The heat is on

Sanne Hensen

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HSF1 integrates cellular stress response pathways

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Uitnodiging

Voor het bijwonen van de openbare verdediging van mijn proefschrift

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HSF1 integrates cellular stress response pathways

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Sanne Hensen
sannehensen@gmail.com

Paranimfen:
Siebe van Genesen
Nicole van de Ven

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HSF1 integrates cellular stress response pathways

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Promotoren:
Prof. dr. N.H. Lubsen
Prof. dr. G.J.M. Pruijn

Manuscriptcommissie:
Prof. dr. G.J.M. Martens
Prof. dr. H.H. Kampinga (Rijksuniversiteit Groningen)
Dr. S.G.D. Rüdiger (Universiteit Utrecht)

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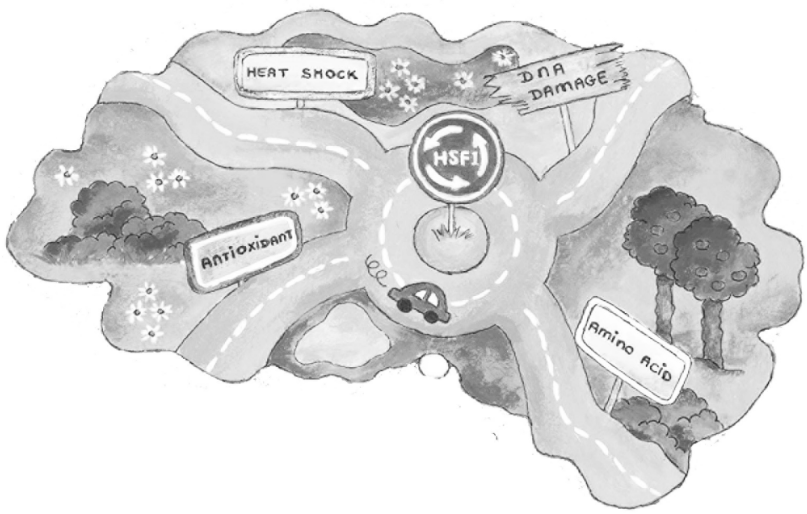
Chapter 1

General introduction



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It did not matter if this interpretation was true or false, it was a working link between imagination and reality, like love." These are the words that the Italian scientist Ritossa used to describe his first interpretation of the results that eventually were the fundamentals for the discovery of the heat shock response [1]. Fifty years ago, Feruccio Ritossa was investigating nucleic acid synthesis in *Drosophila melanogaster*, the fruit fly. When the incubator he used was shifted to the wrong temperature, he noticed a different puffing pattern in the salivary glands of the flies. Apparently, elevated temperatures could strongly increase local transcriptional activity in the cells [2]. Results were rejected by a highly respected journal, saying they lacked biological importance, but eventually turned out to be of great importance in many areas of biology and medicine.

The heat shock response

During lifetime cells have to adapt to and survive all kinds of environmental challenges. These challenges can cause severe damage to a cell and cells thus have to deal with these injuries. When cytoplasmic proteotoxic stress occurs, that is, when proteins are mis- or unfolded in the cytoplasm, cells activate one of the main cellular stress responses: the heat shock response, with heat shock factor 1 (HSF1) as its major transcriptional regulator.

Heat shock factors

So far, six human heat shock factors have been identified: HSF1, HSF2, HSF4, HSF5, HSFX and HSFY [3-6]; HSF3 has only been identified in birds and recently also in mice [7]. HSF2 has been shown to be involved in developmental and differentiation-related processes [8]. Under non-stress conditions it is present as a dimer. Upon activation

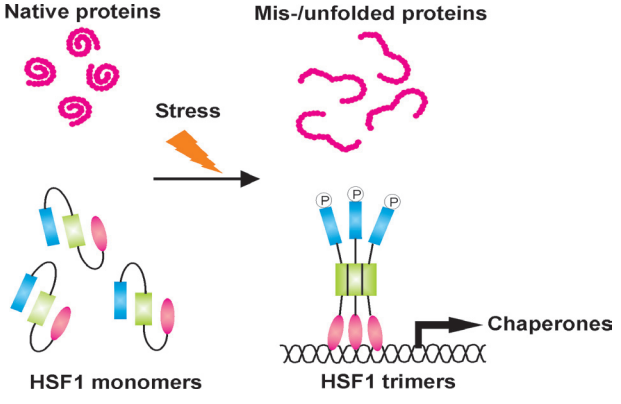


Fig. 1 HSF1 trimerization. Under non-stress conditions HSF1 is mainly present in an inactive monomeric state. Upon proteotoxic stress, proteins tend to mis- or unfold and the heat shock response is activated. HSF1 trimerizes and binds the promoters of heat shock protein genes to activate transcription.

HSF2 forms homotrimers or heterotrimers together with HSF1 and induces the transcription of target genes [9]. HSF4 has been shown to be mainly involved in the development of the lens [8]. Of HSF4 two isoforms exist, HSF4A and HSF4B. HSF4A can repress transcription, whereas HSF4B activates transcription [10,11]. Under non-stress conditions HSF4 exists as a trimer. Human HSF5 has only been validated at the transcript level; the existence at protein level and its functions and characteristics remain to be elucidated. The HSFX gene and the HSFY gene are testis specific and have been found on the X and Y chromosome respectively [12,13]. Even though all these different heat shock factors exist, HSF1 is thought to be the main transcriptional regulator of the heat shock response [6,14]. Under non-stress conditions monomeric HSF1 is maintained in a repressed non-DNA binding state by the interaction with heat shock proteins [14-17]. The proteotoxic stress signals which can initiate HSF1 activation are quite diverse. Heat shock is obviously one of the stresses which activate HSF1, but also oxidative stress, viral and bacterial infections, heavy metals and UV can trigger HSF1 activation. Upon proteotoxic stress HSF1 forms trimers (Fig. 1), is post-translationally modified, and localizes to the nucleus, where it can bind to specific target sequences [3,4,14,17], the heat shock elements (HSEs). These target sequences are inverted repeats of the pentameric sequence nGAAn and are located in the promoters of heat shock protein genes.

Next to its role under stress conditions, HSF1 is also thought to play a role in non-stressed cells. For example, HSF1 knockout mice were shown to have a severely impaired immune response [18]. In addition, a role for HSF1 has been implied in the circadian rhythm: one of the circadian clock genes was reported to be an HSF1 target [19,20]. Very recently it was described that HSF1 regulates a transcriptional program recruited by malignant cells, and this program was distinct from the proteotoxic stress induced one [21]. The role of HSF1 thus appears to be much broader than its best known role in the proteotoxic stress response.

HSF1 structure

The HSF1 protein consists of several functional domains: the DNA binding domain, the trimerization domain, the regulatory domain and the activation domain (Fig. 2). The DNA binding domain is the best conserved domain and belongs to the family of winged helix-turn-helix DNA binding domains [22-24]. It is located in the N-terminal region of the protein and recognizes the pentameric sequence nGAAn in the major groove of the DNA helix [25-27]. A HSF1 monomer has only little affinity for the heat shock element (HSE), but when HSF1 forms trimers, each monomer recognizes one nGAAn sequence which strongly increases the binding affinity. HSF1 therefore prefers HSEs containing at least three inverted repeats: GAAnnTTCnnGAA [28,29].

The oligomerization domain is located adjacent to the DNA binding domain and is responsible for the trimer formation of HSF1. The trimers can be either homotrimers or heterotrimers together with HSF2 [9]. The oligomerization domain is characterized by the presence of hydrophobic heptad repeats (HR-A and HR-B), which have been proposed to form an unusual triple-stranded coiled coil configuration in the HSF1

Fig. 2 Schematic representation of HSF1. The HSF1 protein consists of several functional domains: the DNA binding domain, the oligomerization domain, the regulatory domain and the activation domain (Ad). HR: hydrophobic heptad repeats.

trimeric state through intermolecular hydrophic interactions [30]. Located more towards the C-terminus, HSF1 contains an additional heptad repeat (HR-C), which negatively regulates trimerization. Deletion of the HR-C domain results in spontaneous HSF1 trimerization, whereas deletion of either the HR-A or HR-B domain abolishes trimer formation [31]. In between the HR-A/B and HR-C heptad repeats the regulatory domain is located. This domain represses the transactivation domain and has been proposed to be the sensor for stress signals, since fusing it to the transactivation domain of another protein confers heat inducibility [32,33]. The regulatory domain of HSF1 is subject to various post-translational modifications, which can regulate the repressing ability of this domain and thus HSF1 activity. The transactivation domain is located at the C-terminus and facilitates the transactivation of the promoters of the heat shock protein genes. It contains two activation domains, AD1 and AD2, which are rich in hydrophobic and acidic residues and together ensure a rapid response to stress.

HSF1 activity and post-translational modifications

The precise mechanism of how HSF1 is activated is not completely clear. HSF1 has been described to interact with HSP90, p23 and immunophilins [14-17], which keep it in an inactive, monomeric state. When unfolded proteins appear in the cells HSP90 releases HSF1, allowing it to be converted into an active state [17,34,35]. When HSF1 is activated more heat shock proteins will be produced that can help refolding the misfolded proteins or target them for degradation. When the level of misfolded proteins decreases, an excess of heat shock proteins is present that can subsequently interact again with HSF1 resulting in its inactivation. During heat shock, HSF1 also interacts

with HSP70 and its cochaperones of the HSP40 family, resulting in the inhibition of trans-activating capacity [36]. The interaction of HSF1 with heat shock proteins is thus a negative feedback mechanism to regulate HSF1 activity according to the status of the protein folding environment. Another mechanism proposed to regulate HSF1 activation by stress is that involving a constitutively present non-coding RNA, the heat shock RNA-1 (HSR1) [37]. Shamovsky et al. described that both HSR1 and the translation elongation factor eEF1A were required for HSF1 activation. However, no other group has confirmed this finding so far and a few years after publication of this finding, Kim et al [38] reported that the HSR1 was most likely a contaminant of bacterial origin. HSF1 has also been reported to possess an intrinsic ability to sense stresses. Its DNA binding domain contains two cysteine residues which have been described to form disulfide bonds and mutation of these cysteines prevents HSF1 DNA binding and impairs the heat shock response [39]. We, however, were unable to show decreased HSF1 DNA binding activity when we mutated these two cysteines (unpubl. data).

DNA binding alone is not sufficient for HSF1 to activate transcription of heat shock protein genes. For example, treatment of cells with salicylates induces the binding of HSF1 to the promoters of heat shock protein genes in vivo, but does not lead to transcriptional activation of these genes [40,41]. Other mechanisms must therefore be involved in the activation of HSF1 and these include post-translational modifications. It was already known for a long time that HSF1 is hyperphosphorylated upon stress [42-44] and this has been proposed to, at least in part, promote the activation of HSF1 [45-47]. Conversely, posttranslational modifications of HSF1, including phosphorylation, sumoylation and acetylation, have also been associated with a negative regulation of its activity [48-51]. Guettouche and co-workers analyzed the phosphorylation status of HSF1 in heat stressed cells and found at least 12 residues at which phosphorylation occurred [45]. Phopshorylation of only one of these residues, S326, was found to contribute to the activation of HSF1 upon heat stress. Other studies have also identified residues in the HSF1 molecule which can be phosphorylated [5], of which phosphorylation at S195 [52], S230 [46] and S320 [53] was found to correlate with transcriptional activation. The phosphorylation of T142 has also been described to be associated with transcriptional activation [54], but this could not be confirmed by other studies [45]. Phosphorylation of residues S121 [55], S303 [56,57], S307 [50,57,58] and S363 [56] has been associated with a negative regulation of HSF1 activity. It has been suggested that phosphorylation of S303 blocks the homotrimerization of HSF1 in yeast as well as in mammalian cells [59]. Furthermore, S303 phosphorylation has been shown to serve as a signal for the sumoylation of HSF1 at residue K298 [48]. It remains unclear whether the repressive effect of S303 phosphorylation on HSF1 activity is exclusively mediated through K298 sumoylation or occurs through additional mechanisms.

Next to phosphorylation and sumoylation, acetylation has also been shown to regulate HSF1 activity negatively. Westerheide and colleagues analyzed the acetylation pattern of HSF1 and found at least 9 lysines that were acetylated upon stress [51]. They more thoroughly analyzed the consequences of acetylation at a residue located in the DNA binding domain, K80, because in yeast HSF1 mutation of this residue corresponds with a loss of function phenotype [60,61]. When residue 80 was mutated from a lysine to a glutamine, which mimics constitutive acetylation, HSF1 did form trimers, but failed to bind to the DNA. Mutation to any other amino acid also resulted in defective DNA binding, indicating that lysine 80 is critical for the DNA binding ability of HSF1. Furthermore, they showed that activation of the deacetylase SIRT1 prolonged HSF1 binding to the DNA, implying that SIRT1 deacetylates HSF1 to keep it in a DNA binding competent state.
In summary, the mechanism of how HSF1 activity is regulated has not been fully elucidated so far. It is a complex mechanism that involves the inhibitory role of the chaperones and is, at least in part, regulated by posttranslational modifications.

Heat shock proteins

When HSF1 binds to a heat shock element in the promoter region of heat shock protein genes it activates transcription of these genes, which leads to elevated expression levels of the mRNA for heat shock proteins and ultimately of the heat shock proteins. Heat shock proteins were discovered in 1974 by Tissières and co-workers in the salivary glands of *Drosophila melanogaster* [62]. They examined the salivary glands of control and heat shocked larvae and found a specific set of proteins that was synthesized after a heat shock. More than ten years later the function of the heat shock proteins as molecular chaperones was elucidated and we now know that not only heat can increase heat shock protein levels, but that their levels are increased upon many other types of stresses as well.
As molecular chaperones, heat shock proteins have an important role in the refolding and degradation of mis- and unfolded proteins that accumulate upon stress [63,64]. However, not all heat shock proteins have chaperoning capacity, and not all chaperones are heat inducible. Next to their role in protein folding and degradation, heat shock proteins assist in the assembly and disassembly of macromolecular complexes and regulate translocation of proteins across membranes [65,66]. Based on their sequences heat shock proteins are divided into different families: HSPH (HSP110), HSPC (HSP90), HSPA (HSP70), HSPD (HSP60), DNAJ (HSP40) and the small heat shock protein (shsp) family HSPB. As described above, HSP90 is bound to HSF1 in unstressed cells to keep it in a monomeric inactive state [67,68] and its activity is regulated by ATP binding and hydrolysis. HSP90 is present in high amounts in the cytoplasm under non-stress

conditions, and its expression is further induced upon proteotoxic stress. HSP70 family members are key components of the cellular chaperone network. The expression of some of these proteins is highly inducible upon various stresses (i.e. HSPA1A, HSPA1B and HSPA6), but can also be constitutive (HSC70/HSPA8). HSP70 binds selectively to unfolded short hydrophobic peptides and its activity is controlled by the cycle of ATP binding, hydrolysis and nucleotide exchange [69]. This cycle is regulated by cochaperones such as members of the HSP40 family (i.e. DNAJA1 and DNAJB1), which stimulate ATP hydrolysis and by nucleotide exchange factors, like HSPH members [70-73]. HSP40 members can also interact directly with unfolded proteins and recruit HSP70 to protein substrates [71]. HSP60 typically locates to the matrix of mitochondria and is essential for the folding and assembly of newly imported proteins [74].
Members of the small heat shock proteins also bind to (partially) unfolded proteins and prevent their aggregation until the proteins can be refolded by larger ATP-dependent chaperones or are degraded [75]. The small heat shock protein family consists of ten different members (HSPB1-10) [76], which are characterized by low molecular masses and a common C-terminal motif, the so-called α-crystallin domain. The most studied small heat shock proteins are HSPB1 (Hsp27) and HSPB5 (αB-crystallin). They tend to form oligomers or even multimers of more than 20 subunits and it is thought that these high molecular weight complexes exert chaperoning functions [77,78]. Both proteins can be phosphorylated and their functions are therefore under the control of several signaling pathways. They show rapid phosphorylation that modulates their activities in response to a wide variety of stresses [79]. For at least HSPB1, phosphorylation has been described to result in dissociation of the multimeric complexes [80] and some of the biological activities of HSPB1 are associated with small oligomers, whereas others require the formation of large oligomers [81]. The chaperoning functions of HSPB5 appear to be mediated mostly by the phosphorylated form of the protein [81].

Other cellular stress responses

Cells are also exposed to stresses that do not lead to activation of the heat shock response, and these thus need to be dealt with by other stress responses. Cellular stress responses are not thought to act independently of each other; it is very likely that these pathways interact. For example, the phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α) is a common response to different types of stresses. Insight in the crosstalk between different stress responses would help to understand the consequences for functioning of certain stress pathways when one of them is impaired. Below, three other cellular stress responses will be described: the response to amino

acid deprivation, the unfolded protein response and the antioxidant response, activated respectively upon a lack of amino acids, in response to endoplasmic reticulum (ER) stress and upon oxidative stress.

The amino acid deprivation response

In mammals, nutritional or pathological conditions may affect plasma concentrations of amino acids. Amino acid homeostasis needs to be strongly controlled, in part because of the body's inability to synthesize some of the amino acids. Out of 20 amino acids, about half are essential in mammals and these amino acids therefore need to be obtained from dietary intake. When cells sense a lack of one of the amino acids, the amino acid response is activated. Normally, tRNAs are loaded with their corresponding amino

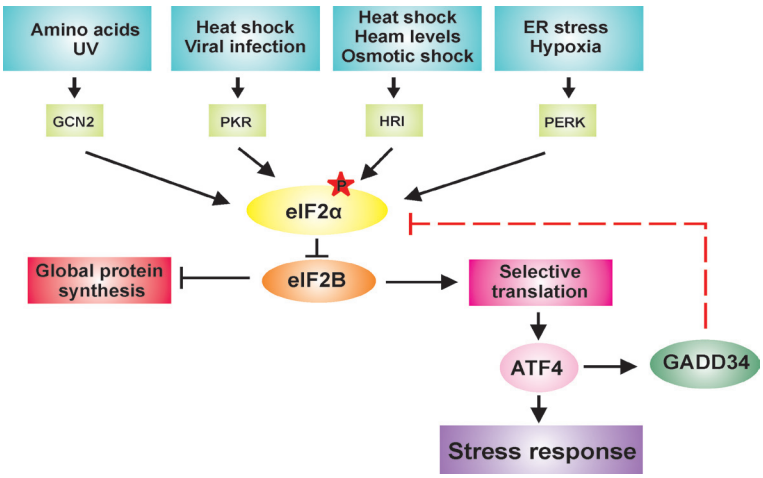


Fig. 3 Various stresses result in eIF2α phosphorylation. Phosphorylation of eIF2α occurs upon several kinds of stresses via different kinases and results in the inhibition of global protein synthesis and the selective translation of mRNA's, like ATF4. ATF4 can subsequently activate specific target genes. ATF4 also initiates a negative feedback loop by inducing the synthesis of GADD34, which results in dephosphorylation of eIF2α.

acid by aminoacyl tRNA-synthetases. However, when a cell is deficient in a specific amino acid, uncharged tRNAs will accumulate and activate the kinase general control non-derepressible 2 (GCN2) [82]. The GCN2 kinase will subsequently phosphorylate eIF2α resulting in the inhibition of the guanine exchange factor eIF2B and impaired formation of the translation initiation complex. The phosphorylation of eIF2α is a common response to various stresses via the activation of different kinases (Fig. 3). It leads to a general inhibition of protein synthesis and a paradoxical increase in translation

of selected mRNA species, amongst which that encoding the transcription factor ATF4 [83]. The ATF4 mRNA contains three upstream open reading frames (uORFs) located 5' to the ATF4 coding sequence, of which two are translated under non-stress conditions. The other uORF overlaps with the open reading frame of ATF4, but is out of frame. During stress, ribosome scanning bypasses this uORF and translation re-initiation occurs at the ATF4 coding region. ATF4 initiates a negative feedback loop by inducing transcription of the growth arrest and DNA damage-inducible 34 (GADD34) gene [84], eventually resulting in the dephosphorylation of eIF2α and thereby permitting translation of the increased stress-responsive mRNAs [85-87].

ATF4 is an important player in the amino acid response. Promoters of amino acid responsive genes often contain an ATF4 binding site and are thus inducible by ATF4. Upon amino acid deprivation ATF4 binds to the amino acid response element (AARE) or the nutrient sensing response unit (NSRU) [88-90] and thereby initiates a complex transcriptional program to adjust several of the cell's physiological functions to the decreased amino acids supply. ATF4 can bind an AARE or NSRU as a homodimer or as a heterodimer together with a member of the CCAAT enhancer binding protein (C/EBP) family. The most studied target genes of ATF4 are asparagine synthetase (ASNS) and CHOP. The ASNS gene encodes an enzyme that is responsible for the biosynthesis of asparagine from aspartate and glutamine [91]. The promoter of the ASNS gene contains the NSRU [90,92], consisting of the NSRE-1 and NSRE-2 sequence, and is very rapidly activated after amino acid starvation. Moreover, a change in histone acetylation status occurs in the ASNS promoter region [93]. ATF4 is the main activator of the ASNS gene. Later ATF3 and C/EBPβ bind, closely correlating with a decline in transcription rate, and these proteins thus act as transcriptional repressors of the ASNS gene [93]. CHOP is a nuclear protein that is related to the C/EBP family of transcription factors [94], which have been implicated in the regulation of several cellular processes such as energy metabolism, cellular proliferation, differentiation and inflammation [95]. CHOP expression after amino acid starvation is both regulated at the transcriptional and the post-transcriptional level [96]. Even though the core sequence of the AARE in the CHOP promoter and the NSRE-1 sequence in the ASNS promoter differ by only two nucleotides, activation of the CHOP promoter needs, next to ATF4, also ATF2, which is constitutively present [89,97].

Autophagy

Autophagy is a cellular process in which lysosomes degrade intracellular components and even whole organelles [98]. It is an important mechanism for protein degradation as it is involved in the clearance of toxic protein aggregates. Amino acid starvation is also known to activate autophagy. During times of nutrient deficiency autophagy is

activated to provide cells with additional free amino acids that can be used for protein synthesis. The three main forms of autophagy are microautophagy, macroautophagy and chaperone mediated autophagy (CMA). Microautophagy and macroautophagy were initially described as mechanisms for "in bulk" degradation of cytoplasmic components. In the case of microautophagy, sequestration of the cargo occurs directly at the surface of the lysosomes. The lysosomal membrane engulfs the cargo by forming vesicles which are then invaginated into the lysosomal lumen where they are rapidly degraded [98,99]. In macroautophagy, vesicles are formed through the formation of a membrane of non-lysosomal origin which engulfs cytoplasmic substrates to form an autophagosome [100]. An autophagosome will subsequently fuse with a lysosome and the sequestered cargo can be completely degraded. Recently it has been described that macroautophagy not only occurs in a non-selective manner, but that selective recognition occurs in the case of organelles and particles such as aggregates and pathogens [101-104]. The third type of autophagy is chaperone mediated autophagy which mediates the selective degradation of soluble proteins. Substrates for this pathway are cytosolic proteins which contain a pentapeptide motif related to KFERQ which is recognized by HSC70 [105]. The substrate protein is then delivered by HSC70 and its cochaperones to a receptor in the lysosomal membrane, the lysosomal-associated membrane protein type 2A (LAMP-2A) [106] and subsequently transported into the lysosmal lumen where it is degraded.

The unfolded protein response

The unfolded protein response (UPR) is induced by the accumulation of mis- or unfolded proteins in the endoplasmic reticulum (ER). Just as the heat shock response does for the cytoplasmic chaperoning capacity, the UPR induces the transcription of chaperones that increase the chaperoning capacity in the ER. The UPR shows some overlap with the response to amino acid deprivation, in that one branch of the UPR involves an eIF2α kinase and activation of the UPR thus also leads to eIF2α phosphorylation and the selective synthesis of ATF4 (reviewed in [107]). The UPR comprises three signalling cascades mediated by different ER-localized transmembrane proteins: PKR (double-stranded RNA-activated protein kinase)-like ER kinase (PERK), inositol requiring 1 (IRE1), and activating transcription factor 6 (ATF6; Fig. 4) [108-110]. Under non-stress conditions, these kinases are kept inactive by the ER-chaperone HSPA5 (BiP). When unfolded proteins accumulate in the ER, HSPA5 dissociates from the kinases and is sequestered by the unfolded proteins to assist in refolding or degradation. PERK is the above mentioned eIF2α kinase of the UPR, and when ER stress occurs, PERK oligomerizes and phosphorylates both itself and eIF2α, thus inhibiting protein synthesis. ATF4 mRNA is translated and ATF4 activates the promoters of

genes encoding ER resident proteins [107]. ATF4 also activates some of the promoters that are induced by the amino acid deprivation response, i.e. CHOP and ASNS [111], but not all amino acid responsive genes are activated [112]. The distinction between the target genes activated by ATF4 in different stress responses is most likely dictated by the binding partners of ATF4. When IRE1 is activated, it cleaves the mRNA encoding X-box binding protein 1 (XBP1), a UPR-specific transcription factor, removing a 26 nucleotide intron. The exons are then ligated and the active form of XBP1 is translated [113,114]. The transcription factor ATF6 is packaged into vesicles upon ER stress and transported and processed in the Golgi apparatus [115]. The N-terminal cytoplasmic domain is cleaved off, moves into the nucleus and binds to ER stress response elements thereby activating the UPR target genes [116]. The UPR thus transiently inhibits protein synthesis and leads to the transcriptional activation of genes coding for ER resident proteins to increase the folding capacity in the ER.

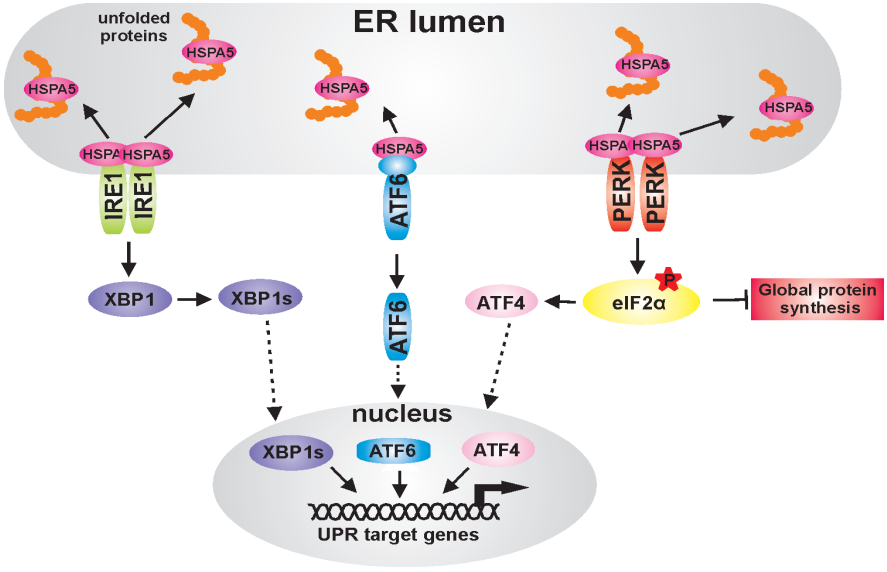


Fig. 4 Various stresses result in eIF2α phosphorylation. Phosphorylation of eIF2α occurs upon several kinds of stresses via different kinases and results in the inhibition of global protein synthesis and the selective translation of mRNA's, like ATF4. ATF4 can subsequently activate specific target genes. ATF4 also initiates a negative feedback loop by inducing the synthesis of GADD34, which results in dephosphorylation of eIF2α.

1

The antioxidant response

Oxidative stress results from the imbalance between the production of reactive oxygen species (ROS) and the inability of the cell to detoxify the highly reactive intermediates. ROS can cause great damage to cellular components, such as lipids, proteins and DNA. Depending on the severity of the oxidative burden and the ability of the antioxidant defence to combat the production of ROS, oxidative stress can result in cell death

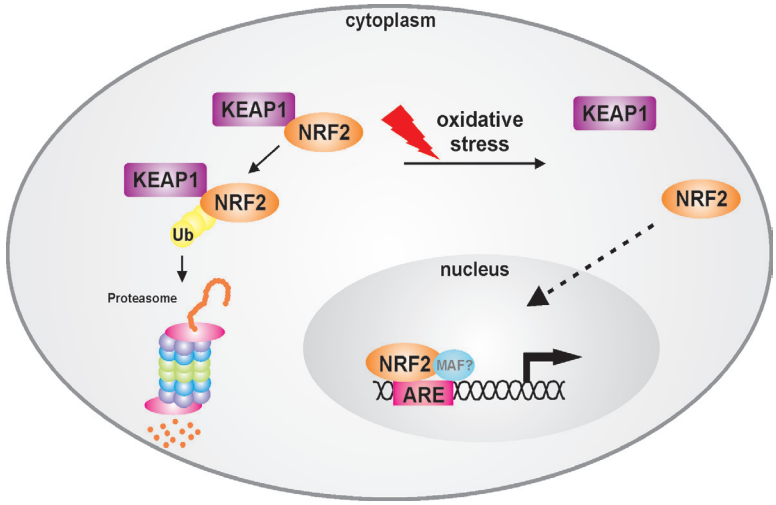


Fig. 5 The antioxidant response. Under non-stress conditions the transcription factor NRF2 is maintained at a low level in the cytoplasm by KEAP1 through KEAP1-dependent ubiquitination and proteasomal degradation. Upon oxidative stress NRF2 is released and translocates to the nucleus to activate transcription from genes containing an antioxidant response element (ARE), possibly together with a small MAF protein.

by inducing apoptosis or necrosis. An important mechanism in the cellular defence against oxidative stress is the activation of the antioxidant response, mediated by NRF2 (nuclear factor (erythroid-derived 2)-related factor 2), a transcription factor that controls the expression of antioxidant response element (ARE)-regulated antioxidant and cytoprotective genes [117,118]. Under non-stress conditions NRF2 is retained in the cytoplasm by the actin-associated KEAP1 (*K*elch-like ECH associated protein 1) protein [119] and maintained at a low level through KEAP1-dependent ubiquitination and proteasomal degradation [120-124] (Fig. 5). Upon oxidative stress, cysteines in the KEAP1 protein are oxidatively modified, resulting in a conformational change and release of NRF2 [121]. NRF2 then translocates to the nucleus where it binds to ARE containing promoters as a heterodimer, usually with a small Maf protein [117], driving expression of these genes. The resulting protein products can subsequently detoxify

the reactive oxidants by enhancing the cellular antioxidant capacity. Murine heat shock protein mRNA levels have been reported to be increased upon NRF2-activating compounds in an NRF2-dependent manner [125,126]. In addition, an NRF2-binding motif was found in the promoter of the Hsp70 gene in zebrafish [127] and this element was conserved between mouse and zebrafish. Heat shock proteins have been shown to be involved in the enhancement of survival, prevention of apoptosis upon oxidative stress and the reduction of damage to DNA, proteins and lipids [128-130].

Proteotoxic stress and aging

Protein homeostasis

Protein homeostasis, also known as proteostasis, is the ability of a cell to maintain the protein balance by regulating several cellular processes, such as protein synthesis, folding, trafficking, and degradation. The maintenance of protein homeostasis is essential for cellular and organismal health and loss of protein homeostasis has been implicated in aging and several protein folding diseases [131-133]. In the dynamic cellular environment, native proteins face numerous challenges to their folded state. Upon all kinds of stresses the cellular protein balance is threatened and cells have to respond to this threat. As described above, the heat shock response is activated when a cell senses cytoplasmic proteoxic stress, and the unfolded protein response is activated upon proteotoxic stress in the ER. The transcription of chaperone genes is induced and will assist the cell in maintaining protein homeostasis (Fig. 6) by refolding misfolded proteins and by targeting irreparable proteins to the proteasome for degradation. These stress responses are thus very important for a cell to sustain protein balance and malfunctioning of these stress signalling pathways can lead to the formation of protein aggregates and eventually even to protein folding diseases.

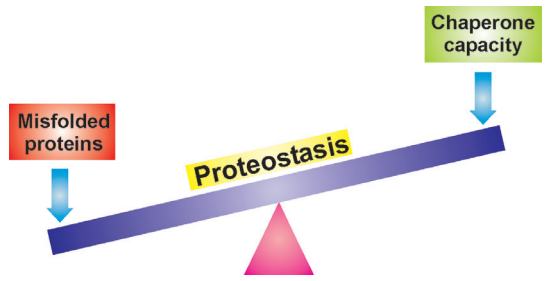


Fig. 6 Protein homeostasis. A cell aims to maintain proteostasis by keeping the balance between the amount of misfolded proteins and the amount of chaperones to refold the misfolded proteins or target them for degradation.

Neurodegenerative diseases

A subclass of protein folding diseases is the group of neurodegenerative diseases, where toxic aggregates are formed in the patient's brain. Among the neurodegenerative diseases are Alzheimer's disease, Parkinson's disease and Huntington's disease, which are all associated with the misfolding of specific proteins. In Alzheimer's disease, the most common form of dementia, misfolded amyloid β protein can adopt a highly stable β-sheet structure and these structures can subsequently multimerize into soluble oligomers. These oligomers generate cellular toxicity by disrupting several cellular processes. The misfolded protein aggregates eventually form insoluble high molecular weight amyloid fibrils, which accumulate in spherical microscopic deposits, the senile plaques. These inclusions were thought to be the major source of cytotoxicity, but recent studies show that larger aggregates are cytoprotective and it could be that the smaller, soluble aggregates are the main source of toxicity [134-136].

In Parkinson's disease, the synaptic protein α-synuclein misfolds to form distinctive protein aggregates in the nerve cells, which are also known as Lewy bodies. As in Alzheimer's disease, it is generally thought that the aberrant soluble α-synuclein oligomers are the toxic species that mediate disruption of cellular homeostasis and neuronal death, through effects on various intracellular targets, including synaptic function. This results in severe motor problems and in advanced stage of the disease dementia commonly also occurs. In both Alzheimer's disease and Parkinson's disease patients, oxidatively modified lipids have been found in the brain [137-139], implying that oxidative stress is also involved in these diseases.

Huntingtons's disease is a polyglutamine disease in which the huntingtin protein contains an abnormally long polyglutamine stretch that eventually causes aggregates of the mutant huntingtin proteins. There is a strong correlation between the length of the glutamine tract and the formation of aggregates, with a pathogenic threshold of 35-40 glutamine residues [140]. These aggregates in the neurons and muscles are highly toxic and have dramatic effects on mobility [141,142].

Aging

Protein folding diseases are highly associated with aging. The age at which neurological symptoms appear varies between different diseases, but generally Alzheimer's and Parkinson's disease are late-onset, while Huntington's disease has an earlier onset, although the onset of this disease is more closely linked with the length of the polyglutamine tract [133]. Protein homeostasis thus seems to be imbalanced in aged cells. The ubiquitin proteasome system (UPS) is the main proteolytic system responsible for protein degradation and it is known that proteosome activity declines with age [143] (Fig. 7). In addition, the efficiency of both macroautophagy and CMA, also major

pathways for protein degradation, has been described to decrease with age [144-147] (Fig. 7). The accumulation of ROS has also been reported to be involved in the aging process (Fig. 7). The main idea is that ROS generation leads to macromolecular damage, including DNA and protein damage [148]. The accumulation of damaged and modified proteins also results from a gradual decay in protein quality control mechanisms.

HSF1 in aging

HSF1 is one of the factors that might be involved in the inability to maintain proteostasis in aged cells (Fig. 7). An age-related decline in heat inducible Hsp70 expression has been shown in cell lines and in vivo [149-155]. Corresponding with decreased Hsp70 levels, the affinity of HSF1 for the DNA decreases with age [156,157]. In human diploid fibroblasts from elderly donors the levels of HSF1 were similar to those in young cells,

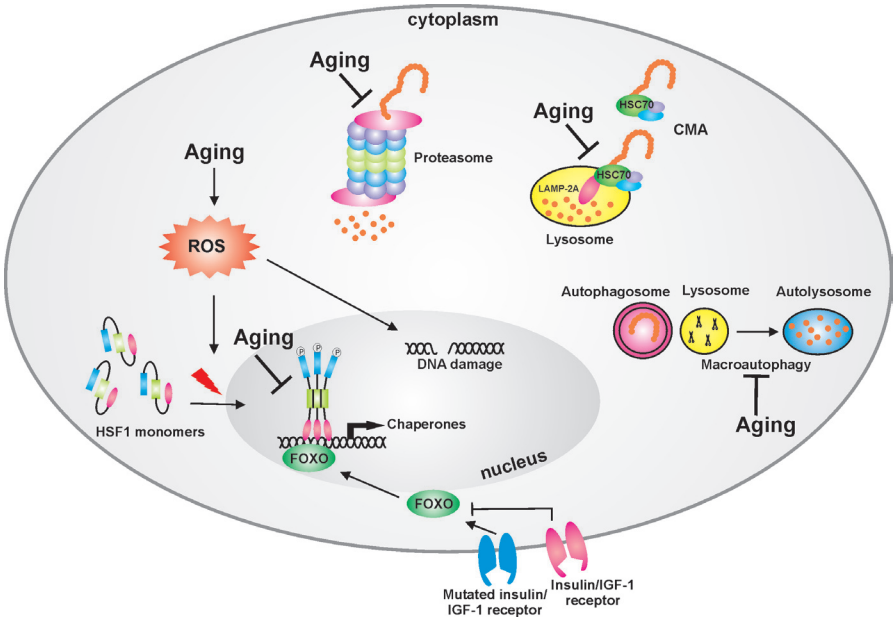


Fig. 7 The effect of aging on cellular mechanisms involved in maintaining proteostasis (adapted from [143]). Aging affects protein degradation pathways, like the proteasome, chaperone mediated autophagy (CMA) and macroautophagy. In addition, the accumulation of reactive oxygen species (ROS) has also been implied in aging, and this can result in DNA damage and damaged proteins. The main stress response that is activated upon proteotoxic stress, the heat shock response, is also inhibited upon aging. HSF1 DNA binding activity decreases in aging cells. Insulin/IGF-1 signalling accelerates aging. When the IGF-1 receptor is mutated or the insulin/IGF-1 signalling pathway is impaired lifespan increases.

whereas the activation of HSF1 changed as a function of age [158]. Downregulation of HSF1 levels decreases lifespan in *C. elegans*, whereas overexpression has been shown to increase lifespan [159]. Furthermore, dietary restriction (moderate caloric restriction with adequate nutrient intake) extends lifespan and protects against proteotoxicity through an HSF1-dependent mechanism in *C. elegans* [160].

Another protein that has been implicated in dietary restriction and longevity is the histone deacetylase SIRT1. Elevated levels of sirtuins increased lifespan in yeast [161], *C. elegans* [162-164] and *Drosophila* [165]. Furthermore, lifespan was increased by dietary restriction in yeast [166], *C. elegans* [167] and *Drospohila* [165] by activating sirtuins. However, the role of sirtuins in dietary restriction and aging is not completely clear. Recently it has been shown that outcrossing in *Drosophila* and *C. elegans* abrogated the positive effect of Sir2 (the ortholog of mammalian SIRT1) overexpression on lifespan [168] and also other studies challenged the positive effects of SIRT1 on lifespan [169,170]. As described before, SIRT1 was also shown to be involved in HSF1 regulation. Activation of SIRT1 maintains HSF1 in a deacetylated, DNA binding competent state [51]. In contrast, downregulation of SIRT levels decreased HSF1 affinity for the heat shock promoters. As SIRT1 protein expression has been described to decrease as a function of age [171], it could well be that the age-related decline of HSF1 DNA binding activity is SIRT1 dependent.

A pathway that has also been implied in the extension of lifespan is the insulin/insulin-like growth factor 1 (IGF1) signalling (IIS) pathway. Mutations in members of this pathway that reduce signalling through this pathway increase lifespan (Fig. 7). In *C. elegans*, a decrease in DAF-2 activity, a hormone receptor similar to the insulin/IGF-1 receptor, doubles lifespan and mutations in the downstream P13K/AKT/PDK kinase cascade also increase longevity [172]. A decrease in IGF-1 signalling also delays proteotoxicity in disease models of aberrant protein aggregation [141,173]. Downstream of AKT is the transcription factor DAF-16, a member of the FOXO family of transcription factors. When AKT is activated, DAF-16 is prevented from moving to the nucleus and transcription from its target genes is thus inhibited. Overexpression of DAF-16 has been shown to increase lifespan in *C. elegans*. Also in *Drosophila*, increasing the activity of FOXO, the *Drosophila* ortholog of DAF-16, has been shown to extend lifespan. Furthermore, in mice an inverse correlation has been found between IGF-1 levels and lifespan [174]. Overexpression of the Klotho gene in mice has been shown to extend lifespan and the Klotho protein inhibited insulin/IGF-1 signalling [175,176]. In humans, mutations in the KLOTHO gene correlated with decreased lifespan [177]. A role for HSF1 has also been implied in the insulin/IGF-1 signalling pathway. HSF1 was shown to be required for lifespan extension resulting from insulin/IGF-1 receptor mutations and DAF-16/FOXO and HSF1 might thus act together to promote longevity [159].

HSF1 in cancer

Besides the beneficial effects of increased HSF1 signalling on prevention of aging and age-related protein folding diseases, increased HSF1 activity also has negative effects on organismal health. HSF1 has been shown to be crucial for tumorigenesis. Mice lacking HSF1 were protected from tumor formation induced by mutations of the RAS oncogene or the tumor suppressor p53 [178]. Furthermore, it was shown that HSF1 activates a transcriptional program that supports highly malignant cells and that is different from the transcriptional program activated upon heat shock [21]. Nuclear HSF1 levels are increased in the tumor cells of patients with breast cancer and this correlates with a poor prognosis [179]. Moreover, HSF1 expression is elevated in human prostate carcinoma cell lines [180]. Corresponding with these findings, increased heat shock protein levels are associated with a wide range of tumors [181]. In breast cancer, overexpression of HSP70, HSP90 and αB-crystallin correlates with poor prognosis [182-184]. Overexpression of HSP70, HSP27 and αB-crystallin might also contribute to drug resistance and a poor response to chemotherapy [185-187]. In addition, Straume and colleagues recently described that HSP27 was significantly upregulated in angiogenic cells in a breast cancer xenograft model, and silencing HSP27 resulted in long term tumor dormancy in vivo [188].

Increased HSF1 activity and increased heat shock protein levels thus aid in the development of cancer. HSF1 and the heat shock proteins therefore seem suitable candidates for molecular targeting to inhibit tumor formation. A lot of research has already been done to investigate the effect of HSP90 inhibitors on cancer cells and this has proven quite successful so far [189]. In addition, HSP70 is also considered as a potential drug target [190]. We do have to stress, however, that HSP90 and HSP70 are very important players in maintaining proteostasis. Even though inhibiting these proteins would have beneficial effects on tumor formation, a decrease in the levels of these proteins might accelerate neurodegenerative processes and aging. On the other hand, activating the heat shock system to inhibit the development of neurodegenerative diseases might have detrimental effects in that it could promote tumor growth. It is thus of major importance to keep in mind the different roles of HSF1 and the heat shock proteins in these diseases when we either wish to inhibit or activate the heat shock system for their treatment.

Outline of this thesis

As described above, HSF1 is a major factor in maintaining protein homeostasis and we hypothesize that its decreased activity is of major importance in the aging process. As

HSF1 and its downstream targets are also involved in several other cellular processes, like development, controlling of life span and maintenance of tumor cell survival, boosting the heat shock system to delay the process of aging may have deleterious effects on cells and we thus need to learn more about the critical nodes of the heat shock system. In addition, we need to study its interaction with other cellular stress systems, as not much is known about the interaction of the heat shock system with other cellular stress systems and it is unlikely that all cellular stress systems act independently of each other. Previous studies have used knockdown of HSF1 with siRNA or HSF1-/- cells to mimic decreased HSF1 activity in aging cells. However, upon aging, HSF1 is still present in the cell, but it cannot activate transcription from heat shock protein genes anymore. In **chapter 2** we describe a cellular model system in which we overexpressed a dominant negative mutant of HSF1 (dnHSF1), which lacks the activation domain and is thus unable to activate transcription. We show that glucocorticoid signalling is affected by the dnHSF1 and that overexpression of the cochaperones DNAJA1 or DNAJB1 can rescue this effect. **Chapter 3** shows that expression of a dnHSF1 was embryonic lethal in *Xenopus laevis*, the African clawed frog, and HSF1 is thus essential for the development of *Xenopus* tadpoles. This chapter also describes that when HSF1 is overexpressed in *Xenopus* tadpoles, this is not detectable in the larval brain, suggesting that HSF1 levels are strictly controlled in neuronal tissue. In **chapter 4** yet another model for mimicking the decreased HSF1 activity in aging cells is illustrated. Upon aging, the binding of HSF1 to the DNA decreases and we created a stable cell line overexpressing a HSF1 mutant that is unable to bind to the DNA, HSF1 K80Q, and analyzed the transcriptome changes in unstressed and stressed cells. In non-stressed cells HSF1 regulates the level of a limited set of largely cell specific transcripts. Unexpectedly, transcript levels of some of the genes that are normally regulated by HSF1, i.e. HSPA1A and HSPA6, increased in HSF1 K80Q cells 24 h after heat shock. Apparently other transcription factors can take over if HSF1 activity is blocked and we identified NRF2 as one of the transcription factors involved.

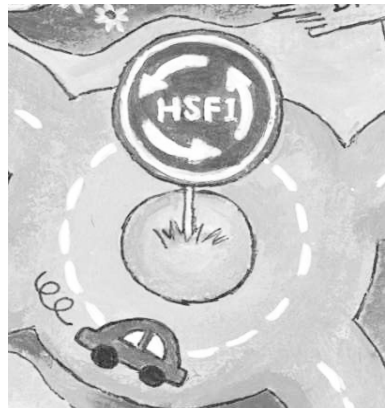
In **chapter 5** we show that HSF1 is inactivated by amino acid deprivation. Upon starvation for either leucine, lysine or glutamine, HSF1 loses its DNA binding activity and heat shock protein mRNA levels are strongly decreased. A lack of amino acids could thus lead to a lower chaperoning capacity and cellular frailty. However, upon methionine starvation HSPA1A and DNAJB1 mRNA levels are increased and in **chapter 6** we describe that this might be caused by the activation of the antioxidant response, as knockdown of NRF2 by siRNA inhibited the increase in HSPA1A mRNA levels in methionine starved cells.

The small heat shock proteins αB-crystallin and HSPB1 have been shown to be involved in the protection against distinct stresses and have been implicated in several diseases,

such as cancer and neurodegenerative diseases. How expression of these proteins exactly regulates cellular physiology is largely unknown. In **chapter 7** we analyzed the transcriptomes of HEK293 cells overexpressing αB-crystallin or HSPB1 and found that expression of αB-crystallin affected the level of a large number of transcripts, whereas the effect of HSPB1 expression was rather small. Overexpression of αB-crystallin might be unfavourable to a cell, as it resulted in higher transcript levels of stress induced genes compared to control cells or cells expressing HSPB1.
Finally, we will summarize and discuss our findings in **chapter 8**.

Chapter 2

Co-chaperones are limiting in a depleted chaperone network



Lonneke Heldens*, Ron P. Dirks*, Sanne M.M. Hensen, Carla Onnekink, Siebe T. van Genesen, François Rustenberg, Nicolette H. Lubsen

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*These authors contributed equally to this work

To probe the limiting nodes in the chaperoning network which maintains cellular proteostasis, we expressed a dominant negative mutant of heat shock factor 1 (dnHSF1), the regulator of the cytoplasmic proteotoxic stress response. Microarray analysis of non stressed dnHSF1 cells showed a two- or more fold decrease in the transcript level of 10 genes, amongst which 4 (co)chaperone genes, HSP90AA1, HSPA6, DNAJB1 and HSPB1. Glucocorticoid signalling, which requires the Hsp70 and the Hsp90 folding machines, was severely impaired by dnHSF1, but could be fully rescued by expression of DNAJA1 or DNAJB1, and partially by ST13. Expression of DNAJB6, DNAJB8, HSPA1A, HSPB1, HSPB8 or STIP1 had no effect while HSP90AA1 even inhibited. PTGES3 (p23) inhibited only in control cells. Our results suggest that the DNAJ co-chaperones in particular become limiting when the chaperoning network is depleted. Our results also suggest a difference between the transcriptomes of cells lacking HSF1 and cells expressing dnHSF1.

Introduction

All cells contain an extensive network of chaperones which together maintain proteostasis, i.e. this network aids in the folding of the primary peptide chain, the refolding of unfolding proteins and the removal of misfolded proteins (for reviews, see [131,132,191-196]). Two of the major nodes in the network are the Hsp70 and Hsp90 folding machines. At the core of these machines are Hsp90 and Hsp70, the proteins that promote folding; the activity and substrate specificity is controlled by a number of co-factors and co-chaperones. For Hsp70 it is the DNAJ (Hsp40) proteins that determine substrate specificity. DNAJs also stimulate ATP hydrolysis by Hsp70. The human genome contains over 40 DNAJ genes [197-199]. Some of these are highly tissue specific, others may be dedicated to a particular substrate or cooperate only with a specific Hsp70 and some may be redundant [200]. The diversity of DNAJs does show that these are important determinants of the activity and specificity of the Hsp70 folding machine.

The chaperoning capacity of the cell is enhanced by additional chaperone synthesis as part of a proteotoxic stress response, either the heat shock response in the case of cytoplasmic stress or the unfolded protein response in the case of ER stress. That an increase in chaperones is required to combat proteotoxic stress suggests that under normal conditions the chaperone capacity of a cell is limiting. Indeed, exogenous expression of aggregation-prone proteins, such as proteins with an expanded glutamine tract (polyQ), is toxic unless chaperones are also over-expressed [201-205]. Cytoplasmic proteotoxic stress signals to heat shock factor 1 (HSF1), which then activates the transcription of a

number of genes encoding a variety of chaperones, together known as the heat shock proteins. In the absence of stress, HSF1 is generally believed to be kept inactive in the cell by direct interaction with Hsp90, p23 and immunophilins (for reviews, see [15-17]). HSF1 null mice show the expected stress-related phenotypes, such as a complete lack of the heat shock response and the inability to develop thermotolerance. However, they also suffer from neuronal, developmental and germ cell defects [206-211], which cannot be directly linked to the heat shock response and which strongly suggests that HSF1 also regulates gene expression under non-stress conditions. Microarray analysis resulted in the identification of 49 genes (19 related to immune response) that are expressed at reduced levels in HSF1 null fibroblasts compared with wild type cells cultured under physiological conditions. The immune response of HSF1 null mice was shown to be severely impaired [18]. More recently, direct evidence for the stress independent regulation of genes by HSF1 was provided in the case of the multi-drug resistance gene 1 [212], and the IL-6 gene [213]. Furthermore HSF1 inhibits heregulin induced transcription in breast carcinoma cells [214].

A number of studies have shown that the quality of the heat shock response diminishes with aging [155,156,215-219], a decrease that may be the result of a decrease in the activity of the deacetylase SIRT1 [51]. Senescence of cultured human fibroblasts is accompanied with a diminishing heat shock response and a reduction in the affinity of HSF1 for the heat shock element (HSE; [155]). Aging-related failure of HSF1 will interfere with an organisms' ability to combat cellular stress and increase the susceptibility to protein folding disease [131,196,201,202,220-222]. Moreover, with accumulating evidence showing that HSF1 also regulates gene expression under non-stress conditions (see above), its decline may already cause phenotypic defects in the absence of exogenous stress [193,194].

Here we have used a dominant negative HSF1 mutant to inhibit HSF1 activity. As expected, a number of chaperone and co-chaperone genes were downregulated by dnHSF1. To test which (co-)chaperone is limiting in dnHSF1 expressing and thus chaperone depleted cells, we used the glucorticoid response to probe the chaperoning network. Maturation of the steroid hormone receptor is known to be controlled by both the Hsp70 and the Hsp90 folding machinery (for review, see [223]) and augmenting the chaperone network by either stress [224] or expression of a constitutively active HSF1 mutant [225] potentiates the glucocorticoid response. We show here that it is, unexpectedly, primarily the DNAJ (Hsp40) proteins which become limiting when the chaperoning network is depleted.

Materials and Methods

Recombinant DNA constructs

Oligonucleotides that were used to generate recombinant DNA constructs are listed in Table 1. Plasmid pLmHSF1SN that contains the code for the HSF448 mutant was kindly donated by Dr. Wang [226]. The 1.36-kb *Xho*I fragment of pLmHSF1SN was cloned into pcDNA5-FRT/TO (Invitrogen), resulting in plasmid pcDNA5-HSF448. The code for the HSF1 mutant HSF379 was PCR amplified from pLmHSF1SN using the HSF379 primer set and cloned into the *Hin*dIII and *Xho*I sites of pcDNA5-FRT/TO, yielding plasmid pcDNA5-HSF379 (dnHSF1). The promoter constructs pGL3-HspB1 (-685/+36), pGL3-DnaJA1 (-464/+167), pGL3-DnaJB1 (-508/+38), pGL3-Hsp90AA1 (-188/+18), pGL3-ST13 (-400/+141), pGL3-STIP1 (-1264/+145), pGL3-PTGES3 (-1108/+104), pGL3-RMB23 (-1265/+189), pGL3-PMVK (-1183/+147), pGL3-BiP (-2742/+202), pGL3-CHOP (-936/+2), and pGL3-HSPA1A (-313/+196) were made by PCR amplifying the promoter fragments from human genomic DNA using the respective "prom" primer sets and cloning the fragments into pGL3-Basic (Promega). The expression plasmids pcDNA5-HSPB1, pcDNA5-HSPB8, pcDNA5-ST13, pcDNA5-STIP1, and pcDNA5-PTGES3 were made by PCR amplifying the cDNAs from HEK293 RNA using the respective "exp" primer sets and cloning the cDNAs into pcDNA5-FRT/TO. Expression plasmids pcDNA5-V5-DnaJA1, pcDNA5-V5-DnaJB1, pcDNA5-V5-DnaJB6, and pcDNA5-V5-DnaJB8 were kindly donated by J. Hageman (University of Groningen, The Netherlands; [227] Expression construct pCMV-SPORT6-Hsp90AA1 was obtained from Imagenes (www.imagenes-bio.de). The Hsp90AA1 coding sequence was completed at the 5' end by inserting the corresponding fragment PCR amplified from human cDNA *SacII-MscI*. Plasmid pOTB7-STIP1 was obtained from Imagenes. The *Eco*RI (blunt) - *Xho*I fragment of pOTB7-STIP1 was cloned into the *Hin*dIII (blunt) and *Xho*I sites of pcDNA5-FRT/TO, resulting in plasmid pcDNA5-STIP1. The glucocorticoid-responsive reporter plasmid pGRE-Luc was made by annealing the GRE primer set and cloning the double stranded oligo into the *Nhe*I and *Bgl*II sites of pGL3-promoter (Promega). The *Drosophila melanogaster* Hsp70-luciferase reporter construct pHL and the Hsp70 expression construct were described earlier [228]. Plasmid pRL-CMV was obtained from Promega. All plasmid constructs were sequence verified.

Table 1. Oligonucleotides that were used to generate recombinant DNA constructs.

Name	Sequence (5' -> 3')
HSF379-for	agctaagcttaccatggatctgcccgtgggcc
HSF379-rev	agctctcgagctacaggcaggctacgctgaggc

PMVKprom-for	agctaagcttactcaggtaaaacaggagatgtg
PMVKprom-rev	agctccatggccaaacagatatggggagaaaag
RBM23prom-for	agctctcgagtatccaagacccaaaggggcc
RBM23prom-rev	agctccatggcagttccgggtccccgcag
STIP1prom-for	agctaagcttgtggggcaggtggaattaaag
STIP1prom -rev	agctccatggcgcagcgcggtccggaacc
HSPB1prom -for	agtcgacaggcatgcaccaccatgcccagc
HSPB1prom -rev	accatggtggctgactctgctctggacgtctg
ST13prom -for	agctaagcttccccttccggcggaggcg
ST13prom -rev	agctccatggtagggaggtggtgg
PTGES3prom -for	agctaagcttaataccttagtgcttattatgaagc
PTGES3prom -rev	agctccatggtgaacggggcaggggggacg
DNAJA1prom-for	agtcgaccacgcgtgaaaaacagcaagac
DNAJA1prom-rev	accatggtggctgaggccggtgtgtgagggga
DNAJB1prom-for	aagtcgaccagacacaggttaggtagttcgtcc
DNAJB1prom-rev	accatggccccctcctgcggcccgccga
CHOPprom-for	tgagctctgtcacccaggctggagtgc
CHOPprom-rev	tagatctctgacctcgggagcgcctggctg
BiPprom-for	tctcgaggtatttttagtagagactgggcac
BiPprom-rev	accatggtgccagccagttgggcagcag
HSP90prom-for	agctaagcttgcgcaggcgctgttcctgg
HSP90prom-rev	agctccatggcgcccggaggccacaccc
HSPA1Aprom-for	aagatcttgaagcgcaggcggtcagca
HSPA1Aprom-rev	aaagcttccggttccctgctctctgtc
HSP90AA1exp-for	tccgcggtcacttagccaagatgcctg
HSP90AA1exp-rev	tggccaatcatagagatatctgcacc
HSPB1exp-for	agctaagcttaccatgaccgagcgccgcgtc
HSPB1exp-rev	agctctcgagttacttggcggcagtctcatcg
HSPB8exp-for	agctaagcttaccatggctgacggtcagatg
HSPB8exp-rev	agctctcgagtcaggtacaggtgacttcctggct
ST13exp-for	agctaagcttaccatggacccccgcaaagtg
ST13exp-rev	agctaagcttaccatggacccccgcaaagtg
PTGES3exp-for	agctggatccaccatgcagcctgcttctgcaaagtg
PTGES3exp-rev	agctctcgagttactccagatctggcatttttttc
GRE-up	ctagcggtacattttgttctagaacaaaatgtaccggtacattttgttct
GRE-low	gatctagaacaaaatgtaccggtacattttgttctagaacaaaatgtacc

Tissue culture, transfections, and reporter gene assays

Flp-In T-REx-293 cells (Invitrogen) were manipulated according to the manufacturer's instructions using the T-REx system (Invitrogen) to generate the stable cell lines HEK-HSF448, HEK-HSF379 and HEK-cDNA5 that carry a single copy of the tetracycline inducible plasmids pcDNA5-HSF448, pcDNA5-HSF379, and pcDNA5-FRT/TO, re spectively. The cells were cultured at 37°C/5% CO_2 in high glucose DMEM medium supplemented with 10% fetal calf serum and 100 units/ml penicillin and 100µg/ml streptomycin. Blasticidin (1.65 µg/ml; Invitrogen) and 100 µg/ml hygromycin were also added to the culture medium during maintenance of the cell lines, but were omitted during experiments. Transient transfections were performed using FuGENE-6 (Roche) according to the manufacturer's instructions. Cells were seeded on 24-well plates and on the next day transfected with ~0.2 µg plasmid per well. For testing the heat shock response in stable HEK293 cell lines, cells were transfected with 160 ng pHL, and 40 ng pCMV-RL. At 48 h after transfection, cells were either left at 37ºC/5% CO_2 (control) or incubated at 45ºC for 30' (heat shock). After 6 h recovery at 37ºC/5% CO_2, cells were harvested for reporter gene analysis. For analysis of promoter activities, cells were transfected with a mixture of 160 ng luciferase reporter plasmid and 40 ng pβactin-β-galactosidase or pCMV-RL per well. For testing glucocorticoid responsiveness, the culture medium of the cells was first replaced with medium supplemented with 10% steroid-free fetal calf serum (Hyclone), and then the cells were transfected with a mixture of 150 ng pGRE-Luc and 50 ng pβactin-β-galactosidase per well. At 24 h after transfection, the culture medium was replaced with medium containing varying concentrations of dexamethasone (Centrafarm). At 48 h after transfection cells were lysed in 200 µl reporter lysis mix (25 mM Bicine, 0.05% Tween 20, 0.05% Tween 80) for 10 min. For the β-galactosidase assay, 40 µl cell lysate was mixed with 100 µl Galacton solution (100 mM Na-phosphate pH 8.2, 10 mM $MgCl_2$, 1% Galacton-Plus (Tropix). After 30 min incubation at room temperature, 150 µl accelerator II (Tropix) was added and luminescence was measured with the Lumat LB 9507 tube luminometer (Berthold). For the luciferase assay, 40 µl cell lysate was mixed with 50 µl luciferin solution and luminescence was again measured with the Lumat luminometer. All reporter gene assays were performed in triplo.

RNA isolation and microarray analysis

HEK-HSF379 or HEK-cDNA5 cells were either left untreated or treated with doxycyclin for 48 hours. Total RNA was isolated using Trizol according to the manufacturer's instructions (Invitrogen) and copied into Cy3-labeled (untreated cells) or Cy5-labeled (doxycyclin treated cells) cRNA using the Agilent Low RNA Input Linear Amp Kit PLUS, or the reverse for the repeat array. Labeled cRNA samples were hybridized

to an Agilent Whole Human Genome Microarray Kit (4 x 44K). The arrays were scanned using an Agilent Microarray Scanner. Image analysis and feature extraction were done with Feature Extraction (version 9.5.1, Agilent). Only genes that passed the GeneSpringGX standard quality control criteria (free trial available at www.genespring.com) were included in the analysis. We used a cut-off level of 2-fold changed expression (average signal intensity across the array) and an arbitrarily chosen signal cut-off of > 50.

Western blot analysis

Cell pellets were homogenized in buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 100 mM NaF, 20 mM $Na_4P_2O_7$, 1 mM PMSF and protease inhibitors (Complete Mini; Roche). Then 4X sample buffer (200 mM Tris-HCl 6.8, 20% β-mercaptoethanol, 8% SDS, 40% Glycerol and 0.4% Bromophenolblue) was added and the lysates were incubated at 95°C for 5 min. For detection of eIF2α phosphorylation, samples were prepared as described [229]. Protein samples were separated in 12% polyacrylamide gels and transferred to nitrocellulose transfer membrane (Protran) using a Bio-Rad Mini-PROTEAN II Electrophoresis cell according to the manufacturer's instructions. For western blot analysis, polyclonal HSF1 antibody (SPA-901; Stressgen) was used at a 1: 15,000 dilution, Hsp70 antibody 4G4 (ab5444; Abcam) was used at a 1:5,000 dilution, polyclonal DnaJB1 antibody (anti-Hsp40; SPA-400; Stressgen) at a 1:10,000 dilution, monoclonal Hsp90 antibody (610418, BD Biosciences) at a 1:1,000 dilution, HSPB1 antibody, obtained from dr. A. Zantema, at a dilution of 1:400, monoclonal eIF2α antibody was at a 1:500 dilution, polyclonal phosphorylated eIF2α antibody (E2152; Sigma) was used at a 1:1,000 dilution, monoclonal V5 antibody (R96025; Invitrogen) was used at a 1:5,000 dilution, polyclonal ST13 antibody (ab13490; Abcam) at a 1:1,000 dilution, polyclonal STIP1 antibody (ab65046; Abcam) a 1:1,000 dilution, monoclonal p23 antibody (ab2814; Abcam) at a 1:1,000 dilution, polyclonal HSPB8 antibody, obtained from dr. W. Boelens, at a dilution of 1:1,000, and monoclonal β-actin antibody (AC-15, Sigma-Aldrich) at a dilution of 1:5,000. Blots were incubated with fluorescent secondary antibodies IRDye® 800 CW conjugated goat (polyclonal) Anti-Rabbit IgG and IRDye™ 680 conjugated goat (polyclonal) Anti-Mouse IgG. (926-32211 and 926-32220 respectively, LI-COR Biosciences) according to the manufacturer's instructions and scanned using a LI-COR Odyssey infrared scanner. Signals were quantified using Odyssey version 2.1 software.

Results

Dominant negative HSF1 mutants

To block HSF1 signalling in human HEK293 cells we decided to use a dominant negative mutant reasoning that, given the interaction of HSF1 with other cellular components, the effect of a transcriptionally inactive mutant could well be different from the effect of HSF1 being completely absent. Two dominant negative HSF1 mutants containing, respectively, the first 379 (HSF379) and first 448 (HSF448) amino acids have been described (reviewed by [230]). HSF379 lacks both the potent trans-activation domain at the extreme C-terminus and the weaker, more N-terminal, trans-activation domain, whereas HSF448 still has the weak trans-activation domain. The heat shock-mediated induction of endogenous Hsp70 was completely abolished by HSF379, showing its potent dominant-negative activity (Fig. 1). Surprisingly, HSF448 was a very poor

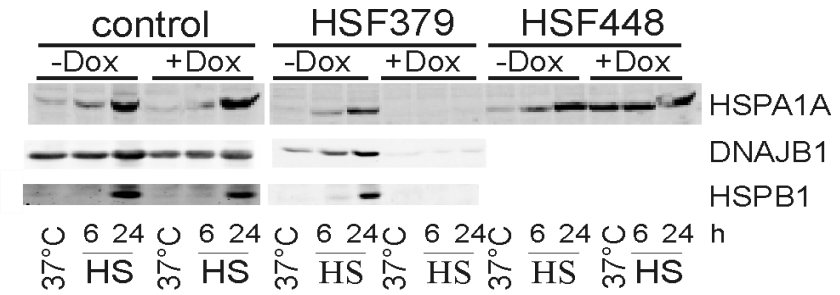

Fig. 1 The HSF1 mutants HSF379 and HSF448 have different effects on basal and heat shock-induced Hsp70 expression. Parental Flp-In HEK293 cells and HEK293 cells carrying a stably integrated copy of the pcDNA5-HSF379 (HEK-HSF379) or pcDNA5-HSF448 (HEK-HSF448) plasmid were cultured in the absence or presence of doxycycline. Cells were exposed to a heat shock (30', 45°C), harvested at the indicated time point (h) after heat shock, and subjected to western blot analysis using an anti-Hsp70 antibody.

inhibitor of heat shock-mediated induction of Hsp70 (data not shown). Moreover, HSF448 caused a significant increase in the basal expression of Hsp70 (Fig. 1). Since this observation was in conflict with earlier data showing the dominant-negative activity of HSF448 [226], we tested the activities of both HSF1 mutants in a luciferase reporter gene assay. As expected, HSF379 completely inhibited the heat shock mediated induction of the *D. melanogaster* Hsp70 promoter (Fig. 2). In the experiments reported below HSF379 was used to inhibit HSF1 activity and will be referred to as dnHSF1.

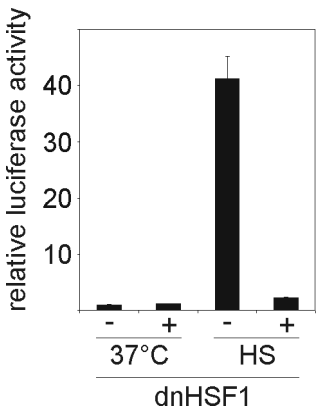

Fig. 2 The effects of dnHSF on basal and heat shock-induced activity of an Hsp70 promoter. HEK293 cells carrying a stably integrated copy of the HSF379 (dnHSF1) were cultured in the absence (-) or presence (+) of doxycycline. Cells were transfected with a mixture of the *Drosophila* melanogaster Hsp70-luciferase reporter (pHL) and the Renilla Luciferase control plasmid pCMV-RL. At 48 hrs after transfection, cells were exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (37°C). When heat shocked, cells were allowed to recover for 6 h and harvested. Hsp70 promoter activities were determined by dividing firefly luciferase values by the corresponding renilla luciferase (experiments using the HSF448 line) or β-galactosidase (experiments using the dnHSF1 line) values to correct for varying transfection efficiencies. The relative luciferase activity in cells cultured at 37°C in absence of the various HSF1 mutants was set at 1. The results are the average of three independent transfections (standard deviations are indicated by error bars).

Table 2. Effect of exogenous expression of dnHSF1 on the transcript levels of the members of the families of heat shock proteins and their co-chaperones.

Gene name	Acc. Nr.	dnHSF1/ Ctrl		Alternative name
		Ave	SD	
HSPH family				
HSPH1	NM_006644	0.78	0.08	heat shock 105kD/110kDa protein 1
HSPH2	NM_002154	0.66	0.04	heat shock 70kDa protein 4
HSPH3	NM_014278	0.61	0.21	heat shock 70kDa protein 4-like
HSPH4	NM_006389	1.19	0.33	hypoxia up-regulated 1
HSPA family				
HSPA1A/B[1]	NM_005345	0.93	0.18	hsp72
HSPA1L	NM_005527	not on array[2]		heat shock 70kDa protein 1-like
HSPA2	NM_021979	1.22	0.20	
HSPA5	NM_005347	1.18	0.35	GRP78, BiP
HSPA6	**NM_002155**	**0.46[3]**	**0.10**	**HSP70B'**
HSPA8	NM_153201	0.87	0.08	HSC70
HSPA9	NM_004134	0.90	0.08	mortalin-2 (mitochondrial protein)
HSPA12A	NM_025015	1.10	0.20	KIAA0417
HSPA12B	NM_052970	nd[4]		
HSPA13	NM_006948	0.57	0.53	STCH

HSPA14	NM_016299	0.85	0.13	
HSP90 family				
HSP90AA1	**NM_005348**	**0.38**	**0.06**	**Hsp90α**
HSP90AB1	NM_007355	0.89	0.06	Hsp90β
HSP90B1	NM_003299	1.16	0.36	Grp94
TRAP1	NM_016292	1.06	0.05	TNF receptor-associated protein 1 (mitochondrial Hsp90)
DNAJ (Hsp40) family				
DNAJA1	NM_001539	0.64	0.10	HDJ2
DNAJA2	NM_005880	1.30	0.42	
DNAJA3	NM_005147	1.00	0.13	
DNAJA4	NM_018602	nd[4]		
DNAJB1	**NM_006145**	**0.25**	**0.05**	**hsp40**
DNAJB2	NM_006736	0.60	0.07	HSJ1
DNAJB3	NM_001001394	nd4		
DNAJB4	NM_007034	0.94	0.09	
DNAJB5	NM_012266	0.97	0.10	
DNAJB6	NM_005494	0.93	0.12	
DNAJB7	NM_145174	nd[4]		
DNAJB8	NM_153330	nd[4]		
DNAJB9	NM_012328	1.22	0.17	
DNAJB11	NM_016306	1.15	0.39	
DNAJB12	NM_001002762	1.04	0.11	
DNAJB13	NM_153614	nd[4]		
DNAJB14	NM_024920	0.87	0.04	
DNAJC1	NM_022365	1.17	0.23	
DNAJC2	NM_014377	0.89	0.06	zuotin related factor 1 (ZRF1)
DNAJC3	NM_006260	0.97	0.18	
DNAJC4	NM_005528	0.99[4]	0.10	
DNAJC5	NM_025219	nd[4]		cysteine string protein (CSP)
DNAJC5B	NM_033105	nd[4]		cysteine string protein beta (CSP-beta)
DNAJC5G	NM_173650	1.053	0.07	
DNAJC6	NM_014787	0.873	0.18	
DNAJC7	NM_003315	1.01	0.15	

DNAJC8	NM_014280	0.92	0.06	
DNAJC9	NM_015190	0.98	0.10	
DNAJC10	NM_018981	1.11	0.24	
DNAJC11	NM_018198	1.12	0.14	
DNAJC12	NM_021800	1.05	0.19	
DNAJC13	NM_015268	0.99	0.17	
DNAJC14	NM_032364	1.08	0.16	
DNAJC15	NM_013238	0.68	0.24	
DNAJC16	NM_015291	1.09	0.10	
DNAJC17	NM_018163	1.04	0.11	
DNAJC18	NM_152686	0.99	0.14	
DNAJC19	NM_145261	0.99	0.13	
DNAJC20	NM_172002	1.07[3]	0.12	J-type co-chaperone HSC20 (RP3-366L4.2)
DNAJC21	NM_194283	0.79	0.18	DnaJA5
DNAJC22	NM_024902	1.06	0.08	hypothetical protein FLJ13236
DNAJC23	NM_007214	0.98	0.08	SEC63
DNAJC24	NM_181706	0.87	0.11	ZCSL3
DNAJC25	NM_001015882	0.99	0.08	DnaJ-like protein (bA16L21.2.1)
DNAJC26	NM_005255	1.07	0.19	cyclin G associated kinase (GAK)
DNAJC27	NM_016544	0.98	0.10	Ras-associated protein Rap1 (RBJ)
DNAJC28	NM_017833	0.73[3]	0.18	C21orf55
DNAJC29	NM_014363	0.93	0.04	sacsin
DNAJC30	NM_032317	1.04	0.06	WBSCR18

HSPB (sHsp) family				
HSPB1	**NM_001540**	**0.29**	**0.13**	**Hsp27**
HSPB2	NM_001541	nd[4]		MKBP
HSPB3	NM_006308	nd[4]		
HSPB4	NM_000394	nd[4]		αA-crystallin (CRYAA)
HSPB5	NM_001885	0.99[3]	0.18	αB-crystallin (CRYAB)
HSPB6	NM_144617	1.04[3]	0.25	Hsp20
HSPB7	NM_014424	nd[4]		cvHsp
HSPB8	NM_014365	nd[4]		HSP22
HSPB9	NM_033194	0.68	0.20	
HSPB10	NM_024410	nd[4]		ODF1

others				
HSPD1	NM_002156	0.88	0.17	Hsp60, chaperonin
HSPE1	NM_002157	0.73	0.08	Hsp10, chaperonin 10
SERPINH1	NM_001235	0.55	0.08	Hsp47
CCT3	NM_005998	0.67	0.17	TCP1, subunit 3 (gamma)

co-chaperones				
AHSA1	NM_012111	0.63	0.07	AHA1 homolog 1
AHSA2	NM_152392	0.51	0.04	AHA1 homolog 2
BAG1	NM_004323	1.03	0.16	
BAG2	NM_004282	1.10	0.13	
BAG3	NM_004281	1.31	0.18	
BAG4	NM_004874	1.283	0.43	
BAG5	NM_001015049	0.99	0.17	
PTGES3	NM_006601	0.88	0.14	p23
ST13	NM_003932	0.63	0.08	HIP
STIP1	NM_006819	0.53	0.06	HOP
STUB1	NM_005861	0.97	0.06	CHIP
AIP	NM_003977	0.94	0.21	
CDC37	NM_007065	nd[4]		
FKBP4	NM_002014	1.00	0.23	
FKBP5	NM_004117	0.98	0.07	
PPID	NM_005038	0.97	0.08	cyclophilin D
PPP5C	NM_006247	1.21	0.30	
SGTA	NM_003021	1.14	0.24	
TOMM70A	NM_014820	1.11	0.25	
TTC4	NM_004623	1.00	0.04	
UNC45A	NM_018671	0.99	0.07	

[1] the array oligonucleotides do not discriminate between the transcripts of these two genes.

[2] none of the oligonucleotides on the array hybridize with the transcript of this gene

[3] the hybridization signal was significant but below 100

[4] the hybridization signal was not significant

Table 3. Non-chaperone encoding genes downregulated by dnHSF1.

Gene name	Acc. nr.	dnHSF1/ Ctrl		Description
		Ave	SD	
PMVK	NM_006556	0.21	0.07	phosphomevalonate kinase
KLRG1	NM_005810	0.35	0.14	killer cell lectin-like receptor subfamily G, member 1
CDKL3	NM_016508	0.39	0.17	cyclin-dependent kinase-like 3
KA21	NM_152349	0.41	0.32	truncated type I keratin KA21
ZNF473	NM_015428	0.48	0.07	zinc finger protein 473
MLH1	NM_00249	0.50	0.17	mutL homolog 1

Transcriptome changes in the presence of dnHSF1

If HSF1 plays a role even in the absence of exogenous stress, then exogenous expression of a dominant negative HSF1 mutant in unstressed cells should change the transcriptome. We therefore compared the transcriptomes of HEK cells with or without doxycycline and with or without dnHSF1using a two-color 44K Agilent Human Expression Profile Array. The transcripts of only 10 genes showed a more than two fold lower level in the presence of dnHSF1 (Table 2 in bold and Table 3). Four of these, namely HSPA6 (hsp70B'), HSP90AA1 (Hsp90), DNAJB1 (Hsp40) and HSPB1 (Hsp27), encode chaperones (Table 2 in bold). The steady state level of the corresponding proteins was also reduced in dnHSF1 expressing cells (Fig. 3; note that the HSPA6 mRNA level is very low in non-stressed HEK293 cells; [231]). Surprisingly, there was a distinct difference between dnHSF1 expressing cells and mouse embryonic fibroblasts lacking HSF1: the hsf-/hsf- MEFs contain wild type levels of Hsp90 and DNAJB1.

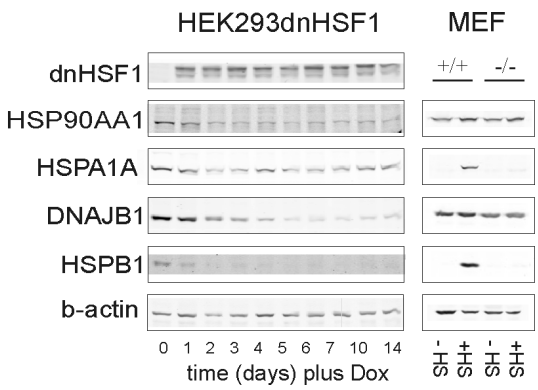

Fig. 3 Left panel. The decay of heat shock protein levels during expression of dnHSF1. HEK-HSF379 cells were treated with doxycyclin for the time indicated and harvested. Right panel. The level of heat shock proteins in MEF wild type cells (+/+) and MEF cells lacking HSF1 (-/-) either before (-HS) or after heat shock and recovery (+HS). Cell lysates were subjected to SDS-PAGE and western blot analysis using the indicated antibodies.

The levels of the transcripts of a number of other chaperone genes did not quite meet the "two fold" lower in the presence of dnHSF1 cut-off, but did come close (AHSA2 for example; Table 2). To test whether HSF responsiveness is a general property of genes encoding (co-)chaperones, we looked at the response of all known members of the HSP gene families (HSPH, HSPA, DNAJ and HSPB) as well as other known (co-) chaperones coding genes expressed in HEK 293 cells (Table 2). Of the HSPA (Hsp70) genes, only HSPA6 responded strongly to dnHSF1. Similarly, very few members of the large DNAJ (Hsp40) family were downregulated by HSF1. This is rather surprising as the DNAJ proteins determine the substrate specificity of and stimulate the activity of the Hsp70 folding machine and are thus critical nodes in the chaperoning network of the cell. Also most of the Hsp70 and Hsp90 co-chaperones are not responsive to dnHSF1. For example, of the 14 Hsp90 co-factors listed in a recent review [232], only the two AHA1 homologs as well as STIP1 and, to a lesser extent, ST13, responded strongly to dnHSF1 (Table 2).

To confirm the effect of HSF1 on the promoter activity of some of the genes downregulated by dnHSF1, we isolated the promoters and compared their activities in HEK-dnHSF1 cells and HEK-cDNA5 cells. The promoters of the STIP1, ST13, DNAJA1, DNAJB1 (see Table 2), and PMVK (selected because it is the strongest downregulated non-chaperone gene, Table 3) genes had significantly reduced activities in HEK-dnHSF1 cells compared with control cells, whereas the promoters of the unfolded protein response target genes CHOP and BiP, two genes with similar expression levels in HEK-dnHSF1 and control cells, were not or only slightly affected by dnHSF (Fig. 4).

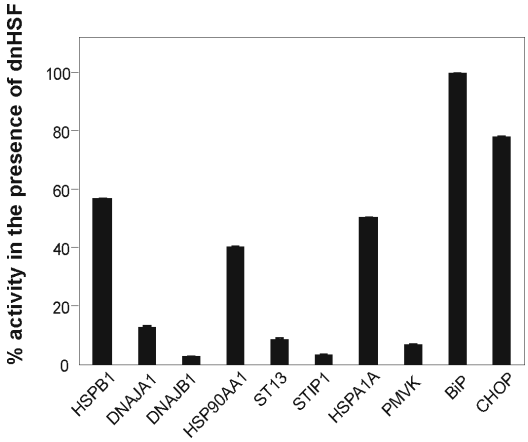

Fig. 4 Inhibition of promoter activity by dnHSF1. Control HEK-cDNA5 cells and HEK-HSF379 cells were treated with doxycyclin. After 3 days, cells were transfected with the indicated promoter reporter constructs (see also Materials and Methods) and a βactin-βgal reporter. At 48 h after transfection, cells were harvested and assayed for reporter gene activities. Promoter activities were determined by dividing luciferase values by the corresponding β-galactosidase values to correct for varying transfection efficiencies. The bars correspond to the % activity of the promoter in the HEK-HSF379 cells compared with the control HEK-cDNA5 cells. The results are the average of three independent transfections (standard deviations are indicated by error bars).

Note that these promoter activities were measured in unstressed cells, explaining why the activity of the promoters of the canonical heat stress inducible HSPA1A (Hsp70) gene is only inhibited by about 50%; note also that the activities of isolated promoter regions do not necessarily reflect the activity of the endogenous promoter which could also be controlled by chromatin structure and/or elements lacking from the isolated promoter region. The HSPB1 gene for example has been reported to have heat shock elements in its first intron as well [233].

Lack of heat shock proteins could cause stress in the cells, which in turn could activate a non-HSF dependent stress response (see also [29]). To determine whether exogenous expression of dnHSF1 caused stress we determined whether expression of dnHSF1 is associated with an increased level of phosphorylated eIF2α. Activation of eIF2α kinases is a common response to a variety of stresses (for review, see [107]). As shown in Figure 5, the basal level of eIF2α phosphorylation is not increased by the expression of dnHSF1. In addition, the decay of eIF2α phosphorylation after a heat shock is not notably affected by expression of dnHSF1 (Fig. 5). This is in accordance with previous reports showing that cells lacking HSF1 are not impaired in their ability to recover from heat stress but do not built up thermostability after a heat stress [211,234].

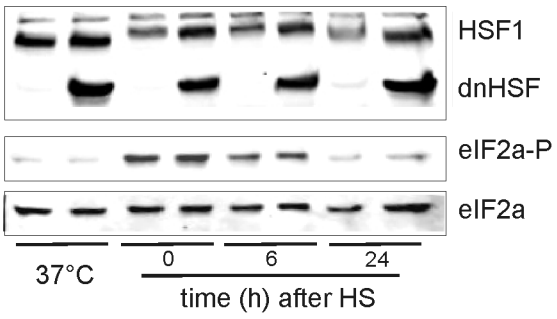

Fig. 5 The effect of exogenous expression of dnHSF1 on eIF2α phosphorylation. HEK-cDNA5 cells and HEK-HSF379 cells were treated with doxycyclin for 48 h. Cells were then exposed to a heat shock of 30' at 45°C (HS) or left at 37°C (37°C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Cell lysates were subjected to SDS-PAGE and western blot analysis using the indicated antibodies.

Glucocorticoid signalling is impaired by dnHSF1 and can be rescued by individual co-chaperones

Expression of dnHSF1 depletes the cell of a number of chaperones and is predicted to decrease the activity of both the Hsp70 and the Hsp90 folding machine. Both are known to be important for maturation and function of steroid hormone receptors (reviewed in [223], [235]) and we thus examined whether expression of dnHSF1 resulted in impaired glucocorticoid hormone signalling. A synthetic glucocorticoid-responsive element (GRE) was linked to a luciferase reporter and used to monitor the response of

HEK-dnHSF1 and HEK-cDNA5 cells to increasing concentrations of dexamethasone. Dexamethasone inducibility of the GRE was at least 50% inhibited in HEK-dnHSF1 cells compared with HEK-cDNA5 cells (Fig. 6). At 10^{-6} M dexamethasone, activity of the GRE was induced by 9-fold in HEK-cDNA5 cells and only by 4-fold in HEK-dnHSF1 cells, and at the highest concentration of dexamethasone the inducibility in HEK-cDNA5 cells was even 13-fold compared with only 5-fold in HEK-dnHSF1 cells.

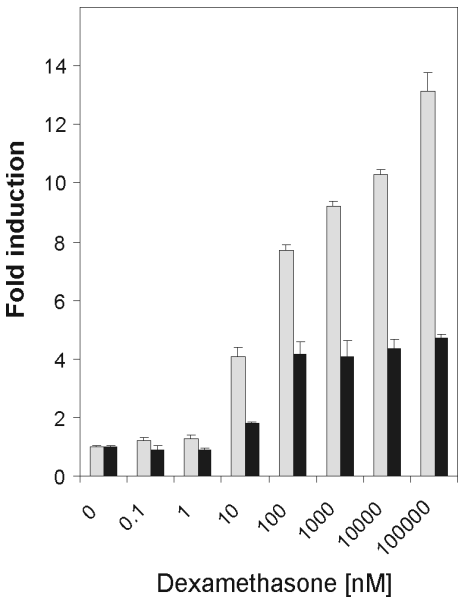

Fig. 6 Exogenous expression of dnHSF1 reduces the glucocorticoid response. Control HEK-cDNA5 cells and HEK-HSF379 cells were treated with doxycyclin. After 3 days, cells were transfected with a glucocorticoid-responsive luciferase reporter (pGRE-Luc) and a βactin-βgal reporter. At 24 h after transfection, cells were either left untreated or exposed to the indicated concentrations of dexamethasone. At 48 h after transfection, cells were harvested and assayed for reporter gene activities. Promoter activities were determined by dividing luciferase values by the corresponding β-galactosidase values to correct for varying transfection efficiencies. The bars correspond to the activity of the glucocorticoid-responsive promoter in the presence of dexamethasone compared to the activity in untreated cells, which was set at 100%. Gray bars show the results for control HEK-cDNA5 cells; black bars those for HEK-HSF379 cells. The results are the average of three independent transfections (standard deviations are indicated by error bars).

If the impaired dexamethasone inducibility in the presence of dnHSF1 is due to a reduction in the expression levels of one or more (co-)chaperone genes, then it should be possible to rescue the glucocorticoid inducibility of the GRE in HEK-dnHSF1 cells by exogenous expression of those (co)-chaperones. We therefore tested the effect of exogeneous expression of different proteins on the glucocorticoid response of the pGRE-Luc reporter in HEK-dnHSF1 cells (Figs. 7 and 8). The chaperone of which the expression is most effected by dnHSF1 is HSPB1. Although HSPB1 is not directly involved in the maturation of the glucocorticoid receptor, its lack may cause overloading of part of the folding network of the cell. However, exogenous expression of HSPB1 or of another sHsp, HSPB8, had no effect (Fig. 7). The level of Hsp90 is also affected by dnHSF1 but is apparently not limiting in the glucocorticoid response, as exogenous expression of Hsp90 was even inhibitory (Fig. 7). PTGES3 (p23)

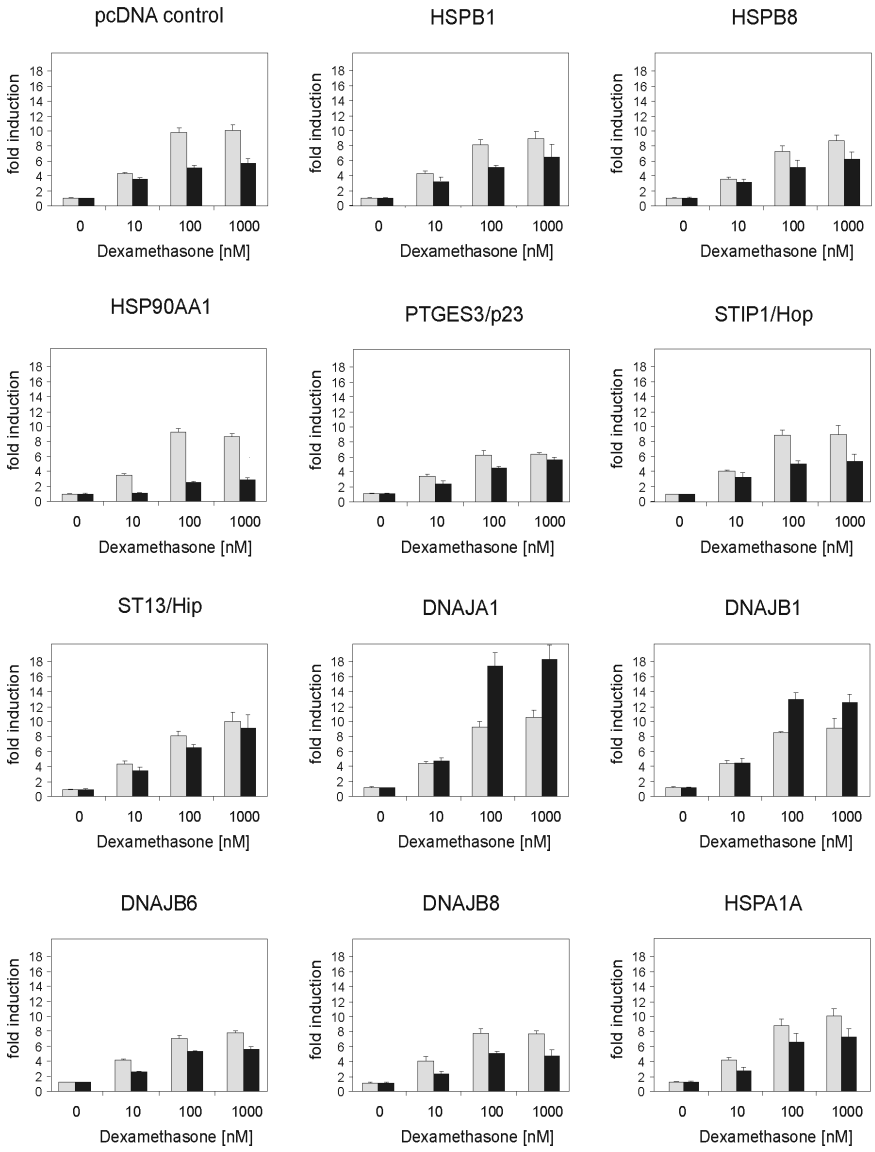

Fig. 7 Effect of over-expression of (co)chaperones on glucocorticoid signaling in HEK-cDNA5 and HEK-dnHSF1 cells. Control HEK-cDNA5 cells (light gray bars) and HEK-HSF379 cells (black bars) were treated with doxycyclin. After 3 days, cells were transfected with a mixture (4:1:5) of glucocorticoid-responsive luciferase reporter (pGRE-Luc), a βactin-βgal reporter, and the expression construct indicated in the Figure. At 24 h after transfection, cells were either left untreated or exposed to the indicated concentrations of dexamethasone. At 48 h after transfection, cells were harvested and assayed for reporter gene activities. Relative luciferase activities and -fold induction were determined as described in the legend to Figure 6. Standard deviations are indicated by the error bars.

inhibited the GRE response in HEK-cDNA5 cells (Table 4) as previously reported [66,236] but increased it slightly in HEK-dnHSF1cells. STIP1 (Hop), which is a co-

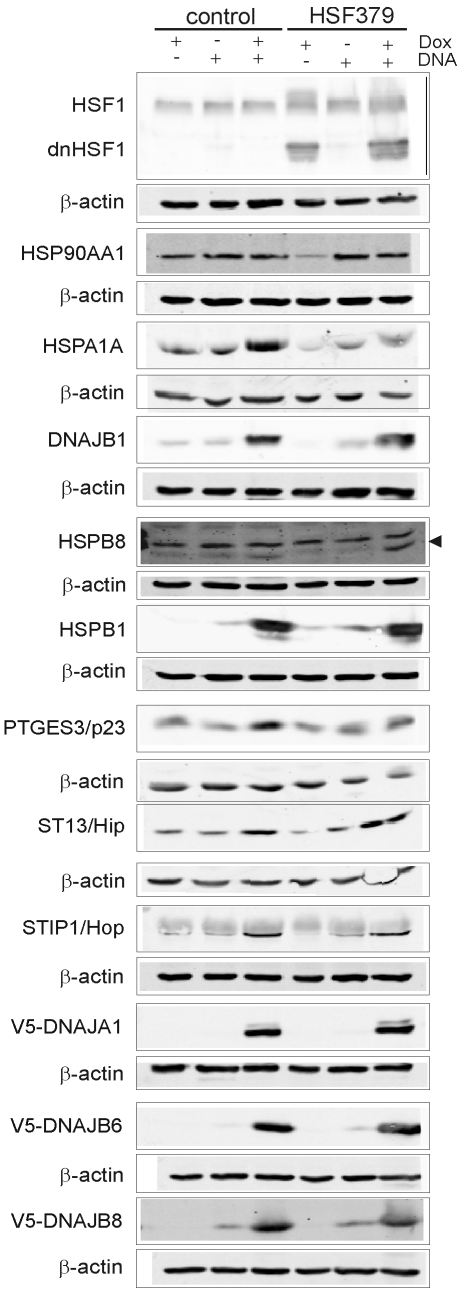

Fig. 8 Levels of exogenous expression of (co) chaperones. Expression plasmids for the (co) chaperones indicated on the left were transfected into either HEK-cDNA cells (control) or HEK-HSF379 cells (+DNA) and expression was induced by adding doxycyclin (+Dox), except for HSP90AA1, of which expression is constitutive. Protein levels were determined by western blotting and staining with the corresponding antibody (see Materials and Methods). The arrowhead indicates HSPB8. Note that in the case of DNAJA1, DNAJB6 and DNAJB8 antibody to the V5-tag carried by the exogenous proteins was used; the endogenous protein is thus not detected. β-actin was used as a loading control.

chaperone of Hsp90 as well as of Hsp70 had no effect, either in HEK-cDNA5 (Table 4) or in HEK-dnHSF cells (Fig. 7). In contrast, ST13 (Hip), an Hsp70 co-chaperone, did restore dexamethasone inducibility to almost the wild type level in HEK-dnHSF cells. Even more effective was exogenous expression of the Hsp70 co-chaperones DNAJA1 (HDJ2) or DNAJB1 (Hsp40): this resulted in even higher dexamethasone inducibility in HEK-dnHSF1 cells compared with HEK-cDNA5 cells (Fig. 7). The rescue effect of DNAJA1 and DNAJB1 was not a general property of Hsp40 family members, since two other members of the DNAJB family, DNAJB6 and DNAJB8, did not show any rescue activity (Fig. 7). Expression of Hsp70 (HSPA1A) itself had no effect (Fig. 7; note that neither overexpression of DNAJ proteins nor overexpression of HSPA1A in HEK-cDNA5 cells affected the GRE response, see Table 4). These data show that it is the primary folding

of the glucocorticoid receptor by the Hsp70 machinery that is most affected in HEK-dnHSF1 cells. As predicted by the wild-type level of DNAJB1 in *hsf1-/hsf1-* MEFs, these cells showed a wild-type glucocorticoid response (data not shown).

Table 4. Relative effect of exogenous expression of (co)-chaperones on glucocorticoid signaling in HEK-cDNA5 cells.

Gene name	**Dexamethasone (nM)**		
	10	100	1000
(co)-chaperones/ control			
HSPB1	1.0 + 0.1	0.8 + 0.1	0.9 + 0.2
HSPB8	0.8 + 0.1	0.7 + 0.1	0.8 + 0.1
HSP90AA1	1.1 + 0.3	0.9 + 0.2	0.9 + 0.3
PTGES3	0.8 + 0.1	0.7 + 0.1	0.6 + 0.1
STIP1	1.0 + 0.1	0.9 + 0.1	0.9 + 0.2
ST13	1.1 + 0.1	0.9 + 0.1	1.1 + 0.2
DNAJA1	1.0 + 0.1	1.0 + 0.1	1.1 + 0.1
DNAJB1	1.0 + 0.1	1.0 + 0.1	1.0 + 0.2
DNAJB6	0.9 + 0.1	0.8 + 0.1	0.8 + 0.1
DNAJB8	0.9 + 0.2	0.9 + 0.1	0.8 + 0.1
HSPA1A	0.8 + 0.2	0.8 + 0.2	1.0 + 0.4

Discussion

Comparison of the transcriptome of embryonic fibroblasts from HSF1 null mice with that of wild type cells identified 49 genes (19 related to immune response) that were not upregulated by a heat shock in wild type cells but nevertheless were expressed at reduced levels in HSF1 null fibroblasts [18]. When HSF1 was depleted by RNA interference in HeLa cells, the expression level of 378 genes changed significantly in the absence of stress [237]. The main effect, surprisingly, was an increase in expression, for 80% of the affected genes, the transcript level increased. In contrast, we found no significant increase in expression in response to dnHSF1; dnHSF1 reduced the expression level of only 10 genes more than two-fold, with a lesser effect on a number of chaperone encoding genes (Tables 2 and 3). The difference between the effect of depleting HSF1 in MEFs and HeLa cells is very likely to be caused by the far greater dependence of

transformed cells on HSF1 [178]. HEK293 are less dependent on HSF1 than HeLa cells [178], but more so than MEFs. The response to blocking HSF1 in HEK293 cells might then be expected to be intermediate in the effect on the transcriptome but it is not. Clearly there is a difference between depleting HSF1 and expressing a dominant negative mutant. In part this difference may be due to a secondary effect: depletion of HSF1 would free the chaperones which are usually complexed with HSF1 while dnHSF1 might capture more chaperones. More importantly is probably the activity ofHSF1 as a repressor of transcription. Recently, it has been shown that HSF1 binds to MTA1, a co-repressor, to form a complex repressing estrogen-dependent transcription in breast carcinoma cells [214]. Similarly, HSF1 has been reported to interact with C/EBPβ, an interaction which represses transcriptional activation [238]. The loss of HSF1 would release repression; expression of dnHSF1 could maintain it.

Expression of dnHSF1 is an efficient way of reducing the chaperoning capacity of the cell, as evidenced by the loss of the basal glucocorticoid response. Since the expression of so many genes playing roles at several stages of glucocorticoid receptor processing was suppressed in HEK-dnHSF1 cells, we did not expect that over-expression of individual proteins would rescue the glucocorticoid response. Nonetheless, the individual co-chaperones DNAJA1, DNAJB1 and ST13/Hip were able to rescue the dnHSF-mediated inhibition of the glucocorticoid response fully; PTGES3/p23 had some effect, whereas over-expression of Hsp90, or STIP1/Hop had no effect. Hsp90 was even inhibitory (Fig. 7). Both DNAJ and ST13/Hop are co-chaperones of Hsp70 and function in the primary folding of the glucocorticoid receptor, but at different levels: DNAJ activates the ATPase of Hsp70, whereas ST13/Hip stabilizes the Hsp70-ADP state (reviewed by [223]). Apparently over-expression of DNAJA1 or DNAJB1 can compensate for a shortage of ST13/Hip and vice versa, as exogenous expression of either protein restores glucocorticoid sensitivity. Together these data show that the limiting node of chaperoning network in dnHSF1 expressing cells is the Hsp70 folding machine, which is in turn is limited not by the level of Hsp70 itself, but rather by its co-chaperones. In vitro folding studies of the glucocorticoid receptor have shown that DNAJB1 is required in catalytic amounts [239]. Our data also show that a lack of DNAJB1 can be compensated for by overexpression of DNAJA1. Functional redundancy between DNAJB1 and another co-chaperone is also implied by the lack of a phenotype of the DNAJB1 knock-out mouse, which has only a minor deficiency in acquired thermotolerance [240]. In the case of the progesterone receptor it has been shown that either DNAJA1 or DNAJB1 can assist in folding but by distinct mechanisms. DNAJA1 bound tightly to the progesterone receptor while DNAJB1 did so only transiently [241].

Heat stress or expression of a dominant positive HSF1 mutant potentiates the

glucocorticoid response [224,225] suggesting that the chaperone network is limiting for this response in normal cells. The chaperone network is also limiting for luciferase refolding as this can be boosted by overexpressing Hsp70, an effect which can be blocked by expressing a dominant negative DNAJB1 mutant [242]. In contrast, exogenous expression of single (co)chaperones did not enhance the sensitivity of HEK-cDNA cells to dexamethasone, indicating that, unlike luciferase refolding, it is either a combination of chaperones and co-chaperones that is limiting or that other proteins are involved. In addition, exogenous expression of a dominant negative DNAJB1 mutant did not block the dexamethasone response significantly (data not shown).

Maintaining proteostasis during aging is expected to prevent or at least ameliorate age-related protein folding and inflammatory disease [131,222]. One possible approach is to prevent the decline in HSF1 activity either by targeting HSF1 directly or by targeting longevity related factors which control HSF1 activity such as SIRT1 [51]. One potential drawback of this approach is that HSF1 also increases the risk of cancer, also an often age-related disease [178]. An alternative is to maintain the capacity of the chaperoning network by boosting a single (co)chaperones. The results reported here show that DNAJA1 and DNAJB1 are promising targets. The finding that MEF cells do have wild-type levels of DNAJB1 in the absence of HSF1 shows that HSF1 can be bypassed in the transcriptional regulation of the DNAJB1 gene.

Acknowledgements

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Chapter 3

Manipulating heat shock factor-1 in *Xenopus* tadpoles: neuronal tissues are refractory to exogenous expression

Ron P. Dirks, Remon van Geel, Sanne M.M. Hensen, Siebe T. van Genesen, Nicolette H. Lubsen

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Background: The aging related decline of heat shock factor-1 (HSF1) signaling may be causally related to protein aggregation diseases. To model such disease, we tried to cripple HSF1 signaling in the *Xenopus* tadpole.
Results: Over-expression of heat shock factor binding protein-1 did not inhibit the heat shock response in *Xenopus*. RNAi against HSF1 mRNA inhibited the heat shock response by 70% in *Xenopus* A6 cells, but failed in transgenic tadpoles. Expression of XHSF380, a dominant-negative HSF1 mutant, was embryonic lethal, which could be circumvented by delaying expression via a tetracycline inducible promoter. HSF1 signaling is thus essential for embryonic *Xenopus* development. Surprisingly, transgenic expression of the XHSF380 or of full length HSF1, whether driven by a ubiquitous or a neural specific promoter, was not detectable in the larval brain.
Conclusions: Our finding that the majority of neurons, which have little endogenous HSF1, refused to accept transgene-driven expression of HSF1 or its mutant suggests that HSF1 levels are strictly controlled in neuronal tissue.

Introduction

In healthy cells, accumulation of abnormally folded proteins in the cytoplasm results in the activation of a stress response system, the heat shock response (HSR). The HSR is essential for maintaining proteostasis. Mouse knockout models have shown that heat shock transcription factor-1 (HSF1) is the key regulator of the HSR [207,210,211]. Under normal physiological conditions, HSF1 is thought to exist as part of an inactive hetero-complex that also includes Hsp90, p23 and immunophilin. Exposure of cells to various stressors results in trimerization and hyperphosphorylation of HSF1, followed by binding of the active trimer to heat shock elements in the promoters of heat shock protein genes and subsequent transcription activation (reviewed by [17]). The expression level and thermostability of HSF1, as well as its affinity for heat shock elements are significantly decreased in aged cells compared with young cells, resulting in low efficiency of the HSR ([156]). As the HSR is already poorly developed in healthy neurons, these cells are particularly vulnerable to damage resulting from decreased activity of HSF1 (reviewed by [243]). Restoring the activity of HSF1 or bypassing the crippled HSF1 may protect the aging cell against toxic protein aggregates [201,220], see also [131,196]. High throughput screens have already resulted in novel compounds that directly or indirectly affect the HSR in cell culture systems (reviewed by [244]). A number of *in vivo* model systems have been described in which the role of a failing HSR in the etiology of neurological diseases can be studied. Mice carrying null mutant HSF1 genes or expressing a dominant-negative HSF1 are attractive model systems, because

the mouse brain closely resembles the human brain; however, small offspring and high costs make rodents less attractive for large scale studies. Invertebrates, such as *C. elegans* and *Drosophila*, are particularly suitable for high throughput experiments, because they have a large offspring and can be easily manipulated. Unfortunately, their nervous system differs considerably from the human brain. Simple vertebrates, such as *Xenopus* and *Danio*, are attractive *in vivo* model systems to study many aspects of human neurological diseases. In addition to the large offspring and low maintenance costs, the basic anatomy of the fish and amphibian brain is highly similar to that of the mammalian brain. Furthermore, tadpoles and zebrafish are translucent, allowing live image analysis of fluorescently labeled proteins. Microinjection of antisense oligonucleotides has already been used to transiently inhibit the expression of HSF1 in zebrafish. This resulted in increased heat shock-induced apoptosis ([245]), reduced basal expression levels of Hsp70 and abnormal eye development ([246]). To be able to study the long term effects of decreased HSF1 activity in neurons and perform high throughput experiments, it would be desirable to develop a model in which the expression of HSF1 is stably inhibited. In this study, we examined if we could mimic the aging-associated decline of the HSR in a stable manner by crippling endogenous HSF1 in *Xenopus* tadpoles. Since a technique for targeted mutagenesis of endogenous genes in *Xenopus laevis* is not (yet) available, we tried to manipulate *Xenopus* HSF1 via three alternative strategies: over expression of heat shock factor binding protein-1 (HSBP1), reported to be a natural inhibitor of HSF1 [247], over expression of a dominant-negative mutant of HSF1, and stable transgene-driven RNA interference (RNAi) directed against HSF1 mRNA. Our surprising finding is that *Xenopus* tadpole brain is largely refractory to exogenous expression of HSF1.

Materials and Methods

Ethics statement

Animal experiments were carried out in accordance with the European Communities Council Directive 86/609/EEC for animal welfare, and were approved by the Radboud University Animal Experimentation Committee (permit TRC 99 / 15072 to generate and house transgenic *Xenopus*).

Animal care

Female South-African claw-toed frogs (*Xenopus laevis*) were obtained from *Xenopus* Express (Cape Town, South-Africa) and kept in water tanks at 18°C at the Central Animal Facility of the Radboud University Nijmegen. Ovulation was induced by

injection of 500 units hCG (Pregnyl; Organon) into the dorsal lymph sac.

DNA constructs

Oligonucleotides that were used to generate recombinant DNA constructs are listed in the on line supplementary information (Table S1). New vectors were constructed to express proteins with N- or C-terminally fused GFP in *Xenopus* A6 cells and tadpoles. The chimeric enhancer/promoter of the *Xenopus* elongation factor-1α (EF1α) gene was PCR amplified from pEF-GFP3 (kindly donated by Dr. Paul Krieg; [257]) using the EF1a primer set, cut with *Sal*I and *Hin*dIII and used to replace the CMV promoter of the *Xenopus* vector pCS2+ ([258]), resulting in pEF2+. The code for GFPdelAUG was PCR amplified from pIRES2-EGFP (Clontech) using the GFPdelAUG primer set, and cloned into the *Bam*HI and *Xba*I sites of pEF2+, yielding pEF-GFPdelAUG. Similarly, the code for GFPdelSTOP was amplified from pIRES2-EGFP using the GFPdelSTOP primer set, and cloned into the *Bam*HI and *Xho*I sites of pEF2+, yielding pEF-GFPdelSTOP. Bicistronic expression vectors, based on viral 2A peptides, were generated as follows: pEF-GFP-T2A-delSTOP was made by annealing the T2A primer set, and cloning the double stranded oligo into the *Eco*RI and *Bgl*II sites of pEF-GFPdelSTOP. pEF-F2A-delAUG-GFP was made by annealing the F2A primer set, and cloning the double stranded oligo into the *Eco*RI and *Xho*I sites of pEF-GFPdelAUG. Adult *Xenopus laevis* brain mRNA was used as a source for XHSBP1, XHSF and XHSF380 cDNA. Total RNA was isolated using the RNeasy kit (Qiagen) and copied into cDNA using the Marathon kit (Clontech). The 243-bp XHSBP1 cDNA was PCR amplified from the *Xenopus* brain cDNA library using the XHSBP1 primer set, and cloned into the *Eco*RI and *Xho*I sites of pEF-GFPdelSTOP, yielding pEF-GFP-XHSBP1. To express native protein, the XHSBP1 cDNA was also cloned into the *Eco*RI and *Xho*I sites of pEF-GFP-T2A-delSTOP, resulting in pEF-GFP-T2A-XHSBP1. The 1143-bp XHSF380 cDNA was PCR amplified from the *Xenopus* brain cDNA library using the XHSF380-C primer set, corresponding to GenBank sequence BC087308 from the NIH *Xenopus* initiative ([259]). XHSF380 was cloned into the *Eco*RI and *Xho*I sites of pEF-GFPdelSTOP, yielding pEF-GFP-XHSF380. In parallel, the XHSF380 cDNA was PCR amplified using the XHSF380-N primer set, cut with *Bgl*II and *Eco*RI, and cloned into the *Bam*HI and *Eco*RI sites of pEF-GFPdelAUG, resulting in pEF-XHSF380-GFP. To drive strong ubiquitous expression of XHSF380-GFP and GFP-XHSF380, the EF1α promoter was replaced with the CMV promoter, resulting in pCMV-GFP-XHSF380 and pCMV-XHSF380-GFP. To drive neuron-specific expression, the EF1α promoter was replaced with the neural β-tubulin (Ntub) promoter, resulting in pNtub-GFP-XHSF380 and pNtub-XHSF380-GFP. Inducible expression constructs were generated by PCR amplifying the TetO promoter from pCS2+[tetO]::GFP using the TetO primer set,

cutting the PCR fragment with *Sal*I and *Hin*dIII and replacing the EF1α promoter with the TetO promoter, resulting in pTetO-GFP-XHSF380 and pTetO-XHSF380-GFP. The Tet-activator plasmid pCS2+rtTA2A-M2 and reporter plasmid pCS2+[tetO]::GFP were kindly donated by Dr. Biswajit Das ([253]). Full length pCMV-GFP-XHSF1 was made by amplifying the 3' part of the XHSF1 code from the *Xenopus* brain cDNA library using the XHSF1 primer set, cutting the PCR fragment with *Eco*RV and *Xho*I and replacing the truncated *Eco*RV-*Xho*I fragment of pCMV-GFP-XHSF380 with the full length fragment.

The bicistronic reporter construct pEF-GFP-T2A-DsRed2 was made by amplifying the code for DsRed2 from pDsRed2-N1 (Clontech) using the DsRed2-C primer set, cutting the PCR fragment with *Bam*HI and *Xho*I, and cloning it into the *Bgl*II and *Xho*I sites of pEF-GFP-T2AdelSTOP. Similarly, pEF-DsRed2-F2A-GFP was made by PCR amplifying the code for DsRed2delSTOP from pDsRed2-N1 using the DsRed2-N primer set, and cloning the PCR fragment into the *Bam*HI and *Eco*RI sites of pEF-F2A-delAUG-GFP. The Hsp70-luciferase reporter construct pHL was described earlier ([228]).

For the purpose of inducing stable transgene-driven RNAi, new vectors were constructed that drive the synthesis of short hairpin RNAs (shRNAs) from an H1 RNA promoter. The human H1 RNA promoter was PCR amplified from genomic DNA using the H1 primer set, cut with *Sal*I and *Hin*dIII and used to replace the CMV promoter of pCS2+, resulting in pH1. To facilitate identification of transgenic tadpoles, a Cac-GFP-tkpolyA cassette, driving expression of the GFP reporter protein from the muscle-specific cardiac actin (Cac) promoter, was cloned into the *Not*I site of pH1, resulting in pH1CG2+. The *Xenopus laevis* RNAse P RNA (accession number X56558) and human H1 RNA sequences were used in a BLAST search of the *Xenopus tropicalis* genome (http://genome.jgi-psf.org), resulting in the identification of multiple copies of the *Xenopus* H1 RNA gene. Based on the alignment of six intact copies of the gene, the XtH1 primer set was designed and used to PCR amplify a *Xenopus tropicalis* H1 RNA promoter (XtH1). The human H1 RNA promoter in the pH1CG2+ vector was replaced with the XtH1 promoter using *Sal*I and *Hin*dIII, resulting in the pXtH1CG2+ vector.

Target sequences for RNAi-mediated decay of XHSF1 mRNA were chosen according to http://www.promega.com/siRNADesigner (Promega) and https://rnaidesigner.invitrogen.com/rnaiexpress (Invitrogen). pH1CG2+XHSF-sh1 was made by annealing of the XHSF-sh1a primer set, and cloning the double stranded oligo into the *Bgl*II and *Hin*dIII sites of pH1CG(A)2+. pXtH1CG2+XHSF1-sh1, -sh2 and –sh3 were made by annealing the XHSF-sh1, -sh2, and -sh3 primer sets, respectively, and cloning the double stranded oligos into the *Bsp*EI and *Hin*dIII sites of pXtH1CG2+.

The control plasmid pEF-GFP was made as follows: GFP cDNA was excised from pIRES2-EGFP (BD Sciences-Clontech) using *Msc*I and *Not*I (filled-in), introduced into the *Eco*RV site of pBluescript SK-, then excised again using *Hin*dIII (filled) and *Eco*RI and introduced into the *Stu*I and *Eco*RI sites of pEF2+.
All plasmids were sequence verified.

Xenopus transgenesis

All expression cassettes, consisting of promoter, cDNA and polyadenylation signal, were excised from their plasmid vector backbones using *Sal*I and *Not*I, separated by agarose gel electrophoresis, and recovered from agarose slices using the GFX gel band purification kit (Amersham). Transgenesis of *Xenopus laevis* was performed according to Kroll and Amaya (1996)[260], with modifications [261]. In summary: 250,000 sperm nuclei were mixed with ~200 ng DNA fragment, incubated for 15 min at room temperature and diluted in 500 µl sperm dilution buffer (250 mM sucrose, 75 mM KCl, 0.5 mM spermidine trihydrochloride, 0.2 mM spermidine tetrahydrochloride, 5 mM MgCl2, pH 7.4). Eggs were dejellied in 2% cystein/1 x MMR (1 x MMR: 0.1 M NaCl, 0.02 M KCl, 0.01 M $MgCl_2$, 0.015 M $CaCl_2$ en 0.5 M HEPES pH 7.5), transferred to 6% Ficoll/0.4 x MMR and injected with 10 nl of the diluted nuclei/DNA mixture at 17°C. At the 4-cell stage, the embryos were transferred to 6% Ficoll/0.1 x MMR and incubated overnight at 17°C. At the gastrula stage, the embryos were transferred to 0.1 x MMR and incubated at 22°C. GFP-positive tadpoles were photographed using a MZ FLIII fluorescence stereomicroscope provided with a DC200 camera (Leica microsystems, Switzerland).

Western blot analysis

Tadpoles or A6 cell pellets were snap frozen in liquid nitrogen and homogenized in 100 µl SDS-PAGE sample mix. Protein samples (25 µl) were separated in 12% polyacrylamide gels and transferred to nitrocellulose transfer membrane (Protran, Schleicher and Schuell) using a Biorad Mini-PROTEAN II Electrophoresis cell according to the manufacturer's instructions (Biorad). For western blot analysis, monoclonal α-GFP antibody (Cat. no. 632375; Clontech) was used at a 1:5000 dilution. Monoclonal α-tubulin antibody (kindly donated by Mr. Huib Croes, Dept. of Cell Biology, NCMLS, Radboud University Nijmegen, The Netherlands) was used at a 1:1000 dilution. Monoclonal anti-Hsp90 antibody (610418, BD Biosciences) was used at a 1:1000 dilution. Proteins were visualized using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce).

Tissue culture, transfections, and reporter gene assays

Xenopus A6 kidney epithelial cells (ATCC CCL-1020) were kindly donated by Ms. Stieneke

van den Brink from the Hubrecht laboratory (Utrecht, the Netherlands). The cells were cultured at room temperature (~25ºC) in 70% Leibovitz medium supplemented with 10% fetal calf serum (FCS) and 25 mM Hepes pH 7.2. Transient transfection was performed using Fugene-6 (Roche) according to the manufacturer's instructions. Cells were seeded on six-well plates (2.5 x 10^5 per well) and on the next day transfected with ~1 μg plasmid per well in serum-free medium. The following day, the transfection mix was replaced with medium supplemented with FCS. For GFP and DsRed fluorescence analysis, cells were cultured on cover slips and fixed in 4% paraformaldehyde at 24 h after transfection. Fluorescence was monitored with a Leica DM RA microscope (Leica Microsystems), coupled to a Cohu high performance CCD camera. For luciferase assays, cells were transfected with a mixture of 100 ng pCMV-β-galactosidase, 200 ng pHL, and 800 ng of the plasmid indicated in the figures. At 48 h after transfection, cells were either left at room temperature (control) or incubated at 33ºC for 1 hour (heat shock). After 6 h recovery at room temperature, cells were lysed in 200 μl reporter lysis mix (2.5 mM, 0.05% Tween 20, 0.05% Tween 80) for 10 min. For the β-galactosidase assay, 40 μl cell lysate was mixed with 100 μl phosphate solution [100 mM Na-phosphate pH 8.2, 10 mM $MgCl_2$, 1% Galacton-Plus (Tropix)]. After 30 min incubation at room temperature, 150 μl accelerator II (Tropix) was added and luminescence was measured with the Lumat LB 9507 tube luminometer (Berthold technologies). For the luciferase assay, 40 μl cell lysate was mixed with 50 μl luciferin solution and luminescence was again measured with the Lumat luminometer. All reporter gene assays were performed *in triplo*.

Results

Xenopus heat shock factor binding protein-1 is not an efficient inhibitor of the heat shock response

HSBP1 is a ~9-kDa polypeptide that was shown to act as a negative regulator of the HSR in cultured mammalian cells and in *C. elegans in vivo* ([247]). Since HSBP1 is highly conserved throughout the animal kingdom ([248]), we examined whether transgene-driven over expression of XHSBP1 could be used to stably inhibit the HSR in *Xenopus* tadpoles. ClustalW alignment of the open reading frames of multiple *Xenopus laevis* HSBP1 ESTs revealed slight variation in the C-terminal part of the protein. However, the central coiled coil region thought to be important for the inhibitory interaction between HSBP1 and HSF1 [248] is identical in all ESTs and nearly identical to that of the human protein (Fig. 1A). The code for XHSBP1 was PCR amplified from adult *Xenopus* brain cDNA and, to allow for quick identification of transgenic tadpoles,

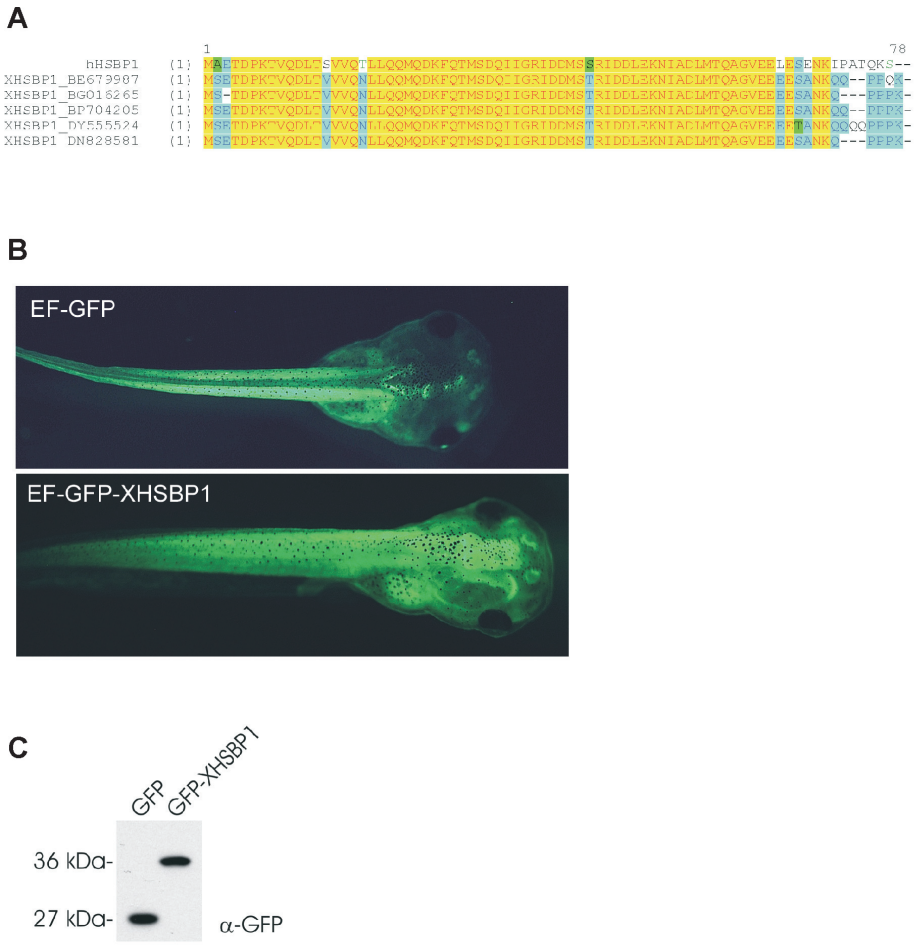


Fig. 1 Ubiquitous transgene-driven expression of XHSBP1 in Xenopus tadpoles. A. ClustalW alignment of human HSBP1 (top) and HSBP1 sequences deduced from five different *Xenopus* laevis ESTs. The central coiled coil region is highly conserved between man and *Xenopus*. **B.** Fluorescence microscope analysis of tadpoles carrying the indicated transgenes. Tadpoles with ubiquitous, high level expression of GFP-HSBP1 look normal. **C.** Western blot analysis of lysates from whole tadpoles carrying the indicated transgenes. Proteins were separated by SDS-PAGE and analyzed with an anti-GFP antibody.

fused to the 3' end of the code for GFP. Tadpoles with ubiquitous expression of the GFP-HSBP1 protein developed normally (Fig. 1B,C). To test whether over expression of GFP-XHSBP1 inhibited the HSR in tadpoles, the endogenous Hsp90 level was monitored by western blot analysis. Basic Hsp90 levels are low in tadpoles as well as in *Xenopus* A6 kidney epithelial cells. Upon heat shock (1 h, 33°C) the level significantly

increases, reaching a maximum after approximately 8 h (Fig. 2A,B). The heat shock-induced Hsp90 level was not significantly different between tadpoles expressing GFP-XHSBP1 and non-transgenic controls (Fig 2B), indicating that GFP-XHSBP1 does not inhibit the activity of HSF1. Subsequently, we monitored the effect of GFP-XHSBP1 on an Hsp70 promoter-luciferase reporter plasmid in transiently transfected A6 cells. Upon heat shock, the activity of the Hsp70 promoter increased tenfold in the presence of GFP alone. Co-expression of GFP-XHSBP1 resulted in only 27% reduction of the

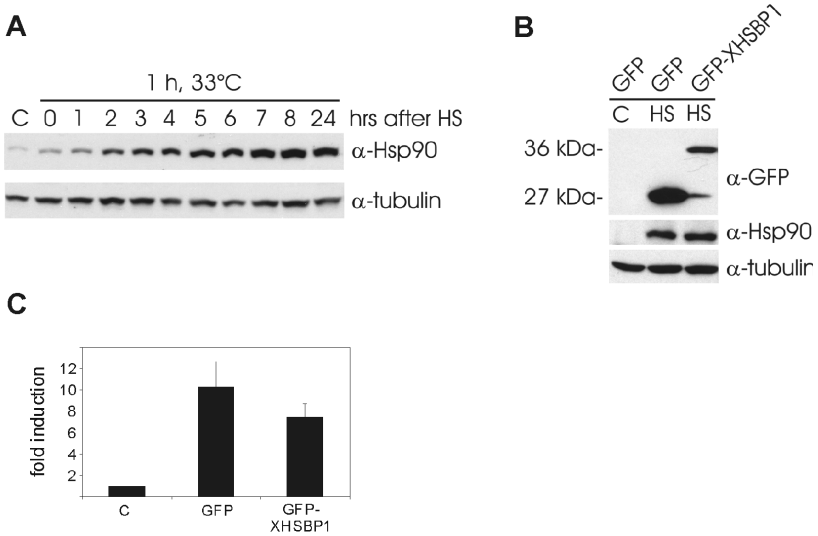


Fig. 2 The effect of exogenous expression of GFP-XHSBP1 on the HSR. A. Heat shock induced expression of Hsp90. *Xenopus* A6 kidney epithelial cells were continuously cultured at room temperature (C) or exposed to a 1 h heat shock (33°C) and then cultured at room temperature for the indicated times. Cell lysates were subjected to SDS-PAGE and western blot analysis using anti-Hsp90 and anti-tubulin antibodies. **B.** Western blot analysis of Hsp90 levels in tadpoles carrying the indicated transgenes. Lysates were made from whole tadpoles before heat shock (C) or after a 1 h heat shock at 33°C followed by 6 h recovery at room temperature (HS). The lysates were subjected to SDS-PAGE and western blot analysis using anti-GFP, anti-Hsp90 and anti-tubulin antibodies. **C.** Reporter gene analysis of the effect of GFP-XHSBP1 on the HSR. A6 cells were transfected with mixtures of an Hsp70-luciferase reporter, a CMV-β-galactosidase reporter and the indicated plasmids. At 48 h after transfection, cells were exposed to a 1 h heat shock at 33°C. Control cells were left at room temperature. Cell lysates were made at 6 h after heat shock and used for reporter gene assays. Hsp70 promoter activities were determined by dividing luciferase values by the corresponding β-galactosidase values to correct for varying transfection efficiencies. The HSR is indicated as fold induction relative to the activity of the Hsp70 promoter in control cells, which was set at 1 (C). The results are the average of three independent transfections (standard deviations are indicated by error bars).

activity of the Hsp70 promoter (Fig. 2C), which is much less than the 80% reduction observed by others ([247]).

HSBP1 is a nuclear protein and the size of the GFP tag might prevent proper transport of the fusion protein into the nucleus. In addition, the GFP tag could cause steric hindrance and thereby interfere with the interaction between XHSBP1 and HSF1. Therefore we sought ways to simultaneously express GFP and native XHSBP1 in tadpoles. Since co-expression from an internal ribosome entry site or from tandemly linked transgenes is unreliable and inefficient in transgenic tadpoles (our unpublished observations), we tested whether a ribosome skip system based on viral 2A peptides ([249]) could be used to simultaneously express two proteins in *Xenopus*. Insertion of the code for either the T2A or F2A peptide between the GFP and DsRed2 open reading frames resulted in ~100% co-fluorescence in A6 cells in both cases (Fig. 3A); however, western blot analysis indicated that ribosomal skipping from the T2A-based bicistronic expression cassette was almost ~100% efficient, whereas the F2A peptide resulted in only ~50% skipping efficiency (Fig. 3B). A control experiment, in which GFP and myc-tagged versions of Hsp27 or dominant-negative HSF1 are expressed from a bicistronic transcription unit further demonstrated that the second part of the T2A-based plasmids is also properly expressed (Fig. 3C). The T2A-based system was then used to test the effect of native HSBP1 on co-transfected Hsp70-luciferase reporter in A6 cells. The activity of the Hsp70 promoter was inhibited, but by only ~30% in the presence of native XHSBP1 (Fig. 3D). In summary, our results indicate that over expression of XHSBP1 is not a successful strategy to inhibit the HSR of *Xenopus*.

RNAi against HSF1 inhibits HSR in A6 cells but is not effective in tadpoles

As an alternative strategy, we tried to reduce the expression of HSF1 by means of stable, transgene-driven RNAi. Earlier efforts to stably inhibit gene expression via RNAi in *Xenopus* tadpoles were only partially successful. Whereas expression of exogenous GFP could be inhibited by co-expressing long GFP dsRNA from RNA polymerase II promoters ([250]) or GFP shRNAs from an RNA polymerase III promoter ([251]), neither polymerase II nor polymerase III promoter-based inverted repeat constructs resulted in stable inhibition of endogenous target genes ([250]). We already showed that the human H1 RNA promoter drives strong and ubiquitous GFP expression in transgenic *Xenopus* tadpoles ([250]). Thus, we selected a 19-mer sequence optimal for HSF1 knockdown and placed the corresponding inverted repeat under the control of the human H1 RNA promoter. To allow for future identification of transgenic tadpoles, the H1-HSF1-sh1 cassette was linked in tandem with a muscle-specific GFP reporter.

To get a first impression of the inhibitory potential of the humH1-HSF1-sh1 plasmid, we tested its effect on the Hsp70-luciferase reporter in A6 cells. The humH1-HSF1-

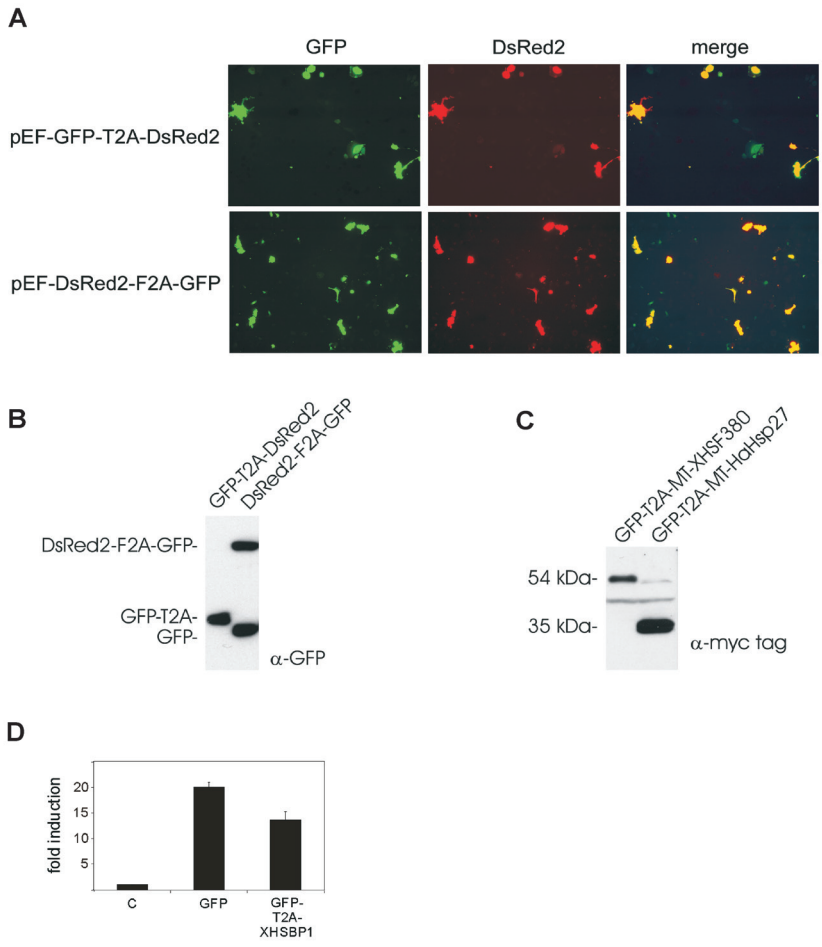


Fig. 3 The effect of exogenous expression of XHSBP1 on the HSR. A. Fluorescence microscope analysis of reporter gene expression from bicistronic plasmids. A6 cells were transfected with the indicated dual reporter gene plasmids based on viral 2A peptides. At 24 h after transfection, GFP and DsRed expression was determined by fluorescence microscope analysis. **B.** Western blot analysis of gene expression from bicistronic plasmids. A6 cells were transfected with the indicated 2A peptide-based plasmids. At 24 h after transfection, cell lysates were made and subjected to SDS-PAGE and western blot analysis using anti-GFP antibodies. **C**. Western blot analysis of gene expression from bicistronic plasmids. A6 cells were transfected with the indicated 2A peptide-based plasmids. At 24 h after transfection, cell lysates were made and subjected to SDS-PAGE and western blot analysis using anti-GFP and anti-myc tag antibodies. **D.** Reporter gene analysis of the effect of native XHSBP1 on the HSR. A6 cells were transfected with mixtures of an Hsp70-luciferase reporter, a CMV-β-galactosidase reporter and the indicated plasmids. Relative luciferase activities and -fold induction were determined as described in the legend to fig. 2C. The results are the average of three independent transfections (standard deviations are indicated by error bars).

sh1 plasmid inhibited the heat shock induced activity of the Hsp70 promoter by only 27% (Fig. 4A). We reasoned that, in *Xenopus* tadpoles, the human H1 promoter may not be recognized by the proper RNA polymerase (polymerase II rather than III), which would result in shRNAs with long single-stranded extensions that do not induce RNAi. This would also explain why GFP expression from this promoter is so efficient ([250]), despite the fact that RNAs transcribed from polymerase III promoters are not usually capped or polyadenylated. Thus, we isolated an H1 (RNAse P) promoter from *Xenopus tropicalis* and expressed the HSF1-sh1 from this promoter. XtH1 promoter-driven HSF1-sh1 reduced the heat shock induced activity of a co-transfected Hsp70-luciferase reporter by almost 50% (Fig. 4A). Similar analyses of HSF1-sh2 and HSF1-sh3, which are directed against other parts of the HSF1 mRNA, resulted in, respectively, 71% and 55% inhibition of the HSR (Fig. 4B).

Since HSF1-sh2 had the strongest inhibitory activity in our *in vitro* system, this inverted repeat was used to generate transgenic tadpoles. Tadpoles carrying the HSF1-sh2 transgene were identified by muscle-specific expression of the GFP reporter. GFP-positive tadpoles developed normally and were equally resistant to sub lethal heat shock as non-transgenic tadpoles (data not shown). Western blot analysis indicated that heat

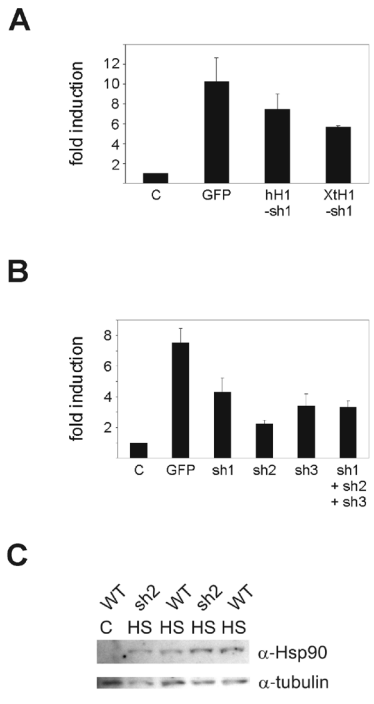


Fig. 4 The effect of XHSF1 shRNAs on the HSR. A,B. Reporter gene analysis of (**A**) the effect of HSF1 shRNAs expressed from the human or *Xenopus* H1 promoter, and (**B**) the effect of different shRNAs expressed from the *Xenopus* H1 promoter, on the HSR. A6 cells were transfected with mixtures of an Hsp70-luciferase reporter, a CMV-β-galactosidase reporter and the indicated plasmids. Relative luciferase activities and -fold induction were determined as described in the legend to fig. 2C. The results are the average of three independent transfections (standard deviations are indicated by error bars). **C.** Western blot analysis of Hsp90 levels in tadpoles carrying the indicated transgenes. Lysates were made from whole tadpoles before heat shock (C) or after a 1 h heat shock at 33°C followed by 6 h recovery at room temperature (HS). The lysates were subjected to SDS-PAGE and western blot analysis using anti-Hsp90 and anti-tubulin antibodies.

shock mediated induction of endogenous Hsp90 expression was not inhibited in GFP positive tadpoles (Fig. 4C). Thus, despite its inhibitory activity on the HSR in A6 cells, HSF1-sh2 was not effective as an inhibitor of the HSR in tadpoles.

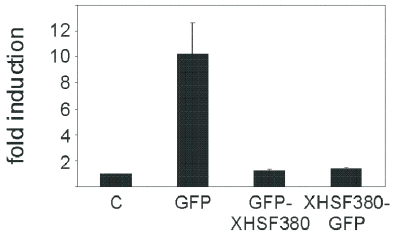
Dominant-negative HSF1 mutant is embryonic lethal and not detectable in transgenic brain

Dominant-negative mutant versions of HSF1 have been successfully used by others to inhibit the HSR in mammalian systems (reviewed by [230]). Therefore, we tried to inhibit the HSR in tadpoles via transgene-mediated expression of dominant-negative HSF1. The first 380 codons of the XHSF1 open reading frame (XHSF380), thus lacking the code for the C-terminal trans activation domain, were PCR amplified from adult *Xenopus* brain cDNA. The XHSF380 cDNA was cloned upstream or downstream of the GFP code and the effect of expression of the GFP-XHSF380 fusion protein on the HSR was first tested via reporter gene analysis of the Hsp70 promoter in transiently transfected A6 cells. Heat shock mediated induction of Hsp70-Luc was completely inhibited by co-expression of GFP-XHSF380 or XHSF380-GFP, indicating that over expression of XHSF380 is a powerful way to inhibit the HSR (Fig. 5A).

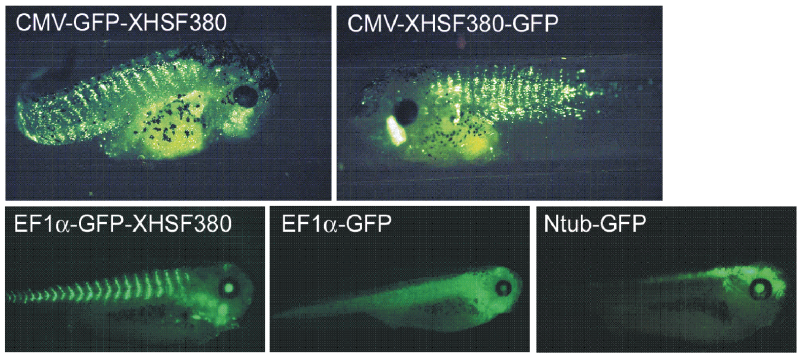
We next used CMV-GFP-XHSF380 and CMV-XHSF380-GFP cassettes to generate transgenic tadpoles. GFP-positive larvae showed clear nuclear fluorescence (Fig. 5B), which is in accordance with an earlier report that *Xenopus* HSF1 is a nuclear protein even in the absence of heat stress ([252]). Surprisingly, CMV promoter-driven expression of GFP-tagged XHSF380 was mainly restricted to skeletal muscle fibers and undetectable in brain and spinal cord (Fig. 5B), despite the fact that the CMV promoter is also highly active in neuronal tissue (data not shown). GFP-positive larvae (n=40, Table 1) developed poorly, remained small, had a curved tail and died before reaching the feeding tadpole stage (Fig. 5B). Embryonic lethality could be due to the high expression level from the strong CMV promoter; however, replacing the CMV promoter with the weaker EF1α promoter, did not prevent the lethal phenotype. Again, GFP fluorescence was not detectable in neuronal tissues (data not shown), although expression of GFP-XHSBP1 from the EF1α promoter resulted in strong fluorescence in brain and spinal cord (Fig. 5B).

In a final effort to target expression of XHSF380 specifically to the brain, the CMV promoter was replaced with the neuron-specific β-tubulin (Ntub) promoter. Injections with Ntub-GFP-XHSF380 or Ntub-XHSF380-GFP cassettes resulted in ~ 300 normal larvae without detectable GFP fluorescence, whereas ~ 10% of the larvae derived from parallel injections with a Ntub-GFP cassette showed strong GFP fluorescence in brain and spinal cord (Fig. 5B, Table 1). We concluded that GFP-tagged XHSF380 cannot be stably expressed in *Xenopus* brain. The observation that both N-terminally and

A


fold induction
12
10
8
6
4
2
C
GFP
GFP-XHSF380
XHSF380-GFP

B


CMV-GFP-XHSF380
CMV-XHSF380-GFP
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EF1α-GFP
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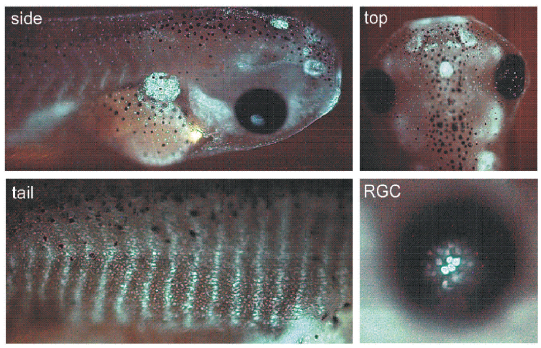
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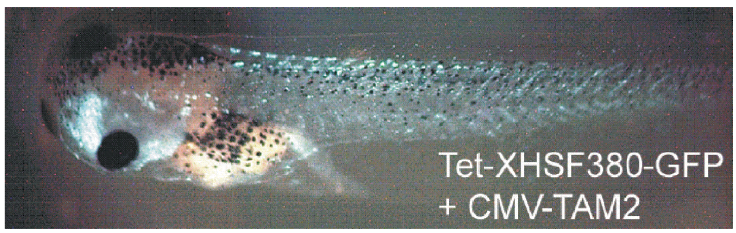

Tet-XHSF380-GFP
+ CMV-TAM2

Fig. 5 Transgene-driven expression of dominant-negative HSF1 in Xenopus larvae. A. Reporter gene analysis of the effect of GFP-XHSF380 or XHSF380-GFP on the HSR. A6 cells were transfected with mixtures of an Hsp70-luciferase reporter, a CMV-β-galactosidase reporter and the indicated plasmids. Relative luciferase activities and -fold induction were determined as described in the legend to fig. 2C. The results are the average of three independent transfections (standard deviations are indicated by error bars). **B.** Fluorescence microscope analysis of GFP-tagged XHSF380 constitutively expressed from either the CMV or the EF1α promoter in transgenic *Xenopus* larvae. Larvae with high constitutive expression of GFP-tagged XHSF380 develop poorly and never reach the feeding tadpole stage. Also shown are transgenic *Xenopus* larvae showing strong neuronal expression of GFP when driven by the EF1α or the Ntub promoter. **C.** Fluorescence microscope analysis of transgenic *Xenopus* larvae expressing full length GFP-tagged XHSF1 from the CMV promoter. Fluorescence is pronounced in kidney, epiphysis, nasal epithelium, olfactory lobes, gills, retinal ganglion cell layer (RGC) and tail muscle nuclei. **D.** Fluorescence microscope analysis of doxycycline-induced expression of GFP-tagged XHSF380 expressed in transgenic *Xenopus* larvae. A mixture of TetO-XHSF380-GFP and CS2+rtTA2A-M2 (CMV-TAM2) cassettes was used to generate transgenic *Xenopus* larvae. The larvae were allowed to develop in the absence of doxycycline until the feeding tadpole stage. GFP-negative larvae were exposed to doxycycline for 20 h and then monitored for GFP fluorescence.

C-terminally GFP-tagged XHSF380 were undetectable in neuronal tissue suggested that this was due to tissue-specific degradation of the fusion protein or the corresponding mRNA, rather than inefficient translation of the transgene-derived mRNA.

To examine whether putative neuron-specific instability of XHSF380 was due to the absence of the C-terminal transcription activation domain, we also generated tadpoles expressing full length GFP-XHSF1 from the CMV promoter. Surprisingly, the expression pattern of GFP-XHSF1 was very similar to that of GFP-XHSF380. Fluorescence was pronounced in nuclei of muscle tissue, but undetectable in most parts of the brain, except for the epiphysis, the olfactory lobes and the retinal ganglion cells (Fig. 5C). In conclusion, the low neuronal expression level of transgene-derived XHSF380 is an intrinsic property of the *Xenopus* HSF1 protein and not a direct result of deletion of the transcription activation domain.

Since expressing XHSF380 was the most effective strategy to inhibit the HSR in *Xenopus* A6 cells, we tried to circumvent the embryonic lethality by suppressing XHSF380 expression during embryogenesis. The Tet-on system has been successfully used to regulate the expression of GFP and thyroid hormone receptor in *Xenopus* tadpoles [253]). GFP-XHSF380 and XHSF380-GFP cassettes under the control of a Tet-responsive CMV promoter were mixed with a Tet-activator expression cassette and used to generate transgenic larvae. Embryos were allowed to develop in the absence of doxycyclin. Subsequently, all normal GFP-negative tadpoles (n ~ 500) were exposed to doxycyclin to induce expression of GFP-tagged XHSF380. After 20 h doxycyclin treatment, ~ 1% of the tadpoles showed nuclear GFP fluorescence (Fig. 5D), predominantly in muscle and again not in the nervous system, in spite of the fact that the TetO system has been

shown to drive strong neuronal expression [253]). Further tadpole development in the presence of doxycyclin remained normal, indicating that the lethal effect of XHSF380 is stage-specific and that it can be circumvented by delaying the expression until after embryogenesis.

Table 1. Expression of XHSF380-GFP, GFP-XHSF380 or GFP from ubiquitous and neuron-specific promoters in transgenic *Xenopus* larvae

Transgene	total number of larvae	number of GFP positive larvae	phenotype of GFP positive larvae
EF1α-GFP	75	8	normal
CMV-XHSF380-GFP	280	28	lethal
CMV-GFP-XHSF380	290	12	lethal
EF1α-GFP-XHSF380	255	13	lethal
Ntub-GFP	160	16	normal
Ntub-XHSF380-GFP	155	0	N.A.
Ntub-GFP-XHSF380	145	0	N.A.
CMV-GFP-XHSF1	225	6	normal

Discussion

To mimic the aging-related decline of the HSR in a *Xenopus* tadpole model system, we tried to inhibit the activity of HSF1 using three different transgene-mediated strategies. XHSBP1 did not inhibit the HSR significantly, neither in A6 cells nor in transgenic tadpoles. Both untagged and GFP-tagged XHSBP1 were poor inhibitors, indicating that the absence of biological activity did not result from poor import into the nucleus due to the GFP tag. Perhaps, the expression level of XHSBP1 was insufficient to exert its inhibitory effect on HSF1, although the GFP fluorescence signal and western blot analysis suggested that the transgene was expressed at a very high level throughout the tadpole. The inhibitory effect of HSBP1 was described in a single study, wherein hemagglutinin-tagged human HSBP1 was shown to inhibit the HSR in COS7 cells and the *C. elegans* orthologue was shown to inhibit the HSR in nematodes *in vivo* ([247]). The discrepancy between the published data and our results could be caused by species-specific differences in the biological activity of HSBP1, although its strong sequence conservation, especially in the hydrophobic core, suggests a conserved function. As our efforts to inhibit the HSR in human HeLa cells using human HSBP1 were also

unsuccessful (unpublished results), the proposed biological function of HSBP1 may have to be reconsidered.

Stable transgene-driven RNAi directed against XHSF1 mRNA was not an effective means to inhibit the HSR in tadpoles. Earlier, it was shown that expression of exogenous GFP can be effectively inhibited by long GFP dsRNAs expressed from RNA polymerase II promoters ([250]) or GFP shRNAs expressed from the U6 RNA polymerase III promoter ([251]). Here, we showed that XHSF1 shRNAs expressed from the *Xenopus tropicalis* H1 RNA promoter (RNA polymerase III promoter) inhibited the HSR by more than 70% in A6 cells. This implies that the RNAi-mediated inhibition of XHSF1 expression in A6 cells is highly efficient. Mice that are heterozygous for a null mutation in the HSF1 gene, still display a 100% HSR, whereas the HSR is completely abolished in homozygous null mutant mice ([210]). There are several reasons why the XHSF1 shRNA might not be effective in transgenic tadpoles, despite its potent inhibitory activity in A6 cells. The heterogeneous genetic background of the parental frogs combined with the pseudotetraploid nature of their genome may result in mismatches between the shRNA sequence and the endogenous XHSF1 sequences, which would negatively influence the RNAi effect. Essential components of the RNAi machinery, such as the Dicer enzyme, may be present at suboptimal levels in tadpoles compared with A6 cells. In addition, activation of the RNAi machinery may require a threshold level of shRNAs that is reached in transiently transfected A6 cells, but not in stably transgenic tadpoles.

The dominant-negative XHSF380 mutant completely abolished the HSR in A6 cells. In tadpoles, its expression from a ubiquitously active promoter resulted in embryonic lethality, indicating that HSF1 signaling is required for normal *Xenopus* development. Surprisingly, HSF1 null mice also show abnormalities that are not directly related to stress, such as defects in female and male germ cells, placenta, and central nervous system ([206-211]). Together with our *Xenopus* data, these results indicate that HSF1 also functions under normal physiological conditions. The basal expression levels of several genes are reduced in specific tissues and embryonic fibroblast derived from HSF1 null mutant mice compared with wild type mice ([18,254]). Whether reduced expression of orthologous genes causes the embryonic lethality observed in tadpoles expressing XHSF380 remains to be determined. HSF1 target genes are expressed during early *Xenopus* development [255]. Embryonic lethality could be successfully circumvented by delaying the expression of XHSF380 until the feeding tadpole stage and a stable *Xenopus* line carrying a tetracyclin-inducible XHSF380 transgene may provide a useful model system to study the *in vivo* effects of a crippled HSR.

Surprisingly, GFP-tagged XHSF380 was not detectable in neuronal tissues, even when a neuron-specific promoter was used to specifically target expression to the brain. Expression from the ubiquitously active CMV or EF1α promoter resulted in clear GFP

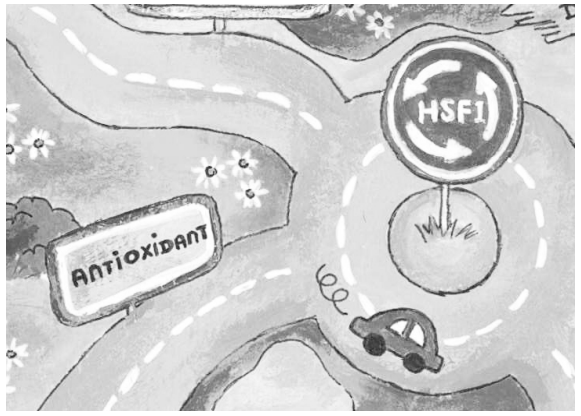
fluorescence in the nuclei of skeletal muscle fibers, indicating that the GFP-XHSF380 cassettes were properly transcribed and translated in non-neuronal tissue. Furthermore, parallel injections with an Ntub-GFP transgene resulted in high neuron-specific GFP fluorescence, indicating that the Ntub promoter functions properly (Fig. 5B). Thus, the absence of detectable GFP-tagged XHSF380 in transgenic brain and spinal cord most likely results from instability of the mRNA or protein. Full length GFP-XHSF1 expressed from the CMV promoter was also undetectable in most parts of the brain, but clearly visible in e.g. muscle and pronephros, again indicating that it is an intrinsic property of HSF1 or its coding sequence that causes the low neuronal expression. Similarly, a dominant positive HSF1 was not expressed in the brain of transgenic mice, in spite of the use of an ubiquitous promoter [220,256]. Our data thus suggest the intriguing possibility that the low HSR in neuronal tissue [243] is due to tissue-specific differences in the half-life of HSF1.

Acknowledgements

We thank Leonie de Wilt for technical assistance, Ron Engels for animal care, Eric Jansen for preparing *Xenopus laevis* sperm nuclei, and Gerard Martens for *Xenopus* facilities.

Chapter 4

A delayed antioxidant response in heat stressed cells expressing a non-DNA binding HSF1 mutant



Sanne M.M. Hensen, Lonneke Heldens, Siebe T. van Genesen, Ger J.M. Pruijn, Nicolette H. Lubsen

Cell Stress and Chaperones 2013; 18(4):455-473

To assess the consequences of inactivation of heat shock factor 1 (HSF1) during aging, we analyzed the effect of HSF1 K80Q, a mutant unable to bind DNA, and of dnHSF1, a mutant lacking the activation domain, on the transcriptome of cells 6 and 24 h after heat shock. The primary response to heat shock (6 h recovery), of which 30% was HSF1 dependent, had decayed 24 h after heat shock in control but was extended in HSF1 K80Q and dnHSF1 cells. HSF1 K80Q, but not HSF1 siRNA treated, cells showed a delayed stress response: an increase in transcript levels of HSF1 target genes 24 h after heat stress. Knockdown of NRF2, but not of ATF4, c-Fos or FosB, inhibited this delayed stress response. EEF1D_L siRNA inhibited both the delayed and the extended primary stress responses, but had off target effects. In control cells an antioxidant response (ARE binding, HMOX1 mRNA levels) was detected 6 h after heat shock; in HSF1 K80Q cells this response was delayed to 24 h. Inactivation of HSF1 thus affects the timing of the antioxidant response and NRF2 can activate at least some HSF1 target genes in the absence of HSF1 activity.

4

Introduction

Cells respond to cytoplasmic proteotoxic stress by producing additional chaperones, the heat shock proteins (HSP). This heat shock response plays an important role in maintaining proteostasis (reviewed in [6,133,262]). The heat shock response is mainly regulated at the level of transcription by heat shock factor 1 (HSF1). Under normal circumstances HSF1 is monomeric and complexed with chaperones. Upon stress, when unfolded proteins accumulate and chaperones become scarce, HSF1 trimerizes, binds to the heat shock element (HSE) and activates transcription (reviewed in [6,14]). HSF1 is required for longevity [159,160] and its inactivation, for example during the DNA damage response [263] or the amino acid starvation response [264], is linked to senescence. During aging, the activity of HSF1 declines [156-158], although the protein is still present. This aging-related failure of HSF1 interferes with an organism's ability to combat proteotoxic stress, which results in increased susceptibility to protein folding diseases [131,196,201,202,220,222]. Furthermore, accumulating evidence indicates that HSF1 also regulates gene expression under non-stress conditions. For example, the circadian clock gene Per2 is an HSF1 target [19,20]. In addition, HSF1 regulates a transcriptional circuit distinct from the proteotoxic stress induced pathway, which has been recruited by malignant cells [21]. Thus, a decline in HSF1 activity may cause phenotypic defects in the absence of exogenous stress [194]. Previously we found that the expression of an HSF1 mutant retaining the DNA binding domain but lacking the activation domain (dnHSF1) reduced the expression level of 10 genes in non-stressed

HEK293 cells, amongst which the genes for the chaperones Hsp90, HSPA6, DNAJB1 (Hsp40) and HSPB1; expression of dnHSF1 did not result in increased transcript levels [265]. HeLa cells treated with siRNA directed against HSF1 showed changed expression levels of 378 genes in the absence of stress [237], where 80% of the affected genes showed increased transcript levels. A comparison of the transcriptome of HSF1-/- mouse embryonic fibroblasts (MEFs) with that of wild type MEF cells resulted in 49 genes (19 related to immune response) that were expressed at reduced levels in MEF HSF1-/- cells [18]. The aging cell differs from the HSF1-/- cells in that the cell still contains HSF1, although not active, and differs from the dnHSF1 cells in that HSF1 is no longer bound to its target promoters. In this study we have investigated the effect of heat stress on the transcriptome changes in two stable cell lines, one with a tet-inducible dnHSF1 mutant and one with tet-inducible expression of an HSF1 mutant in which lysine 80 in the DNA binding region is replaced by glutamine (HSF1 K80Q), thus impairing DNA binding [51]. Unexpectedly, we detected a delayed stress response, i.e. an increase in transcript levels of HSF1 dependent genes in HSF1 K80Q cells 24 h after heat stress, suggesting that there are alternative routes to activation of transcription of these genes when the HSF1 directed transcription fails. We noted that the antioxidant response is delayed in heat stressed HSF1 K80Q cells and found NRF2, a transcription factor directing the antioxidant response, to be responsible for the increase in HSPA1A and HSPA6 mRNA levels in HSF1 K80Q cells 24 h after heat stress.

Materials and Methods

Tissue culture

Flp-In T-REx-293 cells (Invitrogen) were manipulated according to the manufacturer's instructions using the T-REx system (Invitrogen) to generate the stable cell lines HEK-dnHSF1, HEK-HSF1K80Q, HEK-wtHSF1 and HEK-pcDNA5 that carry a single copy of the tetracycline-inducible plasmids pcDNA5-dnHSF1, pcDNA5-HSF1K80Q, pcDNA5-wtHSF1 and pcDNA5-FRT/TO, respectively. The cells were cultured at 37°C/5% CO_2 in high glucose DMEM medium supplemented with 10% fetal calf serum,100 U/ml penicillin and 100 μg/ml streptomycin. Blasticidin (1.65 μg/ml; Invitrogen) and 100 μg/ml hygromycin were also added to the culture medium during maintenance of the cell lines, but were omitted during experiments.

Plasmid construction, transfections and reporter gene assays

The expression vectors pcDNA5-dnHSF1, pcDNA5-wtHSF1 and pcDNA5-HSF1 K80Q have been described earlier [264,265]. Transient transfections were performed

using FuGENE-6 (Roche) according to the manufacturer's instructions. Cells were seeded on 24-well plates and on the next day transfected with 0.2 μg SV40-luc per well and treated with doxycycline to express HSF1 K80Q. 24 h after transfection cells were pre-heat shocked for 30' at 45°C. 14 h later, cells were harvested or heat shocked again for 30' at 45°C in the presence of 20 μg/ml CHX to inhibit translation and harvested immediately or after 1 h of recovery. Cells were lysed in 200 μl reporter lysis mix (25 mM Bicine, 0.05% Tween 20, 0.05% Tween 80) for 10 min. For the luciferase assay, 20 μl cell lysate was mixed with 50 μl luciferin solution (Promega) and luminescence was measured with the Lumat LB 9507 tube luminometer (Berthold). All reporter gene assays were performed in triplicate.

Electrophoretic mobility shift assay

HEK-HSF1K80Q or HEK-wtHSF1 cells were cultured for 48 h in the presence or absence of doxycycline and subsequently heat shocked for 30 minutes at 45°C. Cells were harvested at the indicated times after heat shock and nuclear extracts were prepared using NE-per nuclear and cytoplasmic reagents (Pierce). Extracts were aliquoted and stored at -80°C. Oligonucleotide probes were end-labeled with ^{32}P. The sequences of the oligonucleotides used in EMSA are listed in Table S1. The EMSA protocol was adapted from [266,267]. A mixture containing 5 μg nuclear extract and 3 μg poly dIdC in binding buffer [20 mM HEPES pH 7.9, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 4% (v/v) Ficoll, 1X PhosSTOP (Roche)] was incubated for 20 minutes on ice. 0.01 pmol radiolabeled oligonucleotide was added and the samples were incubated for 20 minutes at room temperature. DNA-protein complexes were separated on a pre-run 4% polyacrylamide gel in 0.25x TBE with recirculation of the buffer. The gel was dried and signals were visualized using a PhosphorImager.

Chromatin immunoprecipitation

HEK-HSF1 K80Q cells were cultured in the absence or presence of doxycycline and heat stressed for 30' at 45°C. After 2 or 18 h of recovery cells were subjected to chromatin immunoprecipitation, performed as described in [268] except that cells were crosslinked for 15 minutes with 1% formaldehyde. After quenching with 125 mM glycine, cells were washed twice with ice cold PBS and resuspended in ice cold lysis buffer (50 mM HEPES-KOH pH 7.6, 140 mM NaCl, 1mM EDTA pH 8.0, 1% (v/v) Triton X-100, 0.1% NaDOC and 1X protease inhibitor complete). Sonicated chromatin was centrifuged for 5 min at 4°C and then incubated overnight in incubation buffer (final concentration; 12 mM HEPES-KOH pH 7.6, 90 mM NaCl, 0.6 mM EDTA pH. 8.0, 0.09% SDS, 0.6% Triton X-100, 0.1% BSA) together with purified anti-HSF1 antibody (SPA-901; Stressgen) and protein A/G beads (Santa Cruz Biotechnology).

Negative control without adding antibody was included. Beads were washed six times with different buffers at 4°C: twice with 0.1% SDS, 0.1% NaDOC, 1% Triton X-100, 150 mM NaCl, HEG (1 mM EDTA, 0.5 mM EGTA and 20 mM HEPES-KOH pH 7.6), once with the same buffer but with 500 mM NaCl, once with 0.25 M LiCl, 0.5% NaDOC, 0.5% NP-40, HEG and twice with HEG. Precipitated chromatin was eluted with 400 µl of elution buffer (1% SDS, 0.1 M $NaHCO_3$), incubated at 65°C for 4 h in the presence of 200 mM NaCl, phenol extracted and precipitated with 20 µg of glycogen at -20°C overnight. ChIP experiments were analyzed by QPCR. Efficiency of ChIP was calculated as percentage of input. The primers used are listed in Table S1.

RNA interference

ATF4 (CREB-2) (sc-35112), HSF1 (sc-35611), c-Fos (sc-29221) and FosB (sc-35403) siRNA's were purchased from Santa Cruz Biotechnology. The control siRNA against luciferase (5'-CGUACGCGGAAUACUUCGAdTdT-3'), NRF2 siRNA (5'-CAGCAUGCUACGUGAUGAAdTdT-3'), NRF2 siRNA#2 (5'-CCAGUGGAUCUGCCAACUAdTdT-3') and EEF1D siRNA (5'-CUGGCUCAGCAAGCCUGCCUAdTdT-3') were purchased from Eurogentec. HEK293 cells were cultured in 6-well plates and transfected with 50 nM siRNA using oligofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. 48 h after transfection cells were re-transfected as described above. Cells were left at 37°C or heat shocked for 30' at 45°C and allowed to recover for 6 h or 24 h. All cells were harvested simultaneously 48 h after re-transfection.

Western blot analysis

Cells were harvested in lysis buffer [25 mM Tris-HCl pH 7.5, 100 mM KCl, 1 mM DTE, 2 mM EDTA, 0.5 mM PMSF, 0.05% NP-40, 1X PhosSTOP (Roche), 1X protease inhibitor cocktail (Complete Mini, Roche)] and protein concentration was determined using a Bradford protein assay (Bio-Rad). Then 4x sample buffer (200 mM Tris–HCl 6.8, 20% β-mercaptoethanol, 8% SDS, 40% glycerol and 0.4% Bromophenolblue) was added and the lysates were incubated at 95°C for 5 min. Protein samples were separated in 10% polyacrylamide gels and transferred to nitrocellulose transfer membrane (Protran). For western blot analysis, the following antibodies were used: mouse monoclonal β-actin antibody (AC-15; Sigma; 1:5000), rabbit polyclonal HSF1 antibody (SPA-901; Stressgen; 1:1000), mouse monoclonal Hsp70 antibody 4G4 (ab5444; Abcam; 1:5000), rabbit polyclonal ATF4 antibody (sc-200X; Santa Cruz Biotechnology) and polyclonal HSPB1 antibody (obtained from Dr. A. Zantema; 1:400). Next, blots were incubated with fluorescent secondary antibodies IRDye® 800CW conjugate goat anti-rabbit IgG and IRDye® 680 conjugated goat anti-mouse IgG (926-32211 and 926-32220

respectively; LI-COR Biosciensces) according to the manufacturer's instructions and scanned using a LI-COR Odyssey infrared scanner.

Quantitative real-time PCR

RNA was isolated with TRIzol (Invitrogen) according to manufacturer's recommendation. 1 µg of RNA was treated with DNaseI (Amplification grade; RNase-free; Invitrogen). Subsequently, 5 mM $MgCl_2$, RT-buffer, 1 mM dNTPs, 18.75 units AMV reverse transcriptase, 20 units RNase inhibitors and 1.25 µM oligo(dT) were added to a total volume of 20 µl. Reverse transcription was performed for 10 minutes at 25°C, 60 minutes at 42°C and 5 minutes at 95°C. For QPCR analysis, cDNA was 10-fold diluted. Quantitative real-time PCR was performed using the StepOnePlus™ Real-Time PCR System with *Power* SYBR® Green PCR Master mix (Applied Biosystems) using the following amplification protocol: 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Per reaction 3 µl of diluted cDNA was used and the DNA was amplified using primers for the sequences of interest, listed in Table S1.

Microarray analysis

HEK-pcDNA, HEK-HSF1 K80Q, HEK-wtHSF1 and HEK-dnHSF1 cells were treated with doxycycline for a total of 48 h. Cells were left at 37°C or heat shocked for 30' at 45°C and harvested either 6 h or 24 h after heat shock. The transcriptomes of HEK-pcDNA cells and HEK-HSF1 K80Q cells, HEK-HSF1 K80Q 6 h after heat shock versus unstressed HEK-HSF1 K80Q cells or HEK-pcDNA5 6 h after heat shock versus unstressed HEK-pcDNA5 cells were compared. HEK-HSF1 K80Q cells or HEK-dnHSF1 cells 24 h after heat stress were compared with control cells under non-stress conditions. Total RNA was isolated using Trizol according to the manufacturer's instructions (Invitrogen) and copied into Cy3-labeled or Cy5-labeled cRNA using the Agilent Low RNA Input Linear Amp Kit PLUS, and reverse labeled for the repeat array. Labeled cRNA samples were hybridized to an Agilent Whole Human Genome Microarray Kit (4 x 44K). The arrays were scanned using an Agilent Microarray Scanner. Image analysis and feature extraction were done with Feature Extraction (version 9.5.1, Agilent). We used a cut-off level of twofold changed expression and an arbitrarily chosen signal cut-off of > 50.

Results

Characterization of the HEK-HSF1 K80Q cell line

In the HSF1 K80Q mutant lysine 80 in the DNA binding region is replaced by glutamine and because of this mutation HSF1 loses its DNA-binding activity [51]. HSF1 K80Q is expected to trimerize with endogenous HSF1, resulting in a strong reduction of binding competent HSF1 trimers. Using nuclear extracts of HSF1 K80Q expressing cells indeed only a weak signal of HSF1 binding to the HSE was detected (Fig. 1A). With nuclear extracts from cells overexpressing wild type HSF1 (wtHSF1) increased binding of HSF1 to the HSE was observed, as expected, even when cells were unstressed (Fig. 1A). The bandshifts shown in Fig. 1A could be supershifted by an antibody to HSF1, indicating that it is indeed HSF1 that was bound (data not shown). Extracts from heat shocked cells showed a more intense bandshift signal and thus an increase in binding competent HSF1. Expression of HSF1 K80Q blocked this increase (Fig. 1A). The loss of binding of HSF1 to the HSE in the presence of HSF1 K80Q was confirmed by chromatin immunoprecipitation (ChIP) (Fig. 1B). In control cells, i.e. HEK-HSF1 K80Q cells cultured in the absence of doxycycline, HSF1 was bound to the HSPA6 promoter region 2 h after heat shock. The binding of HSF1 is transient and as expected we did not observe HSF1 binding 18 h after heat stress. When HSF1 K80Q expression was induced, no bound HSF1 could be detected either 2 h or 18 h after heat shock (Fig. 1B).

When cells are exposed to heat stress they increase their chaperone levels and become more resistant to a subsequent heat stress, a process known as thermotolerance. HSF1 knockout or knockdown or overexpression of a dominant negative HSF1 mutant has been shown to inhibit the acquisition of thermotolerance [211,269,270]. To determine whether expression of HSF1 K80Q also inhibits the development of thermotolerance we heat stressed HEK-HSF1 K80Q cells and analyzed the refolding of luciferase after a second heat shock. As shown in Fig. 1C, pre-heat shocked control cells showed increased refolding activity compared to naïve control cells. In cells overexpressing HSF1 K80Q no difference in refolding activity was found between pre-heat shocked and naïve cells, indicating that these cells do not develop thermotolerance. Expression of HSF1 K80Q also inhibited luciferase refolding after a single heat shock (Fig. 1C), suggesting that HSF1 K80Q expression lowers the chaperoning capacity of the non-stressed cells as well.

We also analyzed the heat-induced expression levels of HSPA1A and HSPB1 and found that their increase was inhibited by expression of HSF1 K80Q, similar to the effect of an HSF1 mutant lacking the activation domains, dnHSF1 (Fig. 1D). Together these data show that the HSF1 K80Q mutant blocks the HSF1 directed transcriptional heat shock

response.

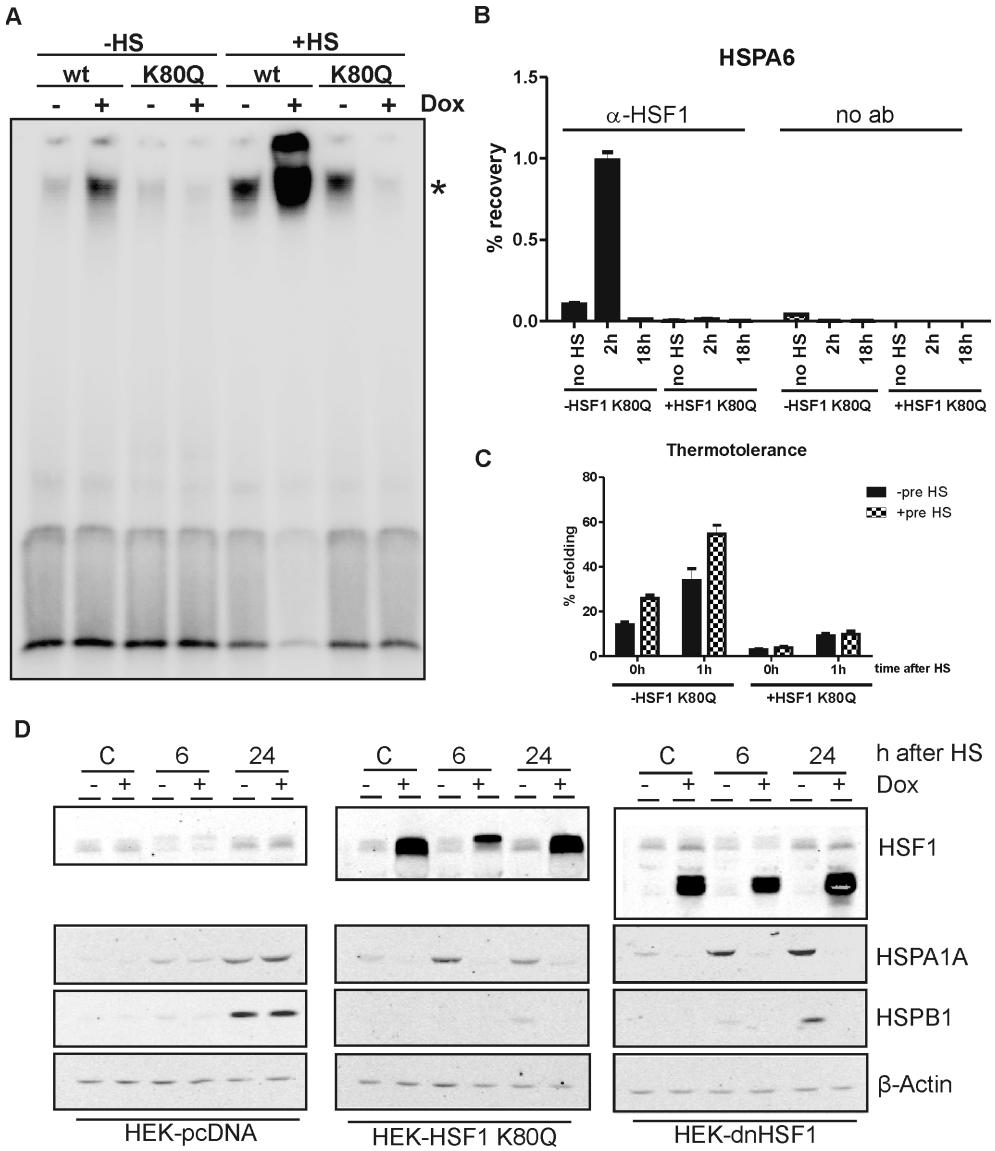


Fig. 1 Characterization of HEK-HSF1 K80Q cells. A. Nuclear extracts were made of HEK-wtHSF1 cells and HEK-HSF1 K80Q cells either non-stressed (-HS) or exposed to heat shock (30' 45°C, +HS). An electrophoretic mobility shift assay was performed with a doublestranded oligo with the HSE sequence. Where indicated (+Dox), doxycyclin was added to induce expression of either wtHSF1 or HSF1 K80Q. **B.** Chromatin immunoprecipitation using nuclear extracts from control and HSF1 K80Q expressing cells was performed with an HSF1 antibody or no antibody added. Bound chromatin was analyzed by QPCR using a primer set surrounding

the HSE of the HSPA6 promoter. Cells were either non-stressed or harvested 2 h or 18 h after heat shock, as indicated. **C.** HEK-HSF1 K80Q cells were transfected with SV40-luc and 24 h after transfection cells were pre-heat shocked for 30' at 45°C. 14 h later, cells were harvested or heat shocked again for 30' at 45°C in the presence of 20 μg/ml CHX to inhibit translation and harvested immediately or after 1 h of recovery and a luciferase assay was performed. **D.** HEK-pcDNA, HEKdnHSF1 or HEK-HSF1 K80Q cells were cultured in the presence or absence of doxycycline and exposed to a heat shock for 30' at 45°C or left at 37°C (C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Cell lysates were subjected to SDS-PAGE and levels of HSF1, HSPA1A, and HSPB1 were determined by western blotting. β-actin was used as a loading control. Error bars represent SD.

Transcriptome changes in the presence of HSF1 K80Q in non-stressed cells

If HSF1 plays a role in the absence of stress, then expression of a non-DNA binding mutant should change the transcriptome. We used microarrays to analyze the effect of HSF1 K80Q on the transcriptome in the absence of stress. As overexpression of the HSF1 protein may have secondary effects, for example by sequestering chaperones, we also analyzed the effect of overexpressing wild type HSF1 on the transcriptome of non-stressed cells. Table 1 shows the list of the 26 genes of which the transcript level changed at least twofold upon expression of HSF1 K80Q in non-stressed cells (relative to the level in both control cells and in cells overexpressing wild type HSF1). For 18

Table 1. Transcriptome changes in non-stressed HEK293 cells upon expression of HSF1 K80Q

Acc. nr.	Gene	K 8 0 Q / Cntrl 370C	Description
NM_004695	SLC16A5	0.1	monocarboxylic acid transporter 6
NM_001163335	SYTL5	0.2	synaptotagmin-like 5
NM_000735	CGA	0.2	glycoprotein hormones, alpha polypeptide
NM_017527	LY6K	0.2	lymphocyte antigen 6 complex, locus K
NM_017671	FERMT1	0.2	fermitin family homolog 1
NM_199441	ZNF334	0.3	zinc finger protein 334
NM_016378	VCX2	0.3	variable charge, X-linked 2
NM_001007125	C20orf201	0.3	chromosome 20 open reading frame 201
NM_002523	NPTX2	0.3	neuronal pentraxin II
NM_001195	BFSP1	0.4	beaded filament structural protein 1, filensin
NM_021785	RAI2	0.4	retinoic acid induced 2

NM_001030059	PPAPDC1A	0.4	phosphatidic acid phosphatase type 2 domain containing 1A
NM_152349	KRT222	0.5	keratin 222
NM_001077489	GNAS	0.5	GNAS complex locus
NM_004750	CRLF1	0.5	cytokine receptor-like factor 1
NM_181803	UBE2C	0.5	ubiquitin-conjugating enzyme E2C
NM_015432	PLEKHG4	0.5	pleckstrin homology domain containing, family G
XM_002345507	LOC100292909	0.5	hypothetical protein
NM_032876	JUB	2.1	jub, ajuba homolog
NM_001673	ASNS	2.2	asparagine synthetase
NM_004563	PCK2	2.3	phosphoenolpyruvate carboxykinase 2 (mitochondrial)
NM_014331	SLC7A11	2.3	cationic amino acid transporter, y+ system
NM_001902	CTH	2.4	cystathionase (cystathionine gamma-lyase)
NM_003714	STC2	2.4	stanniocalcin 2
NM_022445	TPK1	2.9	thiamin pyrophosphokinase 1
NM_005410	SEPP1	37.0	selenoprotein P, plasma, 1

genes we noted a decrease in transcript level; for 8 an increase, particularly in that of the SEPP1 gene. We have not attempted to verify the increase in the SEPP1 transcript level as the signal of this transcript in the microarray was just above background. Note that none of these genes is a canonical heat shock gene (see also Table S2) and that there is no overlap between the transcriptome changes in HSF1 K80Q expressing cells and in dnHSF1 expressing cells [265]. We also compared our microarray data with previously reported results obtained by HSF1 knockdown in HeLa cells [237]. For 16 of the 26 genes, transcript levels were not significantly affected in the siRNA-treated HeLa cells; one (CRLF1, cytokine receptor-like factor 1) had lower transcript levels, like in the HEK-HSF1 K80Q cells, but this could not be confirmed by QPCR (Fig. S2A); for the remaining 9 genes data were not available. Together these data show that HSF1 does control the level of a limited set of transcripts in the non-stressed cells. If depletion of HSF1 by siRNA and blocking HSF1 activity by expression of HSF1 K80Q can be equated (see also below), then this set of genes is largely cell type specific.

Overexpression of wild type HSF1 leads to elevated levels of activated HSF1 and our microarray data thus identify potential HSF1 targets. For 10 genes we found a significantly lower transcript level (relative to that in control cells and in HSF1 K80Q expressing cells) and for 32 an increase in transcript level (Table S2). Eleven of these

genes were also heat shock inducible, whereas for one of these genes the transcript level decreased 6 h after heat shock. Only 3 (CRYAB, DHRS2 and SEPW1) of these 42 potential HSF1 targets in non-stressed HEK293 cells were also identified as HSF1 target genes by exogenous expression of a constitutively active HSF1 mutant in HeLa cells [271]. Transcriptional activation by HSF1 in the absence of stress is thus constrained by cell specific factors.

The effect of HSF1 K80Q and dnHSF1 expression on the transcriptome of heat shocked cells – 6 h after heat shock

The classical role of HSF1 is transcription activation in heat stressed cells. In HEK293 cells allowed to recover from a heat shock for 6 h, we found an increase of at least twofold in the transcript levels of 180 genes. Expression of HSF1 K80Q and/or dnHSF1 inhibited the increase in transcript level of 53 of these genes by at least twofold (Fig. 2A, Table S3). Among these genes are the canonical HSF1 target genes, such as HSPA1A/B, HSPA6, DNAJB1, HSPH1, HSP90AA1, BAG3 and ATF3. For the canonical HSF1 dependent genes, the expression of HSF1 K80Q or dnHSF1 had a similar effect: it inhibited the heat shock induced increase in transcript levels, where expression of dnHSF1 was usually more effective than that of HSF1 K80Q. Note that for the highly expressed heat shock genes, such as HSPA6, the transcript levels still increased significantly in both HSF1 K80Q and dnHSF1 expressing cells, although the increase was far less than in control cells (Table S3). For other genes the effect of HSF1 K80Q or dnHSF1 expression was different. In a number of cases, such as ACTA1 or AOC3, expression of HSF1 K80Q had no effect or even enhanced expression levels, while that of dnHSF1 inhibited (Table S3). Possibly binding of dnHSF1 blocks access of other transcription factors to the promoter; expression of HSF1 K80Q would leave an HSF1 binding site empty.

Paradoxically, for 16 heat shock induced genes, expression levels increased significantly in either HSF1 K80Q or dnHSF1 cells (Table S4). The explanation for this is not clear but it does suggest that HSF1 partially represses stress induced transcription mediated by other transcription factors. This suggestion is supported by the finding that about a third of the genes of which the transcript levels are increased in HSF1 K80Q cells and/or dnHSF1 cells did not respond significantly to heat stress in control cells (Fig. 2B). Alternatively, the impaired chaperoning capacity of the HSF1 K80Q (Fig. 1C) and dnHSF1 cells [270] could result in a stronger stress response.

A heat stress is known to shift the transcription pattern to heat shock genes leading to a decrease in activity of non-heat shock promoters, and it is thus expected that the transcript levels of some genes decrease in heat shocked cells. Indeed, in control cells that had recovered for 6 h from the heat shock, the level of the transcripts of 116 genes

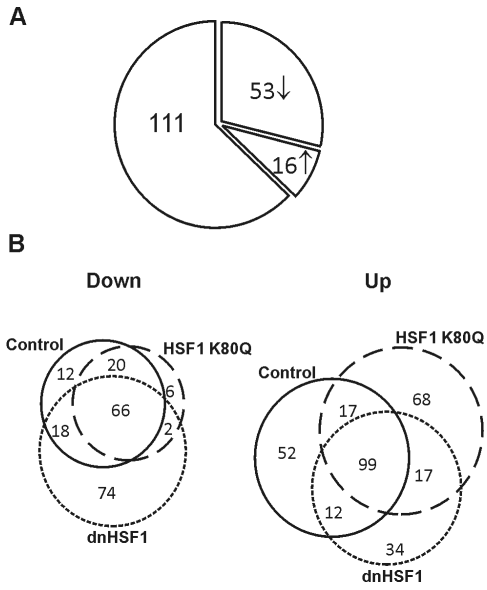


Fig. 2 A. Pie diagram illustrating the effect of expression of HSF1 K80Q and dnHSF1 on the increase in transcript levels in HEK293 cells 6 h after heat shock. An ↓ indicates a ≥ 2-fold decrease in level; an ↑ a ≥ 2 fold increase in level. For details, see Figure S1 and Table S3. **B**. Venn diagram illustrating the overlap in transcriptome changes in control, HSF1 K80Q and dnHSF1 cells 6 h after heat shock. "Down" indicates that the transcript levels were ≥ 2-fold lower than in non-stressed cells; "up" indicates that the transcript levels were ≥ 2-fold higher than in non-stressed cells. The solid line circle represents control cells, the dotted line HSF1 K80Q cells and the dashed line dnHSF1 cells. For details, see Figure S3.

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was at least twofold lower than in non-stressed cells. A response of similar magnitude was seen in HSF1 K80Q cells; in dnHSF1 cells the effect was larger (Fig. 2B and S3).
When we compared our data for HEK293 cells with the published data for heat shocked HeLa cells [237] we found only 20 genes that were heat shock responsive in both cell lines (note that the heat shock conditions differ). The transcript level of 1 gene (ZNF264) decreased in both cell lines (Fig. S1 and Table 2) but we could not confirm this decrease by QPCR in HEK293 cells (Fig. S2A). For 3 genes the transcript levels decreased in HeLa cells, while they increased in HEK293 cells. Of the 16 genes of which the transcript levels increased in both heat shocked HeLa and HEK293 cells, 4 appeared to be not regulated by HSF1 (Table 2). The common set of heat shock responsive genes consists mostly of the well known canonical heat shock genes, such as HSPA1A and DNAJB1, and general stress responsive genes such as GADD45B and PPP1R15A (GADD34).

The effect of HSF1 K80Q and dnHSF1 expression on the transcriptome of heat shocked cells 24 h after heat shock: an extended primary stress response and a secondary stress response

The synthesis of HSF1 dependent chaperones serves as a feedback mechanism to dampen the heat shock response and to restore homeostasis [34,36,68,131,133,272]. As in the HSF1 K80Q and the dnHSF1 cells the expression of these chaperones is inhibited, we analyzed the effect of these HSF1 mutants on the transcriptome of

Table 2. Comparison between the effect of HSF1 K80Q expression in HEK293 cells and that of HSF1 siRNA treatment in HeLa cells

Acc. nr.	Gene	Cntrl 6 h hs	K80Q 6 h hs	dnHSF 6 h hs	HeLa 4 h hs[a]	siRNA 4h hs[a]	Description
HSF1 dependent							
NM_002155	HSPA6	61.9 [b]	*27.4* [c]	*18.1*	11.3	*5.1*	heat shock 70kDa protein 6
NM_006145	DNAJB1	32.6	*3.4*	*0.8*	2.9	*1.5*	DnaJ (Hsp40) homolog, subfamily B, member 1
NM_015675	GADD45B	32.0	29.9	*13.4*	2.0	1.5	growth arrest and DNA-damage-inducible, beta
NM_001885	CRYAB	24.3	*2.4*	*3.6*	2.2	*0.6*	crystallin, alpha B
NM_005345	HSPA1A	13.7	*1.2*	*1.0*	2.0	1.6	heat shock 70kDa protein 1A
NM_006644	HSPH1	9.8	*1.9*	*1.0*	2.8	*1.3*	heat shock 105kDa/110kDa protein 1
NM_004281	BAG3	6.7	*2.2*	*1.3*	4.3	*1.6*	BCL2-associated athanogene 3
NM_001124	ADM	6.2	6.6	3.6	2.3	*1.1*	adrenomedullin
NM_006472	TXNIP	4.4	2.8	4.6	2.6	*0.9*	thioredoxin interacting protein
NM_004419	DUSP5	4.3	2.7	*1.8*	2.6	1.6	dual specificity phosphatase 5
NM_002228	JUN	4.0	*1.8*	*1.4*	2.0	1.8	jun oncogene
NM_001040619	ATF3	3.7	*1.6*	2.5	2.4	2.2	activating transcription factor 3
HSF1independent							
NM_001554	CYR61	7.8	4.0	5.0	2.0	1.9	cysteine-rich, angiogenic inducer, 61
NM_002923	RGS2	19.8	12.4	13.7	2.0	1.1	regulator of G-protein signaling 2, 24kDa
NM_014330	PPP1R15A	8.0	6.9	6.1	2.6	1.6	GADD34; protein phosphatase 1, regulatory (inhibitor) subunit 15A
NM_020127	TUFT1	2.2	2.8	2.0	2.9	2.8	tuftelin 1

a: data taken from [237]

b: numbers indicate the fold change relative to non-stressed control

c: numbers in italics indicate ≥ 2-fold decrease relative to control 6 h after hs (HEK293; 30' 42ºC) or 4 h after hs (HeLa; 90' 43ºC)

cells that had been allowed to recover for 24 h from the heat shock. In control cells the primary transcriptional response to the heat shock had largely decayed: the levels of most transcripts were back to the level in non-stressed cells or at least decreased relative to the level in cells 6 h after heat shock (Fig. 3A and S4, Tables S3 and S4). In HSF1 K80Q and dnHSF1 cells the primary response decayed as well, although to a lesser extent than in control cells (Fig. 3A and S4, Tables S3 and S4). These cells thus

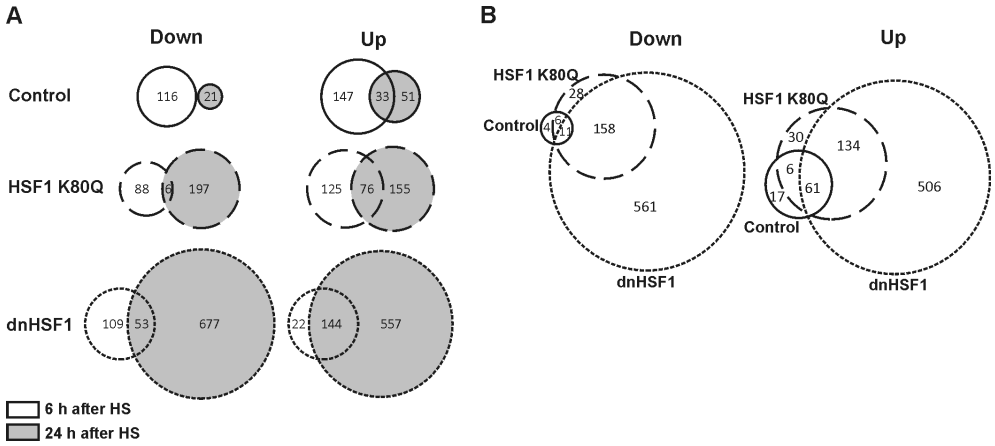


Fig. 3 Venn diagrams illustrating the changes in transcriptome changes in control, HSF1 K80Q and dnHSF1 cells between 6 and 24 h after heat shock (A) and the overlap in transcriptome changes in control, HSF1 K80Q and dnHSF1 cells 24 h after heat shock (B). "Down" indicates that the transcript levels were ≥ 2-fold lower than in non-stressed cells; "up" indicates that the transcript levels were ≥ 2-fold higher than in non-stressed cells. The solid line circle represents control cells, the dotted line HSF1 K80Q cells and the dashed line dnHSF1 cells. The shading indicates 24 h after heat shock. For details, see Figs. S4, S5 and Tables S3, S4.

show an extended primary stress response. We also saw a secondary response, i.e. a change in transcript level of genes which did not respond significantly to a heat shock initially (Fig. 3A), with the transcript level of 21 genes decreased and that of 51 genes increased (Table S5). This relatively small transcriptome change in control cells partially overlapped with the much stronger secondary response in HSF1 K80Q and dnHSF1 cells (Fig. 3B and S5) with the transcript levels of 197 genes down and 155 up in HSF1 K80Q cells and those of 677 genes down and of 551 up in dnHSF1 cells (Fig. 3A). The much larger secondary response in cells lacking active HSF1 shows that HSF1 directed macromolecular synthesis plays a major role in restoring homeostasis. The genes of which the transcript levels are increased in HSF1 K80Q or dnHSF1 cells 24 h after heat stress are enriched for the GO category transcription and transcription regulation

(http://david.abcc.ncifcrf.gov/), suggesting that these cells mount an additional transcriptional response in an attempt to restore homeostasis.

Among the genes of which the transcript levels remained high in HSF1 K80Q cells were a number of ATF4 target genes, e.g. PPP1R15A (GADD34) [273], S100P [274] and ATF3 [275]. We therefore tested whether ATF4 activity was responsible for the high transcript levels of these genes. The level of ATF4 did increase markedly in heat shocked cells, but equally so in control or HSF1 K80Q cells. 24 h after heat stress the level of ATF4 was lower than 6 h after heat stress but still higher than in non-stressed cells (Fig. S6B). To examine the role of ATF4 further, we knocked down ATF4 mRNA with siRNA (Fig. S6, C-D) and determined the effect on the mRNA levels of the

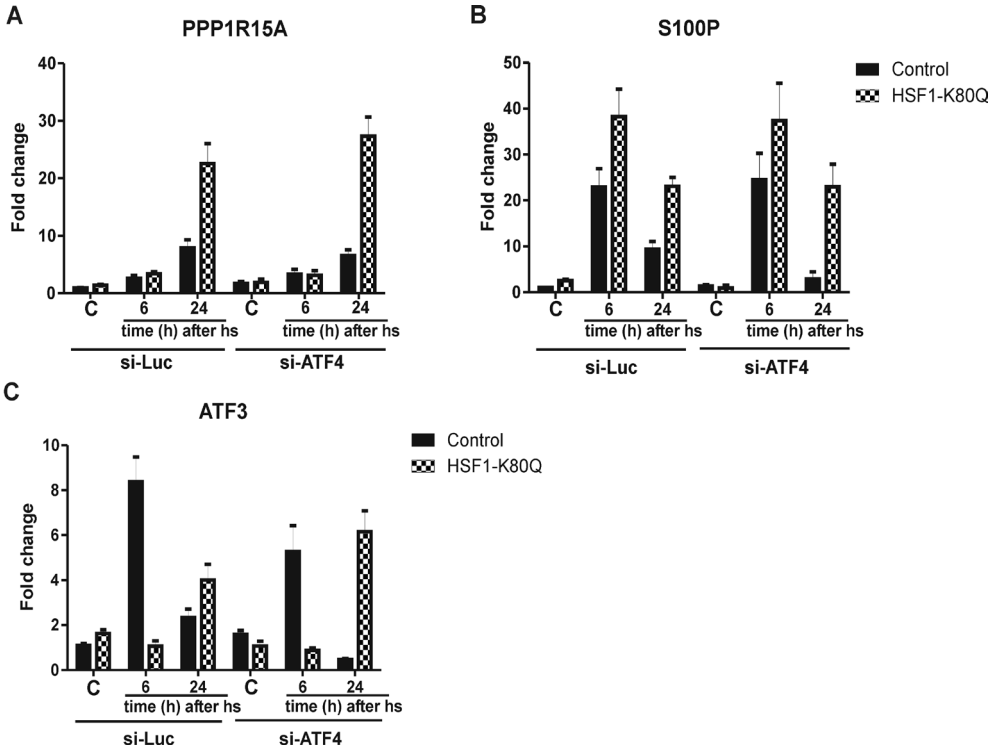


Fig. 4 ATF4 is not involved in the extended primary stress response of its target genes in HSF1 K80Q cells. A-C. HEK-HSF1 K80Q cells were cultured in the presence (HSF1 K80Q) or absence (Control) of doxycycline and transfected for 96 h with siRNA against ATF4 or luciferase as a control with a re-transfection at 48 h. Cells were exposed to a heat shock for 30' at 45°C or left at 37°C (C). When heat shocked, cells were allowed to recover for 6 or 24 h as indicated before harvesting. Total RNA was isolated and transcript levels of the genes indicated relative to GAPDH mRNA levels were measured by QPCR. The fold change of mRNA levels is plotted relative to the level in non-stressed control cells. Error bars represent SD.

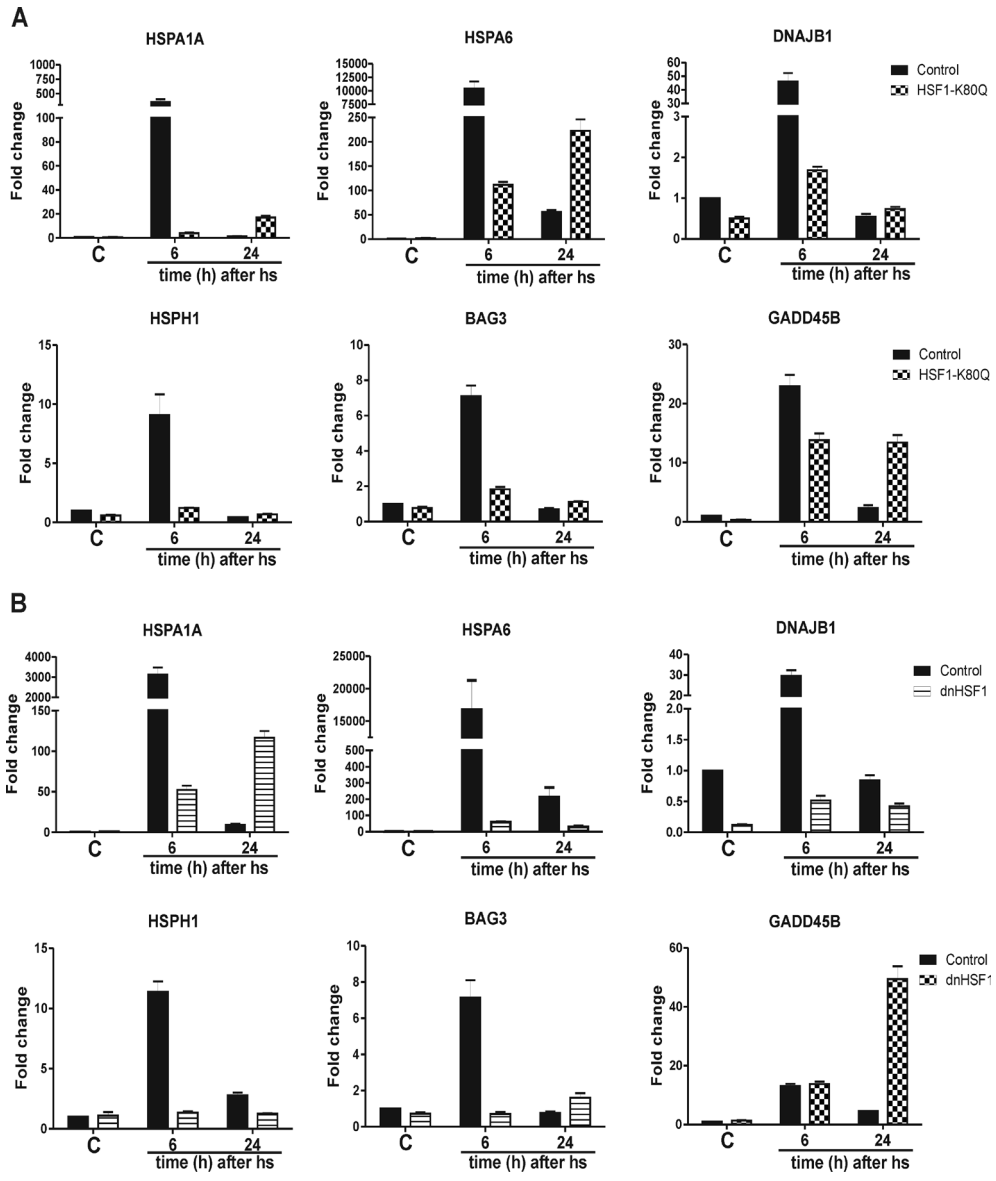


Fig. 5 A delayed stress response in HSF1 K80Q cells. HEK-HSF1 K80Q (**A**) or dnHSF1 (**B**) cells were cultured in the presence (HSF1 K80Q or dnHSF1) or absence of doxycycline (Control) and exposed to a heat shock for 30' at 45°C or left at 37°C (C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and transcript levels relative to GAPDH mRNA levels were measured by QPCR. The fold induction of mRNA levels is plotted relative to the level in non-stressed control cells. Error bars represent SD.

ATF4 target genes PPP1R15A (GADD34), S100P and ATF3 (Fig. 4) by QPCR. ATF4 knockdown did not block the expression of these genes, although the exact effect on the transcript levels varied. These data show that ATF4 does not play a major role in heat shocked cells and suggest that the extended primary stress response of ATF4 target genes in HSF1 K80Q cells is not ATF4 dependent.

The effect of HSF1 K80Q and dnHSF1 expression on the transcriptome of heat shocked cells 24 h after heat shock: a delayed stress response

Surprisingly, the transcript levels of a large fraction of the HSF1 dependent genes stayed the same or were even higher in HSF1 K80Q and dnHSF1 cells 24 h after heat shock, while they decreased in control cells (Table S3). To confirm that at least some apparently HSF1 dependent genes do show a higher transcript level in HSF1 K80Q or dnHSF1 cells 24 h after heat stress, we examined the changes in the transcript level of a set of HSF1 dependent genes by QPCR (Fig. 5). Noteworthy is the increase in transcript level of the HSPA1A and HSPA6 genes in HSF1 K80Q cells. The transcript level of the HSPA1A gene but not that of the HSPA6 gene also went up in dnHSF1 cells. This delayed stress response is not limited to heat shocked cells expressing an inactive HSF1 mutant: arsenite stressed HSF1 K80Q or dnHSF1 cells also showed the delayed stress response (Fig. 6).

These data suggest that HSF1 can be bypassed for at least some HSF1 dependent genes. To investigate which transcription factors could be involved we analyzed the transcriptome changes by Gene Set Enrichment Analysis (GSEA) to assess whether certain transcription factor binding sites were over-represented in the promoter regions of the genes of which the transcript level was elevated 24 h after heat stress. The transcription factors that scored the highest were serum response factor (SRF), octamer transcription factor OCT1 and members of the AP1 family. To determine whether these transcription factors were indeed activated 24 h after heat stress in HSF1 K80Q cells, we analyzed whether they showed increased DNA binding activity. We used probes with the DNA binding sequences for these transcription factors and performed an electrophoretic mobility shift assay with nuclear extracts of control or HSF1 K80Q expressing cells before, 6 or 24 h after heat stress. No differences in signals were found between control and HSF1 K80Q cells either 6 or 24 h after heat shock for the SRE (serum response element) and OCT1 probes (Fig. 7, A-B). When we used a generic AP1 family probe, clear differences were observed (Fig. 7C). A strong band shift signal was found using extracts of control cells 6 h after heat stress but not using similar extracts of HSF1 K80Q cells. When we used extracts isolated from cells 24 h after heat stress, a much stronger band shift signal was detected using extracts of HSF1 K80Q expressing cells compared to extracts of control cells. This complex consistently migrated slightly

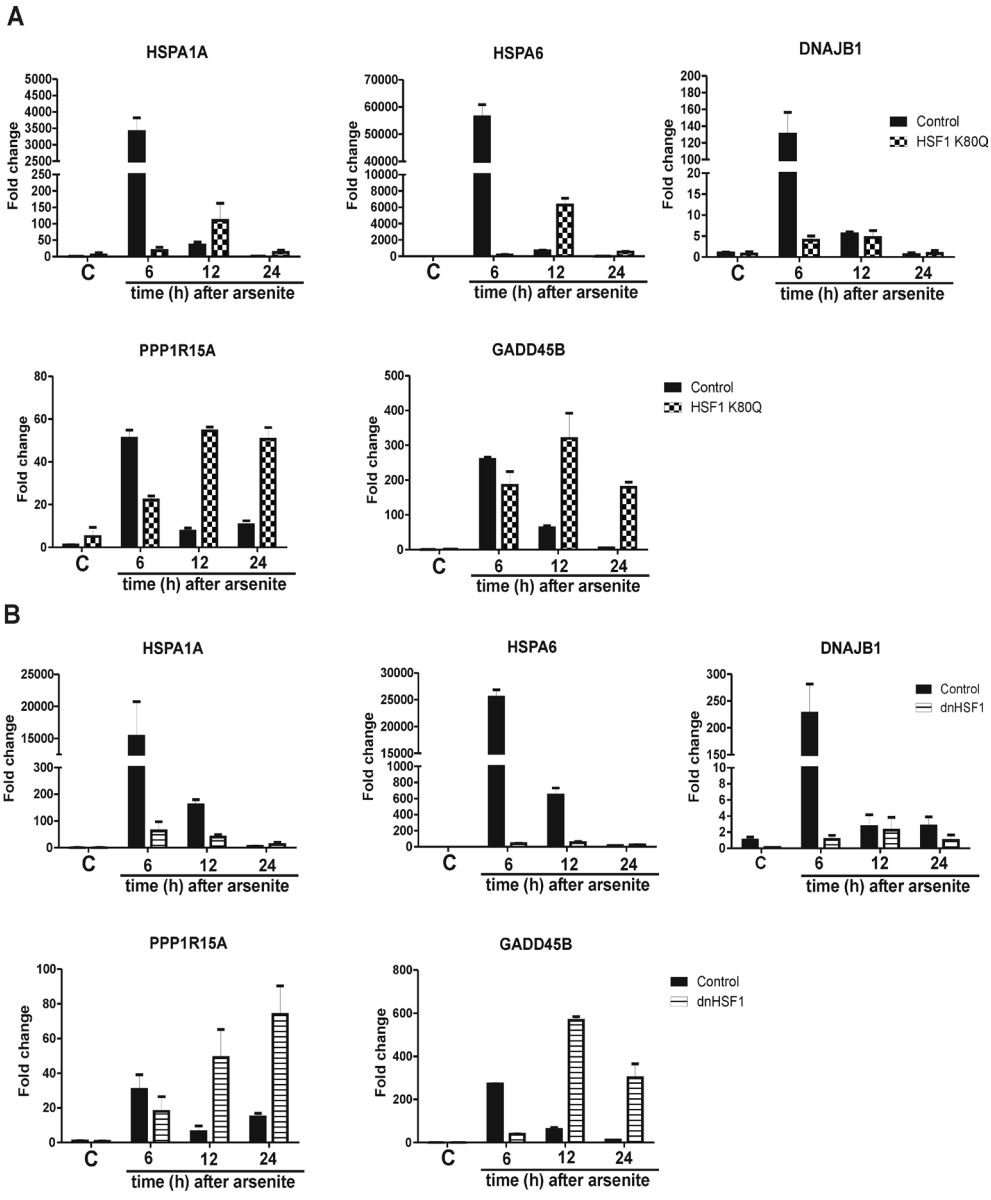


Fig. 6 Relative changes in transcript levels of various genes in arsenite stressed HSF1 K80Q or dnHSF1 cells. HEK-HSF1 K80Q (**A**) or HEK-dnHSF1 (**B**) cells were cultured in the presence (HSF1 K80Q/dnHSF1) or absence of doxycycline (Control) and treated with 0.5 mM of arsenite for 1.5 h. Medium was washed off and cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and transcript levels relative to GAPDH mRNA levels were measured by QPCR. The fold induction of mRNA levels is plotted relative to the level in non-stressed control cells. Error bars represent SD.

faster than the complex detected using extracts from control cells 6 h after heat stress and thus might represent a different DNA-protein complex.

Our microarray data showed that the transcript levels of the AP1 family members c-Fos and FosB were elevated 24 h after heat shock in the presence of HSF1 K80Q (Table S3, Fig. S7, A-C). c-Fos and FosB are both early response genes and have been implicated in the regulation of proliferation and differentiation and their transcript levels increase upon several stimuli, such as growth factors and stress, including oxidative stress [276,277]. To test whether c-Fos or FosB plays a role in the delayed stress response, we knocked down c-Fos and FosB mRNA (Fig. S7, B-C) and examined the effect on HSPA1A and HSPA6 mRNA levels, as well as on the GADD45B and PPP1R15A mRNA levels. Knockdown of c-Fos resulted in an increase in HSPA1A mRNA levels in control cells 6 h after heat shock and in HSF1 K80Q cells 24 h after heat shock (Fig. 8A). c-Fos thus appears to inhibit rather than enhance expression of the HSPA1A gene. Knockdown of c-Fos did not alter the expression pattern of the HSPA6, GADD45B or PPP1R15A genes significantly either in control or in HSF1 K80Q cells, except for

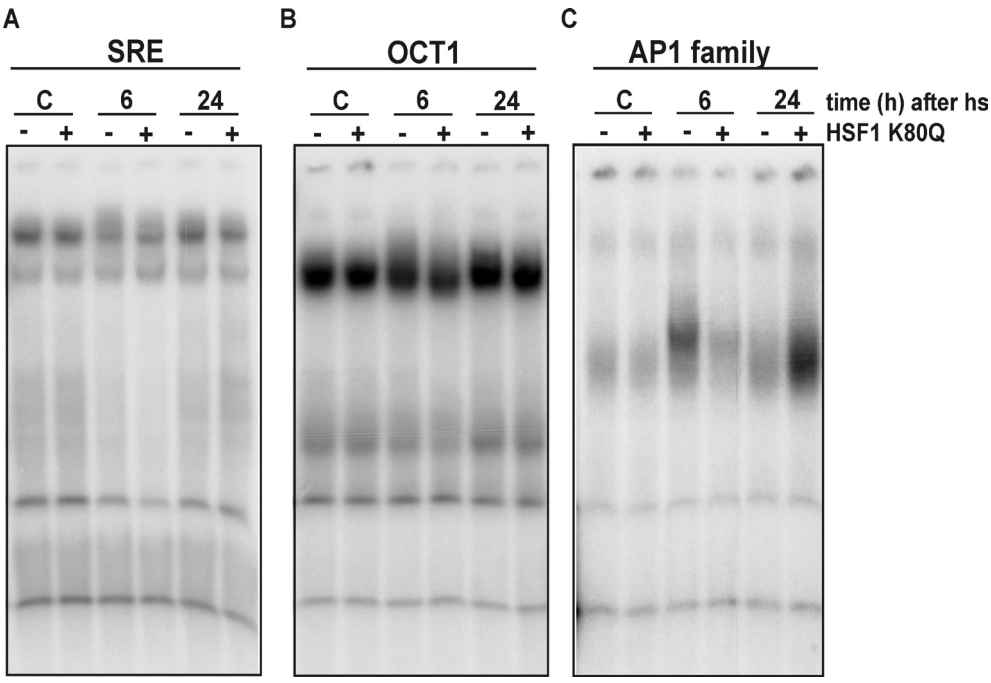


Fig. 7 Binding to the SRF, OCT1 or AP1 consensus sequence. HEK-HSF1 K80Q cells were cultured in the presence (+) or absence (-) of doxycycline and exposed to a heat shock for 30' at 45°C or left at 37°C (C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Nuclear extracts were used in an electrophoretic mobility shift assay with a double-stranded oligonucleotide with the SRE (**A**), OCT1 (**B**) or AP1 family (**C**) sequence.

a slight increase in PPP1R15A mRNA in HSF1 K80Q cells 24 h after heat stress (Fig. 8, B-D). Knockdown of FosB had little effect on HSPA1A, GADD45B or PPP1R15A mRNA levels. Only the increase in HSPA6 mRNA levels 6 h after heat stress, but not that 24 h after heat stress, was slightly inhibited (Fig. 8B). These data show that neither c-Fos nor FosB is responsible for the delayed and/or extended primary stress response in HSF1 K80Q cells.

The generic AP1 probe used in the EMSA shown in Fig. 7 can also be bound by NRF2, a transcription factor involved in the antioxidant response. To determine a possible involvement of NRF2 in the delayed stress response in HSF1 K80Q cells after heat shock, we analyzed the binding to a probe with the antioxidant response element (ARE) sequence, the consensus binding sequence for NRF2, in extracts of heat stressed control or HSF1 K80Q cells and found the same pattern as that seen when the generic AP1 probe was used (Fig. 7 and S8A). The sequence of the CRE (cAMP responsive element;

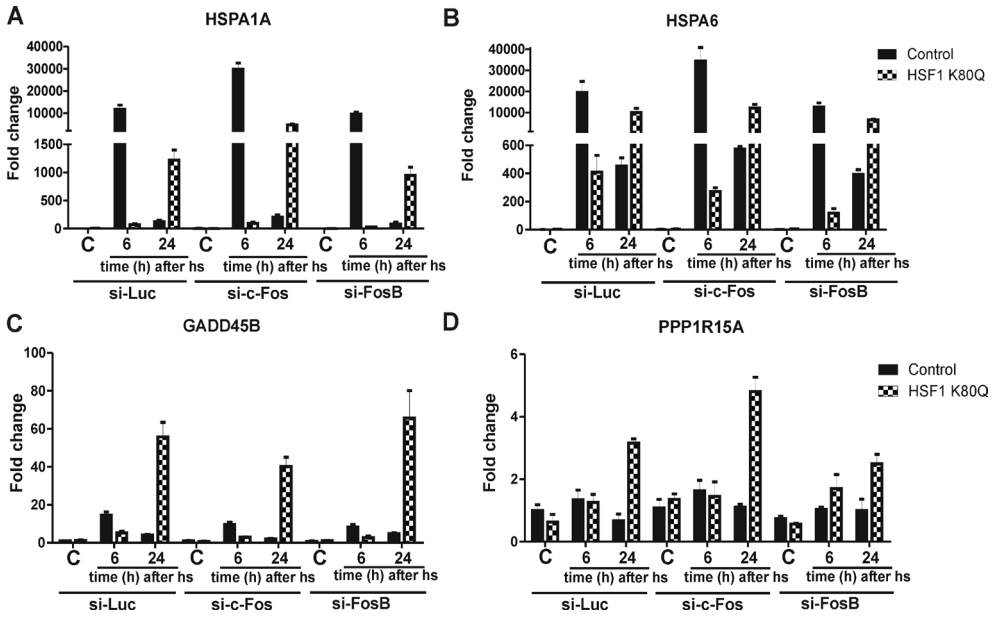


Fig. 8 c-Fos and FosB are not involved in activating transcription of the HSPA1A and HSPA6 genes 24 h after heat shock in HSF1 K80Q cells. A-D. HEK-HSF1 K80Q cells were cultured in the presence (HSF1 K80Q) or absence (Control) of doxycycline and transfected for 96 h with siRNA against c-Fos, FosB or luciferase as a control with a re-transfection at 48 h. Cells were exposed to a heat shock for 30' at 45°C or left at 37°C (C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and transcript levels of the genes indicated relative to GAPDH mRNA levels were measured by QPCR. The fold change of mRNA levels is plotted relative to the level in non-stressed control cells. Error bars represent SD.

CREB protein consensus binding sequence) or TRE (12-*O*-tetradecanoyl phorbol13-acetate (TPA)-responsive element; AP1 consensus binding sequence) overlaps with the ARE. However, competition with a 10-fold molar excess of unlabeled probes for either the CRE or the TRE did not compete away the signal of the ARE complex, whereas the signal almost completely disappeared when unlabeled ARE probe was the competitor (Fig. S8B). These data show that the extracts of control and HSF1 K80Q cells 6 and 24 h after heat stress differ in an ARE binding activity and suggest a role for NRF2 in the stress response of HSF1 K80Q cells. To confirm the differential pattern of NRF2 activity we analyzed the transcript levels of the NRF2 target gene heme oxygenase 1 (HMOX1). HMOX1 transcript levels increased in control cells 6 h after heat stress, but not in HSF1 K80Q cells (Fig. 9A). In control cells 24 h after heat stress the HMOX1 mRNA level had decayed, while in HSF1 K80Q cells the HMOX1 mRNA level had increased. The changes in HMOX1 mRNA levels thus correspond to the changes in the ARE binding pattern. Treatment with NRF2 siRNA blocked the heat shock induced changes in HMOX1 mRNA levels in both control and HSF1 K80Q cells (Fig. 9A and S9), showing that NRF2 indeed regulates HMOX1 expression in response to heat shock. To look further into the role of NRF2 in the delayed and/or extended primary stress response in HSF1 K80Q cells, we investigated the effect of NRF2 siRNA treatment on HSPA1A and HSPA6 mRNA levels, as well as on the GADD45B and PPP1R15A mRNA levels. Knockdown of NRF2 mRNA did not have any effect on HSPA1A and HSPA6 mRNA levels 6 h after heat shock (Fig. 9, B-C). In HSF1 K80Q cells 24 h after heat stress the level of HSPA6 mRNAs was significantly lower in NRF2 siRNA treated cells compared to cells treated with control siRNA. The HSPA1A mRNA level was also decreased, but not as markedly as that of HSPA6 mRNA. These results were confirmed using a second NRF2 siRNA (Fig. S10). These data indicate that NRF2 is involved in the delayed stress response of HSF1 target genes in HSF1 K80Q cells 24 h after heat stress. GADD45B mRNA levels in control cells appeared to be increased when cells were treated with NRF2 siRNA (Fig. 9D); this could, however, not be confirmed with the second siRNA (Fig. S10). The increase in GADD45B transcript 24 h after heat shock in the presence of HSF1 K80Q was not affected by NRF2 knockdown. For the PPP1R15A mRNA, results were also somewhat variable: the first, but not the second, NRF2 siRNA enhanced PPP1R15A transcript levels in control cells 6 h after heat stress (Fig. 9E); the second, but not the first, NRF2 siRNA decreased PPP1R15A mRNA levels in HSF1 K80Q cells both 6 and 24 h after heat stress (Fig. S10). However, irrespective of which NRF2 siRNA was used, the PPP1R15A mRNA level was always higher in HSF1 K80Q cells than in control cells 24 h after heat shock. Together these data indicate that NRF2 is not involved in setting the GADD45B and PPP1R15A mRNA levels in HSF1 K80Q cells 24 h after heat shock.

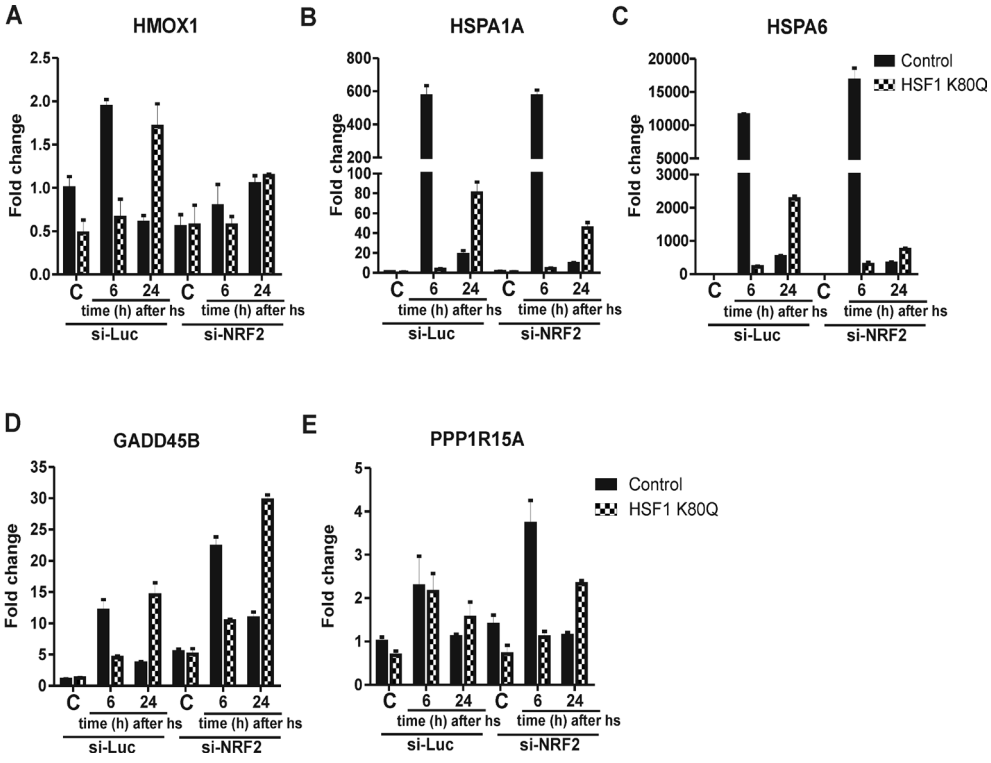


Fig. 9 Transcript levels in NRF2 siRNA treated cells. A-E. HEK-HSF1 K80Q cells were cultured in the presence (HSF1 K80Q) or absence (Control) of doxycycline and transfected for 96 h with siRNA against NRF2 or luciferase as a control with a re-transfection at 48 h. Cells were exposed to a heat shock for 30' at 45°C or left at 37°C (C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and transcript levels of the genes indicated relative to GAPDH mRNA levels were measured by QPCR. The fold change of mRNA levels is plotted relative to the level in non-stressed control cells. Error bars represent SD.

Recently, a shared co-factor for HSF1 and NRF2 has been described. Kaitsuka *et al.* [278] reported that the long spliced variant of the eukaryotic elongation factor 1δ (EEF1D_L) is a heat shock transcription factor. This variant was shown to bind to the HSE in the promoter of the HSPA6 gene and activate transcription. In addition, they showed that the N-terminal domain of EEF1D_L interacts with NRF2 and that EEF1D_L is an essential protein for NRF2-dependent (HMOX1) gene induction. We therefore investigated whether EEF1D_L is involved in the delayed and/or extended primary stress response in HSF1 K80Q cells. We treated cells with the EEF1D_L siRNA described ([278]; Fig. S11) and examined HSPA1A and HSPA6 mRNA levels.

Remarkably, the transcript levels of the HSPA1A and HSPA6 genes responded differently to EEF1D_L siRNA. In contrast to the findings of Kaitsuka *et al.* [278] we did not see an effect of EEF1D_L siRNA on the increase in HSPA6 mRNA levels in control cells 6 h after heat shock (Fig. 10B), but the increase of HSPA1A mRNA levels was diminished (Fig. 10A). In the HSF1 K80Q cells, however, the increase in both the HSPA1A and HSPA6 mRNA levels 24 h after heat stress was strongly inhibited by EEF1D_L siRNA. EEF1D_L siRNA treatment also abolished the increase in GADD45B and PPP1R15A mRNA levels in HSF1 K80Q cells 24 h after heat stress (Fig. 10, C-D), but the data reported above (Fig. 5 and 9) show that HSF1 and NRF2, the suggested partners of EEF1D_L, are not involved. The interpretation of these data is complicated by our finding that EEF1D_L siRNA treatment also decreased HSF1 mRNA levels (Fig.

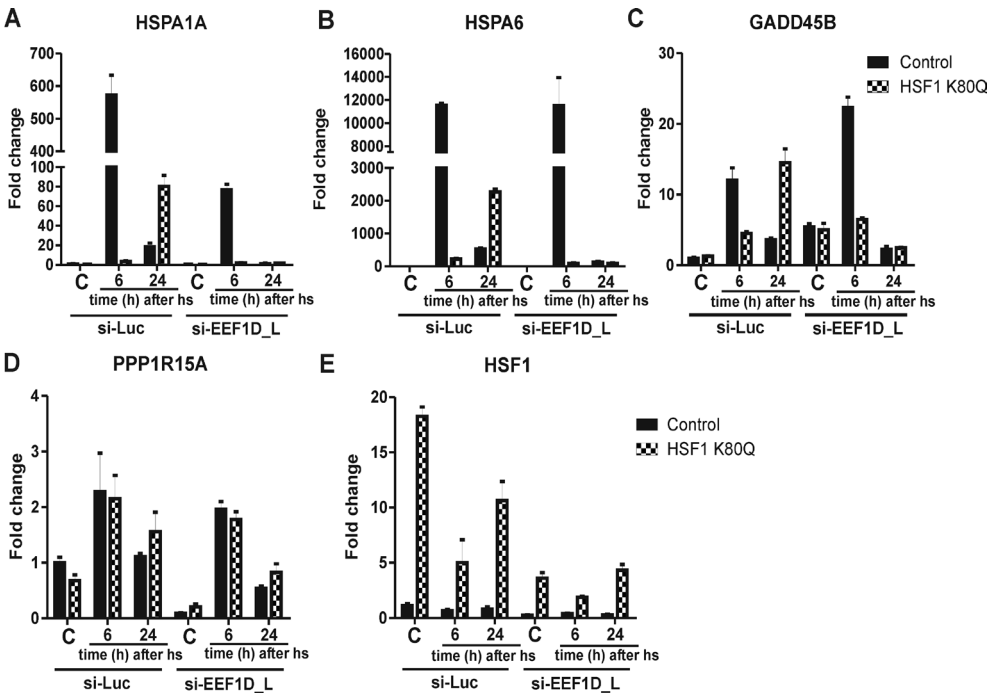


Fig. 10 Transcript levels in EEF1D_L siRNA treated cells. A-E. HEK-HSF1 K80Q cells were cultured in the presence (HSF1 K80Q) or absence (Control) of doxycycline and transfected for 96 h with siRNA against EEF1D_L or luciferase as a control with a re-transfection at 48 h. Cells were exposed to a heat shock for 30' at 45°C or left at 37°C (C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and transcript levels of the genes indicated relative to GAPDH mRNA levels were measured by QPCR. The fold change of mRNA levels is plotted relative to the level in non-stressed control cells. Error bars represent SD.

10E) and EEF1D_L siRNA thus has off-target effects. Unfortunately, we were unable to knockdown EEF1D_L mRNA using other siRNAs, including the second siRNA described by Kaitsuka *et al.* [278].

Comparison of transcriptome changes in the presence of HSF1 K80Q or siRNA HSF1

We demonstrated above (Fig. 5) that the transcript levels of the HSPA6 gene increase in HSF1 K80Q cells but not in dnHSF1 cells 24 h after heat stress. This suggests that the HSE must be free of HSF1 to allow access of other transcription factors to this promoter. To determine if that that is indeed the case, we treated HEK293 cells with HSF1 siRNA, which resulted in efficient knockdown of HSF1 (Fig. 11, A-B). The lack of HSF1 activity in siRNA treated cells was also evident 6 h after heat shock: the increase in the HSPA1A, HSPA6 and DNAJB1 transcript levels was strongly inhibited (Fig. 11C). Surprisingly, we saw no increase in the levels of these transcripts 24 h after heat shock. In fact, these were even lower 24 h after heat shock than 6 h after heat shock. HSF1 siRNA treated cells thus did not show the delayed response of the HSPA1A and HSPA6 genes seen in HSF1 K80Q cells. These data suggest that the delayed response of these HSF1 target genes somehow requires the HSF1 protein.

Next, we investigated whether the HSF1 independent stress response (see Table 2, Table S3) differed between HSF1 siRNA and HSF1 K80Q expressing cells. For the RGS2 (regulator of G-protein signaling 2) and PPP1R15A (GADD34) genes the response was similar, although quantitatively somewhat larger for the PPP1R15A (GADD34) gene in HSF1 K80Q cells (Fig. 11D; for the response in dnHSF1 cells, see Fig. S2B). Finally, we determined whether treatment with HSF1 siRNA also caused an increase in the level of the PCK2 (mitochondrial phosphoenolpyruvate carboxykinase 2) in unstressed HEK293 cells, just as expression of HSF1 K80Q did. The results in Fig. 11D demonstrate that PCK2 transcript levels indeed increased in HSF1 siRNA treated cells. In conclusion, assaying an, admittedly limited, number of transcripts showed no difference between lack of HSF1 and expression of an non-DNA binding HSF1 mutant with the important exception of the delayed stress response seen 24 h after heat shock.

Discussion

HSF1 was originally discovered as the transcription factor required for the synthesis of additional cytoplasmic chaperones, the heat shock proteins, during the proteotoxic stress response. Later HSF1 was also shown to have a physiological role in non-stressed cells, for example the circadian clock gene Per2 is an HSF1 target [19,20]. Tumor

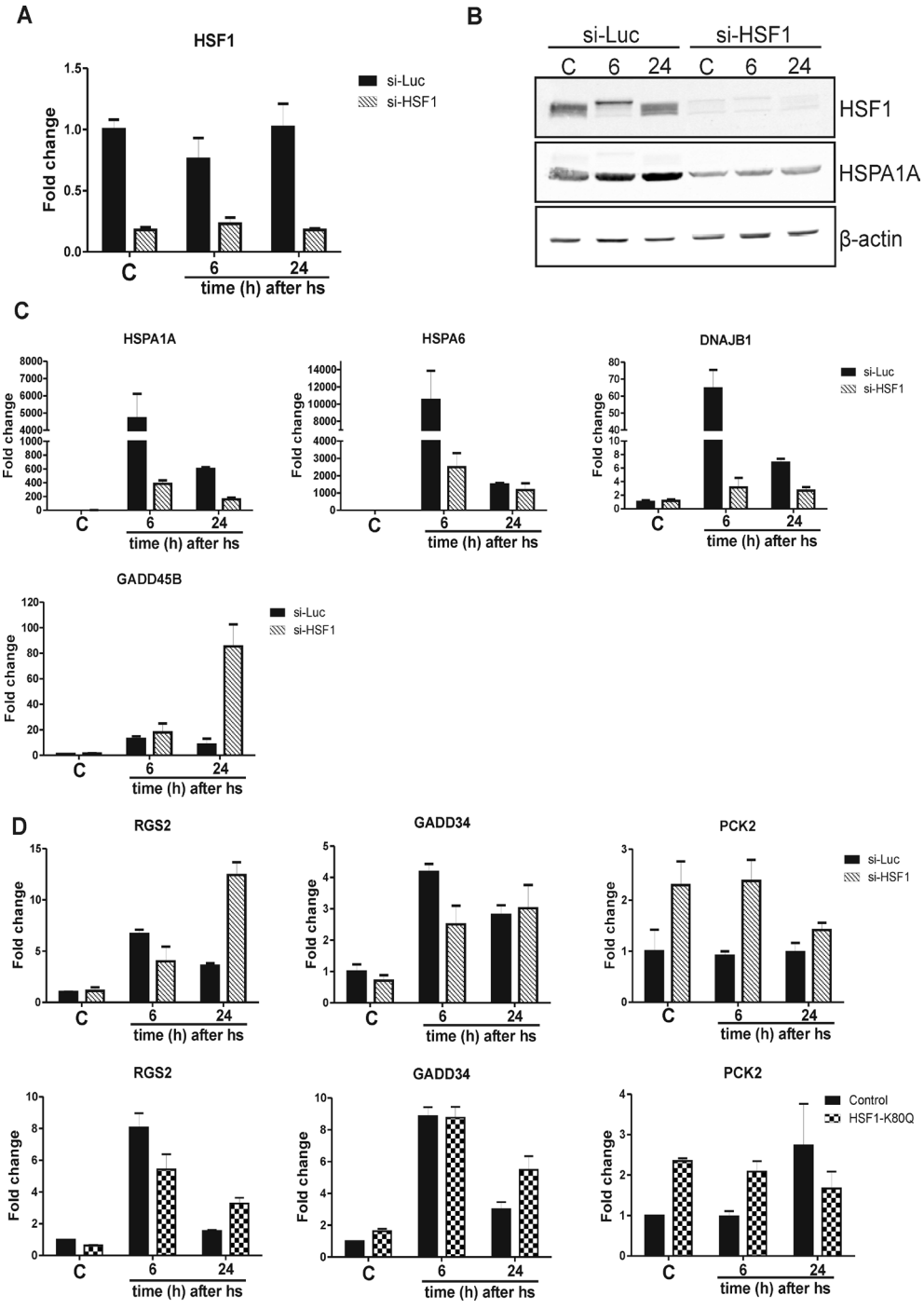

A
HSF1
Fold change
si-Luc
si-HSF1
C
6
24
time (h) after hs
B
si-Luc
si-HSF1
C 6 24 C 6 24
HSF1
HSPA1A
β-actin
C
HSPA1A
HSPA6
DNAJB1
GADD45B
D
RGS2
GADD34
PCK2
Control
HSF1-K80Q

Fig. 11 Relative changes in transcript levels of various genes in stressed and non-stressed HSF1 siRNA treated cells. A. HEK293 cells were transfected with siRNA against luciferase or HSF1 for 96 h to decrease HSF1 levels. Cells were exposed to a heat shock for 30' at 45°C or left at 37°C (C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and HSF1 transcript levels relative to GAPDH mRNA levels were measured by QPCR. The fold induction of mRNA levels is plotted relative to the level in non-stressed control cells. **B.** HSF1 siRNA treated cells were harvested and lysates were subjected to SDS-PAGE and western blot analysis using antibodies against the indicated proteins. C-D. mRNA levels were determined by QPCR analysis and are shown relative to GAPDH mRNA levels. The fold induction of mRNA levels is plotted relative to the level in non-stressed control cells. Error bars represent SD.

cells rely on active HSF1 [21] and blocking HSF1 activity in HeLa cells causes cell death [178]. HEK293 cells are less sensitive to a lack of HSF1 activity [178], possibly because the HSPA1A promoter is activated by E1A [279], the transforming protein in HEK293 cells. Accordingly, blocking HSF1 activity in non-stressed HEK293 cells by overexpression of the non-DNA binding HSF1 K80Q mutant led to only a limited change in the transcriptome – a change that is apparently mostly cell specific as judged from a comparison with the published data for the effect of depletion of HSF1 by siRNA in HeLa cells [237]. None of the canonical HSF1 target genes appear to rely on HSF1 activity for expression in non-stressed HEK293 cells as for none of these genes the transcript level decreased more than two fold (see also Table S2). Yet expression of HSF1 K80Q does affect the chaperoning capacity of the cells adversely as judged from the luciferase refolding assays (Fig. 1C). Possibly overexpression of HSF1 does sequester cytoplasmic chaperones. As expected, HSF1 plays a major role in the response of HEK293 cells to proteotoxic stress and regulates about 30% of genes of which the transcript level increases. The effect of a lack of HSF1 activity is most apparent in cells that had been allowed to recover from a heat stress for 24 h. In control cells the transcriptome has largely returned to normal, while in HSF1 K80Q and particularly in dnHSF1 cells the transcriptome has diverged even further from the non-stressed transcriptome than in cells 6 h after heat shock (Fig. 3). HSF1 directed chaperone synthesis is thus required for heat shocked cells to regain homeostasis. The lack of chaperone synthesis in a cell expressing the HSF1 K80Q mutant is reflected in an extended primary stress response, the continued high level of stress induced transcripts. The simplest model is that the activity of some heat stress activated transcription factor(s) remains high in HSF1 K80Q cells but decays in control cells. Our results do not support such a model, at least as judged from the changes in the transcript levels of the GADD45B and PPP1R15A genes. We could block the increase in level of these transcripts in HSF1 K80Q cells 24 h after heat shock but not the increase in level 6 h after heat shock by treatment with an EEF1D_L siRNA. Different factor(s) must thus

be involved in maintaining these transcript levels in cells 6 and 24 h after heat stress. Which factors regulate transcription of the GADD45B or the PPP1R15A genes in cells either 6 or 24 h after heat stress is not clear. The results reported above show that it is not ATF4, c-Fos, FosB or one of the suggested partners of EEF1D_L, HSF1 or NRF2. In cells 24 h after heat stress it must be either an as yet unidentified partner of EEF1D_L or a factor encoded by a transcript that is also targeted by the EEF1D_L siRNA. Unfortunately, in our hands other EEF1D_L siRNAs were not effective, so we cannot distinguish between these two possibilities. The transcription factor(s) involved in driving HSF1 independent transcription 6 h after heat stress also remain to be identified. In the case of the PPP1R15A gene, we had assumed this to be ATF4, but our results show that that is not the case. The GADD45B promoter has been shown to be targeted by NFY, Sp1, and Egr1 [280]. We have not examined whether any of these factors play a role in the extended primary stress response of HSF1 K80Q cells.

Next to the extended primary stress response, HSF1 K80Q cells mount what we have called a delayed stress response, i.e. an increase in transcript level of HSF1 target genes 24 h after heat shock. Curiously, this response does require HSF1 protein as we could not detect it in HSF1 siRNA treated cells. Our data further show that NRF2 is involved in this delayed stress response. Previously, it has been reported that murine heat shock protein mRNA levels (i.e. Hspb1, Dnajb1 and Hsp90) are increased in an NRF2-dependent manner upon NRF2-activating compounds [125,126]. In addition, an NRF2 binding element has been found in the promoter of the *Hsp70* gene in zebrafish and this element was conserved between mouse and zebrafish [127]. Finally, mammalian heat shock genes have been reported to be enriched in AP1/NRF2/Fos binding sites [21]. The activity of NRF2 is regulated post-translationally. Under non-stress conditions the cysteine rich protein KEAP1 (*K*elch-like ECH associated protein 1) retains NRF2 in the cytoplasm [119] and maintains it at a low level through KEAP1-dependent ubiquitination and proteasomal degradation [120-124]. Upon oxidative stress, cysteines in the KEAP1 protein are oxidatively modified, resulting in a conformational change and release of NRF2 [121]. NRF2 then binds to the ARE usually as a heterodimer with one of the small Maf proteins (for reviews, see [281-283]). The data reported above show that the formation of an ARE binding complex is delayed in HSF1 K80Q cells. Apparently, the formation of active NRF2 in cells recovering from heat stress for 6 h requires HSF1 or an HSF1 regulated function. The difference in mobility of the ARE binding complex formed in extracts of control cells 6 h after heat stress and that in extracts of HSF1 K80Q cells 24 h after heat stress suggests that it is the heteromeric partner of NFR2 that differs, for example because a different small Maf protein is involved. In our microarray analysis we found no significant changes in the levels of the mRNAs encoding the small Maf proteins upon heat stress or upon expression of HSF1

K80Q. However, small Mafs can be (in)activated by post-translational modification [281] and such a modification could be affected by heat stress and HSF1 activity. Our data thus suggest that the delayed stress response in HSF1 K80Q cells is a delayed antioxidant response and point to an interaction between HSF1 or an HSF1 regulated function and a partner of NRF2 in the timing of the antioxidant response. Aging cells are not only impaired in protein homeostasis but also in redox homeostasis. Our results imply that loss of HSF1 activity may be the common cause. Our results also support NRF2 as a promising target in treating age-related disease: it would not only redress the redox balance, but by enhancing the transcript level of at least some HSF1 target genes, also boost resistance to proteotoxic stress.

Acknowledgements

We thank Dr. A. Zantema for the HSPB1 antibody. We thank the microarray facility at the VU UMC (Amsterdam, The Netherlands) for performing the microarray experiments. This work was financially supported by AgentschapNL (project numbers IGE03018 and IGE07004, www.agemtschapnl.nl).

Supplemental tables

Table S1. Oligonucleotides

	Name	Sequence (5'-->3')
ChIP	HSPA6_fwd	ggaaggtgcgggaaggttcg
	HSPA6_rev	ttcttgtcggatgctgga
EMSA	HSE_fwd	ctattctcgttgcttcgagagagcgcgcctcgaatgttcgcgaaaagag
	HSE_rev	ctcttttcgcgaacattcgaggcgcgctctctcgaagcaacgagaatag
	SRF_fwd	cttacacaggatgtccatattaggacatct
	SRF_rev	agatgtcctaatatggacatcctgtgtaag
	OCT1_fwd	tgtcgaatgcaaatcactagaa
	OCT1_rev	ttctagtgatttgcattcgaca
	AP1 family_fwd	gagtaatcgtgagtcatcaattccgagc
	AP1 family_rev	gctcggaattgatgactcacgattactc
	TRE_fwd	cggaatcattgactcatatttactc
	TRE_rev	gagtaaatatgagtcaatgattccg
	ARE_fwd	cggaatgtatgactcagcattactc
	ARE_rev	gagtaatgctgagtcatacattccg
QPCR	GAPDH_fwd	gcagctgaaagaagcccaagt
	GAPDH_rev	tgtcttccatgccaattgca

HSPA1A_fwd	ccgagaaggacgagtttgag
HSPA1A_rev	acaaaaacagcaatcttggaaagg
HSPA6_fwd	cagagatgaactttccctcc
HSPA6_rev	gaagcagaagaggatgaacc
HSPA1B_fwd	cagctctttgctgcttcac
HSPA1B_rev	cttacagtatcaacattaaatgc
HSPA2_fwd	actcaagtcagcgtaaacct
HSPA2_rev	aatagatctcgtacttggcac
DNAJB1_fwd	ttccccagacatcaagaacc
DNAJB1_rev	accctctcatggtccacaac
HSP90_fwd	gttggtcctgtgcggtcact
HSP90_rev	tgggcaatttctgcctgaa
HSPB1_fwd	cgcgctcagccggcaactc
HSPB1_rev	agccatgctcgtcctgccgc
PCK2_fwd	gcagcagaacacaaagggaag
PCK2_rev	tagtgcccgaagttgtagcc
SGK_fwd	cctgggagctgtcttgtatgag
SGK_rev	aggtgtcttgcggaatttgtaa
ATF3_fwd	tgccgaaacaagaagaagg
ATF3_rev	ttagctctgcaatgttccttc
RGS2_fwd	aagattggaagacccgtttgag
RGS2_rev	gcaagaccatatttgctggct
GADD45B_fwd	gacctgcattgtctcctggtc
GADD45B_rev	cagcgttcctgaagagagatgta
PPP1R15A_fwd	cgcttctggcagaccgaa
PPP1R15A_rev	gtagcctgatggggtgcttg
BAG3_fwd	ctccattccggtgatacacga
BAG3_rev	tggtgggtctggtactccc
HSPH1_fwd	aggagttccatatccagaa
HSPH1_rev	cagctcaacattcaccac
HSF1_fwd	agcatgagaatgaggctctgtg
HSF1_rev	gtgctgagccactgtcgttc
ATF4_fwd	gggacagattggatgttggaga
ATF4_rev	acccaacagggcatccaag
S100P_fwd	tcaaggtgctgatggagaa
S100P_rev	acacgatgaactcactgaagtc
c-Fos_fwd	ctactaccactcacccgcagac

c-Fos_rev	ggaatgaagttggcactggag
FosB_fwd	ctcggcctaggtcacgtt
FosB_rev	gccagagtttctagaagcagttt
HMOX1_fwd	ctgtctcaaacctccaaaagcc
HMOX1_rev	tcaaaaaccaccccaaccc
NRF2_fwd	agacggtatgcaacaggac
NRF2_rev	cttctggacttggaaccatg
EEF1D_L_fwd	agacaagcacaagtatgaggagg
EEF1D_L_rev	cctcatcagcgtcctcagg

Table S2. Transcriptome changes in HEK293 cells overexpressing wild type HSF1

Acc. nr.	Gene	wt/Cntrl 37 ° C	K80Q/Cntrl 37 ° C	Cntrl 6 h hs	K80Q 6 h hs	dnHSF 6 h hs	Description
NM_002200	IRF5	0.1	0.9	1.1	1.1	1.1	interferon regulatory factor 5
NM_001034173	ALDH1L2	0.2	0.5	0.7	0.9	1.1	aldehyde dehydrogenase 1 family, member L2
NM_001003702	ARHGEF5L	0.2	0.9	0.9	0.9	1.0	Rho guanine nucleotide exchange factor (GEF) 5-
NM_001024074	HNMT	0.4	1.1	0.9	0.9	*0.2*[a]	histamine N-methyltransferase
NM_198495	CTAGE4	0.4	0.9	0.8	0.9	1.1	CTAGE family, member 4
NM_001145659	CTAGE9	0.4	0.9	0.8	0.9	1.2	CTAGE family, member 9
NM_001008747	LOC441294	0.5	0.9	0.8	0.8	1.0	similar to CTAGE6
NR_027466	RP11-159J2.1	0.5	1.0	0.8	0.9	1.2	CTAGE family, member 5 pseudogene
NM_178561	CTAGE6	0.5	1.0	0.9	0.9	1.2	CTAGE family, member 6
NM_005084	PLA2G7	0.5	0.9	0.9	0.8	1.5	phospholipase A2, group VII
NM_002228	JUN	2.0	0.8	**4.0**[b]	*1.8*	*1.4*	jun oncogene
NM_015167	JMJD6	2.0	1.0	**2.6**	1.6	*0.8*	jumonji domain containing 6
NM_015009	PDZRN3	2.1	0.8	1.0	0.9	*0.4*	PDZ domain containing ring finger 3
NM_001017915	INPP5D	2.1	0.6	1.2	1.0	1.0	inositol polyphosphate-5-phosphatase, 145kDa
NM_017763	RNF43	2.2	1.0	0.4	0.4	*0.2*	ring finger protein 43

NM_003009	SEPW1[c]	2.2	1.0	1.0	1.0	0.8	selenoprotein W, 1
NM_181726	ANKRD37	2.2	1.1	1.7	0.5	0.7	ankyrin repeat domain 37
NM_198391	FLRT3	2.3	1.1	**2.3**	2.0	1.8	fibronectin leucine rich transmembrane protein 3
NR_023388	PRINS	2.3	1.0	0.9	0.8	1.6	psoriasis associated RNA induced by stress (non-protein coding)
NM_004281	BAG3	2.3	1.0	**6.7**	*2.2*	*1.3*	BCL2-associated athanogene 3
NM_006404	PROCR	2.3	0.9	1.0	0.9	0.8	protein C receptor, endothelial (EPCR)
NM_021648	TSPYL4	2.3	1.1	1.1	0.9	0.6	TSPY-like 4
NM_017870	TMEM132A	2.4	0.9	0.8	0.9	1.0	transmembrane protein 132A
NR_028272	NEAT1	2.4	1.2	1.1	0.7	1.1	nuclear paraspeckle assembly transcript 1
NM_006644	HSPH1	2.4	0.8	**9.8**	*1.9*	*1.0*	heat shock 105kDa/110kDa protein 1
NM_001017973	P4HA2	2.5	0.8	1.1	1.0	0.5	prolyl 4-hydroxylase, alpha polypeptide II
NM_002155	HSPA6	2.6	1.2	**61.9**	*27.4*	*18.1*	heat shock 70kDa protein 6 (HSP70B')
NM_033256	PPP1R14A	2.6	1.3	1.0	1.1	1.1	protein phosphatase 1, regulatory (inhibitor) subunit 14A
NM_182908	DHRS2	2.6	1.0	**3.7**	7.7	2.2	dehydrogenase/reductase (SDR family) member 2
NM_004688	NMI	2.7	1.0	1.3	1.2	2.2	N-myc (and STAT) interactor
NM_000905	NPY	2.8	0.7	1.0	0.9	1.0	neuropeptide Y
NM_006366	CAP2	2.8	1.0	1.1	1.1	1.1	CAP, adenylate cyclase-associated protein, 2
NM_181847	AMIGO2	3.0	1.2	1.0	1.0	1.3	adhesion molecule with Ig-like domain 2
NM_003182	TAC1	3.0	1.4	**2.6**	2.0	3.1	tachykinin, precursor 1
NM_203339	CLU	3.0	1.0	**2.3**	1.2	1.2	clusterin
NM_000302	PLOD1	3.6	0.9	1.1	1.0	0.8	procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1
NM_007034	DNAJB4	3.7	1.0	**5.6**	*1.7*	*1.2*	DnaJ (Hsp40) homolog, subfamily B, member 4
NM_021199	SQRDL	3.7	1.3	1.1	1.0	1.3	sulfide quinone reductase-like (yeast)
NM_003248	THBS4	3.9	1.8	0.9	1.0	1.2	thrombospondin 4
NM_000095	COMP	4.0	1.7	0.9	1.0	1.1	cartilage oligomeric matrix protein
NM_001885	CRYAB	5.6	1.1	**24.3**	*2.4*	*3.6*	crystallin, alpha B
NR_024377	FER1L4	7.0	0.6	1.1	1.2	0.6	fer-1-like 4

a: numbers in italics indicate ≥ 2-fold decrease relative to control 6 h after hs

b: numbers in bold indicate ≥ 2-fold increase relative to control before hs

c: genes of which the transcript levels also changed upon HSF1 overexpression in HeLa cells (Hayashida et al. 2010) are underlined

Table S3. List of genes of which the increase in transcript level was inhibited ≥ 2-fold by expression of HSF1 K80Q and/or dnHSF1 6 h after heat shock

Acc. nr.	Gene	Cntrl 6 h hs	K80Q 6 h hs	dnHSF 6 h hs	Cntrl 24 h hs	K80Q 24 h hs	dnHSF 24 h hs	Description
NM_002155	HSPA6	61.9	*27.4*[a]	*18.1*	11.8	20.4	10.4	heat shock 70kDa protein 6 (HSP70B')
NM_006732	FOSB	44.7	25.7	*21.5*	1.3	3.7	**28.7**[b]	murine osteosarcoma viral oncogene homolog B
NM_006145	DNAJB1	32.6	3.4	*0.8*	0.7	0.9	0.6	DnaJ (Hsp40) homolog, subfamily B, member 1
NM_015675	GADD45B	32.0	29.9	*13.4*	3.7	10.1	**40.8**	growth arrest and DNA-damage-inducible, beta
NM_001885	CRYAB	24.3	2.4	*3.6*	17.7	**2.7**	**8.6**	crystallin, alpha B
NM_001100	ACTA1	13.8	14.4	*5.6*	1.5	2.5	**12.8**	actin, alpha 1, skeletal muscle
NM_005345	HSPA1A	13.7	1.2	*1.0*	0.2	0.3	0.9	heat shock 70kDa protein 1A
XM_002346092	LOC100293390	12.2	9.2	*2.7*	2.6	5.5	**12.2**	hypothetical protein
NM_004417	DUSP1	11.8	4.1	*4.4*	1.3	**6.0**	**7.0**	dual specificity phosphatase 1
NM_006644	HSPH1	9.8	1.9	*1.0*	0.7	0.9	**1.1**	heat shock 105kDa/110kDa protein 1
NM_005800	USPL1	6.8	1.9	*1.0*	0.9	1.0	**1.9**	ubiquitin specific peptidase like 1
NM_004281	BAG3	6.7	2.2	*1.3*	0.8	1.1	**2.2**	BCL2-associated athanogene 3
NM_001901	CTGF	6.2	4.4	*2.6*	2.0	**4.4**	**4.0**	connective tissue growth factor
NM_003734	AOC3	6.0	9.9	*1.7*	2.3	2.2	**5.7**	amine oxidase, copper containing 3

NM_182491	ZFAND2A	5.7	*1.8*	*1.4*	1.4	1.6	**3.1**	zinc finger, AN1-type domain 2A
NM_014475	DHDH	5.7	*1.6*	*2.5*	2.1	**1.6**	2.5	dihydrodiol dehydrogenase
NM_007034	DNAJB4	5.6	*1.7*	*1.2*	0.9	1.6	**2.2**	DnaJ (Hsp40) homolog, subfamily B, member 4
NM_018698	NXT2	5.5	*1.7*	*1.0*	1.1	1.5	**1.2**	nuclear transport factor 2-like export factor 2
NR_027709	C10orf110	4.7	6.4	*1.3*	1.4	2.5	**9.7**	chromosome 10 open reading frame 110, non-coding RNA
NM_024584	CCDC121	4.7	*1.3*	*1.9*	0.8	0.9	1.1	coiled-coil domain containing 121
NM_004419	DUSP5	4.3	2.7	*1.8*	1.1	2.3	**3.2**	dual specificity phosphatase 5
BU532663	BU532663	4.3	*2.1*	*1.8*	1.7	**3.1**	**6.4**	cDNA clone IMAGE:6558480
NM_001025366	VEGFA	4.2	3.2	*2.1*	1.6	1.3	**2.1**	vascular endothelial growth factor A
NM_002228	JUN	4.0	*1.8*	*1.4*	1.0	1.7	**5.2**	jun oncogene
NR_003672	SNHG7	3.8	2.1	*1.6*	0.9	0.9	**1.9**	small nucleolar RNA host gene 7 (non-protein coding)
NM_015394	ZNF10	3.7	2.2	*1.4*	1.4	1.6	**2.3**	zinc finger protein 10
NM_001040619	ATF3	3.7	*1.6*	2.5	1.7	**3.5**	**9.1**	activating transcription factor 3
NM_018955	UBB	3.3	*1.2*	*1.0*	0.7	1.1	**1.1**	ubiquitin B
NM_033118	MYLK2	3.3	5.7	*1.3*	1.1	1.6	**10.3**	myosin light chain kinase 2
NM_016449	C22orf43	3.2	*1.6*	2.0	1.0	**1.6**	**2.5**	chromosome 22 open reading frame 43
NM_021009	UBC	3.0	*1.4*	1.6	1.1	**2.1**	**2.3**	ubiquitin C
NR_027795	BTN2A3	2.9	2.2	*1.1*	1.0	1.0	1.0	butyrophilin, subfamily 2, member A3
ENST00000397861	C17orf67	2.9	*0.9*	*0.5*	0.8	**1.9**	**1.3**	Uncharacterized protein C17orf67
NM_001145033	LOC387763	2.8	*0.9*	1.9	1.3	**1.9**	**9.6**	hypothetical protein
NM_133328	DEDD2	2.8	*1.3*	*0.7*	0.9	0.9	**1.1**	death effector domain containing 2

NM_007355	HSP90AB1	2.7	1.4	*1.0*	0.7	0.8	**1.4**	heat shock protein 90kDa alpha (cytosolic), class B member 1
NM_001017963	HSP90AA1	2.7	1.4	*0.5*	0.6	**1.7**	0.5	heat shock protein 90kDa alpha (cytosolic), class A member 1
NM_014412	CACYBP	2.6	*1.2*	*0.8*	0.8	0.7	0.5	calcyclin binding protein
NM_012308	KDM2A	2.6	1.8	*1.0*	0.9	1.3	**1.8**	lysine (K)-specific demethylase 2A
NM_002133	HMOX1	2.6	1.6	*1.0*	1.3	**2.5**	**2.1**	heme oxygenase (decycling) 1
NM_005494	DNAJB6	2.6	*1.2*	*1.0*	0.8	0.9	1.0	DnaJ (Hsp40) homolog, subfamily B, member 6
NM_015167	JMJD6	2.6	1.6	*0.8*	0.9	1.2	**2.2**	jumonji domain containing 6
NM_001077195	ZNF436	2.5	1.7	*1.2*	1.2	**1.8**	**2.3**	zinc finger protein 436
NM_014161	MRPL18	2.4	*1.1*	*0.8*	1.0	**1.1**	0.9	mitochondrial ribosomal protein L18
NM_020861	ZBTB2	2.4	2.3	*1.1*	0.9	1.2	**2.0**	zinc finger and BTB domain containing 2
NM_017541	CRYGS	2.4	2.0	*1.0*	1.1	1.0	**1.6**	crystallin, gamma S
NM_032325	EIF1AD	2.3	1.4	*0.8*	1.0	**1.6**	**2.5**	eukaryotic translation initiation factor 1A domain containing
NM_201286	USP51	2.3	*1.0*	*1.0*	1.1	**1.0**	0.9	ubiquitin specific peptidase 51
XM_002343495	LOC100133337	2.3	*1.0*	*0.9*	0.6	0.8	0.9	hypothetical protein
XM_933296	LOC645955	2.3	1.4	*1.1*	0.9	0.9	**1.3**	hypothetical protein
NM_198261	RSRC2	2.2	2.5	*1.1*	1.0	1.1	**3.4**	arginine/serine-rich coiled-coil 2
NM_012124	CHORDC1	2.1	1.3	*0.7*	0.8	1.1	0.7	cysteine and histidine-rich domain-containing 1
BC043212	LOC100130288	2.0	*0.8*	1.3	0.5	0.7	0.8	cDNA clone IMAGE:5295205

a: numbers in italics indicate ≥ 2-fold decrease relative to control 6 h after hs

b: numbers in bold indicate a transcript level equal or higher to that 6 h after hs

Table S4. List of genes of which transcript level was increased by ≥ 2-fold by expression of HSF1 K80Q and/or dnHSF1 6 h after heat shock

Acc. nr.	Gene	Cntrl 6 h hs	K80Q 6 h hs	dnHSF 6 h hs	Cntrl 24 h hs	K80Q 24 h hs	dnHSF 24 h hs	Description
NM_016084	RASD1	129.1	**256.5**[a]	137.3	*14.0*[b]	*35.1*	190.4	RAS, dexamethasone-induced 1
NM_021076	NEFH	14.3	**31.2**	12.2	*7.2*	18.4	**29.4**	neurofilament, heavy polypeptide
NM_005627	SGK1[c]	11.7	**30.2**	13.5	*3.0*	*7.8*	12.4	serum/ glucocorticoid regulated kinase 1
NM_016378	VCX2	11.1	**35.8**	5.9	12.5	28.4	**43.2**	variable charge, X-linked 2
NM_016379	VCX3A	10.2	**27.5**	5.5	11.8	**25.2**	**43.6**	variable charge, X-linked 3A
NM_013452	VCX	9.7	**34.4**	5.6	9.3	22.6	**40.6**	variable charge, X-linked
NM_024501	HOXD1	8.6	14.3	**18.6**	*1.8*	*4.1*	15.3	homeobox D1
NM_005461	MAFB	4.2	2.5	**8.8**	2.9	3.3	**15.5**	v-maf homolog B
NM_152654	DAND5	3.8	5.1	**8.7**	*0.9*	*1.7*	*4.3*	DAN domain family, member 5
NM_182908	DHRS2	3.7	**7.7**	2.2	**9.1**	**17.5**	**20.1**	dehydrogenase/ reductase
NM_003706	PLA2G4C	3.6	**8.2**	3.1	1.8	3.6	**6.5**	phospholipase A2, group IVC
NM_145239	PRRT2	3.0	2.9	**9.3**	*0.9*	*1.2*	*3.1*	proline-rich transmembrane protein 2
NM_002557	OVGP1	2.7	**7.5**	2.8	1.6	*1.6*	**6.0**	oviductal glycoprotein 1, 120kDa
NM_021979	HSPA2[c]	2.7	**7.0**	**5.5**	1.4	4.7	**8.2**	heat shock 70kDa protein 2
NR_024065	LOH3CR2A	2.1	2.4	**4.8**	1.1	1.6	*2.2*	loss of heterozygosity, 3, chr 2, gene A
NM_000076	CDKN1C	2.0	3.9	**8.4**	**4.3**	**8.2**	**18.8**	cyclin-dependent kinase inhibitor 1C (p57, Kip2)

a: numbers in bold indicate ≥ 2-fold increase relative to control 6 h after hs

b: numbers in italics indicate ≥ 2-fold decrease relative to 6 h after hs

c: validated by Q-PCR (suppl. Fig. S2)

Table S5. The secondary response in cells recovering from a heat shock

Acc. nr.	Gene	Cntrl 6 h hs	K80Q 6 h hs	dnHSF 6 h hs	Cntrl 24 h hs	K80Q 24 h hs	dnHSF24 h hs	Description
NM_000527	LDLR	1.3	1.1	0.7	*0.4*[a]	*0.4*	*0.5*	low density lipoprotein receptor
NM_004265	FADS2	1.0	1.0	0.9	*0.4*	*0.3*	*0.3*	fatty acid desaturase 2
NM_006741	PPP1R1A	1.0	1.0	0.9	*0.4*	*0.5*	*0.5*	protein phosphatase 1, regulatory (inhibitor) subunit 1A
NM_001888	CRYM	1.1	1.1	0.8	*0.4*	*0.4*	1.0	crystallin, mu (CRYM), transcript variant 1
NM_198504	PAQR9	1.1	1.1	1.4	*0.5*	0.9	**3.6**[b]	progestin and adipoQ receptor family member IX
NM_001006943	EPHA8	1.1	1.1	1.0	*0.5*	*0.4*	*0.4*	EPH receptor A8 (EPHA8), transcript variant 2
NM_032880	IGSF21	1.0	1.0	1.0	*0.5*	*0.4*	0.5	immunoglobin superfamily, member 21
NM_001511	CXCL1	1.0	1.1	0.8	*0.5*	0.6	0.7	chemokine (C-X-C motif) ligand 1
NM_177400	NKX6-2	1.2	1.3	0.9	*0.5*	0.6	0.5	NK6 homeobox 2
NM_000860	HPGD	1.2	1.2	0.9	*0.5*	*0.4*	*0.4*	hydroxyprostaglandin dehydrogenase 15-(NAD)
NM_020817	KIAA1407	0.6	0.7	0.9	**2.0**	1.4	0.7	KIAA1407
NM_201400	FAM86A	0.9	0.9	1.0	**2.0**	**2.1**	1.6	family with sequence similarity 86, member A
ENST00000313957	LOC100128398	0.8	0.8	0.8	**2.0**	**3.2**	**2.3**	Hypothetical protein

NR_024060	FAM27A	0.9	1.0	1.1	**2.0**	**3.6**	**3.5**	family with sequence similarity 27, member A, non-coding RNA
NM_001034173	ALDH1L2	0.7	0.9	1.1	**2.0**	0.9	*0.3*	aldehyde dehydrogenase 1 family, member L2
NM_015432	PLEKHG4	0.8	0.8	1.3	**2.1**	**1.8**	1.1	pleckstrin homology domain containing, family G member 4
NM_018271	THNSL2	0.9	1.1	1.6	**2.2**	1.6	1.3	threonine synthase-like 2
XM_002342916	LOC100287241	1.0	0.9	1.0	**2.2**	**4.1**	**3.8**	Hypothetical protein
NM_001001655	ALKBH2	1.0	0.9	0.8	**2.2**	**2.2**	**2.9**	alkB, alkylation repair homolog 2 (E. coli)
NM_033208	TIGD7	0.7	0.7	0.8	**2.2**	**3.1**	**2.7**	tigger transposable element derived 7
NM_030613	ZFP2	0.8	0.9	0.8	**2.2**	**2.2**	1.5	zinc finger protein 2 homolog (mouse)
NM_001348	DAPK3	0.9	0.9	2.1	**2.2**	**2.8**	1.5	death-associated protein kinase 3
NM_001673	ASNS	0.8	0.9	0.9	**2.3**	1.2	*0.5*	asparagine synthetase
NM_020665	TMEM27	1.1	1.0	1.4	**2.3**	**2.1**	**2.1**	transmembrane protein 27
NM_145755	TTC21A	1.0	1.0	1.1	**2.3**	**2.5**	**2.8**	tetratricopeptide repeat domain 21A
NM_003196	TCEA3	1.2	1.1	1.5	**2.5**	1.9	**2.1**	transcription elongation factor A (SII), 3
NM_145170	TTC18	0.8	1.0	1.3	**2.5**	1.4	0.7	tetratricopeptide repeat domain 18
NM_001033953	CALCA	1.1	1.0	1.1	**2.5**	**2.8**	1.2	calcitonin-related polypeptide alpha
NM_198833	SERPINB8	1.3	1.3	2.1	**2.6**	**7.7**	**6.3**	serpin peptidase inhibitor, clade B, member 8
NM_032561	C22orf23	1.1	0.9	0.9	**2.6**	**2.1**	1.0	chromosome 22 open reading frame 23

NM_001042483	NUPR1	1.0	1.4	1.5	**2.8**	1.8	0.9	nuclear protein, transcriptional regulator, 1
NM_001251	CD68	1.3	1.3	2.3	**2.9**	**4.8**	**3.9**	CD68 molecule (CD68), transcript variant 1
NM_000565	IL6R	1.0	1.0	2.6	**2.9**	**7.9**	**8.4**	interleukin 6 receptor
NM_031421	TTC25	0.8	0.8	0.9	**2.9**	**2.3**	**2.1**	tetratricopeptide repeat domain 25
NM_000735	CGA	1.1	1.1	1.6	**3.2**	**8.7**	**4.2**	glycoprotein hormones, alpha polypeptide
NM_001017402	LAMB3	1.1	1.0	1.5	**3.4**	**7.8**	**2.4**	laminin, beta 3
NM_181607	KRTAP 19-1	1.2	1.0	1.2	**5.4**	**10.4**	**10.9**	keratin associated protein 19-1
NM_003064	SLPI	1.2	1.1	1.1	**7.4**	**11.1**	**2.4**	secretory leukocyte peptidase inhibitor

a: numbers in italics indicate ≥ 2-fold decrease relative to control 6 h after hs

b: numbers in bold indicate ≥ 2-fold increase relative to control 6 h after hs

Supplemental figures

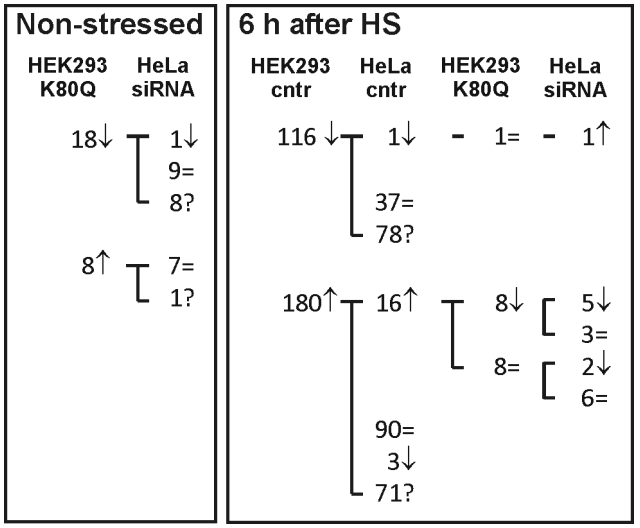


Fig. S1 Comparison of the transcriptome changes between HEK-HSF1 K80Q and HSF1 siRNA treated HeLa cells either in non-stressed cells or cells after heat shock. An ↓ indicates a ≥ 2-fold decrease in level, an ↑ a ≥ 2 fold increase in level relative and = no significant change relative to the transcript level in non-stressed control cells. The data for HSF1 siRNA treated HeLa cells were taken from [237].

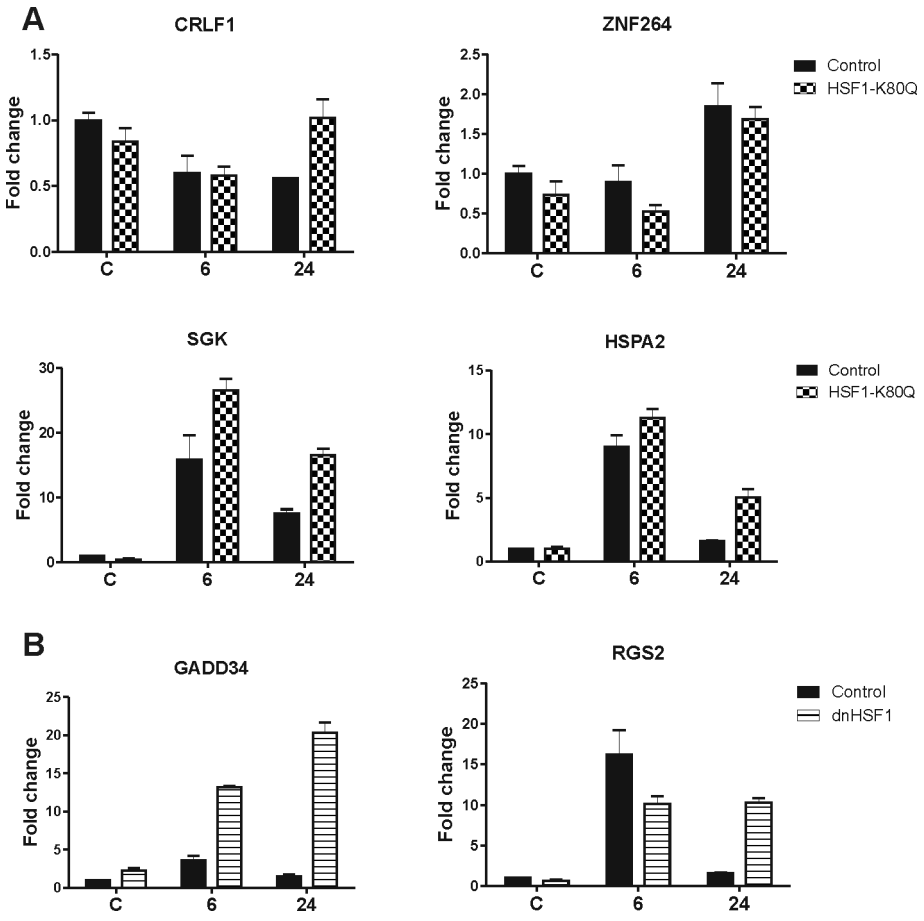


Fig. S2 Relative changes in transcript levels of various genes in stressed and non-stressed HEK dnHSF1 cells. A-B. Cells were cultured in the presence (dnHSF1) or absence of doxycycline (Control) and exposed to a heat shock for 30' at 45ºC or left at 37ºC (C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and transcript levels relative to GAPDH mRNA levels were measured by QPCR. The fold induction of mRNA levels is plotted relative to the level in non-stressed control cells. Error bars represent SD.

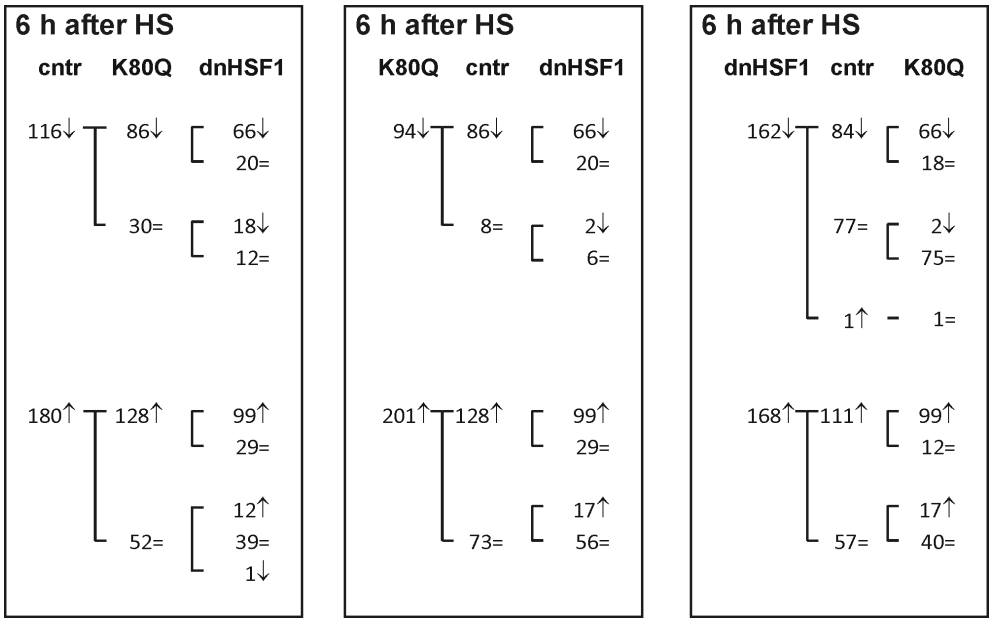


Fig. S3 Comparison of the transcriptome changes between control, HSF1 K80Q and dnHSF1 cells 6 h after heat shock. An ↓ indicates a ≥ 2-fold decrease in level, an ↑ a ≥ 2-fold increase in level and = no significant change relative to the transcript level in non-stressed control cells.

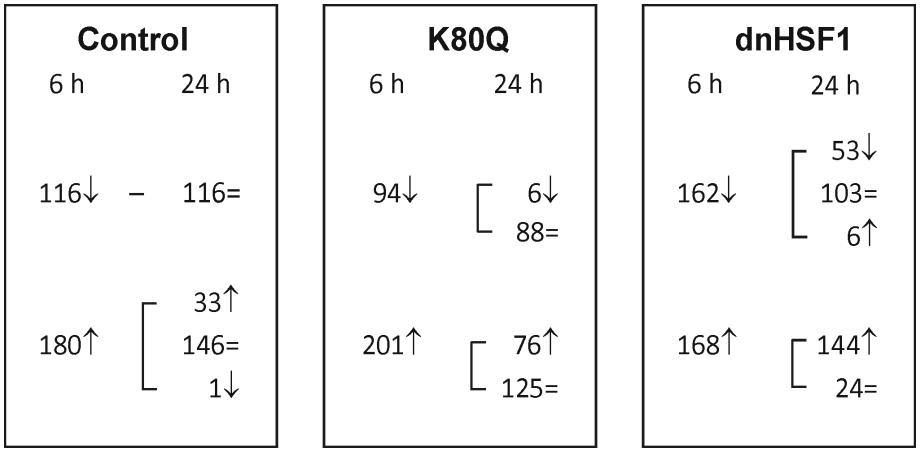


Fig. S4 Comparison of the transcriptome changes in control, HSF1 K80Q and dnHSF1 cells between 6 and 24 h after heat shock. An ↓ indicates a ≥ 2-fold decrease in level, an ↑ a ≥ 2-fold increase in level and = no significant change relative to the transcript level in non-stressed control cells.

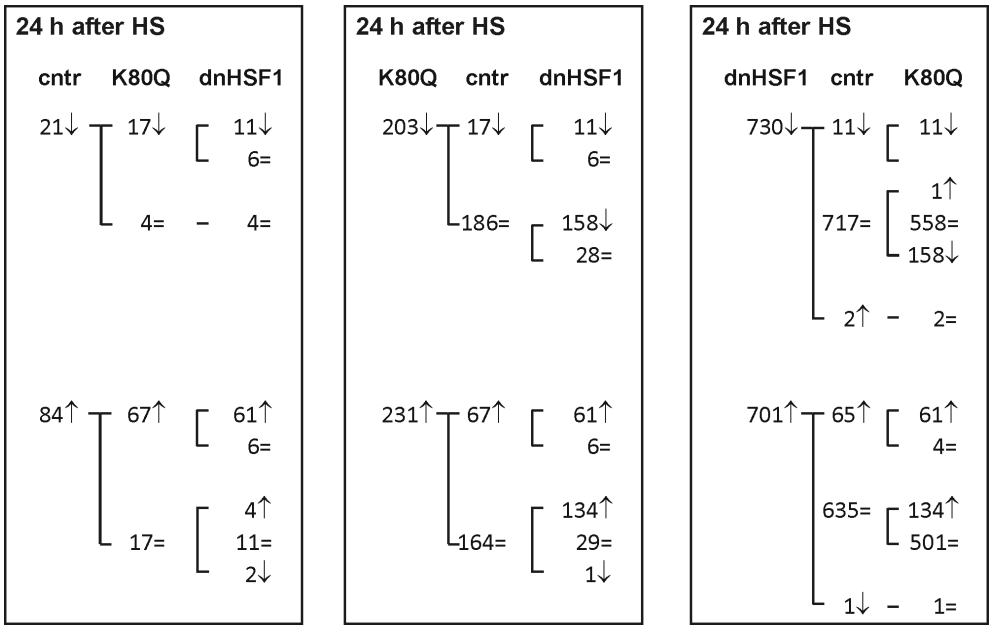


Fig. S5 Comparison of the transcriptome changes between control, HSF1 K80Q and dnHSF1 cells 24 h after heat shock. An ↓ indicates a ≥ 2-fold decrease in level, an ↑ a ≥ 2-fold increase in level and = no significant change relative to the transcript level in non-stressed control cells.

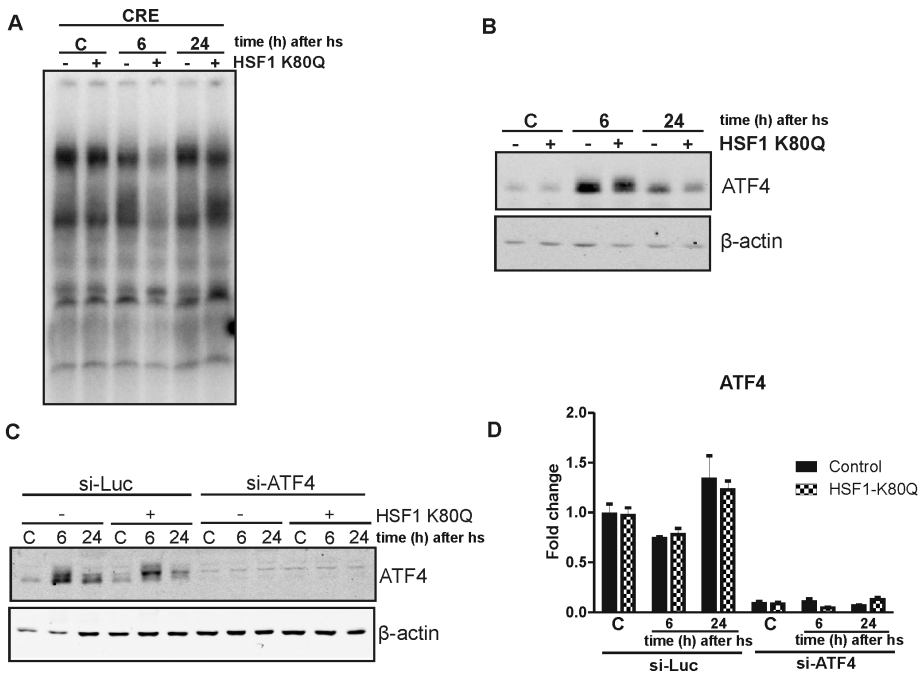


Fig. S6 Knockdown of ATF4 protein and transcript levels. A. HEK-HSF1 K80Q cells were cultured in the presence (+) or absence (-) of doxycycline and exposed to a heat shock for 30' at 45°C or left at 37°C (C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Nuclear extracts were used in an electrophoretic mobility shift assay with a double-stranded oligonucleotide with the CRE sequence. **B.** Cell lysates were subjected to SDS-PAGE and levels of ATF4 were determined by western blotting. β-actin was used as a loading control. **C.** HEK-HSF1 K80Q cells were cultured in the presence (+) or absence (-) of doxycycline and transfected with siRNA against luciferase or ATF4 for 48 h. Cells were exposed to a heat shock for 30' at 45°C or left at 37°C (C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Cells were harvested and lysates were subjected to SDS-PAGE and western blot analysis using antibodies against the indicated proteins. D. ATF4 siRNA treated cells were harvested, total RNA was isolated and ATF4 transcript levels relative to GAPDH mRNA levels were measured by QPCR. The fold change of mRNA levels is plotted relative to the level in non-stressed control cells. Error bars represent SD.

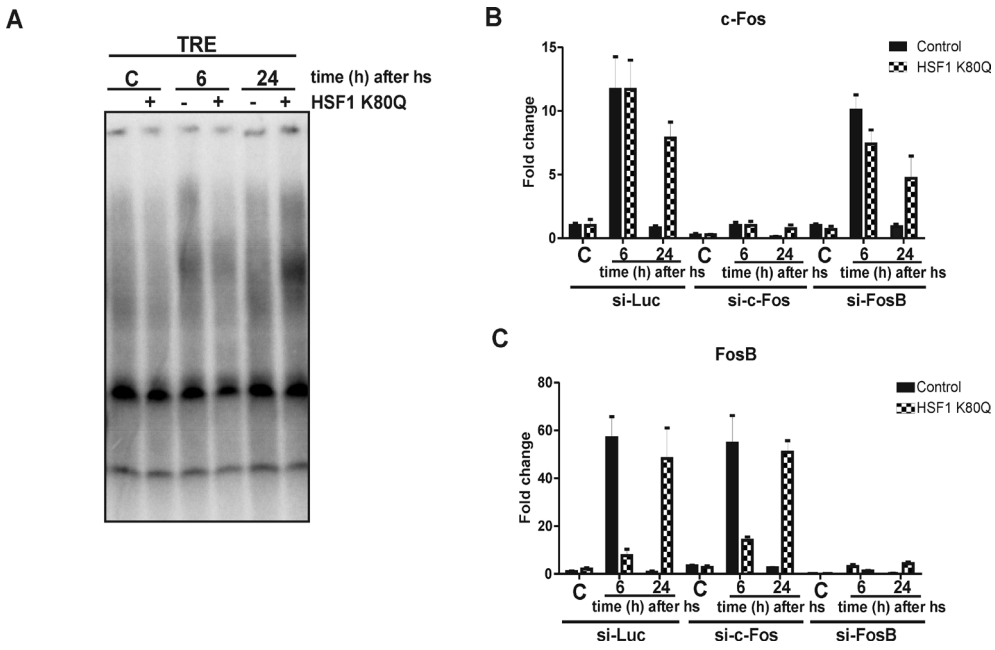


Fig. S7 Knockdown of c-Fos and FosB transcript levels. A. HEK-HSF1 K80Q cells were cultured in the presence (+) or absence (-) of doxycycline and exposed to a heat shock for 30' at 45°C or left at 37°C (C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Nuclear extracts were used in an electrophoretic mobility shift assay with a double-stranded oligonucleotide with the TRE sequence. **B-C.** HEK-HSF1 K80Q cells were cultured in the presence (HSF1 K80Q) or absence (Control) of doxycycline and transfected for 96 h with siRNA against c-Fos, FosB or luciferase as a control with a re-transfection at 48 h. Cells were exposed to a heat shock for 30' at 45°C or left at 37°C (C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and c-Fos or FosB transcript levels relative to GAPDH mRNA levels were measured by QPCR. The fold change of mRNA levels is plotted relative to the level in non-stressed control cells. Error bars represent SD.

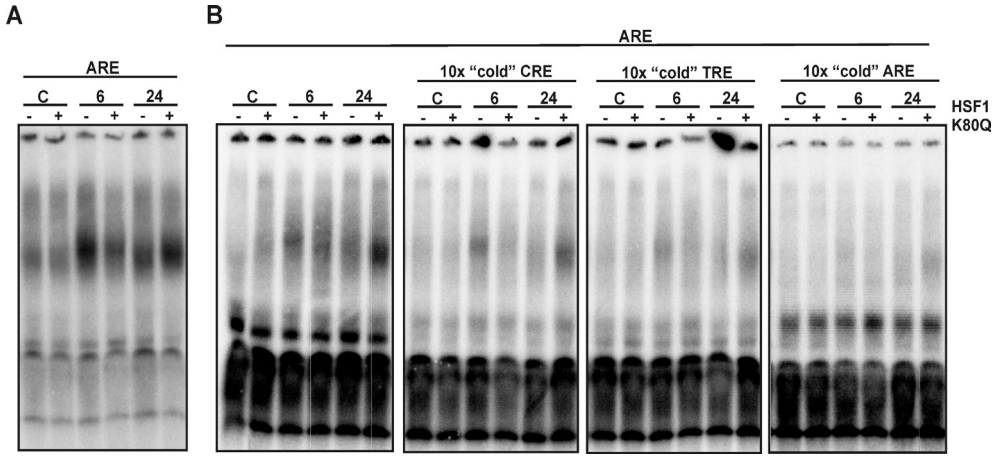


Fig. S8 Increased binding to the ARE sequence in extracts of cells recovered after heat shock for 24 h in the presence of HSF1 K80Q. A. HEK-HSF1 K80Q cells were cultured in the presence (+) or absence (-) of doxycycline and exposed to a heat shock for 30' at 45°C or left at 37°C (C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Nuclear extracts were used in an electrophoretic mobility shift assay with a double-stranded oligonucleotide with the ARE sequence. **B.** Nuclear extracts were used in an electrophoretic mobility shift assay with a double-stranded oligonucleotide with the ARE sequence. A 10-fold molar excess of unlabeled CRE, TRE or ARE probe ("cold") was added to determine the specificity of the signal.

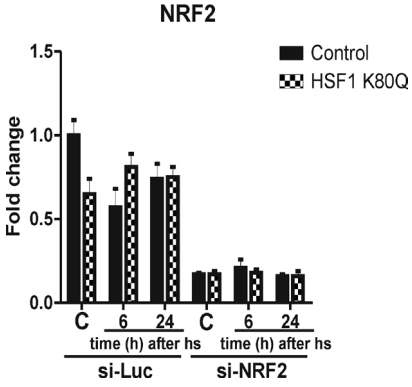


Fig. S9 Knockdown of NRF2 transcript levels. HEK-HSF1 K80Q cells were cultured in the presence (HSF1 K80Q) or absence (Control) of doxycycline and transfected for 96 h with siRNA against NRF2 or luciferase as a control with a re-transfection at 48 h. Cells were exposed to a heat shock for 30' at 45°C or left at 37°C (C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and NRF2 transcript levels relative to GAPDH mRNA levels were measured by QPCR. The fold change of mRNA levels is plotted relative to the level in non-stressed control cells.

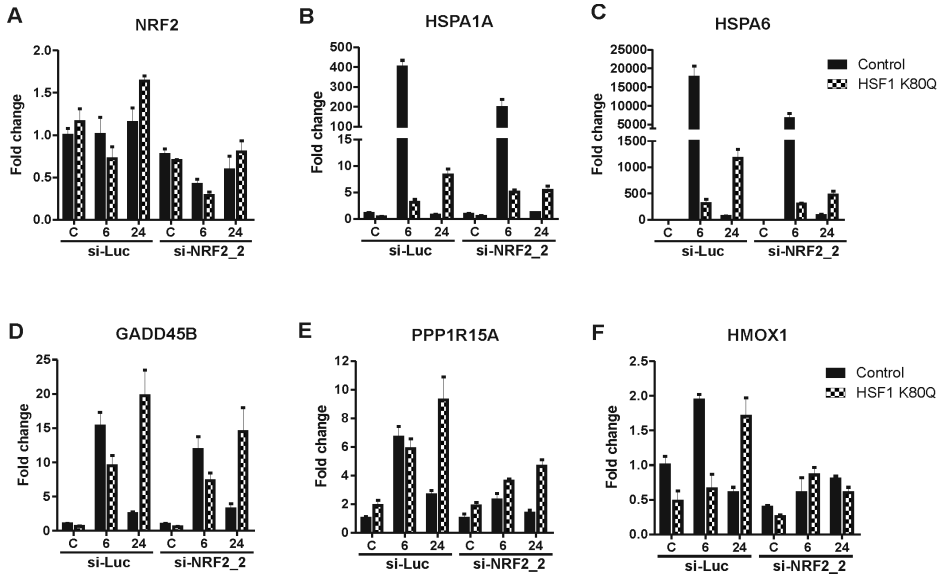


Fig. S10 Transcript levels in cells treated with a second NRF2 siRNA. A-F. HEK-HSF1 K80Q cells were cultured in the presence (HSF1 K80Q) or absence (Control) of doxycycline and transfected for 96 h with siRNA#2 against NRF2 or luciferase as a control with a re-transfection at 48 h. Cells were exposed to a heat shock for 30' at 45°C or left at 37°C (C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and transcript levels of the genes indicated relative to GAPDH mRNA levels were measured by QPCR. The fold change of mRNA levels is plotted relative to the level in non-stressed control cells.

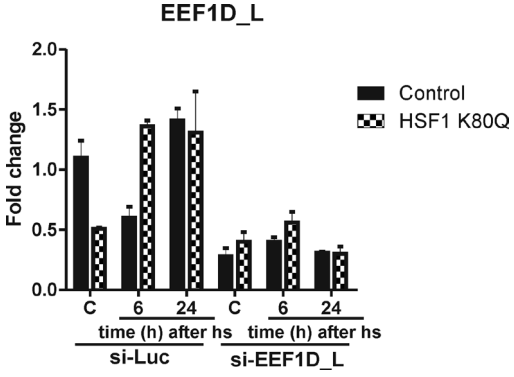
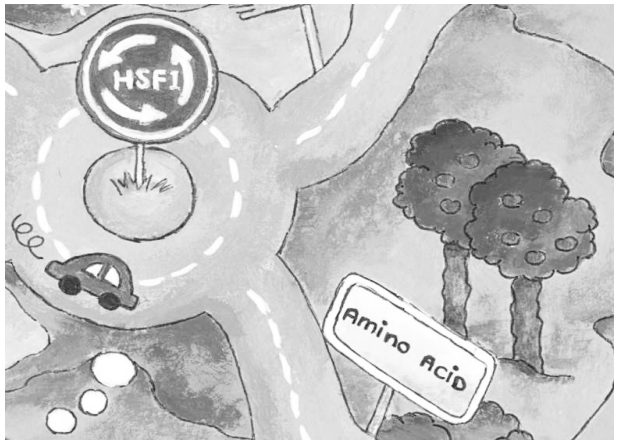


Fig. S11 Knockdown of EEF1D_L transcript levels. HEK-HSF1 K80Q cells were cultured in the presence (HSF1 K80Q) or absence (Control) of doxycycline and transfected for 96 h with siRNA against EEF1D_L or luciferase as a control with a re-transfection at 48 h. Cells were exposed to a heat shock for 30' at 45°C or left at 37°C (C). When heat shocked, cells were allowed to recover for the indicated time before harvesting. Total RNA was isolated and EEF1D_L transcript levels relative to GAPDH mRNA levels were measured by QPCR. The fold change of mRNA levels is plotted relative to the level in non-stressed control cells.

Chapter 5

HSF1 is inactivated by amino acid deprivation



Sanne M.M. Hensen, Lonneke Heldens, Chrissy M.W. van Enckevort, Siebe T. van Genesen, Ger J.M. Pruijn, Nicolette H. Lubsen

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Mammalian cells respond to a lack of amino acids by activating a transcriptional program with the transcription factor ATF4 as one of the main actors. When cells are faced with cytoplasmic proteotoxic stress a quite different transcriptional response is mounted, the heat shock response, which is mediated by HSF1. Here we show that amino acid deprivation results in the inactivation of HSF1. In amino acid deprived cells active HSF1 loses its DNA binding activity as demonstrated by EMSA and ChIP. A sharp decrease in the transcript level of HSF1 target genes such as HSPA1A (Hsp70), DNAJB1 (Hsp40) and HSP90AA1 is also seen. HSPA1A mRNA, but not DNAJB1 mRNA, was also destabilized. Cells cultured with limiting leucine also had less HSPA1A mRNA. Lack of amino acids thus could lead to a lower chaperoning capacity and cellular frailty. We show that the nutrient sensing response unit of the ASNS gene contains an HSF1 binding site, but we could not detect binding of HSF1 to this site in vivo. Expression of either an HSF1 mutant lacking the activation domain (HSF379) or an HSF1 mutant unable to bind DNA (K80Q) had only a minor effect on the transcript levels of amino acid deprivation responsive genes.

Introduction

All cells have a number of distinct programmed responses to cope with various adverse conditions. In eukaryotic cells, lack of amino acids evokes a deceptively simple initial response. Accumulation of uncharged tRNAs activates general control non-derepressible 2 kinase (GCN2) [82] which phosphorylates the eukaryotic translation initiation factor 2α (eIF2α). This results in a general inhibition of protein synthesis and in the selective translation of a few mRNAs amongst which that encoding the transcription factor ATF4 (reviewed in [83]). ATF4 then activates promoters by binding to the amino acid response element (AARE) [88,89] or the nutrient sensing response unit (NSRU) [90,92] and thereby initiates a complex transcriptional program. Two well known targets of ATF4 are the asparagine synthetase (ASNS) and the CHOP (DDIT3) promoters which are the paradigms for the amino acid deprivation response. The activation of these promoters during the amino acid deprivation response has been dissected by Chen and co-workers [93] and Bruhat and co-workers [284]. The binding of ATF4 closely correlates with the transcriptional activation. Later C/EBPβ and/or ATF3, also targets of ATF4, bind and repress ASNS promoter activity. The activation of the CHOP promoter requires not only ATF4 but also ATF2, which is constitutively present [89,97].

The amino acid deprivation response shows some overlap with the unfolded protein response (UPR) elicited by unfolding proteins accumulating in the ER. One branch of

the UPR, PERK, is an eIF2α kinase and activation of the UPR thus also leads to eIF2α phosphorylation and the selective synthesis of ATF4 (reviewed in [107]). ATF4 then activates some, but not all (see for example [112]), of the promoters also activated by the amino acid deprivation response and in addition activates the promoters of genes encoding ER resident proteins. The distinction between the transcriptional program initiated by ATF4 as part of the UPR and that as part of the amino acid response is presumably dictated by auxiliary factors and heteromeric partners.

A third stress response that leads to eIF2α phosphorylation, this time by the PKR and HRI kinases, is that elicited by unfolding proteins in the cytoplasm, the heat shock response. Although ATF4 protein levels are increased upon heat stress and prototypical ATF4 target genes such as *ASNS* and *CHOP* are about 2-fold upregulated during heat shock (see for example the microarray data presented by Page et al. [237]), ATF4 is not thought to play a significant role in the heat shock response. The main actor in this response is heat shock factor 1 (HSF1), which upon stress is phosphorylated and translocated to the nucleus, where it activates the transcription of a number of genes mostly encoding heat shock proteins (reviewed in [3,4,17]). These heat shock proteins act as chaperones for unfolded nuclear and cytosolic proteins, either refolding them or targeting them for degradation.

To date, little is known about the interaction between the heat shock response and the amino acid response. Xie et al. [238] described that under stress conditions HSF1 physically interacts with C/EBPβ, one of the transcription factors involved in the amino acid response. We demonstrate that during leucine deprivation, and also during starvation for lysine or glutamine, nuclear HSF1 loses its DNA binding activity and that the mRNA levels for several heat shock proteins sharply decrease. HSPA1A mRNA is also destabilized (see also [285]). We found that the NSRU of the ASNS promoter does contain an HSE but we could not detect binding to this HSE in vivo. HSF1 did not appear to play a major role in the transcriptional response to amino acid deprivation as evidenced by the changes in transcript levels of amino acid deprivation responsive genes in cells stably expressing either an HSF1 mutant lacking the activation domains or an HSF1 mutant incapable of binding DNA. The physiological role of the inactivation of HSF1 during the amino acid response is thus not clear.

Materials and Methods

Recombinant DNA constructs

The reporter plasmid pGL3-NSRU containing the nutrient sensing response unit (NSRU) was made by annealing the NSRU primers NSRU_fwd and NSRU_rev and

cloning the double stranded oligonucleotide into the NheI and XhoI sites of pGL3-promoter (Promega). pGL3-NSRU1xmut and pGL3-NSRU2xmut were made as the pGL3-NSRU, using the corresponding oligonucleotides. Expression plasmid pcDNA5-HSF1 was made by inserting the Sfo/XhoI fragment of pOTB7-hHSF1 (Imagenes, www.imagenes-bio.de) containing the code for the C-terminal region of HSF1 in pcDNA5-HSF379 (dnHSF1) [265]. The pcDNA5-wtHSF1 (silent mutation) and the pcDNA5-HSF1K80Q mutant were made by performing site-directed mutagenesis on pcDNA5-HSF1 with respectively the HSF1_sil.mut and the HSF1_K80Q primers. Primers are listed in Table 1. All constructs were sequence verified.

Table 1. Primers

	Name	Sequence (5'-->3')
Cloning	NSRU_fwd	ctagcgcatgatgaaacttcccgcacgcgttacaggagcatgatgaaacttcccgcacgcgttacaggag
	NSRU_rev	tcgactcctgtaacgcgtgcgggaagtttcatcatgctcctgtaacgcgtgcgggaagtttcatcatgcg
	NSRU_1xmut_fwd	ctagcgcatgatgaaacaacccgcacgcgttacaggagcatgatgaaacttcccgcacgcgttacaggag
	NSRU_1xmut_rev	tcgactcctgtaacgcgtgcgggaagtttcatcatgctcctgtaacgcgtgcgggttgtttcatcatgcg
	NSRU_2xmut_fwd	ctagcgcatgatgaaacaacccgcacgcgttacaggagcatgatgaaacaacccgcacgcgttacaggag
	NSRU_2xmut_rev	tcgactcctgtaacgcgtgcgggttgtttcatcatgctcctgtaacgcgtgcgggttgtttcatcatgcg
	HSF1_sil.mut	cagaaagtcgtcaacaagcttatccagttcctgatctcactg
	HSF1_K80Q	catgtatggcttccggcaagtggtccacatcgagc
EMSA	HSE_EMSA_fwd	aacgagaatcttcgagaatggct
	HSE_EMSA_rev	agccattctcgaagattctcgtt
	NSRU_EMSA_fwd	gcaggcatgatgaaacttcccgcacgcgttacaggagccag
	NSRU_EMSA_rev	ctggctcctgtaacgcgtgcgggaagtttcatcatgcctgc
	2xNSRU_fwd	ctagcgcatgatgaaacttcccgcacgcgttacaggagcatgatgaaacttcccgcacgcgttacaggag
	2xNSRU_rev	tcgactcctgtaacgcgtgcgggaagtttcatcatgctcctgtaacgcgtgcgggaagtttcatcatgcg
	2xNSRUmut_fwd	ctagcgcatgatgaaacaacccgcacgcgttacaggagcatgatgaaacaacccgcacgcgttacaggag
	2xNSRUmut_rev	tcgactcctgtaacgcgtgcgggttgtttcatcatgctcctgtaacgcgtgcgggttgtttcatcatgcg
ChIP	ASNS_fwd	tggttggtcctcgcaggcat
	ASNS_rev	cgcttataccgacctggctcct
	DNAJB1_fwd	ggatgtcgcgtgtcgctgaa
	DNAJB1_ rev	cgaccagtcccggactctata
QPCR	GAPDH_fwd	ttccccatggtgtctgagc
	GAPDH_rev	atcttcttttgcgtcgccag
	ASNS_fwd	gcagctgaaagaagcccaagt
	ASNS_rev	tgtcttccatgccaattgca
	HSPA1A_fwd	ccgagaaggacgagtttgag
	HSPA1A_rev	acaaaaacagcaatcttggaaagg

DNAJB1_fwd	ttccccagacatcaagaacc
DNAJB1_rev	accctctcatggtccacaac
HSP90_fwd	gttggtcctgtgcggtcact
HSP90 _rev	tgggcaatttctgcctgaa

Tissue culture

Flp-In T-REx-293 cells (Invitrogen) were manipulated according to the manufacturer's instructions using the T-REx system (Invitrogen) to generate the stable cell lines HEK-HSF1K80Q and HEK-wtHSF1 that carry a single copy of the tetracycline-inducible plasmids pcDNA5-HSF1K80Q and pcDNA5-wtHSF1, respectively. T-REx HEK293-pcDNA5 and HEK-HSF379 (dnHSF1) were generated as described before [265]. The cells were cultured at 37°C/5% CO2 in high glucose DMEM medium supplemented with 10% fetal calf serum,100 U/ml penicillin and 100 µg/ml streptomycin. Blasticidin (1.65 µg/ml; Invitrogen) and 100 µg/ml hygromycin were also added to the culture medium during maintenance of the cell lines, but were omitted during experiments. For amino acid starvation experiments cells were washed with PBS and subsequently DMEM/F12 medium (Sigma) with or without leucine, glutamine or lysine, supplemented with 10% dialyzed fetal calf serum, was added for the indicated times.

Transfections and reporter gene assays

HEK293 cells were transiently transfected using Fugene-6 (Roche). Cells were seeded on 24-well plates and on the next day transfected with 0.2 µg plasmid per well: 20 ng pCMV-β-galactosidase and 180 ng luciferase reporter plasmid. Cells were harvested for reporter gene analysis at the time and under the culture conditions indicated. Cells were lysed in 200 µl reporter lysis mix (25 mM Bicine, 0.05% Tween 20, 0.05% Tween 80) for 10 minutes. For the β-galactosidase assay, 10 µl cell lysate was mixed with 100 µl Galacton solution [100 mM Na-phosphate pH 8.2, 5 mM $MgCl_2$, 1% Galacton-Plus (Tropix)]. After 30 minutes incubation at room temperature, 150 µl accelerator II (Tropix) was added and luminescence was measured with the Lumat LB 9507 tube luminometer (Berthold). For the luciferase assay, 10 µl cell lysate was mixed with 50 µl luciferin solution and luminescence was again measured with the Lumat luminometer. All reporter gene assays were performed in triplicate. The activities of the reporter genes were corrected for transfection efficiency on basis of the β-galactosidase activity. Two-tailed student's t-tests were performed to calculate the significance of the data.

Western blot analysis

Cells were harvested in lysis buffer [25 mM Tris-HCl pH 7.5, 100 mM KCl, 1 mM DTE, 2 mM EDTA, 0.5 mM PMSF, 0.05% NP-40, 1X PhosSTOP (Roche), 1X protease inhibitor cocktail (Complete Mini, Roche)] and protein concentration was determined

using a Bradford protein assay (Bio-Rad). For analysis of cytoplasmic and nuclear fractions, extracts were prepared using NE-per nuclear and cytoplasmic reagents (Pierce). Next, 4x sample buffer (200 mM Tris-HCl pH 6.8, 20% β-mercaptoethanol, 8% SDS, 40% glycerol and 0.4% bromophenol blue) was added and the lysates were incubated at 95°C for 5 minutes. Protein samples were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose transfer membrane (Protran). For western blot analysis, the following antibodies were used: mouse monoclonal β-actin antibody (AC-15; Sigma; 1:5000), rabbit polyclonal HSF1 antibody (SPA-901; Stressgen; 1:1000), rabbit polyclonal DNAJB1 antibody (anti-Hsp40; SPA-400; Stressgen; 1:10000), mouse monoclonal Hsp70 antibody 4G4 (ab5444; Abcam; 1:5000) and mouse monoclonal Hsp90 antibody (610418; BD Biosciences; 1:1000). Next, blots were incubated with fluorescent secondary antibodies IRDye® 800CW conjugate goat anti-rabbit IgG and IRDye® 680 conjugated goat anti-mouse IgG (926-32211 and 926-32220 respectively; LI-COR Biosciensces) according to the manufacturer's instructions and scanned using a LI-COR Odyssey infrared scanner.

RNA isolation and microarray analysis

HEK293 cells were cultured for 24 h in the presence or absence of leucine. Total RNA was isolated using Trizol (Invitrogen) and copied into Cy3-labeled or Cy5-labeled cRNA using the Agilent Low RNA Input Linear Amp Kit PLUS (Agilent), or the reverse for the repeat array. Labeled cRNA samples were hybridized to an Agilent Whole Human Genome Microarray Kit (4 x 44K). The arrays were scanned using an Agilent Microarray Scanner. Image analysis and feature extraction were done with Feature Extraction (version 9.5.1, Agilent). We used an arbitrarily chosen signal cut-off of > 50.

Reverse transcription

1 μg of RNA was treated with DNaseI (Amplification grade; RNase-free; Invitrogen). Subsequently, 5 mM $MgCl_2$, RT-buffer, 1mM dNTPs, 18.75 units AMV reverse transcriptase, 20 units RNase inhibitors and 1.25 μM oligo(dT) were added to a total volume of 20 μl. Reverse transcription was performed for 10 minutes at 25°C, 60 minutes at 42°C and 5 minutes at 95°C. For QPCR analysis, cDNA was 10-fold diluted.

Electrophoretic mobility shift assay

T-REx HEK293-pcDNA5 cells were cultured for 24 h in the presence or absence of leucine and subsequently heat shocked for 30 minutes at 45°C, or cultured for 24 h in the presence or absence of lysine or glutamine. T-REx HEK293-wtHSF1 cells were cultured in the presence of doxycycline and exposed to a heat shock for 30 minutes at 45°C. Cells were immediately harvested and nuclear extracts were prepared using NE-per nuclear

and cytoplasmic reagents (Pierce). From the beginning, protease inhibitors were added to the reagents. Extracts were aliquoted and stored at -80°C. Oligonucleotide probes were end-labeled with ^{32}P. The sequences of the NSRU and HSE oligonucleotides used in EMSA are listed in Table 1. After end-labeling, the 5'overhangs of the 2xNSRU oligonucleotide were filled in with unlabeled dNTPs using DNA polymerase I, large (Klenow) fragment. The EMSA protocol was adapted from [266,267]. A mixture containing 5 μg nuclear extract and 3 μg poly dIdC in binding buffer [20 mM HEPES pH 7.9, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 4% (v/v) Ficoll, 1X PhosSTOP (Roche)] was incubated for 20 minutes on ice. 0.01 pmol radiolabeled oligonucleotide was added and the samples were incubated for 20 minutes at room temperature. For supershifts, 1 μg of antibody was added and again the samples were incubated for 20 minutes at room temperature. DNA-protein complexes were separated on a pre-run 4% polyacrylamide gel in 0.25x TBE with recirculation of the buffer. The gel was dried and signals were visualized using a PhosphorImager.

Chromatin immunoprecipitation

T-REx HEK293-dnHSF1, HEK293-HSF1K80Q or HEK293–pcDNA5 cells were cultured for 24 h in the presence or absence of leucine, with or without doxycycline. Chromatin immunoprecipitation was performed as described in [268], except that cells were crosslinked for 15 minutes with 1% formaldehyde. After quenching with 125 mM glycine, cells were washed twice with ice cold PBS and resuspended in ice cold lysis buffer (50 mM HEPES.KOH pH 7.6, 140 mM NaCl, 1mM EDTA pH 8.0, 1% (v/v) Triton X-100, 0.1% NaDOC and 1X protease inhibitor cocktail). Antibodies used for ChIP were rabbit polyclonal ATF4 antibody (sc-200; Santa Cruz) and rabbit polyclonal HSF1 antibody (SPA-901; Stressgen). ChIP samples were analyzed by QPCR with the primer sets listed in Table 1.

Quantitative real-time PCR

Quantitative real-time PCR was performed using the ABI/PRISM 7000 sequence detection system with *Power* SYBR® Green PCR Master mix (Applied Biosystems) using the following amplification protocol: 2 minutes at 50°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Per reaction 4 μl of diluted cDNA or ChIP material was used and the DNA was amplified using primers for the sequences of interest, listed in Table 1. Two-tailed student's t-tests were performed to calculate the significance of the data.

Two-dimensional polyacrylamide gel electrophoresis

For 2D analysis [286], 60 μg protein from nuclear extracts (prepared as described above)

in 30 µl was resuspended in 120 µl ureum buffer [9.3M urea, 0.6M thio-urea, 0.7M β-mercaptoethanol, 4% (v/v) Triton X-100 (electrophoresis grade, Sigma)]. 0.75 µl IPG buffer was added (pH 4-7, GE Healthcare Life Sciences) and samples were centrifuged for 15 minutes at 20ºC. Isoelectric focusing (IEF) was carried out by putting 125 µl of the sample on a 7 cm IPGphor stripholder and adding a Immobuline Drystrip with a pH-gradient of 4 to 7. After covering the Drystrip with cover fluid (GE Healthcare Life Sciences), the strip was passively rehydrated for 12 hours using the Ettan IPGphor II (GE Healthcare Life Sciences) at 20ºC, with a maximum of 50 µA/strip. IEF was performed at 250 V for 250 Vhr, 500 V for 500 Vhr, 1000 V for 1000 Vhr, 5000 V for 30000 Vhr. After IEF, strips were equilibrated for 15 minutes in 5 ml of equilibration buffer (0.1 M Tris-HCl, pH 6.8, 8M urea, 30% glycerol, 1% SDS) containing 5 mg/ml DTT, followed by equilibration for 15 minutes in equilibration buffer containing 45 mg/ml iodoacetamide. Strips were then run on a 12% SDS-polyacrylamide gel, proteins were transferred to nitrocellulose membrane and western blot analysis was performed.

Results

HSF1 loses its DNA binding affinity upon leucine starvation

A microarray analysis of the transcriptome changes in leucine starved HEK293 cells showed a significant loss of HSPA1A (Hsp70) mRNA, with only 18% left after 24 h of leucine deficiency (Table 2). We noted that the transcript levels of some other HSF1 target genes such as *HSPE1, STIP1, DNAJB1* (*Hsp40*) and *DNAJA1* were also lower in leucine starved cells, but their decrease was less than twofold. The decrease in HSPA1A and DNAJB1 mRNA levels was confirmed by QPCR (Fig. 1A). We have previously shown that in HEK293 cells DNAJB1 mRNA levels rapidly drop when HSF1 is inhibited [265] and these data suggested to us that HSF1 might be inactivated in cells deprived of leucine. We thus looked at HSF1 in leucine starved cells. In extracts of unstressed cells, HSF1 is mostly found in the cytoplasmic fraction and that does not change upon amino acid deprivation (Fig. 1B). HSF1 is known to be extensively modified upon activation, primarily by phosphorylation, which results in altered mobility of HSF1 on 1D and 2D gel electrophoresis. The electrophoretic mobility pattern of nuclear HSF1 did not change in leucine starved cells, indicating that there are no major changes in the modification state of HSF1 (Fig. 1B and C).

We then tested whether the nuclear HSF1 is competent to bind the heat shock element (HSE) using EMSA. HSF1 in nuclear extracts from unstressed cells cultured in the presence of leucine bound the HSE: a clear band shift was seen and the band was supershifted by an antibody to HSF1, indicating that it is indeed HSF1 that is bound

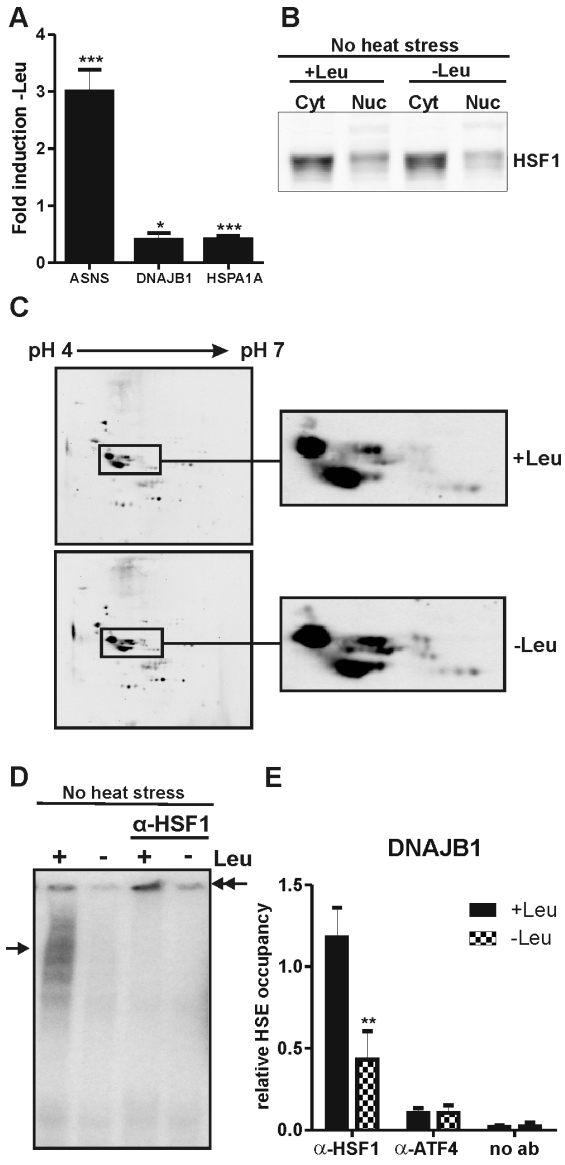

Fig. 1 HSF1 loses its DNA binding affinity upon leucine starvation. A. QPCR validation of ASNS, DNAJB1 and HSPA1A mRNA levels relative to GAPDH mRNA levels upon leucine starvation. Error bars represent SD; *P<0.05; ***P<0.001, relative to +Leu. **B.** HEK293 cells were deprived of leucine for 24 h. Cytoplasmic (cyt) and nuclear (nuc) extracts were made and subjected to SDS-PAGE and western blot analysis using an anti-HSF1 antibody. **C.** Nuclear extracts were subjected to 2D gel electrophoresis and western blot analysis using an anti-HSF1 antibody. **D.** Nuclear extracts were used in an electrophoretic mobility shift assay with a double-stranded oligonucleotide with the HSE sequence. Supershifts were induced with an anti-HSF1 antibody. Single arrows indicate the primary complexes formed; double arrows indicate the supershifted complexes. **E.** Chromatin immunoprecipitation was performed using an anti-HSF1 or an anti-ATF4 antibody. Bound chromatin was analyzed by QPCR using a primer set surrounding the HSE of the DNAJB1 promoter. As a control the ChIP was performed without an antibody. Error bars represent SD; **P<0.01, relative to +Leu.

(Fig. 1D). However, we could not detect a band shift using nuclear extracts isolated from leucine deprived cells, suggesting that HSF1 is unable to bind to the HSE. As a control we measured binding of ATF4 and C/EBPβ to the NSRU sequence of the ASNS promoter using these extracts (Fig. S1A). To show that the loss of DNA binding as assayed by EMSA indeed reflects loss of DNA bound HSF1 we performed a ChIP

Table 2. Genes of which the transcript levels were downregulated more than 2-fold upon leucine starvation (24 h)

Gene name	Acc. nr.	Description	Fold induction -Leu
HSPA1A	NM_005345	Heat shock 70kDa protein 1A	0.18
LDLR	NM_000527	Low density lipoprotein receptor (familial hypercholesterolemia)	0.28
SC4MOL	NM_006745	Sterol-C4-methyl oxidase-like	0.38
IFIT1	NM_001548	Interferon-induced protein with tetratricopeptide repeats 1	0.47
INSIG1	NM_198336	Insulin induced gene 1	0.48
IDI1	NM_004508	Isopentenyl-diphosphate delta isomerase 1	0.50
TBX18	ENST00000330469	T-box 18, mRNA (cDNA clone IMAGE:6023106), partial cds.	0.50
EFHB	BC043212	cDNA clone IMAGE:5295205, with apparent retained intron.	0.50

assay with HEK293 cells that were deprived of leucine for 24 hours, using a primer set surrounding the HSE of the DNAJB1 promoter. The binding of HSF1 to the DNAJB1 promoter was decreased by about 50% upon leucine starvation (Fig. 1E), whereas ATF4 binding to the ASNS promoter was nicely increased in leucine starved cells (Fig. S1B). This confirms that HSF1 loses its binding affinity upon leucine deprivation.

To determine whether the loss of HSF1 activity is an early or a late event during leucine starvation, we followed the decay of DNAJB1, HSPA1A and HSP90AA1 mRNA levels with time after leucine starvation. Within two hours after withdrawal of leucine, these mRNA levels were already strongly decreased (Fig. 2A), while the ASNS mRNA level, indicative of the amino acid response, had not yet risen. In agreement with these findings, the HSF1 binding activity also rapidly decreased after leucine deprivation: less HSF1:HSE complex was detected using nuclear extracts of cells starved for leucine for 3 hours (Fig. 2B). Inactivation of HSF1 is thus an early event in the response to lack of leucine. As it has previously been described that glutamine starvation of U937 cells results in loss of Hsp70 through decreased mRNA stability [285,287], we examined the effect of leucine deprivation on HSPA1A and DNAJB1 mRNA stability in HEK293 cells. Cells were cultured for 30 minutes in medium with or without leucine and subsequently actinomycin D was added to block transcription and the level of the transcripts was analyzed. The rate of loss of the DNAJB1 mRNA levels did not differ between unstarved and starved cells (Fig. 2C). However, HSPA1A mRNA levels did show a faster degradation rate in leucine starved cells compared to control cells,

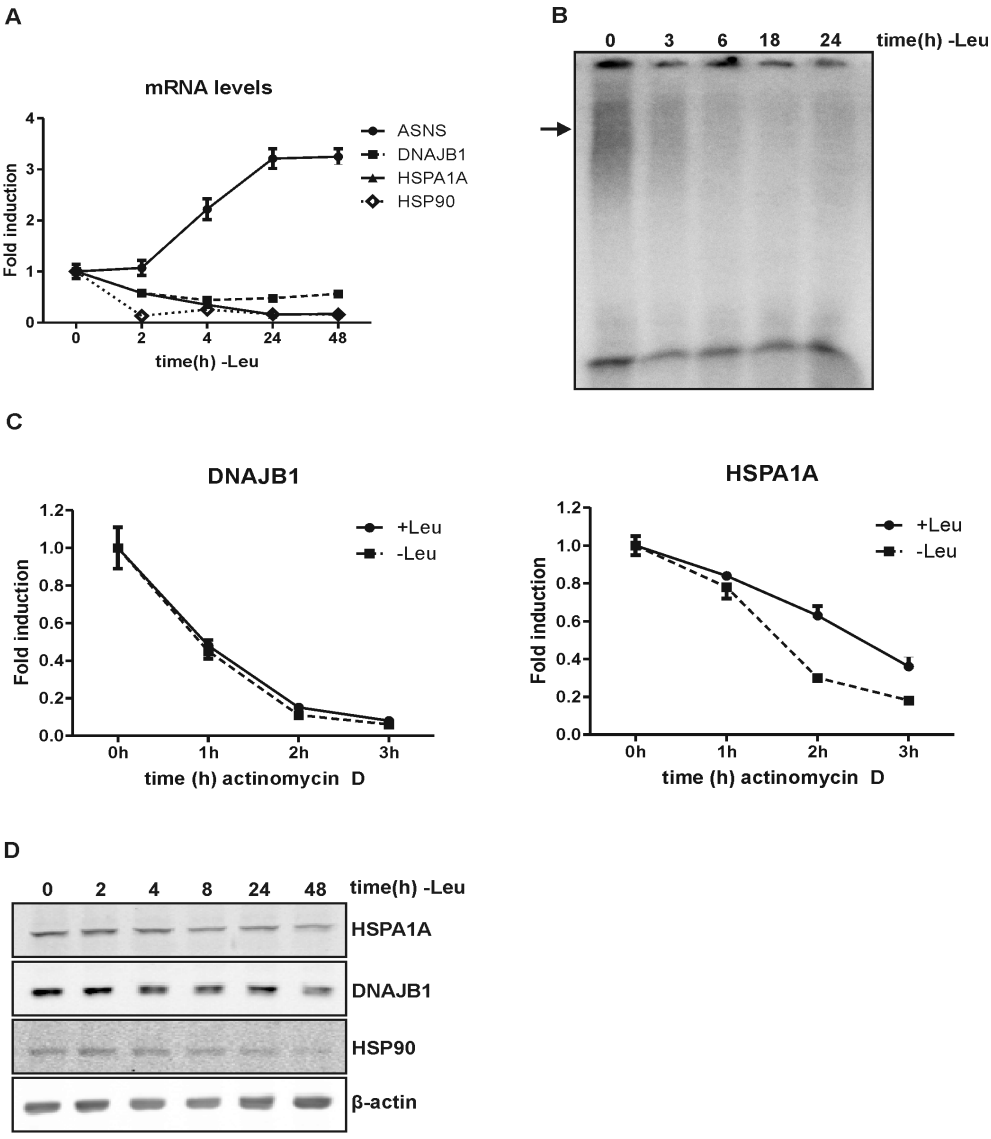

Fig. 2 HSP mRNA and HSP protein levels are decreased upon leucine starvation. A. HEK293 cells were starved for leucine and harvested at the indicated time points. mRNA levels were determined by QPCR analysis and are shown relative to GAPDH mRNA levels. **B.** Nuclear extracts were used in an EMSA with a double-stranded oligonucleotide with the HSE sequence. The arrow indicates the primary complex formed. **C.** HEK293 cells were starved for leucine and after 30 minutes 5 μg/ml actinomycin D was added to block transcription. Cells were harvested at the indicated time points. mRNA levels were determined by QPCR analysis and are shown relative to GAPDH mRNA levels. **D.** Lysates were subjected to SDS-PAGE and western blot analysis using antibodies against the indicated proteins.

indicating decreased mRNA stability. Both the loss in binding activity of HSF1 and decreased stability thus contribute to the lower HSPA1A mRNA levels in leucine starved cells, whereas for DNAJB1 mRNA only the inactivation of HSF1 results in decreased mRNA levels. To see whether reduced heat shock protein mRNA levels also led to a reduction in their corresponding protein levels, we examined HSPA1A, DNAJB1 and HSP90 protein levels upon starvation for leucine. The levels of all three heat shock proteins did decrease upon leucine starvation (Fig. 2D), but not as markedly as the mRNA levels. Presumably these proteins are quite stable.

Leucine starvation thus decreases the endogenous heat shock protein levels, leading to a decreased chaperoning capacity making the cells more sensitive to proteotoxic stress. This raises the question whether leucine starved cells can respond to a proteotoxic insult. To test this, we exposed cells deprived of leucine to a heat stress. The heat shocked leucine deprived cells behaved normally: HSF1 was now found predominantly in the nuclear fraction (Fig. 3A; western blot results obtained with nuclear extracts of unstressed cells are shown in Fig. 1B.) and was HSE binding competent (Fig. 3B). Apparently in unstressed cells it is just the small fraction of active HSF1 that loses DNA binding capacity upon leucine deprivation; the majority of HSF1 is inactive and can still be activated by proteotoxic stress. As HSF1 cycles between the inactive monomeric

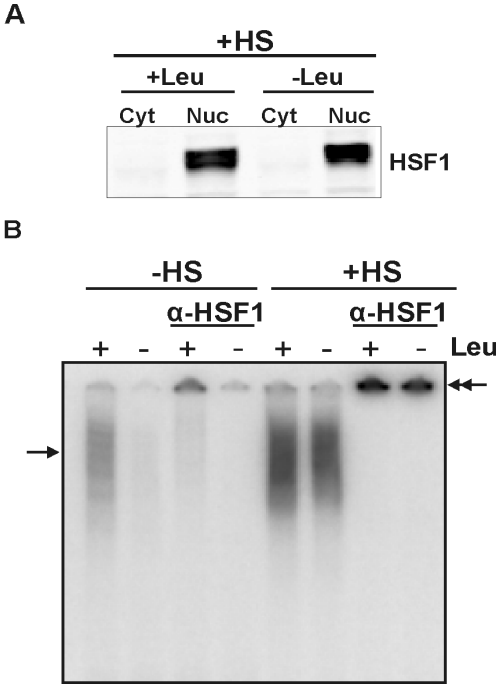

Fig. 3 Leucine starved cells can still respond to a proteotoxic insult. A. HEK293 cells were deprived of leucine for 24 h and exposed to a heat shock for 30' at 45°C. Cytoplasmic (cyt) and nuclear (nuc) extracts were made and subjected to SDS-PAGE and western blot analysis using an anti-HSF1 antibody to determine HSF1 localization. The results obtained with extracts from unstressed cells isolated in parallel are shown in Fig. 1B. **B.** Nuclear extracts were used in an electrophoretic mobility shift assay with a double-stranded oligonucleotide with the HSE sequence. Supershifts were induced with an anti-HSF1 antibody. Single arrows indicate the primary complexes formed, double arrows indicate the supershifted complexes.

state and the active trimeric state [14] it is possible that upon longer periods of leucine starvation more HSF1 is inactivated.

Amino acid starvation in general inactivates HSF1

The amino acid response is a response induced by lack of amino acids and if inactivation of HSF1 is part of the general amino acid response, then the HSF1 DNA binding affinity should also be affected upon starvation for other amino acids. We therefore used EMSA to examine the effects on HSF1 binding upon starvation for two other amino acids: lysine and glutamine. The HSF1:HSE band shift that was detected using nuclear extracts from non-starved cells strongly decreased upon starvation for either of the amino acids (Fig. 4A), indicating that amino acid starvation in general leads to inactivation of HSF1. In a previous study using human monocytic U937 cells glutamine starvation did not affect the binding activity of HSF1 [285]. However, monocytes also use glutamine as an energy substrate so the stress does differ.

Next, we tested the effect of starvation for lysine and glutamine on HSPA1A mRNA levels. ASNS mRNA levels were analyzed as a control for induction of the amino acid response: starvation for either of the two amino acids induced ASNS mRNA levels (Fig. 4B). HSPA1A mRNA levels were decreased upon starvation for both lysine and glutamine, suggesting a general effect of amino acid starvation on heat shock protein

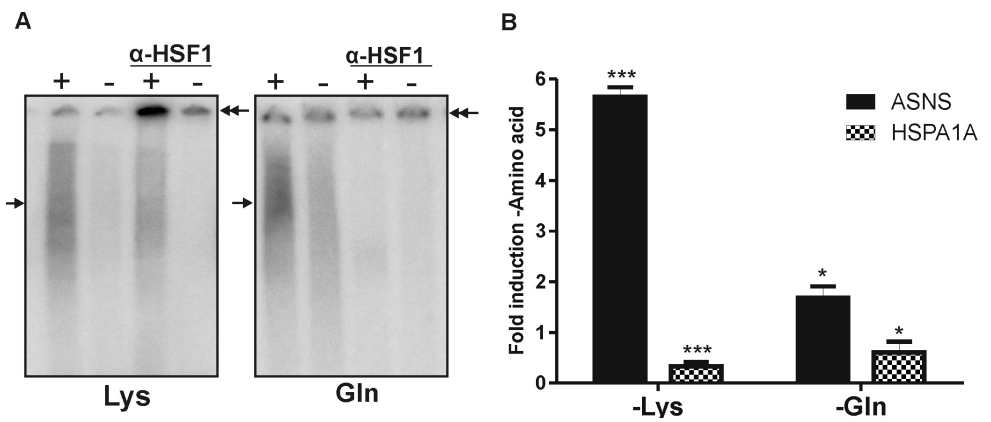

Fig. 4 Lysine and glutamine starvation also inactivate nuclear HSF1. A. HEK293 cells were cultured in the presence of all amino acids (+) or deprived of lysine or glutamine (-) for 24 h. EMSA was performed with a double-stranded oligonucleotide with the HSE sequence. Supershifts were induced with an anti-HSF1 antibody. Single arrows indicate the primary complexes formed, double arrows indicate the supershifted complexes. **B.** ASNS and HSPA1A mRNA levels were determined by QPCR analysis and are shown relative to GAPDH mRNA levels. Error bars represent SD; *P<0.05; ***P<0.001, relative to +amino acid.

mRNA levels, where, at least in the case of HSPA1A mRNA, decreased mRNA stability also plays a role.

Amino acid limitation also affects HSF1 binding affinity and HSP mRNA levels

All experiments reported above were performed with medium completely lacking leucine, lysine or glutamine – an extreme situation. A more physiological situation would be a shortage but not a complete lack of an amino acid. To test whether the HSF1 binding affinity is also lost under conditions of amino acid limitation, we analyzed the binding of HSF1 to a HSE by EMSA using nuclear extracts of cells that were cultured in medium with decreasing leucine concentrations. At one tenth of the normal concentration of leucine in the medium a loss in HSF1:HSE binding was found (Fig. 5A); at this concentration cells were still slowly growing. Culturing the cells for several days in medium containing one tenth of the normal leucine concentration led to a further decrease in HSF1 binding; at the same time NSRU-protein complexes became detectable (Fig. 5B).

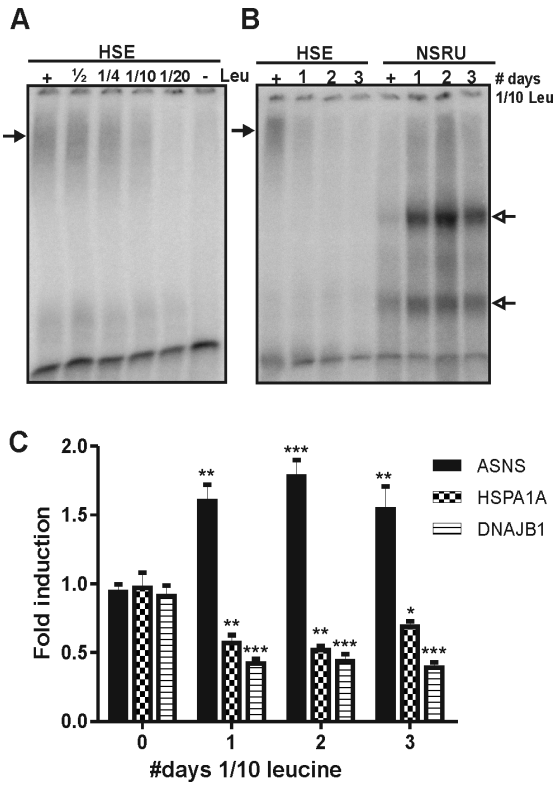

Fig. 5 Amino acid limitation affects HSF1 binding affinity and HSP mRNA levels. A. HEK293 cells were cultured in medium containing limiting amounts of leucine for 24 h. The concentrations are indicated relative to the standard leucine concentration in medium (+), which is 450 μM. EMSA was performed with a double-stranded oligonucleotide with the HSE sequence. The arrow indicates the primary complexes formed. **B.** HEK293 cells were cultured for the indicated times in medium containing 45 μM leucine. As a control, cells were cultured in parallel in standard medium containing 450 μM leucine (+). Medium was changed every day. Nuclear extracts were used in EMSA with a double-stranded oligonucleotide with the HSE or NSRU sequence. The closed arrow indicates the HSE complex formed. Open arrows indicate the NSRU complexes formed. **C.** ASNS, DNAJB1 and HSPA1A mRNA levels were determined by QPCR analysis and are shown relative to GAPDH mRNA levels. Error bars represent SD; *P<0.05; **P<0.01; ***P<0.001, relative to 0 days.

We then determined the effect of culturing the cells in medium with one tenth of the normal leucine concentration on heat shock protein mRNA levels. Already after one day a decrease in HSPA1A and DNAJB1 mRNA levels was detected (Fig. 5C), and this decrease persisted after two and three days of culturing in medium with limiting leucine concentrations. The ASNS mRNA level increased upon leucine limitation. These results indicate that amino acid limitation also affects at least the HSPA1A and DNAJB1 mRNA levels. We were unable to detect a decrease in HSPA1A and DNAJB1 protein levels upon leucine limitation for 3 days (Fig. S2). This was not quite unexpected, as we only found a small effect on protein levels after complete leucine starvation (Fig. 2C). Unfortunately, it is experimentally not possible to test cells cultured for a longer time: passaging cells induces a transient heat shock response.

HSF1 can bind to the NSRU of the ASNS promoter

The results presented above raise the obvious question as to what the role of HSF1 is in the amino acid response: why is it inactivated as part of this response? To answer this question we looked at the NSRU of the ASNS promoter, a canonical amino acid response element, and noted a putative HSF1 binding site (Fig. 6A). To test whether this HSE is functional we inserted a dimer of the NSRU sequence (Fig. 6B) in the pGL3 promoter vector which contains a SV40 promoter driven luciferase gene. The activity of this NSRU-luc construct was inhibited by a dominant negative HSF1, an HSF1 mutant lacking the activation domain [265] (Fig. 6C). Mutation of one of the putative HSEs (NSRU 1xmut; Fig. 6B) increased activity of the NSRU reporter in dnHSF1 expressing cells to about half of that in control cells; mutation of both sites (NSRU 2xmut; Fig. 6B) restored about 75% of the activity. In the absence of dnHSF1 expression, these mutations had no effect (Fig. 6C, -dnHSF1). The HSF1 binding to the putative NSRU HSE is weak: we were unable to detect in vitro binding (EMSA) using a probe containing a single copy of the NSRU. However, when we used a probe containing the NSRU repeat, as present in the NSRU luciferase reporter construct, and nuclear extracts of heat stressed cells overexpressing wtHSF1, complexes could be detected (Fig. 6d). The signals of the faster migrating complexes (indicated by open arrows) decreased when unlabeled oligonucleotides with the mutated HSF1 binding sites (NSRU 2xmut, Fig. 6D) were used and likely represent ATF4 complexes. The slowly migrating complex (indicated by a single closed arrow) could not be competed for by the NSRU 2xmut and was supershifted by an HSF1 antibody (indicated by double arrows). This complex thus represents HSF1 binding. These data demonstrate that HSF1 can indeed bind to the NSRU sequence of the ASNS promoter.

In vivo the NSRU is part of a larger promoter region and the activity of a NSRU-luc construct does not necessarily reflect that of the ASNS promoter. In leucine fed cells

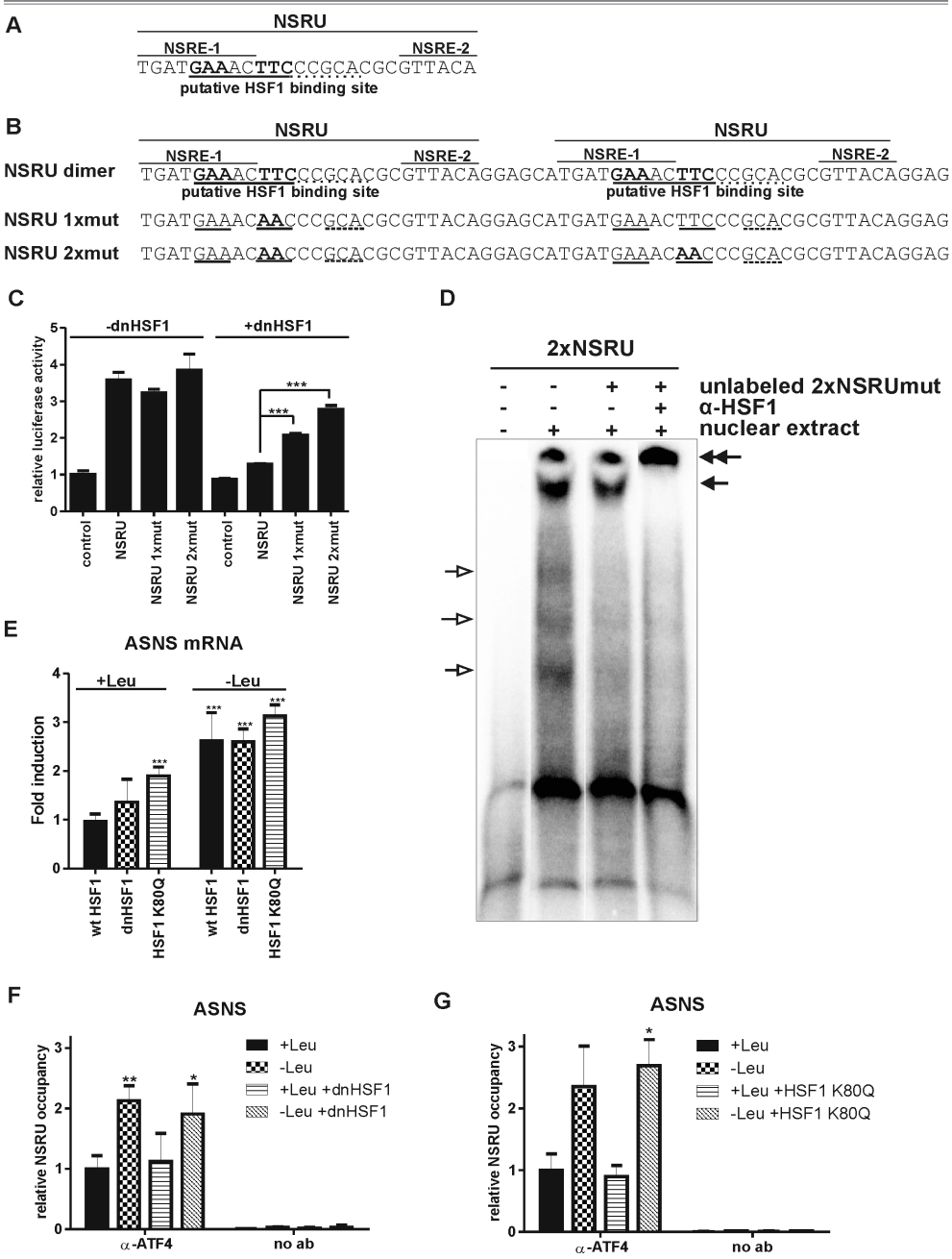

Fig. 6 The ASNS NSRU contains a HSE. A. Sequence of the NSRU of the ASNS promoter. The putative HSF1 binding sequence is underlined. **B.** Sequence of a tandem repeat of the nutrient sensing response unit of the ASNS promoter used in the reporter plasmid. The putative HSF1 binding sequence is underlined. NSRU 1xmut and NSRU 2xmut are the sequences that are mutated for the putative HSE (indicated in bold). **C.** HEK-dnHSF1 cells were transfected

with the indicated NSRU reporter plasmid and treated with doxycycline. Cells were harvested and assayed for reporter gene activities. The results shown are the average of three independent transfections. **D.** HEK-wtHSF1 cells were cultured in the presence of doxycyline and heat stressed for 30' at 45°C. Directly after heat stress nuclear extracts were made. EMSA was performed with a double-stranded oligonucleotide with the 2xNSRU sequence. Where indicated a twofold molar excess of unlabeled double-stranded oligonucleotide with the 2xNSRUmut sequence was added. Supershifts were induced with an anti-HSF1 antibody. Open arrows indicate the ATF4 complexes; closed arrows indicate the HSF1 specific complex and the supershifted complex. **E.** Doxycycline treated HEK-wtHSF1, HEK-HSF1 K80Q and HEK-dnHSF1 cells were starved for leucine. mRNA levels were determined by QPCR analysis and are shown relative to GAPDH mRNA levels. **F.** and **G.** Chromatin immunoprecipitation was performed using an ATF4 antibody. Bound chromatin was analyzed by QPCR using a primer set surrounding the NSRU of the ASNS promoter. In all figures error bars represent SD; *P<0.05; **P<0.01; ***P<0.001.

expressing the dnHSF1 mutant the ASNS transcript level did not decrease but rather increased slightly while the level in leucine starved cells was not affected (Fig. 6E). When we used cells expressing an HSF1 mutant unable to bind DNA (HSF1 K80Q [51]; this mutant also blocks the heat shock induction of HSF1 target genes (Fig. S3), the ASNS mRNA level increased slightly in both leucine fed and leucine starved cells (Fig. 6E). We were also unable to detect binding of HSF1 to the NSRU in vivo by ChIP. Finally, expression of dnHSF1 or HSF1 K80Q did not influence the extent of binding of ATF4, the main activating transcription factor bound to the NSRU, either in the presence or absence of leucine (Fig. 6F and G).

Effect of dnHSF1 or HSF1 K80Q on the transcript levels of amino acid response genes

The results presented above show that even though the NSRU of the ASNS promoter contains an HSE, HSF1 does not appear to regulate expression of the ASNS gene. We therefore looked whether the transcript levels of other amino acid responsive genes are affected in cells expressing HSF1 K80Q or dnHSF1. We identified the amino acid responsive genes in HEK293 cells starved for leucine by microarray analysis (Table S1) and then looked at the change in transcript level of these genes when either HSF1 K80Q or dnHSF1 was expressed. As seen in Fig. S4 and Table 3 in dnHSF1 expressing cells on average the transcript levels increased. A more varied response was seen in HSF1 K80Q cells with an increase in some and a decrease in others. We noted that there appears to be a large cell and even amino acid specific effect to the amino acid deprivation response: a comparison of our results with published microarray studies [288-290] showed that the transcript levels of only 6 genes changed significantly in all cases. Five of these encode transcription factors (ATF3, CEBPB, CEBPG, KLF10, TRIB3) and are all upregulated; one encodes the enzyme ASNS. The transcript level of these canonical amino acid response genes also increased in leucine fed dnHSF1

expressing HEK293 cells; in HSF1 K80Q expressing HEK293 cells only a subset showed an increase (Table 3).
Most, if not all, of the canonical amino acid responsive genes are direct targets of ATF4 and the increase in their transcript level in leucine fed dnHSF1 expressing cells could thus be due to an increase in ATF4 levels. However, we could not detect a significant effect of dnHSF1 on the expression levels of ATF4 or C/EBPβ, another transcription factor involved in the amino acid response, either in the presence or absence of leucine (Fig. S5).

Table 3. Changes in transcript levels of "canonical" amino acid responsive genes upon expression of dnHSF1 or HSF1 K80Q

Gene name	Acc. No.	Description	Fold change	
			dnHSF1	HSF1 K80Q
ASNS	NM_001673	Asparagine synthetase	1.71	1.99
ATF3	NM_004024	Activating transcription factor 3	1.18	0.83
CEBPB	NM_005194	CCAAT/enhancer binding protein (C/EBP), beta	1.54	1.18
CEBPG	NM_001806	CCAAT/enhancer binding protein (C/EBP), gamma	1.29	1.26
DDIT3	NM_004083	DNA-damage-inducible transcript 3 (CHOP)	1.43	1.11
KLF10	NM_005655	Kruppel-like factor 10	1.20	0.74
TRIB3	NM_021158	Tribbles homolog 3 (Drosophila)	1.30	0.93

Discussion

We have shown here that unusually and unexpectedly, HSF1 is not activated but silenced when cells are starved for amino acids. In leucine, glutamine or lysine starved cells HSF1 in the nuclear fraction lost its DNA binding activity. HSF1 activity is regulated via complex regulatory mechanisms, including post-translational modifications. HSF1 can for example be regulated by phosphorylation ([45]; for review, see also [291]). However, we do not see a change in electrophoretic mobility, making extensive changes in the phosphorylation pattern unlikely. It has also been described that acetylation of HSF1 at K80 results in a loss in DNA binding affinity [51]; a distinct possibility is thus that upon amino amino acid deprivation HSF1 is acetylated at K80. We could not show acetylation of HSF1 using an antibody directed against acetylated lysine, but this could well have been an experimental problem.
A possible reason for the inactivation of HSF1 in amino acid starved cells is that HSF1 is directly involved in regulating the activity of amino acid starvation responsive genes

in fed cells, as suggested by the finding of an HSF1 binding site in the NSRU of the ASNS promoter (Fig. 6A), as well as in other amino acid response promoters such as ATF3 [292] and S100P (unpubl. data). However, we could not find direct evidence for HSF1 binding to the NSRU in vivo. Furthermore, the effect of exogenous expression of either a non-DNA binding or a dominant negative mutant of HSF1 on the transcript levels of amino acid starvation responsive genes is not large, while depleting the cells of HSF1 by siRNA had no effect on at least the level of ASNS mRNA (data not shown). Hence, although it is tempting to suggest that HSF1 is a regulatory factor in the amino acid starvation response, we could find no solid evidence that that is indeed the case.

HSF1 is best known for its activation of transcription of the heat shock protein genes during proteotoxic stress, like heat, UV and viral infections but HSF1 also plays physiological role in setting the circadian rhythm [293]. For example the circadian clock gene Per2 is an HSF1 target [19,20]. Intriguingly, one of the genes at the core of the amino acid deprivation response, *KLF10*, is also a target gene of a clock protein [294]. Perhaps the inactivation of HSF1 during the amino acid response is part of the intricate cross-talk between metabolism and the circadian rhythm (for review, see [295]).

Alternatively, inactivation of HSF1 during a non-proteotoxic stress response may be a more general phenomenon and may aid the organism in clearing irreversibly damaged cells. Recently it was shown that HSF1 is also inactivated during the DNA damage response [263], an inactivation that facilitates senescence.

We do see the loss of HSF1 binding during amino acid starvation reflected in the levels of mRNAs of HSF1 target genes: the level of both HSPA1A (Hsp70) and DNAJB1 (Hsp40) mRNAs drops markedly (Fig. 1A). A significant decrease in DNAJB1 mRNA was also noted in leucine starved MEFs [288], while HSPA1A mRNA was reported to decrease significantly in cysteine and histidine starved HepG2 cells [289,290]. In *Drosophila* larvae complete starvation or sugar deprivation led to a strong decrease in Hsp90 mRNA levels [296]. The loss of HSF1 binding affinity and the decrease in HSPA1A and DNAJB1 mRNA levels was not just seen in cells dying from lack of an amino acid, but also in cells fed just enough leucine to continue slow growth (Fig. 5). In the future, it needs to be tested whether whole organisms show the same response when amino acids are limiting. If so, then malnutrition, lack of essential amino acids, would also lead to cellular (and organismal) frailty due to a loss of chaperoning capacity.

Acknowledgements

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Supplemental table

Table S1. Transcript levels induced more than 2-fold upon leucine starvation (24 h)

Gene name	Acc. No.	Description	Fold induction -Leu
FUT1	NM_000148	Fucosyltransferase 1 (galactoside 2-alpha-L-fucosyltransferase, H blood group)	5.33
SLC7A11	NM_014331	Solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	5.31
TRIB3	NM_021158	Tribbles homolog 3 (Drosophila)	5.01
NUPR1	NM_012385	Nuclear protein 1	4.69
DDIT3	NM_004083	DNA-damage-inducible transcript 3	4.12
ALDH1L2	CR749561	mRNA; cDNA DKFZp686A16126	3.99
PCK2	NM_004563	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	3.83
LOC645733	XM_374004	Hypothetical LOC389025	3.44
TXNIP	NM_006472	Thioredoxin interacting protein	3.44
JDP2	NM_130469	Jun dimerization protein 2	3.43
TTC18	NM_145170	Ttetratricopeptide repeat domain 18	3.25
GDAP1L1	NM_024034	Ganglioside-induced differentiation-associated protein 1-like 1	3.24
S100P	NM_005980	S100 calcium binding protein P	3.23
CEBPB	NM_005194	CCAAT/enhancer binding protein beta	3.22
GDF15	NM_004864	Growth differentiation factor 15	3.22
ASNSL1	THC2363646	AJHYNG asparagine synthase (glutamine-hydrolysing) [similarity] - golden hamster	3.10
TAC1	NM_003182	Tachykinin, precursor 1	3.06
ASNS	BC030024	Asparagine synthetase	2.89
OGT	NM_181672	O-linked N-acetylglucosamine (GlcNAc) transferase	2.85
TTC25	NM_031421	Tetratricopeptide repeat domain 25	2.84
TIGA1	NM_053000	TIGA1	2.79
A2LD1	BC001077	A2LD1 AIG2-like domain 1	2.79
LOC285908	NM_181722	NCRNA00174 non-protein coding RNA 174	2.77

LOC729779	LOC729779	Similar to phosphoserine aminotransferase	2.68
RAB39B	NM_171998	RAB39B, member RAS oncogene family	2.60
ATF3	NM_004024	Activating transcription factor 3	2.59
RAPGEF3	NM_006105	Rap guanine nucleotide exchange factor (GEF) 3	2.57
ARRDC4	NM_183376	Arrestin domain containing 4	2.54
SH3BGR	NM_007341	SH3 domain binding glutamic acid-rich protein	2.47
PLEKHG4	NM_015432	Pleckstrin homology domain containing, family G (with RhoGef domain) member 4	2.47
CALCB	NM_000728	Calcitonin-related polypeptide, beta	2.46
AREG	NM_001657	Amphiregulin (schwannoma-derived growth factor)	2.43
LOC100130042	LOC100130042	Similar to methylenetetrahydrofolate dehydrogenase 2	2.43
FCGBP	NM_003890	Fc fragment of IgG binding protein	2.40
INHBA	NM_002192	Inhibin, beta A (activin A, activin AB alpha polypeptide)	2.40
CCDC11	NM_145020	Coiled-coil domain containing 11	2.40
LRRIQ1	NM_032165	Leucine-rich repeats and IQ motif containing 1	2.39
CEBPG	NM_001806	CCAAT/enhancer binding protein gamma	2.39
POPDC2	NM_022135	Popeye domain containing 2	2.37
FLJ31659	NM_153027	TRIM61 tripartite motif-containing 61	2.35
C1orf24	NM_052966	Chromosome 1 open reading frame 24	2.35
ENST00000338358	ENST00000338358	Hypothetical LOC100130691	2.34
KIAA1407	NM_020817	KIAA1407, unknown function	2.34
CR623273	CR623273	METTL12 methyltransferase like 12	2.34
FBXO4	NM_012176	F-box protein 4	2.34
LOC51315	NM_016618	KRCC1 lysine-rich coiled-coil 1	2.32
C21orf69	NM_058189	Chromosome 21 open reading frame 69	2.29
KLF10	NM_005655	Kruppel-like factor 10	2.29
C9orf103	NM_001001551	Chromosome 9 open reading frame 103	2.28
AMT	NM_000481	Aminomethyltransferase	2.28
ZNF33B	NM_006955	Zinc finger protein 33B	2.26
FLJ37035	AK094354	cDNA FLJ37035 fis, clone BRACE2011545	2.25

C6orf26	NM_001039651	Chromosome 6 open reading frame 26	2.24
RBKS	NM_022128	Ribokinase	2.23
LPXN	NM_004811	Leupaxin	2.22
ELAC1	NM_018696	ElaC homolog 1 (E. coli)	2.22
IL23A	NM_016584	Interleukin 23, alpha subunit p19	2.21
DUSP26	NM_024025	Dual specificity phosphatase 26 (putative)	2.21
FLJ10916	NM_018271	THNSL2 threonine synthase-like 2 (S. cerevisiae)	2.20
CHGB	NM_001819	Chromogranin B (secretogranin 1)	2.19
GAS5	NR_002578	Growth arrest-specific 5	2.18
ATP6AP1L	NM_001017971	ATP6AP1L ATPase, H+ transporting, lysosomal accessory protein 1-like	2.18
C2orf74	BC014578	Hypothetical gene supported by AK075484	2.17
CIRBP	AK128423	cDNA FLJ46566 fis, clone THYMU3040829, moderately similar to Cold-inducible RNA-binding protein	2.17
C19orf18	NM_152474	Chromosome 19 open reading frame 18	2.16
SEC63D1	NM_198550	SEC63 domain containing 1	2.14
C1orf97	ENST00000367003	C1orf97 chromosome 1 open reading frame 97	2.12
CHEK1	BX419129	CHEK1 CHK1 checkpoint homolog (S. pombe)	2.11
ENST00000343253	ENST00000343253	Intron CCDC18 coiled-coil domain containing 18	2.11
CCT6B	NM_006584	Chaperonin containing TCP1, subunit 6B	2.11
LOC149134	NM_207326	Hypothetical protein LOC149134	2.10
ZNF688	NM_145271	Zinc finger protein 688	2.10
SAMD13	NM_001010971	Sterile alpha motif domain containing 13	2.10
ENST00000339446	ENST00000339446	Hypothetical LOC387763, mRNA (cDNA clone IMAGE:6272440), partial cds	2.09
RCBTB2	NM_001268	Regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 2	2.09
KIAA1908	AB067495	Hypothetical protein LOC114796	2.09
ADRA1B	NM_000679	Adrenergic, alpha-1B-, receptor	2.08
TCAM1	NR_002947	Testicular cell adhesion molecule 1 homolog (mouse)	2.08

NPAL2	AK025015	cDNA: FLJ21362 fis, clone COL02886	2.08
C21orf6	NM_016940	Chromosome 21 open reading frame 6	2.08
MSTP9	NR_002729	Macrophage stimulating, pseudogene 9	2.08
TncRNA	NR_002802	Trophoblast-derived noncoding RNA	2.08
LOC643684	XM_931745	Hypothetical protein LOC643684	2.07
ADAM12	NM_003474	ADAM metallopeptidase domain 12 (meltrin alpha)	2.07
PPP1R15A	NM_014330	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	2.07
ADAMTS13	NM_139025	ADAM metallopeptidase with thrombospondin type 1 motif, 13	2.07
SYTL1	NM_032872	Synaptotagmin-like 1	2.06
ZC3H6	AK131416	cDNA FLJ16526 fis, clone OCBBF2006987	2.06
WDR78	NM_207014	WD repeat domain 78	2.06
ARHGEF2	NM_004723	Rho/rac guanine nucleotide exchange factor (GEF) 2	2.05
PRIM1	NM_000946	Primase, polypeptide 1, 49kDa	2.05
DKFZp762P2111	AK022976	ZNF783 zinc finger family member 783	2.05
C22orf23	NM_032561	Chromosome 22 open reading frame 23	2.04
ABHD1	BC028378	Abhydrolase domain containing 1	2.04
AF075112	AF075112	L1ME4 repeat	2.04
AX721087	AX721087	SCXA scleraxis homolog A (mouse) AND SCXB scleraxis homolog B (mouse)	2.02
THC2326212	THC2326212	LOC399815 chromosome 10 open reading frame 88 pseudogene	2.02
SMPX	NM_014332	Small muscle protein, X-linked	2.01
LOC388335	NM_001004313	Similar to RIKEN cDNA A730055C05 gene	2.01
LOC285033	CR619653	Hypothetical protein	2.00
TSLP	NM_033035	Thymic stromal lymphopoietin	2.00

Supplemental figures

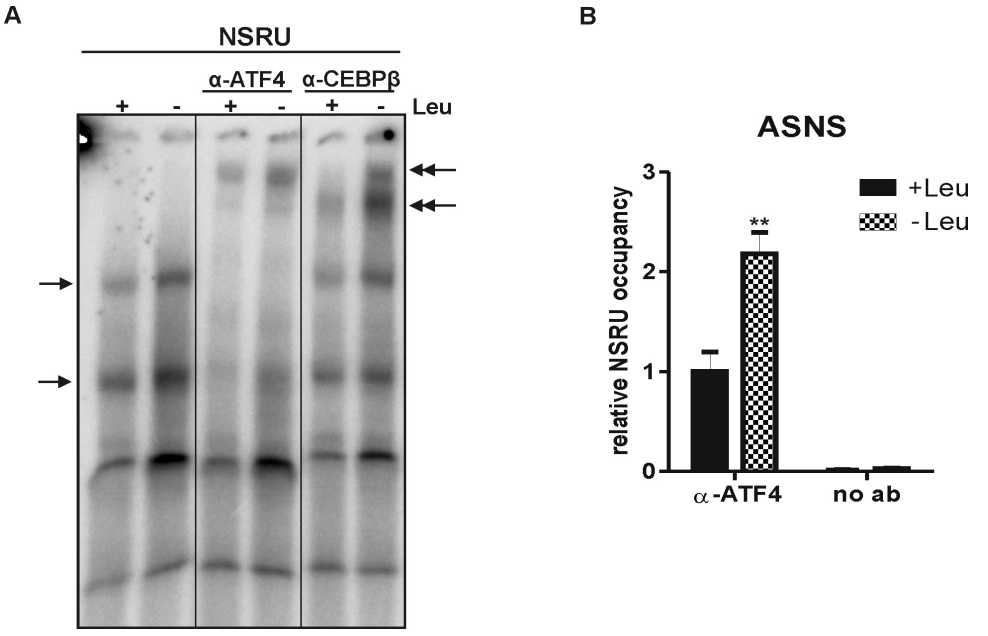


Fig. S1 EMSA and ChIP for the NSRU of the ASNS promoter. A. An electrophoretic mobility shift assay was performed with extracts of leucine deprived HEK293 cells (as used in Fig. 1D) and a double-stranded oligonucleotide for the NSRU sequence. Supershifts were induced with a rabbit polyclonal ATF4 antibody (sc-200; Santa Cruz; 1:1000) and a rabbit polyclonal C/EBPβ antibody (sc-150; Santa Cruz; 1:1000). Single arrows indicate the primary complexes formed, double arrows indicate the supershifted complexes. **B.** Chromatin immunoprecipitation was performed (as in Fig 1E) using an anti-ATF4 antibody. Bound chromatin was analyzed by QPCR using a primer set surrounding the NSRU of the ASNS promoter. As a control the ChIP was performed without an antibody. Error bars represent SD; **P<0.01, relative to +Leu.

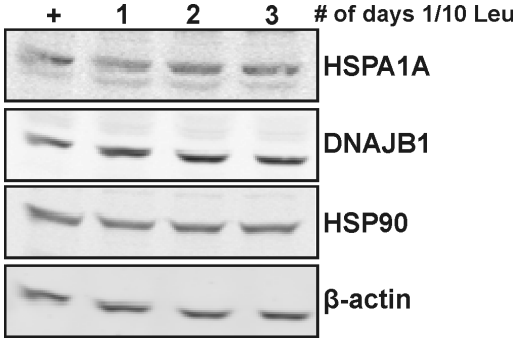


Fig. S2 Heat shock protein levels in cells cultured with limited leucine. HEK293 cells were cultured for the indicated times in medium containing 45 μM leucine. As a control, cells were cultured in parallel in standard medium containing 450 μM leucine (+). Medium was changed every day. Lysates were subjected to SDS-PAGE and western blot analysis using antibodies against the indicated proteins.

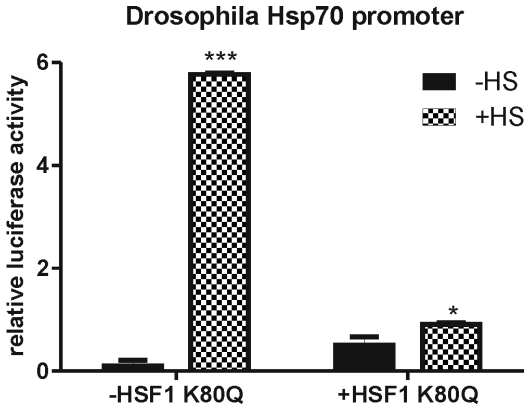


Fig. S3 HSF1 K80Q mutant. HEK-HSF1K80Q cells were transfected with the *Drosophila melanogaster* Hsp70-luciferase reporter plasmid [228] and cultured for 48 h in the presence or absence of doxycycline. Cells were exposed to a heat shock for 30' at 45°C or left at 37°C. Six hours after recovery, cells were harvested and assayed for reporter gene activities. The results are the average of three independent transfections. Error bars represent SD; *P<0.05; ***P<0.001.

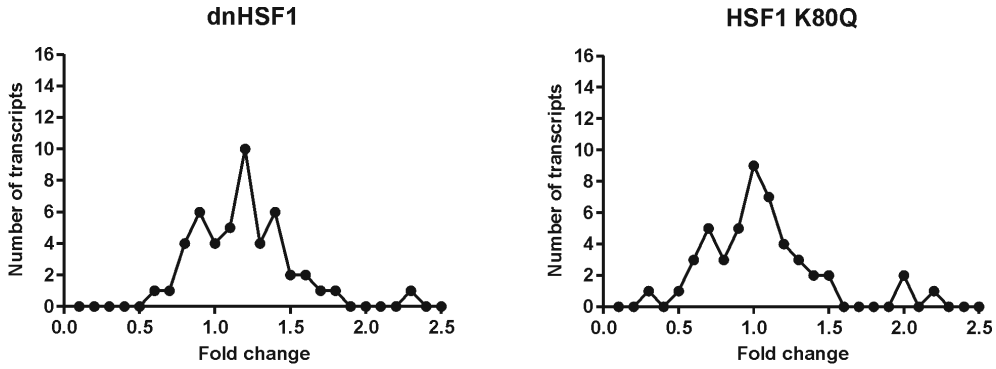


Fig. S4 The levels of amino acid responsive gene transcripts are affected by the inactivation of HSF1. Histograms were made of the fold change in the level of amino acid responsive transcripts upon expression of dnHSF1 or HSF1 K80Q (compared to overexpression of wtHSF1).

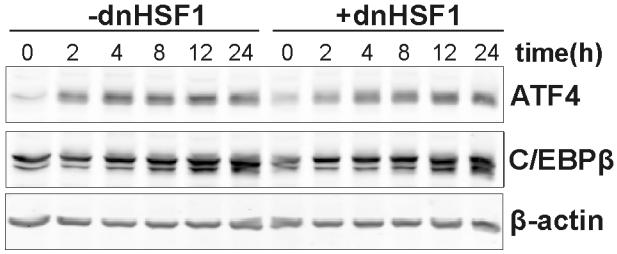
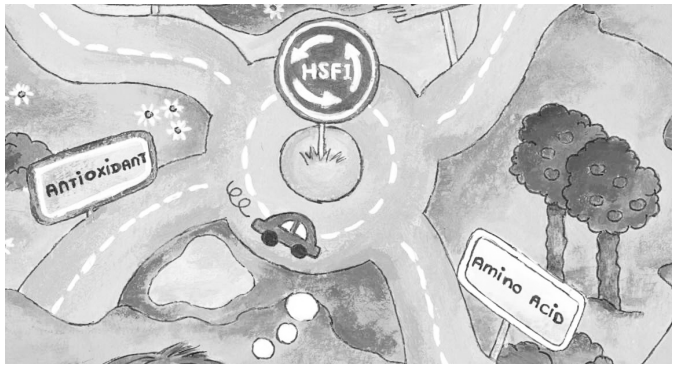


Fig. S5 ATF4 and C/EBPβ expression levels upon leucine starvation are not affected by dnHSF1 expression. HEK-dnHSF1 cells were left untreated or expression of dnHSF1 was induced. At 24 h after induction, cells were deprived of leucine and harvested at the indicated time points. Cell lysates were subjected to SDS-PAGE and western blot analysis using the antibodies against the indicated proteins [rabbit polyclonal ATF4 antibody (sc-200; Santa Cruz; 1:1000), rabbit polyclonal C/EBPβ antibody (sc-150; Santa Cruz; 1:1000), and mouse monoclonal β-actin antibody (AC-15; Sigma; 1:5000).

Chapter 6

Activation of the antioxidant response in methionine deprived human cells results in an HSF1-independent increase in HSPA1A mRNA levels



Sanne M.M. Hensen, Lonneke Heldens, Chrissy M.W. van Enckevort, Siebe T. van Genesen, Ger J.M. Pruijn, Nicolette H. Lubsen

Biochimie 2013; 95:1245-1251

In cells starved for leucine, lysine or glutamine heat shock factor 1 (HSF1) is inactivated and the level of the transcripts of the HSF1 target genes HSPA1A (Hsp70) and DNAJB1 (Hsp40) drops. We show here that in HEK293 cells deprived of methionine HSF1 was similarly inactivated but that the level of HSPA1A and DNAJB1 mRNA increased. This increase was also seen in cells expressing a dominant negative HSF1 mutant (HSF379 or HSF1-K80Q), confirming that the increase is HSF1 independent. The antioxidant N-acetylcysteine completely inhibited the increase in HSPA1A and DNAJB1 mRNA levels upon methionine starvation, indicating that this increase is a response to oxidative stress resulting from a lack of methionine. Cells starved for methionine contained higher levels of c-Fos and FosB mRNA, but knockdown of these transcription factors had no effect on the HSPA1A or DNAJB1 mRNA level. Knockdown of NRF2 mRNA resulted in the inhibition of the increase in the HSPA1A mRNA, but not the DNAJB1 mRNA, level in methionine starved cells. We conclude that methionine deprivation results in both the amino acid deprivation response and an antioxidant response mediated at least in part by NRF2. This antioxidant response includes an HSF1 independent increase in the levels of HSPA1A and DNAJB1 mRNA.

Introduction

Amino acids are the building blocks of proteins and can also serve as intermediates in metabolism. The amino acid availability is closely monitored [297]. When cells sense a lack of one or more amino acids the amino acid response is mounted. Upon accumulation of uncharged tRNAs the general control non-derepressible 2 (GCN2) kinase is activated [82] and subsequently eukaryotic translation initiation factor 2α (eIF2α) is phosphorylated. eIF2α phosphorylation then leads to the inhibition of the global protein synthesis and the selective translation of some mRNAs, e.g. ATF4 mRNA (reviewed in [83]). ATF4 is an important player in the amino acid response: most amino acid responsive genes are ATF4 targets of which asparagine synthetase (ASNS) is the most widely studied one [92,93]. Petti et al. [298] showed that in yeast methionine starvation differs from starvation for other amino acids in that survival was substantially higher compared to for example leucine starvation. Furthermore, methionine has been shown to have a unique effect on fecundity in *Drosophila* upon dietary restriction, a restriction in food intake that does not lead to malnutrition. Grandison et al. [299] described a decrease in fecundity upon reduced food intake, and adding back methionine alone was sufficient to increase fecundity to the same level as did full feeding. Adding back other amino acids did not show this effect. These data thus suggest that starvation for methionine does not equal starvation for other amino acids.

Next to its importance in protein synthesis, the essential amino acid methionine is also involved in the transsulfuration pathway, a pathway in which methionine is converted via the formation of S-adenosylmethionine (SAM) into homocysteine and subsequently cysteine [300]. Cysteine availability is important for the synthesis of glutathione, a molecule that has been shown to have a strong antioxidative effect. A lack of methionine might thus have an effect on the formation of glutathione and can thereby affect the oxidative status of the cell. In literature conflicting results about methionine deprivation and the antioxidant response are described. Erdmann et al. [301] showed that addition of L-methionine reduced free radical formation in endothelial cells through the induction of heme oxygenase 1 (HMOX1). On the other hand it was described that methionine supplementation increases mitochondrial ROS production and mitochondrial DNA oxidative damage in rat liver mitochondria [302] and vice versa that methionine restriction decreases mitochondrial ROS generation and oxidative damage to mitochondrial DNA and proteins, indicating the activation of the antioxidant response [303,304]. Recently, Lin et al. [305] demonstrated that the increased synthesis of glutathione S-transferase P (GSTP) in methionine restricted rat hepatocytes is due to activation of the transcription factor NRF2, a factor involved in the antioxidant response.

We have previously shown that upon starvation for leucine, lysine or glutamine the transcription factor heat shock factor 1 (HSF1), which regulates the proteotoxic stress response, is inactivated and that the mRNA levels of the HSF1 target genes HSPA1A, DNAJB1 and HSP90AA1 levels are strongly decreased [264]. The complex cellular response to methionine starvation made us wonder whether HSF1 is also inactivated in methionine starved cells. We show here that HSF1 indeed also loses its DNA binding affinity in methionine starved cells, but that, unexpectedly and in contrast to what is found during starvation for other amino acids, HSPA1A and DNAJB1 mRNA levels do increase. This increase was not dependent on HSF1 but, at least for HSPA1A, on NRF2. These data show that in methionine starved cells an antioxidant response is superimposed on the amino acid response.

Materials and Methods

Tissue culture

T-REx HEK293-pcDNA5, HEK-HSF379 (dnHSF1) and HEK-HSF1 K80Q cell lines were generated as described before [264,265]. The cells were cultured at 37°C/5% CO_2 in high glucose DMEM medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Blasticidin (1.65 μg/ml; Invitrogen) and 100 μg/

ml hygromycin were also added to the culture medium during maintenance of the cell lines, but were omitted during experiments. For amino acid starvation experiments cells were washed with PBS and subsequently DMEM/F12 medium (Sigma) with or without methionine or leucine, supplemented with 10% dialyzed fetal calf serum, was added for the indicated times.

RNA isolation and reverse transcription

Total RNA was isolated using Trizol (Invitrogen). 1 μg of RNA was treated with DNaseI (Amplification grade; RNase-free; Invitrogen). Subsequently, 5 mM $MgCl_2$, RT-buffer, 1 mM dNTPs, 18.75 units AMV reverse transcriptase, 20 units RNase inhibitors and 1.25 μM oligo(dT) were added to a total volume of 20 μl. Reverse transcription was performed for 10 minutes at 25°C, 60 minutes at 42°C and 5 minutes at 95°C. For QPCR analysis, cDNA was 10-fold diluted.

Chromatin immunoprecipitation

T-REx HEK293–pcDNA5 cells were cultured for 24 h in the presence or absence of methionine. Chromatin immunoprecipitation was performed as described in [268], except that cells were crosslinked for 15 minutes with 1% formaldehyde. After quenching with 125 mM glycine, cells were washed twice with ice cold PBS and resuspended in ice cold lysis buffer (50 mM HEPES.KOH pH 7.6, 140 mM NaCl, 1 mM EDTA pH 8.0, 1% (v/v) Triton X-100, 0.1% NaDOC and 1X protease inhibitor cocktail). Antibodies used for ChIP were rabbit polyclonal ATF4 antibody (sc-200; Santa Cruz) and rabbit polyclonal HSF1 antibody (SPA-901; Stressgen). ChIP samples were analyzed by QPCR with the primer sets listed in Supplemental Table 1.

Electrophoretic mobility shift assay

HEK293 cells were cultured for 24 h in the presence or absence of methionine. Cells were immediately harvested and nuclear extracts were prepared using NE-per nuclear and cytoplasmic reagents (Pierce). Extracts were aliquoted and stored at -80°C. Oligonucleotide probes were end-labeled with ^{32}P. The sequences of the oligonucleotides used in EMSA are listed in Supplemental Table 1. The EMSA protocol was adapted from [266,267]. A mixture containing 5 μg nuclear extract and 3 μg poly dIdC in binding buffer [20 mM HEPES pH 7.9, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 4% (v/v) Ficoll, 1X PhosSTOP (Roche)] was incubated for 20 minutes on ice. 0.01 pmol radiolabeled oligonucleotide was added and the samples were incubated for 20 minutes at room temperature. DNA-protein complexes were separated on a pre-run 4% polyacrylamide gel in 0.25x TBE with recirculation of the buffer. The gel was dried and signals were visualized using a PhosphorImager.

RNA interference

The control siRNA against luciferase (5'-CGUACGCGGAAUACUUCGAdTdT-3'), NRF2 siRNA (5'-CAGCAUGCUACGUGAUGAAdTdT-3') and NRF2 siRNA#2 (5'-CCAGUGGAUCUGCCAACUAdTdT-3') were purchased from Eurogentec. C-Fos (sc-29221) and FosB (sc-35403) siRNAs were purchased from Santa Cruz Biotechnology. HEK293 cells were cultured in 6-well plates and transfected with 50 nM siRNA using oligofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. 48 h after transfection cells were re-transfected as described above. Medium was changed to DME/F12 with or without methionine 24 h after re-transfection and cells were harvested 24 h later.

Quantitative real-time PCR

Quantitative real-time PCR was performed using the StepOnePlus™ Real-Time PCR System with *Power* SYBR® Green PCR Master mix (Applied Biosystems) using the following amplification protocol: 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Per reaction 3 µl of diluted cDNA or ChIP material was used and the DNA was amplified using primers for the sequences of interest, listed in Supplemental Table 1. Two-tailed Student's *t* tests were performed to calculate the significance of the data.

Results and Discussion

Increased HSPA1A and DNAJB1 mRNA levels in methionine deprived HEK293 cells

In a previous study we described that leucine, lysine and glutamine deprivations result in the inactivation of HSF1 and a concomitant decrease in heat shock protein mRNA levels [264]. To determine whether methionine deprivation has a similar effect, we measured HSPA1A and DNAJB1 mRNA levels in methionine starved HEK293 cells and found these, in contrast with the effect of leucine, lysine and glutamine starvation, to be increased (Fig. 1A). Already within 4 hours of methionine starvation an increase in HSPA1A mRNA was seen; the maximal level was reached within 8 hours (Fig. 1B). The mRNA level of DNAJB1 increased only later (Fig. 1C). Note that, although the increase in HSPA1A mRNA level upon methionine deprivation was consistently found, the extent of change in the HSPA1A mRNA levels varied between 3 to 30-fold between different experiments for unknown reasons and in spite of our efforts to keep culture conditions identical between experiments (compare Fig. 1A and B). Note also that compared to the induction levels of HSPA1A mRNA upon heat stress, the

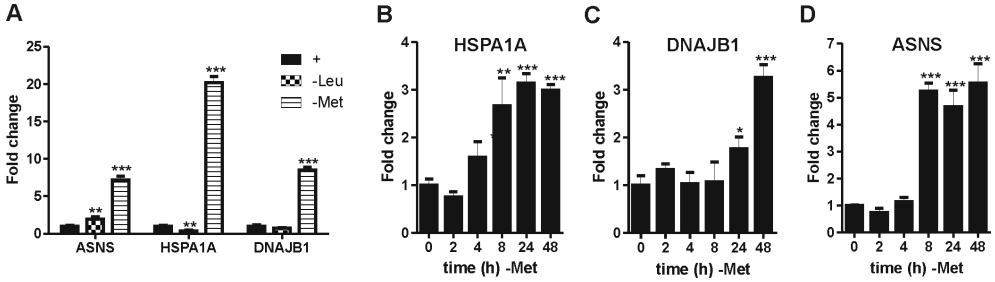


Fig. 1 Methionine starvation increases HSPA1A and DNAJB1 mRNA levels. A. HEK293 cells were deprived of leucine or methionine for 24 h. **B-D.** HEK293 cells were deprived of methionine and harvested at the indicated time points. mRNA levels were determined by QPCR analysis and are shown relative to GAPDH mRNA levels. Error bars represent SD; *P<0.05; **P<0.01; ***P<0.001, relative to fed cells (+ or 0).

effect of methionine deprivation is relatively small. The protein levels of HSPA1A and DNAJB1 did not noticeably increase in methionine starved cells (Fig. S1); presumably the rate of de novo protein synthesis in methionine starved cells is low. ASNS mRNA levels were 5-fold increased upon starvation for methionine (Fig. 1D), indicating that the amino acid response was activated. These data suggest that HSF1 is active when cells are starved for methionine. To determine whether HSF1 is indeed activated upon methionine deprivation, we performed an electrophoretic mobility shift assay with nuclear extracts of methionine deprived cells and examined binding of HSF1 to the heat shock element (HSE). Unexpectedly, this showed that HSF1 binding to the HSE decreases in nuclear extracts of methionine starved cells compared to fed cells (Fig. 2A), just as it does when cells are starved for other amino acids [264]. The HSF1 protein level or cellular location was not affected by methionine deprivation (Fig. S2). As a control we measured binding to the NSRU sequence of the ASNS promoter, which showed increased binding in nuclear extracts of methionine deprived cells compared to fed cells. The loss in HSF1 binding was confirmed by chromatin immunoprecipitation with an anti-HSF1 antibody: less HSF1 was bound to the heat shock element of the DNAJB1 promoter in methionine starved cells (Fig. 2B). At the same time an increase in ATF4 binding to the ASNS promoter was detected (Fig. 2C). Thus, even though HSPA1A and DNJAB1 mRNA levels increase upon starvation for methionine, HSF1 does lose its DNA binding affinity. These results imply that the increase in these HSP mRNA levels upon methionine deprivation is independent of HSF1. To confirm these findings, we used two cell lines stably expressing an HSF1 mutant, either a dominant negative HSF1 (dnHSF1) [265], which lacks the activation domain, or HSF1 K80Q [51], which contains a point mutation in the DNA binding domain and is therefore unable to bind

to the DNA. We measured the HSPA1A and DNAJB1 mRNA levels upon methionine starvation in the presence of either of these HSF1 mutants. In the absence of mutant HSF1 expression, both cell lines showed an increase in HSPA1A and DNAJB1 mRNA levels upon deprivation of methionine (Fig. 2D and E). When doxycycline was added, i.e. when the HSF1 mutants were expressed, HSPA1A and DNAJB1 mRNA levels were still increased relative to methionine fed cells. Note that DNAJB1 mRNA levels are strongly decreased upon expression of dnHSF1 in either fed or starved cells (Fig. 2D); this is in agreement with our previous observations [265], where DNAJB1 mRNA levels were 4-fold decreased in the presence of dnHSF1. All together, these data indicate that the increase in HSP mRNA levels is indeed independent of HSF1.

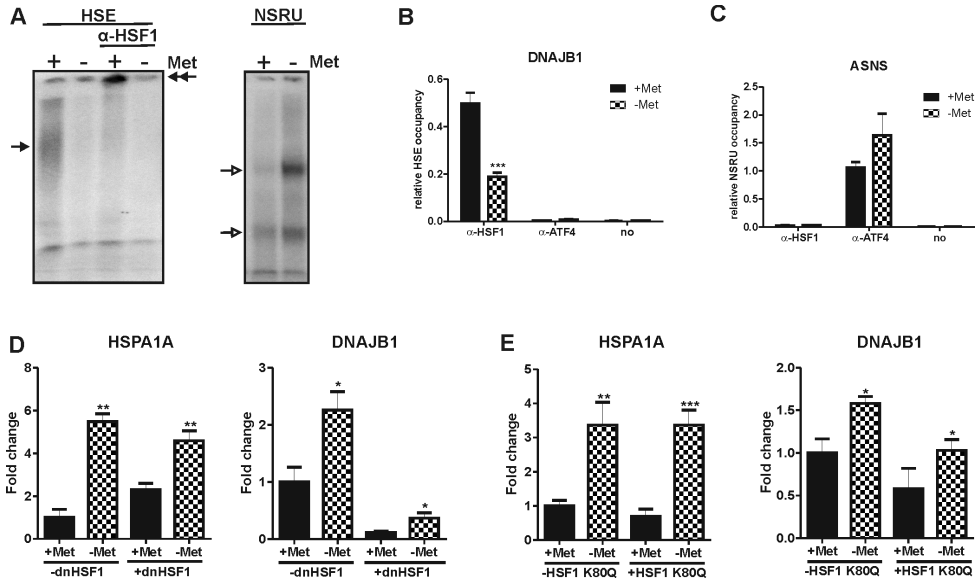


Fig. 2 The increase in HSPA1A and DNAJB1 mRNA levels upon methionine starvation is HSF1 independent. A. HEK293 cells were deprived of methionine for 24 h. Nuclear extracts were used in an electrophoretic mobility shift assay with a double-stranded oligonucleotide with the NSRU or HSE sequence. Supershifts were induced with an anti-HSF1 antibody. Single closed arrows indicate the primary HSE complexes formed; double closed arrows indicate the supershifted HSE complexes. Open arrows indicate the NSRU complexes formed. **B-C.** HEK293 cells were starved for methionine for 24 h. Chromatin immunoprecipitation was performed using an anti-HSF1 or an anti-ATF4 antibody. Bound chromatin was analyzed by QPCR using a primer set surrounding the HSE of the DNAJB1 promoter or the NSRU of the ASNS promoter. As a control the ChIP was performed without an antibody. **D-E.** HEK293-dnHSF1 or HEK293-HSF1 K80Q cells were deprived of methionine in the presence or absence of doxycycline and harvested after 24 h. mRNA levels were determined by QPCR analysis and are shown relative to GAPDH mRNA levels. Error bars represent SD; *P<0.05; **P<0.01; ***P<0.001, relative to +Met.

Activation of the antioxidant response in methionine starved HEK293 cells

As mentioned above, a lack of methionine could have an effect on the oxidative status of the cell, which could signal the increase in the heat shock protein mRNA levels. If so, addition of an antioxidant would counteract these changes. When we deprived cells of methionine and added the antioxidant N-acetylcysteine (NAC) both HSPA1A and DNAJB1 mRNA levels were strongly decreased compared to those in cells that were deprived of methionine without the addition of NAC (Fig. 3A and B). The addition of NAC appears to abolish the amino acid response in that the ASNS mRNA level no longer increased when methionine was withdrawn (Fig. 3C). However it has been shown before that addition of NAC induces ER stress in HeLa cells [306], which in turn activates transcription of the ASNS gene [111]. This would obscure the response of the ASNS promoter to amino acid deprivation. Overall, the fact that addition of the antioxidant NAC completely inhibited the increase in HSPA1A and DNAJB1 mRNA levels in methionine starved cells indicates that this increase is due to oxidative stress caused by the lack of methionine. Indeed we do find an increase in HMOX1 mRNA levels upon methionine deprivation (Fig. 4A), indicating activation of the antioxidant response.

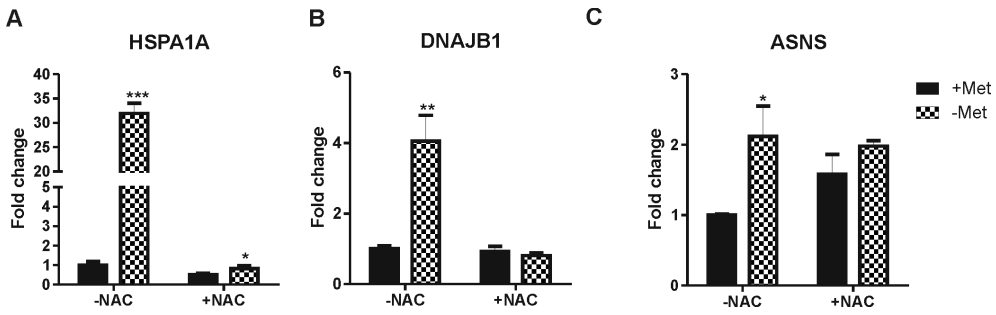


Fig. 3 N-acetylcysteine inhibits the increase in HSPA1A and DNAJB1 mRNA levels upon methionine starvation. A-C. HEK293 cells were deprived of methionine for 24 h in the presence or absence of 10 mM N-acetylcysteine (NAC). mRNA levels were determined by QPCR analysis and are shown relative to GAPDH mRNA levels. Error bars represent SD; *P<0.05; **P<0.01; ***P<0.001, relative to +Met

It has been shown previously that oxidizing agents can induce the transcription of heat shock protein genes via HSF1 activation [307-309]. The results described above, however, demonstrated an HSF1 independent increase in HSPA1A and DNAJB1 mRNA levels, which is most likely mediated by the activation of another transcription factor. An important player in the antioxidant response is NRF2 (nuclear factor (erythroid-derived 2)-related factor 2), a transcription factor that controls the expression of antioxidant

response element (ARE)-regulated antioxidant and cytoprotective genes [117,118], among which is HMOX1. Recently, it has been shown that in rat primary hepatocytes NRF2 is indeed activated upon methionine starvation [305]. Another candidate is the transcription factor AP1, as glutathione depletion has been shown to result in increased expression of c-Fos and FosB, both AP1 family members [310]. AP1 binds to the TRE (TGACTCA; 12-*O*-tetradecanoyl phorbol13-acetate (TPA)-responsive element) of which the sequence is identical to that of the core of the ARE, the NRF2 consensus binding sequence (ATGACTCAGCA). When we examined binding to the ARE in nuclear extracts from methionine starved cells in an EMSA, we found increased complex formation compared to fed cells (Fig. 4B). The signal of the complex binding to the ARE probe was decreased when we competed with a 10-fold molar excess of cold ARE oligo (Fig. 4B). These data show that indeed the activity of NRF2 and/or AP1 is increased in methionine deprived cells.

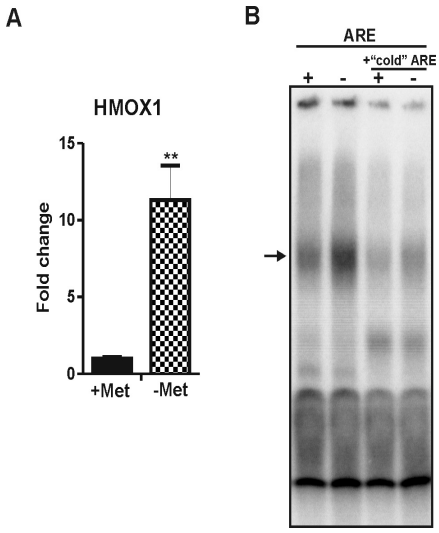


Fig. 4 Increased DNA binding to the ARE sequence in methionine starved cells. A. HEK293 cells were deprived of methionine for 24 h. HMOX1 mRNA levels were determined by QPCR analysis and are shown relative to GAPDH mRNA levels. Error bars represent SD; **P<0.01, relative to +Met. **B.** HEK293 cells were deprived of methionine for 24 h. Nuclear extracts were used in an electrophoretic mobility shift assay with a double-stranded oligonucleotide with the ARE sequence. A 10-fold molar excess of unlabeled ARE probe ("cold") was added to determine the specificity of the signal. The arrow indicates the ARE complexes formed.

To look further into possible roles for AP1 and NRF2 in the increased HSP mRNA levels in methionine deprived cells, we knocked down c-Fos, FosB, or NRF2 mRNA and examined the effect on HSPA1A and DNAJB1 mRNA levels. Methionine deprivation indeed led to a 10- to 15-fold increase in c-Fos and FosB mRNA levels (Fig. S3A, B). Curiously, knocking down c-Fos mRNA resulted in an even larger increase in the FosB mRNA level in methionine deprived cells (Fig. S3B). Conversely, knocking down FosB mRNA enhanced the level of c-Fos mRNA (Fig. S3A). HSPA1A and DNAJB1 transcript levels remained unaffected when either c-Fos or FosB mRNA was knocked

down (Fig. S3C and D). These data suggest that c-Fos and FosB are not responsible for the increase in HSPA1A or DNAJB1 mRNA levels upon methionine deprivation, although we cannot rigorously exclude that the lack of c-Fos is compensated for by enhanced synthesis of FosB or vice versa. The level of NRF2 mRNA increased only slightly in methionine deprived cells (Fig. S4). The activity of NRF2 is regulated post-translationally by the cysteine rich protein KEAP1 (Kelch-like ECH associated protein 1), which acts as a sensor for oxidative stress. Under non-stress conditions KEAP1 retains NRF2 in the cytoplasm [119] and maintains it at a low level through KEAP1-dependent ubiquitination and proteasomal degradation [120-124]. Upon oxidative stress, cysteines in the KEAP1 protein are oxidatively modified, resulting in a conformational change and release of NRF2 [121]. When we depleted NRF2 mRNA by siRNA the increase in mRNA levels of its target gene HMOX1 upon methionine starvation was inhibited by about 50% (Fig. 5A). The increase in HSPA1A mRNA levels was also inhibited by about 50% upon siRNA treatment for NRF2 (Fig. 5B), whereas the increase in DNAJB1 mRNA levels (Fig. 5C) and ASNS mRNA levels (Fig. 5D) was not affected. These results were confirmed using a second NRF2 siRNA (Fig. S5). We did not detect

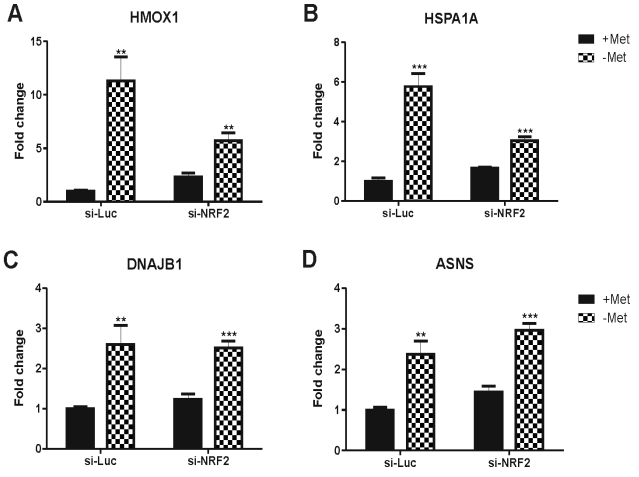


Fig. 5 Transcript levels in NRF2 siRNA treated, methionine deprived cells. A-D. HEK293 cells were transfected for 96 h with siRNA against NRF2 or luciferase as a control with a re-transfection at 48 h. 24 h after re-transfection cells were deprived of methionine for 24 h. mRNA levels were determined by QPCR analysis and are shown relative to GAPDH mRNA levels. Error bars represent SD; **P<0.01; ***P<0.001, relative to +Met.

a change in HSPA1A mRNA stability in methionine deprived cells either in the presence or absence of NRF2 mRNA (data not shown), unlike in leucine or glutamine starved cells where HSPA1A mRNA is destabilized [264,285]. These data thus suggest that the increase in HSPA1A mRNA level is due to NRF2 directed enhanced transcription. Almeida et al. [127] described the presence of an electrophile-responsive element (EpRE), to which NRF2 can bind, in the promoter of the Hsp70 gene in zebrafish and this element was conserved between mouse and zebrafish. We were unable to detect an

involvement of NRF2 in the increase in DNAJB1 mRNA levels in methionine deprived cells, even though the murine Dnajb1 gene has been reported to be a target of Nrf2 [125,126]. The delay in the increase in DNAJB1 mRNA suggests that this increase may be a secondary effect. Apparently, in methionine restricted cells there is a difference between the regulation of transcription of the HSPA1A and DNAJB1 promoter. In contrast to HSPA1A, DNAJB1 is present in high levels in non-stressed cells and not much is known about the regulatory elements of the promoter of the DNAJB1 gene.

Our results show that methionine deprivation results in increased HSPA1A and DNAJB1 mRNA levels, in spite of the inactivation of HSF1, probably due to the activation of the antioxidant response simultaneously with the amino acid deprivation response. The increase in HSP mRNA levels is relatively low compared to that in heat stressed cells and it is not clear whether the increase in the level of the resulting gene products, the heat shock proteins, is sufficient to protect the cell from proteotoxic stress resulting from the change in cellular oxidative status. It could well be that the products of other NRF2 target genes are of more importance in dealing with the damage resulting from the oxidative insult. As described previously [264], leucine starvation results in the inactivation of HSF1 and a subsequent decrease in HSP mRNA levels, which would lead to a loss in chaperoning capacity and enhanced sensitivity to proteotoxic stress, i.e. cellular frailty. Starvation for methionine does also result in the inactivation of HSF1, but heat shock mRNA levels do not decrease, suggesting that methionine deprived cells remain robust.

Our studies were performed using methionine starved tissue culture cells and our results may not apply directly to methionine restriction, which has been shown to increase lifespan in rodent studies [311-314]. The beneficial effects of methionine restriction are likely to be mediated in part by tissue-specific effects on the transcriptome, as in the case of caloric restriction [315] and by systemic factors, such as a lower IGF-1 level [313,316]. Our results do suggest that in methionine restricted cells the antioxidant response is primed and can be quickly called into action to prevent deleterious effects of a complete lack of methionine. However, the loss of HSF1 activity in methionine deprived cells would be predicted to affect longevity adversely [222], but this is perhaps compensated by the increase in the level of at least some heat shock proteins. At least in C. elegans, the loss of HSF1 can be compensated in part by exogenous expression of small heat shock proteins [159]. Intriguingly, methionine restriction also delays tumor growth (for review, see [317]). Malignant cells are dependent upon an HSF1 directed transcriptional program that is distinct from the HSF1 directed heat shock response [21]. It would be of interest to determine whether loss of HSF1 activity in methionine deprived tumor cells plays a role in the inhibitory effect of methionine restriction on tumor growth.

Acknowledgements
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Supplemental Materials and Methods

Western blot analysis
Cells were harvested in lysis buffer [25 mM Tris-HCl pH 7.5, 100 mM KCl, 1 mM DTE, 2 mM EDTA, 0.5 mM PMSF, 0.05% NP-40, 1X PhosSTOP (Roche), 1X protease inhibitor cocktail (Complete Mini, Roche)] and protein concentration was determined using a Bradford protein assay (Bio-Rad). For analysis of cytoplasmic and nuclear fractions, extracts were prepared using NE-per nuclear and cytoplasmic reagents (Pierce). Next, 4x sample buffer (200 mM Tris-HCl pH 6.8, 20% β-mercaptoethanol, 8% SDS, 40% glycerol and 0.4% bromophenol blue) was added and the lysates were incubated at 95°C for 5 minutes. Protein samples were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose transfer membrane (Protran). For western blot analysis, the following antibodies were used: mouse monoclonal β-actin antibody (AC-15; Sigma; 1:5000), rabbit polyclonal HSF1 antibody (SPA-901; Stressgen; 1:1000), rabbit polyclonal DNAJB1 antibody (anti-Hsp40; SPA-400; Stressgen; 1:10000), and mouse monoclonal Hsp70 antibody 4G4 (ab5444; Abcam; 1:5000). Next, blots were incubated with fluorescent secondary antibodies IRDye® 800CW conjugate goat anti-rabbit IgG and IRDye® 680 conjugated goat anti-mouse IgG (926-32211 and 926-32220 respectively; LI-COR Biosciensces) according to the manufacturer's instructions and scanned using a LI-COR Odyssey infrared scanner.

Supplemental table

Table S1. Oligonucleotides

	Name	Sequence (5'-->3')
ChIP	ASNS_fwd	tggttggtcctcgcaggcat
	ASNS_rev	cgcttataccgacctggctcct
	DNAJB1_fwd	ggatgtcgcgtgtcgctgaa
	DNAJB1_ rev	cgaccagtcccggactctata
QPCR	GAPDH_fwd	ttccccatggtgtctgagc
	GAPDH_rev	atcttcttttgcgtcgccag
	ASNS_fwd	gcagctgaaagaagcccaagt

	ASNS_rev	tgtcttccatgccaattgca
	HSPA1A_fwd	ccgagaaggacgagtttgag
	HSPA1A_rev	acaaaaacagcaatcttggaaagg
	DNAJB1_fwd	ttccccagacatcaagaacc
	DNAJB1_rev	accctctcatggtccacaac
	cFos_fwd	ctactaccactcacccgcagac
	cFos_rev	ggaatgaagttggcactggag
	FosB_fwd	ctcggcctaggtcacgtt
	FosB_rev	gccagagtttctagaagcagttt
	NRF2_fwd	agacggtatgcaacaggac
	NRF2_rev	cttctggacttggaaccatg
	HMOX1_fwd	ctgtctcaaacctccaaaagcc
	HMOX1_rev	tcaaaaaccaccccaaccc
EMSA	NSRU_fwd	gcaggcatgatgaaacttcccgcacgcgttacaggagccag
	NSRU_rev	ctggctcctgtaacgcgtgcgggaagtttcatcatgcctgc
	HSE_fwd	ctattctcgttgcttcgagagagcgcgcctcgaatgttcgcgaaaagag
	HSE_rev	ctcttttcgcgaacattcgaggcgcgctctctcgaagcaacgagaatag
	ARE_fwd	cggaatgtatgactcagcattactc
	ARE_rev	gagtaatgctgagtcatacattccg

Supplemental figures

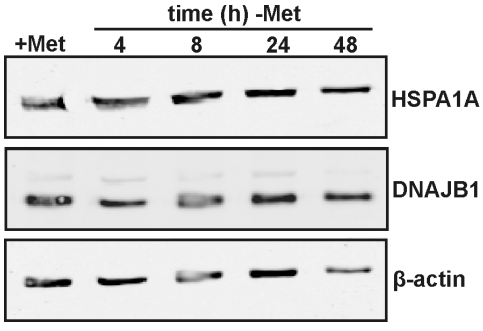


Fig. S1 HSPA1A and DNAJB1 protein expression levels upon methionine deprivation. HEK293 cells were deprived of methionine and harvested at the indicated time points. Cell lysates were subjected to SDS-PAGE and levels of HSPA1A and DNAJB1 were determined by western blotting. β-actin was used as a loading control.

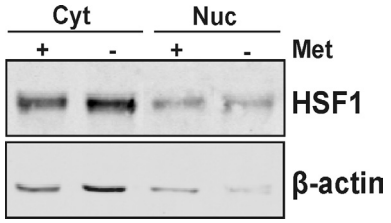


Fig. S2 Expression and localisation of HSF1 upon methionine deprivation. HEK293 cells were deprived of methionine for 24 h. Cytoplasmic (cyt) and nuclear (nuc) extracts were made and subjected to SDS-PAGE and western blot analysis using an anti-HSF1 antibody. β-actin was used as a control. Samples are all from the same gel, but due to clarity reasons, some lanes are omitted.

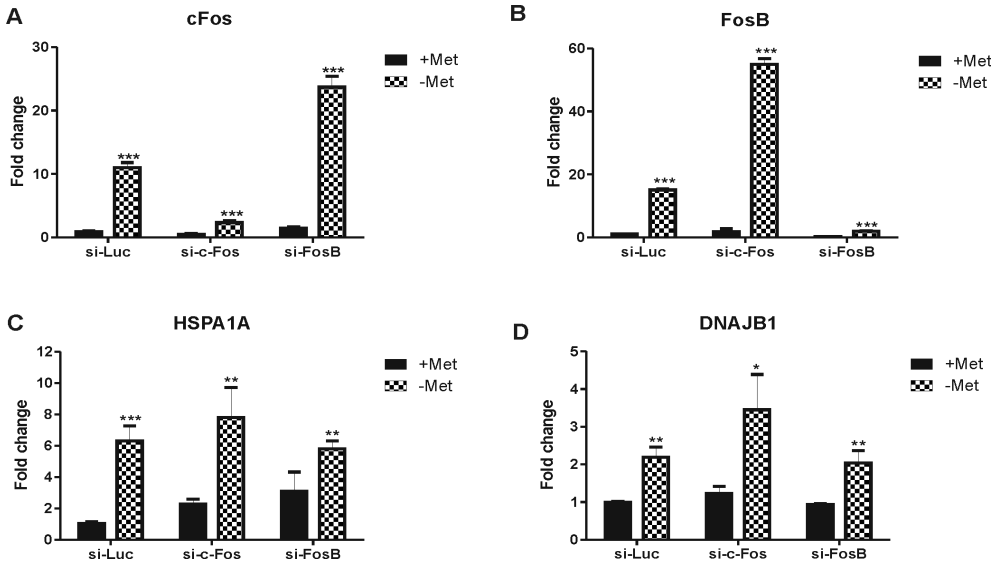


Fig. S3 Transcript levels in c-Fos and FosB siRNA treated, methionine deprived cells. A-D. HEK293 cells were transfected for 96 h with siRNA against c-Fos, FosB or luciferase as a control with a re-transfection at 48 h. 24 h after re-transfection cells were deprived of methionine for 24 h. mRNA levels were determined by QPCR analysis and are shown relative to GAPDH mRNA levels. Error bars represent SD; *P<0.05; **P<0.01; ***P<0.001, relative to +Met.

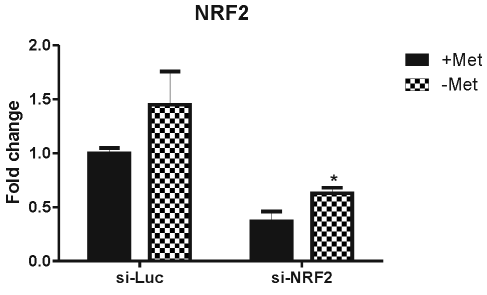


Fig. S4 Knockdown of NRF2 in siRNA treated, methionine deprived cells. HEK293 cells were transfected for 96 h with siRNA against NRF2 or luciferase as a control with a re-transfection at 48 h. 24 h after re-transfection cells were deprived of methionine for 24 h. mRNA levels were determined by QPCR analysis and are shown relative to GAPDH mRNA levels. Error bars represent SD; *P<0.05, relative to +Met.

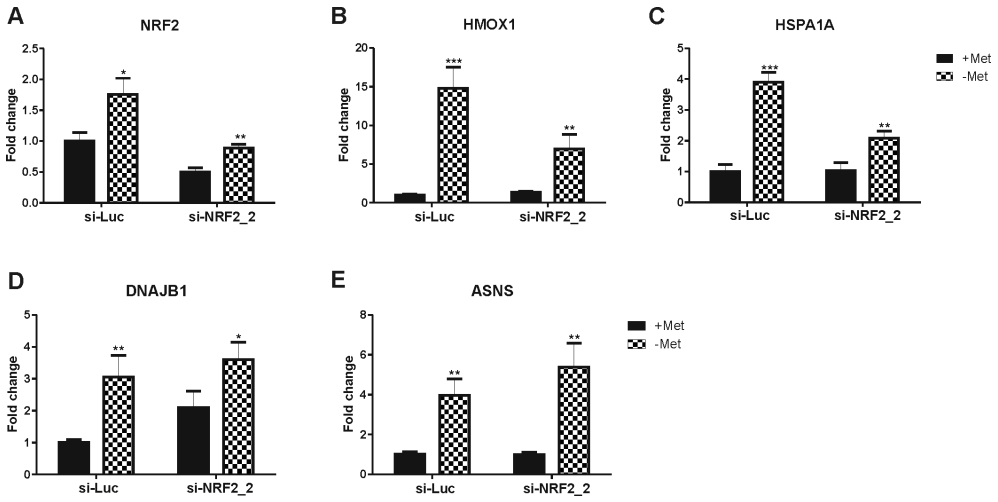


Fig. S5 Transcript levels in methionine deprived cells treated with a second NRF2 siRNA. A-E. HEK293 cells were transfected for 96 h with siRNA#2 against NRF2 or luciferase as a control with a re-transfection at 48 h. 24 h after re-transfection cells were deprived of methionine for 24 h. mRNA levels were determined by QPCR analysis and are shown relative to GAPDH mRNA levels. Error bars represent SD; *P<0.05; **P<0.01; ***P<0.001, relative to +Met.

Chapter 7

Transcriptome changes in HEK293 cells expressing αB-crystallin or HSPB1

Sanne M.M. Hensen, Lonneke Heldens, Siebe T. van Genesen, Nicolette H. Lubsen

The small heat shock proteins αB-crystallin and HSPB1 have been shown to be cytoprotective and are implicated in diseases such as cancer and neurodegenerative diseases. How expression of these proteins do protect the cell is largely unknown. We therefore created T-REx HEK293 cell lines with inducible expression of either one of these proteins and evaluated the transcriptome changes in these cells upon expression. Expression of αB-crystallin affected the level of a large number of transcripts, whereas the effect of HSPB1 expression was rather small. An independently constructed second αB-crystallin T-REx HEK293 cell line showed less severe effects, indicating that different cell lines stably expressing the same protein can have different properties. Both αB-crystallin cell lines did show higher transcript levels of stress induced genes compared to control cells or cells expressing HSPB1 and overexpression of αB-crystallin thus may be unfavourable to a cell.

Introduction

The human small heat shock protein (shsp) family consists of ten different members (HSPB1-10) [76], which are characterized by low molecular masses and a common C-terminal motif, the so-called α-crystallin domain. HSPB1 (Hsp27) and αB-crystallin (HSPB5) have been the most studied small heat shock proteins thus far and are implicated in several disease processes. Both proteins have, for example, been shown to protect against the accumulation of improperly folded proteins in the nervous system in patients suffering from neurodegenerative diseases by preventing intracellular protein aggregation (reviewed in [75]). In addition to the involvement of HSPB1 and αB-crystallin in neurodegenerative diseases, these proteins have also been described to be present in several tumours. Increased HSPB1 expression has been detected in cancers such as breast, ovarian and gastric cancer (reviewed in [318]) and high αB-crystallin levels have been found in, for example, gliomas [319], renal cell carcinomas [320], head and neck cancer, and prostate cancer and these high HSPB1 and αB-crystallin levels mostly correlate with poor prognosis [321,322].

An important property of HSPB1 and αB-crystallin is their protective effect in cells. They are involved in multiple cellular processes and signalling pathways. They have, for example, been described to interact with the cytoskeleton and they appear to play an important role in maintaining the integrity of intermediary and actin filaments ([323], for review see [324]). Accumulating evidence shows that these shsps prevent cytoskeleton aggregation under stress conditions. In addition, HSPB1 has been described to bind to the protein kinase AKT [325,326], a kinase that plays a role in several cellular processes, such as apoptosis and cell proliferation, and the interaction of AKT with HSPB1 was

shown to be necessary for AKT activation under stress conditions. On the other hand, AKT is able to phosphorylate HSPB1, resulting in the disruption of its interaction with HSPB1 [325]. HSPB1 and αB-crystallin are also involved in maintaining the redox balance in a cell by upholding glutathione levels when the ROS production is elevated [327,328]. Furthermore, both HSPB1 and αB-crystallin have been reported to modulate NF-κB activity. αB-crystallin could activate NF-κB, resulting in the protection of muscle myoblasts from TNFα induced cytotoxicity [329]. HSPB1 stimulated the degradation of IκBα and could thereby increase the intracellular level of NF-kB [330]. αB-crystallin has also been reported to be involved in p53 signalling; however these studies are contradictory. Jin et al [331] reported a role for αB-crystallin in p53 degradation and mouse embryonic fibroblasts deficient for αB-crystallin showed elevated levels of p53. On the other hand, Watanabe et al. [332] showed that repression of the αB-crystallin level decreased p53 protein levels in human osteosarcoma cell lines.

Most studies on the effect of either αB-crystallin or HSPB1 reported in the literature use cell lines continuously expressing the shsp investigated. This approach has the disadvantage that cells may adapt their proteome to the expression of the shsp and the effect of the shsp detected could be a secondary rather than a primary effect. In addition, stable cell lines may differ in integration site of the expression construct, with possible consequences for the phenotype unrelated to shsp expression. In principle, these problems can be circumvented by using the Flp-In T-REx system. The site-specific recombination should ensure that the expression construct is always inserted at the same genomic site. The expression of the insert is inducible by adding doxycycline and thus isogenic lines with and without expression can be compared. To investigate how cells respond to the expression of either HSPB1 or αB-crystallin we generated Flp-In T-REx HEK293 cell lines stably transfected with an expression construct for either one of these proteins. To analyze the transcriptome changes upon expression of αB-crystallin or HSPB1 we performed a microarray screen and, surprisingly, found a large difference between the response to overexpression of these proteins. While HSPB1 overexpression had only a small effect on the cells, αB-crystallin expression affected a large number of transcripts. Cells overexpressing αB-crystallin contained higher levels of stress markers than control cells or cells overexpressing HSPB1, suggesting that overexpression of αB-crystallin might be stressful for a cell.

Materials and Methods

Tissue culture

Flp-In T-REx-293 cells (Invitrogen) were manipulated according to the manufacturer's

instructions using the T-REx system (Invitrogen) to generate the stable cell lines HEK-αB-crystallin and HEK-HSPB1 that carry a single copy of the tetracycline-inducible plasmids pcDNA5-αB-crystallin and pcDNA5-HaHsp27 (hamster Hsp27), respectively. T-REx HEK293-pcDNA5 cells were generated as described before [265][18][265]. The cells were cultured at 37°C/5% CO_2 in high glucose DMEM medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Blasticidin (1.65 μg/ml; Invitrogen) and 100 μg/ml hygromycin were also added to the culture medium during maintenance of the cell lines, but were omitted during experiments.

Western blot analysis

Cells were harvested in lysis buffer [25 mM Tris-HCl pH 7.5, 100 mM KCl, 1 mM DTE, 2 mM EDTA, 0.5 mM PMSF, 0.05% NP-40, 1X PhosSTOP (Roche), 1X protease inhibitor cocktail (Complete Mini, Roche)] and protein concentration was determined using a Bradford protein assay (Bio-Rad). Next, 4x sample buffer (200 mM Tris-HCl pH 6.8, 20% β-mercaptoethanol, 8% SDS, 40% glycerol and 0.4% bromophenol blue) was added and the lysates were incubated at 95°C for 5 minutes. Protein samples were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose transfer membrane (Protran). For western blot analysis, the following antibodies were used: mouse monoclonal β-actin antibody (AC-15; Sigma; 1:5000), mouse monoclonal αB-crystallin antibody (1:100), rabbit polyclonal human Hsp28 antibody (1:400) and mouse monoclonal hamster Hsp25 antibody (1:1000). Next, blots were incubated with fluorescent secondary antibodies IRDye® 800CW conjugate goat anti-rabbit IgG and IRDye® 680 conjugated goat anti-mouse IgG (926-32211 and 926-32220 respectively; LI-COR Biosciensces) according to the manufacturer's instructions and scanned using a LI-COR Odyssey infrared scanner.

RNA isolation and microarray analysis

T-REx HEK293-pcDNA5, T-REx HEK293-αB-crystallin and T-REx HEK293-HSPB1 cells were cultured for 48 h in the presence of doxycycline. Total RNA was isolated using Trizol (Invitrogen) and copied into Cy3-labeled or Cy5-labeled cRNA using the Agilent Low RNA Input Linear Amp Kit PLUS (Agilent), or the reverse for the repeat array. Labeled cRNA samples were hybridized to an Agilent Whole Human Genome Microarray Kit (4 x 44K). The arrays were scanned using an Agilent Microarray Scanner. Image analysis and feature extraction were done with Feature Extraction (version 9.5.1, Agilent). We used an arbitrarily chosen signal cut-off of > 50.

Reverse transcription and quantitative real-time PCR

1 μg of RNA was treated with DNaseI (Amplification grade; RNase-free; Invitrogen).

Subsequently, 5 mM $MgCl_2$, RT-buffer, 1 mM dNTPs, 18.75 units AMV reverse transcriptase, 20 units RNase inhibitors and 1.25 μM oligo(dT) were added to a total volume of 20 μl. Reverse transcription was performed for 10 minutes at 25°C, 60 minutes at 42°C and 5 minutes at 95°C. For QPCR analysis, cDNA was 10-fold diluted. Quantitative real-time PCR was performed using the StepOnePlus™ Real-Time PCR System with *Power* SYBR® Green PCR Master mix (Applied Biosystems) using the following amplification protocol: 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Per reaction 3 μl of diluted cDNA was used and the DNA was amplified using primers for the sequences of interest, listed in Table 1.

Table 1. Oligonucleotides

Name	Sequence (5'-->3')
αB-crystallin_fwd	gattgaggtgcatggaaaac
αB-crystallin_rev	aggaccccatcagatgacag
GAPDH_fwd	ttccccatggtgtctgagc
GAPDH_rev	atcttcttttgcgtcgccag
GADD45B_fwd	gacctgcattgtctcctggtc
GADD45B_rev	cagcgttcctgaagagagatgta
GADD34_fwd	cgcttctggcagaccgaa
GADD34_rev	gtagcctgatggggtgcttg
NOV_fwd	ccagatgaggaggattcactgg
NOV_rev	gctgtccactctgtggtctgttc
Bax_QPCRfwd	tggagctgcagaggatgattg
Bax_QPCRrev	gaagttgccgtcagaaaacatg
HIST1H2BK_fwd	ctaagtaaacttgccaaggagg
HIST1H2BK_rev	gcagtagataatgaggtaaccgaag
HIST1H2B_fwd	acctccagggagatccagac
HIST1H2B_rev	ctggtgtacttggtgacggc
HIST1H3A_fwd	gaagtccactgaactgcttattcg
HIST1H3A_rev	ggatgtccttgggcatgatag
LAMB3_fwd	gaagatgtggttgggaacctg
LAMB3_rev	catccgtgtccagaagtcacc
AHR_QPCRfwd	atcacctacgccagtcgca
AHR_QPCRrev	ctctatgccgcttggaagga

Transfections and reporter gene assays

T-REx HEK293-pcDNA5, T-REx HEK293-αB-crystallin and T-REx HEK293-

HSPB1 cells were seeded on 24-well plates with or without doxycycline and on the next day transfected with 0.2 μg plasmid per well: 20 ng pCMV-β-galactosidase and 180 ng *Drosophila* Hsp70 promoter luciferase reporter plasmid using Fugene-6 (Roche). After 24 h cells were treated with the indicated concentrations of arsenite for 24 h and harvested for reporter gene analysis. Cells were lysed in 200 μl reporter lysis mix (25 mM Bicine, 0.05% Tween 20, 0.05% Tween 80) for 10 minutes. For the β-galactosidase assay, 10 μl cell lysate was mixed with 100 μl Galacton solution [100 mM Na-phosphate pH 8.2, 5 mM $MgCl_2$, 1% Galacton-Plus (Tropix)]. After 30 minutes incubation at room temperature, 150 μl accelerator II (Tropix) was added and luminescence was measured with the Lumat LB 9507 tube luminometer (Berthold). For the luciferase assay, 10 μl cell lysate was mixed with 50 μl luciferin solution and luminescence was again measured with the Lumat luminometer. All reporter gene assays were performed in triplicate. The activities of the reporter genes were corrected for transfection efficiency on basis of the β-galactosidase activity.

Results and Discussion

aB-crystallin overexpression increases HSPB1 protein stability

To investigate the effects of overexpression of HSPB1 or αB-crystallin, we created HEK293 cell lines stably transfected with the tetracycline-inducible plasmid pcDNA5-HSPB1 (hamster Hsp27), pcDNA5-αB-crystallin (human αB-crystallin) or empty vector. HEK293 cells have a low, but readily detectable, endogenous level of HSPB1. Upon induction of expression, the HSPB1 level increased markedly in the HEK-HSPB1cell line (Fig. 1A; note that the antibody used to detect human HSPB1 also recognizes hamster Hsp27). Endogenous αB-crystallin was not detectable in either the HEK-pcDNA or the HEK-HSPB1 cell line. We could also not detect αB-crystallin in the HEK-αB-crystallin cell line in the absence of doxycycline (see Fig. 1A), even though the levels of αB-crystallin mRNA were already 200-fold increased in the absence of doxycycline compared to αB-crystallin mRNA levels in the HEK-pcDNA cell line (Fig. 1B). Upon induction αB-crystallin mRNA levels increased another 40-fold and the protein product was now readily detectable on a western blot. Cells expressing high levels of αB-crystallin also had elevated HSPB1 protein levels (Fig. 1A and C). However, we did not find an effect on HSPB1 mRNA levels in our microarray data, suggesting that the HSPB1 protein becomes more stable upon expression of αB-crystallin. To measure the half-life of HSPB1, we treated HEK-pcDNA5 and HEK-αB-crystallin cells with cycloheximide to inhibit translation and harvested the cells at different time points. HSPB1 was indeed degraded more slowly when αB-crystallin was exogenously

expressed (Fig. 1D; tested in two different αB-crystallin cell lines), indicating that αB-crystallin stabilizes HSPB1 protein. Small heat shock proteins are known to be present in the cell as large oligomers and αB-crystallin might capture HSPB1 in hetero-complexes and thereby prevent degradation.

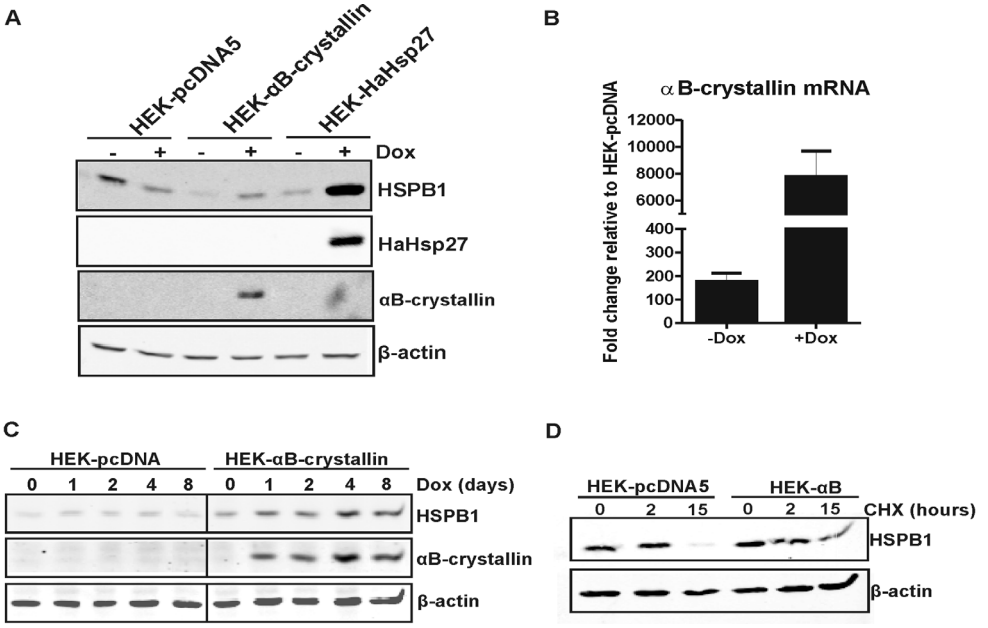


Fig. 1 αB-crystallin overexpression increases HSPB1 protein stability. A. HEK-pcDNA5, HEK-αB-crystallin and HEK-HSPB1 cells were cultured for 48 h in the presence or absence of doxycycline and extracts were subjected to SDS-PAGE and western blot analysis. **B.** HEK-αB-crystallin cells were cultured for 48 h in the absence or presence of doxycycline. Total RNA was isolated, treated with DNaseI and reversed transcribed using AMV-RT. cDNA was subjected to QPCR analysis using primers for αB-crystallin mRNA and for GAPDH mRNA as a control. Fold change is shown relative to αB-crystallin mRNA levels in HEK-pcDNA5 cells. Error bars represent SD. **C.** HEK-pcDNA5 and HEK-αB-crystallin cells were treated with doxycycline for the indicated times. Extracts were subjected to SDS-PAGE and western blot analysis. **D.** HEK-pcDNA5 and HEK-αB-crystallin cells were treated with doxycycline for 48 h and subsequently with 20 μg/ml cycloheximide to inhibition translation. Cells were harvested at the indicated time points after cycloheximide addition and extracts were subjected to SDS-PAGE and western blot analysis.

Transcriptome changes upon overexpression of aB-crystallin or HSPB1

To compare the transcriptomes of the HEK-HSPB1 and HEK-αB-crystallin cell lines with the transcriptome of the control cell line HEK-pcDNA5 we performed a microarray screen. Surprisingly, the effect of αB-crystallin overexpression was significantly larger

than the effect of HSPB1 overexpression. Upon αB-crystallin expression, the transcript levels of in total 127 genes were more than twofold affected (Fig. 2), whereas in the case of HSPB1 only 9 gene transcript levels were altered. αB-crystallin overexpression resulted in the upregulation of 56 genes, 5 of which were also upregulated by HSPB1: NOV, DHRS2, RPRM, TEP1 and RAB6IP2. The transcript level of 71 genes decreased

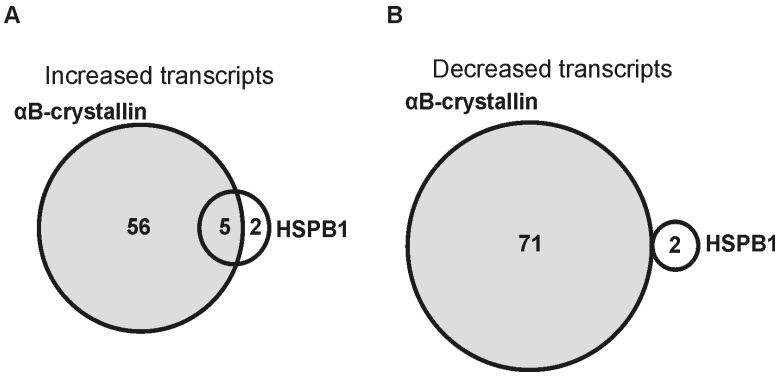


Fig. 2 Venn diagrams of the transcriptome changes in HEK293 cells expressing αB-crystallin and HSPB1. The number of genes of which the transcript level was increased (**A**) and the number of genes of which the transcript level was decreased (**B**) is shown for the overexpression of αB-crystallin and HSPB1.

upon αB-crystallin expression, none of which decreased in the HEK-HSPB1 cell line. Several of the affected genes are known as targets of the nuclear factor kappa B (NF-κB) [333-335]. When, for all NF-κB target genes, the number of genes was plotted against their fold induction upon overexpression of αB-crystallin or HSPB1, we found a normal distribution that slightly shifted to the right for αB-crystallin (Fig. 3A), indicating a small increase in transcript level for several of the NF-κB target genes, among which is growth arrest and DNA damage-inducible, beta (GADD45B). The fact that we found an effect on NF-κB signalling corresponds with the study describing that αB-crystallin modulates NF-κB activity [329]. Overexpression of HSPB1 also resulted in a slight overall increase in the transcript levels of NF-κB target genes, but this shift was less significant than that seen in αB-crystallin expressing cells.

Additionally, we found a significant effect of overexpression of αB-crystallin ($p<0.001$) on targets of the transcription factor and tumour suppressor protein p53 [332,336-338]. When, for all p53 target genes, the number of genes was plotted against their fold induction, the plot for αB-crystallin overexpressing cells showed two peaks (Fig. 3B): a peak with p53 target genes of which transcript levels decrease upon αB-crystallin

overexpression, and a peak with p53 target genes of which transcript levels increase upon αB-crystallin overexpression. For cells overexpressing HSPB1 this effect was not observed. As described above, expression of αB-crystallin has been implicated in p53 signalling before, but results were ambiguous [331,332]. Our study shows a differential effect on p53 target genes; of some the transcript levels increase whereas of others they decrease (Fig. 3B).

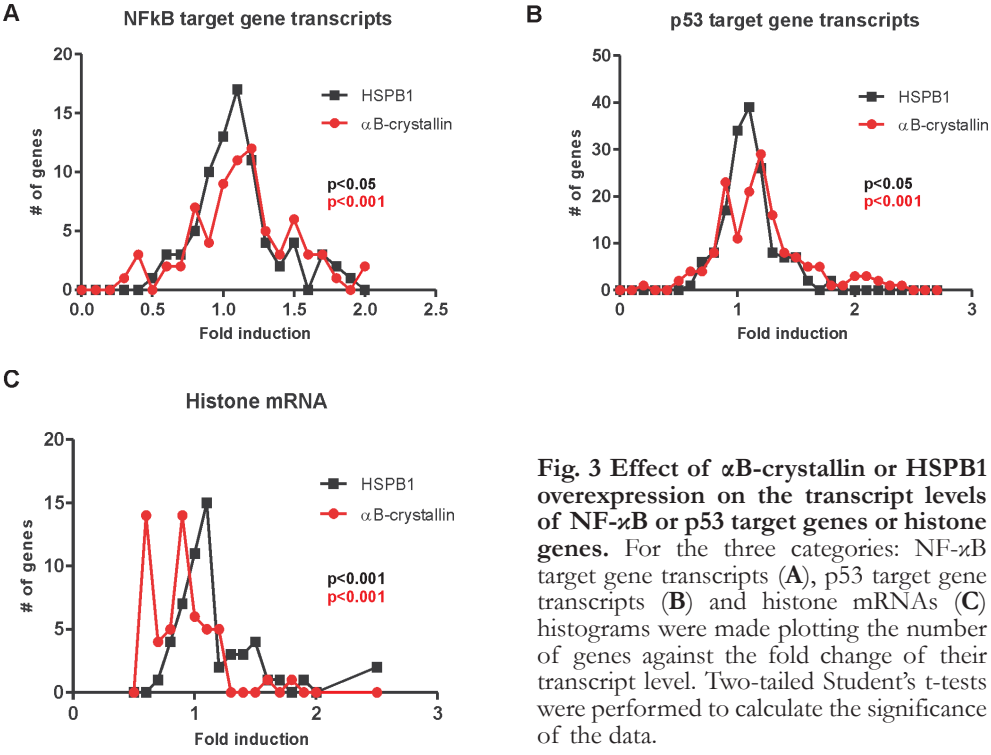


Fig. 3 Effect of αB-crystallin or HSPB1 overexpression on the transcript levels of NF-κB or p53 target genes or histone genes. For the three categories: NF-κB target gene transcripts (**A**), p53 target gene transcripts (**B**) and histone mRNAs (**C**) histograms were made plotting the number of genes against the fold change of their transcript level. Two-tailed Student's t-tests were performed to calculate the significance of the data.

A group of genes of which expression was also significantly affected by the overexpression of αB-crystallin ($p<0.001$) and HSPB1 ($p<0.001$) was the group of the histone genes. On average, overexpression of HSPB1 led to slightly increased histone mRNA levels. Conversely, overexpression of αB-crystallin led to a decrease in a subset of histone mRNA levels. When we looked more specifically at the transcripts of the various subgroups of the histone genes, we found two distinct peaks (Fig. 3C). The peak that was most decreased upon αB-crystallin expression mainly consisted of transcripts of the genes from the histone H2B family, indicating a role for αB-crystallin in the regulation of expression of this histone family. It has previously been described

that αB-crystallin is involved in maintaining the cellular redox balance [328] and that a proper NAD+/NADH redox status is required for optimal histone H2B expression [339].

Validation of the microarray data

Because the effect of overexpression of αB-crystallin was so large we felt that validation in a second, independently created HEK293 cell line stably transfected with an αB-crystallin expression construct was needed and thus isolated a second line. There was no significant difference in αB-crystallin mRNA levels between the two cell lines (compare Figs. 1B and 4A). Next, we analyzed some of the transcripts of which the levels were changed in the first αB-crystallin cell line and investigated whether there were differences in expression patterns between the different cell lines.

When we measured GADD45B mRNA levels by QPCR, these were 4 to 5 fold increased in both αB-crystallin cell lines (Fig. 4B), while overexpression of HSPB1 increased GADD45B mRNA levels by only 1.5-fold. GADD45B is known as a general stress marker of the cell [340]. To determine whether cells overexpressing αB-crystallin have a higher level of other stress induced transcripts as well, we also measured the mRNA levels of another well-known stress marker, growth arrest and DNA damage-inducible 34 (PPP1R15A/GADD34). Indeed PPP1R15A mRNA levels were also elevated in cells overexpressing αB-crystallin, although a strong difference between the two different lines was observed (Fig. 4C).

QPCR showed that mRNA levels of the p53 target gene NOV strongly increased upon either overexpression of αB-crystallin (8-fold and 10-fold in cell line 1 and 2 respectively) or HSPB1 (5-fold; Fig. 4D), and this validates our microarray data. BAX mRNA levels were 3-fold increased upon overexpression of αB-crystallin in cell line 1, whereas these were only slightly increased in cell line 2 or upon overexpression of HSPB1 (Fig. 4E).

Next, we measured histone H2BK mRNA levels by QPCR and found a decrease upon αB-crystallin overexpression in cell line 1, but not in cell line 2 (Fig. 3F). When we used primers recognizing 9 members of the HIST1H2B family and used H3A mRNA levels to correct for cell cycle dependence, we found strongly decreased levels upon overexpression of αB-crystallin in cell line 1 and a slight decrease in cell line 2 and upon overexpression of HSPB1 (Fig. 4G). The effect on histone mRNA levels thus seems to be specific for the first αB-crystallin cell line, and not specific for αB-crystallin overexpression.

Finally, we also measured the mRNA levels of the basement membrane protein LAMB3 (laminin beta 3) and AHR (aryl hydrocarbon receptor) in all cell lines. LAMB3 was strongly increased in the first αB-crystallin cell line (18-fold); in the second cell line only a 4-fold induction was detected (Fig. 4H). For AHR, the second cell line showed

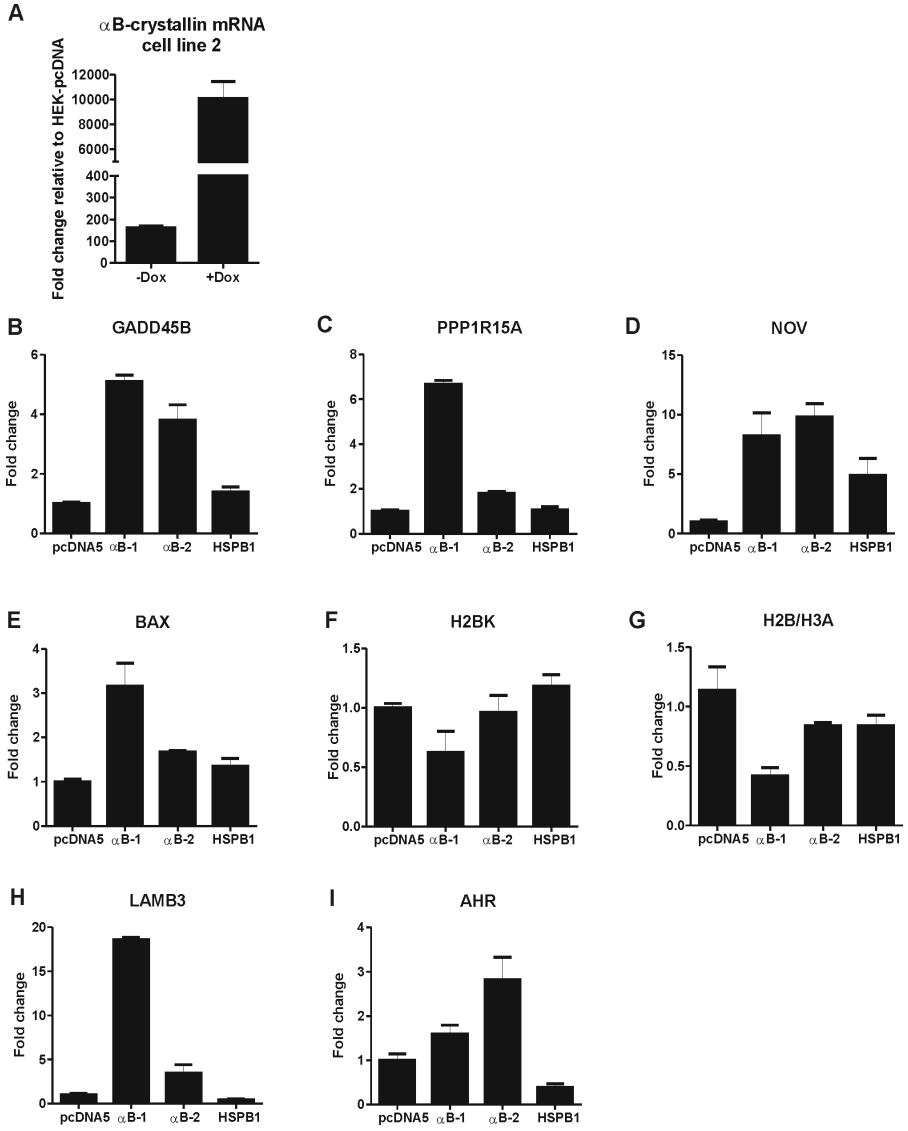


Fig. 4 Differences in gene expression between different stable αB-crystallin cell lines. A. HEK-αB-crystallin cell line 2 was cultured for 48 h in the absence or presence of doxycycline. Total RNA was isolated, treated with DNaseI and reversed transcribed using AMV-RT. cDNA was subjected to QPCR analysis using primers for αB-crystallin mRNA and for GAPDH mRNA as a control. Fold change is shown relative to αB-crystallin mRNA levels in HEK-pcDNA5 cells. **B-I.** HEK-pcDNA5, HEK-αB-1, HEK-αB-2 and HEK-HSPB1 cells were cultured for 48 h in the presence of doxycycline. Total RNA was isolated, treated with DNaseI and reversed transcribed using AMV-RT. cDNA was subjected to QPCR analysis using primers for the indicated transcripts and GAPDH mRNA was used as a control. Fold change is shown relative to HEK-pcDNA5. Primer sequences are listed in Table 1. Error bars represent SD.

a higher increase in mRNA levels (Fig. 4I). Even though we used the FlpIN T-Rex system for stable transfections, allowing integration of the gene at a specific genomic location, we observed a large difference between the two αB-crystallin cell lines. Our second αB-crystallin cell line showed less severe effects on several transcript levels, but according to the elevated GADD45B and GADD34 mRNA levels in both cell lines, αB-crystallin overexpression does seems to result in increased transcript levels of stress induced genes.

Overexpression of aB-crystallin or HSPB1 does not change the sensitivity to arsenite stress

Because αB-crystallin and HSPB1 have been shown to be cytoprotective, we wanted to determine whether cells overexpressing these proteins are indeed more resistant to a stress that induces the heat shock response. To measure the heat shock response we used a luciferase reporter plasmid containing the *Drosophila* Hsp70 promoter, which is highly inducible upon heat or arsenite stress through the activation of heat shock factor 1 (HSF1). We measured the activity of this reporter construct in HEK-pcDNA5, HEK-αB-crystallin or HEK-HSPB1 cells treated with low concentrations of arsenite in the absence or presence of doxycycline. HEK-pcDNA cells only responded slightly to arsenite, and there was, as expected, no difference in response when these cells were cultured in the absence or presence of doxycycline. HEK-αB-crystallin or HSPB1 cells showed, respectively, a 4 or 10-fold higher induction of the *Dropsophila* Hsp70 promoter at higher arsenite concentrations compared to HEK-pcDNA cells (Fig. 5); however, the difference between cells cultured in the absence or presence of doxycycline was small. The higher sensitivity to arsenite

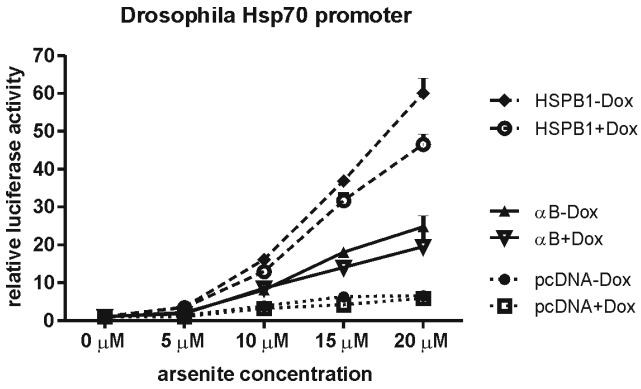


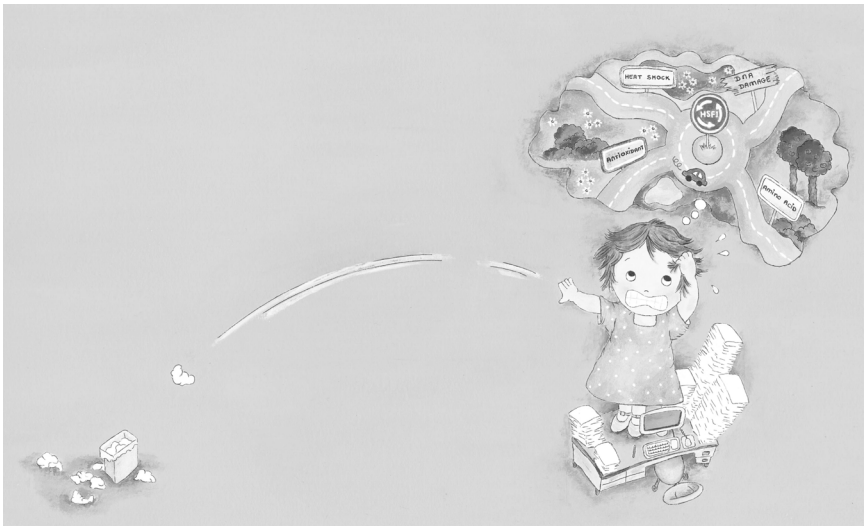
Fig. 5 Arsenite sensitivity of HEK-αB-crystallin, HEK-HSPB1 or pcDNA5 cells cultured with or without doxycycline. HEK-pcDNA5, HEK-αB-crystallin and HEK-HSPB1 cells were cultured in the presence or absence of doxycycline (+/- Dox) and transfected with the *Drosophila* Hsp70 promoter luciferase reporter plasmid. After 24 h cells were treated with the indicated concentrations of arsenite for 24 h and harvested for reporter gene analysis. All reporter gene assays were performed in triplicate. The activities of the reporter genes were corrected for transfection efficiency on basis of the β-galactosidase activity. Error bars represent SD.

stress of the HEK-αB-crystallin or HSPB1 cells relative to that of the HEK-pcDNA line is thus independent of the expression of either αB-crystallin or HSPB1 and must be due to some other property of these lines.

In conclusion, many studies describe protective effects for αB-crystallin or HSPB1 in cells. Our results show that the effect of overexpression of HSPB1 on the transcriptome of HEK293 cells is small, and it thus seems that a protective effect of HSPB1 is not exerted via the regulation of expression of specific transcripts. It must be kept in mind, however, that HEK293 cells already do express HSPB1 and the effect of overexpression may thus be less than in the case of αB-crystallin, which could not be detected in HEK293 cells. Overexpression of αB-crystallin results in increased transcript levels of genes encoding stress markers, which could indicate that cells are stressed. αB-crystallin is never the only shps present in a cell and may be an obligate heteromer in vivo. It is possible that not enough of its small heat shock protein partners are present in the cell to form these heteromers. Thus, in contrast with other studies reporting a protective effect for αB-crystallin, this study suggests that it might not always be beneficial for a cell to have high expression levels of αB-crystallin. Our studies also point to the pitfalls of using tissue culture cells to test for the effect of overexpression of a protein. Even using a well controlled system such as the Flp-In T-REx system, we find significant differences between what should be isogenic cell lines.

Chapter 8

Summary and Discussion



The discovery of the heat shock response by Feruccio Ritossa in 1962 was a nice example of how serendipity has eventually led to great insights in how cells cope with stressful conditions. When cells sense a particular kind of stress they try to adjust their cellular program to survive the unfavourable situation. Stresses like heat, UV or infection cause the mis- and unfolding of proteins, resulting in an imbalanced protein homeostasis. The heat shock response is an important cellular stress system involved in restoring and maintaining the protein balance in the cell by the production of additional chaperones that can help refolding proteins or, when irreparable, target them for degradation. When the heat shock system is impaired unfolded proteins tend to aggregate, which can eventually result in the development of protein folding diseases, e.g. Alzheimer's and Parkinson's disease. It is thus of great importance that a cell possesses a well functioning heat shock system. The main transcription factor responsible for activating transcription of the heat shock protein genes is heat shock factor 1 (HSF1), and decreased activity of HSF1 has been associated with aging [156,157]. In this thesis we used cellular model systems that mimic the age-related decline of HSF1 activity to investigate the critical nodes of the heat shock system and its interaction with other cellular stress systems.

Impaired HSF1 function in the absence of stress

The main function of HSF1 is activating transcription of the heat shock protein genes upon proteotoxic stress. However, accumulating evidence suggests that HSF1 also plays a role in non-stressed cells. For example, HSF1 has been described to be required for embryogenesis [210] and HSF1 knockout mice were shown to have a severely impaired immune response [18]. HSF1 has also been implicated in the circadian rhythm [19,20] and very recently it was described that HSF1 drives a transcriptional program to support highly malignant cells, a program distinct from that initiated upon heat stress [21]. Impaired HSF1 function could thus not only have an effect on stressed cells, but could also have physiological implications for unstressed cells.

In aging cells, the activity of HSF1 decreases, although the protein is still present [156-158,341]. To assess the consequences of this decreased HSF1 activity in aging cells, we created two cellular model systems in which the HSF1 protein was still present but unable to activate transcription of the heat shock protein genes. We used HEK293 cells stably transfected with an HSF1 mutant of which the expression is tetracycline inducible. The first mutant we used was dnHSF1 or HSF379 (Fig. 1, second panel), which lacks the C-terminal activation domain and thus cannot activate transcription of the heat shock protein genes. The other mutant we used, HSF1 K80Q (Fig. 1, third

panel), has a mutation of a lysine to a glutamine in its DNA binding domain, which results in a loss of DNA binding activity and transcription cannot be activated. This mutant more closely mimics the decreased HSF1 activity of the aging cell, as upon aging HSF1 loses its DNA binding affinity.

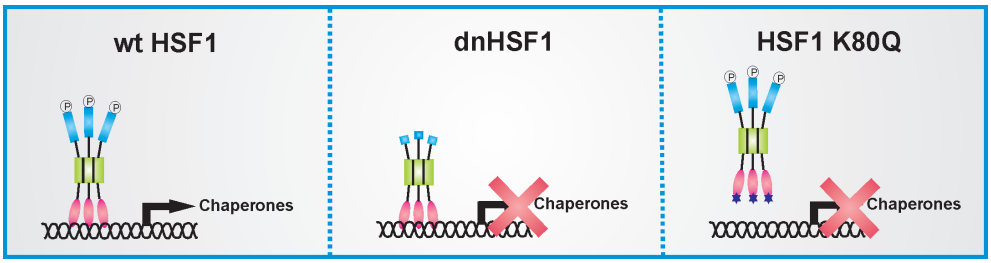


Fig. 1 HSF1 mutants. The dnHSF1 (second panel) lacks the activation domain and is thus unable to activate transcription. HSF1 K80Q (third panel) is mutated in the DNA binding domain and can therefore not bind the DNA.

Chapter 2 describes the transcriptome changes in the absence of stress when the dnHSF1 was expressed. We found only 10 genes of which the transcript level was more than twofold decreased in HEK-dnHSF1 cells compared to control cells and no genes were found to have increased transcript levels. Among the transcripts of which the level was decreased were four canonical HSF1 target genes: DNAJB1, HSPA6, HSP90AA1 and HSPB1. In **chapter 4** we investigated the effect of overexpression of HSF1 K80Q on the transcriptome in the absence of stress. The transcript levels of 18 genes were significantly decreased in HSF1 K80Q expressing cells and those of 8 genes were found to be increased. None of the genes of which the expression level changed in HSF1 K80Q cells was a canonical heat shock gene and the expression of none of these genes was also altered in dnHSF1 cells. This difference might be explained by the fact that dnHSF1 binds the heat shock gene promoters and blocks the binding of possible other transcription factors to the promoter regions, while overexpression of HSF1 K80Q leaves the promoter regions free, as it cannot bind the DNA.

When we compared the HSF1 K80Q data with the published data for HSF1 siRNA in HeLa cells [237], we found only one gene of which the level changed significantly under non-stress conditions in HEK-HSF1 K80Q cells and in HSF1 siRNA treated HeLa cells. The large difference between the changes in transcript levels in unstressed cells HSF1 siRNA treated HeLa cells and HEK293 cells expressing HSF1 K80Q could be due to cell specific factors. Another possibility is that the chaperones that normally interact with HSF1 under non-stress conditions are released when cells are depleted of HSF1, while the overexpression of a HSF1 mutant might capture more chaperones.

Furthermore, HSF1 could possibly also exert functions in which the DNA binding domain is not required. When cells are depleted of HSF1, these functions are also impaired, while HSF1 K80Q overexpression would not affect these.
Overall, we did not find large effects of transcriptionally inactive HSF1 mutants on the transcriptome of unstressed HEK293 cells. However, we only used transformed cells in tissue culture to study the role of HSF1 and we would therefore miss processes that are regulated at an organismal level, like the regulation of the circadian rhythm. To investigate the role of HSF1 in the absence of stress further, we thus need to look at the organismal level. Therefore, we also tried to create an in vivo model system mimicking the aging associated decline of the heat shock response by inhibiting HSF1 activity in *Xenopus* tadpoles (**chapter 3**). Ubiquitous overexpression of *Xenopus* HSF380, the *Xenopus* homolog of the dnHSF1, resulted in embryonic lethality, suggesting that, in correspondence with previously reported results [210], HSF1 is required for normal embryonic development. These data thus also suggest a role for HSF1 in the absence of stress. When a neuron-specific promoter was used, no expression of GFP-labeled HSF380 was detected. Even when wild type GFP-HSF1 was used, no brain specific fluorescence was found. This is in accordance with the recent findings of Hayashida et al. [271] in which they were unable to generate transgenic mice or *Xenopus* expressing high levels of active HSF1 in the brain. These data imply that HSF1 levels in the neuronal tissue of at least mice and *Xenopus* are strictly controlled.

Impaired HSF1 function in stressed cells

HSF1 is mostly known as a transcription factor involved in the transcriptional response to stress and we thus also investigated its role under stress conditions. We analyzed the transcriptomes of dnHSF1 and HSF1 K80Q cells 6 and 24 h after heat stress. Of the genes of which the transcript level was increased in HEK293 cells allowed to recover for 6 h after heat shock, 30% was found to be HSF1-regulated, as overexpression of either dnHSF1 and/or HSF1 K80Q inhibited the increase of their transcript level (**chapter 4**). Expression of dnHSF1 inhibited the increase in transcript level of more genes than did expression of HSF1 K80Q. As in the unstressed situation, expression of dnHSF1 could block the binding of other transcription factors, while expression of HSF1 K80Q leaves the promoter region empty. In HEK293 cells 180 genes showed increased transcript levels after heat stress and when we compared these data with those of HeLa cells [237], we found only 20 genes of which the transcript level was increased upon heat stress in both cell lines. The exact heat shock conditions do differ, but we did not expect the overlap to be so small. Among the common set of heat shock responsive

genes in HEK293 and HeLa cells most were HSF1-regulated, such as HSPA1A, HSPA6 and DNAJB1. The set of common genes also contained general stress responsive genes, such as GADD45B and PPP1R15A (GADD34). The set of genes that is regulated by HSF1 after heat stress thus seems largely cell type specific.

In HEK293 cells allowed to recover for 24 h from heat shock, the effect of overexpression of the HSF1 mutants was much more apparent. Normally, the transcriptome of heat shocked cells that have had the time to recover for 24 h has largely returned to the non-stressed level. However, in cells that express either dnHSF1 or HSF1 K80Q, the transcriptome did not fully return to normal. We found an extended stress response: the level of a number of transcripts remained high 24 h after the heat shock. In addition, we found a secondary response: a response of genes which did not respond to heat stress initially, but which did 24 h after heat stress. As the secondary response is larger in HSF1 K80Q or dnHSF1 cells, this indicates that the products of the HSF1 dependent genes are essential to dampen the secondary response. When we analyzed the genes of which the transcript level was increased 24 h after heat stress in HSF1 K80Q or dnHSF1 cells, they were enriched for the GO category transcription and transcription regulation. Apparently, cells try to restore homeostasis by mounting an additional transcriptional response. Among the genes of which the levels were increased 24 h after heat shock in HSF1 K80Q cells were the HSF1 targets HSPA1A and HSPA6. This was quite unexpected, as HSF1 is not active in these cells. In dnHSF1 cells, the HSPA1A transcript level, but not that of HSPA6, was also increased. We investigated which transcription factors would be responsible for this delayed stress response in HSF1 K80Q cells and found NRF2 to be involved. The binding to an antioxidant response element (ARE) was lower in extracts of HSF1 K80Q cells 6 h after heat shock and increased in HSF1 K80Q cells 24 h after heat shock compared to control cells. NRF2 binds to the ARE and is the main transcription factor involved in the antioxidant response; it thus seems that the HSF1 K80Q cells have a delayed antioxidant response after heat stress. Depleting cells of NRF2 inhibited the increase in HSPA6 and HSPA1A mRNA levels 24 h after heat shock and this thus suggests that NRF2 can somehow activate transcription of these genes.

NRF2 usually binds the ARE as a heterodimer with one of the small Maf proteins (for reviews, see [281-283]). The mobility of the ARE binding complex differed between extracts of cells recovering for 6 or 24 h from heat stress and it could thus be that the heteromeric partner of NRF2 differs in these complexes. We did not find a significant effect of HSF1 K80Q overexpression on the transcript levels of the small Maf proteins in our microarray analysis, but it has been described that the activation of Mafs is regulated by posttranslational modification [281] and it could thus well be that this is affected by heat stress and/or HSF1. It would be very interesting to determine whether

the small Maf proteins are indeed involved, together with NRF2, in the delayed stress response that we detect in HSF1 K80Q cells 24 h after heat shock.

Crosstalk of the heat shock response with other cellular stress systems

Cellular stress responses are mostly described as individual systems acting independently of one another. It is, however, very unlikely that the different systems do not communicate with each other to prevent cellular damage optimally. In **chapter 5** we investigated the interaction between the heat shock response and the amino acid response and we found that, surprisingly, HSF1 is inactivated upon amino acid starvation. This was reflected in the loss of HSF1 DNA binding affinity and a strong decline in heat shock protein mRNA levels upon leucine, lysine or glutamine deprivation. Why would cells want to silence HSF1 when they sense a lack of amino acids? A possibility is that HSF1 is involved in the transcriptional regulation of amino acid responsive genes. HSF1 could for example bind to the promoter region of these genes and inhibit their activation under non-stress conditions. Upon amino acid starvation these genes need to be activated, and HSF1 thus needs to be silenced. We looked for HSF1 binding sites in amino acid responsive genes and found a putative HSF1 binding site in the promoter of the ASNS gene, one of the most studied amino acid responsive genes. We did detect binding of HSF1 to this putative binding site in vitro, but we could not confirm this in vivo. A promoter of another amino acid responsive gene, S100P, also contained a motif that closely resembled an HSF1 binding site. Again an interaction of HSF1 with this promoter was detected in vitro, but could not be confirmed in vivo (see appendix). The S100P transcript level is enhanced upon both heat stress and amino acid starvation, although this seems to be independent of HSF1 activity in both cases. We also determined the effect of overexpression of HSF1 mutants on amino acid responsive genes, but only found small effects. The largest effect was seen on the ASNS transcript level when HSF1 K80Q was expressed (twofold increase). Transcript levels of some other amino acid responsive genes also tended to increase slightly, suggesting an inhibitory role for HSF1, but as we do not find solid evidence for the in vivo binding of HSF1 to amino acid responsive promoters, this is only speculative.

HSF1 can be inactivated by phosphorylation at several sites (**chapter 1**) and it has been reported very recently that glutamine can activate HSF1 [342] via phosphorylation of Ser230. Amino acid starvation could then possibly result in a loss of Ser230 phosphorylation and a subsequent inactivation of HSF1. However, we could not find any differences in the phosphorylation pattern of HSF1 in leucine starved cells

compared to fed cells. The acetylation of HSF1 at K80 has been described to result in a loss of HSF1 DNA binding affinity ([51]; **chapter 4**). With an antibody against acetylated lysine we attempted to investigate whether amino acid starvation results in the acetylation of HSF1 K80, but due to experimental problems we could not confirm whether this was indeed the case. The way in which HSF1 is inactivated upon amino acid deprivation thus remains unclear and needs additional research.
When we starved cells for methionine we also found a loss of HSF1 DNA binding affinity, but the transcript levels of HSPA1A and DNAJB1 were, unexpectedly, enhanced (**chapter 6**). Methionine is involved in the transsulfuration pathway that results in the synthesis of cysteine, and cysteine is required for the synthesis of glutathione, an antioxidant molecule. We could therefore imagine that a lack of methionine results in oxidative stress. Indeed we found the antioxidant response to be activated in methionine deprived cells and this antioxidant response was responsible for the HSF1 independent increase in HSPA1A mRNA levels. NRF2, the main transcription factor involved in the antioxidant response, mediated the increase in HSPA1A mRNA levels, but not the increase in DNAJB1 mRNA levels. When we measured HSPA6 mRNA levels in methionine deprived cells, these were also increased (data not shown). These findings are in accordance with our data of the delayed stress response after heat shock in HSF1 K80Q cells (**chapter 4**), where activation of the antioxidant response results in increased HSPA1A and HSPA6 transcript levels.

A central role for HSF1 in aging

The findings described in this thesis, together with reports in the literature, show that HSF1 is inactivated by other stress responses (Fig. 2), i.e. the response to amino acid deprivation (**chapter 5**) and the DNA damage response ([263]). This inactivation of HSF1 activity would then also affect the antioxidant response. A decline in HSF1 activity would weaken the overall stress resistance of a cell and would hamper recovery from stress. What could be the reason that HSF1 is inactivated when these other stress responses are active? One possibility could be that when cellular damage becomes too large the balance is more easily shifted towards apoptosis, as active HSF1 would protect against apoptosis [343,344].
A consequence of the loss of HSF1 activity is aging. It remains to be elucidated whether aging is programmed, i.e. a pro-active process limiting lifespan by deactivating maintenance and repair systems beyond a species-specific age, or whether it is a passive mechanism resulting from an organism's inability to counteract deteriorative processes through deficient maintenance and repair functions [341]. Passive aging could result

from the inactivation of HSF1 during the response to other stresses. This would mean that those other stress responses are fully activated in aging cells, which seems unlikely. Alternatively, HSF1 could be inactivated as part of a programmed, active aging process by, for example, loss of SIRT1 signalling or changes in insulin/IGF1 signalling (Fig. 2). The mechanism of how HSF1 is inactivated in aged cells is still unclear and to get more insight in the possible mechanisms of aging it is therefore of major importance that the process of HSF1 inactivation is elucidated. However, the inter-individual differences in the ages of death in an isogenic population cannot be explained by the theory of active aging [345]. The phenotypic manifestation of aging is likely to be a combination of passive and active aging. Upon aging inter-individual differences in for example genetic and environmental factors can influence the functionality of repair and maintenance processes in cells and individuals can die earlier due to the consequences of these impaired processes and the inability to counteract deteriorative processes. When an individual lives beyond a specific age, the mechanism of active aging emerges and limits lifespan by the inactivation of certain repair mechanisms.

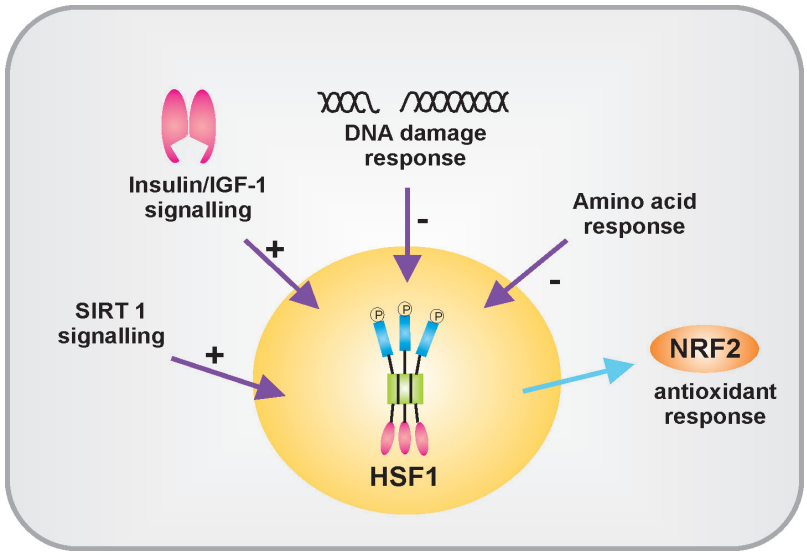


Fig. 2 A central role for HSF1 in aging. All indicated pathways and responses have been implied in aging and the effect on aging appears to go via HSF1. Effects are positive (+) or negative (-).

Increasing the cellular chaperoning capacity in aged cells

To delay the development of protein folding diseases or to ameliorate the consequences thereof, we would need to increase the chaperoning capacity of aging cells. What are the possibilities to do this? As described in this thesis, boosting HSF1 activity could have a lot of side effects next to the intended effect on increased chaperoning capacity. A disadvantage of increasing HSF1 activity is that HSF1 promotes survival. Tumor cells appear to rely on HSF1 activity and activate a transcriptional program that is distinct from the transcriptional program activated upon heat stress [21]. It would thus be safer to boost the expression of individual (co-)chaperones. In that way you could increase the chaperoning capacity of a cell with reduced side-effects. As described in **chapter 2**, glucocorticoid signalling was severely impaired by dnHSF1, but could be fully rescued by the expression of a single co-chaperone (DNAJ). However, luciferase refolding activity, which is affected in cells that are depleted of chaperones, could not be rescued by the expression of a co-chaperone, but required HSPA1A [270]. Thus, for distinct processes or systems, different (co-)chaperones might be needed to compensate for the loss of HSF1 activity.

The small heat shock proteins have been widely reported to inhibit the formation of toxic protein aggregates and are therefore potential candidates for boosting their expression to inhibit age-related diseases. In **chapter 7** we investigated the effect of overexpression of two well-known small heat shock proteins, HSPB1 and αB-crystallin, on the transcriptome of unstressed HEK293 cells. The effect of HSPB1 expression was rather small, but we found a large effect for the expression of αB-crystallin. Cells with high levels of αB-crystallin expression had increased levels of several stress markers, indicating that it might not always be beneficial to have increased αB-crystallin levels. The overexpression of αB-crystallin to increase the chaperoning capacity of a cell would therefore be unfavourable.

In the future we need to determine which parts of the stress systems could be activated to protect against stress and to ameliorate age-related diseases. In addition, it would be interesting to test whether the activation of NRF2 in aging cells could adjust the cellular chaperoning capacity, as we found that NRF2 can activate transcription of some of the heat shock protein genes when HSF1 activity is impaired (**chapter 4**). The HSF1 K80Q system would be a suitable model system to screen for compounds that protect against the harmful effects of stress.

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Samenvatting

De stress respons in cellen

Het menselijk lichaam is opgebouwd uit allerlei soorten cellen. Alle cellen bevatten hetzelfde DNA, de drager van erfelijke informatie – de genen. Van de genen in het DNA wordt eerst boodschapper RNA gemaakt en vanuit deze boodschappers kan het eiwit worden gemaakt. Elk afzonderlijk gen codeert dus voor een specifiek eiwit. Om goed te kunnen functioneren moeten eiwitten een bepaalde vorm hebben; zij moeten op de juiste wijze worden gevouwen. Onze afzonderlijke cellen hebben, net als wij mensen, vaak te maken met stress, zoals hitte (bij bijv. koorts), UV-licht of een virale infectie. De eiwitten in de cellen raken hierdoor beschadigd en kunnen van vorm veranderen. De cel probeert er dan voor te zorgen dat deze beschadigde eiwitten weer in de juiste vorm terug worden gevouwen. De eiwitten die hierbij helpen zijn de chaperonnes, ofwel de heat shock eiwitten. Deze heat shock eiwitten kunnen worden aangemaakt doordat de cel een reparatie systeem aanzet: de heat shock respons. De heat shock respons is een belangrijke stress respons in de cellen en het eiwit heat shock factor 1 (HSF1) speelt hierbij een belangrijke rol. Door stress wordt HSF1 geactiveerd en bindt vervolgens aan specifieke plekken in het DNA: de promotoren (de aandrijvers) van de heat shock genen. Op deze manier zorgt HSF1 ervoor dat er meer heat shock eiwitten worden aangemaakt wanneer cellen gestrest zijn. Deze heat shock eiwitten helpen vervolgens in het repareren van beschadigde eiwitten. Daarnaast zorgen zij ervoor dat als de eiwitten niet meer te repareren zijn, deze eiwitten worden afgebroken. Als het heat shock systeem niet meer goed werkt, dan kunnen eiwitten gaan samenklonteren en dit kan uiteindelijk resulteren in de ontwikkeling van eiwitstapelingsziektes. Bij eiwitstapelingziektes is er sprake van een ophoping van eiwitklonten in bijvoorbeeld de hersenen en dit kan leiden tot de afbraak van zenuwcellen, zoals bij de ziektes van Alzheimer en Parkinson. Het is dus erg belangrijk dat cellen een goed werkend heat shock systeem hebben. Het is reeds bekend dat de activiteit van het HSF1 eiwit afneemt met veroudering en naarmate men ouder wordt is de kans op de ontwikkeling van een eiwitstapelingsziekte ook groter.
Het onderzoek beschreven in dit proefschrift gaat hoofdzakelijk over het HSF1 eiwit en de rol van dit eiwit bij stress en veroudering. Om het effect van inactief HSF1 bij veroudering na te bootsen hebben wij weefselkweekcellen (humane niercellen) gebruikt waarin wij het DNA coderend voor een HSF1 eiwit dat niet meer goed werkt hebben ingebracht. Deze cellen zullen dit eiwit vervolgens gaan produceren. In één model hebben wij een HSF1 gebruikt die het laatste gedeelte mist, dit eiwit noemen wij dnHSF1, en in een ander model hebben wij een verandering aangebracht in het HSF1 eiwit waardoor het niet meer aan het DNA kan binden, dit eiwit noemen wij HSF1 K80Q. Vervolgens hebben wij beide modellen gebruikt om te onderzoeken wat de effecten zijn op de cellen wanneer zij een HSF1 hebben die niet meer goed werkt.

De rol van het HSF1 eiwit in cellen die niet gestrest zijn

Alhoewel HSF1 bekend staat om zijn rol in gestreste cellen, zijn er steeds meer aanwijzingen dat HSF1 ook een rol speelt in de afwezigheid van stress. Wij hebben daarom onderzocht wat het effect is van niet goed werkende, ofwel inactieve, HSF1 eiwitten op cellen die ongestrest zijn. In **hoofdstuk 2** hebben wij gekeken naar het effect van het dnHSF1 eiwit in ongestreste cellen en in **hoofdstuk 4** hebben wij onderzocht wat het effect is van het HSF1 K80Q eiwit in ongestreste cellen. Wij hebben hierbij gekeken naar de boodschapper niveaus van duizenden verschillende genen en deze vergeleken met hun niveaus in cellen met een goed werkend HSF1 eiwit. Er waren maar een paar genen waarvan het boodschapper niveau veranderde in dnHSF1 en HSF1 K80Q cellen. Een inactief HSF1 eiwit lijkt dus niet veel effect te hebben op de genactiviteit van de cellen in de afwezigheid van stress. In deze studie hebben wij alleen weefselkweekcellen gebruikt. Hierdoor is het mogelijk dat wij bepaalde processen missen die niet op het niveau van de cel geregeld zijn, maar waar een heel organisme voor nodig is, zoals bijvoorbeeld het mechanisme van onze biologisch klok. Om de rol van HSF1 in de afwezigheid van stress verder te bestuderen, zouden wij dus moeten kijken naar het hele organisme. Wij hebben hiervoor kikkervisjes van de Afrikaanse klauwpad *Xenopus laevis* gebruikt. Dit is een organisme dat makkelijk is in het gebruik en waarvan de anatomie van de hersenen grote gelijkenis toont met die van de hersenen van de mens. In **hoofdstuk 3** hebben wij geprobeerd om de activiteit van HSF1 te remmen in deze kikkervisjes door het DNA coderend voor dnHSF1 in te brengen in embryo's van deze kikkers. Wanneer wij dit deden overleefden de embryo's het niet, wat aangeeft dat HSF1 nodig is voor de embryonale ontwikkeling en dat HSF1 dus wel degelijk van belang is in de afwezigheid van stress.

De functie van HSF1 in gestreste cellen

Naast de rol van HSF1 in de afwezigheid van stress, hebben wij ook gekeken naar de rol van HSF1 in gestreste cellen. Wij hebben onderzocht wat het effect van een inactief HSF1 eiwit is op de activiteit van genen in cellen die een hitte schok hebben gekregen. Net als de cellen in ons lichaam groeien de weefselkweekcellen die wij voor ons onderzoek gebruiken normaal bij 37ºC. Wij kunnen deze cellen stressen door ze een hitte schok te geven. Dit doen wij door ze een half uur bij een temperatuur van 45ºC te plaatsen. Daarna laten wij ze een tijdje herstellen en kijken dan naar veranderingen in gen activiteit. Op deze manier kunnen wij onderzoeken hoe de cellen zichzelf aanpassen aan deze stress conditie. Nadat wij de cellen 6 uur lieten herstellen van de hitte schok

konden wij bepalen dat 30% van de genen waarvan het mRNA niveau veranderde gereguleerd was door HSF1, omdat de verhoging van de boodschapper niveaus van deze genen geremd werd door dnHSF1 of HSF1 K80Q (**hoofdstuk 4**). Als wij cellen 24 uur lieten herstellen van een hitte schok zagen wij veel grotere effecten van dnHSF1 en HSF1 K80Q. Normaal herstelt een cel goed na het krijgen van een hitte schok. Maar onze resultaten geven aan dat cellen die dnHSF1 of HSF1 K80Q aanmaken en dus geen functioneel HSF1 eiwit hebben niet meer goed kunnen herstellen.
In de cellen waar HSF1 niet werkte waren er ook genen van wie het boodschapper niveau na herstel van 6 uur na een hitte schok niet omhoog ging zoals in gewone cellen, maar waarvan het niveau na 24 uur wel hoger was. Bij deze groep hoorden bijvoorbeeld ook de bekende, door HSF1 gereguleerde, heat shock genen HSPA1A en HSPA6. Dit was een onverwacht resultaat, omdat in deze cellen HSF1 niet actief is en wij dus aannamen dat de boodschappers van deze genen niet aangemaakt zouden kunnen worden. Wij hebben onderzocht welk ander eiwit er dan voor zou kunnen zorgen dat de boodschappers van deze genen aangemaakt worden wanneer HSF1 niet werkt. Wij vonden dat het eiwit NRF2 hierbij betrokken was. NRF2 is een eiwit dat een grote rol speelt in de antioxidant respons, een andere stress respons die volgt op de aanwezigheid van reactieve zuurstofdeeltjes. Reactieve zuurstofdeeltjes ontstaan door bepaalde chemische processen in het lichaam als gevolg van allerlei schadelijke omgevingsfactoren. De antioxidant respons zorgt ervoor dat er dan bepaalde genen worden geactiveerd en de eiwitten die vervolgens gemaakt worden kunnen de reactieve zuurstofdeeltje opruimen. Op deze manier wordt de schade aan de cel beperkt. De antioxidant respons werd ook aangezet na een hitte schok, maar als HSF1 niet werkte, was de antioxidant respons vertraagd. Als de antioxidant respons uiteindelijk dan toch werd aangezet, kon NRF2 de aanmaak van de HSPA1A en HSPA6 boodschappers activeren. Deze resultaten betekenen dat als HSF1 en dus de heat shock respons niet goed werkt, dit ook effect heeft op de antioxidant respons. Door de aanmaak van HSPA1A en HSPA6 onder invloed van NRF2 zijn de cellen dan toch misschien een beetje beschermd.

De interactie van het heat shock systeem met andere cellulaire stress systemen

Cellen hebben verschillende stress systemen om zo de schade na verschillende soorten stressen optimaal te kunnen opruimen. Meestal worden deze stress systemen apart beschreven, maar het is onwaarschijnlijk dat deze systemen onafhankelijk van elkaar werken. Het heat shock systeem en het antioxidant systeem zijn twee belangrijke stress

systemen en zoals hierboven beschreven heeft het heat shock systeem ook invloed op het antioxidant systeem. In **hoofdstuk 5** hebben wij onderzocht wat de interactie tussen de heat shock respons en de respons bij aminozuur starvatie is. Aminozuren zijn de bouwstenen van eiwitten. Er zijn 20 verschillende aminozuren waarvan wij de helft zelf kunnen aanmaken in ons lichaam en de andere helft moeten wij uit onze voeding halen. Aminozuur starvatie treedt op wanneer een cel een tekort aan één of meerdere aminozuren heeft, wat bijvoorbeeld kan komen door ondervoeding. De cel activeert dan een complex mechanisme om de schade door het aminozuurtekort zoveel mogelijk te beperken. Wij vonden dat als de aminozuur respons werd geactiveerd, HSF1 juist geïnactiveerd werd. Het HSF1 eiwit kon niet meer aan het DNA binden als wij cellen niet genoeg aminozuren gaven. Verder werden er ook minder heat shock eiwit boodschappers aangemaakt in cellen die geweekt werden in medium dat de aminozuren leucine, glutamine of lysine miste. Bij een tekort aan aminozuren worden er dus ook minder heat shock eiwitten gemaakt, waardoor cellen gevoeliger zullen zijn voor stress. Wanneer wij ervoor zorgden dat cellen het aminozuur methionine niet konden opnemen, zagen wij dat de boodschapper niveaus van de heat shock genen juist verhoogd waren, terwijl HSF1 ook in dit geval inactief werd (**hoofdstuk 6**). Er moest dus een ander eiwit zorgen voor de aanmaak van deze boodschapper RNA's. Methionine is een aminozuur dat ook nodig is voor de aanmaak van glutathion, een antioxidant molecuul. Het zou dus zo kunnen zijn dat als een cel te weinig methionine heeft er geen glutathion meer kan worden gemaakt en dat er oxidatieve stress ontstaat. In dit geval zou de antioxidant respons worden aangezet. Bij een tekort aan methionine zagen wij dat inderdaad de antioxidant respons werd geactiveerd. Zoals boven beschreven speelt het eiwit NRF2 een grote rol bij de antioxidant respons en ook hier vonden wij dat NRF2 de aanmaak van bepaalde heat shock eiwit boodschappers kon activeren bij een gebrek aan methionine.

Het verhogen van de chaperonne capaciteit in verouderende cellen

De resultaten in dit proefschrift laten zien dat verschillende stress systemen elkaar beïnvloeden. Wij laten bijvoorbeeld zien dat HSF1 niet meer werkt als de aminozuur respons actief is en al eerder is beschreven dat HSF1 niet meer werkt als de stress respons na DNA schade (door bijv. UV-licht) actief is. Daarnaast hebben wij laten zien dat als HSF1 niet meer werkt de antioxidant respons het ook niet meer goed doet. Door een gebrek aan HSF1 kan een cel niet meer goed herstellen van stress. De verkeerd gevouwen en ongevouwen eiwitten die ontstaan door stress kunnen dan niet meer gerepareerd of opgeruimd worden. Deze eiwitten hopen zich vervolgens op in de cel

wat kan leiden tot het ontwikkelen van een eiwitstapelingsziekte.
De activiteit van HSF1 neemt af bij veroudering. De aanmaak van de heat shock eiwitten gaat daardoor ook omlaag. Om de ontwikkeling van eiwitstapelingsziektes tegen te gaan of te remmen, moeten wij de hoeveelheid heat shock eiwitten ofwel chaperonnes in de cellen verhogen zodat de eiwitten die niet goed gevouwen zijn beter worden gerepareerd of worden opgeruimd. Met andere woorden: de chaperonne capaciteit moet worden verhoogd. Hoe zouden wij dit het beste kunnen doen? Wij zouden bijvoorbeeld de activiteit van HSF1 kunnen stimuleren zodat deze ervoor kan zorgen dat er meer heat shock eiwitten kunnen worden gemaakt. Het probleem hierbij is echter dat verhoogde HSF1 activiteit betrokken is bij de ontwikkeling van kanker. Een alternatief zou zijn om de niveaus van afzonderlijke chaperonnes in de cel te verhogen. Zo kunnen wij de chaperonne capaciteit verhogen zonder dat er al te veel bijeffecten plaatsvinden. In **hoofdstuk 2** hebben wij bijvoorbeeld beschreven dat de hormoon signalering in de cel niet meer goed werkte als de cellen een inactief HSF1 eiwit hadden. Wanneer wij bepaalde chaperonnes (DNAJ) toevoegden werkte de hormoon signalering weer. Het was dus mogelijk om met toevoeging van één chaperonne het negatieve effect van inactief HSF1 te compenseren. Bij een ander onderzoek, waarbij gekeken werd naar de vouwing van een bepaald eiwit, werd gevonden dat een andere chaperonne (HSPA1A) nodig was om het negatieve effect van een inactief HSF1 eiwit terug te draaien. Deze studies laten samen zien dat het dus afhankelijk is van het mechanisme waar je naar kijkt welk chaperonne eiwit het effect van het verlies van HSF1 activiteit zou kunnen compenseren.
Een subfamilie van de familie van de heat shock eiwitten is de kleine heat shock eiwit familie. Het is beschreven dat deze kleine heat shock eiwitten de vorming van giftige eiwitklonten kunnen remmen. Deze chaperonne eiwitten zijn dus mogelijke kandidaten voor de remming van eiwitstapelingsziektes. In **hoofdstuk 7** hebben wij daarom onderzocht wat het effect van verhoogde niveaus van de kleine heat shock eiwitten αB-crystalline of HSPB1 op cellen is. Wij hebben de boodschapper niveaus in deze cellen vergeleken met die in normale cellen. Het effect van HSPB1 bleek erg klein te zijn, terwijl het effect van αB-crystalline een stuk groter was. Cellen met verhoogde αB-crystalline niveaus hadden hogere niveaus van boodschappers van algemene stress genen, wat aangeeft dat het niet altijd gunstig is voor een cel om hoge niveaus van αB-crystalline te hebben. Het stimuleren van de chaperonne capaciteit door het verhogen van de αB-crystalline niveaus zou dus niet verstandig zijn.
In de toekomst zullen wij moeten kijken welke delen van het stress systeem geactiveerd zouden kunnen worden om te beschermen tegen stress en om de ontwikkeling van verouderingsziektes af te zwakken. Het zou een interessante mogelijkheid zijn om te testen of het activeren van het NRF2 eiwit in verouderende cellen de chaperonne

capaciteit zou kunnen verhogen. In **hoofdstuk 4** hebben wij namelijk gevonden dat NRF2 voor de aanmaak van heat shock eiwit boodschappers kan zorgen als HSF1 niet functioneert. De model systemen die wij gebruikt hebben om de verlaging van HSF1 activiteit bij veroudering na te bootsen, zouden gebruikt kunnen worden om verder te onderzoeken hoe wij de chaperonne capaciteit kunnen verhogen in verouderende cellen. Op deze manier kunnen wij meer inzicht krijgen in hoe wij de ontwikkeling van eiwitstapelingsziektes bij veroudering kunnen vertragen of voorkomen.

Appendix

Mapping of a HSF1 binding site in the S100P promoter

Sanne M.M. Hensen and Nicolette H. Lubsen

S100P is an EF-hand calcium binding protein that belongs to the S100 family of proteins [1]. Transcription of the S100P gene can be activated upon amino acid deprivation by the transcription factor ATF4 that binds to the amino acid response element in the S100P promoter [2]. In microarray studies, we noted that the increase in S100P mRNA levels upon leucine starvation was strongly inhibited in the presence of a HSF1 mutant lacking the activation domain, dnHSF1 (for QPCR validation, see Fig. 1A). It could thus be that dnHSF1 binds to the S100P promoter and blocks activation of transcription in response to lack of amino acids. Hence, we scanned the S100P promoter sequence for a putative HSF1 binding site. Although we did not find the complete trimeric consensus HSF1 binding sequence (GAAnnTTCnnGAA), we did find a sequence that closely resembles one (-147/-140) (Fig. 1B). To determine whether HSF1 can activate transcription using this putative binding site, we made luciferase reporter constructs containing different parts of the S100P promoter. The -236/+58 and -150/+58 constructs contain both the putative HSF1 binding site and the amino acid response element (AARE) mapped by Namba et al. [2], while the -124/+58 construct lacks the putative HSF1 binding site but retains the AARE, and the -107/+58 construct lacks both. We first determined whether

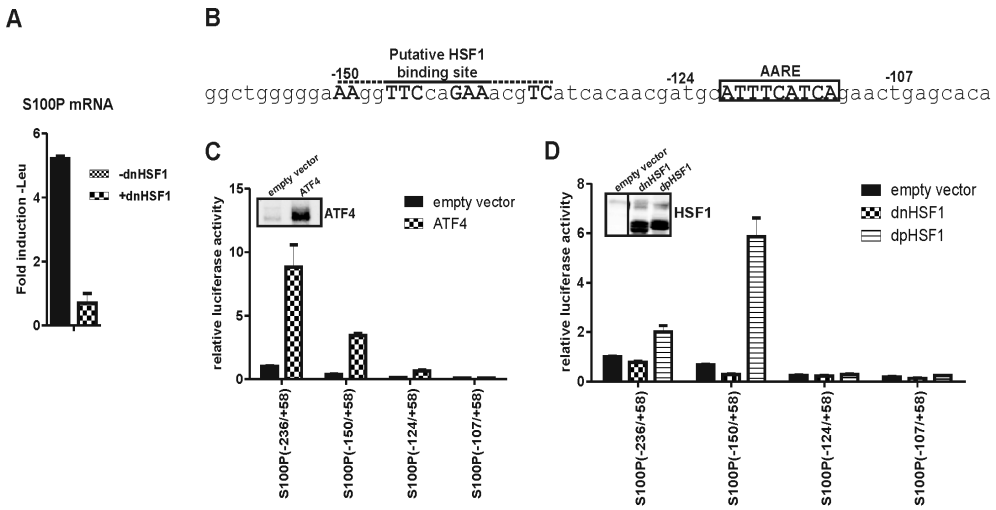

Fig. 1 The S100P promoter contains a putative HSF1 binding site. A. HEK293-dnHSF1 cells were deprived of leucine in the presence or absence of dnHSF1 expression. Total RNA was isolated and mRNA levels were determined by QPCR analysis and are shown relative to GAPDH mRNA levels. **B.** Partial sequence of the S100P promoter with the putative HSF1 binding site and the AARE indicated. **C.** Several S100P promoter constructs were created (for primers see Table 1) and their activity upon expression of ATF4 (shown in the inserted panel) was measured by a luciferase reporter assay. **D.** A luciferase reporter assay was performed to measure the activity of the S100P promoter constructs upon expression of dnHSF1 or dpHSF1 (shown in the inserted panel). Error bars represent SD.

ATF4 could activate the S100P promoter constructs, and therefore co-transfected these with an ATF4 expression plasmid and measured luciferase activity (Fig. 1C; note that the shorter the S100P promoter construct, the lower the "basal activity"). ATF4 indeed activated transcription of all S100P promoter constructs except the -107/+58 construct, indicating that the ATF4 response element binding site is located between nucleotide -124 and -107, which agrees with the location of the AARE reported by Namba et al. [2]. Next, we determined the effect of the expression of dnHSF1 or of a dominant positive mutant of HSF1 (dpHSF1) on the activity of the different S100P reporter constructs. Expression of dnHSF1 slightly repressed the basal activity of the S100P (-236/+58) and S100P (-150/+58) reporter plasmids (Fig. 1D). Overexpression of dpHSF1 increased luciferase activity of the S100P (-236/+58) reporter twofold; surprisingly, the S100P (-150/+58) promoter construct was more sensitive to dpHSF1 and its activity increased almost nine-fold upon overexpression of dpHSF1. The S100P (-124/+58) promoter construct, lacking the putative HSF1 binding site, was not activated by dpHSF1, suggesting that a HSF1 binding site is indeed located between nucleotides -150 and -124.

The data presented above suggest that HSF1 can indeed interact with the S100P promoter. However, we could not confirm in vivo binding using ChIP. We thus tested in vitro binding of HSF1 to the presumptive S100P heat shock element (HSE) of the S100P promoter using an EMSA with a radiolabeled double-stranded oligonucleotide representing the -153/-129 region of the S100P promoter (Fig. 2A). Little binding was seen using an extract from control cells. However, when an extract from heat stressed cells was used, a DNA-protein complex was formed, which supershifted when an HSF1 antibody was added (Fig. 2B). In addition, the (unlabeled) S100P sequence competed for binding with a sequence containing the consensus HSE. This competition was reduced when two nucleotides of the putative S100P HSE (S100P mut1) were mutated and abolished when two other nucleotides of the putative HSE were changed (S100P mut2; Fig. 2A, B). These results show that HSF1 recognizes the putative HSF1 binding site in the S100P promoter in vitro. Binding of dnHSF1 to this site in the S100P promoter might block access or activation by other transcription factors, which could explain why dnHSF1 blocks the amino acid response of the S100P gene.

Above we showed that HSF1 can bind to the S100P promoter and that expression of a dpHSF1 mutant activated a S100P promoter construct. These data suggest that the S100P gene might be a heat shock gene, i.e. a gene of which transcription is activated in heat shocked cells. We thus determined the S100P mRNA levels in heat stressed cells and found that these are indeed strongly increased (Fig. 3). To test whether this induction is HSF1 dependent, we made use of T-REx HEK293 cells which inducibly express either the dnHSF1 mutant or a HSF1 mutant (HSF1 K80Q) which cannot bind

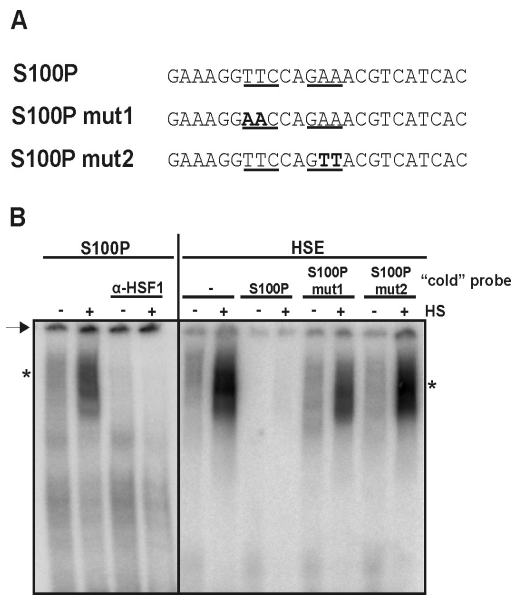

Fig. 2 HSF1 binds to the putative HSE of the S100P promoter in vitro. A. EMSA probes with a S100P sequence containing the putative HSF1 binding site or a mutated putative HSF1 binding site were designed. **B.** Nuclear extracts were used in an electrophoretic mobility shift assay with a double-stranded oligonucleotide with the S100P or HSE sequence (primers are listed in Table 1). Asterisks indicate the primary complexes formed. Supershifts were induced with an anti-HSF1 antibody and are indicated with an arrow. A competition experiment was performed adding a 100-fold molar excess of unlabeled wild type or mutated S100P probe to the radiolabeled HSE probe.

DNA [3]. In the absence of doxycyline, i.e. without expression of the HSF1 mutant, S100P mRNA levels were 7-fold induced in HEK-dnHSF1 (Fig. 3A) and even 30-fold higher in HEK-HSF1K80Q cells (Fig. 3B) 6 h after heat stress. When expression of the HSF1 mutants was induced, the S100P mRNA levels still increased upon heat shock: dnHSF1 inhibited the increase by about 40%, while the HSF1K80Q mutant had very little effect, even though it blocks the heat stress induction of many known HSF1 target promoter (Chapter 4). Collectively, these data suggest that the increase in S100P mRNA levels upon heat stress is not dependent on HSF1. As we also found a putative HSF1 binding site in the promoter of the ASNS gene, a well-known amino acid responsive gene, we could speculate about a role for HSF1 in regulating the promoter activity of

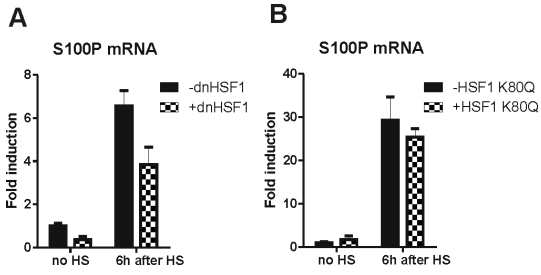

Fig. 3 The increase in S100P mRNA levels upon heat stress is not dependent on HSF1. HEK293-dnHSF1 (**A**) or HEK293-HSF1 K80Q (**B**) cells were cultured in the presence or absence of mutant HSF1 expression and either heat stressed for 30 minutes at 45°C or left at 37°C. After 6 h of recovery cells were harvested and total RNA was isolated. mRNA levels were determined by QPCR analysis and are shown relative to GAPDH mRNA levels. Error bars represent SD.

these genes under non-stress conditions. In both cases, however, we only have in vitro data for the binding of HSF1 to these promoters; we were unable to detect in vivo binding of HSF1 to the putative HSF1 binding sites in these promoters. Thus, even though HSF1 appears to be able to bind the S100P promoter, we did not find direct evidence that it is involved in the regulation of S100P expression.

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Dankwoord

Curriculum Vitae

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Dankwoord

"Ut is gedaon, ut is gedaon…"

Maar niet voordat ik de volgende mensen bedankt heb voor hun steun, hulp en gezelligheid in de afgelopen jaren!

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Curriculum Vitae

Sanne Hensen werd geboren op 21 april 1985 te Venlo. In 2002 behaalde zij haar VWO-diploma aan het Valuascollege te Venlo, waarna zij begon aan de studie scheikunde aan de Radboud Universiteit Nijmegen. Haar bachelorstage voerde ze uit op de afdeling Biochemie NWI binnen de groep van prof. dr. Wilfried de Jong, onder begeleiding van dr. Ron Dirks. Hier deed ze onderzoek naar eiwitstapelingsziektes die gerelateerd zijn aan ouderdom, waarbij *Xenopus laevis* (Afrikaanse klauwpad) als diermodel werd gebruikt. Tijdens haar masteropleiding liep ze stage bij de afdeling Biomoleculaire Chemie onder leiding van prof. dr. Ger Pruijn en werd zij begeleid door drs. Tim Welting. Hier bestudeerde ze een eiwit-RNA-complex dat betrokken is bij cartilage hair hypoplasia: een ziekte die zich kenmerkt door dwerggroei, broos haar en een verzwakt imuunsysteem. Haar tweede masterstage voerde ze uit op de afdeling Reumatologie van het Karolinska Instituut in Stockholm, onder begeleiding van prof. dr. Lars Klareskog, dr. Vivi Malmström, dr. Mona Widhe en dr. Tina Trollmo. Tijdens deze stage ontwikkelde zij een ELISPOT-protocol om B-cellen te detecteren die antilichamen produceren tegen eiwitten die betrokken zijn bij reuma. In januari 2008 behaalde Sanne haar masterdiploma.

Van januari 2008 tot april 2012 werkte ze als promovendus op de afdeling Biomoleculaire Chemie aan de Radboud Universiteit Nijmegen onder begeleiding van prof. dr. Lettie Lubsen. Het onderzoek dat zij in deze periode heeft uitgevoerd staat beschreven in dit proefschrift.

Vanaf april 2012 werkt Sanne als postdoc bij de afdeling Biomoleculaire Chemie, onder leiding van prof. dr. Ger Pruijn, en ontwikkelt zij nieuwe detectiemethodes voor gecitrullineerde eiwitten: eiwitten die betrokken zijn bij reuma.

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*These authors contributed equally to this work