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RESEARCH ARTICLE

Co-chaperones are limiting in a depleted chaperone network

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Abstract 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 are the (co-)chaperone genes HSP90AA1, HSPA6, DNAJB1 and HSPB1. Glucocorticoid signaling, which requires the Hsp70 and the Hsp90 folding machines, was severely impaired by dnHSF1, but 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 in a depleted chaperoning network. Our results also suggest a difference between the transcriptomes of cells lacking HSF1 and cells expressing dnHSF1.

Keywords Heat shock factor 1 · Chaperones ·
Co-chaperones · Aging · Steroid hormone signalling

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Abbreviations
HSF1   Heat shock factor 1
HSE   Heat shock element
GRE   Glucocorticoid-responsive element

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 [1–8]). 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 [9–11]. 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 [12]. 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 overexpressed [13–17]. 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 [18–20]). 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 [21–26], 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 [27]. More recently, direct evidence for the stress-independent regulation of genes by HSF1 was provided in the cass of the multi-drug resistance gene 1 [28] and the IL-6 gene [29]. Furthermore, HSF1 inhibits heregulin-induced transcription in breast carcinoma cells [30].

A number of studies have shown that the quality of the heat shock response diminishes with aging [31–37], a decrease that may be the result of a decrease in the activity of the deacetylase SIRT1 [38]. 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; [34]). Aging-related failure of HSF1 will interfere with an organism's ability to combat cellular stress and increase the susceptibility to protein folding disease [6, 8, 13, 14, 39–41]. 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 [3, 4].

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 glucocorticoid 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 [42]) and augmenting the chaperone network by either stress [43] or expression of a constitutively active HSF1 mutant [44] 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 [45]. The 1.36-kb XhoI 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 HindIII and XhoI 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; [46]). Expression construct pCMV-SPORT6-Hsp90AA1 was obtained from Imagenes (http://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 EcoRI (blunt)—XhoI fragment of pOTB7-STIP1 was cloned into the HindIII (blunt) and XhoI 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 NheI and BglII sites of pGL3-promoter (Promega). The Drosophila melanogaster Hsp70-luciferase reporter construct pHL and the Hsp70 expression construct were described earlier [47]. Plasmid pRL-CMV was obtained from Promega. All plasmid constructs were sequence verified.

Table 1 Oligonucleotides that were used to generate recombinant DNA constructs

Oligo name	Oligo 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	agctaagcttccccttccggccggaggcg
ST13prom-rev	agctccatggtagggaggtggtgg
PTGES3prom-for	agctaagcttaataccttagtgcttattatgaagc
PTGES3prom-rev	agctccatggtgaacggggcagggggacg
DNAJA1prom-for	agtcgaccacgcgtgaaaaacagcaagac
DNAJA1prom-rev	accatggtggctgaggccggtgtgtgaggga
DNAJB1prom-for	aagtcgaccagacacaggttaggtagttcgtcc
DNAJB1prom-rev	accatggcccctcctgcggcccgccga
CHOPprom-for	tgagctctgtcacccaggctggagtgc
CHOPprom-rev	tagatctctgacctcgggagcgcctggctg
BiPprom-for	tctcgaggtatttttagtagagactgggcac
BiPprom-rev	accatggtgccagccagttgggcagcag
HSP90prom-for	agctaagcttgcgcaggcgctgttcctgg
HSP90prom-rev	agctccatggcgccccggaggccacaccc
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	agctctcgagttactccagatctggcattttttc
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, respectively. The cells were cultured at 37°C/5% CO_2 in high glucose DMEM medium supplemented with 10% fetal calf serum and 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. 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 37°C/5% CO_2 (control) or incubated at 45°C for 30′ (heat shock). After 6 h recovery at 37°C/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 triplicate.

RNA isolation and microarray analysis

HEK-HSF379 or HEK-cDNA5 cells were either left untreated or treated with doxycyclin for 48 h. 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 × 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 http://www.genespring.com) were included in the analysis. We used a cut-off level of twofold 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 4× 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 [48]. 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 signaling 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 [49]). 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
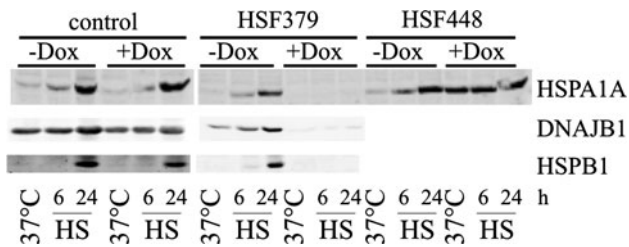
Fig. 1 The HSF1 mutants HSF379 and HSF448 have different effects on basal and heat shock-induced Hsp70 expression. Parental Flp-In HEK293 cells and HEK293 cells carrying a stably integrated copy of the pcDNA5-HSF379 (HEK-HSF379) or pcDNA5-HSF448 (HEK-HSF448) plasmid were cultured in the absence or presence 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
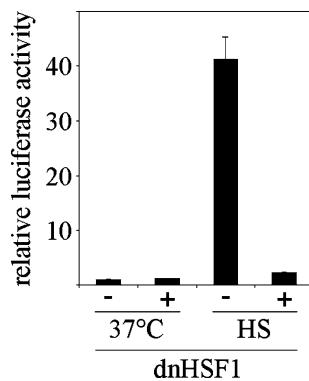

Fig. 2 The effects of dnHSF on basal and heat shock-induced activity of an Hsp70 promoter HEK293 cells carrying a stably integrated copy of the HSF379 (dnHSF1) were cultured in the absence (−) or presence (+) of doxycycline. Cells were transfected with a mixture of the *Drosophila melanogaster* Hsp70-luciferase reporter (pHL) and the Renilla Luciferase control plasmid pCMV-RL. At 48 h 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-galacto-sidase (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*)

induction of endogenous Hsp70 was completely abolished by HSF379, showing its potent dominant-negative activity (Fig. 1). Surprisingly, HSF448 was a very poor 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 [45], 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.

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 dnHSF1 using a two-color 44K Agilent Human Expression Profile Array. The transcripts of only 10 genes showed a more than twofold 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 pro-teins was also reduced in dnHSF1 expressing cells (Fig. 3; note that the HSPA6 mRNA level is very low in non-stressed HEK293 cells; [50]). 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.

The levels of the transcripts of a number of other chaperone genes did not quite meet the "twofold" 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 sub-strate specificity, and stimulate the activity, of the Hsp70 folding machine and are thus critical nodes in the chaper-oning 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 [51], 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

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. no.	dnHSF1/Ctrl		Alternative name
		Ave	SD	
HSPH family				
HSPH1	NM_006644	0.78	0.08	Heat shock 105 kDa/110 kDa protein 1
HSPH2	NM_002154	0.66	0.04	Heat shock 70 kDa protein 4
HSPH3	NM_014278	0.61	0.21	Heat shock 70 kDa protein 4-like
HSPH4	NM_006389	1.19	0.33	Hypoxia up-regulated 1
HSPA family				
HSPA1A/B[a]	NM_005345	0.93	0.18	hsp72
HSPA1L	NM_005527	Not on array[b]	Not on array[b]	Heat shock 70 kDa protein 1-like
HSPA2	NM_021979	1.22	0.20	
HSPA5	NM_005347	1.18	0.35	GRP78, BiP
HSPA6	**NM_002155**	**0.46[c]**	**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[d]	nd[d]	
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[d]	nd[d]	
DNAJB1	**NM_006145**	**0.25**	**0.05**	**hsp40**
DNAJB2	NM_006736	0.60	0.07	HSJ1
DNAJB3	NM_001001394	nd[d]	nd[d]	
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[d]	nd[d]	
DNAJB8	NM_153330	nd[d]	nd[d]	
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[d]	nd[d]	
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[d]	0.10	
DNAJC5	NM_025219	nd[d]	nd[d]	Cysteine string protein (CSP)
DNAJC5B	NM_033105	nd[d]	nd[d]	Cysteine string protein beta (CSP-beta)

Table 2 continued

Gene name	Acc. no.	dnHSF1/Ctrl		Alternative name
		Ave	SD	
DNAJC5G	NM_173650	1.05[c]	0.07	
DNAJC6	NM_014787	0.87[c]	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[c]	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[c]	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[d]	nd[d]	MKBP
HSPB3	NM_006308	nd[d]	nd[d]	
HSPB4	NM_000394	nd[d]	nd[d]	αA-crystallin (CRYAA)
HSPB5	NM_001885	0.99[c]	0.18	αB-crystallin (CRYAB)
HSPB6	NM_144617	1.04[d]	0.25	Hsp20
HSPB7	NM_014424	nd[d]	nd[d]	cvHsp
HSPB8	NM_014365	nd[d]	nd[d]	HSP22
HSPB9	NM_033194	0.68	0.20	
HSPB10	NM_024410	nd[d]	nd[d]	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	

Table 2 continued

Gene name	Acc. no.	dnHSF1/Ctrl		Alternative name
		Ave	SD	
BAG3	NM_004281	1.31	0.18	
BAG4	NM_004874	1.28[c]	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[d]	nd[d]	
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	

[a] The array oligonucleotides do not discriminate between the transcripts of these two genes

[b] None of the oligonucleotides on the array hybridize with the transcript of this gene

[c] The hybridization signal was significant but below 100

[d] The hybridization signal was not significant

Table 3 Non-chaperone encoding genes downregulated by dnHSF1

Gene name	Acc. no.	dnHSF1/Ctrl		Description
		Av.	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_000249	0.50	0.17	mutL homolog 1

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). Note that these promoter activities were measured in unstressed cells, explaining why the activity of the promoters of the two 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 also have heat shock elements in its first intron [52].

Lack of heat shock proteins could cause stress in the cells, which in turn could activate a non-HSF-dependent stress response (see also [53]). 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 [54]). As shown in Fig. 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
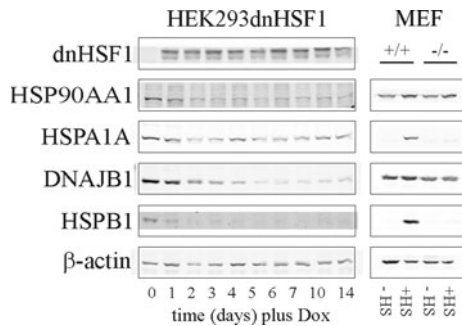
Fig. 3 *Left panel* the decay of heat shock protein levels during expression of dnHSF1. HEK-HSF379 cells were treated with doxycyclin for the time indicated and harvested. *Right panel* the level of heat shock proteins in MEF wild-type cells (+/+) and MEF cells lacking HSF1 (−/−) either before (−HS) or after heat shock and recovery (+HS). Cell lysates were subjected to SDS-PAGE and western blot analysis using the indicated antibodies
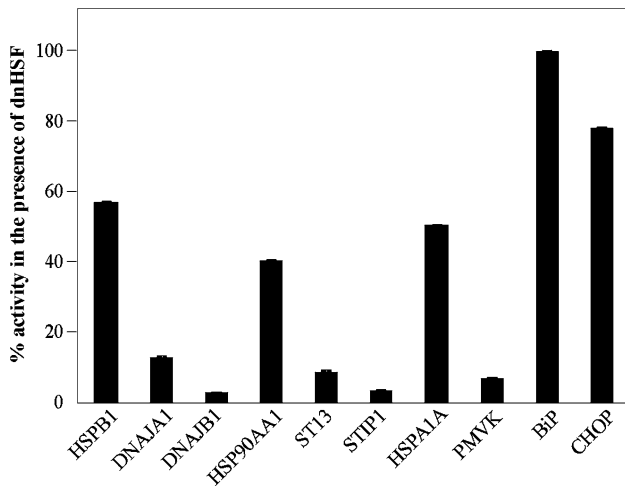

Fig. 4 Inhibition of promoter activity by dnHSF1. Control HEK-cDNA5 cells and HEK-HSF379 cells were treated with doxycyclin. After 3 days, cells were transfected with the indicated promoter reporter constructs (see also "Materials and methods") and a βactin-βgal reporter. At 48 h after transfection, 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*)

from heat stress, but do not built up thermostability after a heat stress [26, 55].

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
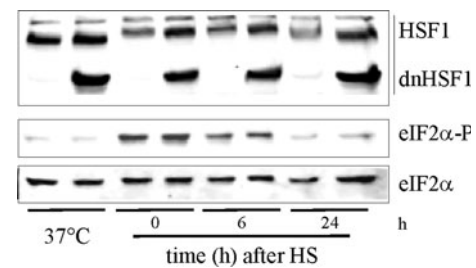

Fig. 5 The effect of exogenous expression of dnHSF1 on eIF2α phosphorylation. HEK-cDNA5 cells and HEK-HSF379 cells were treated with doxycyclin for 48 h. Cells were then exposed to a heat shock of 30′ at 45°C (HS) or left at 37°C (37°C). When heat shocked, cells were allowed to recover for the indicated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

hormone receptors (reviewed in [42], [56]) and we thus examined whether expression of dnHSF1 resulted in impaired glucocorticoid hormone signaling. 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 ninefold in HEK-cDNA5 cells and only by fourfold in HEK-dnHSF1 cells and, at the highest concentration of dexamethasone, the inducibility in HEK-cDNA5 cells was even 13-fold compared with only fivefold in HEK-dnHSF1 cells.

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 affected 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) inhibited the GRE response in HEK-cDNA5 cells (Table 4) as previously reported [57, 58] but increased it slightly in HEK-dnHSF1cells. STIP1 (Hop), which is a co-chaperone of Hsp90 as well as of Hsp70, however, had no effect, either
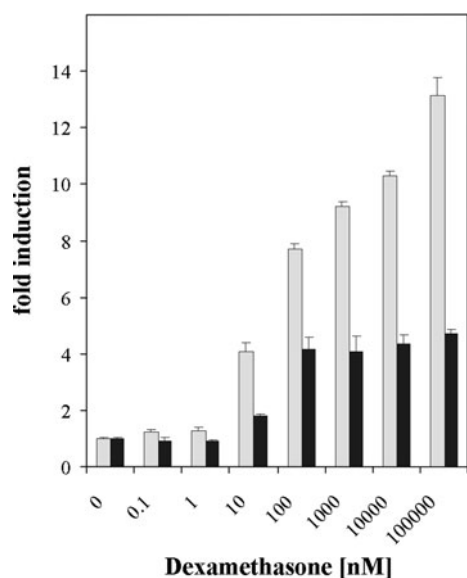
Fig. 6 Exogenous expression of dnHSF1 reduces the glucocorticoid response. Control HEK-cDNA5 cells and HEK-HSF379 cells were treated with doxycyclin. After 3 days, cells were transfected with a glucocorticoid-responsive luciferase reporter (pGRE-Luc) and a βactin-βgal reporter. At 24 h after transfection, 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*)

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).

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 [27]. When HSF1 was depleted by RNA interference in HeLa cells, the expression level of 378 genes changed significantly in the absence of stress [59]. 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 twofold, 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 [60]. HEK293 are less dependent on HSF1 than HeLa cells [60], 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 of HSF1 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 [30]. Similarly, HSF1 has been reported to interact with C/EBPβ, an interaction which represses transcriptional activation [61]. 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 overexpression of individual proteins would rescue the glucocorticoid response. Nonetheless, the individual co-chaperones DNAJA1, DNAJB1 and ST13/Hip were able to fully rescue the dnHSF-mediated inhibition of the glucocorticoid response; PTGES3/p23 had some effect, whereas overexpression 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
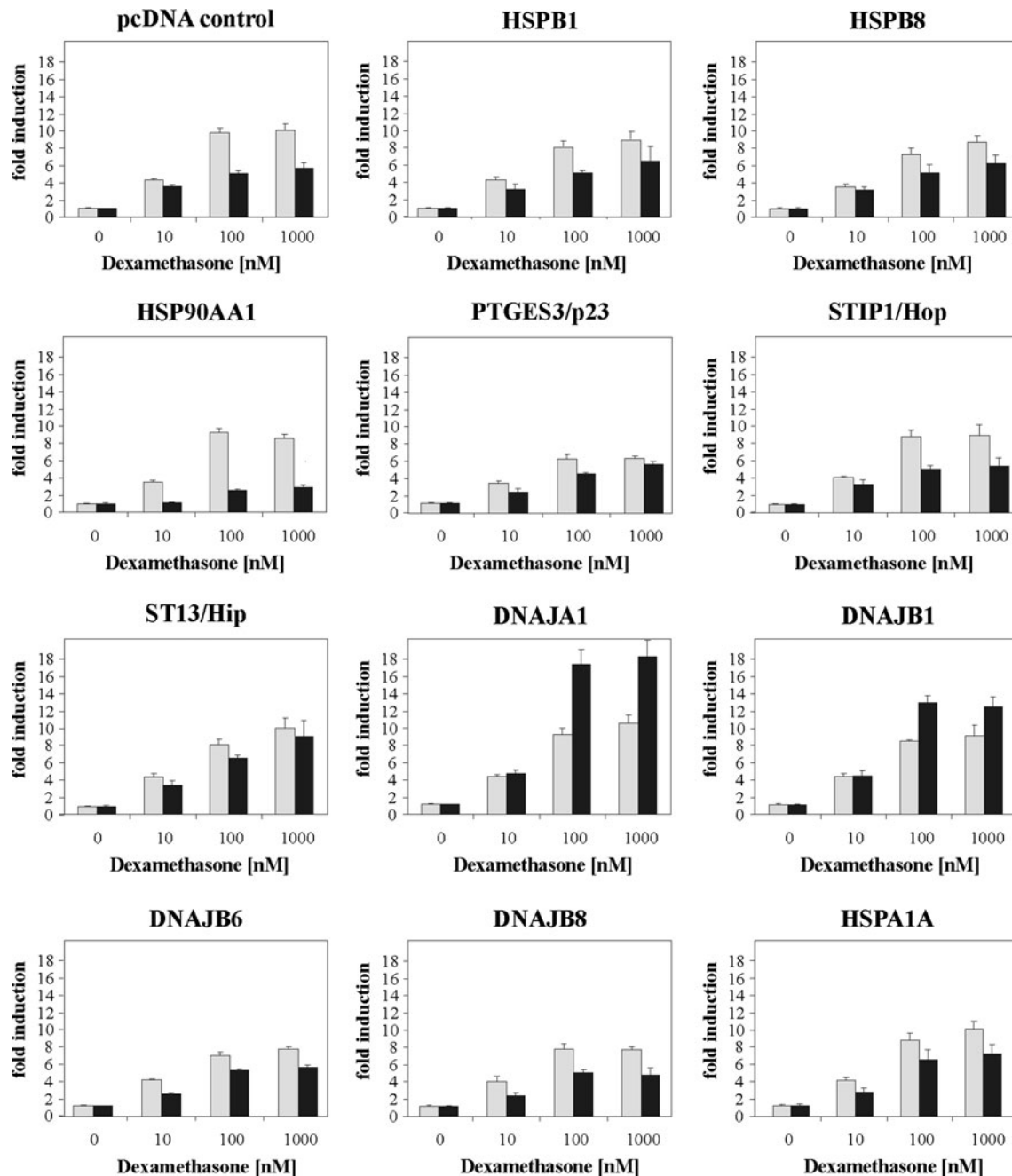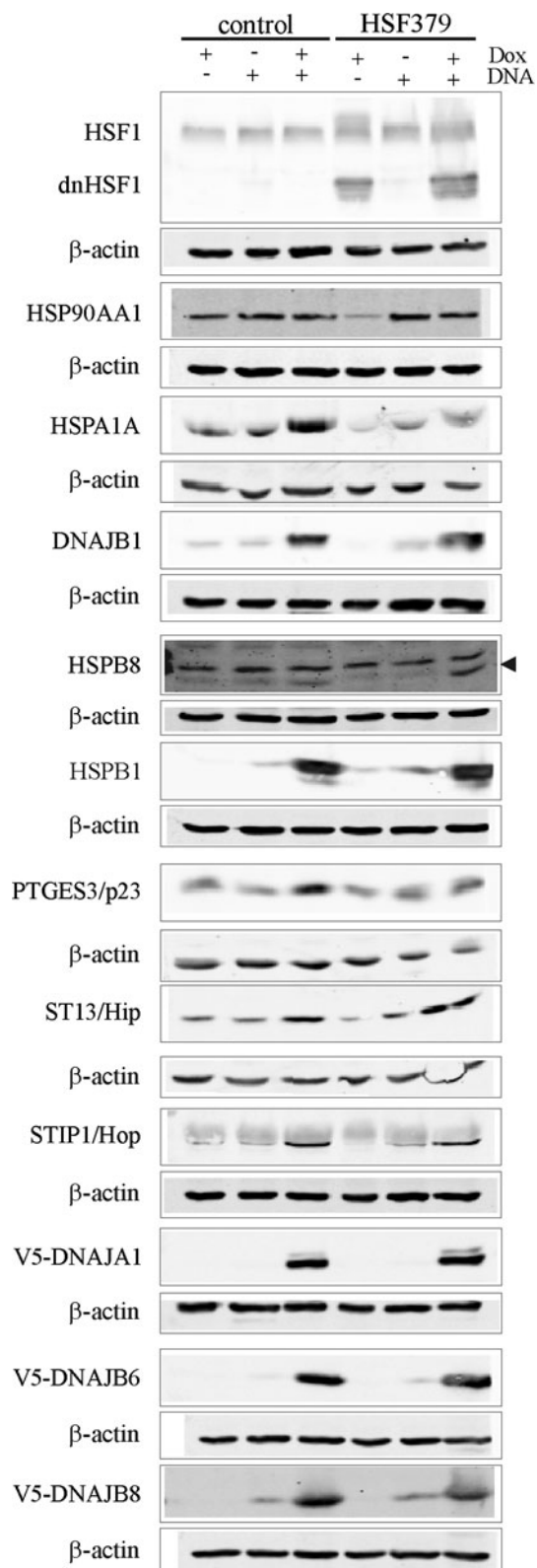
Fig. 7 Effect of overexpression of (co-)chaperones on glucocorticoid signaling in HEK-cDNA5 and HEK-dnHSF1 cells. Control HEK-cDNA5 cells (*light gray bars*) and HEK-HSF379 cells (*black bars*) were treated with doxycyclin. After 3 days, cells were transfected with a mixture (4:1:5) of glucocorticoid-responsive luciferase reporter (pGRE-Luc), a βactin-βgal reporter, and the expression construct indicated. 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 Fig. 6. Standard deviations are indicated by the *error bars*

by [42]). Apparently, overexpression 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 [62]. Our data also show that a lack of DNAJB1 can be compensated for by overexpression of DNAJA1. Functional redundancy between

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

Gene name	Dexamethasone (nM)		
	10	100	1,000
(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

The values represent av. ± SD

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 [64].

Heat stress or expression of a dominant-positive HSF1 mutant potentiates the glucocorticoid response [43, 44], 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 [65]. 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 significantly block the dexamethasone response (data not shown).

Maintaining proteostasis during ageing is expected to prevent or at least ameliorate age-related protein folding and inflammatory disease [6, 41]. One possible approach is

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 [63]. In the case of the progesterone receptor, it has

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 [38]. One potential drawback of this approach is that HSF1 also increases the risk of cancer, another often age-related disease [60]. 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.

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