Published 2007 Wiley-Liss, Inc. genesis 45:487–501 (2007)

ARTICLE

Insights Into Function and Regulation of Small Heat Shock Protein 25 (HSPB1) in a Mouse Model With Targeted Gene Disruption

Lei Huang, Jin-Na Min, Shane Masters, Nahid F. Mivechi, and Demetrius Moskophidis*

Center for Molecular Chaperones/Radiobiology and Cancer Virology, Medical College of Georgia, Augusta, Georgia

Received 2 March 2007; Revised 30 May 2007; Accepted 2 June 2007

Summary: The mammalian small heat shock protein (sHSPs) family is comprised of 10 members and includes HSPB1, which is proposed to play an essential role in cellular physiology, acting as a molecular chaperone to regulate diverse cellular processes. Whilst differential roles for sHSPs are suggested for specific tissues, the relative contribution of individual sHSP family members in cellular and organ physiology remains unclear. To address the function of HSPB1 in vivo and determine its tissue-specific expression during development and in the adult, we generated knock-in mice where the coding sequence of *hspb1* is replaced by a *lacZ* reporter gene. *Hspb1* expression marks myogenic differentiation with specific expression first confined to developing cardiac muscles and the vascular system, and later in skeletal muscles with specific expression at advanced stages of myoblast differentiation. In the adult, *hspb1* expression was observed in other tissues, such as stratified squamous epithelium of skin, oronasal cavity, tongue, esophagus, and uterine cervix but its expression was most prominent in the musculature. Interestingly, in cardiac muscle *hsbp1* expression was down-regulated during the neonatal period and maintained to a relatively low steady-level throughout adulthood. Despite this widespread expression, *hspb1*$^{-/-}$ mice were viable and fertile with no apparent morphological abnormalities in tissues under physiological conditions. However, at the cellular level and under stress conditions (heat challenge), HSPB1 act synergistically with the stress-induced HSPA1 (HSP70) in thermotolerance development, protecting cells from apoptosis. Our data thus indicate a nonessential role for HSPB1 in embryonic development and for maintenance of tissues under physiological conditions, but also shows that it plays an important role by acting synergistically with other HSPs during stress conditions to exert cytoprotection and anti-apoptotic effects. genesis 45:487–501, 2007. **Published 2007 Wiley-Liss, Inc.**†

Key words: *hspb1* (hsp25/27); *hspa1* (*hsp70.1*) and *hspa1b* (*hsp70.3*); knockout mice; small heat shock proteins; muscle development; thermotolerance

INTRODUCTION

Organisms have evolved a range of strategies to increase the reliability of their protein structures. These include heat shock proteins (HSPs), which function as molecular chaperones to ensure correct folding of proteins into their three-dimensional forms that is crucial for biological activity in the cell or promote degradation of misfolded proteins and regulate cell growth and cell signaling pathways that initiate repair, allow adaptation, and ensure survival [for reviews, see references (Agashe and Hartl, 2000; Barral *et al.*, 2004; Benjamin and McMillan, 1998; Bukau *et al.*, 2006; Calderwood *et al.*, 2006; Cohen and Kelly, 2003; Feder and Hofmann, 1999; Jolly and Morimoto, 2000; Lindquist and Craig, 1988; Mosser and Morimoto, 2004; Smith *et al.*, 1998)]. Molecular details of processes, in which HSPs are involved, have emerged from studies on environmental cellular stress. In particular, the rapid induction of HSPs in response to heat has been recognized as a key event that serves to protect cells and tissues against an initial insult, augment recovery, and produce a state of resistance to subsequent stress (thermotolerance) (Gerner and Schneider, 1975; Hildebrandt *et al.*, 2002). This response is remarkably conserved across prokaryotic and eukaryotic cells and plays a pivotal role in host defense and survival (Kregel, 2002; Moseley, 1997). It is therefore not surprising that defective protein folding or failure of the quality control mechanisms compromising essential cellular functions can result in disease. Indeed, defective func-

This article contains supplementary material available via the Internet at http://www.interscience.wiley.com/jpages/1526-954X/suppmat.

*Correspondence to: Demetrius Moskophidis, Center for Molecular Chaperones/Radiobiology and Cancer Virology, 1410 Laney Walker Blvd. CN3143, Augusta, GA 30912.

E-mail: dmoskophidis@mcg.edu

Contract grant sponsor: National Institutes of Health, Contract grant numbers: CA121951, CA062130, CA132640

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/dvg.20319

tion or inappropriate expression of HSPs has been implicated in a wide range of human diseases, including cancer, neurodegenerative conditions, and cardiovascular diseases (Macario and Conway de Macario, 2005). Many members of the multifunctional HSP families are also constitutively expressed, presumably to regulate and enhance the cellular quality control system under physiological conditions. Indeed, more recent studies suggest a broader role for HSPs in regulating the capacity of populations and lineages for evolutionary adaptation to changing environmental conditions (Queitsch *et al.*, 2002; Rutherford and Lindquist, 1998; Sangster *et al.*, 2004; Sollars *et al.*, 2003).

In mammals, there are at least 10 members that comprise the small heat shock protein (sHSP) family. These HSPs are diverse in sequence, size, and function, but share characteristic features, including a conserved α-crystallin domain (80–100 residues in the C-terminal region), a low molecular mass of 12–42 kDa, and a dynamic quaternary structure (Augusteyn, 2004; Ganea, 2001; Haslbeck *et al.*, 2005; Kim *et al.*, 1998; MacRae, 2000; Sun and MacRae, 2005a; Taylor and Benjamin, 2005). The sHSPs are able to assemble into large complexes of 200–400 kDa, and some of them (HSPB1, HSPB6, HSPB8, CRYAA, and CRYAB) have chaperone-like activity (Haslbeck, 2002). Although the principal function of the sHSPs is chaperone activity, other more specific functions, including support of cellular survival under stress conditions by inhibiting apoptosis, stabilization of the cytoskeleton and regulation of cell motility, migration, and muscle contraction, have been also proposed. Consistent with an essential role of sHSPs in muscle physiology is the observation that most of them (HSPB1, HSPB2, HSPB3, CRYAB, HSPB6, HSPB7, HSPB8) are abundant in heart and skeletal muscles, where they may comprise more than 3% of total protein; in particular, their role in humans has been more clearly demonstrated by studies of mutations in the *Cryαb* associated with familial desmin-related myopathy (Chavez Zobel *et al.*, 2003; Sun and MacRae, 2005b; Vicart *et al.*, 1998) or mutations in *hspb1* or *hspb8* considered as cause of the Charcot-Marie-Tooth and distal motor neuropathy (Ackerley *et al.*, 2006; Benndorf and Welsh, 2004; Evgrafov *et al.*, 2004; Irobi *et al.*, 2004).

Perhaps the most widely studied member of the sHSP family is HSPB1. Several studies in the literature assigned a critical role to HSPB1 in mediating protection against stress through maintaining normal cell function by stabilizing the cytoskeleton, facilitating repair or removal of damaged proteins, and inhibiting components of both stress and death-receptor induced apoptotic pathways (Bruey *et al.*, 2000; Didelot *et al.*, 2006; Gerthoffer and Gunst, 2001; Landry and Huot, 1999; Paul *et al.*, 2002; Sreedhar and Csermely, 2004). Various mechanisms have been proposed to account for the ability of HSPB1 to protect stressed cells from apoptotic death, including decreasing the generation of radical oxygen species, directly interacting with cytochrome c released from mitochondria, or facilitating activation of the ubiquitin-proteasome pathway. In addition HSPB1 can also inhibit apoptosis by regulating signaling pathways upstream of apoptosome formation by binding Akt, an interaction necessary for Akt activation in stressed cells (Rane *et al.*, 2003). In contrast to the other major chaperones such as HSPA1 (HSP70i), HSPB1 is ATP independent, yet can efficiently associate with unfolded proteins and maintain them in a folding-competent state. Within unstressed cells HSPB1 levels are generally low, being predominantly comprised of large oligomeric units made up of six tetrameric complexes of HSPB1. During the stress response, increased HSPB1 expression is preceded by phosphorylation-induced reorganization of these multimers (Haslbeck, 2002). Such stress-induced phosphorylation is catalyzed by MAPK-activated protein kinases-2 and -3, which in turn are activated through phosphorylation by p38 MAP kinase (Dorion and Landry, 2002; Gaestel, 2002; Rouse *et al.*, 1994). Importantly, heat-induced nuclear protein aggregation is resolved more rapidly during recovery from severe stress exposure in cells overexpressing HSPB1, indicating that HSPB1 possesses chaperone-like function (Kampinga *et al.*, 1994). In addition, HSPB1 binds to F-actin and can prevent disruption of the cytoskeleton, resulting from either heat stress or cytochalasin D-induced disruption of actin filaments (Guay *et al.*, 1997).

Many of the large gaps in our current understanding of the multifaceted roles of individual sHSPs in mammalian physiology are inherent upon the difficulties in dissecting the functional importance of the various hetero–oligomeric complexes that sHSPs form with each other (Fontaine *et al.*, 2005; Sun *et al.*, 2004). Perhaps the most compelling evidence for a combinatorial function of sHSPs in stress physiology comes from studies with double (HSPB2 and CRYAB) mutant mice, which are viable suggesting that these molecules are dispensable for mouse development, but the mice aged earlier and died with skeletal muscle degeneration (Brady *et al.*, 2001). In this report we establish that this is also the case for HSPB1. Thus using mice where *hspb1* has been deleted and replaced with a reporter gene we demonstrated that HSPB1 is dispensable for embryo development but by acting in concern with other HSPs its function is required for full protection of cell against stress. This model allows assessment of HSPB1 expression and sheds light on this widely studied but poorly understood, member of the sHSP family in vivo.

RESULTS

Generation and Analysis of *hspb1-lacZ* Reporter Mice

To study the physiological role of HSPB1 in the stress response we generated *hspb1* mutant mice using a conventional targeting strategy. In the targeting vector created, the reporter *lacZ* gene was fused in frame with the start codon of *hspb1* to replace ~60% of the *hspb1* coding sequence. The vector was transfected into B6-ES cells, and two clones with correct targeting were microinjected into BALB/c blastocysts, resulting in germline

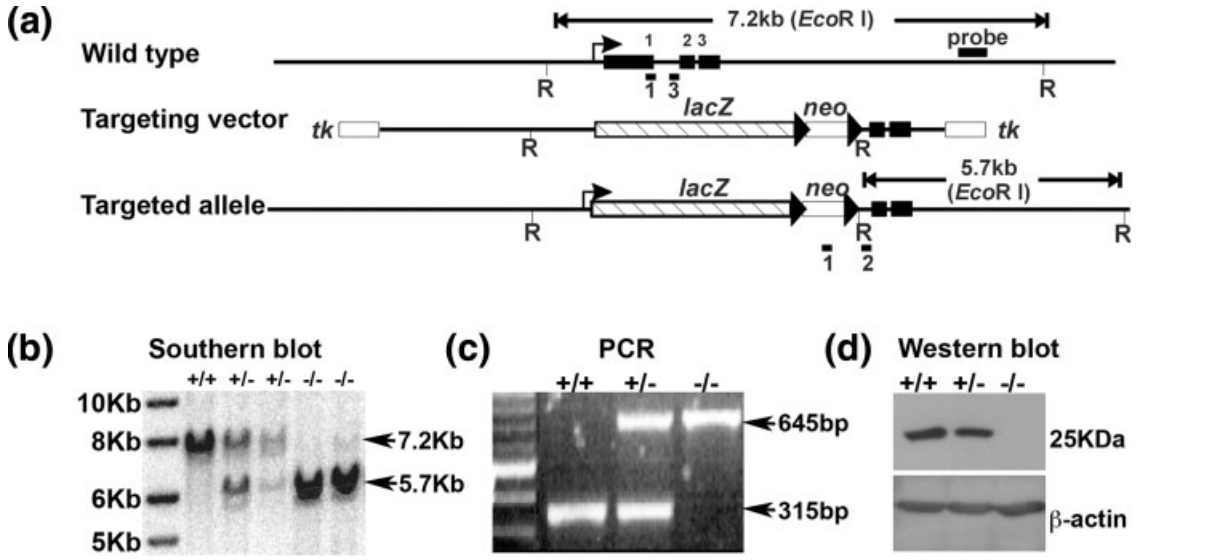

FIG. 1. Generation of *hspb1-lacZ* reporter mice. Diagrammatic representation of (**a**) Genomic structure of *hspb1* locus, targeting vector, and targeted locus following homologous recombination. The targeting vector was constructed by replacing ~0.6 kb of genomic DNA downstream of the start codon, including the first exon and 0.3 kb of the first intron, with a neomycin resistance (*neo*) gene flanked by *loxP* sequences and *lacZ* reporter gene. The vector was designed such that the *hspb1* gene promoter drives reporter expression. The *neo* resistance gene flanked by *loxP* sequences, the thymidine kinase (*tk*) genes, probe for Southern blotting, and primers for PCR are indicated. (**b**) Southern blotting analyses of genomic DNA digested with EcoRI (R) and hybridized with a probe external to the targeting vector yield fragments of 7.2 kb for wild-type and 5.7 kb for targeted alleles, respectively. (**c**) PCR-based genotyping amplifies wild type and targeted *hspb1* locus fragments of 315 and 645 bp, respectively, using primers 1, 2, and 3. (**d**) The absence of HSPB1 at the protein level was confirmed by immunoblotting of tongue homogenates prepared from $hspb1^{+/+}$, $hspb1^{+/-}$, or $hspb1^{-/-}$ mice.

transmitting chimeric mice. A diagrammatic presentation of the targeting strategy, including genotyping of mice by Southern and Western blot analysis of tongue homogenates prepared from $hspb1^{+/+}$ and $hspb1^{-/-}$ mice to verify ablation of HSPB1 protein, is presented in Figure 1. All experiments using homozygous, heterozygous, or wild-type controls were performed on C57BL/6 background. To avoid potential interference of the *tk-neo* marker gene on the expression of the reporter *lacZ* gene under the *hspb1* promoter, mice were bred with transgenic B6-Cre mice to remove the neomycin selection marker. The $hspb1^{-/-}$ mice were viable, born at the expected Mendelian distribution, fertile, and showed no apparent morphological alterations in comparison to heterozygous or wild-type littermates.

Tissue Specific Expression of *Hspb1* During Embryonic Development and in Tissues of Adult Mice

The remarkably widespread, but also poorly conserved, superfamily of sHSPs that in higher multicellular eukaryotes consists of several members (for example 10 genes in human and mouse, 19 in *Arabidopsis thaliana*, 16 in *Caenorhabditis elegans*) led to the proposal that specific functional roles for sHSPs in certain cell types and tissues and during development may have driven this diversification. A better understanding of the common or distinct roles exhibited by the sHSP proteins at the whole organism level requires comparative analyses of their spatial and temporal expression patterns during development and in adult tissues under physiological and stress-induced conditions. In particular, the tissue and cell-specific *hspb1* expression profile, a critical aspect for understanding its function in vivo, has not been well defined, primarily due to the lack of adequate experimental approaches to monitor its expression in vivo.

To address this issue we have performed a detailed analysis of *hspb1* expression by visualizing β-gal activity in organs of $hspb1^{+/-}$ mice that faithfully reproduce the *hspb1* allele transcriptional activity. We first sought to obtain direct evidence for spontaneous *hspb1* expression during embryo development in the absence of defined stress. The onset of *hspb1* expression during embryonic development was observed during early cardiac tube development when looping is not complete. *Hspb1* was not expressed within the late pre-somite stage embryos as revealed by the absence of β-gal staining at E8. β-gal activity, first detected at E8.5, was restricted to the cardiac tube (Fig. 2A), and intense staining was observed in subsequent morphogenesis of the tube, resulting in chamber (ventricles and atria) formation (Fig. 2B). At E10.5, though prominent *hspb1* expression was largely restricted to the cardiac myocardium, staining was also visualized in the developing vascular system (Fig. 2Bc,g). Surprisingly, endocardial cushions did not express *hspb1* at this developmental stage (Fig. 2Be). From the mid-gestational stage (E13.5), strong staining was visualized in the developing skeletal and tissue musculature, whilst overall *hspb1* expression in the cardiac

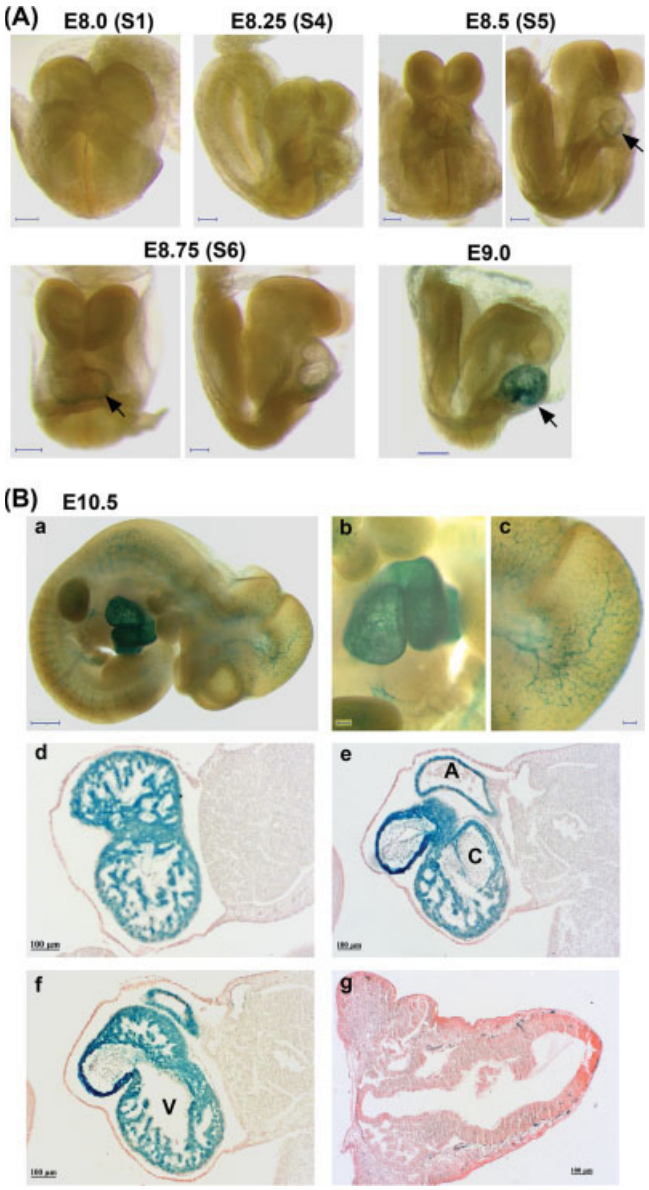

FIG. 2. Tissue-specific constitutive expression of *hspb1* during early embryonic development. *Hspb1*$^{+/-}$ embryos recovered at the indicated embryonic stages were stained with X-gal, paraffin-embedded, and sectioned. Sections were counterstained with hematoxylin and eosin. The number of somites (*S*) is indicated in parentheses. (**A**) Whole-mount X-gal staining of E8 to E9 embryos viewed frontally or from the right side. Expression of *hspb1-lacz* reporter activity (blue indicated as arrow) becomes restricted to cardiac tube at E8.5 (embryo has 5 somites). (**B**) Lateral view of embryos recovered at E10.5 (a) and a high magnification of the cardiac region (b) or brain vasculature (c) is presented. Histological examination of heart (d, e, and f) showing *hspb1* expression in all cardiac components except endocardial cushions that did not express *hspb1*. A, V, and C represent attrial, ventricular, and cushion endocardium, respectively. Histological examination of brain sections from E12.5 embryo (presented in c) showing expression of *hspb1* in the cerebral vasculature (g).

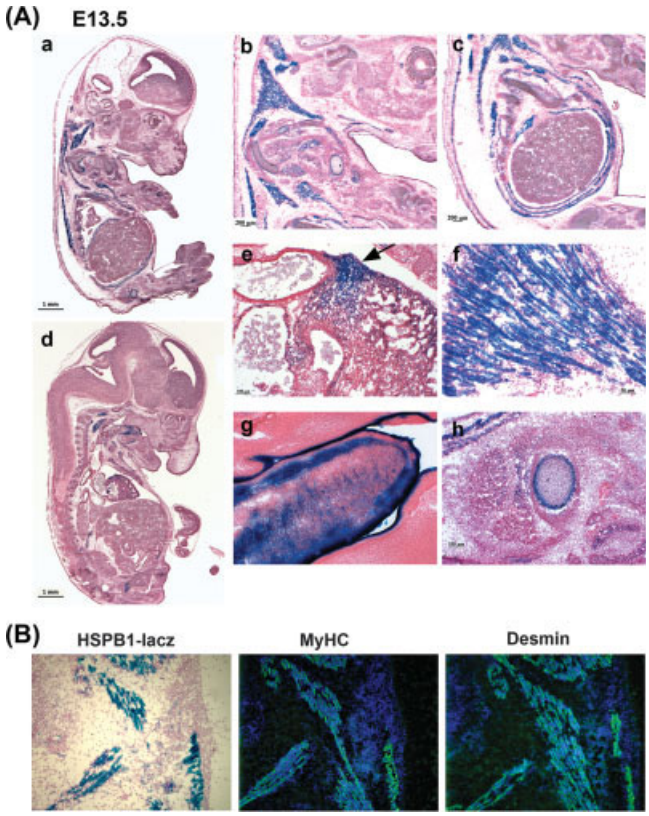

FIG. 3. *Hspb1* expression marks terminal myoblast differentiation to myofibers in skeletal musculature. (**A**) Sagittal sections through E13.5 *hspb1*$^{+/-}$ embryo (a and d) showing *hspb1* expression (blue; β-gal activity). A high magnification of the cephalic/thoracic (b) or abdominal (c) region showing *hspb1* expression in developing musculature is presented. Histological examination of sections obtained from the heart (e) showing *hspb1* expression in the interventricular septum (indicated by arrow) in developing muscles of tongue (f and g) or cartilaginous templates of developing ribs (h). (**B**) Immunostaining of consecutive sections from embryonic skeletal muscles obtained from E13.5 embryo with antibody specific to MyHC (middle panel) or Desmin (righ panel) and β-gal activity (left panel) revealing that *hspb1* expression colocalize with expression of these markers for differentiated myofibers. Sections were stained as follows: Left panel, X-gal for β-gal activity (blue) and counter-stained with hematoxylin and eosin (pink); Middle panel, for MyHC (green) and DAPI (for nuclei in blue); and left panel, for Desmin (green) and DAPI (blue).

musculature became scarce and was restricted to a few cardiomyocytes. Interestingly, we consistently found that a substantial number of β-gal-positive cells persisted in the interventricular septum (illustrated in Fig. 3Ae). Whether these β-gal-positive cells represent a population of differentiated myoblasts from cardiac progenitors (cardioblasts) that have been reported to persist at later developmental stages and in the adult heart, awaits further evaluation (Laugwitz *et al.*, 2005). However, a characteristic picture at this embryonic stage was a prominent up-regulation of *hspb1* expression in the entire developing skeletal musculature. Immunostaining of consecutive sections from embryonic muscles with antibody specific to MyHC or Desmin (markers for differentiated myoblasts) and β-gal activity revealed that *hsb1* expression was remarkably colocalized with the expression of these markers (Fig. 3B). This data suggest that *hspb1* may be a target gene of transcription factors regu-

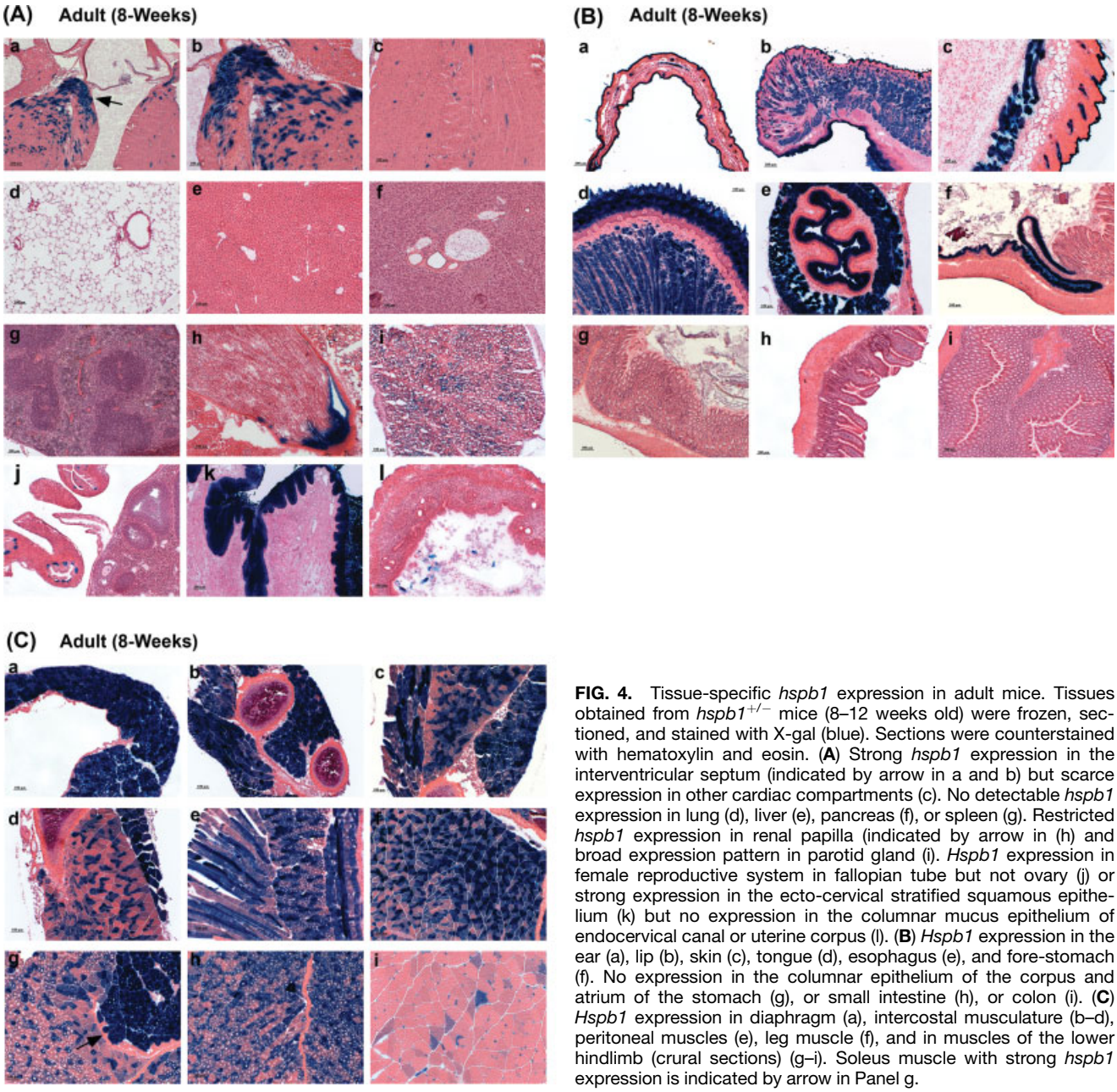

FIG. 4. Tissue-specific *hspb1* expression in adult mice. Tissues obtained from *hspb1*$^{+/-}$ mice (8–12 weeks old) were frozen, sectioned, and stained with X-gal (blue). Sections were counterstained with hematoxylin and eosin. (**A**) Strong *hspb1* expression in the interventricular septum (indicated by arrow in a and b) but scarce expression in other cardiac compartments (c). No detectable *hspb1* expression in lung (d), liver (e), pancreas (f), or spleen (g). Restricted *hspb1* expression in renal papilla (indicated by arrow in (h) and broad expression pattern in parotid gland (i). *Hspb1* expression in female reproductive system in fallopian tube but not ovary (j) or strong expression in the ecto-cervical stratified squamous epithelium (k) but no expression in the columnar mucus epithelium of endocervical canal or uterine corpus (l). (**B**) *Hspb1* expression in the ear (a), lip (b), skin (c), tongue (d), esophagus (e), and fore-stomach (f). No expression in the columnar epithelium of the corpus and atrium of the stomach (g), or small intestine (h), or colon (i). (**C**) *Hspb1* expression in diaphragm (a), intercostal musculature (b–d), peritoneal muscles (e), leg muscle (f), and in muscles of the lower hindlimb (crural sections) (g–i). Soleus muscle with strong *hspb1* expression is indicated by arrow in Panel g.

lating myogenic differentiation. In addition, strong *hspb1* expression was detected in the cartilaginous templates of developing vertebrate and ribs Figure 3Ah, and Figure S3 (Panels h and i), but its expression level declined at later embryonic stages through adulthood.

At later embryonic stages (E17) or during the postnatal period (P0, P5), as shown in supplementary Figures S1, S2, and S3, intense staining was not confined to the skeletal musculature but *hspb1* expression was also observed over the stratified squamous epithelium of skin (composing the epidermis and hair follicles), lip, tongue, esophagus, and forestomach. In tongue and esophagus *hspb1* expression was also detected in the muscular layer. No expression was found in the columnar epithelium of the corpus and atrium of the stomach. In the adult, as shown in Figure 4, the expression pattern established during the postnatal period did not change dramatically, remaining prominently expressed in the skeletal musculature and stratified epithelial layers of skin, oronasal cavity, tongue, esophagus, and forestomach. Strong expression was also observed in the parotid gland (salivary glands) and uterine cervical epithelium and, except for the apex of the renal papilla, the kidney did not show *hspb1* expression. In heart, *hspb1* expression was largely localized in the interventricular septum with moderate expression level in cardiac myocardium. This expression pattern overlaps with *hspa1* expression in certain tissues (e.g., stratified squamous epithelium of

skin, lip, tongue, and esophagus) or is distinct (e.g., *hspb1* is expressed in muscularis mucosa of esophagus, whilst *hspa1* expression is confined to the epithelium; *hspa1* but not *hspb1* is expressed in hippocampus). In the developing eye, *hspb1* expression was restricted to the ocular lens marking secondary fiber cell development during the early postnatal period. Its expression is primarily regulated by heat shock transcription factor 4 (HSF4) (Min *et al.*, 2004), rapidly increased from P0 through P5 to a steady-state level, and was confined to the transitional and nuclear zone of the lens (Supplementary Fig. S4). Lens cuboidal epithelial cells did not express *hspb1*. During development or in the adult (8–12 weeks old) no impressive *hspb1* expression in the brain was observed. We found only a few isolated or scarcely grouped cells in the brain with β-gal staining. In the spinal cord, clearer *hspb1* expression in neuronal cells was observed (data not shown). It is important to note that the expression pattern of the *hspb1* reporter in the different tissues described here is consistent with several reports in the literature where HSPB1 expression in mammalian tissues was studied (Abdelwahid *et al.*, 2001; Chen and Brown, 2007; Duverger and Morange, 2005; Wakayama and Iseki, 1998). However, our finding of restricted *hspb1* expression in the brain contrasts somewhat with published data that demonstrate abundant HSPB1 expression in the central nervous system of an unstressed embryo, as visualized by immunohistochemistry (Loones *et al.*, 2000). Although we cannot entirely eliminate the possibility that removal of the first intron and part of the coding sequence of *hspb1* in our targeting strategy may have affected gene regulation, according to the genome-wide atlas of gene expression in the adult mouse brain of *hsbp1* mRNA was also hardly detectable, which is consistent with our finding (Lein *et al.*, 2007). In conclusion, the spatial and temporarily regulated *hspb1* expression characterize skeletal/cardiac muscle or lens fiber differentiation, indicating that *hspb1* may play a role in the genetic programs responsible for myogenic differentiation and cardiac morphogenesis. In addition, the striking coexpression of *hspb1* with *hspa1* in stratified squamous epithelial layers suggests a common functional role of these structurally diverse molecular chaperones in maintenance of epithelial layers.

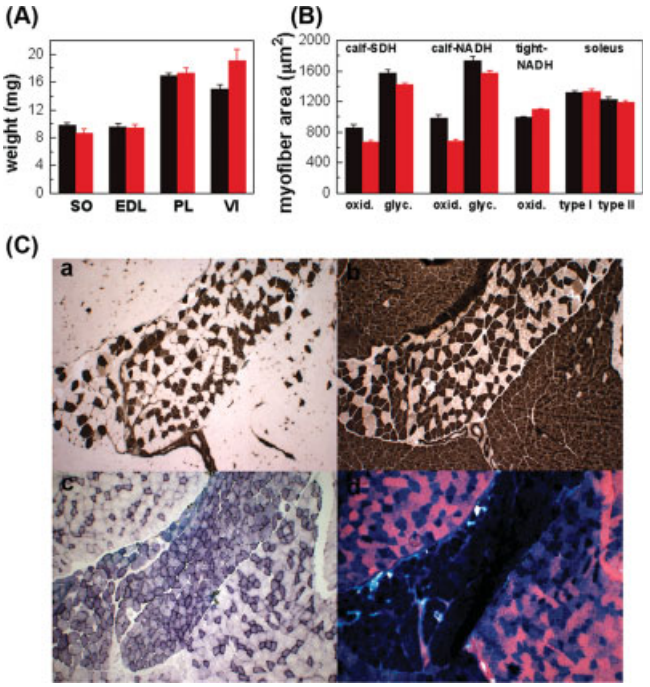

FIG. 5. Normal morphology of skeletal muscles in *hspb1*$^{-/-}$ mice. (**A**) Weight measurements of soleus (SO), extensor digitorum longus (EDL), plantaris (PL), and vastus intermedius muscles from 32-week-old female *hspb1*$^{-/-}$ (black) and B6-control (red) mice. It should be noted that male *hspb1*$^{-/-}$ mice have a similar skeletal muscle phenotype. Mean values ± the standard error of the mean (SEM) of groups of 4 mice. (**B**) Quantification of myofiber area from calf muscles (crural sections) or tight muscles (differentiated to oxidative (oxid.) versus glycolytic (glyc.) type based on the NADH or SDH staining) or from soleus muscle (differentiated to Type I or Type II based on the ATPase staining) from *hspb1*$^{-/-}$ (black) or B6-control (red) mice. Means values ± SEM of ∼100 fibers for two mice per each genotype. (**C**) Near adjacent transverse sections of the lower hindlimb (crural) from 32 week-old *hspb1*$^{-/-}$ mice were stained (a; upper left panel) for myosin ATPase preincubated at pH = 4.6 (Type I fibers stain dark and Type IIa stain light) or (b; upper right panel) pretreated at pH = 10.4 (Type I fiber stain light and Type IIa fibers stain dark), (c; lower left panel) stained for SDH or (d; lower right panel) for β-gal expression (*hspb1*). Note that myofibers in the soleus (SO) muscle stained strongly for β-gal and SDH. Note strong correlation of *hspb1* expression with the pattern of SDH myofiber staining.

HSPB1 Is Not Essential for Tissue and Organ Function Under Physiological Conditions

Although HSPB1 was constitutively expressed in many tissues, no gross morphological phenotypes were apparent in newborn or adult *hspb1*$^{-/-}$ mice of different ages (monitored over a 1-year period). The body weight of male and female *hspb1*$^{-/-}$ mice of different ages (3, 6, or 9 months-old) did not differ significantly from control B6 mice. More detailed analyses have been initiated to study the potential role of HSPB1 in muscle physiology. All skeletal muscles analyzed, including predominantly fast-twitch muscles (extensor digitorum longus and vastus intermedius) or slow-twitch muscles (soleus and plantaris) showed no significant differences with respect to weight and size between *hspb1*$^{-/-}$ and wild type (Fig. 5A). Moreover, ATPase staining that distinguishes between fiber types based on differences in myosin isoforms in response to acid versus alkaline conditions or histological analysis of muscle tissues for succinic dehydrogenase (SDH) activity, an indicator of mitochondria numbers, revealed no obvious abnormalities between *hspb1*$^{-/-}$ and B6 controls. Interestingly, *hspb1* expression visualized by β-gal activity was largely restricted to myofibers with high mitochondrial numbers (SDH-positive with high oxidative capacity), and there was a less clear match with the fiber type (Type I or Type II) differentiated by ATPase staining (Fig. 5B). This finding is interesting because sHSPs, HSPB1 in particular, have been previously reported to function as

antioxidants by augmenting glutathione (GSH) concentration and general protection of mitochondrial integrity, as indicated by the transmembrane potential during oxidative stress (Dalle-Donne *et al.*, 2001; Escobedo *et al.*, 2004; Yan *et al.*, 2005). To determine whether *hspb1* deficiency has similar biochemical effects, we assessed the GSH/glutathione disulfate (GSSG) ratio (an indicator of the intracellular redox state), in the heart. However, our results did not reveal any significant changes in GSH/GSSG ratio, an indicator in assessing the cells' capacity to detoxify free radical species, between *hspb1*$^{-/-}$ mice and wild-type animals (data not shown).

The lack of apparent muscle phenotype may be attributed to compensation by other members of the sHSPs, which are abundantly expressed in heart and skeletal muscles. The fact that muscles of *hspb1*$^{-/-}$ mice in comparison with wild-type B6 mice exhibit similar mRNA expression levels for several relevant sHSPs did not support this notion (Fig. 6). Moreover, as shown in Figure 7, we could not detect significant differences in protein levels of other HSPs in tissues of *hspb1*$^{-/-}$ mice when compared with the wild type under normal conditions, and this again argues against a functional compensation for the loss of HSPB1. Thus, genetic ablation of *hspb1* did not result in apparent morphological alterations in tissues with constitutive expression of this molecular chaperone and, in particular, no alterations in skeletal or cardiac muscle were observed. However, the possibility that HSPB1 is required for protection and maintenance of heart and skeletal muscle function and perhaps of other tissues expressing this molecule under mechanical or other stress conditions awaits further evaluation, and the *hspb1*-deficient mouse model is valuable to address this issue in vivo.

Finally, a functional contribution of HSPB1 in lens physiology is inferred from its strong expression in the ocular lens. In addition, there is evidence in the literature suggesting that HSPB1 may be necessary for maintaining solubility of other crystallins and plays a role in the organization and function of cytoskeletal elements, to enhance cell genomic integrity in lens fiber cells. However, morphological and histological examination of lens did not reveal opacity or other obvious alterations in lenticular structure in *hspb1*$^{-/-}$ mice of different ages (monitored over a 1-year period), demonstrating that the lens can accommodate the loss of HSPB1 function perhaps through compensation by other members of the sHSP family expressed in this organ.

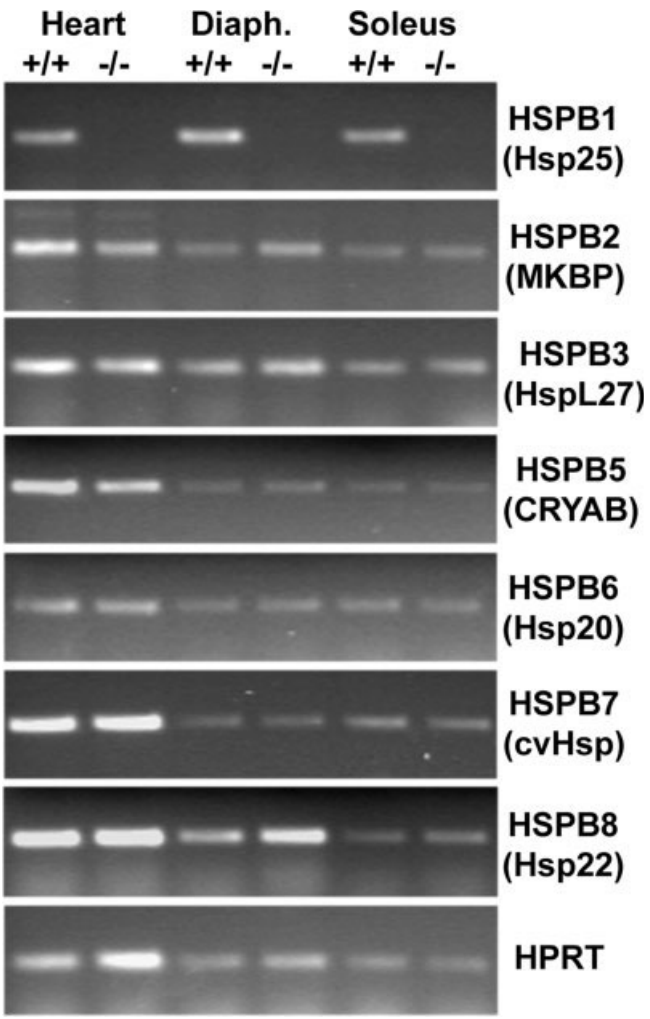

FIG. 6. Loss of *hsp1* function does not alter mRNA levels of sHSPs abundantly expressed in cardiac and skeletal muscles. Semiquantitative RT-PCR analysis of sHSP gene expression (transcripts) in heart, diaphragm, and soleus muscles of *hspb1*$^{-/-}$ (−/−) and wild-type B6- (+/+) mice. As a control HPRT transcripts were detected.

HSPB1 Synergizes With HSPA1 (HSP70.1 and HSP70.3) in Thermotolerance Development, Protecting Cells from Apoptosis

Several studies in the literature have assigned a critical role to HSPB1 and HSPA1 in mediating protection against stress by inhibiting components of both stress and death-receptor induced apoptotic pathways. A logical extension of published studies with HSPA1 (*hsp70.1*$^{-/-}$ or *hsp70.3*$^{-/-}$) or *hsf1*$^{-/-}$ mice (Huang *et al.*, 2001; Xiao *et al.*, 1999; Zhang *et al.*, 2002) was to evaluate the physiological role of HSPB1 alone or in combination with HSPA1 in regulating the cellular stress response and apoptosis. In agreement with earlier reports, we did not observe significant differences in basal or stress-induced levels of several HSPs measured by Western blots of tissues of *hspb1*$^{-/-}$ mice untreated or following whole-body hyperthermic challenge (Fig. 7). This indicates a lack of compensation by other HSPs in *hspb1*$^{-/-}$ mice. Note that except for marked up-regulation of HSPB1 expression in the liver following hyperthermic treatment of wild-type mice, there was no significant change in protein level in other tissues constitutively expressing this molecular chaperone. This is in contrast to HSPA1, which was prominently up-regulated following heat challenge. For a more mechanistic understanding, the requirement of HSPB1 in cellular protec-

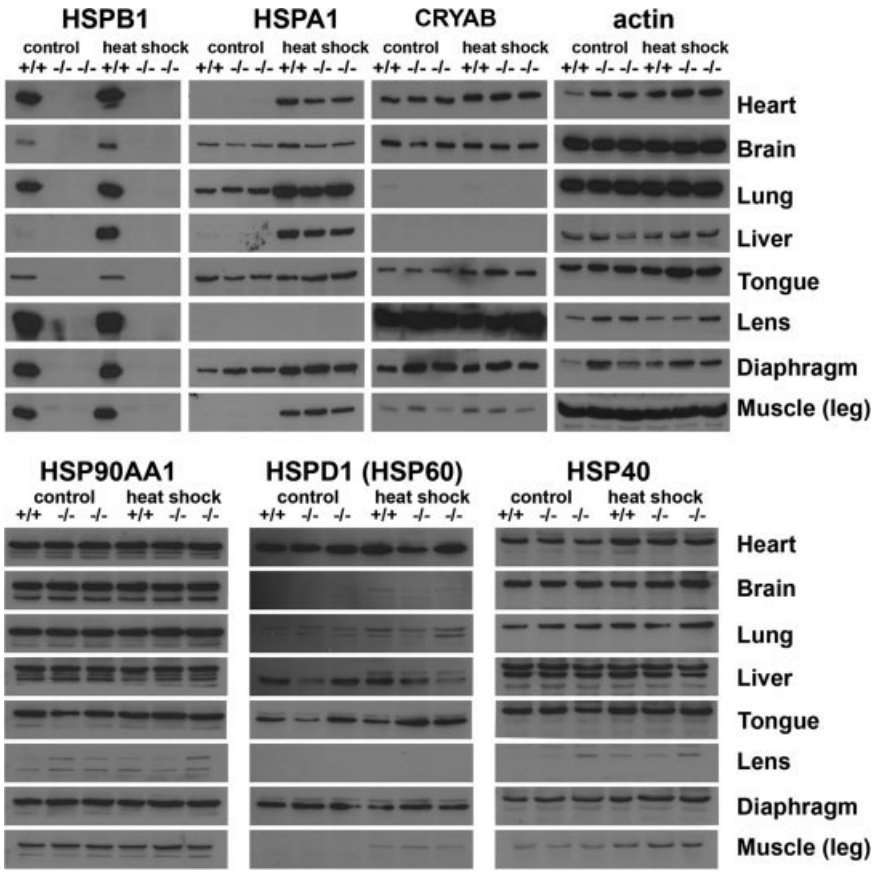

FIG. 7. Tissue-specific HSP induction following whole-body hyperthermic treatment of *hspb1*$^{-/-}$ compared with wild-type adult mice. Tissue-specific constitutive or inducible expression of HSPs in *hspb1*$^{-/-}$ (−/−) mice compared with wild-type B6 mice (+/+). Protein plots showing expression levels of HSPs in tissues (indicated) under normal conditions or following whole-body hyperthermic treatment. Mice were exposed to a mild whole-body thermal stress (42°C for 45 min) and HSP expression was measured 24 h later. As a control for equal protein loading, the blot was probed for actin.

tion against thermal stress was examined with primary murine embryonic fibroblast (MEF) cells or SV40-transformed mouse skin ear fibroblasts (MSF), which differ only in the presence or absence of *hspb1* and/or *hspa1* genes. The growth rate of MSFs from mutant or wild-type mice was similar (data not shown). However, as the data presented in Figure 8A indicate, we observed a substantial reduction in thermotolerance development in *hspb1*$^{-/-}$ MSFs compared with wild-type cells. Extended analyses including *hspa1*$^{-/-}$ or *hspb1*$^{-/-}$ *hspa1*$^{-/-}$ MSFs revealed that HSPA1 and HSPB1 function synergistically in cellular protection against thermal stress. This was most clearly seen for double-mutant (*hspb1*$^{-/-}$ *hspa1*$^{-/-}$) cells, which were very sensitive to apoptotic death following a severe heat challenge (no thermotolerance developed). However, while HSPB1 or HSPA1 protein was not detected in *hspb1*$^{-/-}$ or *hspa1*$^{-/-}$ cells, the level of HSPA1 or HSPB1, respectively, was comparable to that of wild-type MSFs (Fig. 8B). These observations were confirmed using primary MEFs derived from *hspb1*$^{-/-}$ or *hspa1*$^{-/-}$ mice (data not shown).

Next the requirement and extent to which HSPB1 and HSPA1 promote cell survival by inhibiting apoptosis was assessed by examining the cytochrome c/Apaf-1/caspase 9/caspase 3 pathway in wild-type and HSP-deficient MSFs. Western blot analysis confirmed the processing of caspase 9 from the proenzyme (46 kDa) to its active forms (37 and 35 kDa) and an increase in level of cleaved caspase-3 when MSFs preconditioned to heat (43°C for 20 min) were exposed to severe heat shock (45°C for 30 min) (Fig. 8C). However, under conditions of thermotolerance induction, caspase-9 and caspase-3 activation were significantly increased in *hspb1* or *hspa1*-deficient cells compared with wild-type MSFs. This selective increase in caspase-9/3 activity was more dramatic in *hspb1*$^{-/-}$*hspa1*$^{-/-}$ cells that were very heat sensitive (no thermotolerance development). A similar result was observed for poly(ADP-ribose) polymerase (PARP) processing.

The contribution of HSPB1 and HSPA1 in cellular protection against stress was further examined by determining the thermal response of bone marrow (BM) progenitors (CFU-GM) that differ only in the presence or absence of HSPB1 or HSPA1 function. As shown in Figure 9, we observed a substantial reduction in thermotolerance development in HSPB1$^{-/-}$ or HSPA1$^{-/-}$ deficient BM cells compared with wild-type B6 cells. This effect was more pronounced for cells exposed to a severe heat challenge following 24 h of recovery from a relatively mild heat shock. As cells deficient in HSF1 are completely heat sensitive (do not develop thermotolerance) (McMillan *et al.*, 1998; Zhang *et al.*, 2002), the data suggest that coordinate action of both HSPB1 and HSPA1,

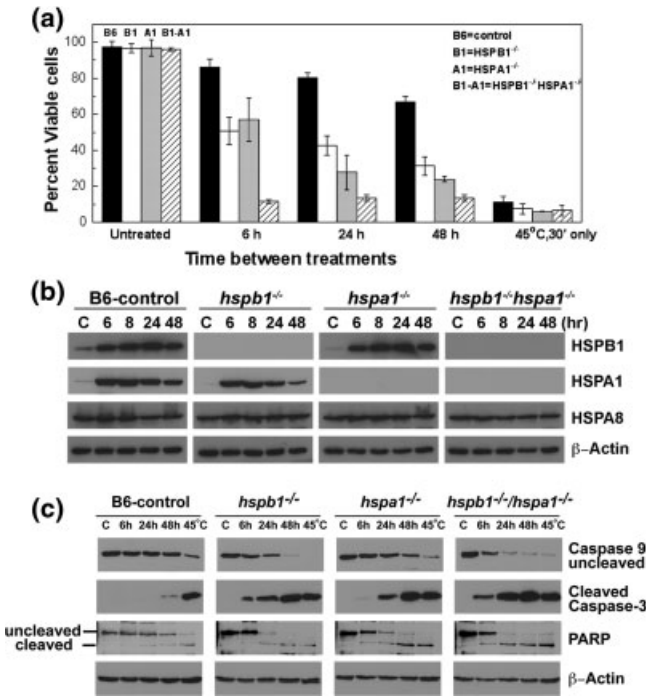

FIG. 8. Expression of *hspb1* and *hspa1* (*hsp70.1* and *hsp70.3*) genes after heat shock is essential for thermotolerance development and maintenance. (**a**) Functional inactivation of *hspb1*, *hspa1* or both *hspb1* and *hspa1* genes results in deficient development and maintenance of acquired thermotolerance. To induce thermotolerance, SV40-transformed mouse MSFs were preconditioned with a relatively mild heat shock (43°C for 20 min) and allowed to recover at 37°C for 6, 24 or 48 h before challenge with a lethal heat shock (45°C for 30 min). Analyses were performed following a further recovery at 37°C for 24 h by staining with Annexin V-FITC and propidium iodide and FACS. Percent cell survival of heat-treated cell populations was calculated based on the staining profile (Annexin and propidium iodide double negative). Control MSFs, black bars; *hspa1*$^{-/-}$ MSFs, open bars; *hspb1*$^{-/-}$ MSFs, grey bars; *hspb1*$^{-/-}$*hspa1*$^{-/-}$ MSFs, hatched bars. Percent cell survival following primary mild heat shock (43°C for 20 min) was >95% for the different MSF populations. (**b**) Exposure of *hspb1*$^{-/-}$, *hspa1*$^{-/-}$ or *hspb1*$^{-/-}$*hspa1*$^{-/-}$ MSFs to sublethal heat shock results in dramatic induction of HSPB1 or HSPA1 expression at comparable levels to that in wild-type MSFs. Cells were exposed to heat (43°C for 20 min) and protein extracted following recovery at 37°C for the indicated time. HSPB1, HSPA1, or HSPA8 (HSC70) protein expression was detected by immunoblotting using specific antibodies. Protein extracted from non-heat-treated cells was used as a control. As a control for equal protein loading the blot was probed for β-actin. (**c**) Inactivation of *hspb1*, *hspa1*, or both *hspb1* and *hspa1* genes increases MSF susceptibility to cell-death through activation of the intrinsic apoptotic pathway. MSFs from wild-type (B6), *hspb1*$^{-/-}$, *hspa1*$^{-/-}$ or *hspb1*$^{-/-}$*hspa1*$^{-/-}$ mice were preconditioned with a relatively mild heat shock (43°C for 20 min) and allowed to recover at 37°C for 6 or 24 h before challenge with a lethal heat shock (45°C for 30 min). Analyses were performed following further recovery at 37°C for 24 h. Procaspase-9, cleaved caspase-3, PARP, and actin as a control for loading were assayed by immunoblotting. Protein extracted from non-heat-treated cells was used as a control (indicated as C). The lane designated 45, 30′ represents protein extracted from cells heated at 45°C for 30 min without preconditioning treatment.

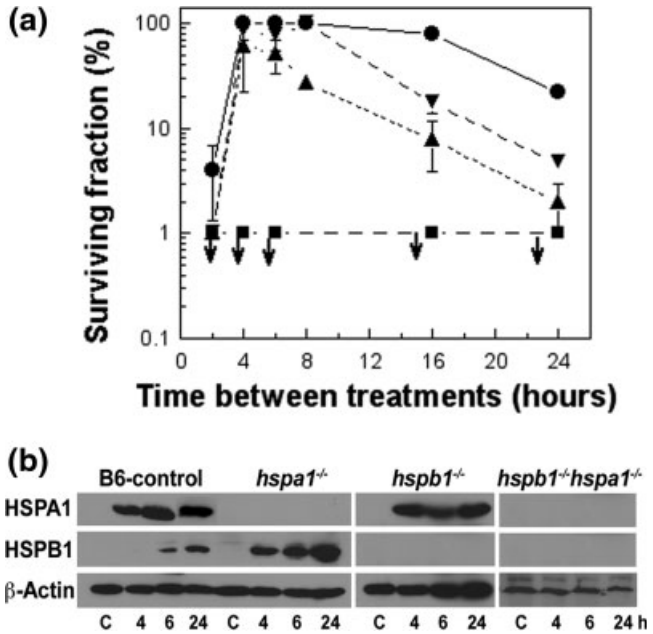

FIG. 9. Induction of HSPB1 and HSPA1 (HSP70.1 and HSP70.3) proteins following thermal stress is essential for thermotolerance induction and maintenance. (**a**) BM progenitors from *hspa1*$^{-/-}$ (double *hsp70.3*$^{-/-}$ and *hsp70.1*$^{-/-}$) (▲), *hspb1*$^{-/-}$ (▼), *hspb1*$^{-/-}$ *hspa1*$^{-/-}$ (■), or wild-type B6 (●) mice were tested for their ability to develop thermotolerance. Cells were preconditioned with a relatively mild heat shock (43°C for 25 min) and allowed to recover at 37°C for 4, 8 or 24 h, before challenge with a lethal heat shock (44°C for 40 min). Cells were subsequently plated for colony formation of granulocytes, macrophages, or a mixture of both granulocytes/macrophages at 37°C for 8 days. The data represent percent survival for each time point calculated as described previously (Huang *et al.*, 2001). Note that BM cells from *hspb1*$^{-/-}$*hspa1*$^{-/-}$ mice do not develop thermotolerance, as revealed by the fact that no colony formation was observed even if a relatively high number of cells (10^6 cells) was plated (indicated by downward arrows). (**b**) Exposure of *hspa1*$^{-/-}$ *or hspb1*$^{-/-}$ BM cells to sublethal heat shock (43°C for 20 min) resulted in dramatic induction of HSPB1 or HSPA1 expression. BM cells derived from *hspa1*$^{-/-}$, *hspb1*$^{-/-}$, *hspb1*$^{-/-}$*hspa1*$^{-/-}$ or wild-type B6 mice were exposed to heat (43°C for 20 min), and protein was extracted following recovery at 37°C for the indicated time. HSPB1 or HSPA1 protein expression was detected by immunoblotting using specific antibodies. Protein extracted from non-heat treated cells was used as a control. As a control for equal protein loading the blot was probed for β-actin.

perhaps with assistance of additional HSPs, is required for cells to become fully resistant to heat challenge. Our results confirmed this notion, showing complete lack of thermotolerance development in double-mutant cells. In addition, exposure of *hspb1*$^{-/-}$ or *hspa1*$^{-/-}$ BM cells resulted in strong induction of HSPA1 or HSPB1 correspondingly, at protein levels comparable to those measured in heat-treated wild-type BM cells (Fig. 9B).

Together, these observations suggest that the cumulative expression of both HSPB1 and HSPA1 proteins after heat shock is essential for maximal levels of thermotolerance development and maintenance, and that lack of either gene product reduces the ability of the cells to maintain a tolerant state. Thus, the function of these mo-

lecular chaperones is additive and is required for the full protection of BM progenitors or MEFs and probably other tissues from stress stimuli.

Sensitivity of Mouse Embryo Fibroblasts Deficient in HSPB1 or HSPA1 to Various Apoptosis-Inducing Agents Other Than Heat Stress

HSPs have been implicated to protect cells against cytotoxic effects of specific drugs. Therefore we investigated the response of cells deficient in HSPB1 or HSPA1 function to treatment with different apoptosis-inducing regimes, including ionizing radiation (X-ray) or etoposide (p53-dependent apoptosis), serum starvation, or treatment with the oxidative stress inducer menadione. In summary, our results did not reveal significant changes in the sensitivity of MSFs or MEFs deficient in HSPB1 or HSPA1 function to these apoptosis-inducing treatments (Supplementary Fig. S5).

DISCUSSION

Significant advances in elucidating biochemical and structural features characteristic to individual HSP family members have been made in recent years. However, our understanding of HSP biology at the organismal level is very limited. Many questions remain about the mode and extent of functional synergy between individual members of the same or different HSP families. For example, the sHSP family, consisting of at least 10 structurally conserved members in mammals, represents a good model for study of this issue, which may lead to a more comprehensive understanding of how HSP chaperones function in vivo. In this context, this study was undertaken to explore the contribution of HSPB1 in mammalian physiology using a genetic approach, which allows function and tissue expression of this molecular chaperone to be studied in parallel under normal and environmental stress conditions. We demonstrated that HSPB1 expression is not ubiquitous but rather is tightly regulated in a tissue-specific manner. Furthermore, its inactivation does not affect mouse development, but the data clearly demonstrate a remarkably functional contribution of both HSPB1 and HSPA1 in cellular protection against thermal stress. This provides further support for the model of functional synergism among diverse HSP chaperones in cell physiology. The model developed based on the data presented here makes three further predictions.

The first is that distinct transcriptional programs are involved in regulating spatial and temporal *hspb1* expression during cardiac and skeletal muscle development, expression in differentiated lens fibers as well as its coexpression with *hspa1* in stratified epithelial layers. The emerging model based in the literature and supported by the data of this study predicts that *hspb1* is likely a target gene for transcription factors regulating late-stage myogenic differentiation, lens fiber formation, and keratinocyte development. Although the transcriptional programs regulating tissue and cell-specific expression patterns of *hspb1* remain unexplored, there is evidence to support that its expression in muscles may involve myogenic differentiation factors. In vertebrates, myogenic differentiation proceeds through a gradual increase in expression of muscle function-specific genes on cell-cycle-arrested precursor cells (myoblasts), leading to fusion of myoblasts into multinucleate myofibers (Bassel-Duby and Olson, 2006; Buckingham, 2001; Kassar-Duchossoy *et al.*, 2004). Muscle-specific gene expression and myogenesis are regulated by combinatorial associations between myogenic basic helix-loop-helix transcriptions factors and MEF2 factors (Molkentin and Olson, 1996). In particular, the first steps in myogenic determination involve expression of Myf5, MRF4, and MyoD, which subsequently leads to expression of myogenin and MEF2 isoforms that promote conversion of myoblasts to myotubes. Interestingly, a recent study identified hspb1 as a transcriptional target of MEF2 (Blais *et al.*, 2005). It is therefore tempting to speculate that MEF2 factors, in conjunction with MyoD and other myogenic regulatory factors, may be involved to up-regulate *hspb1* during heart development and in differentiated skeletal muscles. Indeed, expression of MEF2 factors, (all isoforms are detected in the heart at E8.5 and later in somites) are restricted to the regions of skeletal and cardiac muscle differentiation and overlap with the pattern of *hspb1* expression during development, as described in this study (Subramanian and Nadal-Ginard, 1996). In addition, constitutive *hspb1* expression under normal conditions in adult is largely confined to muscles with high mitochondrial activity (SDH-positive) and possibly involves signaling pathways including MEF2/histone deacetylase (HDAC) signaling, which plays a role in the transformation of myofibers in response to intracellular calcium changes, an effect of external physiological signals (Chin *et al.*, 1998). Next, the MEF2/HDAC and calcineurin pathways induced by exercise may participate in the expression of proteins associated with oxidative stress, including HSPs such as HSPB1. In contrast, *hspb1* expression in differentiated ocular lens fibers is tightly regulated by HSF4, whose expression in ocular lens detected during the early postnatal period (P2–P5) parallel HSPB1 protein synthesis in the ocular lens (Min *et al.*, 2004). Monitoring *hspb1* expression in lens using *hsf4*$^{-/-}$*hspb1*$^{+/-}$*-lacZ* reporter mice, we have confirmed that ablation of HSF4 activity completely eliminated HSPB1 expression in the lens (data not shown). Finally, the remarkable overlapping expression pattern of *hsbp1* and *hspa1* in the stratified epithelial layers suggest the existence of common transcriptional regulatory programs stimulating expression of these chaperones. Although we do not know the identity of transcription factors involved in this process, the physiological relevance and implication of HSPB1 and HSPA1 coexpression seem to be more apparent. For example, considering that the majority of human cancers are of epithelial origin, the constitutive expression of HSPB1 and HSPA1, which can promote tumor cell survival exhibiting anti-apoptotic properties, may represent a major hallmark in

epithelial cancer development and perhaps are a valid target for treatment of this and other diseases involving such tissues.

The second prediction is that loss of HSPB1 function seems not to have apparent consequences on normal development and ontogeny or tissue physiology. This is surprising, given the functional versatility ascribed to HSPB1, which is one of the best characterized members of the sHSP family. In particular, expression of HSB1 in mouse embryo during the preimplantation stage led to the proposal that its function was essential for development, an assumption not supported by our data (Kim *et al.*, 2002). In addition, according to human genetic studies showing the existence of mutations in the *hspb1*-featured motor neural diseases, it was predicted that the *hspb1*$^{-/-}$ mice may develop neuropathy. Although this interesting aspect is under further investigation, morphological examination of the mutant mice over a period of 18 months did not indicate signs of neurological disease. However, this finding does not necessarily diminish the importance of HSBP1 mutations in neuronal diseases. Furthermore, the lack of *hspb1* expression under physiological stress conditions in the brain does not exclude the possibility that its expression is induced in neurodegenerative diseases where it plays a role in ameliorating the disease pattern. One plausible explanation for this functional redundancy could be the unusual complexity in the sHSP system, in which individual members of the sHSP family may act in concert as well as display distinct functions in tissues expressing these chaperone molecules. For example, in addition to HSPB1, several other sHSPs (HSPB2, HSPB3, CRYAB, HSPB6, HSPB7, and HSPB8) are abundant in cardiac or skeletal muscle (Sun *et al.*, 2004; Verschuure *et al.*, 2003). In such cells, evidence was provided that heterodimers and hetero-oligomers of sHSPs may form (e.g., HSPB1, HSPB6, and CRYAB form one type of complex, while HSPB2 and HSPB3 form a different type) (Sugiyama *et al.*, 2000) and fulfill specific and perhaps overlapping functions. Thus, the functional consequences of HSPB1 ablation could easily be compensated by formation of structural units of other interacting sHSPs. However, it is possible that the high level of chaperones in muscle tissue is needed to prevent cellular damage during stress conditions or may play an important function during contractile activity. Functional analysis of HSPB1-deficient muscles under stress conditions (exercise) will be informative, and such studies are underway in our laboratory. Another avenue to further evaluate the combinatorial functions of sHSPs both under normal and stress conditions is to perform comparable analyses on mouse lines where multiple sHSPs are eliminated. Indeed, crossing *hspb1*$^{-/-}$ mice with double *hspb2*$^{-/-}$*cryab*$^{-/-}$ mice (Brady *et al.*, 2001), we generated mice deficient in three major sHSPs abundant in muscle. Our findings that the deletion of three sHSP has no apparent effects on embryo development although the mice died early in adult developing severe muscle degeneration (not seen in *hspb1*$^{-/-}$ mice or developing much later at 7–9 months in *hspb2*$^{-/-}$*cryab*$^{-/-}$ mice) support our hypothesis that indeed *hspb1* acting together with other sHSPs is required to sustain at least cardiac and skeletal muscle function under physiological stress conditions. The key question whether HSPB1 has a different role compared with HSPB2 and CRYAB in disease progression, or alternatively, the observed effects reflect the high abundant of HSPB1 in muscle, remains elusive. Likewise the possible effects of combined sHSP deletion on neuronal function and development of neuropathies, the diseases caused by mutations in human sHSP, requires further investigation. These studies with the mutant mice are expected to produce insightful information for the proposed functional convergence between these sHSPs in vivo.

Finally, the demonstration that functional cooperation is required between HSPA1 and HSPB1 molecules with common functional but also distinct structural features for an efficient cellular response to severe environmental stress stimuli is a remarkable property of the cellular molecular chaperone machinery. A possible scenario to explain such functional cooperation is based on the cardinal function of these HSPs in inhibiting components of both stress and death-receptor-induced apoptotic pathways through their anti-apoptotic effects involving distinct molecular mechanisms (Beere *et al.*, 2000; Concannon *et al.*, 2003; Garrido *et al.*, 2001; Paul *et al.*, 2002; Sreedhar and Csermely, 2004). It is well known that stressful conditions, including heat shock, may lead to cell death by apoptosis and necrosis. While little is known about the mechanisms of stress-induced reproductive death by necrosis, the initial damage in apoptosis does not directly kill the cell but initiates specific signaling pathways that lead to "cellular suicide." In this regard, there is compelling evidence that resistance to stress by cells primed with a mild heat shock that induces HSPs (especially HSPA1 and HSPB1) may be due to down-regulation of the signaling events that initiate apoptosis. This scenario does not exclude the possibility that additional functional features of HSPB1 in mediating protection against stress occur by stabilizing the cytoskeleton or by facilitating repair or removal of damaged proteins. Consequently, these proteins are regarded as complementary protective proteins and our results support this view.

In summary, our study in conjunction with published data on the phenotype of double-mutant HSPB2/CRYAB-deficient mice (Brady *et al.*, 2001) demonstrate that there is functional cooperation among sHSPs and other HSPs in sustaining normal cell and tissue physiology. The availability of *hspb1*-deficient mice provides an opportunity to further explore the extent to which HSPB1 activity can be compensated for by members of the small and other HSP families, or whether HSPB1 has a unique function in cellular protection from environmental stress. Thus, these mutant mice provide a valuable experimental model to achieve a better understanding of the fundamental cellular processes, in which sHSP chaperones engage in the response to environmental

stresses, as well as to determine their role in clinically relevant pathologies in humans.

EXPERIMENTAL PROCEDURES

Generation of Mice With Targeted Disruption of *hspb1*

The *hspb1* targeting vector was constructed from one 15-kb fragment of genomic DNA isolated from a 129/SvJ mouse genomic DNA phage library (Lambda fixII Library; Strategene, La Jolla, CA) using mouse *hspb1* cDNA as a probe (Frohli *et al.*, 1993). Approximately 0.6 kb of genomic DNA downstream of the start codon, including the first exon and 0.3 kb of the first intron, was replaced by a 3.5-kb fragment containing the *lacZ* coding sequence with the bovine growth hormone poly(A) signal and a 1.2-kb neomycin resistance (*neo*) gene cassette flanked by *loxP* sites to enable its later removal by Cre-recombinase. The targeting vector containing 5.4 kb of 5′ flanking (left arm) and 2.7 kb of the 3′ flanking (right arm) sequence homology flanked by thymidine kinase genes for positive/negative selection of embryonic stem (ES) cells, was linearized at the unique SalI restriction for transfection into B6-ES cells. Genomic Southern blotting of 142 G418/FIAU resistant colonies yielded 11 positive clones. Two clones with correct targeting were microinjected into BALB/c blastocysts, resulting in germline-transmitting chimeric mice (Medical College of Georgia Mouse Embryonic Stem Cell and Transgenesis Facility). The resulting chimeric mice were crossed with C57BL/6 (B6) females. Mice with germ-line transmission of the targeted mutant allele were detected by Southern blotting analysis of tail DNA digested with EcoRI (R) and hybridized with a probe external to the targeting vector, which yields fragments of 7.2 kb for wild type and 5.7 kb for targeted alleles, respectively, or by PCR-based genotyping, amplifying wild type and targeted *hspb1* fragments of 315 and 645 bp, respectively, using primers P1 (CAG GAT GAT CTG GAC GAA GAG), P2 (CTG AGA TTT GGG ATG CGA AG), and P3 (GGA GCC CGC ATC ATT CTC).

Histology, Immunohistochemistry, and β-Galactosidase Staining

Embryos recovered at early and middle embryonic stages (E8 to E13.5) were whole-mount stained for β-galactosidase (β-gal) activity with X-gal (Molecular Probes, Eugene, OR), paraffin-embedded, and sectioned. Other embryos at later embryonic stages, or newborns or tissues harvested from adult mice were embedded in Optimal Cutting Temperature compound (OCT, Tissue Tek), snap-frozen in a liquid nitrogen 2-methyl-butane bath, sectioned, air-dried, and fixed in 0.2% glutaraldehyde in PBS (pH = 7.3) with 2 mM MgCl2 for 10 min. Sections were stained with X-gal, counter-stained with hematoxylin and eosin, and subjected to gross and microscopic pathologic analysis.

For immunofluorescence microscopy, snap-frozen tissues were sectioned and fixed with acetone at −20°C. Sections were incubated with monoclonal antibody specific to embryonic muscle myosin heavy chain (MHCd; clone RNMy2/9D2; Novocastra; MA) or specific to desmin (clone DE-U-10; Sigma, St. Louis, MO) for 1 h at room temperature (20°C), and antigen–antibody complexes were detected with FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) as specified.

Histochemical determination of $Ca2^{+}$-dependent myofibrillar (myosin heavy chain) adenosine triphosphatase (mATPase) and SDH activity were performed on unfixed 10 μm cryostat sections following the modified procedures described previously (Muller and Vogell, 1974; Nolte and Pette, 1972; Quiroz-Rothe and Rivero, 2001). For mATPase staining, sections were incubated at 37°C in acetic acid buffer solution (pH = 4.3 or pH = 4.6) containing 1.8 mM $CaCl_2$ for 5 min or in 2-amino 2-methyl 1-propanol (AMP) buffer solution (pH = 10.4; 36 mM $CaCl_2$) for 14 min. The sections were then rinsed with AMP buffer (pH = 9.4; 18 mM $CaCl_2$) and further incubated at 37°C for 30 min (acid preincubation at pH = 4.3 or pH = 4.6) or for 14 min (alkaline preincubation at pH = 10.4) in 0.15% ATP buffer solution. Sections were then washed with 1% $CaCl_2$ (three-times) and 2% $CaCl_2$ (once), and enzymatic activity was visualized by applying a 3% ammonium sulfide solution. For SDH activity, staining cryostat sections were incubated at 37°C for 30 min with 50 mM sodium succinate/PBS buffer containing 0.5 mg/ml Nitroblue Tetrazolium.

Western Blot Analysis for HSP-Protein Expression

Whole-cell extracts (35 μg of total protein) were subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter (Bio-Rad, Hercules, CA). Immunodetection using the enhanced chemiluminescence (ECL) method (ECL kit; Amersham, Piscataway, NJ) was performed according to the manufacturer's instructions. The membrane was probed first with appropriate primary antibody and horseradish peroxidase (HPR)-conjugated secondary antibody and visualized as described previously (Huang *et al.*, 2001). Immunodetection was performed with antibodies specific to HSPB1 (HSP25; #SPA-801), CRYAB (#SPA-223), HSP40 (#SPA-400), HSP90AA1 (HSP90a; #SPA-771), HSP110 (#SPA-1103), mouse monoclonal antibody specific to HSPD1 (HSP60; clone LK-1/#SPA-806), HSPA1A/A1B (HSP70; clone C92F3A-5/#SPA-810), or rat monoclonal antibody specific to HSPA8 (HSC70; clone 1B5/#SPA-815) and were purchased from Stressgen (Ann Harbor, MI). As a control for equal protein loading, the blot was probed for β-actin with specific rabbit polyclonal antibody (Sigma).

RT-PCR

RNA preparation and cDNA synthesis were as described previously (Min *et al.*, 2004). One twentieth of this reaction was used as template for PCR amplifica-

tion using the primers listed in Supplementary Table 1. As a control, mouse HPRT (hypoxanthine phosphoribosyltransferase) transcripts were detected.

Whole-Body Hyperthermic Challenge

Wild-type or *hspb1*$^{-/-}$ mice were semi-immersed in a circulating water bath at 42°C for 45 min and left to recover for 6–8 h before euthanization. Tissues were prepared for histological examination as described earlier. Untreated mice were used as controls.

Thermal Response and Development of Thermotolerance in BM

BM cells derived from *hspa1*$^{-/-}$ (double *hsp70.1*$^{-/-}$/*3*$^{-/-}$) (Hunt *et al.*, 2004), *hspb1*$^{-/-}$ (*hsp25*$^{-/-}$), *hspa1a*$^{-/-}$-*hspb1*$^{-/-}$ (*hsp70.1*$^{-/-}$/*3*$^{-/-}$ and *hsp25*$^{-/-}$) or wild-type B6 mice were tested for their ability to develop thermotolerance (Mivechi and Li, 1986). Cells were preconditioned with a relatively mild heat challenge (43°C for 25 min) and challenge with more severe (lethal for untreated cells) heat (44°C for 40 min) at the times indicated during the recovery period at 37°C. Cells were subsequently plated and incubated at 37°C in 5% CO_2 for 8 days. Colonies of granulocytes, macrophages, or a mixture of granulocytes and macrophages were counted microscopically. The colony forming efficiency of untreated CFU granulocytes/macrophages (CFU-GM) was $\sim 2/10^3$ nucleated cells. Cells were plated at various concentrations ($1 \times 10^5 - 100 \times 10^5$ cells per dish), depending on the given treatment. The percentage survival was calculated by the following formula: percent survival = [(number of colonies after severe heat challenge/number of cells plated)/(number of colonies after primary heat/number of cells plated)] × 100 as described previously (Huang *et al.*, 2001).

Apoptosis and Survival of Mouse Fibroblasts Deficient in Expression of hspb1, hspa1, or Both hspb1 and hspa1 Following Thermal Challenge

Mouse embryonic fibroblasts (MEFs) were prepared from Day 13–14 embryos and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. SV40-transformed murine skin fibroblasts (MSFs) were generated from ears and used for heat-shock experiments as indicated. To induce thermotolerance, cells were preconditioned with a relatively mild heat shock (43°C for 20 min) and allowed to recover at 37°C for 24 h before challenge with a lethal heat shock (45°C for 30 min). Analyses were performed following further recovery at 37°C for 24 h. Cell viability and the level of apoptosis after heat challenge were measured by staining with annexin V-FITC and propidium iodide (Apoptosis Detection Kit, R & D systems, Minneapolis, MN), according to the manufacturer's instructions, and analyzed using a FACS-calibur cytometer (Becton Dickinson).

Measurement of Caspase Activity

Apoptotic caspase activity was assayed by immunoblotting of total cell lysates (50 μg of protein) either with cleaved caspase-3 (Asp175) monoclonal antibody (Cell Signaling, Beverly, MA) or with antibody specific to caspase-9 (AAP; StressGen, Victoria, BC, Canada) or antibody (H-250; Santa Cruz Biotech, Santa Cruz, CA) to one of the caspase substrates, PARP.

Measurement of Fiber Diameter

Quantitation of myofiber diameter was calculated by measuring the narrowest diameter of the fiber with the ruler function of Adobe Photoshop software CS2. Approximately 100 fibers from two 8-week-old mice of each genotype were analyzed.

GSH Assay

Total glutathione (GSH/GSSG) was measured using the DTNB-GSSG Reductase recycling assay (Anderson, 1985) with minor adaptation for 96-well plate readers.

ACKNOWLEDGMENTS

We thank Dr. Graeme Price for helpful discussions and suggestions and Monica Meyer for expert technical assistance.

LITERATURE CITED

Abdelwahid E, Eriksson M, Pelliniemi LJ, Jokinen E. 2001. Heat shock proteins, HSP25 and HSP70, and apoptosis in developing endocardial cushion of the mouse heart. Histochem Cell Biol 115:95–104.

Ackerley S, James PA, Kalli A, French S, Davies KE, Talbot K. 2006. A mutation in the small heat-shock protein HSPB1 leading to distal hereditary motor neuronopathy disrupts neurofilament assembly and the axonal transport of specific cellular cargoes. Hum Mol Genet 15:347–354.

Agashe VR, Hartl FU. 2000. Roles of molecular chaperones in cytoplasmic protein folding. Semin Cell Dev Biol 11:15–25.

Anderson ME. 1985. Determination of glutathione and glutathione disulfide in biological samples. Methods Enzymol 113:548–555.

Augusteyn RC. 2004. α-Crystallin: A review of its structure and function. Clin Exp Optom 87:356–366.

Barral JM, Broadley SA, Schaffar G, Hartl FU. 2004. Roles of molecular chaperones in protein misfolding diseases. Semin Cell Dev Biol 15:17–29.

Bassel-Duby R, Olson EN. 2006. Signaling pathways in skeletal muscle remodeling. Annu Rev Biochem 75:19–37.

Beere HM, Wolf BB, Cain K, Mosser DD, Mahboubi A, Kuwana T, Tailor P, Morimoto RI, Cohen GM, Green DR. 2000. Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. Nat Cell Biol 2:469–475.

Benjamin IJ, McMillan DR. 1998. Stress (heat shock) proteins: Molecular chaperones in cardiovascular biology and disease. Circ Res 83:117–132.

Benndorf R, Welsh MJ. 2004. Shocking degeneration. Nat Genet 36: 547–548.

Blais A, Tsikitis M, Acosta-Alvear D, Sharan R, Kluger Y, Dynlacht BD. 2005. An initial blueprint for myogenic differentiation. Genes Dev 19:553–569.

Brady JP, Garland DL, Green DE, Tamm ER, Giblin FJ, Wawrousek EF. 2001. αB-crystallin in lens development and muscle integrity: A gene knockout approach. Invest Ophthalmol Vis Sci 42:2924–2934.

Bruey JM, Ducasse C, Bonniaud P, Ravagnan L, Susin SA, Diaz-Latoud C, Gurbuxani S, Arrigo AP, Kroemer G, Solary E, Garrido C. 2000. Hsp27 negatively regulates cell death by interacting with cytochrome c. Nat Cell Biol 2:645–652.

Buckingham M. 2001. Skeletal muscle formation in vertebrates. Curr Opin Genet Dev 11:440–448.

Bukau B, Weissman J, Horwich A. 2006. Molecular chaperones and protein quality control. Cell 125:443–451.

Calderwood SK, Khaleque MA, Sawyer DB, Ciocca DR. 2006. Heat shock proteins in cancer: Chaperones of tumorigenesis. Trends Biochem Sci 31:164–172.

Chavez Zobel AT, Loranger A, Marceau N, Theriault JR, Lambert H, Landry J. 2003. Distinct chaperone mechanisms can delay the formation of aggresomes by the myopathy-causing R120G αB-crystallin mutant. Hum Mol Genet 12:1609–1620.

Chen S, Brown IR. 2007. Neuronal expression of constitutive heat shock proteins: Implications for neurodegenerative diseases. Cell Stress Chaperones 12:51–58.

Chin ER, Olson EN, Richardson JA, Yang Q, Humphries C, Shelton JM, Wu H, Zhu W, Bassel-Duby R, Williams RS. 1998. A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. Genes Dev 12:2499–2509.

Cohen FE, Kelly JW. 2003. Therapeutic approaches to protein-misfolding diseases. Nature 426:905–909.

Concannon CG, Gorman AM, Samali A. 2003. On the role of Hsp27 in regulating apoptosis. Apoptosis 8:61–70.

Dalle-Donne I, Rossi R, Milzani A, Di Simplicio P, Colombo R. 2001. The actin cytoskeleton response to oxidants: From small heat shock protein phosphorylation to changes in the redox state of actin itself. Free Radic Biol Med 31:1624–1632.

Didelot C, Schmitt E, Brunet M, Maingret L, Parcellier A, Garrido C. 2006. Heat shock proteins: Endogenous modulators of apoptotic cell death. Handb Exp Pharmacol 2006:171–198.

Dorion S, Landry J. 2002. Activation of the mitogen-activated protein kinase pathways by heat shock. Cell Stress Chaperones 7:200–206.

Duverger O, Morange M. 2005. Heat shock protein 25 plays multiple roles during mouse skin development. Cell Stress Chaperones 10:268–277.

Escobedo J, Pucci AM, Koh TJ. 2004. HSP25 protects skeletal muscle cells against oxidative stress. Free Radic Biol Med 37:1455–1462.

Evgrafov OV, Mersiyanova I, Irobi J, Van Den Bosch L, Dierick I, Leung CL, Schagina O, Verpoorten N, Van Impe K, Fedotov V, Dadali E, Auer-Grumbach M, Windpassinger C, Wagner K, Mitrovic Z, Hilton-Jones D, Talbot K, Martin JJ, Vasserman N, Tverskaya S, Polyakov A, Liem RK, Gettemans J, Robberecht W, De Jonghe P, Timmerman V. 2004. Mutant small heat-shock protein 27 causes axonal Charcot-Marie-Tooth disease and distal hereditary motor neuropathy. Nat Genet 36:602–606.

Feder ME, Hofmann GE. 1999. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. Annu Rev Physiol 61:243–282.

Fontaine JM, Sun X, Benndorf R, Welsh MJ. 2005. Interactions of HSP22 (HSPB8) with HSP20, αB-crystallin, and HSPB3. Biochem Biophys Res Commun 337:1006–1011.

Frohli E, Aoyama A, Klemenz R. 1993. Cloning of the mouse hsp25 gene and an extremely conserved hsp25 pseudogene. Gene 128:273–277.

Gaestel M. 2002. sHsp-phosphorylation: Enzymes, signaling pathways and functional implications. Prog Mol Subcell Biol 28:151–169.

Ganea E. 2001. Chaperone-like activity of α-crystallin and other small heat shock proteins. Curr Protein Pept Sci 2:205–225.

Garrido C, Gurbuxani S, Ravagnan L, Kroemer G. 2001. Heat shock proteins: Endogenous modulators of apoptotic cell death. Biochem Biophys Res Commun 286:433–442.

Gerner EW, Schneider MJ. 1975. Induced thermal resistance in HeLa cells. Nature 256:500–502.

Gerthoffer WT, Gunst SJ. 2001. Invited review: Focal adhesion and small heat shock proteins in the regulation of actin remodeling and contractility in smooth muscle. J Appl Physiol 91:963–972.

Guay J, Lambert H, Gingras-Breton G, Lavoie JN, Huot J, Landry J. 1997. Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. J Cell Sci 110 (Part 3):357–368.

Haslbeck M. 2002. sHsps and their role in the chaperone network. Cell Mol Life Sci 59:1649–1657.

Haslbeck M, Franzmann T, Weinfurtner D, Buchner J. 2005. Some like it hot: The structure and function of small heat-shock proteins. Nat Struct Mol Biol 12:842–846.

Hildebrandt B, Wust P, Ahlers O, Dieing A, Sreenivasa G, Kerner T, Felix R, Riess H. 2002. The cellular and molecular basis of hyperthermia. Crit Rev Oncol Hematol 43:33–56.

Huang L, Mivechi NF, Moskophidis D. 2001. Insights into regulation and function of the major stress-induced hsp70 molecular chaperone in vivo: Analysis of mice with targeted gene disruption of the hsp70.1 or hsp70.3 gene. Mol Cell Biol 21:8575–8591.

Hunt CR, Dix DJ, Sharma GG, Pandita RK, Gupta A, Funk M, Pandita TK. 2004. Genomic instability and enhanced radiosensitivity in Hsp70.1- and Hsp70.3-deficient mice. Mol Cell Biol 24:899–911.

Irobi J, Van Impe K, Seeman P, Jordanova A, Dierick I, Verpoorten N, Michalik A, De Vriendt E, Jacobs A, Van Gerwen V, Vennekens K, Mazanec R, Tournev I, Hilton-Jones D, Talbot K, Kremensky I, Van Den Bosch L, Robberecht W, Van Vandekerckhove J, Broeckhoven C, Gettemans J, De Jonghe P, Timmerman V. 2004. Hot-spot residue in small heat-shock protein 22 causes distal motor neuropathy. Nat Genet 36:597–601.

Jolly C, Morimoto RI. 2000. Role of the heat shock response and molecular chaperones in oncogenesis and cell death. J Natl Cancer Inst 92:1564–1572.

Kampinga HH, Brunsting JF, Stege GJ, Konings AW, Landry J. 1994. Cells overexpressing Hsp27 show accelerated recovery from heat-induced nuclear protein aggregation. Biochem Biophys Res Commun 204:1170–1177.

Kassar-Duchossoy L, Gayraud-Morel B, Gomes D, Rocancourt D, Buckingham M, Shinin V, Tajbakhsh S. 2004. Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. Nature 431:466–471.

Kim KK, Kim R, Kim SH. 1998. Crystal structure of a small heat-shock protein. Nature 394:595–599.

Kim M, Geum D, Khang I, Park YM, Kang BM, Lee KA, Kim K. 2002. Expression pattern of HSP25 in mouse preimplantation embryo: Heat shock responses during oocyte maturation. Mol Reprod Dev 61:3–13.

Kregel KC. 2002. Heat shock proteins: Modifying factors in physiological stress responses and acquired thermotolerance. J Appl Physiol 92:2177–2186.

Landry J, Huot J. 1999. Regulation of actin dynamics by stress-activated protein kinase 2 (SAPK2)-dependent phosphorylation of heat-shock protein of 27 kDa (Hsp27). Biochem Soc Symp 64:79–89.

Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, Woodard S, Lin LZ, Cai CL, Lu MM, Reth M, Platoshyn O, Yuan JX, Evans S, Chien KR. 2005. Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 433:647–653.

Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A, Boe AF, Boguski MS, Brockway KS, Byrnes EJ, Chen L, Chen L, Chen TM, Chin MC, Chong J, Crook BE, Czaplinska A, Dang CN, Datta S, Dee NR, Desaki AL, Desta T, Diep E, Dolbeare TA, Donelan MJ, Dong HW, Dougherty JG, Duncan BJ, Ebbert AJ, Eichele G, Estin LK, Faber C, Facer BA, Fields R, Fischer SR, Fliss TP, Frensley C, Gates SN, Glattfelder KJ, Halverson KR, Hart MR, Hohmann JG, Howell MP, Jeung DP, Johnson RA, Karr PT, Kawal R, Kidney JM, Knapik RH, Kuan CL, Lake JH, Laramee AR, Larsen KD, Lau C, Lemon TA, Liang AJ, Liu Y, Luong LT, Michaels J, Morgan JJ, Morgan RJ, Mortrud MT, Mosqueda NF, Ng LL, Ng R, Orta GJ, Overly CC, Pak TH, Parry SE, Pathak SD, Pearson OC, Puchalski RB, Riley ZL, Rockett HR, Rowland SA, Royall JJ, Ruiz MJ, Sarno NR, Schaffnit K, Shapovalova NV, Sivisay T, Slaughterbeck CR, Smith SC, Smith KA, Smith BI, Sodt AJ, Stewart NN, Stumpf KR, Sunkin SM, Sutram M, Tam A, Teemer CD, Thaller C, Thompson CL, Varnam LR, Visel A, Whitlock RM, Wohnoutka PE, Wolkey CK, Wong VY, Wood M, Yaylaoglu MB, Young RC, Youngstrom BL, Yuan XF, Zhang B, Zwingman TA, Jones AR. 2007. Genome-wide atlas of gene expression in the adult mouse brain. Nature 445:168–176.

Lindquist S, Craig EA. 1988. The heat-shock proteins. Annu Rev Genet 22:631–677.

Loones MT, Chang Y, Morange M. 2000. The distribution of heat shock proteins in the nervous system of the unstressed mouse embryo suggests a role in neuronal and non-neuronal differentiation. Cell Stress Chaperones 5:291–305.

Macario AJ, Conway de Macario E. 2005. Sick chaperones, cellular stress, and disease. N Engl J Med 353:1489–1501.

MacRae TH. 2000. Structure and function of small heat shock/α-crystallin proteins: Established concepts and emerging ideas. Cell Mol Life Sci 57:899–913.

McMillan DR, Xiao X, Shao L, Graves K, Benjamin IJ. 1998. Targeted disruption of heat shock transcription factor 1 abolishes thermotolerance and protection against heat-inducible apoptosis. J Biol Chem 273:7523–7528.

Min JN, Zhang Y, Moskophidis D, Mivechi NF. 2004. Unique contribution of heat shock transcription factor 4 in ocular lens development and fiber cell differentiation. Genesis 40:205–217.

Mivechi NF, Li GC. 1986. Lack of development of thermotolerance in early progenitors of murine bone marrow cells. Cancer Res 46:198–202.

Molkentin JD, Olson EN. 1996. Combinatorial control of muscle development by basic helix-loop-helix and MADS-box transcription factors. Proc Natl Acad Sci USA 93:9366–9373.

Moseley PL. 1997. Heat shock proteins and heat adaptation of the whole organism. J Appl Physiol 83:1413–1417.

Mosser DD, Morimoto RI. 2004. Molecular chaperones and the stress of oncogenesis. Oncogene 23:2907–2918.

Muller W, Vogell L. 1974. Temporal progress of muscle adaptation to endurance training in hind limb muscles of young rats. A histochemical and morphometrical study. Cell Tissue Res 156:61–87.

Nolte J, Pette D. 1972. Microphotometric determination of enzyme activity in single cells in cryostat sections. I. Application of the gel film technique to microphotometry and studies on the intralobular distribution of succinate dehydrogenase and lactate dehydrogenase activities in rat liver. J Histochem Cytochem 20:567–576.

Paul C, Manero F, Gonin S, Kretz-Remy C, Virot S, Arrigo AP. 2002. Hsp27 as a negative regulator of cytochrome C release. Mol Cell Biol 22:816–834.

Queitsch C, Sangster TA, Lindquist S. 2002. Hsp90 as a capacitor of phenotypic variation. Nature 417:618–624.

Quiroz-Rothe E, Rivero JL. 2001. Co-ordinated expression of contractile and non-contractile features of control equine muscle fibre types characterised by immunostaining of myosin heavy chains. Histochem Cell Biol 116:299–312.

Rane MJ, Pan Y, Singh S, Powell DW, Wu R, Cummins T, Chen Q, McLeish KR, Klein JB. 2003. Heat shock protein 27 controls apoptosis by regulating Akt activation. J Biol Chem 278:27828–27835.

Rouse J, Cohen P, Trigon S, Morange M, Alonso-Llamazares A, Zamanillo D, Hunt T, Nebreda AR. 1994. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell 78:1027–1037.

Rutherford SL, Lindquist S. 1998. Hsp90 as a capacitor for morphological evolution. Nature 396:336–342.

Sangster TA, Lindquist S, Queitsch C. 2004. Under cover: Causes, effects and implications of Hsp90-mediated genetic capacitance. Bioessays 26:348–362.

Smith DF, Whitesell L, Katsanis E. 1998. Molecular chaperones: Biology and prospects for pharmacological intervention. Pharmacol Rev 50:493–514.

Sollars V, Lu X, Xiao L, Wang X, Garfinkel MD, Ruden DM. 2003. Evidence for an epigenetic mechanism by which Hsp90 acts as a capacitor for morphological evolution. Nat Genet 33:70–74.

Sreedhar AS, Csermely P. 2004. Heat shock proteins in the regulation of apoptosis: New strategies in tumor therapy: A comprehensive review. Pharmacol Ther 101:227–257.

Subramanian SV, Nadal-Ginard B. 1996. Early expression of the different isoforms of the myocyte enhancer factor-2 (MEF2) protein in myogenic as well as non-myogenic cell lineages during mouse embryogenesis. Mech Dev 57:103–112.

Sugiyama Y, Suzuki A, Kishikawa M, Akutsu R, Hirose T, Waye MM, Tsui SK, Yoshida S, Ohno S. 2000. Muscle develops a specific form of small heat shock protein complex composed of MKBP/HSPB2 and HSPB3 during myogenic differentiation. J Biol Chem 275:1095–1104.

Sun X, Fontaine JM, Rest JS, Shelden EA, Welsh MJ, Benndorf R. 2004. Interaction of human HSP22 (HSPB8) with other small heat shock proteins. J Biol Chem 279:2394–2402.

Sun Y, MacRae TH. 2005a. Small heat shock proteins: Molecular structure and chaperone function. Cell Mol Life Sci 62:2460–2476.

Sun Y, MacRae TH. 2005b. The small heat shock proteins and their role in human disease. FEBS J 272:2613–2627.

Taylor RP, Benjamin IJ. 2005. Small heat shock proteins: A new classification scheme in mammals. J Mol Cell Cardiol 38:433–444.

Verschuure P, Tatard C, Boelens WC, Grongnet JF, David JC. 2003. Expression of small heat shock proteins HspB2, HspB8, Hsp20 and cvHsp in different tissues of the perinatal developing pig. Eur J Cell Biol 82:523–530.

Vicart P, Caron A, Guicheney P, Li Z, Prevost MC, Faure A, Chateau D, Chapon F, Tome F, Dupret JM, Paulin D, Fardeau M. 1998. A missense mutation in the αB-crystallin chaperone gene causes a desmin-related myopathy. Nat Genet 20:92–95.

Wakayama T, Iseki S. 1998. Expression and cellular localization of the mRNA for the 25-kDa heat-shock protein in the mouse. Cell Biol Int 22:295–304.

Xiao X, Zuo X, Davis AA, McMillan DR, Curry BB, Richardson JA, Benjamin IJ. 1999. HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. EMBO J 18:5943–5952.

Yan LJ, Rajasekaran NS, Sathyanarayanan S, Benjamin IJ. 2005. Mouse HSF1 disruption perturbs redox state and increases mitochondrial oxidative stress in kidney. Antioxid Redox Signal 7:465–471.

Zhang Y, Huang L, Zhang J, Moskophidis D, Mivechi NF. 2002. Targeted disruption of hsf1 leads to lack of thermotolerance and defines tissue-specific regulation for stress-inducible Hsp molecular chaperones. J Cell Biochem 86:376–393.