydrolysis in the catalysis of SIRT1 deacetylation activity (6). Additionally, SIRT3, SIRT4, and SIRT6 possess ADP-ribosyltransferase activity capable of transferring the O-acetyl-ADP-ribose onto acceptor protein residues (5).

Interest in sirtuins has grown with their association with longevity. It was found that an extra copy of Sir2 transformed into yeast resulted in a reduction in recombination of ribosomal DNA and a subsequent 30% increase in lifespan of the yeast mother cell (7). The Sir2 homolog Sir2.1 was later discovered to extend lifespan in C. elegans, suggesting that Sir2 homologs and the NAD\(^+\)/NADH ratio may also be responsible for extending lifespan in higher eukaryotes such as mammals (8). While several studies have supported the role of Sir2 in longevity, recently the impact of sirtuins in fly and worm lifespan extension has been questioned due to confounding issues with genetic background (9). However, while new experiments using C. elegans with identical genetic backgrounds show that the original effect on lifespan by Sir2 overexpression was overestimated, there is still a 10-14% lifespan extension observed by increased Sir2 levels, thus continuing to support a role for SIRT1 as a longevity factor (10).

As might be expected for a factor that can extend lifespan, SIRT1 has been characterized to promote cytoprotection upon exposure to stressful conditions. Here, we review the regulation of SIRT1 activity and levels by stress and the role of SIRT1 in facilitating cytoprotection. We discuss the dual role of SIRT1 as both an oncogene and a tumor suppressor. Finally, we speculate on the role of SIRT1 in the induction of the heat shock response and molecular chaperones as a mechanism for cancer promotion.

**Regulation of SIRT1 activity by stress**

The deacetylase activity of SIRT1 has been well-documented in the regulation of several stress-induced transcription factors including p53 (11, 12), HSF1 (13-15), NF-κB (16-18), PGC-1α (19, 20), and the FOXO family of transcription factors (21-23). The activation of SIRT1 upon stress may therefore be an evolutionarily conserved process to
drive cellular homeostasis by invoking a variety of stress response pathways. As a critical stress sensor, it follows that SIRT1 activity and levels are modulated by multiple cellular stresses, thus allowing for the coordination of the appropriate cellular response.

**Regulation of SIRT1 activity by metabolic stress**

Sirtuins are linked to metabolism based on their unique ability to breakdown NAD\(^+\) during protein deacetylation, resulting in the formation of nicotinamide and O-acetyl-ADP-ribose (24). Therefore, a prime mode of SIRT1 activity regulation is through metabolic stress. SIRT1 depends on NAD\(^+\) as a critical cofactor for enzymatic activity, therefore indicating SIRT1 as an energy sensor. Changes in levels of NAD\(^+\), NADH, or their ratio, resulting from changes in diet or metabolic status, can lead to changes in SIRT1 activity.

Various enzymes in the NAD\(^+\) salvage pathway have also been implicated in the regulation of SIRT1 activity (Figure S10). Nicotinamide phosphoribosyltransferase (NAMPT) is an enzyme that converts nicotinamide to nicotinamide mononucleotide (NMN), which then reacts with ATP to regenerate NAD\(^+\) (25). Increased NAMPT activity has been shown to activate SIRT1 activity in human vascular smooth muscle cells (26). Interestingly, NAMPT is induced after some forms of stress including complete serum removal, thus linking metabolic stress pathways with other stress pathways (26). NAMPT levels decrease as cells age, and the resulting decline in NAD\(^+\) levels may contribute to the lowered SIRT1 activity observed upon aging (26).

In yeast, worms, and flies nicotinamide is recycled back to NAD\(^+\) in 4 steps that compose the NAD\(^+\) biogenesis pathway. The first step is nicotinamide catalysis by PNC1 to produce nicotinic acid. PNC1 is upregulated by environmental stressors, leading to increased stress resistance and lifespan in worms and flies (27-29). Thus, PNC1 can promote cell survival in response to environmental stress.

Caloric restriction (CR) is a 30-40% decrease in dietary intake with maintained nutrition that has been shown to increase longevity and protect against age-related disease (30). The life extension effects of CR were first established as early as 1935 using
calorically-restricted rats (31) and have now been extended to yeast (32, 33), nematodes (34, 35), flies (36, 37), and mice (38). In fasted mice, an increase in NAMPT activity correlates with an increase in NAD⁺ levels and enhances SIRT1 transcriptional activity (39). Interestingly, it has been reported that a 10-fold change in cellular NAD⁺ concentration is required to affect Sir2 activity in yeast (40). However, caloric restriction studies in yeast have shown that Sir2 activity is regulated by a reduction of NADH, a competitive inhibitor of Sir2 (41, 42). CR was found to increase the replicative lifespan of budding yeast by Sir2 activation due to decreasing the NADH levels resulting in an increase in the NAD⁺/NADH ratio (41). Furthermore, the deletion of Sir2 blocks the beneficial effects of CR on yeast lifespan (43).

AMP-activated protein kinase (AMPK), activated in response to increasing amounts of AMP, functions as an energy sensor that responds to cellular metabolic stress including CR (44). SIRT1 and AMPK are therefore two evolutionary conserved energy sensing molecules that are vital in the regulation of energy homeostasis, with SIRT1 sensing change to the NAD⁺/NADH ratio and AMPK sensing change in the AMP/ATP ratio (45). AMPK increases the NAD⁺/NADH ratio in skeletal muscle after exposure to an AMPK activator (AICAR) (45) proving AMPK to be an indirect activator of SIRT1. The role of AMPK as an energy sensor and regulator of metabolism led to the investigation of its function in the lifespan extension effects observed in a low glucose environment. A change in AMPK activity coupled with increased NAD⁺ levels was found 18-24 hours and 30-36 hours after glucose restriction, respectively, in skeletal muscle. This indicates that AMPK activity precedes the change in NAD⁺ levels in mammalian cells resulting in an increase in SIRT1 activity in a low glucose environment (46). Together, AMPK and SIRT1 may lead to improved fitness in C. elegans, as AMPK and SIRT1 homologs are important regulators of lifespan (47, 48).

Not only is SIRT1 regulated in part by metabolism, but SIRT1 is also required for the CR phenotype. CR produces complex behavioral changes in mammals, including increased physical activity and an increase in distance coverage, which is most likely associated with foraging behavior (49). Moreover, wild-type mice and SIRT1 knockout (KO) mice demonstrate vastly different phenotypes upon CR, with the SIRT1 KO mice exhibiting a
phenotype very similar to wild-type control mice that are not on a restricted diet (50). Recently, it was shown that CR can synergize with heat shock stress to promote increased thermotolerance and fitness in a sir2.1-dependent manner (51). sir2.1, the mammalian SIRT1 homolog, is also necessary for the cytoprotection conferred by CR and heat shock to preserve movement in a C. elegans polyglutamine neurodegenerative disease model (51). SIRT1, therefore, is an energy sensor that can coordinate the response to metabolic stress at both the cellular and the organismal level.

Regulation of SIRT1 activity by protein modulators

As SIRT1 is a critical coordinator of cellular stresses, including metabolic stress, its activity must be finely regulated. Two proteins that are known to function as SIRT1 modulators are Deleted in Breast Cancer 1 (DBC1) and Active Regulator of SIRT1 (AROS). DBC1 was initially cloned from a region homozygously deleted in 3.5% of breast cancers (52). DBC1 has been found to directly interact with SIRT1 and inhibit deacetylase activity both in vivo and in vitro (53). DBC1-mediated down-regulation of SIRT1 deacetylates p53 and inhibits p53 transcriptional activity. The repression of SIRT1 activity by DBC1 leads to an increase in p53 acetylation, and therefore an upregulation of p53-dependent apoptotic activity. Likewise, the RNA interference of DBC1 results in an increase of SIRT1-mediated deacetylation, thus inhibiting p53-dependent apoptosis (54). In a similar manner, DBC1 has also been shown to regulate the acetylation status of the SIRT1 targets PPARγ and HSF1 (14, 55). Interestingly, the DBC1-SIRT1 interaction increases following DNA damage and oxidative stress. The stress-induced DBC1-SIRT1 interaction requires ATM-dependent phosphorylation of DBC1 at Thr454, thus creating a second binding site for SIRT1 (56). Therefore, stressful conditions do not only activate SIRT1, but can also blunt SIRT1 activity, suggesting that the cell has the ability to fine-tune the control of this factor.

AROS, a small ribosomal binding protein, has been shown to interact with SIRT1 and modulate its activity. In contrast to DBC1, AROS increases the deacetylase function of SIRT1. AROS enhances SIRT1-mediated deacetylation of p53, thus inhibiting p53-mediated transcriptional activity (57). Recently, it was shown that AROS, like DBC1,
also has an impact on HSF1 acetylation status and the heat shock response (14). The AROS-SIRT1 interaction is likely another way in which the cell can fine-tune stress-induced SIRT1 activity. It will be interesting to investigate which cellular stresses can regulate the AROS-SIRT1 interaction.

Regulation of SIRT1 activity by stress-inducible post-translational modifications

SIRT1 is highly modified by post-translational modifications and these modifications are regulated by cellular stress. Mass spectrometry has identified 13 phosphorylation sites within SIRT1 (58). One of the kinases identified to phosphorylate SIRT1 is the c-Jun N-terminal kinase (JNK). JNK inducibly binds to and phosphorylates SIRT1 upon oxidative stress at Ser27, Ser47, and Thr530, resulting in increased nuclear localization of SIRT1 (59). JNK belongs to the mitogen-activated protein kinase family and is responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock (60). This could explain, in part, how these various stress stimuli lead to an increase in SIRT1 activity on its nuclear targets.

Mammalian target of rapamycin (mTOR) is another stress-inducible kinase that has been identified to phosphorylate SIRT1. mTOR phosphorylates SIRT1 on Ser47, thus inhibiting SIRT1 activity (61). mTOR is a key cellular sensor of nutrient and energy levels as well as redox status, and is activated by increased levels of insulin, growth factors, amino acids, and oxidative stress (62). SIRT1 physically interacts with the mTOR-Raptor complex, and a single amino acid substitution in the TOR signaling motif in SIRT1 prevents Ser47 phosphorylation (61). Under conditions of caloric restriction, mTOR activity is inhibited, thus leading to enhanced SIRT1 activity.

Casein Kinase II (CK2) is another stress-activated kinase implicated in SIRT1 regulation. Four CK2 phosphorylation sites have been identified in murine SIRT1 at Ser154, Ser649, Ser651, and Ser683 (63). Two of these sites are also phosphorylated in human SIRT1 at the corresponding amino acids Ser659 and Ser661 (64). Ser659 and Ser661 lie within the Essential for SIRT1 Activity (ESA) region of human SIRT1 (65). The ESA region interacts with the catalytic domain of SIRT1, thus activating its catalytic activity and increasing the affinity for SIRT1 substrates. Phosphorylation at Ser659 and Ser661 has
been proposed to enhance the interaction of the ESA region with the catalytic core. It is possible that this would affect DBC1 binding to SIRT1, as the binding site for ESA in the catalytic domain is also the binding site for DBC1 (53). Therefore, the control of SIRT1 function by its own C-terminal domain and the regulation of this interaction by CK2 may be an important regulatory mechanism for SIRT1 activity.

Aside from phosphorylation, SIRT1 can be modified by sumoylation, methylation, and transnitrosylation. SIRT1 is sumoylated at Lys734, and SUMO1/Sentrin-Specific Peptidase 1 (SENP1), a nuclear desumoylase, removes this modification (66). Sumoylation of SIRT1 increases its catalytic activity as measured by p53 deacetylation and desumoylation by SENP1 reduces its deacetylase activity (66). Stress-inducing agents, including UV radiation and hydrogen peroxide, were found to promote the association of SIRT1 with SENP1 (66). Therefore, certain stress-inducing agents may counteract the anti-apoptotic activity of SIRT1 by recruiting SENP1 to SIRT1, resulting in the desumoylation and inactivation of SIRT1.

SIRT1 is methylated via the methyltransferase Set7/9, which methylates SIRT1 at Lys233, Lys235, Lys236, and Lys238. SIRT1 interacts with Set7/9 both in vitro and in vivo, and the interaction is increased upon DNA damage. SIRT1 methylation may inhibit SIRT1 deacetylation activity, as DNA damage inhibits the interaction of SIRT1 and p53, leading to an increase in p53 acetylation levels (67).

SIRT1 has also been found to be transnitrosylated by nitrosylated GAPDH (68). Transnitrosylation of SIRT1 inhibits SIRT1 deacetylase activity, as measured by an increase in levels of acetylation of the SIRT1 target PGC-1α. Cys387 and Cys390 within the catalytic core of SIRT1 are targeted by nitrosylation. These cysteines are involved in the coordination of Zn2+ binding and nitrosylation of these sites may result in SIRT1 protein misfolding. Multiple types of post-translational modifications, therefore, are involved in fine-tuning SIRT1 activity in response to stress.
Regulation of SIRT1 levels by stress

Stress-responsive regulation of SIRT1 expression levels by transcription factors

In addition to regulation of SIRT1 at the activity level, SIRT1 can also be regulated at the expression level. SIRT1 expression is altered by various stress-inducible transcription factors including p53, c-Myc, FOXO family members, and certain PPAR family members. p53 is inducible by a myriad of stresses, including DNA damage and oxidative stress. p53 has been shown to regulate SIRT1 expression by binding to the sirt1 promoter at two binding sites to repress sirt1 mRNA expression (69). As p53 is a SIRT1 deacetylation target, this provides a feedback loop to regulate SIRT1 expression by modulating the acetylation status and thus the activity state of this protein. Consistent with a negative role for p53 in regulating SIRT1 expression, p53 null mice show increased basal expression of SIRT1 in certain tissues (70). The negative regulation of SIRT1 by p53 could also be an explanation for why SIRT1 levels can be higher in tumors that have lost p53.

The proto-oncogene c-Myc is activated through the mitogen-activated protein kinase (MAPK) pathway, and is responsive to various mitogenic and stress signals. c-Myc induces SIRT1 expression by binding to the sirt1 promoter and activating transcription (71). As SIRT1 deacetylates c-Myc, resulting in decreased c-Myc stability, a c-Myc-SIRT1 feedback loop likely exists that may be relevant in cancer.

FOXO transcription factors are SIRT1 targets that can also increase SIRT1 expression. FOXO1, which can be regulated by insulin levels and is thus under metabolic control, can increase SIRT1 expression by binding to FOXO1 binding sites in the sirt1 promoter (70, 72). SIRT1-mediated deacetylation of FOXO1 leads to an increase in the transcriptional activity of FOXO1, thus indicating a positive feedback loop between SIRT1 and FOXO1 (72). FOXO3, on the other hand, translocates into the nucleus in the absence of nutrients and interacts with p53, inhibiting its suppressive activity on SIRT1 transcription, and thus leading to increased SIRT1 levels (70). FOXO3 is also a deacetylase target of SIRT1, but unlike FOXO1, SIRT1 can either activate or inhibit the transcriptional activity of this factor depending on the circumstances (22).
Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that are also regulated by metabolic state. The three subtypes, PPARα, PPARγ, and PPARβ/δ, are expressed in multiple organs and regulate different physiological functions such as energy metabolism, insulin action, and inflammation. Fasting can induce PPARα activity in mice and induce SIRT1 expression through a PPARα binding site in the sirt1 promoter (39). While PPARα may not be a direct target of SIRT1, SIRT1 can regulate the activity of its coactivator PGC-1α, thus creating a positive feedback loop (73). PPARβ/δ can also induce SIRT1 expression by inducing Sp1 binding to the sirt1 promoter (74). PPARγ, in contrast to PPARα and PPARβ/δ, represses SIRT1 expression through direct interactions with the sirt1 promoter (55). As PPARγ is a deacetylation target of SIRT1, this suggests the presence of a negative feedback loop.

Hypermethylated in cancer 1 (HIC1) is a transcriptional repressor that can bind to the sirt1 promoter and negatively regulate sirt1 mRNA expression (75). The repressive effect of HIC1 is dependent on carboxyl-terminal-binding protein (CtBP), an NAD+/NADH redox sensor (76). Nutrient deprivation decreases the interaction of CtBP with HIC1, thus decreasing the repressor function of HIC1 and increasing SIRT1 expression (76). HIC1 is also a deacetylase target of SIRT1, thus providing another negative feedback loop mechanism (77). Therefore, multiple stress-responsive transcription factors can regulate SIRT1 levels and thus influence the ability of SIRT1 to deacetylate target proteins.

Stress-responsive regulation of SIRT1 expression levels by RNA stability and translation control

Regulation of RNA stability and translation are other ways in which stress can regulate SIRT1 levels. A stress-regulated mRNA binding protein, as well as specific miRNAs, have been demonstrated to regulate SIRT1 expression levels. HuR, a ubiquitously expressed mRNA binding protein, increases SIRT1 levels by binding to the 3’ UTR of p53 mRNA, thus stabilizing it (78). Oxidative stress triggers the dissociation of the HuR-SIRT1 mRNA complex, thereby promoting SIRT1 mRNA degradation, decreasing SIRT1 transcriptional levels, and lowering cell survival. The cell cycle checkpoint kinase
Chk2 is implicated in this process, as it is activated by hydrogen peroxide, interacts with HuR, and is predicted to phosphorylate HuR at residues Ser88, Ser100, and Thr118. Mutation of these residues reveals a complex pattern of HuR binding, with Ser100 appearing to be important for HuR dissociation from SIRT1 mRNA after hydrogen peroxide treatment (78).

Several stress-regulated miRNAs control SIRT1 expression by binding to sirt1 mRNA and suppressing translation or reducing mRNA stability. The first miRNA described to regulate SIRT1 was miR-34a (79). This miRNA was first discovered as a tumor suppressor in neuroblastoma (80). miR-34a is regulated by metabolism, as its levels were shown to increase in mice fed a high-fat diet (81). miR-195, also regulated by metabolism, has been shown to regulate SIRT1 expression in cardiomyocytes (82). miR-199a, induced by hypoxia, targets both SIRT1 and the hypoxia-inducible factor HIF-1α (83). Therefore, interactions with RNA binding proteins and miRNAs can influence SIRT1 expression levels and thus the cellular response to stress.

**SIRT1, stress, and cellular fitness**

SIRT1 can function in either a cytoprotective manner or a pro-apoptotic manner by activating stress response pathways through a number of factors including p53 (11), FOXO (22), HSF1 (13, 15), Nrf2 (84), and various DNA damage repair factors (85-91) (Figure S11). Under moderate stress, the result of modulation of these pathways is to promote metabolic homeostasis, prevent apoptosis, and repair oxidative cellular damage and damaged DNA. Under chronic or extreme stress conditions, however, SIRT1 can promote cell death by inducing apoptosis.

**SIRT1 promotes cytoprotection upon moderate stress**

Under moderate stress conditions, SIRT1 promotes cytoprotection via modulation of a number of cell survival regulators, including p53, FOXO, HSF1, NF-κB and DNA damage response proteins. p53 is a well-characterized substrate of SIRT1 that is critical in cell cycle checkpoint regulation, apoptosis, and tumor suppression (11, 12). p53 is transcriptionally activated by a number of protein kinases including ATM (92), ATR
The activity of p53 is mediated by its acetylation status, where deacetylation reduces activity, thus allowing cells to bypass p53-mediated apoptosis (75, 95, 96). SIRT1 null mice show that p53 is hyperacetylated when compared to wild-type mice (97), while overexpression of SIRT1 in cell lines suppressed p53-dependent apoptosis and increased cell survival after exposure to oxidative stress (12). In addition, the p53 family member p73 is also deacetylated by SIRT1, resulting in suppression of apoptosis (98).

The FOXO family of transcription factors has proven to be important in many biological functions, implicating it as an essential factor in cellular fitness and protection. These functions include checkpoint regulation (99) and detoxification (100). In response to oxidative or genotoxic stress, FOXO proteins translocate to the nucleus and activate target genes involved in cytoprotection and DNA repair (22, 101). FOXO interactions with SIRT1 are controlled during stress stimuli. Specifically, FOXO3 and SIRT1 have been shown to form a complex upon stress, allowing for SIRT1-mediated deacetylation of this transcription factor (22). FOXO3 deacetylation potentiates cell cycle arrest and induces the transcription of DNA repair target genes while simultaneously attenuating FOXO-mediated apoptosis, thus allowing the cell to avoid apoptosis and maintain stress resistance. Deacetylation of FOXO family members leads to an increase in DNA binding affinity of this transcription factor, resulting in the transcription of cytoprotective target genes (102, 103), thereby allowing for cytoprotection and cell survival (22, 104, 105).

The heat shock response regulator HSF1 has recently been established as a SIRT1 substrate (13, 15). The heat shock response is the cell’s cytoprotective response to protein damaging stress that results in the induction of molecular chaperones to restore proteostasis and promote cell survival. SIRT1 deacetylates HSF1 and thus promotes DNA binding (13). The interaction of SIRT1 with HSF1 has been shown to be induced by heat shock (15). Induction of the heat shock response by SIRT1, therefore, is another way to promote cytoprotection. The regulation of HSF1 by SIRT1 and implications in cancer will be discussed later in this review.
In addition to the regulation of transcription factor activity, SIRT1 has an established role in interactions with DNA damage response proteins. Deacetylation of the DNA repair proteins NBS1, Werner syndrome protein (WRN), xeroderma pigmentosum group A protein (XPA), and Ku70 by SIRT1 have all been shown to control genomic stability (86-91). The MRE11-RAD50-NBS1 (MRN) complex is a conserved nuclease complex that can sense damaged DNA and regulate the cellular response to DNA double-strand breaks (106). The regulatory subunit of this complex, NBS1, is mutated in the human genetic disease Nijmegen breakage syndrome, a chromosomal instability disorder that results in multiple symptoms including microcephaly, radiation sensitivity, and predisposition to lymphoid malignancy (107). SIRT1 maintains NBS1 in a hypoacetylated state, thus allowing it to be responsive to DNA damage-induced phosphorylation by the ATM kinase (91). Phosphorylation activates NBS1, leading to the initiation of DNA damage repair (91).

WRN, a RecQ DNA helicase family member, helps to maintain genome stability and is another target of SIRT1 (107). Werner syndrome, an autosomal recessive disorder associated with premature aging and cancer predisposition, is caused by mutations in the WRN gene (108). SIRT1 interacts with WRN in a manner that is enhanced after DNA damage (89). The acetylation of WRN decreases its helicase and exonuclease activities, while deacetylation by SIRT1 reverses this effect (89).

SIRT1 also interacts with XPA, a nucleotide excision repair (NER) factor that is essential for the NER process (109). The interaction occurs in a UV irradiation-inducible fashion (87). Complementation experiments using XPA null cells with an acetylation mimic of XPA shows that SIRT1-mediated deacetylation of XPA is required for optimal NER (87).

Ku70 is another DNA repair factor that can interact with SIRT1. Upon exposure to radiation, SIRT1 enhances both the DNA repair capacity of the cell and the deacetylation of Ku70 (88). Overexpression of SIRT1 increases the repair of DNA strand breaks induced by radiation, while SIRT1 siRNA decreases this repair activity (88). SIRT1 forms a complex with Ku70 leading to deacetylation, suggesting that SIRT1 may
modulate DNA repair activity through regulating the acetylation status of repair protein Ku70 following DNA damage (88).

Interestingly, in addition to its function in DNA repair, Ku70 is one of several proteins that inhibit apoptosis by sequestering the pro-apoptotic factor BAX, a BCL-2 family member, from the mitochondria (110). SIRT1 has been implicated in promoting cytoprotection through Ku70 deacetylation, thus strengthening its ability to sequester the pro-apoptotic BAX protein (86). Upon non-stress conditions, BAX is localized in the cytosol complexed with Ku70 (85, 111). Upon DNA damage-inducing stress, Ku70 becomes acetylated, which breaks the interaction with BAX, allowing it to translocate to the mitochondria (85). SIRT1, through deacetylation of Ku70, can strengthen the interaction of Ku70 with BAX, thus preventing apoptosis and promoting cytoprotection (85). Therefore, SIRT1 can lead to enhanced cytoprotection through deacetylating transcription factors, DNA repair factors, and a factor directly involved in preventing apoptosis.

**SIRT1 as a pro-apoptotic factor**

While SIRT1 has been well-established as a cytoprotective factor upon moderate stress, in certain cases SIRT1 can induce apoptosis. High levels of reactive oxygen species (ROS), for instance, can cause SIRT1 to activate apoptosis through a number of different routes including Nrf2, NF-κB, and p53-controlled pathways. The transcription factor Nuclear Factor Erythroid-derived 2-Related Factor 2 (Nrf2) is a central regulator of the cellular response to oxidative stress (112). High ROS levels cause Nrf2 to bind to antioxidant response elements (ARE) within promoter regions of phase II detoxification enzymes, allowing for upregulation of superoxide dismutase (SOD) and glutathione S-transferase (gst) genes, among other antioxidant gene targets (113). Acetylation of Nrf2 promotes DNA binding and target gene transcription, while deacetylation of Nrf2 disengages it from the ARE resulting in transcriptional termination (114). Therefore, SIRT1 is a negative regulator of Nrf2 and consequently down-regulates this adaptive response to oxidative stress (84).
NF-κB is another transcription factor that can be activated by ROS and other cellular stress conditions. As NF-κB transcriptionally upregulates a number of survival genes, such as the IAP family of apoptotic inhibitors, the over-activation of NF-κB is common in tumorigenesis and chemotherapy resistance [reviewed in (115)]. SIRT1 has been shown to form a complex with the RelA/p65 subunit of NF-κB, deacetylating Lys310 (116). This decreases NF-κB activity and down-regulates survival genes, thus resulting in increased apoptosis (116).

As a distinct mechanism to induce apoptosis at the mitochondrial level, high ROS can direct cytosolic p53 to induce apoptosis via the SIRT1-induced targeting of p53 to this organelle (117). In this pathway, deacetylated p53 binds to the outer-membrane of the mitochondria and outcompetes BAX for binding to BCL-2 proteins. The subsequent activation of BAX then leads to the release of cytochrome c from the mitochondria and the induction of apoptosis (118). SIRT1, therefore, has dual functions of both promoting cytoprotection and apoptosis, depending on the nature and severity of the stress.

**Activation of the heat shock response by SIRT1 and implications in cancer**

As certain conditions can cause SIRT1 to be cytoprotective while others can cause SIRT1 to induce cell death, SIRT1 has been implicated both as an oncogene and as a tumor suppressor [reviewed in (119, 120)]. Here a role for SIRT1 as an oncogene through activation of HSF1 and the cytoprotective heat shock response is explored.

**SIRT1-dependent regulation of HSF1**

As mentioned earlier, HSF1 has recently been established as a SIRT1 substrate (13, 15) and is the master regulator of the heat shock response, the cell’s cytoprotective molecular reaction to protein damaging stress (Figure S12). While the classic inducer of this adaptive stress response is heat shock, other stressors can also induce this response, including oxidative stress, heavy metals, and various pathophysiological states (121). The heat shock response results in the induction of molecular chaperone genes, called heat shock proteins (HSPs), which function to restore proteostasis and promote cell survival. In non-stressed cells HSF1 exists in a monomeric form. Upon stress, HSF1
forms trimers that accumulate in the nucleus and bind to heat shock elements in the promoters of target genes (122). Hyperphosphorylation leads to transcriptional activity and hsp genes are induced (123).

SIRT1 regulates the heat shock response through deacetylation of HSF1 (13). HSF1 can be acetylated at multiple sites, and the acetylation of Lys80 within the DNA-binding domain inhibits DNA binding ability (13). Activation of SIRT1 leads to the deacetylation of HSF1, resulting in prolonged binding of HSF1 to the hsp70 promoter and induction of the heat shock response. Conversely, down-regulation of SIRT1 promotes the attenuation of the heat shock response via increased HSF1 acetylation and decreased HSF1 DNA binding ability. Interestingly, the interaction of SIRT1 with HSF1 has been shown to be regulated by stress, as endogenous SIRT1 co-immunoprecipitates with HSF1 only upon heat shock in mouse embryonic fibroblasts (MEFs) (15).

The regulation of HSF1 and the heat shock response by SIRT1 has been shown to be biologically significant in a number of ways. As expected for a cytoprotective factor, the overexpression of SIRT1 was found to confer increased tolerance to high temperature heat shock in 293T cells (13). As HSF1 and SIRT1 are both aging factors, it is interesting to note that WI-38 human fibroblast cells at late passage numbers show a decreased heat shock response and reduced activation of HSF1 DNA binding by heat shock that correlates with a reduced abundance of SIRT1 (13).

SIRT1 has also been shown to work together with HSF1 to protect against α-synuclein pathology, an aging-related disorder (15). In this study, a transgenic mouse was used bearing the human α-synuclein gene with the A53T mutation, which causes familial early-onset Parkinson’s disease (15). When the A53T mice were crossed with SIRT1 transgenic mice, SIRT1 was found to prolong the lifespan of the mice and to decrease α-synuclein aggregates in the mouse brain. SIRT1 was found to deacetylate HSF1 and increase HSP70 levels in the brains of the A53T mice, but not in brains of mice not expressing α-synuclein A53T. Therefore, these combined results suggest that SIRT1 deacetylates HSF1 and activates chaperone expression only under stress conditions, including heat shock stress or aggregation-induced stress.
Association of HSPs and HSF1 with cancer

Heat shock proteins (HSPs), the transcriptional targets of HSF1, are elevated in a number of cells and tissues from a variety of cancers including prostate (124), lung (125), pancreas (126), bladder (127), and breast (128). HSPs comprise several distinct classes of molecular chaperones that maintain proper protein function in the cell by facilitating the folding, translocation, and proteolytic turnover of many critical regulators of cell growth and cell death (129-131). Multiple studies have shown the upregulation of HSPs in cancers and its association with increased aggression and lower survival rates (132-135). Consequently, elevated HSP expression is associated with poor prognosis and resistance to chemotherapy (136) and is often used as a biomarker for tumor progression and prognosis, though not all cancers (i.e. renal cell carcinoma) present this correlation (137). In particular, overexpression of HSP27, HSP70, and HSP90 seems to play crucial roles in cancer cell survival (138, 139). For instance, overexpression of HSP27 in rat colon adenocarcinoma cells injected into syngeneic animals increased tumorigenicity resulting in increased tumor size and a delay in tumor regression (140). Overexpression of HSP70 in Rat-1 fibroblasts can lead to reversible oncogenic transformation (141). In tumor cells, knockdown of HSP70 with antisense oligomers led to the induction of apoptosis and the inhibition of tumor cell proliferation (142). The inhibition of HSP90 by small molecule inhibitors has also shown promising anti-cancer results [reviewed in (143)]. These combined results suggest that tumors may take advantage of chaperone-based cell-survival mechanisms, thus allowing the cancer cells to proliferate.

The manner in which increased expression of HSPs promotes cancer is likely to occur through multiple cytoprotective mechanisms, including effects on maintaining proteostasis, preventing apoptosis, and facilitating growth-promoting signaling pathways. Rapidly proliferating tumor cells have increased dependency on protein folding for survival, due in part to an increase in expression of multiple proteins for oncogenic signaling pathways (144). In addition, the mutagenic nature of tumor cells results in an increase in polymorphic variants of essential proteins that may have folding difficulties, thus requiring increased assistance with protein folding for cell survival (143). HSPs,
through their effects of promoting protein folding and maintaining proteostasis, are therefore likely to assist cancer cells in this regard.

In addition to effects on proteostasis, HSPs can act at several points in the apoptotic pathway to inhibit apoptosis, including preventing cytochrome c release, regulating the apoptosome, and preventing caspase activation (139). HSP70 has been widely studied with regard to apoptosis, and has been shown to interfere with the assembly of the apoptosome by preventing the recruitment of procaspases 9 and 3 (145). The mechanism by which HSP70 is cytoprotective against apoptosis may also be through inhibitory binding and sequestration of pro-apoptotic proteins.

Facilitating growth-promoting signaling pathways is another key way in which HSPs may aid in tumor progression. HSP90 is highly relevant in this regard due to its oncogenic client proteins, including mutant p53, NF-κB, HER2, B-Raf and AKT (131). In fact, certain tumors have been found to be dependent on HSP90, and HSP90 inhibitors can selectivel target these cancer cells (146). The overexpression of multiple HSPs in cancer cells could therefore help promote cell survival and aid cancer progression through multiple mechanisms.

HSF1, the master regulator of chaperones, has itself been implicated in cancer in a number of experiments. For instance, HSF1 was found to promote Ras-induced transformation, as HSF1 null MEFs produced fewer Ras-induced foci than wild-type MEFs (147). HSF1 is also required for transformation induced by HER2 in MCF-10A cells (148) and was shown to cooperate with HER2 to promote mammary tumorigenesis and metastasis (149). In addition, HSF1 promotes lymphomas in p53-deficient mice (150). Oncogenesis has in fact recently been shown to be an inducer of the heat shock response due to activation of HSF1 through unknown mechanisms (151). It may be that the variety of stresses common to cancer, such as hypoxia, lack of nutrients, ATP depletion, and acidosis, combine to promote HSF1 activity. HSF1 genome occupancy in cancer cells was found to include genes involved in cell-cycle regulation, signaling, metabolism, adhesion and translation, as well as known hsp target genes (151). This set of gene targets could all be significant in promoting oncogenesis.
Implications of SIRT1-induced activation of the heat shock response in cancer

It is possible that the upregulation of SIRT1 observed in many cancers may facilitate HSF1 activation that is commonly observed in tumorigenesis. It is interesting that DBC1, the negative regulator of SIRT1, has been found to be homozygously deleted in certain breast cancers. DBC1 has been shown experimentally to inhibit HSF1 activity and the heat shock response (14). Therefore, deleting this inhibitor of SIRT1 may indirectly promote HSF1 activity in cancer as well as promote the deacetylation of p53, thus enabling cancer cell survival. Developing an understanding of how HSF1, SIRT1, and SIRT1 modulators influence cancer may lead to the discovery of new cancer therapies.

Final thoughts

The role of SIRT1 in the activation and regulation of stress response mechanisms may represent a vital evolutionarily conserved function in cellular homeostasis. A better understanding of the role of SIRT1 in stress may provide insight into diseases marked by a dysfunction in critical adaptive stress responses including cancer.

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We would like to thank the members of the Westerheide laboratory for helpful discussion over the course of this review.

Declaration of Conflicting Interests

The authors declare that there are no potential conflicts of interest with respect to the authorship and/or publication of this article.
Figure S10. Model of SIRT1-dependent deacetylation of substrate proteins.

SIRT1 binds to NAD$^+$ and an acetylated substrate protein in order to catalyze the transfer of the acetyl group to the NAD-backbone, thus resulting in the deacetylated substrate protein, nicotinamide (NAM), and O-acetyl-ADP-ribose in a 1:1:1 ratio. Upon triggering of the NAD$^+$ salvage pathway, nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the conversion of NAM to nicotinamide mononucleotide (NMN). NMN is then converted back to NAD$^+$ by nicotinamide mononucleotide adenylyltransferase 1-3 (NMNAT1-3). Caloric restriction has been shown to trigger the NAD$^+$ salvage pathway by activating NAMPT and AMPK promotes an intracellular increase in NAD$^+$ levels.
Figure S11. SIRT1 is activated and inhibited by stress-responsive factors, and plays a dynamic role in regulating cytoprotection, and apoptosis.

SIRT1 levels and activity are mediated by metabolism and interactions with various stress-responsive regulators. SIRT1 activation by caloric restriction, HuR, NAD⁺, and AROS influences the activity of several targeted factors including HSF1, nuclear p53, FOXO, and DNA repair proteins. Deacetylation of these substrate proteins results in a decrease of apoptosis and an increase in stress adaptation. Conversely, SIRT1-mediated deacetylation of Nrf2, NF-κB, and cytoplasmic p53 can lead to a pro-apoptotic environment due to increased reactive oxygen species and decreased p53 activity. These dynamic processes of SIRT1 may be inhibited by its interactions with DBC1, NAM, HIC-1, and miRNAs. (¹ nuclear, ² cytoplasmic).
HSF1 exists as an inactive monomer in the cytoplasm. Upon induction of the heat shock response by denaturing stress, it trimerizes and translocates to the nucleus where it binds to heat shock elements (HSEs) found in the promoter regions of HSP genes. Transcription of hsp genes is promoted by the hyperphosphorylation of HSF1, while the attenuation of the heat shock response is regulated by a dual mechanism involving negative feedback inhibition from HSPs and acetylation at a critical lysine residue within the DNA-binding domain of HSF1 causing loss of affinity for DNA. SIRT1 is a NAD$^+$-dependent HDAC that deacetylates HSF1, thus promoting stress-induced HSF1 DNA binding ability and increasing HSP expression. Protein modulators AROS and DBC1 have recently been shown to impact hsp70 transcription, HSF1 acetylation status, and HSR1 recruitment to the DNA (14).
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Appendix F. Protocols

In addition to the methods provided in each study, at the request of my committee (and in dedication to the members of the Westerheide lab) I have included my commonly used protocols in this dissertation.
Tissues culture and biochemistry protocols used in these studies

1. Counting cells, pg. 223
2. RNA isolation for tissue culture, pg. 224
3. Reverse transcription reaction, pg. 225
4. Quantitative PCR with sybr green + rox (cat # 172-5101), pg. 226
5. Western blot analysis, pg. 228
6. Flag pull-down using anti-flag m2 affinity gel beads (sigma f2426), pg. 231
7. Co-immunoprecipitation (co-IP), pg. 233
8. NAD/NADH quantification, pg. 234
9. Chromatin immunoprecipitation (CHIP), pg. 236
10. Protein expression using bl21(de3) competent E. coli (NEB, cat#c2527h), pg. 240
11. GST pull-down assay, pg. 241
12. Immunocytochemistry, pg. 244
1. COUNTING CELLS

☐ Aspirate off the old media from the plate and add half as much 1X PBS.
☐ Gently rotate the plate and aspirate off 1X PBS.
☐ Add Trypsin and gently swirl around the plate.  *(For a 60 mm plate, add 100 µL of Trypsin. For a 100 mm plate, add 250 µL of Trypsin.)*
☐ Swirl around the plate and place in 37°C CO2 incubator for about 5 min.
☐ Add media to the trypsinized plate (5 mL to a 60 mm plate; 10 mL to a 100 mm plate) and transfer the trypsinized cells to a 15 mL conical.
☐ Pipette 100 µL of cells and 100 µL trypan dye to a 1.5 mL tube and vortex briefly.
☐ Add 10 µL of trypan mixture to a hemocytometer slide.
☐ Count 2 boxes of 4x4 squares.

Figure S13. Counting cells using a hemocytometer.

☐ Determine the appropriate dilution to achieve the desired concentration of cells.
*If the total of the 2 boxes is 52, then the cell count is 0.52 \times 10^6 cells/mL. Use dimensional analysis to calculate the number of desired cells per well.*
☐ Transfer to a reservoir and pipet 100-200 µL to a 96 well plate.
☐ Let cells grow ON prior to transfection or drug exposure.
2. RNA ISOLATION FOR TISSUE CULTURE

Use appropriate sterile molecular techniques for RNA extraction. Pipets and bench should be cleaned with RNase Away and sterile filter pipet tips should be used.

- Add 1 mL Trizol reagent to previously harvested cell pellet (from a 100 mm plate of cells). *(If cell pellets are freshly collected, a freeze/thaw in the -80°C may aid in lysis.)*
- Pipet up and down until viscous.
- Place tubes on a nutator at RT for 5 min.
- Let sit at RT for 2-3 min.
- Add 200 µL of chloroform.
- Shake tubes vigorously for 10-15 sec. *(DO NOT VORTEX.)*
- Let sit at RT 2-3 min.
- Add 200 µL of chloroform.
- Centrifuge at 12,000 x g at 4°C for 15 min.
- Transfer the supernatant (500-550 µL) to a new tube. *(The RNA will be contained in the upper clear aqueous layer and is approximately 60% the volume of Trizol added.)*
- Add 500 µL of isopropanol and vortex briefly.
- Let sit at RT for 10 min. Precipitate at -80°C for 30 min. *(Alternatively, this can be a stopping point.)*
- Centrifuge at 12,000 x g at 4°C for 10 min. *(A white pellet will form.)*
- Aspirate the supernatant.
- Wash pellet with 1 mL cold 70% EtOH (made with RNase-free H₂O).
- Centrifuge at 9,000 x g at 4°C for 2 min.
- Aspirate, but leave about 200 µL. *(Do not touch the pellet! Remove the rest of the liquid with a P200 or stretched glass pipet!)*
- Invert tubes to dry, but be careful not to overdry.
- Depending on the size of pellet, resuspend in 15-30 µL of RNase-free H₂O.
3. REVERSE TRANSCRIPTION REACTION USING ABI’S HIGH CAPACITY REVERSE TRANSCRIPTION KIT (CAT # 4368814)

Table S4. Reverse transcription master mix recipe.

<table>
<thead>
<tr>
<th>Recipe</th>
<th>1 rxn</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X RT buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>25X dNTPs</td>
<td>0.8</td>
</tr>
<tr>
<td>10X RT random primers</td>
<td>2.0</td>
</tr>
<tr>
<td>RT enzyme</td>
<td>1.0</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>4.2</td>
</tr>
<tr>
<td><strong>Total=</strong></td>
<td><strong>10 µL</strong></td>
</tr>
</tbody>
</table>

- Make up MM in 0.1 or 0.2 mL tube. *(Make a half reaction more than needed.)*
- Transfer 10 µL of MM to 0.1 mL tubes.
- Add 10 µL of the appropriate RNA plus RNase-free dH$_2$O to all sample tubes. *(RNA should not exceed 2µg.)*
- Vortex and short spin.

Cycle conditions:
1. 25°C for 10 min
2. 37°C for 120 min
3. 85°C for 5 min
4. 4°C for $\infty$
4. QUANTITATIVE PCR WITH SYBR GREEN + ROX (CAT # 172-5101)

- Make a working stock of 50 ng/µL for your template DNA. *(For ChIP experiments, it is usually best to not dilute the template due to the low yields of most ChIP DNA.)*
- Add 35 µL of ROX (cat # 172-5858) to 1 mL of iTaq fast SYBR Green Mix before starting.

**Table S5. Quantitative PCR master mix recipe.**

<table>
<thead>
<tr>
<th>Recipe</th>
<th>1 rxn (1 well)</th>
<th>3.3 rxn (3 wells)</th>
<th>x 7 lanes (pre-MM)^</th>
<th>x ___ lanes (pre-MM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR green (with ROX)</td>
<td>10.0</td>
<td>33.0</td>
<td>231</td>
<td></td>
</tr>
<tr>
<td>F primer (0.9 µM)</td>
<td>0.2</td>
<td>0.66</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>R primer (0.9 µM)</td>
<td>0.2</td>
<td>0.66</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>DNA template (50 ng/µL)</td>
<td>1.0</td>
<td>3.3</td>
<td>(23.1)</td>
<td></td>
</tr>
<tr>
<td>dH₂O</td>
<td>8.6</td>
<td>28.38</td>
<td>198.7</td>
<td></td>
</tr>
<tr>
<td><strong>Total =</strong></td>
<td>20 µL</td>
<td>66 µL</td>
<td>462 µL</td>
<td></td>
</tr>
</tbody>
</table>

^One pre-MM for each primer set (i.e. GOI & endogenous control). There should be enough left over for a 20 µL negative control. Generally make 0.5-1 rxn more than needed to ensure precision pipetting.

*Primer amounts are based on 10 µM working stocks.

- Make up a pre-MM in a 1.5 mL tube. *(This is the reaction cocktail.)*
- Transfer 63 µL of the pre-MM to 0.5 mL tubes for each triplicate set.
- Add 3.3 µL of the appropriate cDNA or ChIP DNA to each tube.
- Vortex and short spin.
- Transfer 20 µL to three vertical wells to obtain triplicates.
- Cover plate with adhesive and short spin. Seal the plate well to avoid evaporation.
- Set cycle parameters:
  - 95°C for 45 sec
  - 95°C for 3 sec
  - 55-60°C for 30 sec**
  - x 35-45 cycles
  - Perform melting curve according to instrument instructions

**Data collection. The annealing and extension phase is performed concurrently, which is why small amplicons of 80-200 bps are necessary when designing primers.
*Record the primers and temperature settings used. Use the 96 well plate template to record sample location.

A.  

<table>
<thead>
<tr>
<th>Primers</th>
<th>Temperature (°C)</th>
</tr>
</thead>
</table>

B.  

Figure S14. A) Recipe template for qPCR. B) Plate layout template for qPCR.

Tips:

*The SYBR green mix can be very viscous. It is best to avoid mixing by pipetting up and down so as not to lose volume. Due to the viscosity, it is good practice to pause 3 or so seconds before going to the second stop while pipetting so that all the volume can be expelled from the tip.

*When pipetting 20 µL into the plate, it is best to NOT go to the second stop in order to avoid a possible bubble that may interfere with data collection.
5. WESTERN BLOT ANALYSIS

Making the gels:

☐ Make up the separating gel and stacking gel mix. Do not add TEMED or 10% APS until each gel is ready to be poured.

Table S6. SDS-PAGE separating and stacking gel recipes by percent acrylamide.

<table>
<thead>
<tr>
<th>Component</th>
<th>6%</th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
<th>15%</th>
<th>Component</th>
<th>4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M Tris (pH 8.8)</td>
<td>2.5 mL</td>
<td>2.5 mL</td>
<td>2.5 mL</td>
<td>2.5 mL</td>
<td>2.5 mL</td>
<td>1 M Tris (pH 6.8)</td>
<td>1.25 mL</td>
</tr>
<tr>
<td>40% Acrylamide</td>
<td>1.5 mL</td>
<td>2.0 mL</td>
<td>2.5 mL</td>
<td>3.0 mL</td>
<td>3.8 mL</td>
<td>40% Acrylamide</td>
<td>1.25 mL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 µL</td>
<td>100 µL</td>
<td>100 µL</td>
<td>100 µL</td>
<td>100 µL</td>
<td>10% SDS</td>
<td>100 µL</td>
</tr>
<tr>
<td>10% APS</td>
<td>100 µL</td>
<td>100 µL</td>
<td>100 µL</td>
<td>100 µL</td>
<td>100 µL</td>
<td>10% APS*</td>
<td>100 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>4 µL</td>
<td>4 µL</td>
<td>4 µL</td>
<td>4 µL</td>
<td>4 µL</td>
<td>TEMED*</td>
<td>10 µL</td>
</tr>
<tr>
<td>dH₂O</td>
<td>5.8 mL</td>
<td>5.3 mL</td>
<td>4.8 mL</td>
<td>4.3 mL</td>
<td>3.5 mL</td>
<td>dH₂O</td>
<td>7.3 mL</td>
</tr>
</tbody>
</table>

kDa Range: 60-200 60-70 12-45

☐ Cast the separating gel by pipetting into the Biorad gel apparatus using a P1000 and quickly overlay with 1 mL of hydrated Butanol. (Leave ¼ space for the stacking gel.)

☐ Let sit for 30 min.

☐ After polymerization, remove the Butanol overlay via gravity and rinse with dH₂O.

☐ Make up the stacking gel and pipet over the separating gel.

☐ Insert the comb and let sit to polymerize for at least 30 min.

☐ Place the gel in the electrophoresis chamber, cover with running buffer, remove the comb, and rinse the wells with buffer to remove unpolymerized acrylamide.

Preparing the samples:

☐ Add Laemmli buffer to aliquoted samples.

☐ Incubate the sample at 95°C for 5 minutes to denature the proteins.

☐ Mix by vortexing, short spin, and load onto the gel. Load from left to right.

☐ Add Laemmli buffer into all empty wells for an even dye front

☐ Run the gel at 180V until the dye front has emerged from the gel (about 1 hour).

(If smaller kDa proteins are being analyzed, the gel may be stopped before the dye front has run off.)

Blotting:

☐ Remove the stacking layer from the gel.
☐ Activate a PVDF membrane in 100% methanol or wet a nitrocellulose membrane in a tray filled with dH₂O.

☐ Fill a large tray with transfer buffer and assemble the blot. (*Place the gel so that the marker is on the right. Use 1 piece of thick blotting paper or 2 pieces of thin blotting paper.*)

*Blotting paper*

*Membrane*

*Gel*

*Blotting paper*

☐ Flip the blot assembly over and place in a semi-dry blotter membrane-side down.

☐ Set to 300 mA. Blot for one hour. (*Add more time to transfer large kDa proteins or for multiple gels on the blotter.*)

☐ Stain with 0.1% Ponceau S (in 1% glacial acetic acid) for a few seconds in order to visualize protein bands.

☐ Rinse the blot with dH₂O to remove background staining.

☐ Scan or photograph the blot to confirm equal loading in each lane. (*The Ponceau rinses off of nitrocellulose membranes with TBST. For PVDF membranes, add NaOH dropwise into TBST until staining dissipates. It should only take 1-2 drops.*)

**Blocking:**

☐ Block in 5% non-fat dry milk in TBST at RT for 30 min. Rinse briefly in TBST and proceed to immunostaining.

**Immunochemical Staining:**

*Note the primary antibody, species, and antibody dilution.*

☐ Incubate the blot at 4°C ON with primary antibody diluted in 1% milk or BSA. (*The next day, recover the antibody solution in a 15 mL conical. Add 0.02% NaAzide to stem bacterial growth. Freeze at -20°C and reuse 5-15 times. Do not recover HRP-conjugated antibodies as NaAzide acts as an inhibitor to the reaction.*)

☐ Wash the blot with TBST for 5 min x 3.

☐ Incubate the blot with secondary antibody diluted in 5% milk in TBST for 1 ½ to 2 hours at RT.

☐ Wash the blot with TBST for 5 min x 3.

**Antibody detection:**

☐ Prepare ECL Plus/Prime reagents (2 mL solution A + 50 µL solution B).

☐ Remove the blot to a transparency sheet in a cassette and quickly pipette 1 mL ECL Plus per blot.
Incubate for 5 minutes at RT.

Sandwich the blot between a second transparency and expose to film. *(A good exposure should capture any subtle differences in expression between samples. Start with a 30 sec and 1 min exposure and proceed from there, adding more or less time as needed. The ECL will begin to wear off within 45 min-1 hr.)*

**Table S7. Western Blot stock solutions.**

<table>
<thead>
<tr>
<th>10X Tris/glycine buffer (2L, pH 8.3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.92 M Glycine 288 g</td>
</tr>
<tr>
<td>250 mM Tris 60.6 g</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>10X TBS (2 L, pH 8.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M NaCl 175.32 g</td>
</tr>
<tr>
<td>0.5 M Tris 121.14 g</td>
</tr>
</tbody>
</table>

*Autoclave or filter. Fungus grows in this in Florida!

**10% Ammonium Persulfate**

Make up a 1 g APS in 10 mL sterile dH2O and divide into 0.5 mL aliquots. Store at -20°C.

*As APS degrades, it begins to acidify. To test the integrity of the APS aliquot, spot onto pH indicator paper. If the solution soaking into the paper does not change the color to acid past the initial spot, then the APS has not degraded.*

**Ponceau S Dye**

(0.1% Ponceau in 10% acetic acid)

Dissolve 0.5 gram of Ponceau in 400 mL dH2O

Add 50 mL of glacial acetic acid dH2O to 500 mL

*Ponceau may be reused by pouring back into the original bottle.*

**Table S8. Western Blot working solutions.**

<table>
<thead>
<tr>
<th>1X SDS Running Buffer</th>
<th>1X Transfer buffer</th>
<th>1X TBST (0.5% Tween)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mL 10X tris/glycine buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>895 mL dH2O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mL 20% SDS (Final SDS is 0.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mL 10X tris/glycine buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mL methanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>800 mL dH2O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mL 10X TBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>890 mL dH2O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mL 10% Tween-20 (Final Tween is 0.05%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6. FLAG PULL-DOWN USING ANTI-FLAG M2 AFFINITY GEL BEADS (SIGMA F2426)

Make sure to use a FLAG empty vector as a control in overexpression transfection experiments.

Protein extraction:

- Lyse cell pellets in 250-500 uL of M-PER buffer (Thermo Scientific, catalog # 78501) + protease inhibitors.
- Rotate for 15 min at 4°C.
- Centrifuge at 13,000 rpm for 10 min at 4°C.
- Quantitate protein via Bradford assay.
- Assess the highest amount of protein to use so that each sample is normalized.
- Save the excess as input and prepare tubes containing 20 ug of total protein for WB analysis.

Preclear:

- Add 20 uL mouse IgG agarose beads (Sigma, cat#A0919).
- Rotate 30 min 4°C.
- Spin 1 min at 4°C at 1000 rpm
- Transfer supernatant to new tubes

Immunoprecipitate:

- Equilibrate beads by washing 40 uL/IP of anti-Flag M2 affinity gel beads (Sigma, cat#F2426) in 500 uL TBS 2X.
- Add 40 uL of equilibrated beads to normalized cell lysates.
- Rotate overnight at 4°C.

Wash beads:

- Centrifuge at 1000 rpm for 1 min at 4°C.
- Transfer supernatant to new tubes labeled “flow through” to test for pull-down efficiency.
- Add 500 uL of 1X PBS, vortex briefly, and rotate for 5 min at 4°C.
- Centrifuge at 1000 rpm for 1 min at 4°C and remove the supernatant. Repeat 3 times.
Elution with FLAG peptide:

- Add 3 uL of 5 mg/mL 3X FLAG peptide (Sigma, cat#F-4799) to 100 uL PBS for FLAG elution solution.
- Add 25 uL FLAG elution solution to each sample.
- Incubate shaking for 30 min at 4°C. *(Do not rotate or the small volume will cause the beads to stick to the sides.)*
- Centrifuge at 1000 rpm for 1 min at 4°C.
- Transfer the supernatant to a new tube, being careful not to pipet up the beads.
- Add 7 uL 5X SDS loading dye. *(Store at -80°C.)*

Western blot analysis:

*Follow standard Western blot protocol. After initial Western blot analysis, strip the blot and immunostain using a FLAG antibody. The signal is robust, so abbreviated incubations should be utilized.*

- **Primary Antibody:** anti-FLAG M2 (Sigma, cat#F3165) 1:500 for 30 min at RT
- **Secondary Antibody:** anti-mouse HRP 1:10,000 in 5% milk for 30 min at RT
7. CO-IMMUNOPRECIPITATION (CO-IP)

Collect cells:

☐ Aspirate media and rinse the cells two times with ice-cold 1X PBS.
☐ Scrap cells in 1 mL 1X PBS and pipet to a 1.5 mL eppendorf tube.
☐ Centrifuge at 2,000 rpm for 5 minutes at 4°C. Aspirate and discard the supernatant.
☐ Lyse cell pellets in 250-500 uL of M-PER buffer (Thermo Scientific, catalog # 78501) + protease inhibitors.
☐ Rotate for 15 min at 4°C.
☐ Centrifuge at 13,000 rpm for 10 min at 4°C.
☐ Quantitate protein via Bradford assay.
☐ Assess the highest amount of protein to use so that each sample is normalized.
☐ Save the excess as input and prepare tubes containing 20 ug of total protein for WB analysis.
☐ Retain 20 µg of the cell lysate for the input sample.

Immunoselection:

☐ Preclear normalized samples with 20 µL of washed antibody-binding beads (see wash procedure below).
☐ Rotate at 4°C for 30 min.
☐ Centrifuge at 2000 rpm for 1 min at 4°C to pellet the beads.
☐ Transfer the cell lysate to a fresh tube.
☐ Add 5-10 µL of primary antibody to the precleared cell lysate.
☐ Rotate at 4°C ON.

Immunoprecipitation:

☐ Wash the antibody-binding beads. Make sure to pipette using a cut P1000 tip.
  ☐ Transfer protein G Sepharose 4B beads to a 1.5-2 mL tube. (Use 40 µL of beads per IP.)
  ☐ Add 500-1000 µL 1X PBS
  ☐ Invert the tubes twice to mix
  ☐ Centrifuge at 2000 rpm for 1 min 4°C at to pellet the beads.
  ☐ Aspirate the supernatant
☐ Add 40 µL antibody-binding beads to each IP and rotate ON at 4°C.
☐ Centrifuge at 2000 rpm for 2 min at 4°C.
☐ Retain supernatant for “flow through” analysis.
☐ Wash beads 1x with lysis buffer and 2 times with ice-cold 1X PBS. Vortex briefly and rotate for 5 min between washes.
☐ Elute beads with 50 µL of 5% SDS sample buffer. Heat beads at 95°C for 5 minutes prior to loading on an SDS-PAGE gel.
8. NAD/NADH QUANTIFICATION
(CELL TECHNOLOGY, FLUORESCENT NAD/NADH DETECTION KIT
CATALOG # FLNADH 100-2)

This experiment is LIGHT sensitive. You must have two plates of cells for each variable
(i.e. one for the NAD extraction and one for the NADH extraction). The pellets can be
combined when collected as long as they are

Sample preparation:

☐ Heat shock the cells at 42°C or treat as desired.
☐ Wash a 100 mm plate of cells with 1X PBS.
☐ Collect in 0.5-1 mL of 1X PBS using a cell scraper.
☐ Centrifuge at 2000 rpm for 5 min at 4°C.
☐ Remove the supernatant and add 200 µL NAD or NADH Extraction Buffer (Part# 3046 and 3047).
☐ Gently vortex, then add 200 µL NAD/NADH Lysis Buffer (Part# 3045) and homogenize by pipetting up and down.
☐ Gently vortex the samples and heat them at 60°C for 15 min.
☐ Cool on ice and add 100 µL of NAD/NADH Reaction Buffer (Part # 3044).
☐ Add 200 µL of the OPPOSITE extraction buffer and vortex, to neutralize the homogenates.
☐ Centrifuge at 5000-8000 x g for 5 min. (Use immediately or freeze at -80°C; freezing may lead to slight loss of NAD/NADH.)

☐ Make up the reaction cocktail and put on ice:

Table S9. NAD/NADH reaction recipe.

<table>
<thead>
<tr>
<th>Reaction cocktail</th>
<th>10 rxns</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD/NADH reaction buffer (Part # 3044)</td>
<td>1 mL</td>
</tr>
<tr>
<td>Enzyme mix (Part# 6021)</td>
<td>10 µL</td>
</tr>
<tr>
<td>NADH detection reagent (Part# 4018)</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

NAD/NADH Standard Curve:

☐ Label tubes 1-8.
☐ For tube 1, add 997 µL of NADH Standard Diluent (Part# 3051) and 3 µL of reconstituted NADH standard (Part# 7013, 3000 nM).
☐ For tubes 2-8, add 250 µL of NADH Standard Diluent.
☐ Add 250 µL of tube 1 to tube 2.
☐ Vortex well and add 250 µL of tube 2 to tube 3, and so on to tube 7.
☐ Tube 8 is the blank control.
Table S10. Serial dilution for the NADH standard.

<table>
<thead>
<tr>
<th>Tube #</th>
<th>NADH Concentration in tubes.</th>
<th>Final NADH Concentration in wells.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3000 nM</td>
<td>1000 nM</td>
</tr>
<tr>
<td>2</td>
<td>1500 nM</td>
<td>500 nM</td>
</tr>
<tr>
<td>3</td>
<td>750 nM</td>
<td>250 nM</td>
</tr>
<tr>
<td>4</td>
<td>375 nM</td>
<td>125 nM</td>
</tr>
<tr>
<td>5</td>
<td>187.5 nM</td>
<td>62.5 nM</td>
</tr>
<tr>
<td>6</td>
<td>93.75 nM</td>
<td>31.25 nM</td>
</tr>
<tr>
<td>7</td>
<td>48.87 nM</td>
<td>15.625 nM</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

NAD/NADH Assay:

- Add 50 µL of the standard or sample to a 96 well, black plate in triplicate. *(In order to ensure that the samples NADH levels fall within the range of the standard curve, it is a good idea to make a set of triplicates consisting of 25 µL of the sample + 25 µL NADH Standard Diluent.)*
- Pipette 100 µL of the Reaction Cocktail to all the wells.
- Incubate at RT in the DARK for 1-1.5 hours. *(It is helpful to create an aluminum foil lid and store the plate in a drawer.)*
- Take a reading at excitation 530-570 nm and emission 590-600 nm. Use the NADH standard to make a standard curve of RFU vs NADH (nm). Solve for x to determine the concentration of NADH. *(For the NAD samples, the NAD was reduced to NADH and a fluorescent detection regent at a 1:1 ratio.)*

Figure S15. Plate layout template for NAD/NADH assay
9. CHROMATIN IMMUNOPRECIPITATION (CHIP)

Cell collection:

*Grow up, treat, and harvest 1X10^7 total cells per condition to be tested. This corresponds to about two 80% confluent 100 mM plates of HeLa cells and will allow for 5 IP’s per sample. This can equate to combining the pellets of duplicate experiments.

☐ Add formaldehyde at a 1% concentration (270 µL of 37% formaldehyde) to each 100mm plate (10 mls of cell culture media).
☐ Incubate for 10 min at RT with occasional swirling.
☐ Add glycine at a 125mM concentration (500 µL of 2.5M glycine).
☐ Incubate for 5 min at RT with occasional swirling.
☐ Aspirate media and wash cells 2X with ice-cold 1X PBS.
☐ Scrape cells in 1mL 1X PBS and collect in a 1.5mL tube
☐ Centrifuge at 4°C at 2000 rpm for 5 min.
☐ Aspirate the supernatant. *(This may be a stopping point. Store pellets at -80°C.)*

*WORK AT 4°C UNTIL IMMUNOPRECIPITATION IS COMPLETED!

Cell lysis:

☐ Add 1mL of Cell Lysis Buffer with protease inhibitors to each tube and pipet up and down to resuspend the pellet.
☐ Incubate the tubes on ice for 10 min with occasional tapping or on a nutator at 4°C.
☐ Centrifuge at 4°C at 2000 rpm for 5 min to pellet the nuclei.
☐ Aspirate or decant supernatant
☐ Add 300 µL of Nuclei Lysis Buffer to each tube containing the nuclear pellet and resuspend by pipetting up and down.
☐ Incubate the tubes on ice for 10 min with occasional tapping or on a nutator at 4°C.

Chromatin shearing (via a Diagenode Bioruptor):

*Do not tilt the Bioruptor tank!*
*Check that the temperature in the bath stays below 10°C throughout the sonication time! This machine will break if over heating occurs.*
*Empty the tank after each use by using a hand held water pump!*
*Change water after every 15 min of sonication!*

☐ Fill the Bioruptor tank with cold water layered with 0.5 cm of crushed ice 15 min prior to sonication.
☐ Place the 1.5 mL tubes into the tube holder, filling any empty spots with blank tubes.
Sonicate tubes on HIGH for a total of 45 minutes, cycling 30 sec on and 30 sec off. *(The Bioruptor needs a 20 min break for every hour of use!)*

Spin at 13,000 rpm for 10-15 min and transfer the supernatant to a new tube. *(Use sheared chromatin immediately or aliquot and store at -80°C.)*

**Optional step to test sonication:**

- Add 75 µL of Crosslink Reversal Buffer to 25 µL of sonicated sample. *(Crosslink Reversal Buffer: 100 mM Tris-HCL pH 8.0, 10 mM EDTA, 1% SDS.)*
- Incubate in a heat block ON at 65°C
- **Next day:** Phenol/chloroform extract the samples by adding 400 µL of phenol/chloroform, vortexing, and centrifuging at 14,000 rpm at 4°C for 10 minutes.
- Transfer the top clear phase to a new tube, avoiding the white interface.
- EtOH precipitate the samples by adding one-tenth volume (40 µL) of 3M NaOAc, pH 5.2 and two volumes (800 µL) of cold 95% ethanol.

- Place at -80°C for at least 30 minutes or ON.
- Resuspend in 40 µL of TE and add 8 µL of 6X dye.
- Analyze different amounts (1 µL to 10 µL) of sheared chromatin by 1% agarose gel. *(Run the gel slowly at 70V or less for the best separation.)*
- Note the sonication conditions that produce heavy DNA smearing between 200-500 bp

**Immunoselection:**

- To 300 µL of sonicated chromatin, add 2.7 mL of ChIP dilution buffer plus protease inhibitors. *(The chromatin suspension must be diluted at least 1:10 to reduce the concentration of SDS.)*
- Aliquot 0.5 mL to six 1.5 mL tubes. *(Each tube can be used for 1 IP.)*
- Preclear by adding 15 µL Salmon Sperm DNA/protein A agarose-50% slurry. *(This reduces non-specific background.)*
- Rotate for 30 min at 4°C.
- Centrifuge for 1 min at 4°C at 1000 rpm.
- Transfer the 500 µL of supernatant to fresh tubes.
- Add 5-10 µL of antibody to the samples and vortex for 5 sec. *(Make sure to include an IgG negative control. It may also be useful to include a positive control using a histone antibody if this is a new ChIP experiment.)*
- Rotate overnight at 4°C.

- **Next day:** Add 30 µL Salmon Sperm DNA/protein A agarose-50% slurry.
- Rotate for 1 hour at 4°C (or ON).
- Centrifuge for 1 min at 4°C at 1000 rpm.
Carefully remove 500 µL of the supernatant and save as “input”. *(The input samples are used as an endogenous control for the qPCR.)*

Perform the following series of washes with 1 mL of each buffer for 10 min at RT.
- Low salt buffer 1 time
- High salt buffer 1 time
- LiCl was 1 time
- 1X Tris EDTA 2 times

**Immunoprecipitation:**

- Add 250 µL of IP elution buffer.
- Vortex for 30 sec.
- Rotate for 15 min at RT.
- Centrifuge for 5 min at 5000 rpm.
- Transfer the supernatant to a new tube.
- Repeat the elution with another 250 µL of IP elution buffer and then pool with the first eluate.
- Add 20 µL of 5M NaCl to IP sample and input samples.
- Incubate at 65°C for 4 hours to ON. *(Set another heat block to 45°C.)*

**Next day:** Add 10 µL 0.5 M EDTA, 20 µL 1M Tris (pH 6.5), and 2 µL of 10 mg/mL proteinase K.
- Incubate at 45°C for 1 hour.

**DNA purification:**

- Add 300 µL of phenol/chloroform/isoamyl alcohol (25:24:1).
- Vortex and centrifuge for 5 min at 13,000 rpm.
- Transfer aqueous phase to a new tube.
- Add 15 µL 5M NaCl and 1 µL glycogen. Vortex briefly.
- Add 1 mL 95% ethanol.
- Precipitate at -80°C for 1 hour or ON.
- Centrifuge for 20 min at 4°C at 13,000 rpm.
- Remove ethanol and wash pellets with 1 mL 75% ethanol.
- Centrifuge for 10 min at 4°C at 13,000 rpm.
- Remove 75% ethanol and dry the pellets briefly.
- Resuspend with 30 µL of sterile dH₂O.
- Add sterile dH₂O µL of sterile water to new tubes labeled “input 1:150”
- Transfer 1 µL from the input tubes to new 1:150 tubes. *(Use diluted input for qPCR.)*
Table S11. Chromatin Immunoprecipitation buffers.

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell Lysis Buffer (100 mL)</strong></td>
<td>5 mM PIPES pH 8.0 1M PIPES- 0.5 mL 85 mM KCl 1M KCl- 8.5 mL 0.5% NP40 10% NP-40- 5 mL</td>
</tr>
<tr>
<td><strong>Nuclei Lysis Buffer (100 mL)</strong></td>
<td>50 mM Tris-HCl pH 8.1 1M Tris- 5 mL 10 mM EDTA 0.5 M EDTA- 2 mL 1% SDS 20% SDS- 5 mL</td>
</tr>
<tr>
<td><strong>ChIP dilution buffer (100 mL)</strong></td>
<td>0.01% SDS 10% SDS- 0.1 mL 1.1% Triton X 100 1.1 mL 1.2 mM EDTA 0.5 M EDTA- 0.24 mL 16.7 mM Tris-HCl pH 8.1 1M Tris-HCl (pH 8)- 1 mL 167 mM NaCl 5M NaCl- 3.34 mL</td>
</tr>
<tr>
<td><strong>Low salt wash (50 mL)</strong></td>
<td>0.1% SDS 10% SDS- 0.5 mL 1% Triton X-100 0.5 mL 2 mM EDTA 0.5M EDTA- 0.2 mL 20 mM Tris-HCl pH 8.1 1M Tris-HCl (pH 8)- 1 mL 150 mM NaCl 5M NaCl- 1.5 mL</td>
</tr>
<tr>
<td><strong>High salt wash (50 mL)</strong></td>
<td>0.1% SDS 10% SDS- 0.5 mL 1% Triton X-100 0.5 mL 2 mM EDTA 0.5M EDTA- 0.2 mL 20 mM Tris-HCl pH 8.1 1M Tris-HCl (pH 8)- 1 mL 500 mM NaCl 5M NaCl- 5 mL</td>
</tr>
<tr>
<td><strong>LiCl/Detergent Wash Buffer (50 mL)</strong></td>
<td>0.25 M LiCl 0.529g LiCl salt 1% NP-40 10% NP-40- 5 mL 1% deoxycholate 10% deoxycholate- 5 mL 1 mM EDTA 0.5 M EDTA –0.1 mL 10 mM Tris-Hcl pH 8 1 M Tris-HCl – 0.5 mL</td>
</tr>
<tr>
<td><strong>ChIP elution buffer (50 mL)</strong></td>
<td>50 mM NaHCO3 1M NaHCO3- 2.5 mL 1% SDS 10% SDS- 5 mL</td>
</tr>
</tbody>
</table>
10. PROTEIN EXPRESSION USING NEB BL21(DE3) COMPETENT E. COLI (CAT # C2527H)

Transformation:

- Thaw the cells on ice for 10 min. As little as 50 µL of cells may be used for transformation and may be aliquoted to multiple tubes using a wide (or cut) pipette tip.
- Add 1 pg - 100 ng of plasmid DNA (not to exceed 5 µL) to the cells. Tap the tube gently to mix. Do not vortex or mix by pipetting.
- Incubate the mixture on ice for 30 min. Expect a 2-fold loss in transformation efficiency for every 10 minutes you shorten this step.
- Heat shock the cells in a heat block at exactly 42°C for 10 sec and place the tube on ice for 5 min.
- Add 250 µL of room temperature SOC to the cell mixture.
- Incubate the tube at 250-300 rpm at 37°C for 1 hr. Warm selection plates to 37°C for 20-30 min prior to plating.
- Invert the cells to mix and tap on the bench to bring the volume to the bottom of the tube.
- Using a sterile spreader, spread a series of volumes on at least 3 plates and incubate at 37°C ON (i.e. 25 µL, 50 µL, 100 µL). Alternatively, incubate at 30°C for 24–36 hours or at 25°C for 48 hours.

Protein expression:

- After antibiotic selection, pick one colony from the plate and resuspend in 10 mL of liquid culture with antibiotic. Ideally, try to have 90% air in the liquid culture flask/conical.
- Incubate at 37°C until OD₆₀₀ reaches 0.4–0.8. A culture started in the morning should be ready for induction 6 hours later.
- Induce expression with 1 mM IPTG for 2 to 6 hours at 37°C.
- For expression analysis, lyse a cell pellet with SDS sample buffer and run on a 10% SDS-PAGE gel. Expression can be verified by Coomassie staining to verify induction of proteins at the appropriate molecular weight. (If this is the first time these plasmids are used, multiple induction times are beneficial.)
11. GST PULL-DOWN USING PROMEGA’S MAGNEGST™ PULL-DOWN SYSTEM (PART# TM249)

TNT synthesis of Prey Protein:

*The TNT® T7 Quick Coupled transcription/translation reaction can be performed using any plasmid with a T7 primer sequence. Qiagen preps typically result in suitable purification for plasmids used in this application.*

**Table S12. Recipe for the TNT® T7 Quick Coupled transcription/translation reaction.**

<table>
<thead>
<tr>
<th>Components</th>
<th>1 Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNT® T7 Quick Master Mix</td>
<td>40 µL</td>
</tr>
<tr>
<td>Methionine, 1mM</td>
<td>1 µL</td>
</tr>
<tr>
<td>plasmid DNA template(s) (0.5 µg / µL)</td>
<td>2 µL</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>7 µL</td>
</tr>
<tr>
<td><strong>Total=</strong></td>
<td><strong>50 µL</strong></td>
</tr>
</tbody>
</table>

☐ Thaw the TNT® T7 Quick Master Mix by hand-warming or on ice. The other components can be thawed at room temperature and stored on ice.

☐ Assemble the reaction components in a 1.5 mL tube.

☐ Incubate the reaction at 30°C for 60–90 minutes.

Expression of GST-fusion protein in bacterial cells:

*Verify that plasmids are appropriate for expression in bacteria.*

☐ Inoculate 5 mL of LB with either the GST control plasmid or the GST-fusion protein(s) of interest. *(Make sure to use an appropriate strain of E. coli that is ideal for protein expression (i.e. BL21). Once your plasmids are transformed, this experiment may be started from glycerol stocks.)*

☐ The next day, induce plasmid expression with 1mM IPTG for 4-6 hours.

☐ Collect 1-2 mL of culture for expression analysis and 1-2 mL of culture for pull-down assay.

☐ For expression analysis, lyse a cell pellet with SDS sample buffer and run on a 10% SDS-PAGE gel. Expression can be verified by by Coomassie staining to verify induction of proteins at the appropriate molecular weight. *(If this is the first time these plasmids are used, multiple induction times are beneficial.)*
Cell Lysis:
- Pellet 1-2 mL of induced bacterial culture at 8,000 rpm for 3 min.
- Remove supernatant and incubate at -80°C for 30 min.
- Thaw pellet and add 200 µL of the MagneGST™ Cell Lysis Reagent and resuspend by pipetting.
- Add 2 µL of RQ1 RNase-free DNase.
- Incubate at RT for 20-30 min on a rotating platform.

Particle Equilibration:
- Pipet 20 µL of MagneGST™ Particles into a 1.5 mL tube.
- Place the tube in the magnetic stand and remove supernatant. *(Magnetic capture takes only a few seconds.)*
- Remove the tube from the stand, add 250 µL of MagneGST™ Binding/Washing buffer, and resuspend by inverting.
- Remove supernatant and repeat wash x3.

Binding of the GST-fusion protein(s) to magnetic beads:
- Resuspend the particles in 90 µL of MagneGST™ Binding/Washing buffer and 10 µL of 10% BSA.
- Add 200 µL of cell lysate containing the GST-fusion protein of interest.
- Incubate with CONSTANT mixing for 30 min at RT on a rotating platform.
- Place the tube in the magnetic stand and remove supernatant. *Save for gel analysis.*
- Remove the tube from the stand, add 250 µL of MagneGST™ Binding/Washing buffer, and resuspend by inverting. Incubate at RT on a rotating platform for 5 min.
- Place the tube in the magnetic stand and remove supernatant. Repeat wash (without 5 min incubation) x5.
- After the last wash, resuspend the particles in 20 µL of MagneGST™ Binding/Washing buffer. Aliquot 5 µL to four 1.5 mL tubes.

Capture of Prey protein:
- To each 5 µL aliquot add 20 µL of TNT reaction.
- Add 155 µL of MagneGST™ Binding/Washing buffer and 20 µL of 10% BSA.
- Incubate for 1 hr at RT on a rotating platform.
- Vortex briefly and place the tube in the magnetic stand and remove supernatant. *Save for gel analysis.*
- Add 400 µL of MagneGST™ Binding/Washing buffer and mix by inverting. Incubate at RT on a rotating platform for 5 min.
Place the tube in the magnetic stand and remove supernatant. Save for gel analysis.
Add 400 µL of MagneGST™ Binding/Washing buffer and mix by inverting.
Place the tube in the magnetic stand and remove supernatant. Repeat wash x5.

Elution:
Add 20 µL of SDS sample buffer.
Incubate at 5 min at RT.
Place the tube in the magnetic stand and remove eluate for analysis by 10% SDS-PAGE. Use 50% of sample for analysis.

Stronger signals in the experimental samples compared to the GST control lanes indicate that the prey is specifically pulled down by the bait protein. Some background is expected.
12. IMMUNOCYTOCHEMISTRY

**Culture cells:**

- In the fume hood, soak glass cover slips in a dish filled with 100 % ethanol.
- Touch to sterile gauze and air dry.
- Place a cover slip at the bottom of each well in a 6 well plate.
- Split cells 1:2 with a total volume of 2 mL and incubate ON.

**Fix Cells:**

- Aspirate the media then, cover cells to a depth of 2-3 mm with 4% formaldehyde in PBS.
- Allow cells to fix for 15 min at RT with occasional swirling.
- Aspirate fixative and rinse 3x in 1XPBS for 5 min each.

**Immunostaining:**

- Block specimen in Blocking Buffer for 1 hr.
- While blocking, prepare primary antibodies by diluting in Antibody Dilution Buffer. *If using two different antibodies, make sure they are hosted in different species.*
- Aspirate blocking solution and apply diluted primary antibody. Incubate at 4°C ON.
- The next day, rinse 3x in 1X PBS for 5 min each.
- Incubate specimen in Alexa Fluoro secondary antibodies diluted in Antibody Dilution Buffer for 1-2 hours at room temperature. *Cover with aluminum foil!* Dilute at 1-10 µg antibody/mL buffer (2µg/mL works well).
  - **Alexa Fluoro #555** (red) (Species: Rabbit)
  - **Alexa Fluoro #488** (green) (Species: Mouse)
- Rinse 3X in PBS for 5 min each
- Remove the coverslips from the wells with tweezers and place upside down onto a slide into Vector Shield + DAPI.
- Seal slides by painting around edges of coverslips with clear nail polish.
- For best results, examine specimens immediately using the appropriate excitation wavelength. *For long-term storage, store slides flat at 4°C protected from light.*

<table>
<thead>
<tr>
<th>Table S13. Immunocytochemistry buffers.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blocking buffer:</strong></td>
</tr>
<tr>
<td>2.5 mL 10X PBS</td>
</tr>
<tr>
<td>1.25 mL FBS</td>
</tr>
<tr>
<td>21.25 mL dH2O</td>
</tr>
<tr>
<td>*stir, then add 75 µL Triton</td>
</tr>
</tbody>
</table>
C. elegans protocols used in these studies

13. Spectrophotometer calibration, pg. 246
14. Making nematode growth media plates, pg. 247
15. Freezing and thawing C. elegans, pg. 249
16. Synchronization of C. elegans via bleaching, pg. 250
17. RNA isolation for C. elegans, pg. 253
18. Genomic DNA Extraction for C. elegans, pg. 254
13. SPECTROPHOTOMETER CALIBRATION

☐ Inoculate LB with OP50 and incubate at 250-300 rpm at 37°C ON.
☐ Measure and record the OD at 600 nm with the spectrophotometer.
☐ Label 1.5 mL tubes 1-7.
☐ Pipette 900 µL of fresh LB or 1X PBS to each tube.
☐ Add 100 µL of cell culture to tube 1 and serially dilute to each of the following tubes. *(Briefly vortex tube 1 and pipette 100 µL of tube 1 to tube 2. Continue through tube 7. Tube 1 is a 10⁻¹ dilution, tube 2 is a 10⁻² dilution, etc.)*
☐ Label three LB agar plates (without antibiotic) as samples 5-7.
☐ Pipette 100 µL from tubes 5, 6, and 7 to the corresponding plate, spread, and incubate at 37°C ON.
☐ Count the number of colonies per plate and calculate the number of cells per mL. *For example, for plate 7, 15 colonies per 10⁻⁷ equates to 1.5 x 10⁸ cells/mL. You may choose to average the results for plates 5-7. This corresponds to the original OD₆₀₀ measurement and the number of cells/mL for 1 OD₆₀₀ can be calculated."

Figure S16. Bacterial serial dilution for spectrophotometric calibration.
14. MAKING NEMATODE GROWTH MEDIA PLATES

Table S14. NGM plate recipe.

<table>
<thead>
<tr>
<th>Recipe</th>
<th>Standard</th>
<th>Rich</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>1.5 g</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Bacto-peptone</td>
<td>1.25 g</td>
<td>3.75 g</td>
</tr>
<tr>
<td>Agar</td>
<td>10 g</td>
<td>10 g</td>
</tr>
<tr>
<td>dIH₂O</td>
<td>500 mL</td>
<td>500 mL</td>
</tr>
</tbody>
</table>

☐ Make up NGM agar in a 1 L flask.
☐ Autoclave for 45 minutes on a liquid setting.
☐ Cool to 55°C, then add the following:
  • 0.5 mL cholesterol (5 mg/mL)
  • 0.5 mL 1M CaCl₂
  • 0.5 mL 1M MgSO₄
  • 12.5 mL KH₂PO₄, pH 6.0

Pouring NGM plates:

☐ Pipette 10 mL of NGM agar per 60 mm plate or 25 mL per 100 mm plate. Plates should be less than half full. *(Thick agar results in escaping worms.)*
☐ Once plates have solidified, turn them upside down to continue drying. Let plates dry on the bench at RT for ideally 2 days. *(Once plates are dry, they may be seeded with bacteria or stored at 4°C for 1 month.)*

Seeding NGM plates:

☐ On the day before the plates finish drying, inoculate LB broth with OP50 (or an RNAi bacteria) from a glycerol stock. *(1 mL of culture is required per 60 mm plate. If contamination is a reoccurring issue, streak the bacteria onto a plate and inoculate cultures from an isolated colony. This method is preferred for making RNAi plates.)*
☐ Let shake at 250-300 rpm at 37°C ON.
☐ Pellet the bacteria by centrifuging at 3,500 g for 10 minutes.
☐ Create a 10X concentration of bacteria by removing 90% of the supernatant.
☐ Resuspend the pellet in the remaining 10% of broth. Resuspension may also be done in S-basal in lieu of LB.
☐ Pipette 100 μL of bacteria per 60 mm plate or 200 μL of bacteria per 100 mm plate.
Create a circular, symmetrical bacterial lawn that is central to the plate allowing a border of no bacterial growth. The best way to do this is to use the bottom of an autoclaved 1.5 mL tube or a small spreader that can be made from a bent glass pipet.

Let the plates dry at RT ON. (Plates may be stored at 4°C for 1 month.)
15. FREEZING AND THAWING C. ELEGANS

Freezing:

☐ Transfer 20-25 adults to a 10 cm plate that has been seeded with 200 µL of 10X OP50 bacteria.
☐ Let grow for 3-4 days at 22-25°C. (Worms should now be starved with eggs present and lots of L1’s and L2’s. The early larval stages survive upon freezing.)
☐ Wash the plate with 5-10 mL of S buffer.
☐ Swirl plates and then tilt on the lid to allow pooling to one side.
☐ Collect the worms in a 15 mL conical.
☐ Pellet the worms for 1 minute at full speed in a swing bucket centrifuge.
☐ Aspirate buffer and perform at least one more 5 mL wash. If the liquid is cloudy due to uptake of OP50 bacteria, do one or more 10 mL washes until clear.
☐ Aspirate all but 2 mL of S buffer.
☐ Resuspend and distribute 0.5 mL to four 1.8 mL cryovials.
☐ Add an equal amount (0.5 mL) of S buffer + 30% glycerol.
☐ Put the tubes in a rack and place in a Styrofoam container at -80°C ON.
☐ The next day, the cryovials may be transferred to a destination box in the -80°C freezer.

Thawing:

Test thaw the worms to ensure a successful freeze. A successful freeze should have a few worm survivors that are capable of producing progeny. This is also the protocol for thawing for experimental use.

☐ Remove a cryovial from the freezer and sit on a bench to thaw completely at room temperature.
☐ Allow worms to collect at the bottom of the tube and pipette them with as little liquid as possible.
☐ Transfer to the edge of the bacterial lawn on a 60 mm plate. (A good method is to pipet the worms in a circle around the bacterial lawn.) Worms should be active after just a few minutes.
☐ After 2-3 days, transfer the surviving animals to separate plates and ensure that they can produce progeny.

Table S15. S buffer recipe.

<table>
<thead>
<tr>
<th>S buffer</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>K\textsubscript{2}HPO\textsubscript{4}</td>
<td>0.05 M</td>
</tr>
<tr>
<td>KH\textsubscript{2}PO\textsubscript{4}</td>
<td>0.05 M</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.85 g</td>
</tr>
</tbody>
</table>
16. SYNCHRONIZATION OF C. ELEGANS VIA BLEACHING

Bleach Synchronization:

- Chunk worms onto a seeded 100 mm NGM plate. *(Allow the worms to grow 2-3 days so that there are lots of eggs and gravid adults on the plate.)*
- Add 5mL of M9 onto the plate and gently swirl to dislodge the worms.
- Transfer the worms to a 15mL conical tube.
- Centrifuge for 1 minute at 5,000 rpm to pellet the worms.
- Aspirate most of the M9 without disturbing the worm pellet.
- Add 15mL of 20% alkaline hypochlorite solution to the tube. *(8.25 mL dH2O + 3.75 mL 1M NaOH + 3 mL bleach)*
- Mix the tube gently by inverting (or on a rotating platform) for approximately 5 minutes or until you see a decrease in the number of intact adult worms. *(Do not bleach for much longer than this or you will kill the eggs.)*
- Centrifuge for 1 minute at 5,000 rpm.
- Aspirate the 20% alkaline hypochlorite solution without disturbing the worm pellet.
- Add 15mL of M9 to the tube and mix by inverting.
- Centrifuge for 1 minute at 5,000 rpm.
- Aspirate most of the M9 without disturbing the worm pellet.
- Repeat M9 wash 2x.
- Add 7mL of fresh M9 and pipet up and down to resuspend the pellet.
- Transfer to a 50 mL flask and place on the shaker at RT.
- Let the eggs hatch ON with gentle rocking (24-34 hours). *(Since there is no food, the larvae should be halted at the L1 stage.)*
- Collect in a 15 mL conical.
- Centrifuge for 1 minute at 5,000 rpm and remove all but 200 µL. *(For liquid culture, remove all but 4 mL and split between AL and CR flasks.)*
- Distribute 200 µL onto seeded NGM plates (around the bacterial lawn) or 2 mL into liquid culture.

Liquid Culture:

Table S16. *C. elegans* liquid culture components.

<table>
<thead>
<tr>
<th>S-Basal complete</th>
<th>100 mL mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-basal</td>
<td>96.4 mL</td>
</tr>
<tr>
<td>1M MgSO₄</td>
<td>300 µL</td>
</tr>
<tr>
<td>1M CaCl₂</td>
<td>300 µL</td>
</tr>
<tr>
<td>100X trace metals</td>
<td>1 mL</td>
</tr>
<tr>
<td>1M potassium citrate</td>
<td>1 mL</td>
</tr>
</tbody>
</table>

*For ad libitum (AL) cultures, use 1.9 x 10¹⁰ cells/mL. For caloric restriction (CR) cultures, use 2.6 x 10⁹ cells/mL. Subtract the volume of bacteria to be added from the total volume of S-basal.*
Inoculate 100-200 mL of LB broth with OP50 the previous day and shake at 250-300 rpm at 37°C ON.

Measure optical density at OD$_{600}$. *(Each spectrophotometer should be calibrated to obtain a conversion factor for cells/mL of E. coli.)*

Add the appropriate amount of bacterial culture to 100 mL S-basal complete medium in order to achieve the desired level of food source.

Add the synchronized worms to the prepared volume of complete S-basal medium + OP50.

Shake the inoculated cultures at 200 rpm at 22-24°C.

Take a sample each day to monitor worm development. *AL worms reach adulthood in ~3 days. CR worms may be delayed. Depending on the experiment, it may be better to start the CR worms one day early.*

**Figure S17. C. elegans life cycle at 22°C.**

Aartwork by Altun and Hall, © Wormatlas.

### Table S17. C. elegans solutions for synchronization.

<table>
<thead>
<tr>
<th>S-basal (1 L)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>5.9 g</td>
</tr>
<tr>
<td>1 M KH$_2$PO$_4$, pH 6.0</td>
<td>50 mL</td>
</tr>
<tr>
<td>cholesterol (5 mg/mL in ethanol)</td>
<td>1 mL</td>
</tr>
<tr>
<td>ddH$_2$O to 1L</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M9 (1 L)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$•7H$_2$O</td>
<td>5.8 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>3.0 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0 g</td>
</tr>
<tr>
<td>MgSO$_4$•7H$_2$O</td>
<td>0.25 g</td>
</tr>
<tr>
<td>ddH$_2$O to 1L</td>
<td></td>
</tr>
</tbody>
</table>
Table S17 continued from previous page.

<table>
<thead>
<tr>
<th>Trace Metals Solution (500mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSO$_4 \cdot 7$H$_2$O</td>
</tr>
<tr>
<td>Na$_2$EDTA</td>
</tr>
<tr>
<td>MnCl$_2 \cdot 4$H$_2$O</td>
</tr>
<tr>
<td>CuSO$_4 \cdot 5$H$_2$O</td>
</tr>
<tr>
<td>ddH$_2$O to 500mL</td>
</tr>
</tbody>
</table>

*Store in the dark.*
17. RNA ISOLATION FOR *C. ELEGANS*

Use appropriate sterile molecular techniques for RNA extraction. Pipets and bench should be cleaned with RNase Away and sterile filter pipet tips should be used.

- Wash at least one 100 mm plate with 10 mL of M9 buffer.
- Swirl the plates and then tilt on the lid to allow pooling to one side.
- Collect the worms in a 15 mL conical.
- Pellet the worms for 1 minute at 5,000 rpm in a swing bucket centrifuge.
- Aspirate the buffer and do at least one more 10 mL wash. *(If the liquid is cloudy due to uptake of OP50 bacteria, do one or more 10 mL washes until clear. Alternatively, bacteria can be completely filtered out using 30 µm spin columns from Thermo Scientific cat 69725.)*
- Aspirate all but 0.5 mL of M9 buffer.
- Transfer to a 1.5 mL tube.
- Centrifuge at 12,000 x g at 4°C for 2 min.
- Remove most of the supernatant.
- Add 1 mL Trizol reagent.
- Pipet up and down with a P200 until viscous.
- Vortex by hand for about 30 seconds.
- Vortex at 4°C for 20 min until the worms dissolve.
- Place on a nutator at RT for 10 min.
- Add 200 µL of chloroform.
- Shake tubes vigorously for 10-15 sec. *(DO NOT VORTEX.)*
- Let sit at RT 2-3 min.
- Add 200 µL of chloroform.
- Centrifuge at 12,000 x g at 4°C for 15 min.
- Transfer the supernatant (500-550 µL) to a new tube. *(The RNA will be contained in the upper clear aqueous layer and is approximately 60% the volume of Trizol added.)*
- Add 500 µL of isopropanol and vortex briefly.
- Let sit at RT for 10 min. Precipitate at -80°C for 30 min. *(Alternatively, this can be a stopping point.)*
- Centrifuge at 12,000 x g at 4°C for 10 min. *(A white pellet will form.)*
- Aspirate the supernatant.
- Wash pellet with 1 mL cold 70% EtOH (made with RNase-free H₂O).
- Centrifuge at 9,000 x g at 4°C for 2 min.
- Aspirate, but leave about 200 µL. *(Do not touch the pellet! Remove the rest of the liquid with a P200 or stretched glass pipet!)*
- Invert tubes to dry, but be careful not to overdry.
- Depending on the size of pellet, resuspend in 15-30 µL of RNase-free H₂O.
18. GENOMIC DNA EXTRACTION FOR C. ELEGANS

If synchronized populations are desired, culture five plates of worms. If unsynchronized populations are desired, worms can be propagated further by supplementing the plate with additional 10X OP50 every few days. **Optional:** The Morimoto lab recommends overlaying the NGM media with 1% agarose to avoid potential inhibition of subsequent enzymatic manipulations.

- Culture two 100 mm plates of worms on NGM plates.
- Add 5mL of M9 onto the plate and gently swirl to dislodge the worms.
- Transfer the worms to a 15mL conical tube.
- Centrifuge for 1 minute at 5,000 rpm to pellet the worms.
- Aspirate most of the M9 without disturbing the worm pellet.
- Repeat M9 wash 2x or more as needed.
- Flash freeze the pellet at -80°C or in liquid N₂. *(Stopping point.)*
- Add five volumes of worm genomic DNA lysis buffer and incubate at 65°C for 1-2 hours.
- Incubate at 95°C for 20 min to deactivate the proteinase K.
- Add RNase A to 0.1mg/mL and incubate at 37°C for 1 hour.
- Add 1 volume of phenol/chloroform.
- Mix gently and spin at 4,000 rpm for 5 min at RT. *(Do not perform at 4°C to avoid precipitation of SDS.)*
- Transfer the supernatant (aqueous phase) to a new tube.
- Repeat the phenol/chloroform extraction until the white precipitate at the aqueous/organic interface is no longer visible.
- Add 0.1 volume of 3M sodium acetate and 2 volumes of 100% ethanol.
- Incubate at -80°C for 1 hour. *(Stopping point.)*
- Centrifuge at 14,000 rpm at 4°C for 15 min.
- Aspirate the supernatant.
- Wash pellet with 1 mL cold 70% EtOH.
- Centrifuge at 14,000 rpm at 4°C for 2 min.
- Repeat 70% EtOH wash 2X.
- Aspirate, but leave about 200 µL. *(Do not touch the pellet! Remove the rest of the liquid with a P200 or stretched glass pipet!)*
- Invert tubes to dry, but be careful not to overdry.
- Depending on the size of pellet, resuspend in 30 µL of RNase-free H₂O or TE. *(If the white precipitate does not dissolve, let the suspension sit for 15 min at 37°C and then remove the supernatant, leaving the white pellet.)*
- Prep should yield an OD₂₆₀/OD₂₈₀ ratio of approximately 1.8.

### Table S18. *C. elegans* solutions for genomic DNA extraction.

<table>
<thead>
<tr>
<th>M9 (1 L)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄•7H₂O</td>
<td>5.8 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>3.0 g</td>
</tr>
</tbody>
</table>
Table S18 continued from previous page.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>5.0 g</td>
</tr>
<tr>
<td>MgSO(_4)•7H(_2)O</td>
<td>0.25 g</td>
</tr>
<tr>
<td>ddH(_2)O to 1L</td>
<td></td>
</tr>
</tbody>
</table>

**Worm Genomic DNA Lysis Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>200 mM</td>
</tr>
<tr>
<td>Tris-HCl (pH 8.5)</td>
<td>100mM</td>
</tr>
<tr>
<td>EDTA (pH 8.0)</td>
<td>50mM</td>
</tr>
<tr>
<td>ddH(_2)O to 10 mL</td>
<td></td>
</tr>
</tbody>
</table>

*Aliquot at -20°C*

*Add 0.1 mg/mL of proteinase K (10 mg/mL) and 0.5% SDS (from 20%) before use.*
ABOUT THE AUTHOR

Rachel R. Raynes was raised in the south and grew up primarily in Jacksonville, Florida where she attained a Bachelor of Science degree in Biology from the University of North Florida in 2005. After graduation with her Bachelor’s degree, Rachel worked for two years in veterinary medicine and pharmacy compounding. Upon graduation with her Ph.D. in Biology with a concentration in Cell and Molecular Biology from the University of South Florida in 2013, she has obtained a postdoctoral position at the University of Southern California, Davis School of Gerontology in the lab of Dr. Kelvin Davies where she will be studying the oxidative stress response and proteasomal degradation. Rachel plans to continue a career in academic science with a focus on adaptive stress responses upon completion of her postdoctoral training (NIH willing).
Worm humor

Cytoprotection!

Whoa. That was close. Wanna make some HSPs?

OMG! It burns!

Good call on the pre-conditioning!

....Alex?

33°C Heat Shock

36°C Heat Shock

No Preconditioning.

So what are you doing later?

You know, trolling for males.

I figure my chances are 1 in 1000.

...Hello?

25°C Homeostasis

36°C Heat Shock

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