Heat Shock Factor 1 Is a Powerful Multifaceted Modifier of Carcinogenesis

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SUMMARY
Heat shock factor 1 (HSF1) is the master regulator of the heat shock response in eukaryotes, a very highly conserved protective mechanism. HSF1 function increases survival under a great many pathophysiological conditions. How it might be involved in malignancy remains largely unexplored. We report that eliminating HSF1 protects mice from tumors induced by mutations of the RAS oncogene or a hot spot mutation in the tumor suppressor p53. In cell culture, HSF1 supports malignant transformation by orchestrating a network of core cellular functions including proliferation, survival, protein synthesis, and glucose metabolism. The striking effects of HSF1 on oncogenic transformation are not limited to mouse systems or tumor initiation; human cancer lines of diverse origins show much greater dependence on HSF1 function to maintain proliferation and survival than their nontransformed counterparts. While it enhances organismal survival and longevity under most circumstances, HSF1 has the opposite effect in supporting the lethal phenomenon of cancer.

INTRODUCTION
The heat shock response is one of the most ancient and evolutionarily conserved protective mechanisms found in nature. Environmental insults provoke a variety of adaptive physiological responses to help organisms cope with specific stressors. The dramatic induction of heat shock proteins (HSPs) is an important unifying component of most of these responses, and this induction has proven to be essential for survival under stressful conditions. Work over the last three decades has revealed that the major HSPs are molecular chaperones that guard against “illicit or promiscuous interactions” between other proteins. Their basal expression facilitates normal protein folding and guards the proteome from the dangers of misfolding and aggregation. In the face of proteotoxic stressors including heat, hypoxia/ischemia, free radicals, ATP depletion, and acidosis, the importance of HSPs in preventing the aggregation and promoting the refolding of other proteins becomes acute. When misfolding exceeds a certain threshold, other HSPs disaggregate proteins and refold them or divert them to the proteasome for destruction (Whitesell and Lindquist, 2005).

Regulation of HSP expression is intricate, with multiple layers of redundancy and feedback control, but a small family of transcription factors called heat shock factors (HSFs) are the primary regulators of stress-inducible expression in eukaryotic cells. The structure and function of HSFs have been conserved for more than a billion years. They bind consensus heat shock elements (HSEs) within the promoter regions of HSP genes (Westerheide and Morimoto, 2005), and this binding is critical to HSP induction. Several HSFs are present in mammalian cells, but HSF1 is clearly the dominant factor controlling cellular responses to stress. Deletion of Hsf1 in mammalian cells allows normal basal expression of HSPs but completely abrogates induction in response to heat shock and a variety of other stresses (Xiao et al., 1999). In mice and Drosophila, HSF1 is dispensable for growth and survival under controlled laboratory conditions but essential for survival following stresses such as high temperature and endotoxin challenge (Jedlicka et al., 1997; Xiao et al., 1999). Hsf1-deficient mouse embryos suffer from defects in placental development and are recovered from crosses in lower numbers than expected by Mendelian segregation. Other than being ~20% smaller than wild-type mice, however, they display no overt organ system abnormalities and, in the absence of acute stress, live to late adulthood (Xiao et al., 1999; A. Steele and S.L., unpublished data).

Although less well understood, the activities of HSF1 extend far beyond the classical induction of HSPs. In yeast, HSF1 has now been shown to regulate up to 3% of the genome and impact genes ranging in function from energy production to signal transduction, from small molecule transport to carbohydrate metabolism, and from cytoskeletal organization to vesicular transport (Hahn et al., 2004). Immunolocalization and chromatin
immunoprecipitation indicate that HSF1 binds to a similarly broad array of non-HSP genes in Drosophila (Westwood et al., 1991; Birch-Machin et al., 2005) and human erythroleukemia cells (Trinklein et al., 2004).

The HSF1-mediated stress response and the activity of specific HSPs have both been implicated in protecting organisms from a broad range of pathophysiological conditions including thermal injury, ischemia/reperfusion, and age-related neurodegeneration (Christians et al., 2002; Westerheide and Morimoto, 2005). Intriguingly, in nematodes, HSF1 promotes longevity under stable laboratory conditions (Hsu et al., 2003; Morley and Morimoto, 2004). Much less is known about the role of HSF1 in cancer. It has long been noted that HSP levels increase in a wide range of tumor types (Jolly and Morimoto, 2000). Many of the signaling pathways and transcription factors that are frequently deranged in cancers display a striking dependence on the chaperone machinery, especially HSP90 (Whitesell and Lindquist, 2005). Moreover, HSF1 expression is elevated in human prostate carcinoma cell lines (Tang et al., 2005). But whether the multifaceted HSF1-mediated stress response plays a causal, supportive, or inhibitory role in mammalian oncogenesis is unknown.

On the one hand, given its prominent role in helping cells cope with stressful insults, HSF1 might promote oncogenesis by facilitating cellular adaptation to the malignant lifestyle. On the other hand, given its general role in enhancing longevity, HSF1 might assist organisms in combating malignancy. To investigate these possibilities, we used both whole-animal and cell-culture models in which HSF1 expression could be disrupted by genetic techniques. We find that HSF1 is a remarkably potent modifier of tumor-free survival in whole animals. Further, it modulates oncogenesis by coordinating a diverse array of core cellular functions and supports the aberrant proliferation and survival of human tumor cell lines carrying a wide range of molecular genetic defects. As a very ancient adaptive mechanism, the HSF1-dependent stress response has evolved to enhance survival in the face of environmental challenges from without and disease processes within such as ischemic injury and neurodegeneration. These broadly recognized beneficial effects, however, contrast sharply with its lethal role in the phenomenon of cancer that we now report.

RESULTS

HSF1 Deficiency Suppresses Chemical Skin Carcinogenesis in Mice

To begin investigating the role of Hsf1 as a modifier of tumorigenesis, we used a classical multistep chemical skin carcinogenesis protocol. In this mouse model, somatic mutations are induced in epidermal cells by a single topical application of the mutagen dimethylbenzantracene (DMBA). Tumor promotion is then achieved by repeated applications of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). Early on, the overwhelming majority of the resulting skin tumors are benign papillomas. A small portion of these tumors spontaneously progress to become malignant squamous cell carcinomas, which are invasive and sometimes metastatic (Yuspa, 1994). When Hsf1 wild-type mice (Hsf1+/+) and their Hsf1 null littermates (Hsf1−/−) were treated with DMBA and TPA, no obvious skin damage or irritation was noticed in either genotype after topical application of the chemicals. There was, however, a striking difference in carcinogen-induced tumorigenesis. Hsf1−/− mice were far more resistant to tumor formation than Hsf1+/+ mice (Figure 1A), and this difference was manifested in several ways. First, the latency period before the development of any tumors was 5 weeks longer in Hsf1−/− mice than in Hsf1+/+ mice (Figure 1B). Second, Hsf1−/− mice exhibited a marked reduction in tumor incidence (Hsf1+/+ 93.1% versus Hsf1−/− 60.9% at week 24, p = 0.0047, chi-square test) (Figure 1B). Third, they had a much lower overall tumor burden. This applied both to the number of tumors that arose (Figure 1C) and to the size the tumors achieved (Figure 1D). Fourth, and most importantly, Hsf1−/− mice survived much longer than their wild-type counterparts (Figure 1E).

To further investigate the extraordinary resistance of Hsf1−/− mice to carcinogen-induced tumorigenesis, an independent experiment was performed in which Hsf1+/− mice and their wild-type littermates were treated with a second mutagen, NMMG (N-methyl-N’-nitro-N-nitrosoguanidine), at week 25 to promote tumor progression. Once again, Hsf1−/− mice developed many fewer tumors. They also had a very strong survival advantage over Hsf1+/+ mice (see Figure S1A in the Supplemental Data available with this article online). Thus, in sharp contrast to the many circumstances under which Hsf1-deficient organisms are at a survival disadvantage relative to wild-type organisms, in survival after chemically induced skin carcinogenesis they have a profound advantage.

Nature of Carcinogen-Induced Tumors

Although there was a large difference in the number of tumors formed in the Hsf1+/+ and Hsf1−/− mice, the percentage of benign versus malignant tumors (papilloma versus squamous cell carcinoma) was comparable (Figures S1C and S1D). Next we asked if the tumors harbored mutations in the H-Ras proto-oncogene. This gene is almost always activated during chemical skin carcinogenesis. Furthermore, activating mutations of RAS occur in approximately 30% of all human cancers including skin cancers (Balmain et al., 1984; Sebti and Adjei, 2004). Fourteen skin lesions were randomly sampled in both genotypes. All harbored activating mutations in H-Ras. Strikingly, these occurred at positions that are known hot spots in human malignancies (Table S1).

HSF1 Deficiency Suppresses Tumorigenesis Driven by Mutant p53

To test the generality of the detrimental effects of HSF1 on tumor-free survival, we examined its impact on the
development of tumors in mice carrying a germline mutation in the tumor suppressor *p53*, the most frequently mutated gene in human cancers. We crossed mice heterozygous for a clinically relevant hot spot mutation (*p53*<sup>R172H</sup>; Olive et al., 2004) with *Hsf1*<sup>+/−</sup> mice. Three genotypes, all heterozygous for *p53*<sup>R172H</sup>, were examined: (1) *Hsf1*<sup>+/+</sup>, (2) *Hsf1*<sup>+/−</sup>/C<sup>0</sup>, and (3) *Hsf1*<sup>/−/−</sup>/C<sup>0</sup>. Mice were allowed to age with no intervention and were monitored for tumor formation and overall survival. Moribund mice were sacrificed and subjected to full necropsy to detect potential tumor formation at the gross and microscopic levels in all major tissues and organs.

Tumor-free survival was dramatically prolonged in *Hsf1*<sup>/−/−</sup> mice carrying a mutant *p53* allele (Figure 2A). Intriguingly, in this model, compromise of *Hsf1* function appeared to alter the distribution of tumor types. *Hsf1*<sup>/−/−</sup> mice had an increase in carcinoma frequency and a decrease in sarcoma frequency compared to *Hsf1*<sup>+/+</sup> mice (Figure 2C). Note, however, that changes in the tumor spectrum of *Hsf1*<sup>/−/−</sup> mice could not be determined because so very few mice of this genotype developed tumors.

**Hsf1 Status Does Not Alter Intrinsic Cell Growth Rates**

To pursue observations made in mice at a cellular and molecular level, we examined the effect of *Hsf1* status on several classical parameters of neoplastic transformation in cell culture. First, we examined freshly isolated mouse embryonic fibroblasts (MEFs). *Hsf1*<sup>+/+</sup> and *Hsf1*<sup>/−/−</sup> cells had comparable growth rates in vitro (data not shown). Moreover, staining cells for DNA content followed by flow cytometry revealed similar cell-cycle profiles in both genotypes (Figure S1B). Thus, as with the skin carcinogenesis model, *Hsf1-*deficient mice had a surprising and profound survival advantage compared to wild-type animals.

**Nature of p53 Mutant Tumors**

Histopathological review revealed that both *Hsf1*<sup>+/+</sup> and *Hsf1*<sup>/−/−</sup> mice produced a broad spectrum of tumor types when carrying the *p53*<sup>R172H</sup> hot spot mutation. These included sarcomas, lymphomas, and carcinomas (Figure 2B). Intriguingly, in this model, compromise of *Hsf1* function appeared to alter the distribution of tumor types. *Hsf1*<sup>/−/−</sup> mice had an increase in carcinoma frequency and a decrease in sarcoma frequency compared to *Hsf1*<sup>+/+</sup> mice (Figure 2C). Note, however, that changes in the tumor spectrum of *Hsf1*<sup>/−/−</sup> mice could not be determined because so very few mice of this genotype developed tumors.

**Figure 1. HSF1 Deficiency Suppresses Chemical Skin Carcinogenesis**

(A) Representative images of mouse skin tumors 25 weeks after topical DMBA application.

(B) Lower skin tumor incidence and longer incubation time in *Hsf1*<sup>/−/−</sup> mice (p < 0.0001, two-way ANOVA).

(C) Lower tumor burden in *Hsf1*<sup>/−/−</sup> mice. The data are presented as the number of skin tumors per mouse (mean ± SE, p < 0.0001, two-way ANOVA).

(D) Smaller tumor volumes in *Hsf1*<sup>/−/−</sup> mice (the lines indicate geometric means; p = 0.0003, Mann Whitney test).

(E) The survival curves of *Hsf1*<sup>/−/−</sup> and *Hsf1*<sup>/−/−</sup> mice following skin carcinogenesis (median survival: *Hsf1*<sup>/−/−</sup> 41 weeks; *Hsf1*<sup>/−/−</sup> undefined; p = 0.0073, Logrank test).
Figure 2. HSF1 Deficiency Suppresses Tumorigenesis Driven by Mutant p53

(A) Tumor-free survival curves of p53R172H knockin mice (Hsf1+/+ versus Hsf1−/−, p = 0.0001; Hsf1+/− versus Hsf1−/−, p = 0.0185; Hsf1+/+ versus Hsf1+/−, p = 0.0387; Logrank test).

(B) Representative micrographs of tumors (scale bars, 160 μm). (Ba) Carcinoma, (Bb) lymphoma, (Bc) soft tissue sarcoma, (Bd) teratoma (A, adipose; B, bone; M, muscle; RE, respiratory epithelium).

(C) Tumor spectra of Hsf1+/+ and Hsf1+/− mice (Hsf1+/+, n = 15; Hsf1+/−, n = 10).
(McMillan et al., 1998) and transformed them with a variety of oncogenes.

**HSF1 Enables Cellular Transformation Initiated by Oncogenic RAS**

To directly investigate susceptibility to transformation by RAS, MEFs were transduced with retroviruses encoding green fluorescent protein (GFP), mouse Hsf1, or oncogenic H-RASV12D, a RAS mutation commonly found in human cancers. After several weeks in culture, cells were fixed and stained to visualize the transformed foci that had arisen as a consequence of the classic RAS-mediated loss of contact inhibition of growth.

Transduction with H-RASV12D induced high rates of focus formation in wild-type cells, but such foci were rare in cells derived from mice carrying the germline deletion (Hsf1−/− cells; Figure 3A and Figure S2A). Cells from both the abundant Hsf1+/− foci and the rare Hsf1−/− foci were fully transformed as measured by soft agar cloning and tumor formation following subcutaneous injection into nude mice (data not shown). As expected, transduction with virus encoding GFP did not induce focus formation. Importantly, focus formation was also not observed with Hsf1 overexpression (Figure 3A and Figure S2A).

Thus, by this criterion, Hsf1 acts as a powerful modifier of tumorigenesis rather than as an oncogene per se.

Reduced transformation of Hsf1−/− cells was not due to an intrinsic growth defect, nor was it due to reduced viral transduction efficiency. Both immortalized cell lines displayed comparable saturation densities (Figure 3A, mean ± SD, Hsf1−/−: 2832 ± 267 nuclei per field versus Hsf1+/−: 3411 ± 275, n = 5, p = 0.0086) and proliferation characteristics (Figure S4). If anything, Hsf1−/− MEFs displayed slightly greater gene transfer efficiencies as measured by flow cytometry after transduction with GFP-encoding retrovirus (mean ± SD, Hsf1+/−: 9.0% ± 0.6% positive versus Hsf1−/−: 12.7% ± 1.4% positive, n = 4, p = 0.003).

To control for the unlikely possibility that reduced transformation in Hsf1−/− MEFs was due to a potent but unknown polymorphism that happened to be closely linked to the HSF1 gene, we took advantage of short hairpin RNA interference (shRNAi) technology. Independent to the HSF1 gene, we took advantage of short hairpin RNA interference (shRNAi) technology. Independent

**HSF1 Enhances Proliferation and Survival in Response to Diverse Oncogenic Stimuli**

Neoplastic stimuli can increase the rates of cell proliferation, cell survival, or both. To determine which of these processes is influenced by HSF1, immortalized Hsf1−/− and Hsf1+/− MEFs were transduced with retroviruses encoding GFP or several mechanistically distinct oncogenes. Retroviral transduction of H-RAS and PDGF-B drove a marked increase in cell number in Hsf1+/− cells, but not in Hsf1−/− cells (Figure 3C). This was due to increased proliferation of Hsf1−/− cells rather than increased death in Hsf1−/− cells (Figure 3E).

Unlike RAS and PDGF-B, which act as mitogenic signal transducers, c-MYC and LTA act primarily as regulators of cell-cycle progression and might not be expected to dramatically increase proliferation in these already-immortalized cell lines. Indeed, c-MYC and SV40 Large T Antigen (LTA) did not significantly increase cell accumulation (Figure 3D) or induce focus formation (data not shown). LTA and c-MYC can, however, predispose cells to apoptosis (Evan et al., 1992; Yin et al., 1997). Indeed, in contrast with RAS and PDGF, c-MYC and LTA sharply increased cell death in Hsf1−/− cultures, but not in Hsf1+/− cultures (Figure 3E). Thus, depending upon the nature of the oncogenic stimuli involved, HSF1 enables oncogenic transformation in at least two ways, by permitting increased cell proliferation and/or by decreasing cell death.

**HSF1 Modulates Signal Transduction**

The ability to sustain dysregulated signaling is crucial to human cancers. In light of our observation that Hsf1−/− MEFs are resistant to RAS-driven transformation, we sampled downstream effectors in the RAS/MAPK signaling pathways. HSF1 deficiency caused a marked reduction in the levels of kinase suppressor of RAS 1 (KSR1) protein, both in Hsf1−/− MEFs and in shRNAi knockout lines (Figure 4A). Furthermore, activation of the downstream effector, ERK, was blunted in Hsf1−/− MEFs following serum stimulation (Figure 4B).

We also asked whether Hsf1 affects the G protein-coupled receptor (GPCR) pathway, which increases cAMP levels, drives PKA activation, and is implicated in many human cancers (Bossis et al., 2004). A marked reduction in the phosphorylation of endogenous PKA substrates was observed in cells with germline Hsf1 deletion (Figure 4C). shRNAi-mediated Hsf1 knockdown in wild-type cells produced a similar effect. (In this case, dosage
sensitivity was apparent.) These differences in PKA activity were verified by directly measuring the phosphorylation of a standard peptide substrate. Lysates from Hsf1−/− cells demonstrated less than half the PKA activity of lysates from Hsf1+/+ cells (mean ± SD, Hsf1+/+ 2452 ± 451 versus Hsf1−/− 917 ± 100, n = 2, p < 0.05) (Figure 4D). Having found that HSF1 modulates at least two classical oncogenic signaling pathways, we asked if it affects other crucial, but more recently recognized, cancer-related processes: ribosomal biogenesis and translation control.

Figure 3. HSF1 Enables Cellular Transformation Initiated by Oncogenic RAS and PDGF-B
(A) Hsf1−/− MEFs are relatively resistant to focus formation driven by oncogenic H-RASV12D. Immortalized MEFs were plated and transduced with retroviruses encoding the genes indicated. Foci were fixed and visualized by dye staining. The number of foci per well was quantified as shown in Figure S2. All experiments were repeated once with similar results.
(B) Hsf1−/− MEFs are relatively resistant to focus formation driven by the proto-oncogene PDGF-B.
(C) Hsf1−/− MEFs are refractory to proliferation driven by oncogenic RAS and PDGF-B. Equal numbers of immortalized Hsf1+/+ and Hsf1−/− MEFs were transduced with retroviruses encoding GFP, H-RASV12D, or PDGF-B. The cells were fixed on the indicated days and the number of cells per well determined by fluorescent DNA staining. Relative cell number was calculated by normalizing the values against the GFP-transduced group at each time point (mean ± SD, n = 5, ***p < 0.001, two-way ANOVA).
(D) Expression of c-MYC and LTA does not drive marked proliferation in immortalized Hsf1+/+ and Hsf1−/− MEFs.
(E) Hsf1−/− MEFs show no enhanced survival in response to RAS and PDGF/B expression but reduced survival in response to c-MYC and LTA expression. Viability of immortalized Hsf1+/+ and Hsf1−/− MEFs was determined by flow cytometry 36 hr after transduction. The data are presented as percent nonviable cells (mean ± SD, n = 5, *p < 0.05; ***p < 0.001, Student’s t test).
HSF1 Modulates the Translation Machinery

The dysregulated growth of cancer cells requires growth factor independence in the control of ribosome biogenesis and protein translation. To reveal a potential role for HSF1 in this process, we cultured MEFs under growth factor-depleted conditions—that is, serum starvation. HSF1 status caused no difference in the levels of eIF4E, an mRNA cap-binding protein, β-actin, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). However, HSF1-deficient MEFs consistently had reduced levels of the three ribosomal subunits tested, L26RP, L28RP, and S6RP, whether they were deficient from germline knockout or from an shRNAi (Figures 5A and 5B). Furthermore, much lower levels of phosphorylated ribosomal protein S6 kinase (p70 S6K), a potent regulator of translational activity, were observed in Hsf1−/− cells compared to wild-type cells (Figures 5A and 5B). Importantly, in both germline-deleted and shRNAi knockdown cells, comparable levels of these ribosomal proteins were restored when the cells were returned to culture in serum-replete medium (Figures 5A and 5B). Thus, HSF1 deficiency enforces tighter growth factor dependency on the translational machinery.

Inhibition of mTOR function by rapamycin impairs protein translation and reduces cell size. Eliminating its downstream effector, p70 S6K, recapitulates this phenotype (Fingar et al., 2002). The lower levels of p70 S6K phosphorylation in Hsf1−/− cells, together with the smaller size of Hsf1−/− mice (Xiao et al., 1999; and data not shown) led us to ask if individual cells derived from Hsf1−/− mice are smaller than Hsf1+/+ cells. Indeed, the mean cell volume (μL) of Hsf1−/− MEFs was 20% less than that of Hsf1+/+ MEFs (1925 ± 49 versus 2401 ± 184, mean ± SD, n = 3, p < 0.05). This suggested that the
mTOR pathway might be affected by Hsf1 status. Indeed, 
Hsf1<sup>-/-</sup> cells were significantly more sensitive than 
Hsf1<sup>+/+</sup> cells to rapamycin-induced growth inhibition (Figure 5C 
and Figure S5A). The hypersensitivity of 
Hsf1<sup>-/-</sup> cells was not due to increased cell death (data not shown). 
Instead, rapamycin caused the same type of cell-cycle 
arrest in G1 typical of mTOR inhibition, but it was more 
profound in Hsf1<sup>-/-</sup> than in Hsf1<sup>+/+</sup> cells (Figure 5D). Nota-
bly, rapamycin neither induced a heat shock response nor 
impaired it (Figure S5B). Thus, independently of a classic 
proteotoxic stress response, HSF1 maintains the activity 
of the translation machinery and permits continued cell-
cycle progression in immortalized but nontransformed 
cells under growth factor-reduced conditions in a manner 
that likely involves the role of mTOR in regulating protein 
translation.

**HSF1 Modulates Glucose Metabolism**

Unlike normal cells, virtually all cancer cells preferentially 
catabolize glucose by glycolysis, even under normoxic 
conditions, and thereby produce high levels of lactic acid 
(Bissell et al., 1976; Gatenby and Gillies, 2004). Recent ev-
eidence indicates that increased glycolysis is a conse-
quence of oncogenic transformation and is advantageous 
to tumor growth and survival (Fantin et al., 2006; Matoba 
et al., 2006). To determine whether Hsf1 status alters glu-
cose metabolism, we first examined glucose uptake, 
which is almost universally increased in cancers.
Hsf1+/+ and Hsf1−/− MEFs were cultured overnight in the presence of a fluorescent, noncleavable glucose analog, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose (2-NBDG). Hsf1−/− MEFs accumulated much less 2-NBDG than did Hsf1+/+ cells (Figure 6A). Hsf1 knockdown MEFs exhibited the same trend, although greater variability was observed, as expected in the heterogeneous population of knockdown cells (Figure 6B).

Tumor cells, which utilize glucose at a much higher rate than normal cells, are generally more sensitive to glucose deprivation. We asked if Hsf1−/− cells are less addicted to glucose and less sensitive to its deprivation. Culturing Hsf1+/+ MEFs for 4 days in glucose-reduced medium led to a drastic decrease in total cell number and cell viability relative to cells cultured in standard high-glucose medium (Figures 6C and 6D). Hsf1−/− MEFs tolerated low glucose conditions much better. Thus, HSF1 deficiency leads to reduced dependence on glucose to support cellular energy needs and/or more efficient use of the sugar. Two other findings support this hypothesis. First, the nonmetabolizable glucose analog 2-deoxy-glucose (2-DG), which competes with glucose for the key glycolytic enzyme phosphohexose isomerase, killed Hsf1+/+ cells more efficiently than Hsf1−/− cells (Figure S6A). Second, under glucose-replete conditions, Hsf1−/− and shRNAi C2 cells generated significantly less lactate and possessed lower lactate dehydrogenase (LDH) activity than Hsf1+/+ cells (Figures S6B and S6C). Thus, the resistance of Hsf1-deficient cells to malignant transformation that we have demonstrated is associated with a pattern of basal glucose metabolism that could make it more difficult for them to undergo the glycolytic shift characteristic of cancers.

HSF1 Maintains the Transformed Phenotype

Having established in whole mice and cultured mouse cells that HSF1 is required in multifaceted ways for the initiation of transformation, we asked if it was also required for the maintenance of transformed phenotypes in established oncogenic cell lines and, at the same time, whether it was relevant to human cancers. We chose human lines of varying malignant potential, lines created in the laboratory with known oncogenes and lines derived from naturally occurring tumors with a diversity of histopathological origins and a broad spectrum of molecular genetic
To modulate HSF1 expression, we evaluated three independent human HSF1-targeted shRNAi constructs, hA8, hA9, and hA6, and two control constructs (a GFP-targeted shRNAi and an shRNAi with no known homology within the human genome), all of the same infectious titers (Figure S8). Only the three HSF1-targeted vectors lowered HSF1 levels (Figure 7A); the most efficacious, hA6 and hA9, were selected for further analysis.

We first examined three breast cell lines representing progressively more oncogenic states (Figure 7B): (1) primary human mammary epithelial (PHME) cells; (2) human mammary epithelial (HME) cells made immortal, but nontumorigenic, by expression of hTERT (telomerase); and (3) HME cells rendered fully transformed and tumorigenic by introduction of LTA and H-RAS in addition to hTERT (HMLER) (Elenbaas et al., 2001). PHME cells were little affected by transduction with HSF1-targeted constructs. Tumorigenic HMLER cells were strongly affected. Immortalized, but nontumorogenic, cells (HME) were intermediate in sensitivity, suggesting a correlation between oncogenic state and HSF1 dependence (Figure 7B).

Next we examined a diverse collection of breast cell lines derived from spontaneous human tumors (Figure 7C). The lines varied with regard to p53 status, carrying wild-type (MCF-7) or various mutant alleles (BT-474, MDA-MB-231, and T47D) (International Agency for Research on Cancer TP53 Database) and with regard to HER2 overexpression, estrogen sensitivity, and metastatic potential (MD Anderson Breast Cancer Cell Line Database). All were strongly affected by both of the HSF1-inhibitory hairpins; none was affected by the control hairpins.

Finally, we examined malignant cells of diverse histological origins either derived from human tumors (HeLa [cervix], PC-3 [prostate], and S462 and 90-8 [peripheral nerve sheath]) or derived by in vitro transformation (293T [kidney]). All were strongly inhibited by HSF1 knockdown (Figure 7D). Where a difference in sensitivity to the two targeting hairpins was observed, it was always the hairpin that inhibited HSF1 expression the most severely (hA6; Figure 7A) that had the stronger effect (Figures 7B and 7C). This strong correlation between the extent of HSF1 inhibition and phenotypic effects, together with the fact that...
similar results were obtained with independent human and mouse targeting sequences and with a mouse germline knockout, argue strongly against “off-target” factors being responsible for these effects. Note also that the hairpins had no effect on normal diploid human fibroblasts (WI-38; Figure 7D). Therefore, we conclude that, in addition to its enabling role in tumor initiation, HSF1 function helps to maintain the growth and survival of human cancer cells with diverse underlying malignant defects.

DISCUSSION

Modulation of Oncogenesis by HSF1

As master regulator of the heat shock response, HSF1 enhances organismal survival and longevity in the face of environmental challenges. In sharp contrast to its widely appreciated beneficial effects, we now report that HSF1 can act to the detriment of organisms by supporting malignant transformation. In mice, HSF1 deficiency dramatically limited spontaneous tumor formation initiated by a common, dominant-negative mutation of the p53 tumor suppressor gene. It had a similar effect on chemical-induced skin carcinogenesis associated with activating mutations of the H-Ras proto-oncogene. The reduced incidence of tumors in these animals, and their associated effects on long-term survival, cannot be due to an effect of HSF1 on mutation rates. HSF1 deficiency rendered cultured cells highly refractory to transformation initiated by mutated H-RAS and by PDGF-B overexpression. Most importantly, the depletion of HSF1 in diverse previously established human cancer cell lines strongly impaired their growth and survival while having little effect on normal cells.

Tumor cells undergo a drastic shift of intracellular milieu during transformation. Gross alterations in signal transduction, energy production, and the metabolism of nucleic acids and protein are inevitable. In addition, cells within a tumor mass are frequently exposed to harsh and rapidly changing microenvironments that include such stressors as hypoxia, acidosis, nutrient deprivation, and immune attacks from the host. To survive and prosper, like organisms living in the wild, tumor cells within a host must be able to adapt effectively. Our data indicate that HSF1, the master regulator of one of the most ancient and evolutionarily conserved adaptive mechanisms, plays a prominent role in enabling cells to accommodate such drastic alterations, survive initial oncogenic stresses, and successfully adapt to the malignant state. In this setting, HSF1 function strongly reduces life span of the host.

Concurrent with our findings, expression of the oncoproteins heregulin beta1 and Ras has recently been reported to activate HSF1. This activation protects such cells against apoptosis and enables anchorage-independent growth (Khaleque et al., 2005; Stanhill et al., 2006). During the preparation of this manuscript, another group reported that HSF1 deficiency alters the spectrum of tumors arising in p53 knockout mice (Min et al., 2007). In contrast with our findings, however, they found no significant effect either on overall tumor incidence or on tumor-free survival. Several factors may account for this major discrepancy. First, Min et al. used a different transgenic strategy to knock out Hsf1, which resulted in unanticipated recombination events and only deleted the second exon of the gene. Although wild-type HSF1 protein was not detected, a truncated Hsf1 message was produced whose biological consequences are unknown (Zhang et al., 2002). The knockout mouse strain used to generate our data expresses no Hsf1 message. Furthermore, to address potential artifacts of transgenesis, our key findings using Hsf1 knockout cells were confirmed using a variety of independent mouse and human shRNAi constructs. Second, Min et al. evaluated the effects of HSF1 deficiency in a p53 null mouse model while we looked at a clinically relevant p53 missense mutation. Missense mutations in the p53 DNA-binding domain are the most clinically relevant lesions and have been reported in more than 50 different types of human cancer. Though complete deletions are commonly associated with human malignancy for other tumor suppressor genes, they occur rarely for p53, and individuals with germline homozgyous deletions are unknown, raising questions as to the relevance of homozgyous p53 deletion as a cancer model (Olive et al., 2004). Perhaps most importantly, we avoided model-specific limitations by examining a variety of mechanistically distinct oncogenic stimuli other than p53 alteration and demonstrated consistent effects in whole animals, mouse cell cultures, and diverse human cancer cell lines. Given these considerations, we believe our findings capture the role of HSF1 in oncogenesis on a broader, more biologically and clinically relevant level.

Given the global nature of the alterations in cellular physiology that occur during malignancy, rather than deeply interrogating individual pathways, we pursued a broad survey of potential mechanisms whereby HSF1 might modify tumorigenesis. We found moderate but highly significant and readily detectable effects of HSF1 on a broad array of cellular functions, all of which play a role in successful malignant transformation. First, depending on the nature of the oncogenic stimulus, HSF1 enhances cell proliferation and/or survival. Second, HSF1 modulates both of the major cancer-promoting signal transduction cascades that we tested, the RAS/MAPK and cAMP/ PKA pathways, and likely affects many more. Third, HSF1 maintains efficient ribosomal biogenesis and p70 S6K activation under growth factor-limited conditions, which are particularly relevant to malignant states. Lastly, HSF1 promotes glycolysis, a key metabolic pathway for tumor growth and survival.

Our data indicate that HSF1 itself does not act as a classical oncogene or tumor suppressor. Neither enforced overexpression nor knockout directly drives transformation. Instead, HSF1 orchestrates a broad network of cellular functions that act globally to support tumorigenesis (Figure S9). Individually, any one of these effects might modify tumorigenesis. We found moderate but highly significant and readily detectable effects of HSF1 on a broad array of cellular functions, all of which play a role in successful malignant transformation. First, depending on the nature of the oncogenic stimulus, HSF1 enhances cell proliferation and/or survival. Second, HSF1 modulates both of the major cancer-promoting signal transduction cascades that we tested, the RAS/MAPK and cAMP/ PKA pathways, and likely affects many more. Third, HSF1 maintains efficient ribosomal biogenesis and p70 S6K activation under growth factor-limited conditions, which are particularly relevant to malignant states. Lastly, HSF1 promotes glycolysis, a key metabolic pathway for tumor growth and survival.

Our data indicate that HSF1 itself does not act as a classical oncogene or tumor suppressor. Neither enforced overexpression nor knockout directly drives transformation. Instead, HSF1 orchestrates a broad network of cellular functions that act globally to support tumorigenesis (Figure S9). Individually, any one of these effects might have only a modest influence on malignant transformation. We suggest that the profound effects of HSF1 on tumor initiation and transformation are due to their acting in...
concert with each other and, indeed, with yet other mechanisms still to be discovered.

As a therapeutically relevant extension to our findings on tumor initiation, we find that HSF1 also promotes cancer cell maintenance. Hsf1 knockout is not lethal, or even detrimental to normal life span, in mice. Not surprisingly, we find that HSF1 knockdown has a minimal effect on the proliferation/survival of normal primary human cells in culture. In marked contrast, compromise of HSF1 profoundly impaired a wide variety of established human malignant as well as premalignant cell lines without relationship to the diversity of their known underlying molecular genetic defects.

**Coupling Cellular Physiology to Environmental Contingency by HSF1**

The induction of HSPs by HSF1 can potentiate oncogenesis in a variety of ways. For example, HSP90 chaperones many signal transducers, and certain oncoproteins are particularly dependent upon it (Whitesell and Lindquist, 2005). Further, HSP70 and other chaperones interface with the apoptotic machinery with important functional consequences (Jolly and Morimoto, 2000). It is essential to realize, however, that the effects of HSF1 extend far beyond HSP induction.

Befitting a survival factor highly conserved for more than 1 billion years and broadly deployed to combat a wide variety of physiologically distinct toxicities, HSF1 is emerging as the coordinator of an extensive array of cellular pathways at a system-wide level. For example, heat stress activates the RAS/MAPK pathway in an HSF1-dependent manner (Mivechi and Giaccia, 1995). Given that intracellular cAMP levels rise upon heat stress and the cAMP/PKA pathway negatively regulates HSF1 (Ferguson et al., 2005; Sawaji et al., 1999), crosstalk between the cAMP/PKA pathways and other HSF1-mediated response pathways seems almost certain. In yeast, HSF1 regulates 3% of the genome and occupies the promoter regions of a wide variety of genes, including those for several key glycolytic enzymes (Hahn et al., 2004). It appears to regulate a similarly broad spectrum of genes in Drosophila and man (Westwood et al., 1991; Birch-Machin et al., 2005; Trinklein et al., 2004). Our data indicate the involvement of HSF1 in regulating translation, ribosome biogenesis, and glucose metabolism in mammalian cells. For this emerging, system-wide regulation of growth and survival pathways by HSF1, our work establishes a vital functional consequence in oncogenesis.

**Balancing Aging, Longevity, and Cancer Risk**

Strikingly, our data now make it clear that HSF1 plays opposite roles in the complex diseases that plague aging populations. It powerfully potentiates the development of cancers. But it has also been implicated in protection against ischemia/reperfusion injury, neurodegenerative disorders, and other broad-ranging physiological processes affecting life span, such as aging and senescence (Auluck et al., 2005; Cohen et al., 2006; Gutsmann-Conrad et al., 1998; Hsu et al., 2003; Morley and Morimoto, 2004). In aging cells and organisms, alterations in energy metabolism, signal transduction, and protein homeostasis are accompanied by blunting of the stress response, ultimately leading to compromised viability.

Such an astonishing duality for the effects of HSF1 mirrors the surprising double-edged roles recently reported for the tumor suppressors p53 and p16<sup>INK4a</sup>. On the one hand, expression of p53 or p16<sup>INK4a</sup> shortens life span by accelerating cellular senescence and limiting the regenerative capacity of stem and progenitor cells; on the other hand, their activities extend life span by suppressing the emergence of life-threatening cancers (Beausejour and Campisi, 2006; Tyner et al., 2002). Our findings establish that an even more ancient survival factor, HSF1, has a similar duality, in reverse: its normal functions extend life span but in the case of cancer have a gravely deleterious impact on organismal survival. At a fundamental level, the ability of HSF1 to enable lethal malignancies is an unfortunate legacy of its ancient role in enhancing the survival of normal cells exposed to diverse acute and chronic stresses.

Our findings also have important therapeutic implications. An expanding array of small, drug-like compounds are becoming available with potent HSF1-modulating activity in cells and whole organisms. Therapeutic induction of the HSF1-mediated stress response by nontoxic exposure to agents such as HSP90 inhibitors and celastrol is being explored in hypoxic-ischemic injury and protein misfolding disorders such as Huntington’s and Parkinson’s disease (Lu et al., 2002; Wang et al., 2005). Whether the therapeutic activation of HSF1 would increase the likelihood of oncogenic transformation is unknown, but it seems imperative to determine. Conversely, based on our findings with human cell lines, inhibiting HSF1 activation could provide a multifaceted and broadly effective cancer chemopreventive as well as chemotherapeutic strategy but might accelerate neurodegenerative processes and aging. A profound dichotomy holds true for other centrally poised drug targets such as the proteosome, whose inhibition is associated with promising anticancer activity in certain malignancies (Richardson et al., 2006) but in some situations might exacerbate neurodegenerative processes (McNaught et al., 2004). Using compounds that do not cross the blood-brain barrier is just one of many possible strategies to maximize benefits and reduce risks. We have much to learn about how the ancient adaptive function of HSF1 operates in the maze-like interface between genotype and environment to modify malignancy and other complex “diseases of civilization.” At the heart of this maze lies the promise of far more effective therapeutic intervention.

**EXPERIMENTAL PROCEDURES**

**Skin Carcinogenesis Study**

Hsf1<sup>−/−</sup> mice (BALB/c x 129SvEv), a gift from Irvor J. Benjamin, were intercrossed. DMBA (Sigma) (100 µg) dissolved in acetone was...
applied topically to each mouse. One week later, 20 μg of TPA (Sigma) was applied to each mouse twice a week for 24 weeks. Skin tumor formation was monitored weekly, and tumor dimensions were measured by caliper. The tumor volume is calculated as \[\text{volume} = \frac{\text{width}^2 \times \text{length}}{2}.\]

**Tumorigenesis Study of p53R172H Mice**

p53R172H/+ mice (129S4/SvJae), a gift from Tyler Jacks, were crossed with Hsf1−/− mice. Moribund mice or mice with severely compromised health conditions were euthanized, and all major tissues were harvested and fixed in 10% formalin (soft tissues) or Bouin’s fixative (bones). Tumors were identified and diagnosed by an experienced veterinary pathologist (A.B.R.). Mice harboring no tumors were considered as censored subjects.

**Focus Formation Assay and Image Analysis**

Cells (5 x 10⁴ or 1 x 10⁵) were incubated with viral supernatants overnight. After 2 or 3 weeks, cells were fixed in cold 100% methanol. Foci were visualized by staining cells with 0.1% toluidine blue solution followed by destaining with 1% acetic acid. Images were documented with a FluorChem Imaging System (Alpha Innotech). The numbers and sizes of foci were further measured using CellProfiler software.

**Lentiviral shRNA Knockdown Experiment**

Lentiviral pLKO.1-puro shRNA constructs were obtained from the RNAi Consortium. Specific hairpin sequences and detailed viral production procedures can be found in the Supplemental Data.

**Cell Proliferation Assay**

Cell number and mass were quantitated by DNA (Hoechst 33342) and protein (sulforhodamine B) staining, respectively, using standard procedures. Detailed procedures are described in the Supplemental Data.

**Cell Counting and Viability Assay**

Cell number and viability were quantitated by microcapillary flow cytometer (Guava EasyCyte System, Guava Technologies) using the ViaCount reagent (Guava Technologies).

**In-Cell Western**

Cells were fixed with 10% formalin/PBS solution for 20 min. Wells were blocked with Odyssey blocking buffer (LI-COR Biosciences) for 2 hr at RT and incubated with phospho-p44/42 MAPK (Thr202/Tyr204) Ab (4377, Cell Signaling Technology) at 4°C overnight. After washing, cells were stained with IRDye 800CW Donkey Anti-Rabbit (LI-COR Biosciences) and TO-PRO-3 iodide (Invitrogen) for 1 hr at RT. After washing, microplates were scanned using the Odyssey Infrared imaging system (LI-COR Biosciences).

**PKA Activity Assay**

PKA activity was measured with the PepTag Non-Radioactive cAMP-Dependent Protein Kinase Assay kit (Promega).

**Glucose Uptake Analysis**

After incubation with medium containing 100 μM 2-NBDG (Invitrogen) overnight, cells were washed with PBS and detached. The relative fluorescence intensity of cells was measured using a Guava EasyCyte cytometer, and histograms were plotted using WinMDI software.

**Cell Survival Assay**

Relative cell growth/survival was measured using the CellTiter-Blue Cell Viability Assay Reagent (Promega).

**Statistical Analysis**

All statistical analyses were performed using Prism 4 (GraphPad Software).

**Supplemental Data**

Supplemental data include Supplemental Experimental Procedures, nine figures, and one table and can be found with this article online at http://www.cell.com/cgi/content/full/130/6/1005/DC1/.

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