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THE DIVERSE MEMBERS OF THE MAMMALIAN HSP70 MACHINE SHOW DISTINCT CHAPERONE-LIKE ACTIVITIES

Research Article

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SYNOPSIS

Humans contain many HSP70/HSPA and HSP40/DNAJ encoding genes, most of which are localized in the cytosol. To test for possible functional differences or/and substrate specificity, we assessed the effect of overexpression of each of these HSPs on refolding of heat-denatured luciferase and on the suppression of aggregation of a non-foldable polyglutamine (poly-Q) expanded Huntingtin fragment. Overexpressed chaperones that suppressed poly-Q aggregation were found not to be able to stimulate luciferase refolding. Inversely, chaperones that supported luciferase refolding were poor suppressors of polyQ aggregation. This was not related to client specificity per se, as the poly-Q aggregation inhibitors often also suppressed heat-induced aggregation of luciferase. Surprisingly, the exclusively heat-inducible HSPA6 lacks both luciferase refolding and poly-Q aggregation-suppressing activities. Furthermore, whereas overexpression of HSPA1A protected cells from heat-induced cell death, overexpression of HSPA6 did not. Inversely, siRNA mediated blocking of HSPA6 did not impair the development of heat-induced thermotolerance. Yet, HSPA6 has a functional substrate-binding domain and possesses intrinsic ATPase activity that is as high as that of the canonical HSPA1A when stimulated by J-proteins. *In vitro* data suggest that this may be relevant to substrate specificity as purified HSPA6 could not chaperone heat-unfolded luciferase but was able to assist in reactivation of heat-unfolded p53. So, even within the highly sequence-conserved HSPA family, functional differentiation is larger than expected with HSPA6 being an extreme example that may have evolved to maintain specific critical functions under conditions of severe stress.

INTRODUCTION

The HSP70 family of chaperone proteins is one of the most conserved protein families in evolution. It is found in all organisms excluding some hyperthermophilic archaea [12]. HSP70 proteins function in de novo protein folding and the suppression of protein aggregation under stress conditions [10,17,44]. After the sequencing of the human genome, it became apparent that the human HSP70 family (or HSPA Family) consist of 13 different proteins (excluding the four homologous HSP110/HSPH proteins) [2] (for the nomenclature of the human HSP members and some of their characteristics, see supplemental table 1). Although some of the gene expansion may have resulted to serve cellular compartmentalization, recent data obtained with protein localization prediction methods suggest that most of the HSPA proteins localize to the cytosolic and nuclear compartment [7,14] and it is currently unclear why eukaryotic cells need so many cytosolic/nuclear HSPA proteins.

In addition to 13 HSPA genes, over 49 different HSP40/DNAJ encoding genes have been found in the human genome [20]. DNAJ proteins have in common a conserved J-domain and were subdivided into three distinct groups (DNAJA, DNAJB and DNAJC) based on the presence certain structural motifs [6] that, however, do not edify functional properties [19]. All DNAJ proteins that have been investigated so far accelerate the ATPase activity of HSPA proteins; in conjunction with other cofactors such as BAG-1, HSPBP1, HSPH/HSP110, CHIP and HIP, they tightly regulate the activity of the HSPA machine. As for the HSPA proteins, most of the DNAJ proteins are predicted to localize to the cytosolic or nuclear compartment [14]. A recent detailed study in yeast revealed that cytosolic and nuclear DNAJ proteins show complex overlapping as well as unique properties [37]. However, for the mammalian situation, details on the regulation of HSPA proteins by DNAJ proteins are largely lacking.

HSP proteins in yeast have been divided in stress chaperones and “housekeeping“ or “CLIPS” (chaperones linked to protein synthesis)([1]. Indeed, chaperones have been found that are only expressed after (severe) stress conditions (e.g. HSPA6). In line with this, HSPA8 has been proposed as a “housekeeping” or CLIPS chaperone. However, other chaperones such as the human HSPA1A/B and DNAJB1 genes show basal expression under normal conditions as well as induction upon proteotoxic stress and the existence of a distinct CLIPS and HSP network in humans is currently unclear.

Recently, we have cloned the major part of the cytosolic/nuclear HSPH, HSPA, DNAJA and DNAJB family [14]. In this study, we used two potential chaperone clients with fundamentally different biochemical properties to compare the functionalities of these chaperones in living mammalian cells: (I) heat inactivated luciferase, known to be refoldable after heat denaturation both in vitro and in vivo [26-28]; (II) the genetically unstable, expanded polyglutamine (polyQ) containing Huntingtin, an aggregation prone protein that is unable to reach the native state [8]. Our data demonstrate that some HSP members that can stimulate luciferase cannot suppress polyQ aggregation. Inversely, several other members that suppress polyQ aggregation cannot stimulate luciferase refolding. This clearly suggests some degree of functional differentiation between the cytosolic/nuclear HSP. The most extreme example of this concerns HSPA6. This most strongly heat-inducible member of the human HSP70 family that show high sequence homology with HSPA1A lacks the canonical properties of HSP70s as a rather non-selective chaperone. Given its exclusive expression under conditions of heat shock and its high intrinsic, merely DNAJ-independent ATPase activity, we speculate that HSPA6 might serve to chaperone certain critical substrates under extreme stress conditions.

MATERIALS AND METHODS

Cell lines, cell culture and transient transfections

Both the hamster O23 cell line and Human embryonic kidney 293 cells stably expressing the tetracycline (tet) repressor (Flp-In T-Rex HEK293, Invitrogen) were grown in DMEM (Gibco) supplemented with 10% foetal bovine serum (Sigma) and 100 units/ml penicillin and 100µg/ml streptomycin (Invitrogen). Flp-In T-Rex HEK293 were kept under selective pressure for the tetracycline repressor and the Flp-in recombination site by adding 5 µg/ml Blasticidine (Sigma) and 100µg/ml of Zeocin (Invitrogen) to the culture medium. Cultures were maintained at 37°C and 5% CO_2 in a humidified incubator. For transient transfections, cells were grown to 50-60% confluence in 35 mm-diameter dishes coated with 0.001% of poly-L-lysine (Sigma) and/or on coated coverslips for confocal microscopy analyses. Cells were transfected with a total of 1 µg of DNA using Lipofectamine (Invitrogen) according to the manufacturer instructions.

Gene Cloning

Detailed information about the plasmids used in this study can be found in supplemental table 2 and 3. Briefly, tetracycline-inducible HSP expression plasmids were constructed as follows. First, the GFP and the V5 tag, harbouring a Kozak consensus ATG initiation codon and lacking a stop codon, were cloned in the pcDNA5/FRT/TO vector. Subsequently the coding sequence of the different chaperones was amplified using the primers listed in supplemental table 2. As a template source, cDNA was made from total RNA as previously described [13]. As a source of total RNA, QPCR Human reference Total RNA (Stratagene) was used. DNAJB4, DNAJB5 and DNAJB8 were amplified from cloned full-length cDNAs purchased from Open Biosystems (clone ID: DNAJB4: 4340658, DNAJB5: 4684829 and DNAJB8: 5296554). The fragments were cloned in pcDNA5/FRT/TO GFP no stop, and the presence of the correct gene was sequence verified. Protein expression was verified by Western blotting. Subsequently, fragments were subcloned to pcDNA5/FRT/TO V5 and pcDNA5/FRT/TO.

peGFP-HDQ23 and peGFP-HDQ74 were kindly provided by David Rubinsztein, Cambridge, UK.

siRNA

HSPA1A and HSPA6 gene knockdown was performed using siGENOME SMARTpool siRNA from Dharmacon. siRNA was transfected using lipofectamine 2000 at a final concentration of 50 nM. Cells were transfected with siRNA 96 hours before heat treatment and were again transfected with siRNA in combination with DNA 48 hours prior to heat treatment. Pre-heat treatment (to aquire thermotolerance) was given 18 hours before, so 78 and 30 hours after the two siRNA treatments respectively.

Focused Gene Arrays

Oligo GEArray Human Toxicology & Drug Resistance microarrays (OHS-401) were purchased from Superarray. RNA was isolated from HEK-293 cells 0, 1, 3 and 6 hours after a 30 minutes 45°C heat shock as described previously [16]. Subsequently, 3 µg of RNA was used in the first strand cDNA synthesis reaction as described by the manufacturer. Visualization was performed with Enhanced Chemiluminescence and Hyperfilm (ECL, Amersham) and the resulting images were scanned and processed.

Quantitative PCR

Relative changes in transcript level were determined on the Icycler (BioRad) using SYBR green supermix (Bio-Rad) as previously described [13]. Primers were designed with Perl primer. Calculations were done using the comparative C_T method according to User Bulletin 2 (Applied Biosystems). For each set of primers, the PCR efficiency was between 90 and 105%. Primer sequences are listed in supplemental table 2.

Cell extracts and samples preparation
24 or 48 hours after transfection cells were recovered by trypsinization, pelleted and resuspended in 1 ml of PBS. The cell suspension was centrifuged at 6000 rpm for 5 min at RT and the pellet was resuspended in 75-100 µl of RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 2% SDS plus protease inhibitors) and sonicated. Protein content was determined with the DC protein assay (Bio-RAD). Western blot samples were prepared at a final concentration of 1µg/µl in SDS-PAGE loading buffer and heated for 5 min at 100 ºC. Filter trap samples were prepared at a final concentration of 100 ng/µl, 20 ng/µl and 4 ng/µl in FTA buffer (10 mM Tris-Cl pH 8.0, 150 mM NaCl and 50 mM dithiothreitol) + 2% SDS and heated for 5 min at 100 ºC. Samples were used immediately or kept frozen at -20ºC.

Western blot analysis
Equal amounts of protein were loaded on 10% or 12,5% SDS-PAGE gels. Proteins were transferred onto nitrocellulose membranes and probed with mouse anti-GFP antibody JL-8 (Clontech) at a 1:5000 dilution, mouse anti-V5 antibody (Invitrogen) at a 1:5000 dilution, rabbit AR (N20) antibody (Santa Cruz) at a 1:2000 dillution. GAPDH was used a loading control and was detected with a mouse antibody (RDI Research Diagnostics) at 1:10000 dilution. Blots were subsequently incubated with HRP-conjugated anti-mouse secondary antibody (Amersham) at 1:5000 dilution and visualization was performed with Enhanced Chemiluminescence and Hyperfilm (ECL, Amersham).

Filter trap assay
To determine protein aggregates, the filter trap assay was performed as previously described [5]. Briefly, 10, 2 and 0.4 µg of protein extracts were applied onto 0,2 µm pore Cellulose Acetate membrane prewashed with FTA + 0.1% SDS. Mild suction was applied and the membrane was washed 3 times. Aggregated proteins trapped in the membrane were probed with mouse anti-GFP antibody JL-8 (Clontech) at a 1:5000 dilution and mouse anti-V5 antibody (Invitrogen) at a 1:5000 dilution followed by HRP-conjugated anti-mouse secondary antibody (Amersham) at 1:5000 dilution. Visualization was performed using enhanced chemiluminescence and Hyperfilm (ECL, Amersham).

Luciferase measurements
Cell lysis and luciferase activity measurements were done as previously described [27]. Luciferase activity after treatments were expressed relative to the activity in unheated control cells (=100%). Error bars in plots represent standard deviations. In the case of a single experiment, the standard deviation was calculated according to the rules of error propagation. In the case of multiple experiments, standard deviations were calculated directly from the differences in the percentages of luciferase activity compared to an unheated control.

Ni-NTA precipitation of His tagged proteins
Cells were trypsinized and washed with PBS. Subsequently, the cross-linker DSP was added to a final concentration of 1 mM and incubated for 30 minutes on ice. The cross-linking was stopped by the addition of glycine to a final concentration of 2 mM and incubated for 15 minutes on ice. Cells were washed with PBS and the cell pellet was harvested in 0.5 ml of lysis buffer containing 150 mM NaCl, 50 mM NaH2PO4, 10 mM imidazole, 0.5% NP-40 (igepal), 1.5 mM $MgCl_2$, 3% glycerol 0.9 mM DTT and protease inhibitors, pH 8.0. Cells were lysed by passage through a 26G needle for 5 times and subsequently centrifuged twice at 14000 rpm for 15 minutes. Then, 30 µl of supernatant was taken and mixed with 30 µl 2X Laemli sample buffer. The rest of the supernatant was transferred to a new tube and 10 µl Ni-NTa agarose beads (Qiagen) was added and incubated at 4 °C for 1 hour with slow agitation. Next, the beads were

washed 4 times with wash buffer containing 300 mM NaCl, 50 mM NaH_2PO_4, 20 mM imidazole, 0.5% NP-40 (igepal), 1.5 mM $MgCl_2$, 3% glycerol, pH 8.0. The pellet was eluted in 30 µl lysis buffer and 30 µl 2X Laemli sample buffer and boiled. 10 µl was subsequently loaded for Western blot analysis.

Immunolabelling and confocal microscopy

16-24 hours after transfection indirect immunofluorescence of the V5 tag was performed to detect the chaperones. Cells were fixed with 3.7% formaldehyde for 15 minutes, washed three times with Phosphate-Buffered Saline (PBS), permeabilized with 0.2% Triton-X100 and blocked during 30 minutes with 0.5% BSA and 0.1% glycine in PBS. Incubation with mouse anti-V5 monoclonal antibody (Invitrogen) 1/100 dilution was performed overnight at 4ºC followed by a 1 hour incubation with CY5-conjugated anti-mouse secondary antibody (Jackson) at 1:200 dilution. Aggregates were visualized using the eGFP tag. To visualize nuclei, cells were stained 10 minutes with 0.2 µg/ml 4',6-diamidino-2-phenylindole (DAPI). Coverslips were mounted in Citifluor. Images of CY5 and DAPI fluorescence were obtained using the Leica confocal laser scanning microscope (Leica TCS SP2, DM RXE) with a 63X/1.32 oil lens. The captured images were processed using Leica Confocal Software and Adobe Photoshop.

In vitro ATPase, luciferase refolding and citrate synthase aggregation protection assay

HSPA1A and HSPA6 were cloned in the pET151 prokaryotic expression system (Invitrogen) and isolated according to the manufactures procedures. DNAJB1 was purified as described before [21]. ATPase experiments were performed as previously described [11]. Luciferase refolding assay was a modified version of the one described [42]. Luciferase (Promega, 12.66 mg/ml) was diluted 100 times in the mixture containing 10 µM HSP90, 25 mM Tris, pH 7.8, 8 mM $MgSO_4$, 1% BSA, 10% glycerol, 0.25% Triton X-100 and incubated for 20 min at 40 ºC. Then the renaturation process was carried out at room temperature in the buffer containing the following components at final concentrations: 40 mM TRIS (7.4), 50 mM KCl, 5 mM DTT, 15 mM $MgSO_4$, 100 µg/ml creatinine kinase, 20 mM creatinine phosphate, 0.015%BSA, 5 mM ATP, 80 nM denatured luciferase, 0.4 µM HSP90, 1 µM DNAJB1 and appropriately 2.4 µM HSPA1A or HSPA6. At time points 0-80 min, 5-µl aliquots were taken, and the activity of renatured luciferase was measured in a luminometer (BMG Labtechnologies) after addition of the Bright-Glo substrate (Promega).

The citrate synthase aggregation protection assay was performed as previously described [38].

Cellular survival assays

Overexpression: Cells stably expressing HSPA1A and HSPA6 under the tetracycline inducible promoter were treated with tetracycline to induce expression and incubated for 24 hours. Knockdown: cells were treated with siRNA as stated above and incubated for 72 hours. Subsequently, cells were heat shocked for 30 minutes at 45 °C or left at 37 °C and incubated at 37 °C for 18 hours to acquire maximal thermotolerance as previously shown [16]. Subsequently, cells were treated with a second heat shock of 45 °C for 15, 30, 45, 60 and 120 minutes and plated in triplicate (200 cells) on 10-cm petri dishes in 10 ml of culture medium containing tetracycline and allowed to grow for 14 days. The colonies were fixed and stained, and colonies containing >50 cells were scored.

Bioinformatics

Primary amino acid alignments were performed in ClustalX2 using the neighbour-joining algorithm and Blosum matrixes at the default settings [22]. Bootstrap analysis was performed using 1000 random number generator seeds and 1000 bootstrap trials. Phylograms were made by importing the homology tree output of ClustalX in TreeView [35].

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RESULTS

Expansion of DNAK-like and DNAJ-like chaperones in the three branches of life

The reason for having high chaperone gene numbers in human cells is currently unclear. Possible evolutionary forces to drive chaperone gene duplication are compartmentalization, emergence of multi-domain proteins, multi-cellularity, cellular specialization and development (Figure 1A). The HSPA/DNAK/DNAJ gene families are the most abundant HSP encoding gene families found in the human genome. DNAK and DNAJ genes were lacking in the archea *T. kodakaraensis* and *M. jannaschii* (Figure 1B and C) but were found in other archea such as *T. acidophilum* and *M. mazei*. Both DNAK and DNAJ sequences were found in all eubacteria analysed. The number of DNAK/HSPH/HSPA dramatically increased in eukaryotes (Figure 1B), which was partially due to an increase in the number of HSPA genes (Figure 1B). However the number of HSPA genes did not change markedly within eukaryotes while the DNAJ gene numbers differed from around 20 genes in yeast up to over 49 in humans and over 80 in plants (Figure 1C). These data suggest that a higher number of DNAJ proteins seem to specialize the HSPA chaperone machines [19].

Humans contain a highly homologues subfamily of cytosolic HSPA family members

One reason for increases in the gene numbers of DNAK/HSPA/HSPH between prokaryotes and eukaryotes could be cellular compartmentalization. Neighbour-joining phylogeny analysis and computational subcellular localization prediction methods show that all the HSPH/HSP110-like members cluster together in one subfamily and are cytosolic/nuclear proteins (Figure 2A) except HSPH4/Grp170, which is expressed in the ER. HSPA13/HSPA14 and HSPA12A and HSPA12B are distinct members of the HSPA family and are poorly studied. A very homologous subfamily includes HSPA1A/B, HSPA1L, HSPA2, HSPA6, and HSPA8 that all are predicted to be cytosolic/nuclear. The slightly more distantly related members HSPA5 and HSPA9 are the ER and the mitochondrial equivalents of HSPA8 respectively. Ectopic expression of the V5-tagged members was in line with the predicted localization (Figure 2A). Thus, although part of the HSPA family did expand due to cellular compartmentalization, most of the expansion was independent of this event and these members may exert their specialized functions within the cytosolic/nuclear compartment.

Many DNAJ members are predicted to localize to the cytosolic and nuclear compartment

A recent study in yeast showed that most DNAJ members are cytosolic proteins [37]. Bioinformatics predicts that three of the four human DNAJA members localize in the cytosol (Figure 2B). Neighbour-joining phylogeny analysis pointed to the existence of two subfamilies within the DNAJB family. The first family shows homology to DNAJB1, DNAJB4 and DNAJB5 whilst the second consists of the members DNAJB2, DNAJB6, DNAJB7 and DNAJB8 (Figure 2C). For both subfamilies, most members were predicted to be in the cytosol or nucleus (Figure 2C). For the members that we use in this study this could be confirmed for the ectopically expressed V5-tagged proteins (Figure 2C). These data indicate that also in cultured human cells most DNAJ members are cytosolic/nuclear proteins.

Expression profiles of HSPH, HSPA, DNAJA and DNAJB chaperones

As most of the HSP proteins are located in the cytosolic/nuclear compartment, we used qPCR to precisely analyze the constitutively expressed levels and the heat inducibility of HSPH, HSPA, DNAJA and DNAJB transcripts in HEK-293 cells (Figure 3A-C). These results show that HSPH1 is the major heat inducible HSPH gene (Figure 3A) and also the most abundant member under non-heat conditions (Figure 3B). HSPA1A/B, HSPA1L and HSPA6 are the major heat inducible HSPA genes within the HSPA family (Figure 3A). Interestingly, messenger RNAs

for both HSPA1A and HSPA6 are not detected under non-stress conditions but constitute a significant part of the HSPA transcript levels after heat stress (Figure 3B). Although HSPA1L shows high heat-inducibility, the transcript levels after heat shock remain relatively low (Figure 3B). HSPA1B is expressed at high levels and is heat inducible, while HSPA8 and HSPA9 are also expressed at high basal levels but are hardly heat inducible (Figure 3A and 3B).

For the DNAJ family, we found DNAJB1 and DNAJB4 to be the major heat inducible DNAJ genes; DNAJB6, DNAJB9 and DNAJB11 were only mildly inducible and the rest of the members investigated were not upregulated after heat stress at all (Figure 3A). Under normal growth conditions, DNAJA1 was found to be the most abundant DNAJ transcript (Figure 3C). DNAJA3, DNAJB1, DNAJB6 and DNAJB11 were also found to be expressed under non-stress conditions but only at moderate levels (Figure 3C). Interestingly, DNAJB1 was found to be by far the most abundant DNAJ transcript after heat stress (Figure 3C). These data are consistent with previous studies that show that DNAJB1 is the major partner of the heat inducible HSPA1A/B protein. Whether it also serves as a co-factor for HSPA6 is currently unknown. So surprisingly, only a minority of the HSPA and DNAJ family is expressed in HEK-293 cells and only a small number of these genes are heat inducible.

To test the generality of these findings in HEK-293 cells, we measured the global constitutive expression pattern of various HSPH, HSPA and DNAJ encoding genes in various cell lines and human tissues. For this purpose, dedicated mini arrays were used which contain a relative large number of chaperones. Although expression levels for some members such as HSPA1A, DNAJC8 and HSPA9 varied widely between cell lines, we found that the profile of chaperone expression was surprisingly similar amongst the cell types investigated indicative that our findings in HEK-293 cells are representative and do not largely deviate from the other cell-lines tested (Figure 3D). Also the heat-inducibility pattern of the investigated HSPs in Hela cells [14] was comparable as that observed in HEK-293 cells (Figure 3 A-C; supplemental figure 1). In fact, data from the public data bases on HEp2, a human epithelial cell line and U397, a human lymphoma from myelomonocytic origin also revelead the same pattern of (non) heat-inducibility of the various HSP members used here (supplemental figure 1), strongly supporting the idea that the heat-inducibility of the selected HSP genes is the widely conserved.

Stimulation of protein refolding and aggregation suppression is facilitated by distinct chaperone members

To explore putative differences in function, we expressed each of the selected chaperones in HEK-293 cells and performed two functional tests. Firstly, firefly luciferase was co-expressed with the chaperones and cells were heated to measure the refolding of luciferase after the heat stress. Expression of chaperones such as HSPA1A have been found to enhance the refolding of heat-denatured luciferase [26,29]. Secondly, Huntingtin fragments with expanded polyglutamine were co-expressed with the selected chaperones. These polyglutamine expanded proteins are known to aggregate and expression of chaperones such as DNAJB1 have been found to suppress its aggregation [18,36]. We used versions of luciferase and the expanded polyQ proteins that are expressed in both the cytosol as well as the nucleus simultaneously [16] because all cloned chaperones except DNAJA3 and DNAJB9 were predicted or found expressed in the cytosol and/or nucleus (Figure 2B, C and data not shown).

As shown in Figure 4A, HSPA1A showed the highest activity on luciferase refolding whilst various HSPA paralogs such as HSPA1L, HSPA2 and HSPA8 showed some, albeit lower activity. HSPA14 did not show an effect on refolding, but this was not unexpected as this member does not contain the canonical substrate binding domain and is known to have a specialized function in translation [19]. Unexpectedly, however, also the HSPA6 paralog showed no activity on luciferase refolding (see below). In addition, the *E. coli* DNAK or *Sacharomyces cerevisiae* SSA-1, both well known to support luciferase refolding in the host organisms [4,39]

did not support luciferase refolding in Hek293 cells (Figure 4A). Wilst SSA1 was not expressed to comparable levels as the other chaperones, DnaK was expressed at similar levels as HSPA1A. It's lack of refolding stimulating activity may suggest that co-factors of the mammalian HSPA machine may be unable to cooperate with non-mammalian chaperones. In addition to HSPA members, overexpression of the DNAJA2 and DNAJA4 also resulted in stimulation of luciferase refolding. All other DNAJ members tested were not effective or even slightly suppressed refolding, although for some members (DNAJA3, DNAJB4 and DNAJB5) the data were inconclusive as they were only expressed at low or undetectable levels.

We next studied the capacity of the same set of chaperones to suppress the aggregation of a 74 glutamine-containing Huntingtin exon-1 fragment. As shown in Figure 4C, the most active aggregation suppressors were DNAJB1, DNAJB2, DNAJB6 and DNAJB8. Interestingly, these best preventers of polyQ aggregation did not support refolding (Figure 4A). Inversely, none of the members that enhanced luciferase refolding (figure 4A) could suppress polyQ aggregation (figure 4C). One possibility to explain these results is substrate specificity. To test whether this could explain the differential effects of the chaperones on refolding luciferase or on poly-Q aggregation suppression, we tested the effect of the various chaperones on luciferase aggregation. Hereto, cells were heated, lysed and the lysates were run on SDS PAGE. Heat denatured luciferase can be detected as SDS insoluble aggregates in the stacking gel (Figure 4D). These aggregates physically differ from polyQ aggregates, as they are not retained in a filter trap assay (data nor shown). Yet, the subset of chaperones that were most potent in suppressing polyglutamine aggregation (DNAJB1, DNAJB2a, DNAJB6 and DNAJB8) also substantially suppressed luciferase aggregation (Figure 4D) without supporting its refolding (Figure 4A). Inversely, however, the two strongest supporters of refolding (HSPA1A and DNAJA4) also suppressed luciferase aggregation, yet were rather ineffective in preventing polyglutamine aggregation. It must be noted that their reduction of luciferase aggregation might merely be a reflection of their stimulating effect on luciferase refolding. So, the difference of the various members to prevent aggregation or stimulate refolding is not due to difference in substrate specificity; rather, the data suggest that the different HSPs have different client affinity and/or differently affect client processing.

To exclude the possibility of cell-type specific effects, we next tested the effects of the subset of chaperones on refolding and polyglutamine aggregation suppression in hamster lung fibroblast cells (O23) (Figure 5A and B). In the O23 hamster fibroblasts, refolding of luciferase (Figure 5A) and suppression of polyQ aggregation (Figure 5B) was supported by the same set of different chaperones as in HEK-293 cells, with the exception of DNAJB5, which was active for the suppression of polyQ aggregation in O23 but not HEK-293 cells (Figure 5B). Together, these data indicate that higher eukaryotic cells seem indeed equipped with at least two molecular distinct chaperones activities. One set to suppress protein aggregation and one set able to support refolding of denatured substrates (Figure 5C).

HSPA6 is a heat inducible HSP70 member distinct from HSPA1A and HSPA1B

One very striking observation was that the strongly heat-inducible HSPA6 (Figure 3), one of the least investigated HSPA members and probably the most recently evolved member that exists only in large mammals [32], did not support luciferase folding like the strongly heat-inducible HSPA1A. Yet, HSPA6 shows high sequence homology to HSPA1A (81% identity) and has been linked to cellular heat responses especially when grown at low cell densities (26). Compared to HSPA1A, HSPA6 shows the highest degree of divergence in the C-terminal lid domain (Figure 6A). To investigate whether this C-terminus may have evolved to serve different functions of HSPA6, aside from those tested here, we constructed chimeras of HSPA1A and HSPA6 using two shared restriction sites (Figure 6B). One site (Van91I) is positioned in the middle of the ATPase domain and the other (PstI) is located near the outer boundary of the ATPase domain (Figure 6B). Surprisingly, the chimeric proteins containing the C-terminus of HSPA6 fused to the

N-terminal ATPase fragment of HSPA1A were found to be fully active in supporting luciferase refolding. In contrast, both chimeric proteins containing the ATPase domain from HSPA6 were ineffective (Figure 6C). We next repeated this experiment in a HEK-293 cells depleted for HSPA1A and HSPA1B using shRNA and found essentially the same results (Figure 6D). In addition, experiments in hamster lung fibroblast O23 lacking basal expression of HspA1A/B, showed similar results (data not shown), implying that the effect of the chimera were not due to formation of functional interactions with the wild type endogenous HSPA1 members. These data thus show that the complete C-terminus including the spacer domain, the substrate binding domain as well as the lid domain from HSPA6 are functional and can recognise firefly luciferase as a client like HSPA1A. Yet, efficient refolding is not supported likely due to a difference in the more homologous ATPase domain One possibility is that the HEK293 cells do not contain the required (heat-inducible) co-factors needed for adequate regulation of the HSPA6 ATPase that leads to refolding activity. To test whether HSPA6, which is exclusively expressed under heat shock conditions, depends on other heat co-inducible factors, we primed cells with a short heat shock in the presence or absence of ectopically expressed HSPA6 or HSPA1A. Such priming leads to a resistance to second heat treatments, referred to thermotolerance (TT) which amongst others effects, improves luciferase refolding [16,29] (Figure 6E). Ectopically expressed HSPA1A lead to an even further improvement of refolding in thermotolerant cells but this effect was not seen after over-expression of HSPA6 (Figure 6E). Thus also after the induction of “all” heat-inducible factors, HSPA6 remained dysfunctional in refolding luciferase. Inversely, we blocked the heat shock induced expression of HSPA6 or HSPA1 by RNAi during thermotolerance development. As shown in supplemental Figure 2, the expression of both HSPA1A and HSPA6 could be blocked selectively and efficiently. Using these selective RNAi molecules, we found that RNAi against HSPA1A significantly reduced luciferase refolding not only in control or HSPA1A transfected cells but also in thermotolerant cells (Figure 6F). In contrast, the knockdown of exogenous HSPA6 expression did not show a reduction in refolding under all these conditions (Figure 6F). Thus, HSPA1A but not HSPA6 contributes to enhanced luciferase refolding during thermotolerance and the absence of HSPA6 activity for this endpoint seems not to be due to a lack of heat inducible co-factors.

It still could be argued that the HEK293 cells may lack the correct DNAJ partner, even after priming the cells with a heat shock, required to support HSPA6. Therefore, we tested if there were differences in the spectrum of DNAJ proteins that could physically interact with either HSPA1A or HSPA6. We used Ni-NTA precipitation followed by Western blot analysis to investigate this. Like HSPA1A, also HSPA6 was found to physically interact with all DNAJ members tested (Figure 7) in a specific manner since none of the V5-tagged DNAJ proteins bound to the Ni-NTA beads (Figure 7). HSPA1A and HSPA6 did not interact with either Troponin I or Troponin T, two proteins unrelated to molecular chaperones further demonstrating that the interaction is specific. These results not only indicate that the DNAJ domain is the main determining factor for binding of DNAJ to HSPA proteins, but also reveal that differences in specificity for DNAJ proteins does not underlie the observed difference between HSPA1A and HSPA6 in simulating luciferase refolding..

We next set up a series of *in vitro* cell free experiments to directly analyze the functionality and ATPase activity of HSPA6. HSPA1A and HSPA6 were purified and incubated alone or together with purified DNAJB1 to measure their *in vitro* steady state ATP hydrolysis activities. Surprisingly, the ATPase of HSPA6 was found not be defective, but its activity was even higher than that of HSPA1A (Figure 8A). DNAJB1 stimulated the ATPase of HSPA1A, but even than, the activity was lower than for HSPA6 without DNAJB1. Moreover, the ATPase activity of HSPA6 was not (further) enhanced by DNAJB1 (Figure 8A). Differences in the ATPase activity of Hsp70 members were previously to exist for the yeast Hsp70s, Ssa and Ssb, and these differences were suggested to be potentially relevant for the functional uniqueness of these Hsp70s [24]. To test whether such may indeed be true, we compared the activity of

purified HSPA1A and HSPA6 on 3 different substrates. First, we heat denatured purified firefly luciferase in the absence or presence of HSPA1A and HSPA6 with and without DNAJB1 Consistent with our cellular data, HSPA6 either alone or together with DNAJB1 showed no detectable activity on luciferase refolding after thermal denaturation, whereas HSPA1A together with DNAJB1 clearly supported refolding (Figure 8B). We next measured the ability of HSPA6 to suppress aggregation of the thermosensitive enzyme citrate synthase using a citrate synthase aggregation protection assay. As shown in Figure 8C, the presence of recombinant HSPA1A suppressed the heat-induced aggregation of citrate synthase while recombinant HSPA6 did not (Figure 8C). Finally, we used tumour suppressor p53 for which it was recently shown that HSPA1A can restore its activity after heat shock [42,43]. This chaperone activity involves a remodelling of the p53 tetrameric complex, which might depend on mechanistically distinct chaperone characteristics than assisting in the renaturation of heat-unfolded monomeric proteins. Interestingly, now HSPA6, like HSPA1A, did show a chaperone-like activity as it was found to rescue the heat-induced impairment of human recombinant wild-type p53 to bind to the *p21/WAF1* promoter (Figure 8D). In fact, HSPA6 was even more efficient in rescuing p53 activity and, unlike HSPA1A, did not rely on the presence of J-proteins.

Since HSPA6 is exclusively expressed under conditions of severe stress [31,33] we wondered how its rescue of p53 activity and maybe other critical clients or structural elements in cells would affect cell survival after a severe heat shock. Hereto, HEK293 cell lines were generated that stably express either HSPA1A or HSPA6 under control of tetracycline-regulated promoters (Figure 9A). Whereas expression of HSPA1A reduced heat-induced mortality, measured using the colony formation assay, expression of HSPA6 did not show such cytoprotective effects (Figure 9B). To test whether HSPA6 might require co-factors that are only expressed in heat stressed cells to protect cells, we also used thermotolerant HSPA1A and HSPA6 expressing cell lines. Thermotolerant cells over-expressing “all” heat-inducible HSPs showed a high level of resistance towards heat induced cell killing (Figure 9B) that was much higher than the resistance seen for HSPA1A overexpression as published before [29]. The over-expression of HSPA1A (or HSPA6) in thermotolerant cells did not show a supertolerant phenotype indicating that a maximal survival is reached by the general induction of heat stress proteins under these thermotolerant conditions (Figure 9B). Therefore, the effects of HSPA6 expression in thermotolerant cells could not be assed using over-expression. We therefore selectively depleted either HSPA1A or HSPA6 or blocked their induction during thermotolerance development by the selective RNAi described above (Figure 9C). Whilst siRNA against HSPA1A strongly enhanced heat-induced cell killing in both control and thermotolerant cells (Figure 9D), the knockdown of HSPA6 showed no effect for both conditions. Together, our data show that the strongly heat-inducible HSPA6 appears to play no significant role in protecting HEK-293 cells against heat-induced cell death.

DISCUSSION

Human cells contain many members of the HSP70/HSPA and DNAJ/HSP40 family and most members of the HSPA and DNAJ family, that form the core of HSP70 machines, were found to reside in the cytosolic and nuclear compartment of cells. Except for those that could not be studied due to poor expression, we found that their ectopic overexpression resulted in different phenotypes with regards to the handling of two fundamentally different clients: refoldable, heat denatured luciferase versus non-refoldable, rapidly aggregating polyQ proteins. One subset shows a high activity to stimulate luciferase refolding while the second subset was very effective in suppressing polyQ aggregation. This was not related to client specificity per se, as the poly-Q aggregation inhibitors also suppressed heat-induced aggregation of luciferase. The probably largest functional differentiation was seen for HSPA6 that, despite its high sequence homology to HSPA1A, could not handle a typical Hsp70 client like heat-unfolded luciferase or citrate synthase, but that did rescue p53 activity after heat denaturation.

Although our comparison involved only two different clients, they prompt us to propose a (admittedly simplified) model on how different HSP members may primarily act on two fundamentally different classes of substrate, i.e. those that are potentially (re)foldable (generated by acute stresses) and those that are non-(re)foldable proteins (due to chronic stresses). Many acute stresses (like heat shock) will cause unfolding of normally native proteins that, upon stress relief, can ultimately regain their original native state. Strikingly, most of the HSPs that were effective in refolding luciferase (Figure 4 and 5) were heat inducible (Figure 3 and supplemental Figure 1). In contrast, aggregation of non-refoldable proteins like polyQ proteins is prevented by other members, most of which are not (strongly) heat inducible. (Figure 3-6, supplemental Figure 1). In fact, we recently found a similar paradigm for members within the HSPB family of small HSP in which the strongest heat-inducible HSPB1 supported luciferase refolding, without showing a major effect on polyQ aggregation whereas members like HSPB7, HSPB8, and HSPB8 did not support luciferase refolding but strongly suppressed polyQ aggregation [41]. Intriguingly, all these three polyQ aggregation inhibiting HSPB members were directly involved in protein degradation pathways [40,41]. So, it appears that a specialized subset of cytosolic chaperones may have evolved to dispose (rather than refold) non-foldable proteins as those linked to inherited genetic mutations (e.g. polyglutamine expanded Huntingtin, CFTR, or alpha-synuclein). What the molecular basis for this differentiation in client recognition and processing is, remains to be elucidated. However, it is striking to note that for stimulating luciferase refolding Hsp70 is often limited [25]. Also, including for effects of HSPB1, refolding always entirely dependent on an adequately regulated nucleotide cycle of the Hsp70 machine because accelerating this cycle with the Hsp70 nucleotide exchange factor BAG1 inhibits refolding [3,30]. In contrast, for polyQ the effects of the best inhibitors of aggregation in both DNAJ families and HSPB families are largely independent of Hsp70 and not inhibited by BAG-1 [15,41].

The most striking functional diversity found in this study concerns HSPA6. This Hsp70 member that is only found in large mammals is not expressed at 37°C but shows a strong induction after heat-stress [31]; this report) and after proteasome inhibition [33] especially when cells are grown at low density [34]. Yet, HSPA6 was unable to assist refolding of heat-unfolded luciferase (in cells and *in vitro*) or prevent aggregation of citrate synthase (*in vitro*), activities that were seen for all other cytosolic HSPA members (except HSPA14 that lacks the full peptide binding domain) Domain swapping experiments showed that HSPA6 has a functional peptide binding domain but an abnormal N-terminal ATPase domain that was related to its inability to chaperone luciferase. *In vitro* experiments next revealed that the (intrinsic) basal ATPase activity of HSPA6 is much higher than that of HSPA1A again pointing to the possibility that the nucleotide cycle is critical for the general chaperone-like activity of Hsp70 proteins (see above). Indeed, also for DnaK, stimulation of luciferase refolding was reduced when the ATPase activity was too high [23]. Similarly, deleting the C-terminal EEVD motif from HSPA1A resulted in an

increased ATPase activity and loss of chaperone activity *in vitro* [9] and reduced activity in cells [25]. Intriguingly, the ATPase activity of the ΔEEVD mutant and its (residual) chaperone activity could no longer be boosted by DNAJ proteins [9,25] resembling the effects we show here for the ATPase activity of HSPA6 (Figure 8A). Strikingly, however, HSPA6 was able to rescue p53 activity after thermal denaturation even more effectively than HSPA1A. So, higher rates of intrinsic ATPase activity of Hsp70 may be disadvantageous for certain substrates (luciferase, citrate synthase) while being advantageous for other substrates (p53). Also in yeast, the ATPase activities amongst different Hsp70s were found to vary significantly [24]. Whereas, these differences were mainly attributed to differences in the C-terminal domains, our domain swapping experiments suggest that for HSPA1A and HSPA6 the functional differences we found are intrinsic to the ATPase domain itself. Yet, irrespective of the mechanism responsible for the different ATPase activities, these differences in the ATPase activity may be relevant to the functional uniqueness of Hsp70s. Another striking feature of the purified HSPA6 was that – unlike HSPA1A- it did not require additional J-proteins to rescue heat denature p53 activity. Although we do not completely rule out that our purified HSPA6 contain traces of prokaryotic DNAJ proteins, this would suggest that, given its higher basal ATPase activity than HSPA1A, HSPA6 might be able to chaperone a subset of critical, special clients under conditions where the availability of J-proteins is limited.

Whatever the physiological function of HSPA6 and its chaperone action on p53 (and maybe other) clients, HSPA6 upregulation or its depletion did not affect the clonogenic ability of naïve or primed (thermotolerant) HEK293 cells after heat shock. As such, it clearly differs from HSPA1A, implying that, after heat shock, canonical protein damage-repair type of activities as stimulated by HSPA1A are more critical compared to the more specialized activities of HSPA6 for the reproductive ability of cells like HEK293.

In summary, we have shown that cells seem to be equipped with many functionally distinct subsets of chaperones, some of which that seem dedicated to (re)folding and some that may have evolved to dispose non-foldable proteins. Even more so, within the highly sequence conserved HSPA family, functional differentiation is larger than expected with HSPA6 being an extreme example, lacking both the generic chaperone-like properties of other HSP70s and suggesting that it has evolved to maintain specific critical functions under conditions of severe stress.

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FIGURE LEGENDS

Figure 1. DNAJ but not HSPA genes have been duplicated during the evolution of eukaryotic life.
(A) Hypothetical events during evolution responsible for chaperone gene duplication and specialization.
(B) Number of DNAK/HSPA like genes as determined by genome BLAST and literature.
(C) Number of DNAJ like genes as determined by genome BLAST and literature.

Figure 2. Most HSPH/A and DNAJ proteins are expressed in the cytosol or nucleus. Phylogenetic relationship and subcellular localization of HSPH, HSPA, DNAJA and DNAJB proteins.
Phylograms (left panels) were made from primary amino acid sequences using ClustalX2. Bootstrap values of 1000 random trials are indicated on each node of the phylogram. Combined data from literature, the human protein reference database and protein localization prediction programs were used to predict subcellular localizations (middle panels). Experimentally determined localization was performed by immunolabeling of transfected V5 tagged HSP constructs followed by confocal microscopy image analysis (right panels). (A) HSPH/HSPA family, (B) DNAJA family, (C) DNAJB family.

Figure 3. Expression of the HSPH/A and DNAJ under normal growth conditions and after heat stress.
(A) Heat inducibility of HSPH/A and DNAJ members in HEK-293 cells measured by qPCR analysis. Samples were taken without heat shock, after 1 hour, 3 hours and 6 hours of heat shock of 45 °C for 30 minutes. Relative transcript expression levels were normalized to GAPDH.
(B) Pie representation of semi-quantitative HSPH/HSPA transcript level determination using subtractive Ct comparison. Heat shock condition refers to 30 minutes of heat shock at 45 °C followed by 3 hours recovery at 37 °C. The surface area for GAPDH was set equal for the minus heat shock and plus heat shock condition.
(C) Pie representation of semi-quantitative DNAJ transcript level determination using subtractive Ct comparison. Heat shock condition refers to 30 minutes of heat shock at 45 °C followed by 3 hours recovery at 37 °C. The surface area for GAPDH was set equal for the minus heat shock and plus heat shock condition and the GAPDH of the HSPH/HSPA pie chart from (B).
(D) Mini oligo gene array showing HSPH/HSPA and DNAJ transcript levels in various human cell lines and tissues.

Figure 4. Luciferase refolding and suppression of aggregation is mediated by distinct subsets of molecular chaperones in HEK-293 cells.
(A) Luciferase encoding plasmids were co-transfected with plasmids encoding mRFP (control) or encoding the indicated V5 tagged chaperones. Cells were heated for 30 min at 45 °C to inactivate luciferase and after a recovery period at 37 °C for 2 hours luciferase activity was measured. Luciferase activity was plotted relative to the activity of unheated control samples.
(B) Western analysis of transfected luciferase, chaperones and eGFP-HDQ74 levels. Luciferase-eGFP and eGFP-HDQ74 were detected using an anti-GFP antibody and chaperones were detected using an anti-V5 antibody.
(C) Filter trap assay in samples from cells overexpressing eGFP-HDQ74 together with the indicated V5 tagged chaperones. Samples were prepared 48 hours after cotransfection. Serial five-fold dilutions were loaded on cellulose-acetate membranes and probed with an anti-GFP antibody.
(D) Western blot analysis of heat denatured luciferase. High molecular weight fractions in the stacking gel (upper bands) or soluble fractions (lower bands) at 45 ºC and 37 ºC. 24 hours after

transfection with luciferase alone or with the indicated V5-tagged chaperones cells were heated for 30 minutes at the indicated temperature and directly lysed hereafter. Luciferase-eGFP was detected using an anti-GFP antibody. Co-expression of chaperones was verified using an anti-V5 antibody. GAPD (bottom panel) is shown for loading control.

Figure 5. Luciferase folding and aggregation suppression is mediated by distinct subsets of molecular chaperones in O23 cells.
(A) Luciferase encoding plasmids were co-transfected with plasmids encoding mRFP (control) or encoding the indicated V5 tagged chaperones. Cells were heated for 30 min at 45 °C to inactivate luciferase and after a recovery period at 37 °C for 2 hours luciferase activity was measured. Luciferase activity was plotted relative to the activity of an unheated control.
(B) Filter trap assay in samples from cells overexpressing eGFP-HDQ74 together with the indicated V5 tagged chaperones. Samples were prepared 48 hours after cotransfection. Serial five-fold dilutions were loaded on cellulose-acetate membranes and probed with an anti-GFP antibody.
(C) Summarized data for chaperones showing the highest efficacy for the stimulation of refolding and suppression of aggregation.

Figure 6. HSPA6 shows no activity on either the stimulation of refolding or on the suppression of aggregation despite having a functional substrate binding domain.
(A) Primary amino acid alignment for HSPA1A and HSPA6 using ClustalX2. Putative domain boundaries are indicated.
(B) Domain mapping of the Van91I and PstI restriction sites in HSPA1A and HSPA6 proteins.
(C) Activity of the chimeric HSPA1A-HSPA6 and HSPA6-HSPA1A proteins on the stimulation of luciferase refolding. Cells coexpressing luciferase-eGFP and the indicated (chimeric) chaperones were heated at 43 °C followed by a 2 hours recovery period at 37 °C. Luciferase activity was plotted relative to the activity of an unheated control.
(D) Activity of the chimeric HSPA1A-HSPA6 and HSPA6-HSPA1A proteins on the stimulation of luciferase refolding in a HEK-293 cell line depleted for HSPA1A/B. Cells coexpressing luciferase-eGFP and chaperones were heated at 45 °C followed by a 2 hours recovery period at 37 °C. Luciferase activity was plotted relative to the activity of an unheated control.
(E) Activity of HSPA1A or HSPA6 on the stimulation of luciferase refolding in thermotolerant versus non-thermotolerant cells. Cells were transfected with the indicated chaperone encoding plasmids and pre-heat shocked at 45 °C for 30 minutes or left untreated. Chaperone expression was induced using tetracycline. After 24 hour incubation, cells were heat shocked at 45 °C for 30 minutes or left untreated. After another 2 hours recovery period at 37 °C, cells were lysed and luciferase activity was measured. The activity of luciferase was plotted relative to the activity of an unheated control.
(F) Effect of HSPA1A or HSPA6 depletion or blockage of expression by siRNA on the refolding capacity in non-thermotolerant or thermotolerant cells. Cells were transfected with the indicated siRNA and chaperone encoding plasmids (see methods section) and pre-heat shocked at 45 °C for 30 minutes or left untreated. After 24 hours incubation, cells were heat shocked at 45 °C for 30 minutes or left untreated. After another 2 hours recovery period at 37 °C, cells were lysed and luciferase activity was measured. The activity of luciferase was plotted relative to the activity of an unheated control.
In panels C,E and F, GAPD (always bottom panels) is shown for loading control.

Figure 7. Both HSPA1A and HSPA6 bind the same plethora of DNAJ proteins.
Cells coexpressing His tagged HSPA1A or HSPA6 together with the indicated V5 tagged DNAJ members were lysed and precipitated with Ni-NTA beads. Expression of bound DNAJ proteins was verified with anti-V5 antibodies. DNAJ expression alone showed no signal and also no

signal was seen when co-expressing either Troponin I or Troponin T indicating that the HSPA/DNAJ association was specific.

Figure 8. Comparison of ATPase activity and chaperone like activities of purified HSPA1A and HSPA6.
(A) Steady-state ATP hydrolysis rates for HSPA1A and HSPA6 *in vitro.* [-^{32}P]ATP was added to reaction mixtures containing HSPA1A, HSPA6 and DNAJB1 as indicated. Subsequently, samples were loaded on thin layer chromatography plates and autoradiographed and the region corresponding to spots was excised and counted in a liquid scintillation counter. The percentage of hydrolyzed ATP/min is plotted as a function of time.
(B) Reactivation of *in vitro* denatured firefly luciferase. Luciferase was denatured in the presence of purified HSPA1A, HSPA6 and DNAJB1 as indicated (see methods for details). The percentage of reactivated luciferase is plotted as a function of time. 100% refers to the luciferase activity prior to denaturation.
(C) Suppression of thermal induced citrate synthase aggregation. Heat induced aggregation of citrate synthase (150nM) caused light scattering. HSPA1A (150nM) did prevent the citrate synthase aggregation, whereas HSPA6 (150 nM) did not. All conditions included ATP (1mM) and DNAJB1 (50 nM). Control experiments showed that DNAJB1 with or without the addition of ATP did not prevent heat induced citrate synthase aggregation (not shown).
(D) Remodelling of the heat-inactivated p53 tetrameric complex. Human recombinant wild-type p53 was incubated at room temperature or at 40°C for 20 minutes in the absence or presence of the indicated chaperone proteins. Hereafter, the ability of p53 to bind to the *p21/WAF1*-promoter was measured using electrophoretic mobility shift assays (EMSA) as described before [43]. The heat treatment resulted in complete loss of p53 promoter binding activity. This could be partially rescued by HSPA1A when either DNAJA1 or DNAJB1 were present. HSPA6 also rescued p53 activity and does not rely on the addition of J-proteins,

Figure 9. HSPA6 does not contribute to enhanced cellular heat resistance.
(A) Confocal micrographs showing tetracycline induced expression of HSPA1A or HSPA6 in stable cell lines. HSPA1A and HSPA6 was detected by immunolocalization using the anti-V5 antibody.
(B) Clonogenic survival of HSPA1A and HSPA6 overexpressing, non-thermotolerant or thermotolerant (TT) cells. TT cells were pre-heat shocked at 45 °C for 30 minutes 24 hours before the second heatshock. Cellular survival is plotted as a function of time at 45 °C and normalized against an unheated control.
(C) Western blot analysis showing knockdown of HSPA1A and HSPA6 as indicated. GAPD (bottom panel) is shown for loading control.
(D) Clonogenic survival of HSPA1A and HSPA6 depleted, non-thermotolerant or thermotolerant (TT) cells. Cells were transfected with the indicated siRNA (see methods section) and pre-heat shocked at 45 °C for 30 minutes (TT) or left untreated. Cellular survival is plotted as a function of time at 45 °C and normalized against an unheated control.

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Figure 1

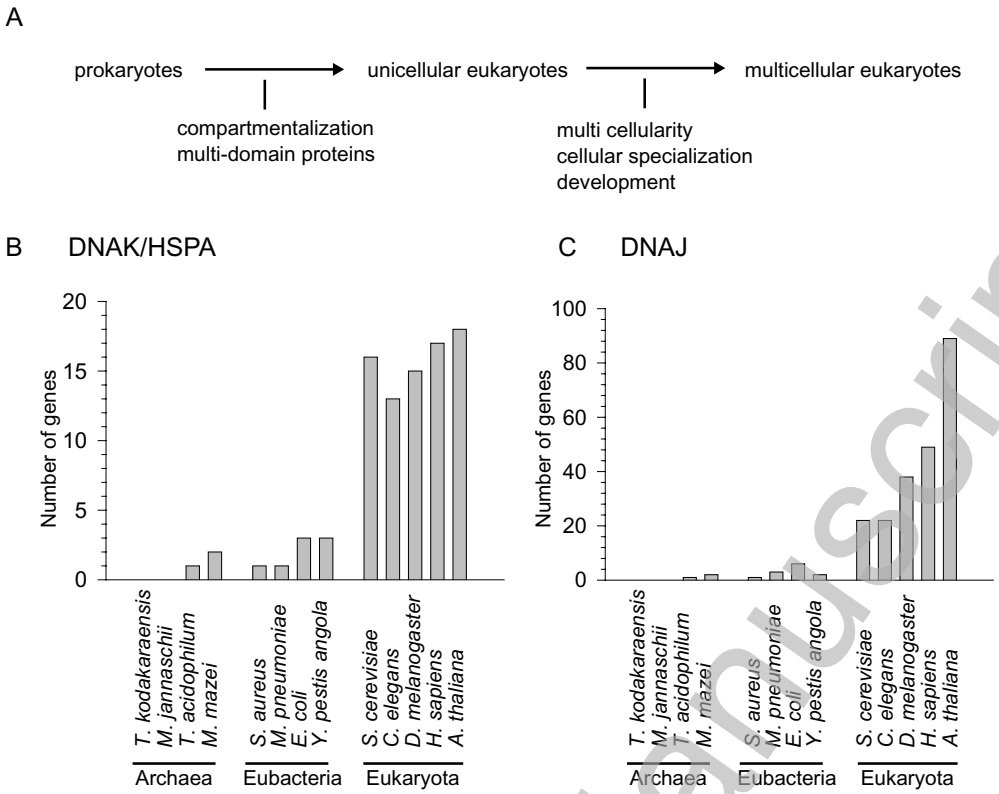

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Figure 2

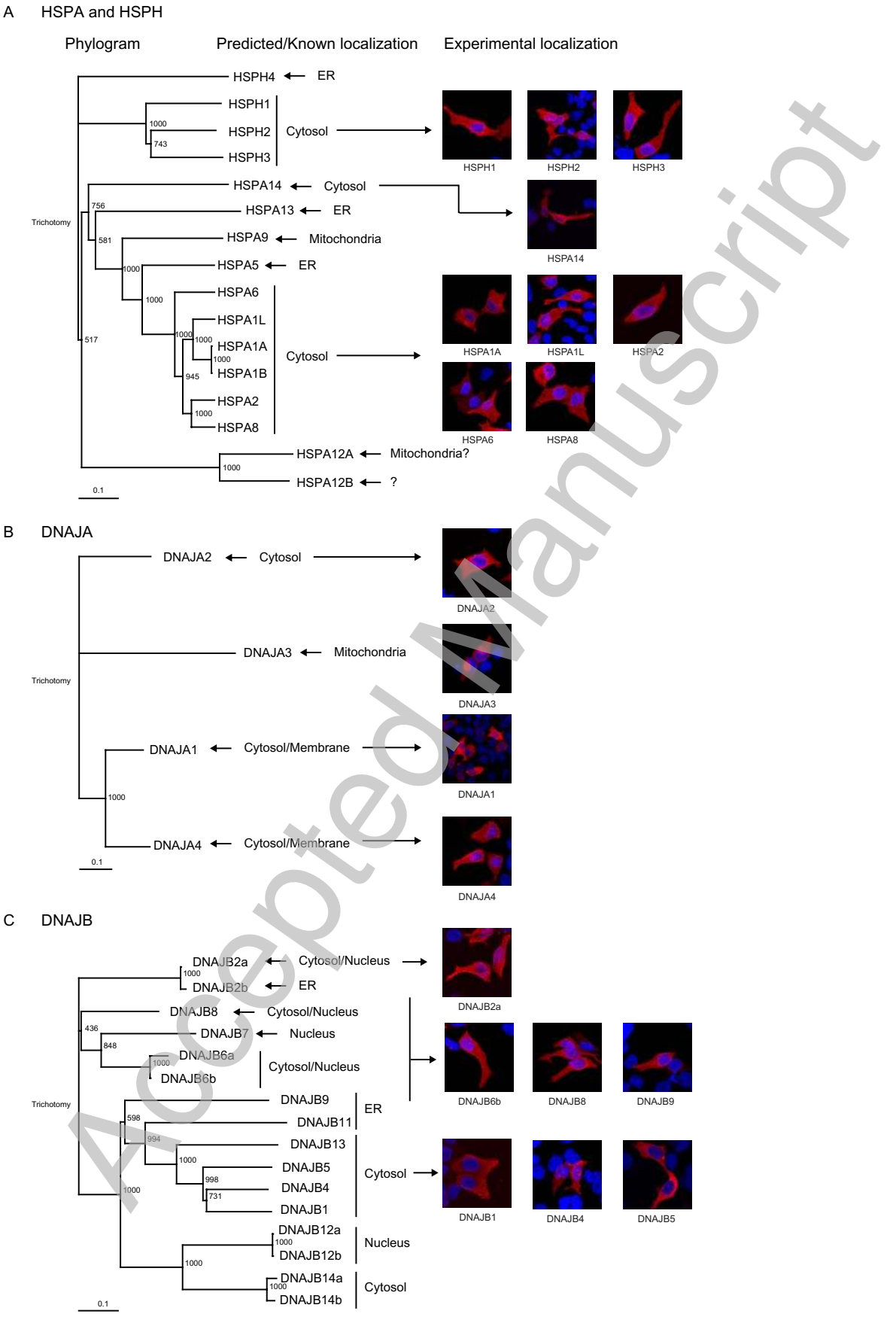

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Figure 3

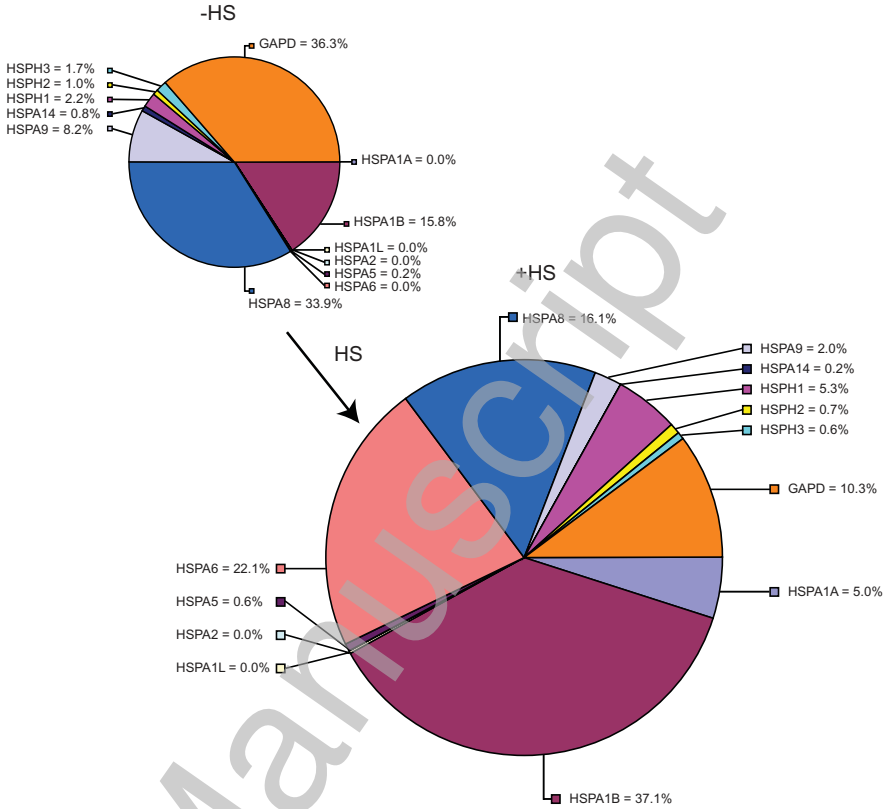
A Hek-293 Inducibility

	Gene	control	1 hour	3 hours	6 hours
HSPH	HSPH1	1,0	1,9	8,6	10,0
	HSPH2	1,0	1,5	2,6	1,8
	HSPH3	1,0	0,5	1,2	1,4

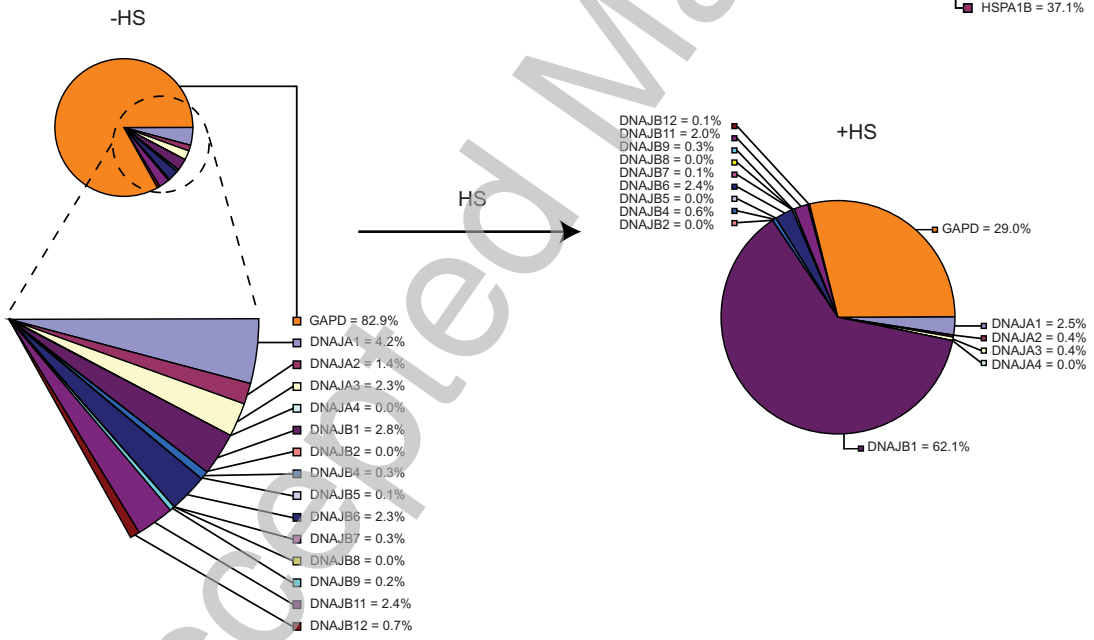
	Gene	control	1 hour	3 hours	6 hours
HSPA	HSPA1A	1,0	495,7	3121,0	401,7
	HSPA1B	1,0	3,0	8,0	1,1
	HSPA1L	1,0	13,5	112,0	8,0
	HSPA2	1,0	1,0	1,1	1,4
	HSPA5	1,0	2,7	10,2	7,0
	HSPA6	1,0	832,2	4588,5	157,7
	HSPA8	1,0	1,1	1,7	0,8
	HSPA9B	1,0	0,8	0,9	0,9
	HSPA14	1,0	0,8	0,8	0,9

	Gene	control	1 hour	3 hours	6 hours
DNAJ	DNAJA1	1,0	1,2	2,1	3,0
	DNAJA2	1,0	1,4	1,1	1,3
	DNAJA3	1,0	1,4	0,7	1,2
	DNAJA4	1,0	1,2	1,1	0,8
	DNAJB1	1,0	30,5	79,6	4,8
	DNAJB2	1,0	1,3	0,8	0,9
	DNAJB4	1,0	2,4	6,5	2,3
	DNAJB5	1,0	1,4	1,1	1,4
	DNAJB6	1,0	1,8	2,9	2,4
	DNAJB7	1,0	1,4	0,8	1,0
	DNAJB8	1,1	1,4	1,6	5,3
	DNAJB9	1,0	1,5	3,9	1,4
	DNAJB11	1,0	1,5	2,4	2,5
	DNAJB12	1,0	1,3	0,6	1,2

B Hek-293 Abundance for HSPH/HSPA



C Hek-293 Abundance for DNAJ



D Various Cell-lines/Tissues

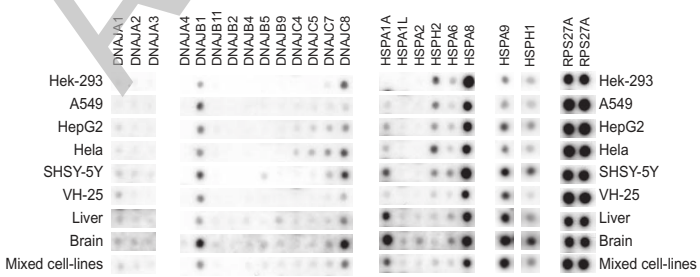


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Figure 4

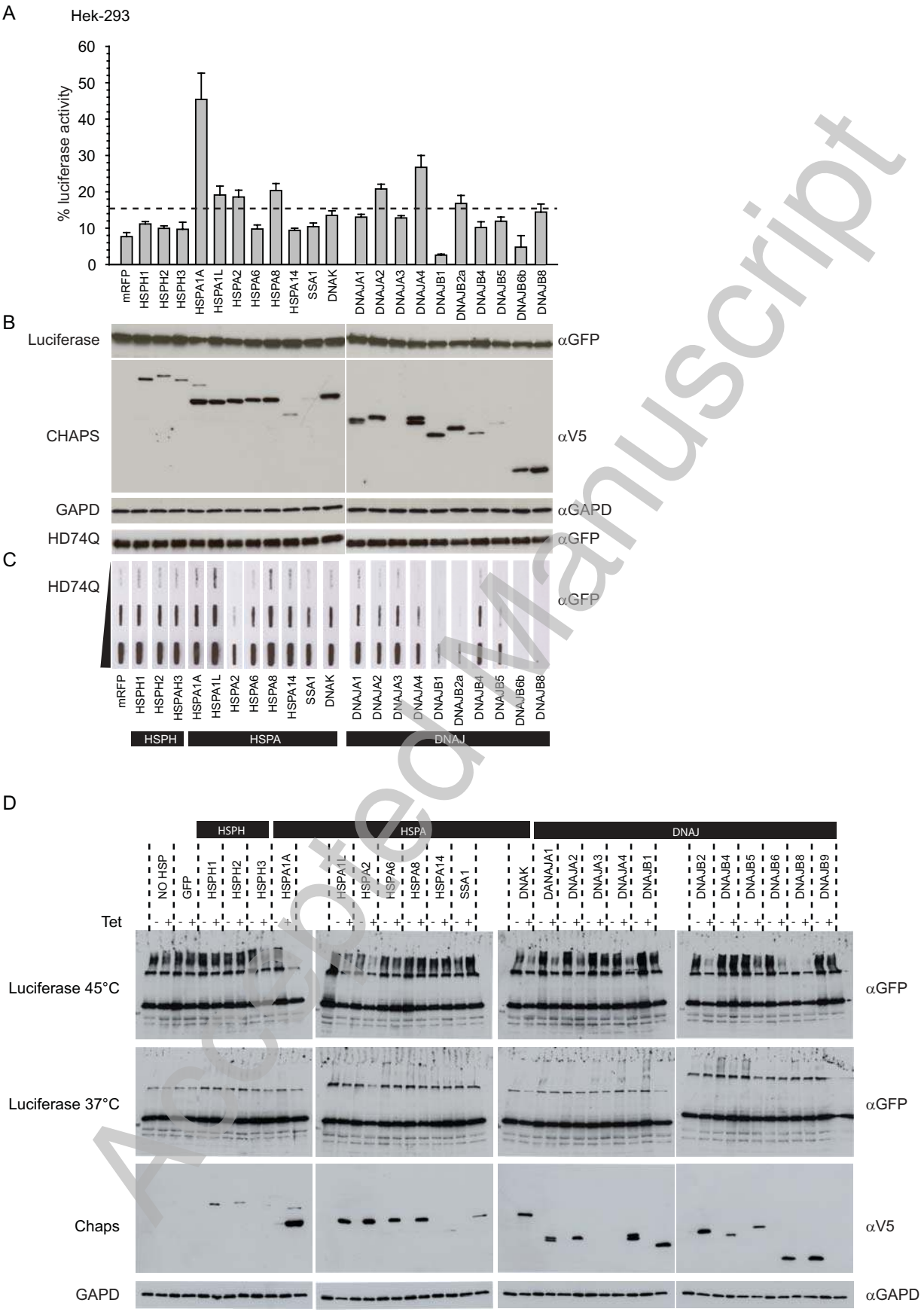

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Figure 5

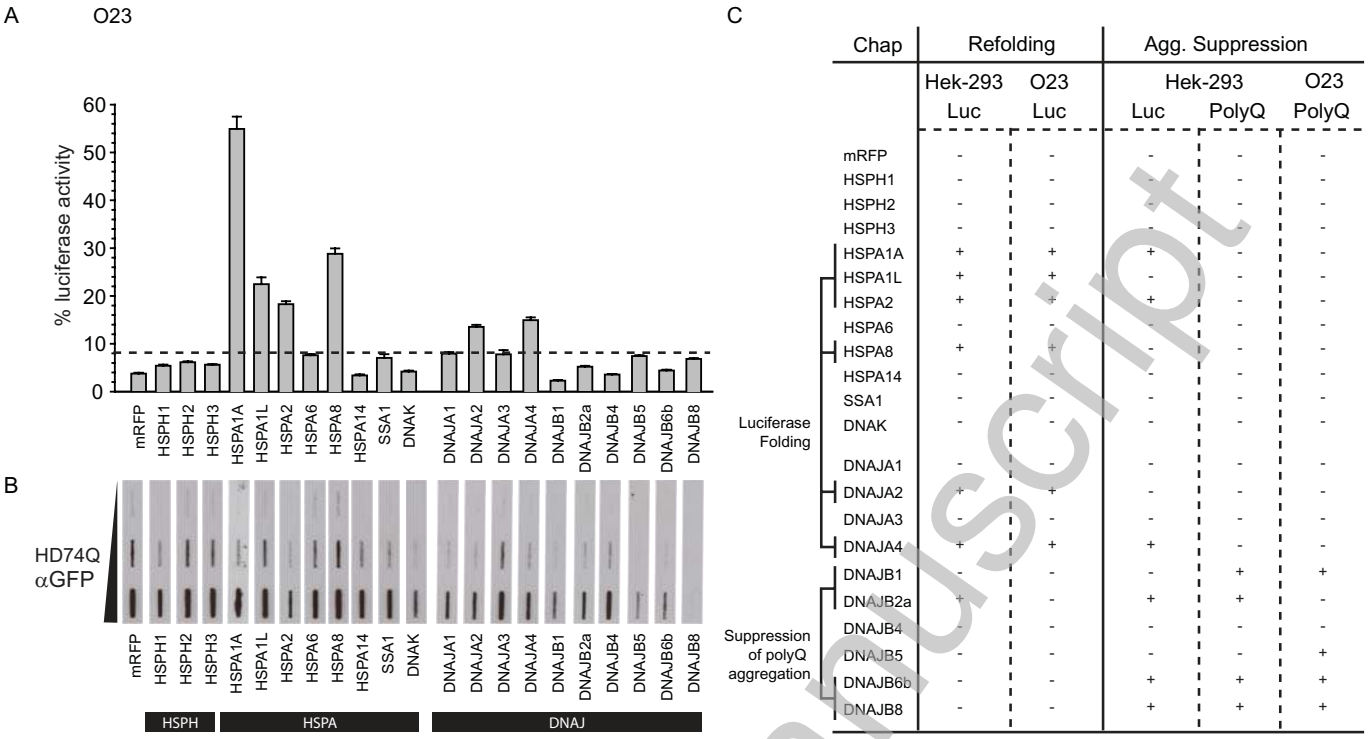

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Figure 6

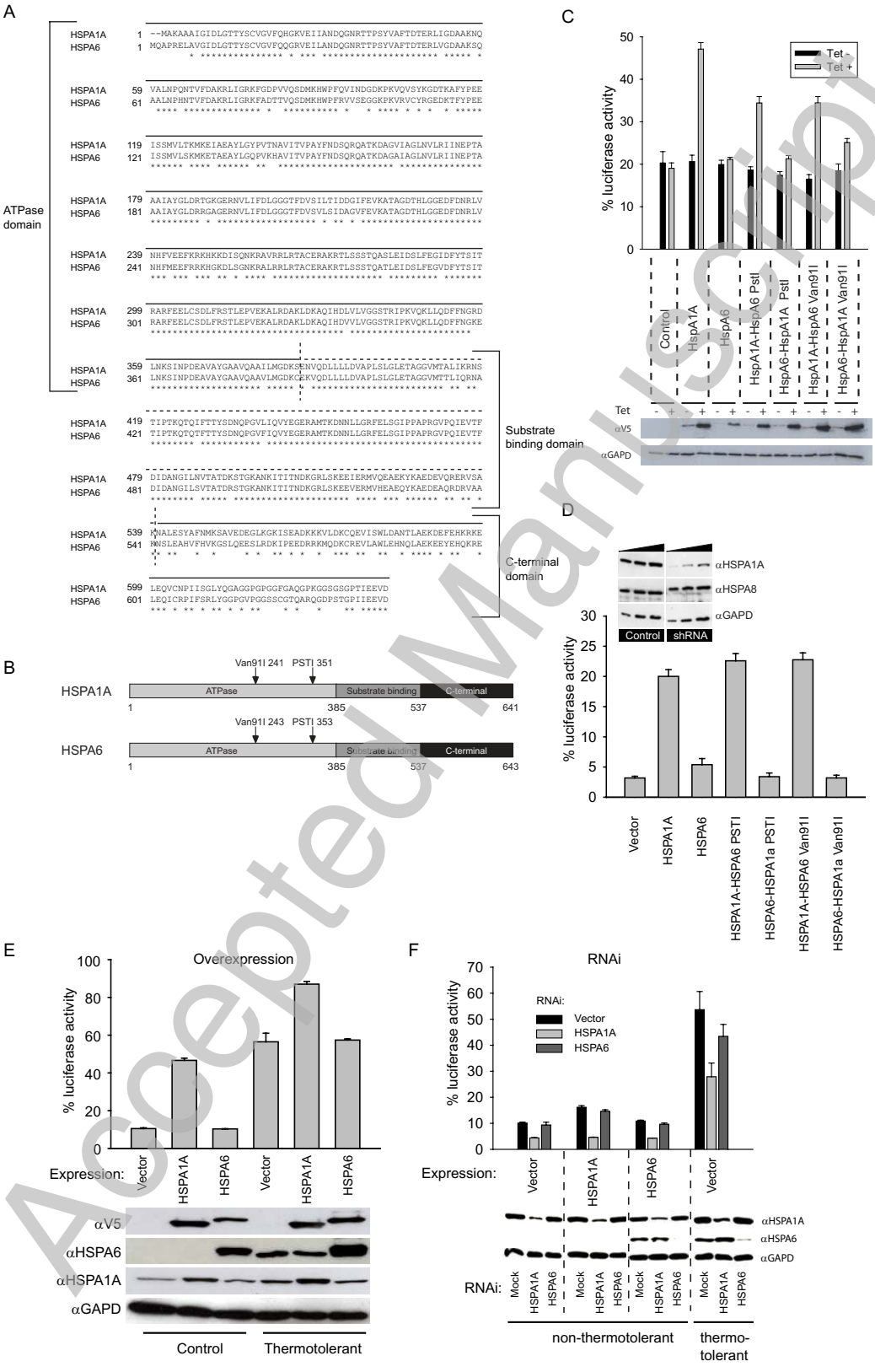

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Figure 7

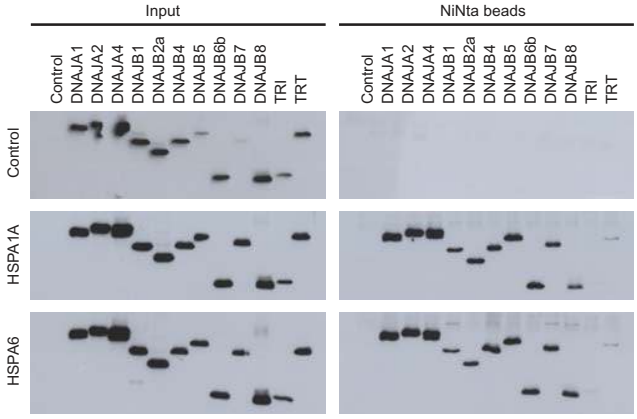

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Figure 8

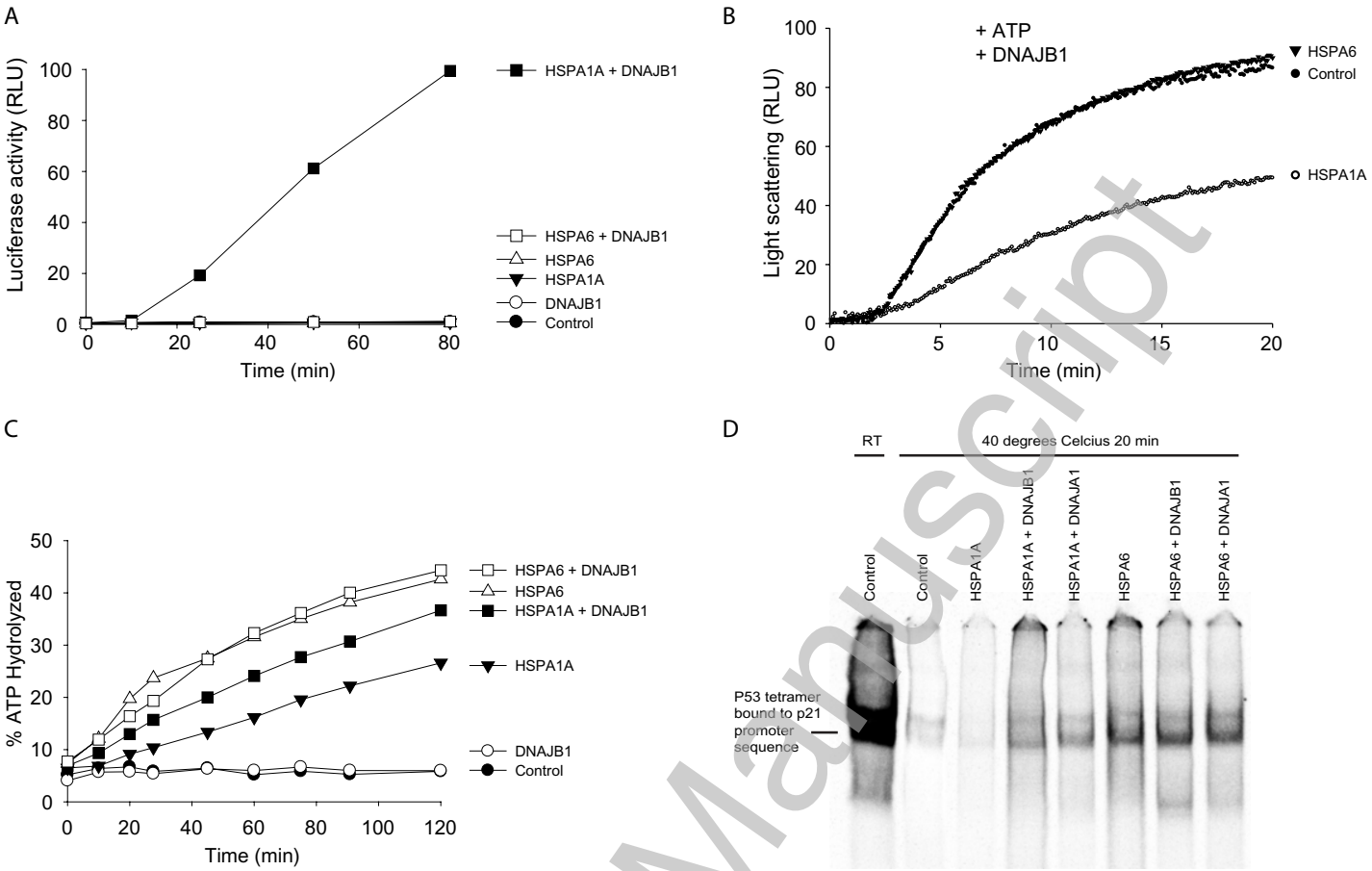

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Figure 9