High-throughput screening system for inhibitors of human Heat Shock Factor 2

Levi M. Smith¹ · Dwipayan Bhattacharya¹ · Daniel J. Williams¹ · Ivan Dixon¹ · Nicholas R. Powell¹ · Tamara Y. Erkina¹ · Alexandre M. Erkine¹

Received: 17 December 2014 / Revised: 6 April 2015 / Accepted: 12 May 2015 / Published online: 24 May 2015
© Cell Stress Society International 2015

Abstract Development of novel anti-cancer drug leads that target regulators of protein homeostasis is a formidable task in modern pharmacology. Finding specific inhibitors of human Heat Shock Factor 1 (hHSF1) has proven to be a challenging task, while screening for inhibitors of human Heat Shock Factor 2 (hHSF2) has never been described. We report the development of a novel system based on an *in vivo* cell growth restoration assay designed to identify specific inhibitors of human HSF2 in a high-throughput format. This system utilizes a humanized yeast strain in which the master regulator of molecular chaperone genes, yeast HSF, has been replaced with hHSF2 with no detrimental effect on cell growth. This replacement preserves the general regulatory patterns of genes encoding major molecular chaperones including Hsp70 and Hsp90. The controlled overexpression of hHSF2 creates a slow-growth phenotype, which is the basis of the growth restoration assay used for high-throughput screening. The phenotype is most robust when cells are cultured at 25 °C, while incubation at temperatures greater than 30 °C leads to compensation of the phenotype. Overexpression of hHSF2 causes overexpression of molecular chaperones which is a likely cause of the slowed growth. Our assay is characterized by two unique advantages. First, screening takes place in physiologically relevant, *in vivo* conditions. Second, hits in our screen will be of medically relevant potency, as compounds

that completely inhibit hHSF2 function will further inhibit cell growth and therefore will not be scored as hits. This caveat biases our screening system for compounds capable of restoring hHSF2 activity to a physiologically normal level without completely inhibiting this essential system.

Keywords HSF1 · HSF2 · Molecular chaperones · HSF inhibitor · Protein homeostasis

Introduction

Cancer cells encounter a variety of internal and external stresses not commonly encountered by normal cells. These include an imbalance of biosynthetic pathways as a result of mutations and chromosomal rearrangements, oxidative stress caused by abnormal cellular growth, and hypoxia due to improper angiogenesis, as well as other imbalances (Solimini et al. 2007). The principal cellular safeguard combating these stresses is an array of molecular chaperones, the expression of which is regulated by Heat Shock Factors (HSFs). HSF1 was first identified as a promising target for anti-cancer therapy when HSF1 knockout mice demonstrated delayed tumor development, reduction in tumor size, and prolonged survival following exposure to carcinogens (Dai et al. 2007; Whitesell and Lindquist 2009). The recognition of HSF1 as a new target for drug development (Whitesell and Lindquist 2009) led to several attempts to develop a screening system for the identification of HSF1 inhibitors (Au et al. 2009; Santagata et al. 2012; Yoon et al. 2011). Only one screen resulted in identification of a compound likely affecting HSF1 directly (Yoon et al. 2011). Other screening systems identified indirect inhibitors of HSF1, likely because the function of molecular chaperones is closely connected with other cellular stress response systems including protein degradation and protein

Electronic supplementary material The online version of this article (doi:10.1007/s12192-015-0605-0) contains supplementary material, which is available to authorized users.

✉ Alexandre M. Erkine
aerkine@butler.edu

¹ Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, Butler University, Indianapolis, IN 46208, USA

homeostasis in general (Santagata et al. 2012). The greatest successes in the hunt for inhibitors of the molecular chaperone system are several inhibitors of the major molecular chaperone HSP90 (Sidera and Patsavoudi 2014; Whitesell et al. 2012; Xiao et al. 2006). Despite these successes, major regulators of this system remain entirely undrugged. The paucity of success in developing specific inhibitors of HSFs advocates for the development of new screening strategies.

While HSF1 is the major regulator of stress response genes, it does not function alone. In addition to *HSF1*, the human genome contains both the *HSF2* and *HSF4* genes as well as the non-functional pseudogene *HSF3* (Akerfelt et al. 2007). Historically, the functions of HSF2 and HSF4 were believed to be tissue specific, with HSF2 functioning in corticogenesis and spermatogenesis (Akerfelt et al. 2007; Chang et al. 2006; Wang et al. 2003, 2004) and HSF4 in lens development (Fujimoto et al. 2004). Recent data implicate a more extensive role for HSF2 and demonstrate its cooperation with HSF1 in the activation of molecular chaperone genes (Ostling et al. 2007; Sandqvist et al. 2009). Involvement of HSF2 in carcinogenesis was also suggested via a mechanism that includes p53 (Lecomte et al. 2010). The functional cooperation of HSF1 and HSF2 and their co-involvement in carcinogenesis, taken together with the identification of HSF1 as an attractive anti-cancer drug target, strongly implicates HSF2 in carcinogenesis (Lecomte et al. 2010; Scherz-Shouval et al. 2014). Despite this strong narrative, to date, no inhibitors of HSF2 are known and no attempts to develop a screening system for HSF2 inhibitors have been reported.

The function of HSFs in the regulation of molecular chaperones is extremely conserved among eukaryotes. The extent of this conservation is illustrated by the fact that the single and essential *HSF1* gene in yeast can be substituted with human *HSF2* (*hHSF2*) or *HSF4* (*hHSF4*) without creating a significant phenotype (Liu et al. 1997). Surprisingly, substitution with human *HSF1* (*hHSF1*) is impossible, presumably due to a defect in trimerization of hHSF1 in yeast. This deficit is ameliorated by three point mutations in *hHSF1* that give rise to a constitutively trimerized hHSF1 (Liu et al. 1997; Neef et al. 2013). The high conservation of HSF function and the interchangeability of human and yeast HSFs open the possibility of creating a screening system for HSF inhibitors using humanized yeast strains.

Here, we report the development of an in vivo screening system optimized for high-throughput applications. The assay utilizes a humanized yeast strain that expresses hHSF2 as the sole source of HSF in the cell. The strain harbors *hHSF2* on two distinct plasmids: one expresses hHSF2 at a basal level sufficient to sustain growth and is regulated by a constitutively active promoter; the second expresses hHSF2 under an inducible promoter that allows overexpression of hHSF2 in a controlled manner. We found that overexpression of hHSF2 in yeast creates a slow-growth phenotype that allows

identification of inhibitors of hHSF2 by restoration of normal cell growth.

Materials and methods

Yeast strains and growth conditions The DNY47 strain (MATa *ade2 trp1 leu2 his3 ura3 hsf1Δ::LEU2* (*yep50gal-yhsf1 URA3-CEN4-ARSI*) *ERG6::loxp-KanMX-loxp PDR5Δ::loxp SNQ2Δ::loxp*) and p413GPD-*hHSF2* (human HSF2a) plasmid were obtained from Denis Thiele's group (Neef et al. 2010). The strain harbored the p413GPD-*hHSF2* plasmid (*HIS3* marker) and the *Yep50gal-yhsf1* plasmid (*URA3* marker). The latter was shuffled out by counter-selection on 5-FOA. The resultant strain was transformed with either the pYES-*hHSF2* vector or by pYES2/CT vector (pYES-empty). For construction of pYES-*hHSF2*, the *hHSF2* ORF (human HSF2a) was amplified by PCR using the following primers forward 5' GCCCCCATGAAGCAGAGTTTCAACGTGCCGGCTTTCCT 3' and reverse 5' GAGGCGGTGATGTAAGCGTGACATAACTAATTACATG 3', and ligated into pYES2.1/V5-His-TOPO vector (Invitrogen) between the *GAL1* promoter and *CYC1* terminator according to the manufacturer's protocol. The construct was verified by restriction digestion analysis and by direct DNA sequencing. The protein expression was verified by Western blotting.

Strains were cultivated in –his –ura synthetic dropout media containing either glucose or galactose as a carbon source.

Protein isolation and Western blotting For protein isolation, we followed the previously published protocol (Kushnirov 2000). Briefly, harvested cells were incubated in 0.1 M NaOH for 10 min, washed with water, resuspended in sample buffer (1 M Tris–HCl, 80 % glycerol, 10 % SDS, 1 % bromophenol blue), and transferred to lysing matrices. Lysis was carried out using a Thermo FastPrep FP120 cell disruptor at speed 6 for 40 s, repeated twice. Lysates were then placed in a boiling water bath for 5 min and then briefly centrifuged. For Western blotting, antibody dilutions of 1:500 for anti-HSF2 (Assay Biotech; Cat# C10871), 1:20,000 for anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Acris antibodies; Cat# AP21309AF-N), and 1:10,000 for anti-rabbit IgG, DyLight 550 (Thermo Scientific; Cat# 84541) were prepared in TTBS. Blotting was performed using PVDF membrane. Antibody incubation duration was overnight at room temperature for primary antibodies and 1 h at room temperature for the secondary antibody.

RNA isolation and RT-qPCR RNA was isolated from cells following 16 h of incubation. Heat shock treatment was performed by placing cultures in a pre-warmed 39 °C hot water bath for 15 min. Sodium azide was added to cells at a final concentration of 20 mM. RNA isolation was performed using

mechanical disruption of cells using a Thermo Fast Prep FP120 on speed 6 for 3 cycles of 40-s runs followed by the Qiagen® RNeasy kit protocol with on-column DNase digestion using Qiagen's RNase-Free DNase set (Cat# 79254). Reverse transcriptase quantitative real-time PCR (RT-qPCR) was carried out using Power SYBR Green RNA to Ct (part # 4389986). Primers used were the following: *ACT1* forward 5' CCG GTT TTG CCG GTG ACG ACG CTC CTC 3', *ACT1* reverse 5' AAA GTC AAG ATA CCT CTC TTG GAT TGA GCT TCA TCA CCA ACG 3', *SSA1* forward 5' TCG ATG ACA AGT TGA AGG AGT TGC AAG A 3', *SSA1* reverse 5' GCA CCA CCT GGG AAA CCG CCT GGA GCA CCA CC 3', *HSC82* forward 5' GCA ATT TGA CAA ATT TTA CTC TGC CTT CG 3', *HSC82* reverse 5' AAT CAG TCA AGG AAG TCA ATT CAT CGA CAG ATT TA 3', *HSP82* forward 5' ATT CTG AGT TCG TGG CCT ACC CAA TCC AAT TAG TC 3', *HSP82* reverse 5' GTC GTC TTC ATC CTT CTT TTC CTC ATC CTT C 3', *SSA4* forward 5' CGC GTT TAC TTT GAA AAA TTC TGT GAG CGA AAA TAA 3', *SSA4* reverse 5' GTA TTC CTC GGT GGA GGC CGC TTG CGA AGC A 3'.

Growth curve development Cultures of LNL4 pYES-empty and LNL4 pYES-h*HSF2* α were grown in histidine- and uracil-deficient medium containing 2 % raffinose to mid-log phase, washed twice with sterile water, and resuspended in uracil-deficient medium containing 2 % galactose at a calculated starting OD₆₀₀=0.1. Alternatively, cultures were pre-grown in galactose-containing medium for 20 h and then diluted to OD₆₀₀=0.1 prior to starting the growth curve measurements. Cultures were incubated for 24 h with OD₆₀₀ measurements made every hour.

High-throughput screening protocol Cultures of LNL4 pYES-empty and LNL4 pYES-h*HSF2* α (h*HSF2* α is a functional isoform of human *HSF2* gene later in the text called *hHSF2*) were grown in uracil-deficient medium containing 2 % raffinose to mid-log phase and washed twice with sterile water. Cells from these cultures were used for subsequent inoculation of uracil-deficient media containing 2 % galactose with the following consideration. When preparing cultures containing 2 % galactose, the LNL4 pYES-h*HSF2* cultures were inoculated with twice as many cells as the LNL4 pYES-empty cultures so that the OD₆₀₀ measurements following overnight growth would be similar. Following overnight incubation, OD₆₀₀ measurements of the two cultures were required to be within 0.2 of each other in order to proceed. If cultures were sufficiently similar, fresh cultures were prepared in uracil-deficient media containing 2 % galactose with an initial calculated OD₆₀₀ of 0.01 for both strains. Experimental compounds were prepared as 1 mM stock solutions in DMSO. Compounds were tested at a concentration of 10 μ M. Cultures were incubated with compounds for 22 h before the final OD₆₀₀. Tween 20 was added to each well at

a final concentration of 0.5 % prior to final OD₆₀₀ measurement to reduce error in OD measurements due to variable menisci in the small volume wells.

Spot dilution assay Cultures of LNL4 pYES-h*HSF2* α were grown to log phase in uracil-deficient media containing 2 % raffinose. A 1-mL aliquot was taken from each culture and was washed twice with sterile water by centrifuging samples for 5 min at 1000 $\times$ g. Washed cells were resuspended in sterile water to an OD₆₀₀=0.3. Fivefold serial dilutions were created in sterile 96-well plates. Using a replica plater, the diluted cultures were transferred to solid-media plates containing either 2 % glucose or 2 % galactose. Plates were incubated at 25, 30, 33, and 36 °C. Images were taken after 42 h of incubation, and the same imaging settings were used for all captures.

Results

Previous attempts to create a screening system for hHSF1 modulators have resulted in the identification of a single inhibitor, KRIBB11 (Yoon et al. 2011). The consistent failure of these systems has demonstrated the significant challenges associated with systems based on human cell line assays (Santagata et al. 2012). Noting these points, we pursued an alternative strategy based on the growth inhibition caused by the overexpression of the target protein in yeast cells (Baldi and Roberge 2009). We were optimistic that our strategy would produce a robust phenotype based on prior knowledge that 30 % of human proteins inhibit yeast cell growth when overexpressed (Tugendreich et al. 2001). Specifically, we hypothesized that overexpression of hHSF2 would cause a slow-growth phenotype due to the overexpression of molecular chaperones and that this phenotype could be alleviated by the inhibition of hHSF2 (Fig. 1). Highly efficient efflux pumps pose a particular challenge to drug screening in yeast-based systems. To ensure sufficient retention of compounds within cells, it is a common practice to introduce inactivating mutations within the genes encoding the major efflux pump components (Baldi and Roberge 2009). Our strain construction began with the DNY47 strain provided

Fig. 1 Concept of the screening strategy. *Left to right*: yeast cells expressing normal amount of HSF (black ovals) growing rapidly; overexpression of HSF causes a slow-growth phenotype; small molecule inhibitors of HSF (red stars) restore cell growth to normal

by the D. Thiele group. This strain has already been adapted for screening by the mutation of several efflux pumps (Neef et al. 2010). DNY47 was particularly suited to our needs, as it descended from a strain originally created by Peter Sorger (1990), who deleted the single yeast *HSF1* (*yHSF1*) gene from its normal chromosomal location and replaced it with a plasmid constitutively expressing this essential gene. The subsequent strain allows convenient shuffling of plasmids encoding alternative HSFs (see “Materials and methods”). Capitalizing on these advantages, we first replaced the plasmid constitutively expressing *yHSF1* with the p413-hHSF2 construct, which constitutively expresses hHSF2. This strain was then transformed with pYES-hHSF2 plasmid, which encodes the *hHSF2* gene under the *Gal1* promoter, so that overexpression can be regulated by exchange of carbohydrate in the growth medium. The newly created strain was assigned the name LNL4. In these cells, episomally encoded hHSF2 is the sole source of HSF, replacing the master regulator of heat shock genes—yHSF. The combination of two hHSF2-encoding plasmids created a system in which one plasmid provides the constitutive level of hHSF2 necessary for survival, and a second plasmid allows for inducible overexpression of hHSF2.

We first examined the expression of hHSF2 from pYES-hHSF2 or p413-hHSF2 plasmids transformed individually into the W303 strain (Fig. 2a). The Western blotting of samples from these strains demonstrated that there is no cross-reactivity of the anti-hHSF2 antibody with the yHSF1 present in W303 strain (panel A, lane 11), that the p413-hHSF2 plasmid provided stable background levels of hHSF2 (panel A, lane 12), and that the pYES-hHSF2 plasmid was able to significantly increase hHSF2 expression in a galactose-dependent manner. The maximum intensity of hHSF2 levels was reached after 8–16 h of exposure to galactose (Fig. 2a, lanes 6 and 8).

We then used our newly created LNL4 strain transformed with both plasmids (Fig. 2b) to test the kinetics of the hHSF2 overexpression. LNL4 pYES-hHSF2 cells cultivated in glucose-containing medium expressed only the background level of hHSF2 permitted by constitutive expression from the p413-hHSF2 plasmid. Alternatively, growth in the presence of galactose increased hHSF2 expression by 20-fold after 16 h.

We next examined whether hHSF2 overexpression produces a growth phenotype. Using spot dilution assay, we saw that in glucose-containing media, strains LNL4 pYES-empty and LNL4 pYES-hHSF2 grow similarly, while in galactose-containing media, the growth phenotype differs between two strains (Fig. 3). This difference is most clearly seen at 25 and 30 °C and begins to diminish at 33 °C. At 36 °C, the phenotype appears to reverse, favoring the overexpression of hHSF2 in this high-stress environment. The attenuation and reversal of phenotypes with increasing temperature were not unexpected as the overexpression of molecular chaperones,

Fig. 2 Overexpression of HSF2 from the pYES-hHSF2 plasmid. Western blots using anti-hHSF2 and anti-GAPDH antibody are shown. **a** Expression of hHSF2 in W303 yeast strain from the pYES-hHSF2 or p413-hHSF2 plasmids. Samples were collected after 0, 4, 8, 16, or 24 h after switching to galactose as the carbon source. **b** Expression of hHSF2 in the LNL4 yeast strain from both pYES-hHSF2 and p413-hHSF2 plasmids grown either in glucose-containing medium or after switching to galactose for 0, 4, 6, 8, 16, or 24 h. Position of molecular weight markers are shown on the right

while detrimental at lower temperatures, may be beneficial at higher temperatures when they are needed for maintenance of protein homeostasis by chaperoning misfolded proteins.

Fig. 3 Slow-growth phenotype is compensated by higher cultivation temperatures. The serial spot dilution assay for cells bearing either empty pYES vector or pYES-hHSF2 overexpressing construct is shown for glucose- or galactose-containing media at indicated growth temperatures

The dramatic retardation of growth at 25 °C is most useful for our screening application and was utilized in subsequent experiments. The interesting observations made at elevated temperatures are intriguing and warrant further investigation of the ability of hHSF2 to independently mediate the heat shock response, a characteristic not previously attributed to hHSF2 in human cells.

Establishing a connection between the overexpression of molecular chaperones, caused by the overexpression of hHSF2, and the growth phenotype was important, because it is the protective function of molecular chaperones that is thought to contribute to carcinogenesis (Dai et al. 2007). To test if the pattern of molecular chaperone expression is affected by overexpression of hHSF2, we chose four differentially regulated genes: *SSA1*, *SSA4*, *HSC82*, and *HSP82*. These genes encode major molecular chaperones of the Hsp70 and Hsp90 superfamilies. *SSA1* and *SSA4* encode almost identical variants of Hsp70, while *HSC82* and *HSP82* code for Hsp82 protein variants (Hsp82 is the yeast ortholog of human Hsp90). The expression of *SSA1* and *HSC82* genes is minimally influenced by stress and is considered constitutive (Erkine et al. 1996; Slater and Craig 1989). Alternatively, the expression of *SSA4* and *HSP82* is highly inducible and stress dependent (Boorstein and Craig 1990; Erkine et al. 1999).

We were interested in determining whether hHSF2 is capable of independently regulating chaperone expression in response to heat shock, because previous reports suggest that human HSF2 is not independently responsible for the heat stress regulation in mammalian cells (Bjork and Sistonen 2010; Fiorenza et al. 1995; Ostling et al. 2007). When LNL4 pYES-empty cells, which constitutively express hHSF2 from the p413GPD-hHSF2 plasmid, were exposed to elevated temperatures, the expression of the selected molecular chaperones responded surprisingly similarly to strains with wild-type yHSF regulation. Upon heat stress in glucose-containing media, *SSA1* expression changed only 1.8-fold, *HSC82* expression changed insignificantly, while *SSA4* expression increased 50-fold and the expression of *HSP82* was up 11-fold (Fig. 4b). The expression patterns observed in our humanized strain were consistent with the expression pattern of these genes when regulated by the native activator (Boorstein and Craig 1990; Erkine et al. 1996, 1999; Slater and Craig 1989). Thus, the expression profiles of these genes did not change significantly when yeast HSF was replaced by human HSF2. This by itself is an interesting and novel observation contradicting to previous reports, which claim that human HSF2 is incapable of independently regulating the response to heat stress (Ostling et al. 2007); the substitution of yeast HSF1 with human HSF2 not only secures the molecular chaperone expression necessary for housekeeping functions but also provides proper gene regulation in response to heat stress.

We then wanted to test whether the expression of heat shock genes is affected by the shift from glucose-containing

media to galactose, which is necessary for induction of hHSF2 overexpression. We saw that in the strain where heat shock genes are regulated only by constitutively expressed hHSF2 (Fig. 4a, LNL4 pYES-empty), the expression of all four genes was not significantly affected by the change of the carbon source from glucose to galactose at normal cultivation temperature, while the stress response was mildly enhanced (Fig. 4b LNL4, pYES-empty). We attribute the especially mild increase in reaction to heat stress to potentiation of the stress system due to the utilization of galactose, which is an unusual carbon source and significantly changes metabolism.

Next, we wanted to see whether expression levels of *SSA1*, *SSA4*, *HSC82*, and *HSP82* were affected by the overexpression of hHSF2. As expected, the strain containing the overexpression vector (LNL4 pYES-hHSF2) and grown in glucose showed almost identical levels of expression for all four genes in comparison to the strain containing empty vector and grown in glucose-containing medium. The induction of hHSF2 overexpression by growth in galactose, however, resulted in a dramatic increase in HSP expression. In fact, we saw significant increases in the expression of all four genes (Fig. 4a). The observed increase was so great that there was a very limited change in expression in response to subsequent heat shock, indicating that the expression of HSPs was likely close to the maximal level. This in turn indicates that hHSF2-overexpressing cells at normal cultivation temperature have the high stress levels of molecular chaperone expression without the possibility of attenuation by homeostatic mechanisms. This consistently high expression level of molecular chaperones is likely the cause of the observed slow-growth phenotype of the hHSF2-overexpressing strain (Fig. 3).

For the development of the growth restoration screening assay, it was essential to find the growth conditions that would maintain the greatest difference between growth in the hHSF2 overexpressing (slow growth) and constitutive low hHSF2 expression (fast growth) conditions. Utilizing a more dramatic difference in phenotype allows stratification of hits by the degree of hHSF2 inhibition, rather than simply identifying compounds as inhibitory or not. The data in Fig. 3 suggest that 30 °C is the optimal temperature but did not provide a quantitative measure of the difference in growth. To quantify the difference in growth rates, cultures were grown at 30 °C and OD₆₀₀ measurements were made every hour. To maximize the magnitude of the slow-growth phenotype, we assessed whether preliminary stimulation of hHSF2 overexpression affects the discriminating power of the assay. We compared the difference in growth of LNL4 pYES-empty and LNL4 pYES-hHSF2 cultures cultivated before assay initiation in either glucose- or galactose-containing media. We observed that the cell densities of the pYES-empty and pYES-hHSF2 cultures grown in galactose-containing media differed by only 1.6-fold after 17 h of growth (Fig. 5). By pre-growing cultures overnight in the presence of galactose and

Fig. 4 Overexpression of hHSF2 causes overexpression of molecular chaperones. **a** mRNA expression levels for the *SSA1*, *HSC82*, *HSP82*, and *SSA4* genes relative to actin mRNA are determined by RT-qPCR. The LNL4 pYES-empty and LNL4 pYES-hHSF2 strains were grown either in glucose- or galactose-containing medium. **b** Fold of induction calculated by normalization of heat shock (15 min at 39 °C) mRNA levels to the non-heat shock mRNA levels (**a**) for the corresponding genes. The extremely small values are indicated above the columns. Values represent mean $\pm$ S.D. ($n \geq 3$)

subsequently diluting cultures to the same density at time zero of the assay, we observed a greater difference of twofold after 14 h of growth. These results were consistent with the kinetics of hHSF2 overexpression which reached maximum after 8–16 h of switching the carbon source (Fig. 2). Using the optimized growth conditions, we calculated the Z-factor (Zhang et al. 1999) of our assay to be 0.75, indicating an excellent assay for high-throughput screening. We also optimized a detailed protocol for implementation of the assay (see [Supplemental material](#)).

To test if the growth restoration assay is applicable to other forms of HSF, we created similar strains overexpressing yeast HSF from the same expression vector. The spot dilution

growth assay results (Fig. 6) demonstrate that in the presence of galactose, overexpression of yHSF1 creates an even more robust phenotype in comparison to our hHSF2-expressing strains. This dramatic phenotype points to the possibility of utilizing yHSF1 overexpression in the initial stages of the screening process and/or for the discrimination between the pan-HSF- and hHSF2-specific inhibitors.

In an attempt to test if the only reported direct human HSF1 inhibitor KRIBB11 (Yoon et al. 2011) affects the phenotype of the hHSF2- or yHSF1-overexpressing strains, we obtained the compound and assessed its activity in our assay. Using concentrations within the range of 1–200 μ M, similar to described in Yoon et al. (2011), we found that the compound lacked

Fig. 5 Pre-growth on galactose-containing medium increases the discriminating power of the assay. Growth curves for the LNL4 pYES-empty and LNL4 pYES-hHSF2 strains after switching from glucose- to galactose-containing medium (a) or with preliminary growth of cultures for 20 h in galactose-containing media (b). X-axis: time in hours; Y-axis: OD₆₀₀ reading

activity both in human HSF2 and yeast HSF1 overexpression strains, as well as the empty vector control strains (data not shown). Considering that KRIBB11 affects predominantly pTEFb–hHSF1 interactions (Yoon et al. 2011), we think that in the yeast context, KRIBB11 simply lacks its functional target pair.

Discussion

The development of inhibitors of protein homeostasis regulators is a challenging task (Dai et al. 2007; Whitesell and Lindquist 2009). Although the need for direct HSF inhibitors is clearly stated (Whitesell and Lindquist 2009), only a single direct inhibitor (Yoon et al. 2011) and a few indirect inhibitors have been reported (Whitesell and Lindquist 2009). The majority of reported protein homeostasis inhibitors target either major molecular chaperones such as HSP90 in the case of the geldanamycin family (Whitesell et al. 2012), or pathways indirectly affecting HSF, as in the cases of triptolide (Phillips et al. 2007; Westerheide et al. 2006), quercetin (Hansen et al. 1997), and others (Whitesell and Lindquist 2009). KRIBB11

reportedly acts via a direct interaction with HSF1 but requires micromolar concentrations even in simplified in vitro systems (Yoon et al. 2011). It is likely that a significant barrier to identifying direct and therapeutically useful inhibitors of protein homeostasis is the vital nature of this system to cell survival. The heat shock response is critical not only for surviving cellular stresses but also for housekeeping processes such as protein synthesis and gene regulation. All in vitro assays for inhibitors of essential proteins suffer from not only downstream permeability and stability challenges not selected against in the original screen, but also toxicity issues inherent to targeting an essential process. By design, our in vivo assay requires that compounds be amenable to absorption and not lethally potent. Candidates that exhibit cellular toxicity due to excessive target inhibition will further exacerbate the slow-growth phenotype and will be eliminated as therapeutically useful inhibitors of HSF2 during the screen. Alternatively, compounds that incompletely inhibit HSF2 activity and permit physiologic stress responses are the most likely hits in our screen. This type of selection is especially important for essential proteins such as HSFs.

The protocol that we have developed (see [Supplemental material](#)) yields an excellent value for Z-factor and utilizes the simple readout of cell density, measured by OD₆₀₀. While it is more common to utilize a luciferase-based assay that relies on cellular ATP as a surrogate for metabolic activity (BacTiter-Glo, Promega), we found that these systems introduced more noise to the system and consequently decreased the Z-factor (data not shown).

Since no specific inhibitors of any HSF isoform have been reported (Whitesell and Lindquist 2009), identifying even a pan-HSF inhibitor is of considerable clinical interest. In addition, screening procedures usually include either secondary screens to investigate specificity or to sort and categorize the initial pool of hits. The choice of the initial screen is often dictated either by a specific need or by robustness. Considering that overexpression of yeast HSF1 creates a stronger phenotype than overexpression of human HSF2, a high-throughput screening sequence might begin with the assay based on the overexpression of yeast HSF with the goal of identifying pan-HSF inhibitors. Hits would then be subjected to a secondary screen using the hHSF2 overexpression assay to identify hHSF2-specific inhibitors. Unfortunately, the master regulator of human stress genes, hHSF1, is incapable of functionally substituting for yeast HSF1. This defect has previously been attributed to the inability of hHSF1 to trimerize in yeast cells (Liu et al. 1997). Recently, a number of mutant forms of human HSF1 capable of supporting yeast cell growth have been reported (Neef et al. 2013). These mutant forms may be utilized in secondary discriminating assays, provided they manifest a slow-growth phenotype when overexpressed.

One potential argument against the value of our assay for inhibitors of hHSF2 is that utilization of yeast cells in our

Fig. 6 Overexpression of yeast HSF creates strong slow-growth phenotype. The serial spot dilution assay for cells bearing either pYES-empty vector or the pYES-yHSF1 construct is shown for glucose- or galactose-containing media at indicated growth temperatures

screening system might be considered too non-native. However, among eukaryotes, HSFs are rather rare examples of near-complete functional evolutionary conservation. The fact that yeast cells survive and even maintain relatively normal growth rates when reliant upon human HSFs (Liu et al. 1997) demonstrates that humanized yeast strains expressing different isoforms of human HSF are appropriate for screening for compounds' ability to modulate human HSF function. Our demonstration that the expression pattern of constitutive and highly inducible heat shock genes is maintained largely unchanged by the substitution of yeast HSF1 with human HSF2 (Fig. 4) further supports this concept. The existence of three specialized isoforms of human HSFs and their abilities to functionally substitute for the single essential yeast HSF, with the caveat that hHSF1 requires modest mutation (Liu et al. 1997; Neef et al. 2013), provide flexibility for further development of our high-throughput screening assay.

Acknowledgments We thank Myrna Nisenbaum for the help with the manuscript preparation and Pam Crowell for the critical reading of the manuscript and helpful suggestions. We are grateful to Denis Thiele and Daniel Neef for the DNY47 strain and for the p413GPD-hHSF2 plasmid. This work was supported by the National Science Foundation [MCB-1029254], the National Institutes of Health [R03 MH097538], and the Holcomb Award to AME from Butler University.

References

- Akerfelt M, Trouillet D, Mezger V, Sistonen L (2007) Heat shock factors at a crossroad between stress and development. *Ann N Y Acad Sci* 1113:15–27. doi:10.1196/annals.1391.005
- Au Q, Zhang Y, Barber JR, Ng SC, Zhang B (2009) Identification of inhibitors of HSF1 functional activity by high-content target-based screening. *J Biomol Screen* 14:1165–1175. doi:10.1177/1087057109347472
- Balgi AD, Roberge M (2009) Screening for chemical inhibitors of heterologous proteins expressed in yeast using a simple growth-restoration assay. *Methods Mol Biol* 486:125–137. doi:10.1007/978-1-60327-545-3_9
- Bjork JK, Sistonen L (2010) Regulation of the members of the mammalian heat shock factor family. *FEBS J* 277:4126–4139
- Boorstein WR, Craig EA (1990) Structure and regulation of the SSA4 HSP70 gene of *Saccharomyces cerevisiae*. *J Biol Chem* 265:18912–18921
- Chang Y et al (2006) Role of heat-shock factor 2 in cerebral cortex formation and as a regulator of p35 expression. *Genes Dev* 20:836–847. doi:10.1101/gad.366906
- Dai C, Whitesell L, Rogers AB, Lindquist S (2007) Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. *Cell* 130:1005–1018. doi:10.1016/j.cell.2007.07.020
- Erkine AM, Adams CC, Diken T, Gross DS (1996) Heat shock factor gains access to the yeast HSC82 promoter independently of other sequence-specific factors and antagonizes nucleosomal repression of basal and induced transcription. *Mol Cell Biol* 16:7004–7017
- Erkine AM, Magrogan SF, Sekinger EA, Gross DS (1999) Cooperative binding of heat shock factor to the yeast HSP82 promoter in vivo and in vitro. *Mol Cell Biol* 19:1627–1639
- Fiorenza MT, Farkas T, Dissing M, Kolding D, Zimarino V (1995) Complex expression of murine heat shock transcription factors. *Nucleic Acids Res* 23:467–474
- Fujimoto M et al (2004) HSF4 is required for normal cell growth and differentiation during mouse lens development. *EMBO J* 23:4297–4306. doi:10.1038/sj.emboj.7600435
- Hansen RK, Oesterreich S, Lemieux P, Sarge KD, Fuqua SA (1997) Quercetin inhibits heat shock protein induction but not heat shock factor DNA-binding in human breast carcinoma cells. *Biochem Biophys Res Commun* 239:851–856. doi:10.1006/bbrc.1997.7572
- Kushnirov VV (2000) Rapid and reliable protein extraction from yeast. *Yeast* 16:857–860. doi:10.1002/1097-0061(20000630)16:9<857::AID-YEA561>3.0.CO;2-B
- Lecomte S, Desmots F, Le Masson F, Le Goff P, Michel D, Christians ES, Le Drian Y (2010) Roles of heat shock factor 1 and 2 in response to proteasome inhibition: consequence on p53 stability. *Oncogene* 29:4216–4224. doi:10.1038/ncr.2010.171
- Liu XD, Liu PC, Santoro N, Thiele DJ (1997) Conservation of a stress response: human heat shock transcription factors functionally substitute for yeast HSF. *Embo J* 16:6466–6477
- Neef DW, Turski ML, Thiele DJ (2010) Modulation of heat shock transcription factor 1 as a therapeutic target for small molecule intervention in neurodegenerative disease. *PLoS Biol* 8, e1000291. doi:10.1371/journal.pbio.1000291
- Neef DW, Jaeger AM, Thiele DJ (2013) Genetic selection for constitutively trimerized human HSF1 mutants identifies a role for coiled-coil motifs in DNA binding. *G3 (Bethesda)* 3:1315–1324. doi:10.1534/g3.113.006692
- Ostling P, Bjork JK, Roos-Mattjus P, Mezger V, Sistonen L (2007) Heat shock factor 2 (HSF2) contributes to inducible expression of hsp genes through interplay with HSF1. *J Biol Chem* 282:7077–7086. doi:10.1074/jbc.M607556200
- Phillips PA et al (2007) Triptolide induces pancreatic cancer cell death via inhibition of heat shock protein 70. *Cancer Res* 67:9407–9416. doi:10.1158/0008-5472.CAN-07-1077
- Sandqvist A et al (2009) Heterotrimerization of heat-shock factors 1 and 2 provides a transcriptional switch in response to distinct stimuli. *Mol Biol Cell* 20:1340–1347. doi:10.1091/mbc.E08-08-0864
- Santagata S et al (2012) Using the heat-shock response to discover anticancer compounds that target protein homeostasis. *ACS Chem Biol* 7:340–349. doi:10.1021/cb200353m
- Scherz-Shouval R et al (2014) The reprogramming of tumor stroma by HSF1 is a potent enabler of malignancy. *Cell* 158:564–578. doi:10.1016/j.cell.2014.05.045
- Sidera K, Patsavoudi E (2014) HSP90 inhibitors: current development and potential in cancer therapy. *Recent Patents Anti-Cancer Drug Discov* 9:1–20
- Slater MR, Craig EA (1989) The SSA1 and SSA2 genes of the yeast *Saccharomyces cerevisiae*. *Nucleic Acids Res* 17:805–806
- Solimini NL, Luo J, Elledge SJ (2007) Non-oncogene addiction and the stress phenotype of cancer cells. *Cell* 130:986–988. doi:10.1016/j.cell.2007.09.007
- Sorger PK (1990) Yeast heat shock factor contains separable transient and sustained response transcriptional activators. *Cell* 62:793–805
- Tugendreich S et al (2001) A streamlined process to phenotypically profile heterologous cDNAs in parallel using yeast cell-based assays. *Genome Res* 11:1899–1912. doi:10.1101/gr.191601
- Wang G, Zhang J, Moskopidis D, Mivechi NF (2003) Targeted disruption of the heat shock transcription factor (hsf)-2 gene results in increased embryonic lethality, neuronal defects, and reduced spermatogenesis. *Genesis* 36:48–61. doi:10.1002/gene.10200
- Wang G et al (2004) Essential requirement for both hsf1 and hsf2 transcriptional activity in spermatogenesis and male fertility. *Genesis* 38:66–80. doi:10.1002/gene.20005

- Westerheide SD, Kawahara TL, Orton K, Morimoto RI (2006) Triptolide, an inhibitor of the human heat shock response that enhances stress-induced cell death. *J Biol Chem* 281: 9616–9622
- Whitesell L, Lindquist S (2009) Inhibiting the transcription factor HSF1 as an anticancer strategy. *Expert Opin Ther Targets* 13:469–478. doi: [10.1517/14728220902832697](https://doi.org/10.1517/14728220902832697)
- Whitesell L, Santagata S, Lin NU (2012) Inhibiting HSP90 to treat cancer: a strategy in evolution. *Curr Mol Med* 12:1108–1124
- Xiao L, Lu X, Ruden DM (2006) Effectiveness of hsp90 inhibitors as anti-cancer drugs. *Mini Rev Med Chem* 6:1137–1143
- Yoon YJ et al (2011) KRIBB11 inhibits HSP70 synthesis through inhibition of heat shock factor 1 function by impairing the recruitment of positive transcription elongation factor b to the hsp70 promoter. *J Biol Chem* 286:1737–1747. doi: [10.1074/jbc.M110.179440](https://doi.org/10.1074/jbc.M110.179440)
- Zhang JH, Chung TD, Oldenburg KR (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* 4:67–73