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Targeted Disruption of *hsf1* Leads to Lack of Thermotolerance and Defines Tissue-Specific Regulation for Stress-Inducible Hsp Molecular Chaperones

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Abstract The rapid synthesis of heat shock proteins (Hsps) in cells subjected to environmental challenge is controlled by heat shock transcription factor-1 (Hsf1). Regulation of Hsps by Hsf1 is highly complex and, in the whole organism, remains largely unexplored. In this study, we have used mouse embryo fibroblasts and bone marrow progenitor cells from *hsf1*$^{-/-}$ mice as well as *hsp70.3-lacZ* knock-in mice bred on the *hsf1*deficient genetic background (*hsf1*$^{-/-}$-*hsp70.3*$^{+/-}$-*lacZ*), to further elucidate the function of Hsf1 and its participation as a transcriptional activator of Hsp70 synthesis under normal or heat-induced stress conditions in vitro and in vivo. The results revealed that heat-induced Hsp70 expression in mouse tissue is entirely controlled by Hsf1, whereas its activity is not required for tissue-specific constitutive Hsp70 expression. We further demonstrate that Hsf1 is critical for maintaining cellular integrity after heat stress and that cells from *hsf1*$^{-/-}$ mice lack the ability to develop thermotolerance. This deficiency is explained by the elimination of stress-inducible Hsp70 and Hsp25 response in the absence of Hsf1 activity, leading to a lack of Hsp-mediated inhibition of apoptotic cell death via both caspase-dependent and caspase-independent pathways. The pivotal role of the Hsf1 transactivator in regulating rapid synthesis of Hsps as a critical cellular defense mechanism against environmental stress-induced damage is underlined. J. Cell. Biochem. 86: 376–393, 2002.

Key words: heat shock factor 1; heat shock protein 70; gene disruption; knock-in; apoptosis

A critical role in the cellular response to acute stress situations has been ascribed to the Hsp70 protein family of molecular chaperones, which play an essential role in translocation, degradation, and folding of proteins due to their unique ability to bind and stabilize the conformation of proteins that are in a non-native state [Hartl, 1996; Bukau and Horwich, 1998]. In mammals, the Hsp70 family consists of both constitutively expressed (Hsc70) and stress-inducible Hsp70 proteins encoded by the *hsp70.3* and *hsp70.1* genes, which are located within the major histocompatibility complex class III locus with the *Hsct* gene, a testis-specific heat shock cognate [Gaskins et al., 1990; Gunther and Walter, 1994; Christians et al., 1997; Feder and Hofmann, 1999]. The complex regulatory functions attributed to the Hsp70 family of proteins as well as to other inducible Hsps have led to the speculation of their potential involvement in multiple disease processes such as cancer, neurodegenerative diseases such as Alzheimer's, artheroscelerosis, sepsis, and febrile illness [Benjamin and Mcmillan, 1998; Smith et al., 1998]. In addition, a critical anti-apoptotic role has been assigned to Hsp70 and other stress-inducible Hsps (such as Hsp25 and Hsp84/86) [Xanthoudakis and Nicholson, 2000].

The expression of heat shock proteins (Hsps) is mainly regulated at the transcriptional level by heat shock transcription factors (Hsfs) [Kingston et al., 1987; Sorger, 1991;Westwood and Wu, 1993]. In lower organisms, such as yeast and *Drosophila*, regulation of the heat shock response is accomplished by a single *hsf* gene [Wu, 1995]. In higher eukaryotes, however, there are multiple Hsfs that respond to

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diverse environmental insults [Wu, 1995]. The three members of this family that have been identified in the mouse (Hsf1, Hsf2, and Hsf4) have 40% sequence conservation including a homologous winged helix-turn-helix DNA binding domain and an adjacent hydrophobic heptad repeat (HR A/B) that is required for oligomerization of the molecule [Sarge et al., 1991; Tanabe et al., 1999]. Once activated, Hsf1, Hsf2, or Hsf4 bind to heat shock elements (HSEs), consisting of several inverted arrays of 5′-nGAAn-3′ binding sites present upstream of the transcription initiation site [Wu, 1995]. Optimal pentanucleotide binding sites of different Hsfs on consensus HSEs may differ, suggesting perhaps that the various Hsfs in higher eukaryotes have different target gene specificities, leading to different biological functions [Kroeger and Morimoto, 1994; Wu, 1995]. Mammalian Hsf1 exists as a latent monomer that trimerizes upon stress, and binds DNA, resulting in chromatin remodeling followed by transcriptional competence [Sarge et al., 1993; Wu, 1995; Brown and Kingston, 1997]. Hsf1 is ubiquitously expressed and is the most effective transactivator of stress-induced expression of Hsp70 genes, and associates with multiple proteins during its activation and inactivation processes suggesting that Hsf1 activity is regulated at multiple levels [Morimoto, 1998]. In contrast, Hsf2 activates transcription of Hsps once the protein accumulates in cells, for example when cells are treated with agents that inhibit the ubiquitin-proteosome pathway [Sistonen et al., 1992; Mathew et al., 1998; Zhu and Mivechi, 1999], and its expression at high levels in embryogenesis suggests a specific role during embryo development. The function of the most recently described Hsf4 is unknown [Nakai et al., 1997; Frejtag et al., 2001; Zhang et al., 2001]. Overall, regulation of Hsps by Hsf1, Hsf2, or Hsf4 along with their biological functions in tissues and specific cell types in the whole organism is poorly understood. Establishment of in vivo model systems to probe the function and tissue-specific expression of Hsfs and Hsps will enhance our understanding of the mechanism of their participation in such diverse functions.

Targeted disruption of *hsf1* results in embryonic lethality due to defects in chorioallantoic placenta and prenatal lethality in specific genetic backgrounds [Xiao et al., 1999]. Furthermore, fibroblasts generated from these mice exhibit a lack of thermotolerance [Mcmillan et al., 1998]. However, the mechanism of cell death and whether *hsf1*-deficient cells exhibit sensitivity to heat or other cytotoxic agents have not been addressed. To further understand the role of Hsf1 in apoptosis and the molecular mechanisms underlying transcriptional control of Hsps by Hsf1 in the whole organism, we have generated *hsf1*-deficient mice and have bred these mice with *hsp70.3*-deficient mice, in which the entire first exon of the gene has been replaced with the *LacZ* gene (β-galactosidase) [Huang et al., 2001]. We demonstrate that *hsf1*-deficient bone marrow cells or MEFs undergo rapid apoptosis, and that these cells are unable to develop thermotolerance. We further demonstrate that in adult *hsf1*$^{-/-}$-*hsp70.3*$^{+/-}$-*lacZ* mice, inducible Hsp70 is constitutively expressed in cells and tissues that are exposed to the environment. However, Hsf1 appears to be the sole transcriptional regulator of inducible Hsp70 expression following the exposure of the animals to heat stress.

MATERIALS AND METHODS

Generation of Mice With Targeted Disruption of the *hsf1* Gene

The cloning of the murine *hsf1* gene from a 129/SvJ mouse genomic library in lambda fix II vector (Stratagene, La Jolla, CA) and its exon-intron structure has been previously reported [Zhang et al., 1998]. A genomic clone containing an 18-kb *hsf1* fragment (portion of intron 1 plus exons 2–13) was used to construct a targeting vector containing the mutant *hsf1* gene. The targeting vector contained a proximal 3.2 kb fragment with homology to *hsf1* (portion of intron 1 to exon 2), which was PCR amplified using the isolated 18 kb *hsf1* clone as a template using primers: 5′-CTG CAG AAC CAA TGC ATT GGC GGC CGC TCG AGA ACA CAG CAT TC TTG AAA GAA A-3′ that also included *Bst*XI, *Not*I, *Eag*I, and *Xho*I restriction enzyme sites, and 5′-GAA TCG GCC GTG GTC AAA CAC GTG GAA GCT GTT-3′ containing an *Eag*I restriction enzyme site. This PCR-amplified fragment was digested with *Eag*I and was subcloned into plasmids containing an *IRES-lacZ-neomycin* cassette. The resulting plasmid was then digested with *Xho*I/*Nru*I to release the 3.2-kb proximal arm of *hsf1-IRES-lacZ-neomycin* fragment. For the distal 3.7-kb fragment homology to *hsf1*, the 18-kb genomic clone was

digested with *Nhe*I to release a 7.5-kb fragment, which was subcloned into pBlueScript at *EcoR*V site. This plasmid was then digested with *HindIII* to remove a 3.8-kb fragment. The remaining 3.7 kb fragment (portion of exon 2–9) was subsequently released by *Sma*I/*Xho*I and was inserted together with the 3.2 kb *hsf1-IRES-lacZ-neomycin* fragment into the final targeting vector (λDASHII-254-2TK) at the *Xho*I site (Fig. 1A). The fidelity of all clones was confirmed by sequencing. This cloning strategy deleted 55 bp of exon 2, 5′ to the *Nhe*I restriction enzyme site. The *neomycin* gene was flanked by Cre recombinase recognition sequences (*loxP*) to allow removal of the *neomycin* gene in the mutant mice. The *neomycin* gene (positive selectable marker) was driven by the PKG promoter and contained an SV40 poly-(A) signal, and a stop codon. The *lacZ* gene contained sequences of the picornaviral Internal Ribosomal Entry Site (IRES) at its 5′-end and a poly A signal and a stop codon at its 3′-end (clones encoding sequences for *lacZ* and *IRES* were the gift of Dr. A. Smith (Univ. of Edinburgh, Scotland). The targeting vector contained sequences of two thymidine kinase (*TK*) genes that were used as a negative selectable marker.

Thirty micrograms of the targeting vector linearized with *Not*I was electroporated (BioRad Gene Pulse, 250 V, 950 μF) into Embryonic Stem (ES) cells (D3; Incite Genomics, Genome Systems). ES cells were cultured in the presence of neomycin (200 μg/ml) and gangciclovir (2 μM), and resistant clones were selected and expanded. Genomic DNA was isolated from 167 individual clones and 5 μg of DNA from each clone was digested with *Pst*I and analyzed by Southern blotting using a 1-kb fragment as a probe (indicated as outside probe in Fig. 1A). Two ES clones were positively identified to contain a mutant *hsf1* gene by a homologous recombination event. The two positive clones were microinjected into C57BL/6 blastocysts and germline transmitting chimeric mice were obtained. Germ line transmission of the mutant allele was verified by Southern blotting and PCR analysis of tail

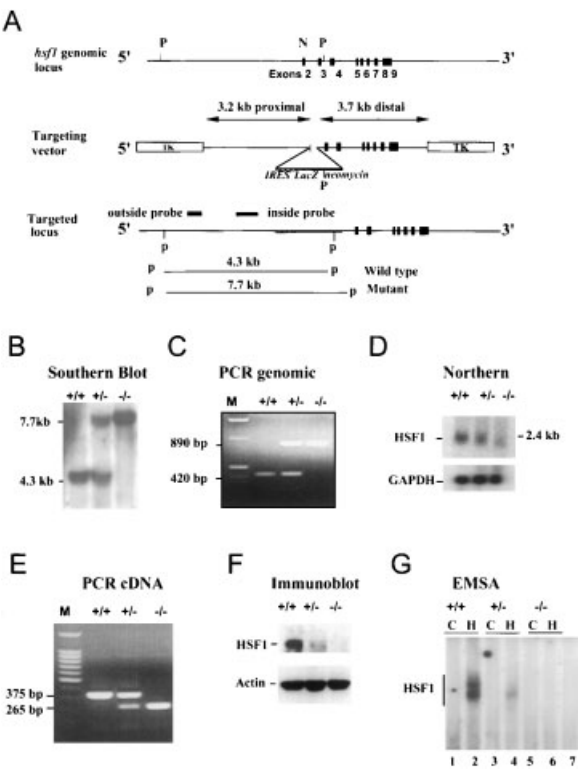

Fig. 1. Targeted disruption of *hsf1* by homologous recombination. (**A**) Schematic of segment of the *hsf1* locus, targeting construct, and targeted locus. Coding exons are boxes in black, beginning with exon 2 [Zhang et al., 1998]. Targeting construct replaces the coding region for 55 bp of exon 2. *LoxP* flanked, PKG-neomycin cassette with upstream *IRES-LacZ* are indicated. Two thymidine kinase genes were used for negative selection. ''Outside probe'' was used for screening embryonic stem cell clones to distinguish between endogenous and targeted alleles. The ''inside probe'' was used for genotyping. The 3.2- and 3.7-kb fragments are the proximal and distal *hsf1* homologous segments in the targeting vector. The final insert between the two thymidine kinase genes (*TK*) is 13 kb. N and P represent *Nhe*I and *Pst*I sites, respectively. (**B**) Southern blot analysis of genomic DNA prepared from tails of wild-type (+) and targeted mutant mice (−). The 7.7 and 4.3 kb are *Pst*I digested fragments corresponding to the targeted (−) and wild-type (+) alleles, respectively. (**C**) PCR analysis of tail DNA derived from wild type (+) and targeted mutant mice (−) showing 420- and 890-bp fragment derived from wild-type and targeted alleles, respectively. (**D**) Northern blot analysis of total RNA derived from MEFs of wild-type (+) or mutant (−) mice. Full-length murine *hsf1* cDNA was used as a probe. Hsf1 gene rates an approximately 2.4-kb fragment. GAPDH is shown for equal loading. (**E**) cDNA from wild-type (+) and *hsf1* mutant (−) mice were amplified using forward primer located in exon 1 and reverse primers located in exon 3. Sequencing the 375 bp (+) and 265 bp (−) fragment indicated normal splicing of exons 1, 2, and, 3 and splicing of exon 1 to exon 3, respectively. (**F**) Immunoblot analysis of extracts of MEF derived from wild-type (+) or mutant (−) *hsf1* analyzed by SDS–PAGE using antibody to Hsf1. Actin is shown as an indicator of loading. (**G**) Electrophoretic mobility shift assays (EMSA). Nuclear extracts of wild-type (+) or *hsf1* mutant (−) MEFs were prepared from untreated control (C) or heated (43°C for 20 min plus 30 min recovery at 37°C to ensure Hsf1 activation). **Lanes 1** and **2**, **3** and **4**, **5** and **6** represent untreated control (C) and heated (H) samples, respectively. **Lane 7** is the same extracts as in lane 2 but with 200 times excess cold HSE to show specificity.

DNA from F1 offspring. The heterozygous mice were fertile and normal in size.

Genotype analysis. Genomic DNA from a small fragment of mouse-tail was prepared. Multiplex PCR analysis was used to genotyping the mice in reactions which included one common primer (5′-GAG ATG ACC AGA ATG CTG TGG GTG-3′), a reverse primer (5′-GCA AGC ATA GCA TCC TGA AAG AG-3′) to identify the wild type, and a reverse primer (5′-CAG CTC TGA TGC CGC CGT GTT CC-3′) for the *neomycin* gene to identify the mutant mice.

To identify the sequences of the transcripts made in *hsf1*-deficient mouse embryo fibroblasts (MEFs) in comparison to the transcripts made in wild-type MEFs, PCR primers were designed to amplify a fragment between exon 1 and exon 3. The sequences of the PCR primers were as follows: 5′-GCG ACG ACA CTA GCT CAG CCT T-3′, and 5′-GCT CAA TGT GGA CTA CTT TTC GG-3′.

Gene Targeting and Generation of *hsp 70.3-lacZ*-Deficient Mice

The generation and gene targeting strategy for the *hsp70.3* gene has been reported previously [Huang et al., 2001]. The targeting vector was constructed so that it replaced the entire coding region of the gene with a *lacZ-neomycin* cassette. The $hsf1^{-/-}$-$hsp70.3^{+/-}$-*lacZ* mice were generated by crossing $hsf1^{-/-}$ males with $hsp70.3^{-/-}$-*lacZ* female mice [Huang et al., 2001].

Whole-Body Hyperthermic Challenge

Wild type (+/+), heterozygous (+/−) or homozygous (−/−) *hsf1* adult mice were semi-immersed in a circulating water bath at 42°C and left to recover for 6–8 h before euthanization [Li et al., 1983]. Tissues were prepared for histological examination as described below. Untreated mice were used as controls. For in vitro heating of the bone marrow, cells were extracted from the femurs and tibias and were diluted to 1×10^6 cells per ml and the cell suspension was heated in a temperature-controlled circulating water bath. For MEFs, a monolayer of cells cultured in 6 cm tissue dishes was heated while immersed in the temperature-controlled circulating water bath.

Cell Culture

MEFs were prepared by trypsin digestion of individual 14-day-old embryos. MEFs were cultured in Dulbecco's Minimal essential Medium (DMEM) plus 5% heat inactivated fetal calf serum (HI-FCS), frozen in passages 1 or 2, and were used between passages 2 and 3. Bone marrow cells were extracted and suspended in alpha-Minimal Essential Medium (αMEM) supplemented with 20% HI-FCS and treated as described in the Results, pelleted by centrifugation, rinsed two times with PBS, and were diluted in SDS-sample buffer for immunoblotting. For immunoblot analysis of various tissues, organs were sonicated in cold PBS containing protease inhibitors (1 mM PMSF, 20 μg of leupeptin, 5 μg of pepstatin), centrifuged to remove debris, and then diluted in six times SDS sample buffer for immunoblotting.

Thermal Response and the Kinetics of Thermotolerance Induction in CFU-GM Obtained From *hsf1*-Deficient Mice

Bone marrow cells from +/+, +/−, or −/− *hsf1* mice were tested for their ability to develop thermotolerance [Mivechi and Li, 1987]. Bone marrow cells were heated at 43°C for 20 min and challenged with a more severe heat (44°C for 40 min) at times indicated during the recovery period at 37°C. Cells were subsequently plated on soft agar and incubated at 37°C/5% CO_2 for 8 days. Colonies of granulocytes, macrophages, or a mixture of granulocytes/macrophages were counted microscopically. The colony forming efficiency of untreated CFU-GM was approximately $2/10^3$ nucleated cells. Cells were plated at varying concentration ($1–16 \times 10^5$ cells per dish) depending on the given treatment. The percentage survival was calculated by the formula: percentage survival = ((number colonies after severe heat challenge/number of cells plated)/(number of colonies after primary heat/number of cells plated)) × 100.

Immunoblotting and Immunofluorescence Analysis

Bone marrow, MEFs, or homogenized tissues were suspended in SDS-sample buffer and heated to 95°C for 5 min. A fraction of each sample was TCA precipitated and the amount of protein was estimated using bicinchoninic acid (Pierce, Rockford, IL). Immunoblot analysis was performed using standard protocols [He et al., 1998]. Antibodies to various proteins were purchased from the following manufacturers: constitutive and stress-inducible Hsp70 species (3A3), Affinity Bioreagents (Golden,

CO); β-galactosidase, Promega, (Madison, WI); actin, Sigma, (St. Louis, MO); inducible hsp70 (C92), hsp105, hsp86, hsp60, and hsp25, StressGen, (Vancouver, Canada); pro-caspase 9 and poly-ADP-ribose polymerase (PARP), Santa Cruz, (Santa Cruz, CA).

Immunofluorescence analysis to detect cytochrome c was performed as previously reported [He et al., 1998]. Antibody to cytochrome c (sc7159) was purchased from Santa Cruz (Santa Cruz, CA).

Histology

Tissues harvested from adult mice, were embedded in OCT compound, snap-frozen in a dry ice 2-methyl-butane bath, sectioned, air-dried and fixed in 0.2% glutaraldehyde in PBS (pH 7.3) with 2 mM $MgCl_2$ for 10 min. Sections from each tissue specimen were stained with either hematoxylin, or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) for β-galactosidase activity (Molecular Probes, Eugene, OR). All sections were counter-stained with eosin, and subjected to gross and microscopic pathological analysis.

Cell-Death Determination

For detection of apoptotic cells, Annexin V staining was performed on treated cells using Apoptosis Detection kit according to the manufacturer's instructions (R and D Systems, Minneapolis, MN). Briefly, MEFs were trypsinized and pelleted at 500 rpm for 5 minutes, rinsed once with PBS, and resuspended in buffer containing Ca^{2+} plus 2% BSA. Annexin V and propidium iodide (5 μg/ml) were added to each treatment group (10^5 cells) and incubated at 25°C for 15 minutes before flow cytometric analysis using a FACS Caliber cytometer (Becton Dickinson, San Diego, CA). To determine the mitochondrial membrane potential, treated cells were incubated in PBS containing 2% BSA and 40 nM of 3,3′-dihexylocarbocyanine iodide (DioC6) (Molecular Probes, Eugene, OR) for 30 minutes at 37°C. FACS analysis was performed following addition of 5 μg/ml propidium iodide to each sample [Petit et al., 1995]. Cellular DNA fragmentation was determined using ELISA (Roche, Mannheim, Germany).

Northern Blotting and Gene Microarray Analysis

Total 10 μg of RNA isolated from MEFs by trizol reagent (Gibco-BRL) was analyzed for Northern blotting using HSF1 cDNA as a probe [Sarge et al., 1991].

For mouse oligonucleotide microarray analysis, mice were treated with whole body hyperthermia (42°C for 45 min) and left to recover for 5 h ad libitum. Mice were then anesthetized, organs were perfused with sterile PBS, livers were excised, and total RNA was isolated from each sample. Samples were analyzed using an Affymetrix Gene Chip Murine Genome U74A Set (Affymetrix, Inc., Santa Clara, CA), which allows monitoring of the relative abundance of greater than 11,000 murine mRNA transcripts from genes and ESTs including 6,000 known genes.

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays using whole cell extracts have been described in detail previously [He et al., 1998; Zhang et al., 1998; Zimarino and Wu, 1987].

RESULTS

Generation of Mice Deficient in *hsf1*

To disrupt the mouse *hsf1* locus, the targeting vector shown in Figure 1A was constructed. Integration of the mutant *hsf1* gene by homologous recombination inserts an *IRES-LacZ-neomycin* gene into the *hsf1* open reading frame, replacing 55 bp of the *hsf1* exon 2 and disrupting the DNA binding domain [Zhang et al., 1998]. The position of the outside probe used to detect the homologous recombination is indicated in Figure 1A. This generates a 4.3-kb fragment in the wild type and a 7.7-kb fragment for the targeted locus following *Pst* I digestion. Southern blot analysis of DNA extracted from the tail of wild type, heterozygous, or homozygous *hsf1*-deficient mice is shown in Figure 1B. Routine genotyping of the mutant mice was performed using primers that generated a 420-bp fragment for the wild type and an 890-bp fragment for the mutant (Fig. 1C).

Northern blot analysis of total RNA obtained from MEFs derived from wild-type or mutant mice is presented in Figure 1D. A 2.4-kb sized transcript was detected in MEFs derived from the wild-type or $hsf1^{+/-}$ mice. A slightly faster migrating transcript was detected in MEFs derived from $hsf1^{-/-}$ mice. To confirm the identity of the transcripts in MEFs derived from $hsf1^{-/-}$ mice and to ensure that a portion of the DNA binding domain was deleted, an area between exons 1–3, spanning the deleted

region, was amplified by PCR using cDNA obtained from MEFs of wild-type or mutant mice. The PCR amplified fragments for the wild type and *hsf1* mutant were 375 and 265 bp, respectively (Fig. 1E). Sequencing of the 375-bp fragment indicated the presence of an intact *hsf1* gene containing exons 1, 2, and 3. However, sequencing of the 265-bp fragment indicated splicing of exon 1 to exon 3 directly, with entire deletion of exon 2 (data not shown). Splicing of exon 1 to exon 3 leads to an out-of-frame Hsf1 protein [Zhang et al., 1998] and lack of Hsf1 protein expression in *hsf1*-deficient cells was confirmed by immunoblotting (Fig. 1F).

Electrophoretic mobility shift analysis (EMSA) of nuclear extracts from control or heated mouse embryo fibroblasts (MEFs) derived from wild-type, heterozygous, or homozygous *hsf1*-deficient mice showed absence of any heat-inducible Hsf1 DNA binding activity in the nuclear extracts derived from $hsf1^{-/-}$ MEFs. Nuclear extracts derived from $hsf1^{+/-}$ MEFs exhibited Hsf1 DNA binding activity, but at reduced levels when compared to wild-type MEFs (Fig. 1G). Essentially, all DNA binding activity that is usually observed in the heated samples is contributed by HSF1, as detected by supershift of antibody-Hsf1 complexes (data not shown, [Zhang et al., 2001].

An analysis of homozygous $hsf1^{-/-}$ males crossed with $hsf1^{-/-}$ or $hsf1^{+/-}$ female mice indicated female infertility in homozygous *hsf1* deficient females on the C57BL/6 genetic background. Therefore, $hsf1^{-/-}$ males were routinely crossed with $hsf1^{+/-}$ females and the progeny were individually genotyped. Litter sizes were normal and there was no evidence of significant embryonic lethality on this genetic background.

The *LacZ* gene included in the targeting vector was inserted into the *hsf1* locus so that its expression was controlled by the *hsf1* promoter. However, β-galactosidase is not expressed in the *hsf1* mutant mice, as evidenced by the lack of mRNA detected by RT-PCR analysis of MEFs (data not shown) as well as the lack of any β-galactosidase expression in any of the tissues examined by histological staining with X-gal. To ensure that the absence of β-galactosidase expression driven by the *hsf1* promoter in *hsf1*-deficient mice is not due to insertion of the neomycin gene at the 3′-end of the *LacZ* gene, we removed the neomycin gene flanked by two *loxP* sites by crossing *hsf1*-deficient mice with transgenic mice expressing Cre-recombinase [Koni et al., 2001]. This, however, did not result in β-galactosidase expression driven by the *hsf1* promoter in any tissues (see Fig. 2A,B, middle panel). Lack of β-galactosidase expression driven by the *hsf1* promoter could be attributed to its insertion in the second exon, which we found to be entirely deleted in the mutant mice (Fig. 1E).

To ensure that the levels of Hsps were reduced in $hsf1^{-/-}$ mice, we determined the gene expression profile of hepatocytes derived from $hsf1^{-/-}$ mice versus the wild-type mice. For this, mice were heated at 42°C for 45 min and allowed to recover for 5 h before the perfused livers were extracted for analysis. The mRNA expression pattern was analyzed using Affymetrix GeneChip. Hsf1 appears to uniquely regulate the expression of Hsp105, Hsp86, Hsp84, Hsp70, Hsp60, and Hsp40 and, to a lesser extent, the inducibility of the DNAJ-like protein and chaperonin 10 (Table 1). Hsp25, a major heat-inducible Hsp in the liver that is detected by immunoblotting (see Fig. 2C) is absent from the microarray analysis. Our data indicate that the mRNA level for Hsp25 was not significantly higher in livers derived from wild-type mice compared to the *hsf1*-deficient mice. Only a small number of non-heat shock genes were expressed at elevated levels in the wild-type mice (data not shown). Interestingly, many genes were upregulated in $hsf1^{-/-}$ liver cells following heat shock. A number of these genes are specific to liver and a few are associated with protein degradation pathways, suggesting that there may be an increased level of protein degradation in heat-treated hepatocytes of $hsf1^{-/-}$ mice (data not shown).

Inducible Hsps Exhibit Basal Expression in Different Tissues That Are not Controlled by Hsf1

Hsf1 mRNA, as detected by Northern blot analysis, is specifically abundant in tissues such as heart, kidney, liver, ovary, placenta, and, to a lesser extent, testis, spleen, and perhaps other tissues [Sarge et al., 1991] (and Mivechi, unpublished data). Furthermore, it has been postulated that Hsf1 is activated by various physiological and pathological conditions, and following stresses such as heat, oxidative stress, hormones, and high pH [Yellon and Marber, 1994; Benjamin and McMillan, 1998; Morimoto, 1998; Smith et al., 1998; Jolly and Morimoto, 2000]. It is conceivable to assume therefore, that Hsf1 could potentially be constitutively active

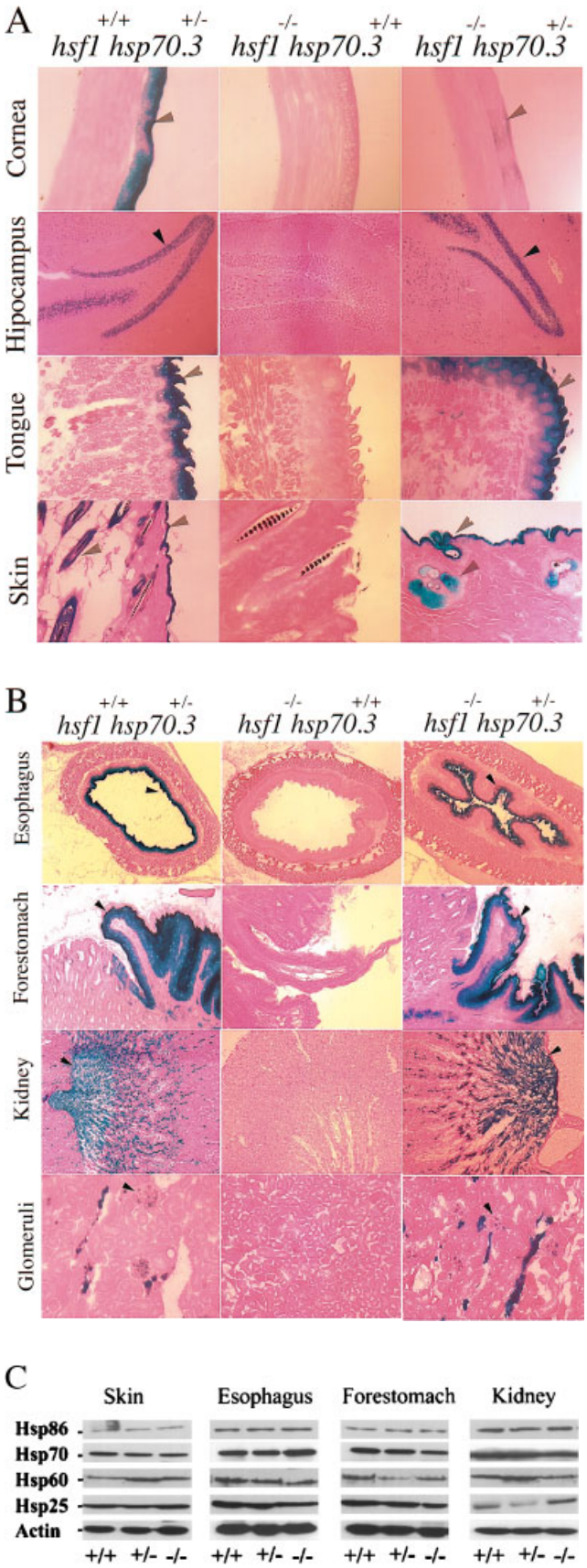

Fig. 2. Hsf1 does not control constitutive expression of inducible HSPs in vivo. (**A**, **B**) An 8–12-week-old adult *hsf1*$^{+/+}$-*hsp70.3*$^{+/-}$-*LacZ*, *hsf1*$^{-/-}$-*hsp70.3*$^{+/+}$, and *hsf1*$^{-/-}$-*hsp70.3*$^{+/-}$-*LacZ* mice were euthanized and tissues were frozen, sectioned, and stained with hematoxylin/eosin (data not shown) or with X-gal to detect β-galactosidase expression. Panel A demonstrates specific sections of the cornea, hipposcampus, tongue, and skin. Panel B demonstrates specific sections of the esophagus, forestomach, kidney tubules and glomeruli. (**C**) Immunoblot analyses of extracts prepared from skin, esophagus, forestomach, and kidney obtained from adult mice using antibody to Hsp105, Hsp86, inducible Hsp70, Hsp60, and Hsp25 and actin. Actin is shown as an indicator of loading.

TABLE I. Analysis of Hsp Gene Expression in *hsf1*$^{-/-}$ Versus *hsf1*$^{+/+}$ Liver Hepatocytes Following Whole Body Hyperthermia (42°C, 45 min Plus 5 Hours of Recovery)

Acc. no	Fold change	Protein name	Function[1]
M12571	−34.6	Hsp70	Binding to nascent polypeptide chain, refolds denatured proteins *in vitro* by preventing aggregation; antiapoptotic protein; regulating thermotolerance
AF109906	−21	Hsp70.1/70.3 (MHClass III region RD gene)	
L40406	−17	Hsp105	Protects cells and proteins from cytotoxic effect of heat
AB028272	−10.5	Hsp40	Hdj1 & Hdj2, Cochaperones for HSP70 and increasing its ATPase activity and release of substrate
J04633	−8.6	Hsp86	Interacts with steroid hormone receptor; disaggregation and refolding of proteins; binds HSF1 and regulates heat shock repsonse
M18186	−2.8	Hsp84	
X53584	−2.3	Hsp60	Help to refolds denatured proteins *in vitro* by preventing aggregation; could participate in protein degradation process by acting as a cofactor in proteolytic systems
AF055664	−2.2	Hsp DNA J-like 2	Hsp40-like
U09659	−1.8	Chapcronin 10	Mitochondrial matrix protein; assist folding of nonnative polypeptide

[1]Hartl, 1996; Jolly and Morimoto, 2000; Li and Mivechi, 1986, 1998; Li et al., 1983; Morimoto, 1998.

in vivo in specific cells and tissues. To determine the extent to which Hsf1 regulates inducible Hsp gene expression in different cells, tissues, and organs, we crossed *hsf1*$^{-/-}$ males with *hsp70.3*$^{-/-}$ females [Huang et al., 2001]. The progenies were backcrossed to generate *hsf1*$^{-/-}$*hsp70.3*$^{+/-}$*-lacZ* mice. Histological analyses were performed to compare the pattern of *hsp70.3*-driven β-galactosidase expression in *hsf1*$^{+/+}$*hsp70.3*$^{+/-}$*-lacZ*, *hsf1*$^{-/-}$*hsp70.3*$^{+/+}$, or *hsf1*$^{-/-}$*hsp70.3*$^{+/-}$*-lacZ* adult mice. The results show that in *hsf1*$^{+/+}$*hsp70.3*$^{+/-}$*-lacZ* mice, β-galactosidase is expressed in multiple tissues under physiological conditions (Fig. 2A,B). Tissues expressing β-galactosidase include those that are exposed to the external environment, such as the epithelial layers of the skin, cornea, tongue, esophagus, and forestomach. Other tissues that express Hsp70.3 constitutively are hippocampus, kidney tubules, glomeruli, and bladder (data not shown). However, in the *hsf1*$^{-/-}$*hsp70.3*$^{+/-}$*-lacZ* mice, β-galactosidase expression patterns are unaltered in these tissues, suggesting that under physiological conditions, Hsf1 does not control the transcription of Hsp70 mRNA in most tissues we have tested. It is important to note, however, that in certain tissues, such as the epithelial layers of the cornea (Fig. 2A) and bladder (data not shown), there was a consistent reduction in β-galactosidase expression in *hsf1*$^{-/-}$*hsp70.3*$^{+/-}$*-lacZ* mice, suggesting either that Hsf1 is constitutively active in particular tissues or, alternatively that Hsf1 may influence the transcription of inducible Hsps by other transcription factors, possibly other Hsf family members.

To confirm the pattern of expression for Hsps other than Hsp70 in the wild-type and *hsf1*-deficient mice, immunoblot analyses were performed using skin, forestomach, esophagus, and kidney derived from wild type, *hsf1*$^{+/-}$, or *hsf1*$^{-/-}$ mice. Results indicate that in these tissues, expression of Hsp105 was absent (data not shown), however, Hsp86, Hsp70, Hsp60, and Hsp25 were expressed at different levels under physiological conditions and that such expression was not regulated by Hsf1 (Fig. 2C).

Inducible Expression of Hsps In Vivo Is Entirely Controlled by Hsf1 Transcription Factor

Among the Hsf family members, Hsf1 is a transcription factor that is activated in tissue culture cells and uniquely regulates the expression of the inducible Hsps following exposure of cells to heat stress [Abravaya et al., 1991; Sarge et al., 1991; Green and Reed, 1998; He et al., 1998; Dai et al., 2000]. However, this phenomenon has not been adequately examined in specific tissues and cells in the whole organism in vivo. For this, we compared the heat-inducible expression pattern of Hsp70 in all tissues in *hsf1*$^{+/+}$*hsp70.3*$^{+/-}$*-lacZ* and *hsf1*$^{-/-}$*-hsp70.3*$^{+/-}$*-lacZ* mice. These mice were treated with mild whole body hyperthermia (42°C, 45 min) and following 6 h of recovery, β-galactosidase expression was examined in tissues by histological analyses. The results demonstrate that in heat-treated *hsf1*$^{+/+}$*-hsp70.3*$^{+/-}$*-*

lacZ mice, β-galactosidase expression is robustly elevated in tissues that do not express Hsp70 constitutively. These tissues include liver, intestine, adrenal gland, pancreas, and testis. Inducible β-galactosidase expression was essentially absent from the analogous tissues in *hsf1*$^{-/-}$-*hsp70.3*$^{+/-}$-*lacZ* mice (Fig. 3A). These same differences in heat-inducible β-galactosidase expression between *hsf1*$^{+/+}$-*hsp70.3*$^{+/-}$-*lacZ* and *hsf1*$^{-/-}$-*hsp70.3*$^{+/-}$-*lacZ* mice were also clearly evident for all other tissues, such as heart, lung, brain, kidney, skeletal muscle, and skin (data not shown). Interestingly, heat-induced β-galactosidase expression in most tissues was limited to specific cells. For example, in the pancreas most acini cells expressed β-galactosidase, but cells in the Islets of Langerhans lacked any β-galactosidase expression. In testis, heat-inducible expression of β-galactosidase is strikingly absent in the seminiferous tubules, and expression is limited only to the interstitial tissues. Similar results were obtained when *hsf1*$^{+/+}$-*hsp70.3*$^{+/-}$-*lacZ* and *hsf1*$^{-/-}$*hsp70.3*$^{+/-}$-*lacZ* mice were heated, and euthanized 24 hours later, although in this case β-galactosidase expression in *hsf1*$^{+/+}$-*hsp70.3*$^{+/-}$-*lacZ* mice had begun to decay, and no inducible expression of β-galactosidase was detected in *hsf1*$^{-/-}$-*hsp70.3*$^{+/-}$-*lacZ* mice. When *hsf1*$^{+/+}$-*hsp70.3*$^{+/-}$-*lacZ* mice were heated every 24 h multiple times, β-galactosidase expression remained elevated (a similar observation is normally obtained for expression of the inducible Hsps in similarly heated tissue culture cells (Mivechi, unpublished data). However, β-galactosidase was not inducibly expressed when *hsf1*$^{-/-}$-*hsp70.3*$^{+/-}$-*lacZ* mice were similarly treated (data not shown).

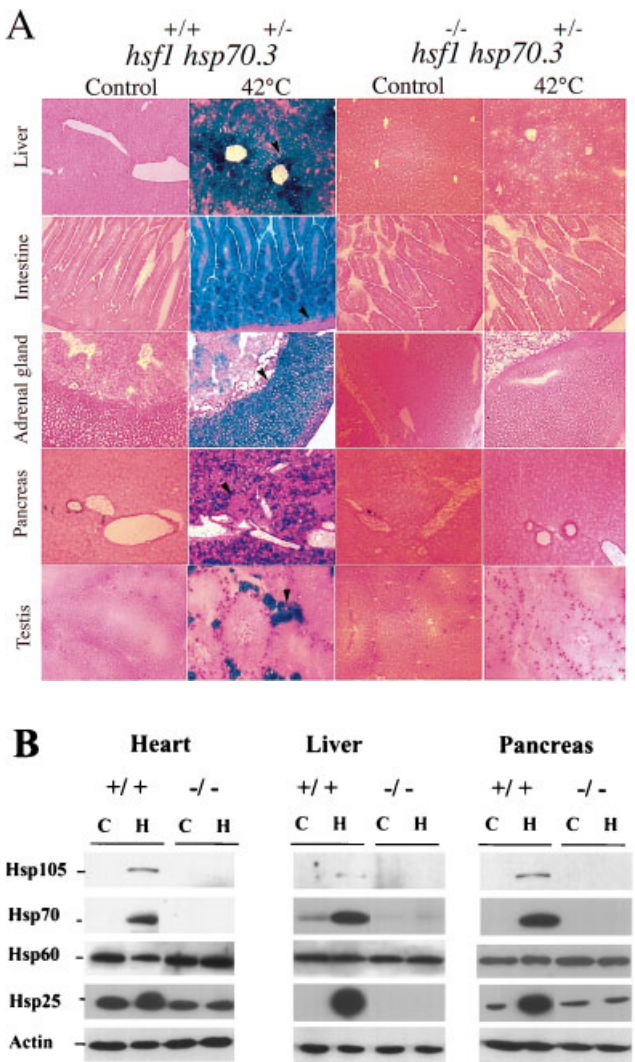

Fig. 3. Hsf1 regulates the heat-inducibility of all Hsps in organs of the adult mice. (**A**) An 8–12-week-old adult *hsf1*$^{+/+}$-*hsp70.3*$^{+/-}$-*LacZ* or *hsf1*$^{-/-}$-*hsp70.3*$^{+/-}$ untreated or heated (42°C, 45 min plus 6 h of recovery ad libitum) mice were euthanized and tissues were frozen, sectioned, and stained with hematoxylin/eosin (data not shown) or with X-gal to detect β-galactosidase expression. Panel A demonstrates specific sections of the liver, pancreas, small intestine, adrenal gland, and testis under physiological conditions or after mice received a whole body hyperthermia. (**B**) Immunoblot analyses of extracts prepared from heart, liver, or pancreas of untreated or heated (42°C, 45 min plus 6 h of recovery ad libitum) mice. Blots were probed using antibody to Hsp105, inducible Hsp70, Hsp60, and Hsp25 and actin. Actin is shown as an indicator of loading.

Immunoblot analyses were performed to examine the level of heat-inducible Hsps in various tissues from untreated and heat-treated wild-type or *hsf1*$^{-/-}$ mice. The results indicate that expression of Hsps 105, Hsps 70, and Hsps 25 were induced in heart, liver, and pancreas obtained from wild-type or *hsf1*$^{-/-}$ mice. Some basal expression for Hsp25 was observed in the heart and pancreas in the wild-type and *hsf1*$^{-/-}$ mice. Hsp60 is constitutively expressed in these tissues and its levels did not change significantly (Fig. 3B). These results indicate that Hsf1 is the transcription factor that uniquely regulates the heat inducibility of the stress-inducible Hsps in all tissues tested.

hsf1-Deficient Bone Marrow Cells Exhibit Increased Thermal Sensitivity and Lack the Ability to Develop Thermotolerance

One of the most intriguing phenomena in thermal biology is the response of heated cells to subsequent heat challenges. This response is manifested as a transient resistance to subsequent heat stress and is called thermotolerance [Li and Mivechi, 1986, 1998]. At the molecular level, the enhanced synthesis of inducible Hsps after primary heat stress has been correlated with the increased levels of resistance that cells develop in a matter of hours to subsequent stress treatments [Li et al., 1991, 1995; Li and Mivechi, 1998]. Therefore, we chose to examine whether bone marrow cells derived from *hsf1*$^{-/-}$ mice exhibit increased thermal sensitivity and whether these cells demonstrate a reduced ability to develop thermotolerance. Thus, we examined the inducible expression of Hsp70 and Hsp25 in bone marrow derived from wild-type or *hsf1*$^{-/-}$ mice by immunoblotting (Fig. 4A). The results indicate that *hsf1*$^{-/-}$ bone marrow cells lack inducible expression of both Hsps, whilst the level of the constitutively expressed species, Hsc70, remained unaffected (Fig. 4A).

Bone marrow contains undifferentiated myeloid progenitor cells that have the capacity to expand once cells are treated with GM-CSF; they form individual colonies, known as CFU-GM, when plated on soft agar [Mivechi and Li, 1987]. Therefore, we determined the ability of CFU-GM to respond to a single dose of heat in the bone marrow obtained from *hsf1*$^{+/+}$, *hsf1*$^{+/-}$, or *hsf1*$^{-/-}$ mice. Extracted cells were heated at either 43°C or 44°C in vitro and plated for colony formation. The results indicate that CFU-GM derived from *hsf1*$^{-/-}$ mice exhibit increased thermal sensitivity compared to CFU-GM from wild-type mice. The surviving fraction of CFU-GM was reduced 50–300-fold when cells were exposed to 43°C for 60 min, or 44°C for 30 min (Fig. 4B,C). These results indicate that the presence of the *hsf1* gene confers a survival advantage to bone

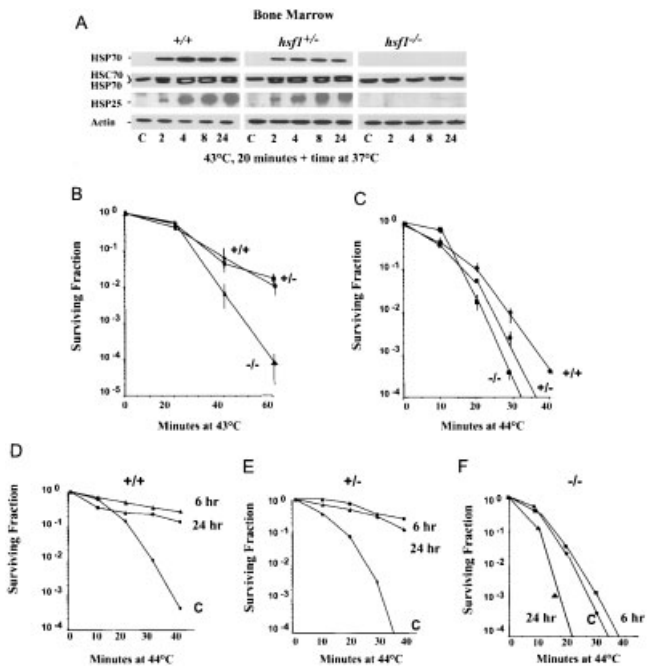

Fig. 4. CFU-GM derived from *hsf1*$^{-/-}$ mice exhibit lack of inducible Hsps, increased thermal sensitivity, and lack of development of thermotolerance. (**A**) Bone marrow was extracted from adult wild-type (+) or mutant (−) mice. Cells were left untreated (**C**) or heated at 43°C for 20 min and incubated at 37°C for 2, 4, 8, or 24 h before they were processed and analyzed by immunoblotting. Membranes were probed with antibody to inducible Hsp70 (c92) (top row), antibody to both inducible and constitutive Hsp70 proteins (3A3) (second row), or antibody to Hsp25. Actin was used for indicator of equal loading. (**B** and **C**) Bone marrow from wild-type (+) or mutant (−) mice were heated in vitro at 43°C (B) or 44°C (C) for increasing periods of time, collected, and plated for CFU-GM analysis. (**D–F**) Bone marrow from wild-type (+) or mutant (−) mice were either heated at increasing time at 44°C (control) or were heated at 43°C for 20 min left at 37°C for 6 h or 24 h for thermotolerance development before they received a challenging heat dose at 44°C for increasing period of time.

marrow-derived CFU-GM even after a single heat stress.

To examine the ability of CFU-GM to develop thermotolerance, bone marrow cells derived from wild-type, *hsf1*$^{+/-}$, or *hsf1*$^{-/-}$ mice were exposed to mild heat treatment (43°C for 20 min) to precondition the cells and allow thermotolerance development. Cells were then challenged with a more severe heating to 44°C. Results indicate that CFU-GM derived from wild-type and *hsf1*$^{+/-}$ mice developed 500-fold more thermotolerance than CFU-GM derived from *hsf1*-deficient mice, which entirely lack any ability to develop thermotolerance (Fig. 4D–F). *hsf1*-deficient CFU-GM exhibit even higher levels of heat sensitivity 24 h after the primary heat treatment than cells that did not receive a preconditioning heat treatment, suggesting that the lack of an ability to express the inducible Hsps confers a further detriment to survival in preheated cells (Fig. 4F).

Mouse Embryo Fibroblasts Deficient in *hsf1* Undergo Rapid Apoptosis in Response to Heat Stress

Apoptosis is necessary for maintaining tissue homeostasis in higher organisms [Budihardjo et al., 1999; Strasser et al., 2000], and there is accumulating evidence that Hsps play a critical role in regulating programmed cell death [Xanthoudakis and Nicholson, 2000]. When overexpressed in cells, the individual inducible Hsps protect cells against the cytotoxic effects of heat [Li et al., 1991; Oh et al., 1997; Fortin et al., 2000; Mosser et al., 2000]. Since tissues derived from *Hsf1*-deficient mice lack the ability to induce any Hsps, we asked whether *hsf1*-deficient MEFs undergo increased apoptotic cell death. Thus, we examined the Hsp expression profile of MEFs, by immunoblotting, following a single heat treatment (Fig. 5A). The results indicate that *hsf1*-deficient MEFs lack heat-inducible expression of Hsp105, Hsp70, and Hsp25 after heat shock. The levels of Hsp86 and Hsp60 remained relatively unchanged between untreated control and heated MEFs (Fig. 5A).

To further examine heat-induced apoptosis in MEFs derived from *hsf1*$^{-/-}$ mice, we selected conditions where cells were given a mild heat shock to induce thermotolerance. Heat-induced apoptosis was then examined following a severe challenging heat dose, using Annexin V staining which detects early apoptotic cells (Fig. 5B). The results indicate that MEFs deficient in *hsf1* lack the ability to develop any thermotolerance. MEFs derived from heterozygous *hsf1*$^{+/-}$ mice also exhibit a severe reduction in thermotolerance development. This is in contrast to MEFs derived from wild-type mice, where tolerance is fully developed at 6 h and is maintained at 24 and 42 h after primary heat treatment.

A reduction in mitochondrial transmembrane potential ($\Delta\Psi$m) is one of the changes that takes place during the reversible stages of apoptosis; it precedes cytochrome c release in a number of cell types [Green and Reed, 1998; Budihardjo et al., 1999] and can be examined with 3,3′ dihexyloxacarbocyanine iodide (DioC6), a cationic dye that strongly labels the mitochondria. A decrease in mitochondrial membrane potential ($\Delta\Psi$m) in cells undergoing apoptosis is associated with a reduction of DioC6 uptake as detected by FACS analysis. To determine the changes in mitochondrial membrane potential in *hsf1*$^{-/-}$ cells and compare it to wild-type cells, MEFs were given a primary heat shock. After 6, 24, or 42 h, cells received a severe challenging heat shock and mitochondrial membrane potential was measured. The data show that wild-type cells develop thermotolerance, as indicated by maintenance of $\Delta\Psi$m, while MEFs from *hsf1*$^{+/-}$ or *hsf1*$^{-/-}$ mice showed a sharp reduction in $\Delta\Psi$m, indicating a lack of thermotolerance (Fig 5C). Since mitochondria play a pivotal role in initiating the cascade of caspase activation by releasing cytochrome c following apoptotic signals, the inability of *hsf1*-deficient MEFs to maintain $\Delta\Psi$m indicates that inducible Hsps maintain mitochondrial membrane integrity as part of their function.

We then determined whether *hsf1*$^{-/-}$ MEFs showed other hallmarks of apoptosis, such as generation of active caspase 9 from procaspase 9, and cleavage of PARP, which is a substrate for caspase 3, which is activated following caspase 9 activation [Budihardjo et al., 1999]. For this, untreated control cells, cells preconditioned to develop thermotolerance, or cells receiving a severe challenging heat treatment without the preconditioning heat treatment were analyzed for activation of caspase 9 and cleavage of PARP. In the wild type, 6 h thermotolerant cells exhibited minimal caspase 9 activation and PARP cleavage (Fig. 5D). In contrast, caspase 9 activation and PARP cleavage were seen in thermotolerant MEFs derived from *hsf1*$^{-/-}$ and *hsf1*$^{+/-}$ mice. Thus, there appears to be an absence of thermotolerance in *hsf1*-deficient

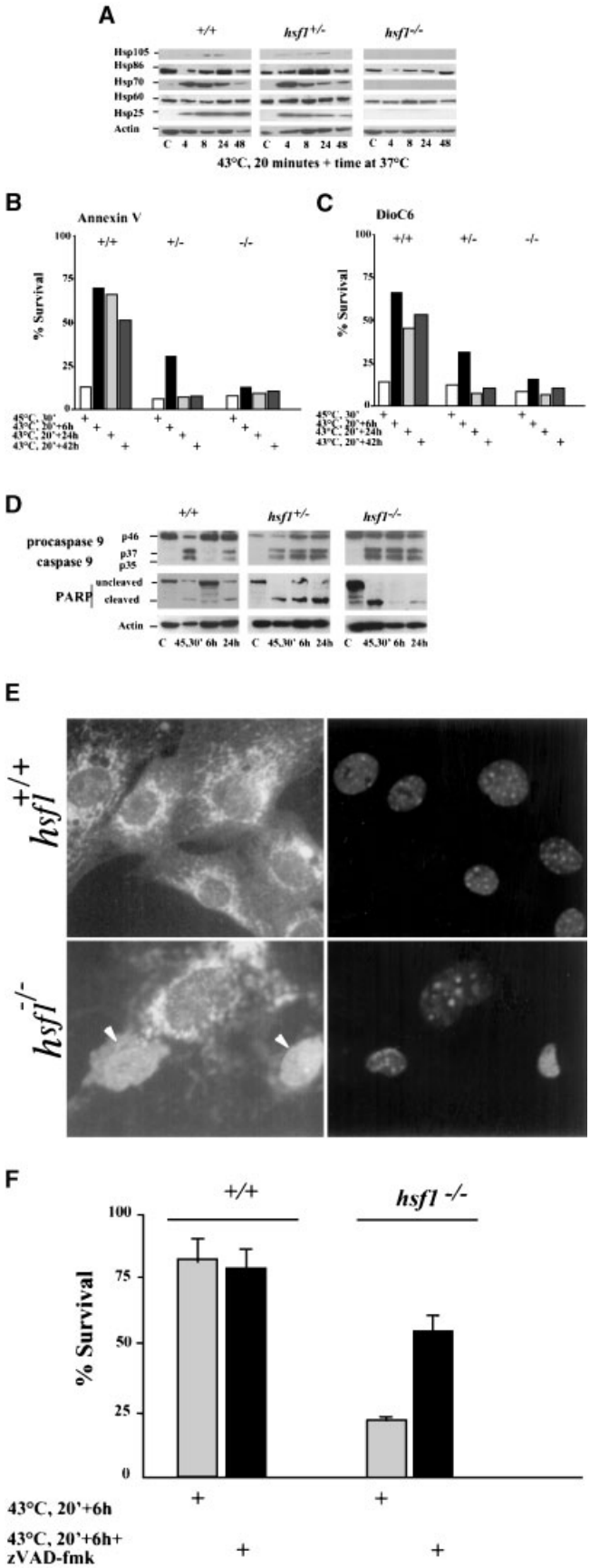

Fig. 5. *hsf1*$^{-/-}$ MEFs lack inducible Hsps expression and undergo extensive apoptosis after heat shock. (**A**) MEFs derived from wild-type (+) or mutant (−) mice received no treatment (C) or were heated at 43°C for 20 min and left at 37°C for 4, 8, 24, or 48 h followed by immunoblot analysis using antibody to Hsp105, Hsp86, inducible Hsp70, Hsp60, Hsp25, and actin. Actin is shown for equal loading. (**B** and **C**) MEFs derived from wild-type (+) or mutant (−) mice were given a mild preconditioning heat shock at 43°C for 20 min to induce thermotolerance. After 6, 24, or 42 h recovery at 37°C, cells were given a more severe heat challenge (45°C for 30 min) followed by 24 h recovery at 37°C to test for thermotolerance development. Cells were then analyzed using Annexin V-FITC staining (B) or DioC6 staining (C) in the presence of propidium iodide. Percent cell survival of heat treated cell populations was calculated based on the staining profile (Annexin and PI double negative) (B) or PI negative cells (C). 45°C, 30′, indicate cells received the challenging heat shock (45°C, 30 min) only. (**D**) MEFs derived from *hsf1* wild-type (+) or mutant (−) mice were left untreated (C), or were given a mild preconditioning heat shock at 43°C for 20 min and after 6 or 24 h at 37°C they were given a challenging heat shock at 45°C for 30 min and incubated at 37°C for an additional 24 h before cell lysates were prepared and immunoblot analyses performed using antibody to procaspase 9 (p46) that also recognizes caspase 9 (p35, p37) and uncleaved and cleaved PARP. 45°C, 30′ represents cells that received 45°C for 30 min heat challenge only. (**E**) MEF derived from wild-type (+) or mutant (−) *hsf1* received a preconditioning heat treatment (43°C, 20 min) followed by recovery at 37°C for 6 h to induce thermotolerance. Cells were challenged at 45°C for 30 min and cytochrome c release into the cytoplasm was detected after 3 h by Immunofluorescence analysis. Cytochrome c in the mitochondria normally stains as punctate and once released show as diffuse staining (indicated by arrow). Under conditions used, less than 1% of wild-type and more than 20% of *hsf1*$^{-/-}$ cells showed cytochrome c release into the cytoplasm. (**F**) MEF derived from wild-type (+) or mutant (−) mice were given a preconditioning heat shock at 43°C for 20 min to induce thermotolerance. After 4 h incubation as 37°C, some groups received 50 μM of z-VAD.fmk and after an additional 2 h at 37°C, all groups received 45°C for 30 min. After 20 h of incubation at 37°C to induce apoptosis, cells were analyzed using Annexin V. Percent cell survival of heat treated cell populations was calculated based on the staining profile (Annexin and PI double negative). Experiments were performed three times and results are means ± S.D.

cells based on caspase 9 activation or PARP cleavage.

Cytochrome c is normally sequestered in the mitochondria, but following treatment of cells with apoptotic stimuli, it is released into the cytoplasm. Once in the cytoplasm, cytochrome c binds Apaf1 and then binds and activates caspase 9 [Budihardjo et al., 1999]. Cytochrome c release from the mitochondria can be detected by immunoblotting of cytoplasmic fraction or by immunofluorescence. To show that cytochrome c is released in *Hsf1*-deficient cells that lack thermotolerance development, wild-type or *hsf1*-deficient thermotolerant MEFs were given a challenging heat shock and cytochrome c release from mitochondria was detected by immunofluoresence 1, 3, or 6 h later. The results show that MEFs deficient in *hsf1* released cytochrome c into the cytoplasm (detected as diffuse staining) in large numbers when compared to wild-type MEFs (Fig. 5E, only data for 3 h post heat challenge is presented).

Hsp70 has been implicated in protecting cells against apoptosis by binding to apoptosis protease activating factor 1 (Apaf-1), thereby inhibiting the formation of Apaf-1/caspase 9/cytochrome c complex which leads to the activation of capsase 3 and ultimately cell death [Beere et al., 2000]. Furthermore, Hsp70 has been shown to bind to a caspase-independent apoptotic molecule known as apoptosis inducing factor (AIF) and protect cells against events occurring during apoptosis [Ravagnan et al., 2001]. Other heat shock proteins such as Hsp25 and Hsp84/86 have also been shown to prevent cell death by binding to cytochrome c or to Apaf1, respectively [Xanthoudakis and Nicholson, 2000]. To investigate whether *hsf1*-deficient cells also undergo caspase-independent apoptosis, $hsf1^{+/+}$ and $hsf1^{-/-}$ MEFs were given a primary heat shock to induce thermotolerance and cells were then treated with general caspase inhibitor Z-VAD.fmk followed by a severe heat treatment to induce cell death. Apoptosis was determined using Annexin V staining (Fig. 5F). Results indicate that $hsf1^{+/+}$ cells exhibit development of thermotolerance and cell survival was 85–90%. However, $hsf1^{-/-}$ cells lack development of thermotolerance and cell survival was 20–24% following the severe heat treatment. In contrast, while $hsf1^{+/+}$ cells treated with Z-VAD.fmk did not show any increase in cellular survival following the severe heat stress, since cell survival is approaching maximal, $hsf1^{-/-}$ cell survival was increased by at least two-fold ($54 \pm 5\%$). These results indicate that heat-induced cell death in $hsf1^{-/-}$ cells occurs in part through a caspase-dependent mechansim.

Sensitivity of Mouse Embryo Fibroblasts Deficient in *hsf1* to Various Apoptosis Inducing Agents Other Than Heat Stress

Heat shock proteins have been implicated to protect cells against cytotoxic effects of specific drugs. Since $hsf1^{-/-}$ cells lack the ability to synthesize inducible Hsps, we investigated the response of $hsf1^{-/-}$ cells to killing by drugs that induce Hsps (such as ethanol) as well as those that do not induce Hsps (such as actinomycin D and X-irradiation). We also tested the sensitivity of $hsf1^{-/-}$ cells to ER stress (thapsigargin) and to serum deprivation. Thapsigargin is known to induce Glucose regulated proteins, Grps and cell death occurs via activation of caspase 12 [Nakagawa et al., 2000]. Serum deprivation-induced apoptosis is thought to occur via a caspase-independent mode of cell death [Ravagnan et al., 2001]. Our results show that $hsf1^{-/-}$ cells exhibit increased sensitivity and undergo apoptosis when exposed to ethanol, adriamycin, menadione, serum deprivation, and thapsigargin compared to $hsf1^{+/+}$ cells (Fig. 6A–E), but do not show increased sensitivity when treated with X-irradiation or actinomycin D. When MEFs are preconditioned to accumulate Hsps, $hsf1^{+/+}$ cells exhibit tolerance to ethanol and adriamycin, but not to any other agents tested. However, $hsf1^{-/-}$ cells were unable to develop tolerance to any of the drugs that were tested (Fig. 6A–G).

The results of these experiments indicate that $hsf1^{-/-}$ cells exhibit small amount of increased sensitivity to some of the apoptosis inducing agents that cause cell death through caspase-dependent mechanisms (such as ethanol and adriamycin), caspase-independent mechanisms (such as serum deprivation), or agents causing ER stress and activation of the caspase 12 apoptotic pathway (such as thapsigargin). Furthermore, $hsf1^{-/-}$ cells lack the ability entirely to develop tolerance to any chemotherapeutic agents or other toxic stimuli that were tested.

DISCUSSION

It is well documented that Hsf1 regulates expression of the stress-induced Hsps [Lindquist,

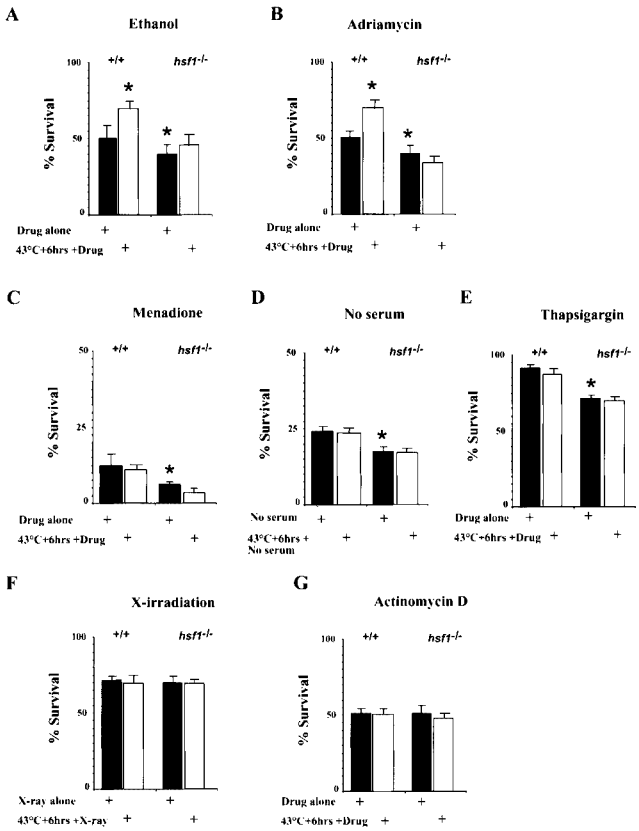

Fig. 6. Increased sensitivity and lack of tolerance of *hsf1*$^{-/-}$ MEFs to specific chemotherapeutic agents. 2×10^5 MEFs were cultured overnight and left untreated or were heated at 43°C for 20 min to induce thermotolerance. After 6 hours incubation at 37°C to allow expression of Hsps and thermotolerance, cells were treated continously (**A**) with 6% ethanol for 24 h, (**B**) with 3 μg/ml adriamycin for 48 h, (**C**) with 150 μM of menadione for 48 h, (**D**) medium without serum for 48 h, (**E**) with 2 μM of thapsigargin for 24 h, (**F**) X-irradiation (2000 Rad) followed by 48 h incubation at 37°C, (**G**) with 1 μM of actinomycin D for 24 h. Cells were then analyzed using Annexin V staining. Percent cell survival of treated cell populations was calculated based on the staining profile (Annexin and PI double negative). Black bars indicate nontolerant cells treated with apoptotic inducing agents, and open bars indicate thermotolerant cells treated with apoptotic inducing agents. Error bars indicate the standard deviation of the means for three replicates. Asterisks indicate significant differences ($P<0.02$) in panels A and B between +/+ and *hsf1*$^{-/-}$ groups that were drug treated, as well as between +/+ tolerant and +/+ non-tolerant groups that were drug treated; in panels C, D, and E significant differences ($P<0.02$) were observed between +/+ and *hsf1*$^{-/-}$ groups that were drug treated.

1986; Kingston et al., 1987; Wu, 1995; Morimoto, 1998; Dai et al., 2000]. Furthermore, *hsf1*-deficient mice exhibit female infertility and embryonic lethality on specific genetic backgrounds [Xiao et al., 1999]. However, our results indicate that *hsf1*-deficient mice on the C57BL/6 genetic background are viable, but females are infertile. Furthermore, our work confirms and extends the previous studies and suggests that in the whole organism Hsf1 alone regulates the heat inducibility of Hsps in all tissues and cells. Surprisingly, the total abolition of this heat inducibility in *hsf1*-deficient mice indicates that the other Hsf family members (Hsf2 and Hsf4) play different roles than that of Hsf1. It is conceivable that in vivo, Hsf2 and Hsf4 require the cooperation of Hsf1 to induce Hsps by heat, and hence lack of Hsf1 renders one or both of these factors non-functional. This cooperation and functional interdependency between Hsf family members has been previously proposed [Tanabe et al., 1998].

In addition, we found that the inducible Hsps, such as Hsp25 and Hsp70, are expressed constitutively in specific tissues and in particular cell types, as specifically demonstrated by the *LacZ* reporter gene that replaced the inducible *hsp70.3* gene in knockout mice.

Multiple and diverse functions have been attributed to various inducible Hsps, e.g., participation in protein folding, protein degradation and repair of damaged proteins [Hartl, 1996; Bukau and Horwich, 1998]. Such diversity in function could dictate the complex regulatory pattern of expression that is observed for these various stress inducible Hsps in different tissues in vivo. We found that inducible Hsps are constitutively expressed in such tissues as the epithelial layer of the cornea, skin, esophagus, forestomach and bladder. These tissues are either exposed directly to the external environment (e.g., the epithelial layer of the skin), or have specialized functions, some of which may require the presence of Hsps. For example, Hsps

could be critical in maintenance and prevention of protein damage in the cornea, which is in constant contact with moving fluids, or in the epithelial layer of the bladder, which is a barrier to the rapid diffusion of salt and water. We have shown recently that this basal expression of Hsps is developmentally regulated, since mice at embryonic day 17 show inducible Hsp70 expression in the epithelial layer of the skin, as well as other tissues such as tongue and forestomach [Huang et al., 2001].

Other critical roles attributed to inducible Hsps are prevention of apoptosis and participation in development of transient heat resistance, referred to as thermotolerance [Li and Mivechi, 1986, 1998; McMillan et al., 1998]. Our data indicate that *hsf1*-deficient cells do not develop thermotolerance, clearly suggesting that inducible Hsps are essential for this function. Studies indicate that overexpression of various Hsps, such as Hsp84/86, Hsp70 [Mosser et al., 1997; Jaattela et al., 1998; Beere et al., 2000; Saleh et al., 2000], and Hsp25 [Bruey et al., 2000], leads to resistance to apoptosis induced by various toxic stimuli [Xanthoudakis and Nicholson, 2000]. Hsp70 and Hsp84/86 interact with Apaf-1 and prevent the formation of the cytochrome c/Apaf-1/caspase 9 complex, thereby inhibiting the intrinsic apoptotic pathway [Beere et al., 2000; Xanthoudakis and Nicholson, 2000]. Hsp25 binds to cytochrome c and inhibits the formation of an apoptosome. Furthermore, a fraction of procaspase 3, which is present in the mitochondria, has been found in complexes with Hsp60 and Hsp10 [Samali et al., 1999]. The release of these Hsps from procaspase 3 occurs simultaneously with release of cytochrome c, before the mitochondrial transmembrane potential is lost [Samali et al., 1999]. Other anti-apoptotic functions for Hsps include the Hsp70 interaction with Bag1 [Takayama et al., 1995, 1997], which was identified originally as an anti-apoptotic protein that interacts with other molecules such as Bcl2 and Bcl-XL [Takayama et al., 1997, 1999]. Furthermore, Hsp70 prevents JNK activation [Gabai et al., 2000]. Increased JNK activity has been associated with increased apoptosis in number of cell types [Chen et al., 1996; Davis, 2000; Gabai et al., 2000]. Our results using MEFs derived from $hsf1^{-/-}$ mice indicate that these cells are extremely susceptible to heat-induced apoptosis, and perhaps other modes of cell death after heat stress.

A number of pathways of cell death have been identified. These include clonogenic cell death, apoptosis, and necrosis [Li et al. 1996; Mosser et al., 1997, 2000; Gabai et al., 2000]. Heat can induce both apoptosis as well as necrosis, depending on the severity of heat stress. Our results indicate that death of MEFs derived from $hsf1^{-/-}$ mice can be prevented when cells were pretreated with Z-VAD.fmk, a general caspase inhibitor. This indicates that heat-induced cell death in $hsf1^{-/-}$ MEFs occurs to certain extent via caspase-dependent apoptosis. A caspase-independent mode of cell death requiring JNK activation has also been proposed for heat-induced cell death, as found in a study using antisense RNA to decrease Hsp70 expression in human tumor cells [Gabai et al., 2000]. Our results indicate that there is only a small amount of increase in JNK activity in heat-treated *hsf1*-deficient MEFs when compared to wild-type MEFs. However, the increase in JNK activity is sustained for longer period of time in *hsf1*-deficient cells when compared to wild type MEFs (data not shown). Accumulation of Hsps during thermotolerance development could be critical for suppression of the JNK signaling pathway. Perhaps this leads to inhibition of phosphorylation of critical proteins involved in various death-inducing pathways [Park et al., 2001]. However, involvement of JNK in heat-induced apoptosis could be more complex, since *JNK*-deficient cells seem to also possess a reduced ability to maintain thermotolerance (Mivechi, manuscript in preparation). Precisely how Hsf1 functions to prevent apoptosis in wild-type cells is complex and needs further investigation. It appears that there may be a certain degree of stability in specific pro-survival protein complexes in cells possessing the *hsf1* gene. In the absence of Hsf1, these protein complexes could become unstable and fall apart. It is clear that although Hsf1 increases the levels of Hsps after heat stress, cells deficient in *hsf1* are more susceptible to even a single heat shock, as manifested by an increase in cell death (Fig. 4). If Hsf1 does not contribute to basal levels of Hsps under physiological conditions, then HSPs synthesized during the first few minutes of heat shock might play critical roles in preventing the early events in apoptosis, such as cytochrome c release from the mitochondria. The *hsp70.3*- and *hsp70.1*-deficient cells undergo increased apoptosis, although to a lesser extent than $hsf1^{-/-}$ cells [Huang et al., 2001]. *hsp70.1* and

hsp70.3-deficient cells are also unable to maintain thermotolerance as efficiently as wild-type cells [Huang et al., 2001]. All the anti-apoptotic functions proposed for Hsps fail in $hsf1^{-/-}$ cells, and these cells are unable to develop any adaptation or tolerance to heat shock. $hsf1^{-/-}$ cells are also unable to develop thermotolerance to any of the cytotoxic agents we have tested including those shown in Figure 6. Furthermore, $hsf1^{-/-}$ MEFs show some increase in apoptosis compared to +/+ MEFs in response to treatment with ethanol (which has been shown to induce Hsps and cause damage to proteins), adraimycin, menadione and thapsigargin [Li and Mivechi, 1986, 1998; Li et al., 1995; Ravagnan et al., 2001]. These agents cause DNA damage and exert oxidative and ER stress to cells, respectively. Overexpression of Hsp70 was recently shown to protect cells from apoptosis induced by serum starvation. Apoptosis induced by serum deprivation appears to lead to cell death via a caspase-independent cell death and Hsp70 was shown to interact with apoptosis inducing agent, AIF [Ravagnan et al., 2001]. $hsf1^{-/-}$ cells show increase apoptosis when cultured in serum free medium when compared to $hsf1^{+/+}$ MEFs. However, wild type or $hsf1^{-/-}$ thermotolerant MEFs did not show increased levels of protection to apoptosis when grown in the absence of serum. Large amounts of overexpression of Hsp70 could be the cause of resistance that was observed previously, although Hsp70 interaction with AIF was clearly demonstrated [Ravagnan et al., 2001]. Furthermore, $hsf1^{-/-}$ cells do not show increased sensitivity to apoptosis by agents such as X-irradiation, actinomycin D or any other DNA damaging agents we have tested (e.g., VP16, Cisplatinum, data not shown). Our studies indicate that $hsf1^{-/-}$ cells are specifically sensitive to damaging effect of heat and are vastly deficient in development of thermotolerance to heat stress. $hsf1^{-/-}$ increased sensitivity to apoptosis in response to treatment with agents other than heat are much less pronounced. This is the case for even those agents that are known to cause protein damage.

In conclusion, in this study we demonstrate that Hsf1 solely regulates heat-inducible expression of Hsps in vivo. Furthermore, we show that inducible Hsp70 as well as other inducible Hsps are constitutively expressed in specific tissues, and this constitutive expression is not regulated by Hsf1. Finally, Hsf1 is essential for maintenance of thermotolerance, which appears to be a state in which cells become highly resistant to cell death induced by heat shock. Lack of Hsf1 renders cells extremely susceptible to heat-induced apoptosis through the intrinsic apoptotic pathway.

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