

The α Crystallin Domain of Small Heat Shock Protein b8 (Hspb8) Acts as Survival and Differentiation Factor in Adult Hippocampal Neurogenesis

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Adult hippocampal neurogenesis is to a large degree controlled at the level of cell survival, and a number of potential mediators of this effect have been postulated. Here, we investigated the small heat shock protein Hspb8, which, because of its pleiotropic prosurvival effects in other systems, was considered a particularly promising candidate factor. Hspb8 is, for example, found in plaques of Alzheimer disease but exerts neuroprotective effects. We found that expression of Hspb8 increased during differentiation *in vitro* and was particularly associated with later stages (48–96 h) of differentiation. Gain-of-function and loss-of-function experiments supported the hypothesis that Hspb8 regulates cell survival of new neurons *in vitro*. In the dentate gyrus of adult mice *in vivo*, lentiviral overexpression of Hspb8 doubled the surviving cells and concomitantly promoted differentiation and net neurogenesis without affecting precursor cell proliferation. We also discovered that the truncated form of the crystallin domain of Hspb8 was sufficient to affect cell survival and neuronal differentiation *in vitro* and *in vivo*. Precursor cell experiments *in vitro* revealed that Hspb8 increases the phosphorylation of Akt and suggested that the prosurvival effect can be produced by a cell-autonomous mechanism. Analysis of hippocampal Hspb8 expression in mice of 69 strains of the recombinant inbred set BXD revealed that *Hspb8* is a *cis*-acting gene whose expression was associated with clusters of transcript enriched in genes linked to growth factor signaling and apoptosis. Our results strongly suggest that Hspb8 and its α -crystallin domain might act as pleiotropic prosurvival factor in the adult hippocampus.

Introduction

Adult hippocampal neurogenesis is dominantly regulated at the level of the survival of newborn neurons. In 26 strains of the BXD recombinant inbred panel of mice, 85% of the variability in adult neurogenesis was explained by cell survival, whereas precursor cell proliferation explained only 19% (Kempermann et al., 2006). Learning and environmental complexity recruit more new neurons by increasing survival.

Relatively little is known about the molecular mechanisms underlying this survival-promoting effect, although specific sur-

vival effects have been seen in many experiments (e.g., Pieper et al., 2010; Sahay et al., 2011). Reported mediators include neurotrophic factors, most notably BDNF, hormones, and cytokines (Rossi et al., 2006; Galea, 2008; Mueller et al., 2008; Peng et al., 2008; Pinnock and Herbert, 2008; Mastrangelo et al., 2009; Pawluski et al., 2009; Zhang et al., 2009; Koo et al., 2010; Leuner et al., 2011). We have reported that melatonin has an almost exclusive effect on survival and maturation of newborn neurons in the adult dentate gyrus (Ramírez-Rodríguez et al., 2009, 2011). At the intracellular level, the role of antiapoptotic factors, such as Bcl2, have been described and functional studies suggest that the activity-dependent promotion of survival is linked to the active elimination of new cells (Perera et al., 2007).

Small heat shock proteins are interesting candidate molecules to orchestrate complex functions because of their pleiotropic effects (Wang et al., 2004; Yew et al., 2005; Depre et al., 2006; Quraishe et al., 2008; Gurusamy et al., 2009; Sui et al., 2009; Karch and Borchelt, 2010; Gonzalez-Malerva et al., 2011; Kirbach and Golenhofen, 2011). They are well suited as evolutionarily conserved mediators of “activity,” considering their name-giving ability to upregulate their expression in response to stressors. Hspb8 has mainly been studied in the heart, where it exerts a plethora of prosurvival functions, including the stimulation of

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glycogen synthesis (Wang et al., 2004), potentiation of BMP signaling (Sui et al., 2009), and buffering oxidative stress, among other mechanisms by modifying SOD (Karch and Borchelt, 2010). Overexpression of Hsp8 caused cardiac hypertrophy by promoting cell proliferation and survival through an Akt-mediated pathway (Sui et al., 2009).

Hsp8 is expressed in the brain; and according to the Allen Brain Atlas, its expression is concentrated in (but not limited to) the neurogenic niche of the hippocampus (www.brain-map.org; Hsp8-Sagittal-b04-0153), where its expression is upregulated as an early response to hypoxia (David et al., 2006). In addition, Hsp8 is expressed in cultured hippocampal neurons (Kirbach and Golenhofen, 2011).

Hsp8 is linked to neuronal survival by its interaction with Bag3 to induce macroautophagic removal of misfolded proteins (Yew et al., 2005; Gurusamy et al., 2009). This chaperone activity has been shown for amyotrophic lateral sclerosis (Crippa et al., 2010) and proposed for Alzheimer disease (Wilhelmus et al., 2006). Mutations of Hsp8 are involved in the hereditary peripheral neuropathy of Charcot-Marie-Tooth neuropathy type 2 (Tang et al., 2005; Irobi et al., 2010). Based on our preliminary observation and this literature, we set out to investigate Hsp8 as potential pleiotropic survival factor in adult hippocampal neurogenesis.

Hsp8 is also known as H11 kinase, Hsp22, Hsp20-like, or α C Crystallin (Cryac). It is not to be confused with Hsp27/Hspb5 (α B Crystallin) on which a larger literature exists (e.g., Hagemann et al., 2009).

Materials and Methods

Animals. C57BL/6 mice were obtained from Charles River. They were held in standard laboratory cages with a light cycle of 12 h lights on and 12 h lights off. The animals had access to food and water *ad libitum* at the animal facility of the Max Delbrück Center for Molecular Medicine Berlin-Buch, Germany. A total of 80 female mice, 8 weeks old at the beginning of the experiment, were used. All animal work was performed according to the rules of directive of the European Union and was approved by the responsible authority, Landesamt für Gesundheit und Technische Sicherheit Berlin.

Isolation of adult hippocampal precursor cells (AHPCs). AHPCs were isolated from the hippocampus of adult female mice as previously reported (Babu et al., 2011). Briefly, animals were killed by cervical dislocation. Brains were removed from the skull and placed in cold artificial CSF (aCSF) containing 124 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 25 mM NaHCO₃, 10 mM D-glucose. Hippocampal coronal slices (300 μ m) were obtained using a vibratome to dissect out the dentate gyrus. Dentate gyri were dissociated by enzymatic digestion and cell suspension separated by centrifugation using a Percoll gradient.

Precursor cells were plated on laminin-precoated coverslips or 96 multiwell plates and cultured with 20 ng/ml of human EGF and 20 ng/ml of human FGF-2 (both from PeproTech) in Neurobasal medium supplemented with B27 (Invitrogen), for 24 h.

Western blot (immunoblotting). Precursor cells were lysed as reported previously (Babu et al., 2009; Ramírez-Rodríguez et al., 2009). Total lysate from AHPCs was obtained with RIPA buffer (150 mM NaCl, 10% glycerol, 0.5 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, 25 μ g/ml leupeptin, 25 μ g/ml aprotinin, and 1 mM sodium ortho-vanadate in 50 mM Tris-HCl, pH 7.6) and homogenized with an ultrasonic homogenizer for 30 s. Cellular debris was removed by centrifugation at 14,000 \times g. Total protein content was quantified using Bradford reagent (Bio-Rad). Protein separation was performed by the Laemmli method (Cleveland et al., 1977) and transferred to PVDF or nitrocellulose paper. Membranes were blocked with 5% skim milk in 0.05% Tween 20-TBS and incubated with the goat anti-Hsp8 1:500 (Abcam); rabbit-anti-Hsp8 1:3000 (a kind gift from Dr. Roelfs, Radboud University Nijmegen, Nijmegen, The Netherlands); goat- or mouse-anti-GFP (1:500, Abcam); rabbit-anti-phospho AKT or rabbit anti-AKT (1:1000, Cell Signaling) or with the

mouse anti-GAPDH antibody 1:5000 (Millipore Bioscience Research Reagents, Hampshire, England). Blots were washed 3 times with Tween 20-TBS and incubated for 1 h in a 1:3000 dilution of phosphatase-conjugated donkey anti-mouse; donkey anti-goat; donkey anti-rabbit antibodies. Proteins were visualized with the enhanced chemiluminescence detection system. Autoradiograms were scanned with a GS-800 densitometer and PDQuest Advanced Software (Bio-Rad).

Standard RT-PCR and qRT-PCR. The expression of Hsp8 in AHPC was analyzed by RT-PCR. RNA was isolated using RNeasy (QIAGEN), and cDNA was generated using the Superscript system (Invitrogen). Products were separated on 1% agarose gels. Primer sequences for Hsp8 (forward, TGAATTCGACCAACATCATGGCTGAC; reverse, GAAGTCGACCAAGGCTGACGTCTTAG) were from BioTez.

For analyzing expression changes of Hsp8 during neural precursor differentiation in culture, RNA was extracted as was mentioned above at 0, 12, 24, 48, and 96 h, respectively. RNA samples were adjusted to 1 μ g/ μ l and stored at -80°C . Three independent reverse transcriptase (RT) reactions were performed for each RNA sample using oligo(dT) primers and Superscript II RNase H reverse transcriptase, followed by incubations with RNase H (Invitrogen) for 20 min at 37°C . Primer sequences were as follows: forward, CATCTCAAGCCACATCACCTTG; reverse, GGCCAGGAGAGGAGAGC. Quantitative PCR was performed in a reaction mix containing SYBR Green (Quantitect SYBR Green PCR kit, QIAGEN). The products were detected with an Opticon DNA Engine (MJ Research). A melting curve analysis verified the specificity of the reaction. The PCR protocol was as follows: 95°C for 15 min; 94°C for 30 s; 58°C for seconds; 72°C for seconds; 40 cycles from steps 2–4 followed by a melting curve from 55°C – 90°C . The relative amount of the tested transcript was normalized to the level of *Gapdh* using specific primers set all obtained from BioTez.

Plasmid constructs. The full-length of Hsp8 was amplified from cDNA of neural precursor cells by PCR with primers that flank the open reading frame (forward, TGAATTCGACCAACATCATGGCTGAC; reverse, GAAGTCGACCAAGGCTGACGTCTTAG) and ligated into the pIRES2-EGFP vector at the EcoRI/SalI sites. The pIRES2-EGFP vector enables Hsp8 to be expressed together with the EGFP from a single bicistronic mRNA. For cloning the Hsp8 truncated in the 120–145 residues corresponding to the α crystallin domain (Δ Hsp8), we used two primer sets (forward, TGAATTCGACCAACATCATGGCTGAC; reverse, CTCTGCAGTATCCATCCTTGGTCTTTAC; and reverse, TTCCTGCAGAAGTGATCCAGCCA; forward, GAAGTCGACCAAGGCTGACGTCTTAG), both fragments were digested using the enzymes EcoRI/PstI and PstI/SalI, respectively, and ligated into the pIRES2-EGFP vector at the EcoRI/SalI. The sequences of the full-length and the truncated Hsp8 (Δ Hsp8) were verified by sequencing.

Precursor cell electroporation (overexpression and knock-down). Electroporation of precursor cells with the p-Hsp8-IRES2-eGFP and p- Δ Hsp8-IRES2-eGFP was performed as described previously (Lie et al., 2005; Jessberger et al., 2008). Briefly, precursors were detached, and 1×10^6 cells were transfected with 10 μ g of the respective DNA using the Nucleofector II electroporation device according to the manufacturer's instructions (Amaxa). After transfection, cells were plated at 2.5×10^4 or 1×10^5 densities, depending on the parameter that would be evaluated. After 48 h, cells cultured in proliferation conditions were induced to differentiate during 4 additional days. Proportions of neurons that expressed GFP were identified by MAP2 expression. Protein expression was verified 36 h after transfection.

For knocking down expression of Hsp8, AHPCs were electroporated with sense and antisense siRNA oligonucleotides. The control siRNA was from Ambion, and Hsp8-siRNA was from MWG-Biotech. The Hsp8-siRNA sequence was as follows: sense, CAACGAGCUUCCUCAAGAC; antisense, GUCUUGAGGAAGCUCGUUG. The resuspended duplexes were aliquoted and stored at -20°C . The electroporation of precursor cells with siRNA was performed according to the protocol provided by Amaxa. Briefly, before electroporation, cells were detached and 1×10^6 cells were mixed with 160 nM of siRNA duplexes and 2 μ g of control GFP plasmid DNA (pmaxGFP, Amaxa). Then, AHPCs were plated and cultured under proliferation conditions during 48 h. Thus, cells were induced to differentiate during 4 additional days. The proportion of neurons that coexpress GFP

and β III-tubulin were identified in randomized fields. Silencing of *Hspb8* in precursor cells was confirmed by immunoblotting.

Approximately 200–300 cells were quantified. The analyzer was blinded for the experimental conditions. Every experiment was performed at least three times as duplicates. Additionally, conditioned media (CM) from precursor cells expressing the pIRES2-EGFP, p-Hspb8-IRES2-eGFP, or from cells transfected with the control siRNA or Hspb8-siRNA was collected to determine a possible cell-autonomous or non-cell-autonomous effect of Hspb8 on precursor cell proliferation or survival determined by WST-1 and BrdU incorporation using ELISA-based assay.

Immunocytochemistry. Cultures were fixed with 4% paraformaldehyde in 0.1 M PBS, pH 7.4, for 20 min. After PBS washing, cells were permeabilized with 0.5% Triton X-100 in PBS for 30 min. Nonspecific sites were blocked with 5% donkey serum (Millipore Bioscience Research Reagents) containing 0.5% Triton X-100 (blocking buffer), free-aldehyde groups were reduced with 1 M glycine for 20 min. Primary antibodies were diluted in blocking buffer and monolayer incubated overnight at 4°C. After washes with PBS, incubation with secondary antibodies was allowed for 2 h at room temperature. The primary antibodies were as follows: goat anti-Hspb8 1:500 and monoclonal mouse anti-Hspb8 1:500 (Abcam); monoclonal mouse anti-Nestin 1:400 (BD Biosciences); monoclonal mouse anti- β III-tubulin 1:1000 (Promega); rabbit anti- β III-tubulin (TuJ1) 1:1000 (Covance); guinea-pig anti-GFAP 1:1000 (Advanced Immunochemistry); rat anti-BrdU 1:500 (Biozol); and mouse anti-eGFP 1:500 (Abcam). Secondary antibodies raised in donkey (Jackson ImmunoResearch-Dianova) were used at 1:250. Colabeled cells were quantified in randomized fields using a Leica TCS SP2 confocal microscope (Leica). All analyses were done in sequential scanning mode to avoid false positive colabeling.

Lentiviral vectors. The control vector was pCH_CMV_eGFPWS_Isa and to generate the Hspb8 and Δ Hspb8 vectors, we cloned their cDNA upstream of the IRES and EGFP and inserted the bicistronic cassette in place of the GFP sequence in the pCH_CMV_eGFPWS_Isa vector. The empty vector was generated inserting the bicistronic cassette (IRES2-EGFP) in place of the GFP sequence. Concentrated lentiviral vectors (LV) stocks, pseudotyped by the vesicular stomatitis viral envelope, were produced as described previously (Geraerts et al., 2006). Expression titers, determined in 293T cells by FACS analysis, were 1.65×10^8 to 3×10^8 transducing units/ml with a p24 concentration of 1.38×10^6 to 2.95×10^6 pg/ml.

Surgical procedure and immunohistochemistry. Mice were stereotactically injected with 2 μ l of the LV containing Hspb8 or Δ Hspb8 and GFP into the right hemisphere of the dentate gyrus. Coordinates from bregma in mm are as follows: anteroposterior -2.3 , mediolateral -1.7 , dorsoventral -1.7 . For control group, 2 μ l of control GFP expressing LV was injected. The group size was $n = 5$ or 6 mice.

To study the effects of Hspb8 and Δ Hspb8 expression on cell proliferation in the hippocampus, adult mice were injected three times with BrdU (50 mg/kg body weight i.p.; Sigma-Aldrich) 6 h apart, starting the first injection at 3 weeks after lentiviral injection. Mice were killed 2 h after the last BrdU injection. For survival/differentiation studies, the same BrdU injection protocol was applied and animals were killed at 3 weeks after the last BrdU administration.

Mice were deeply anesthetized with ketamine and perfused transcardially with 0.9% sodium chloride followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed from the skulls and postfixed overnight. After fixation, brains were transferred into 30% sucrose. Brains were cut into 40 μ m coronal sections on a sliding microtome (Leica) and cryoprotected. Sections were stained free-floating with antibodies diluted in Tris-buffered saline containing 3% donkey serum and 0.1 Triton X-100. For BrdU, DNA was denatured in 2N HCl for 30 min at 37°C. To study the distribution of Hspb8 and its coexpression in the course of adult hippocampal neurogenesis, we used a Nestin-GFP mouse.

Primary antibodies were applied in the following dilutions: rabbit anti-Hspb8 (1:100; ProteinTech); goat anti-double-cortin (1:200; Santa Cruz Biotechnology); monoclonal anti-calretinin (1:500; Swant); monoclonal mouse anti-NeuN (1:100; Millipore Bioscience Research Reagents); rabbit anti-S100 β (1:250; Swant); goat anti-GFP (1:500, Abcam); and rat anti-BrdU (1:500, Biozol). Fluorophore-coupled sec-

ondary antibodies were as follows: anti-rat TRITC, anti-mouse Cy5, anti-rabbit Cy5, and anti-goat FITC. All secondary antibodies were raised in donkey and diluted 1:100 or 1:250 (Jackson ImmunoResearch-Dianova). Sections were coverslipped in polyvinyl alcohol with diazabicyclo-octane as antifading agent.

Phenotypic analysis in vivo. Areas transduced by LV were identified by expression of GFP. The proliferation and survival rates were expressed as mean of BrdU/GFP⁺ cells per animal. For phenotypic analysis of lentiviral-labeled cells, one-in-12 series of sections were triple-stained with immunofluorescence as described above. Fifty BrdU-labeled cells within the subgranular and granule cell layer were analyzed for coexpression of the different markers.

The colabeling of BrdU/GFP/NeuN and BrdU/GFP/S100 β , respectively, identified new neurons and new astrocytes in the hippocampus. Analysis was done by 3D confocal microscopy in sequential scanning mode. Newly generated neurons and astrocytes in infected areas are shown as fraction related to the control group (Kempermann et al., 2004).

Bioinformatic analysis. Quantitative trait locus (QTL) mapping was done using data and online tools available from the GeneNetwork repository (<http://www.genenetwork.org>). The analysis used a hippocampal mRNA expression dataset measured using Affymetrix M430 version 2 microarrays and normalized with the PDNN method (Overall et al., 2009). This dataset is accessible as “Hippocampus Consortium M430v2 (Jun06) PDNN” from the GeneNetwork website. Gene enrichment analysis was done with the DAVID online database (<http://david.abcc.ncifcrf.gov>) (Huang da et al., 2009a, 2009b) using all genes present on the microarray as background. The interaction network was generated using the STRING tool (<http://string.embl.de>) (Snel et al., 2000; Szklarczyk et al., 2011).

Statistical analysis. Analysis was performed using SigmaStat 3.1 and Statview 5.0.1 software. Results are presented as mean \pm SEM. Statistical analysis from the *in vitro* and *in vivo* data was performed using one-way ANOVA, followed by appropriated *post hoc* test. Differences were considered statistically significant at $p = 0.05$.

Results

Hspb8 is expressed during adult neural precursor cell differentiation

Immunohistochemical investigations with antibodies against Hspb8 revealed that the protein is expressed in the dentate gyrus, the CA fields, and the hilus of the hippocampus of adult mice (Fig. 1A,B), confirming the mRNA distribution seen in the Allen Brain Atlas. The expression of Hspb8 in the dentate gyrus was corroborated by PCR and Western blot after microdissection (Fig. 2B,C). To analyze the expression of Hspb8 in the hippocampus, we performed immunostainings in tissue sections from Nestin-GFP reporter mice (Fig. 1). We detected that Hspb8 is widely expressed in the hippocampus, including in the wall of blood vessels (Fig. 1). To investigate which cells would express Hspb8 in the course of adult hippocampal neurogenesis, we colabeled Hspb8 with different markers of neurogenesis (Fig. 1). We detect Hspb8-staining in some Nestin-positive type 1 and type 2a cells (Fig. 1C). However, we found more colabeling with Hspb8 in type 2b cells (Nestin/DCX-positive; Fig. 1C). Also, postmitotic new neurons expressing calretinin (Fig. 1D) colabeled with Hspb8. Strongest expression of Hspb8 was seen in the population of mature granule cell neurons that also coexpressed NeuN (Fig. 1E). These results indicated that Hspb8 is expressed during various developmental stages in the course of adult hippocampal neurogenesis.

We next analyzed the expression of Hspb8 in neural precursor cells isolated from the dentate gyrus of adult mice. Neural precursor cells cultured under proliferation conditions are polygonal and bipolar with plump and short processes (Fig. 2A). Hspb8 showed a punctuate pattern in the cytoplasm of precursor cells (Fig. 2A3). Again the presence of Hspb8 was confirmed by PCR and Western blot (Fig. 2B,C). Western blot autoradiograms showed low levels of Hspb8 in proliferating precursor cells (Fig. 2D).

We next investigated the changes of mRNA expression and protein during the process of differentiation *in vitro*. In the course of differentiation, the cells started to extend long thin neurites after 12–24 h (Fig. 3A1) and reached their maximal neurite length at 96 h after the induction of differentiation. Most of these neurites made connections with adjacent cells creating a network (Fig. 3A2). *Hspb8* mRNA was significantly increased at 48 and 96 h after induction of cell differentiation compared with earlier time points ($p < 0.0002$; Fig. 3B). In addition to the changes in the transcript, the levels of Hspb8 protein increased. Hspb8 protein levels were low under proliferation conditions, similarly to those shown in Figure 2C; and also at 12 h after the initiation of differentiation. However, Hspb8 slightly increased after 24 h of differentiation reaching a maximum at 96 h (Fig. 3C). This time course of expression suggested a primary relevance of Hspb8 during relatively more advanced stages of differentiation, presumably including but not limited to survival per se.

Hspb8 is required for precursor cell differentiation and survival *in vitro*

To further test for the functional involvement of Hspb8 in differentiation and survival, adult hippocampal precursor cells *in vitro* were transfected with Hspb8, and the effect on neuronal differentiation was evaluated at 4 d after the induction of differentiation (Fig. 4A). Overexpression of Hspb8 was confirmed by immunoblotting of GFP as cotransfected reporter and Hspb8 (Fig. 4B1). To analyze the effects of Hspb8 on cell differentiation, we performed immunocytochemistry against GFP and MAP2ab on the cells *in vitro* (Fig. 4B2). MAP2ab was used as marker for relatively mature neurons *in vitro* (Babu et al., 2009). Overexpression of Hspb8 significantly increased neuronal differentiation by ~66% compared with cells nucleofected with the control plasmid ($p = 0.001$; Fig. 4C). The effect of Hspb8 on neuronal differentiation was accompanied by an increase of 22% in cell survival after 4 d of differentiation ($p = 0.001$; Fig. 4D).

We next silenced *Hspb8* with specific siRNA sequences by nucleofection and verified protein expression after the induction of differentiation (Fig. 5A–C). Western blot analysis confirmed a decrease in Hspb8 protein expression after siRNA transfection (Fig. 5C). Silencing *Hspb8* caused a significant decrease in neuronal differentiation of cells that coexpressed GFP and the early pan-neuronal marker β III-tubulin by ~60% ($p = 0.011$, Fig. 5D), and also in cell survival ($p = 0.004$, Fig. 5E).

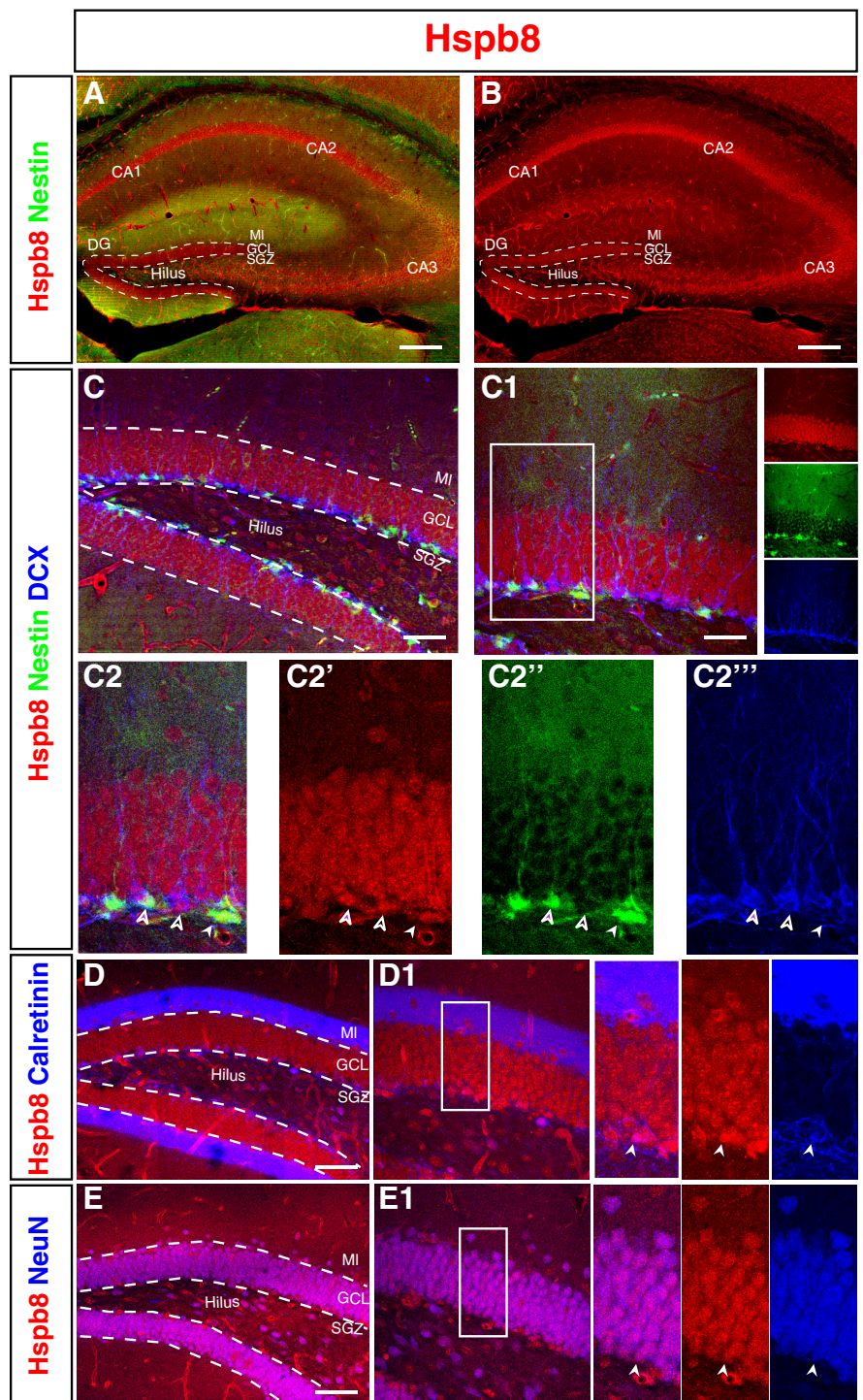


Figure 1. Hspb8 is expressed in the dentate gyrus (DG) of adult mice. Immunofluorescent labeling reveals the expression of Hspb8 in the hippocampus of adult mice. **A, B**, The protein (red) appears to be more concentrated in the DG, CA3, CA2, CA1, and in the hilus. **C**, Hspb8 (red) shows colocalization with type 1 cells (small arrowhead). Also, Hspb8 colocalizes with type 2 cells identified by coexpression of Nestin-GFP (green) with double-cortin (blue). **C2**, High-power confocal images. **C2'–C2'''**, Representative images of each protein marker (arrowheads). Also, Hspb8 colocalized with calretinin in some cells (**D**). **D1**, High-power images (arrowheads indicate Hspb8/calretinin double-positive immature neurons). **E, E1**, Representative images show that Hspb8 predominantly colocalizes with mature NeuN-positive neurons in the hippocampus. The colocalization is shown in high-power images (arrowhead; right). **A–D**, The molecular layer (ML), granular cell layer (GCL), and subgranular zone (SGZ). Scale bars: **A, B**, 150 μ m; **C**, 75 μ m; **C1**, 45 μ m; **D, E**, 60 μ m.

Control sequences did not affect cell differentiation ($p = 0.44$) or survival ($p = 0.75$) compared with the control group.

Given that overexpression of Hspb8 increased precursor cell differentiation and survival, that the silencing of the Hspb8

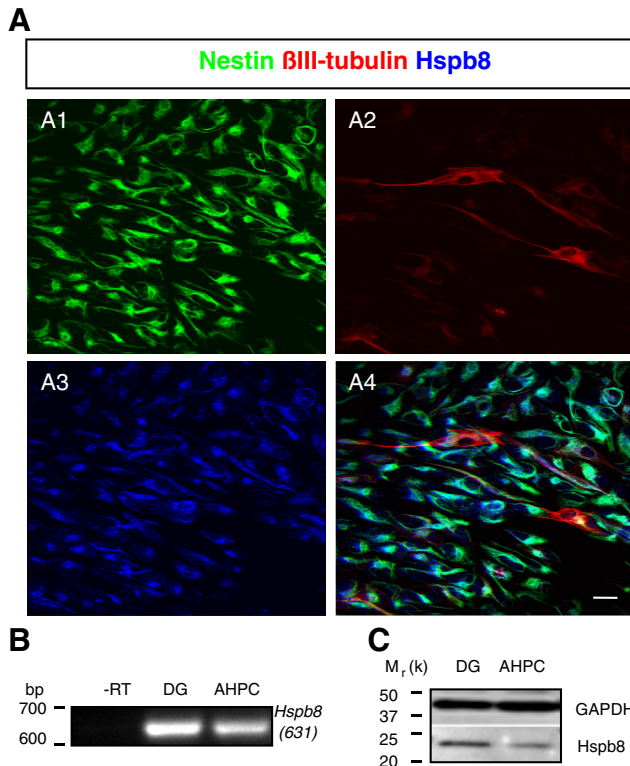


Figure 2. Hspb8 is expressed in adult hippocampal precursor cells and in the dentate gyrus of adult mice. **A**, Precursor cells express Hspb8. Immunofluorescence staining depicting the expression of Hspb8 (**A3**, blue) in precursor cells in culture. Precursor cells in culture were identified by nestin expression (**A1**, green) and β III-tubulin (**A2**, red). Scale bar: **A4**, 20 μ m. **B**, RT-PCR of Hspb8 using RNA derived from mouse adult precursor cells showed that the transcript is expressed in the precursor cells and in the dentate gyrus (DG). **C**, Expression of Hspb8 protein was also found in precursor cells (AHPC) and in the DG.

mRNA reduced neuronal differentiation and cell survival, and that during differentiation the levels of Hspb8 increased at 24, 48, and 96 h after induction of differentiation, Hspb8 appears to regulate survival and differentiation of new neurons *in vitro*.

Hspb8 does not alter precursor cell proliferation *in vivo*

Based on both our *in vitro* results and the expression of Hspb8 in the course of adult hippocampal neurogenesis *in vivo*, we next addressed the functional effects of Hspb8 on adult neurogenesis *in vivo*. We overexpressed Hspb8 using a lentiviral vector and stereotaxic injection. Proliferating cells were permanently labeled with three injections of BrdU, 6 h apart, at 3 weeks after viral injection (Fig. 6A). Proliferating transduced cells were identified by the codetection of both BrdU and GFP (Fig. 6B). Quantification of BrdU/GFP-labeled cells revealed that overexpression of Hspb8 or Δ Hspb8 did not change the number of proliferating cells in the dentate gyrus (LVGFP, 68 ± 16 ; LV-Hspb8, 60 ± 13.86 ; LV- Δ Hspb8, 72 ± 18 ; $p = 0.87$; Fig. 6C).

Hspb8 promotes survival and neurogenesis *in vivo*

In contrast, overexpression of Hspb8 increased survival of newborn cells in the dentate gyrus. Surviving cells were labeled with BrdU 3 weeks after viral vector injection (Fig. 6D) and identified another 3 weeks later (Fig. 6E). Overexpression of Hspb8 significantly increased the number of BrdU/GFP-labeled cells compared with the control group (LVGFP; Fig. 6F). The number of surviving cells in the Hspb8 transduced dentate gyrus increased significantly by 63% (LVGFP, 88 ± 14.42 ; LV-Hspb8, 144 ± 17.66 ; $p = 0.022$; Fig. 6F).

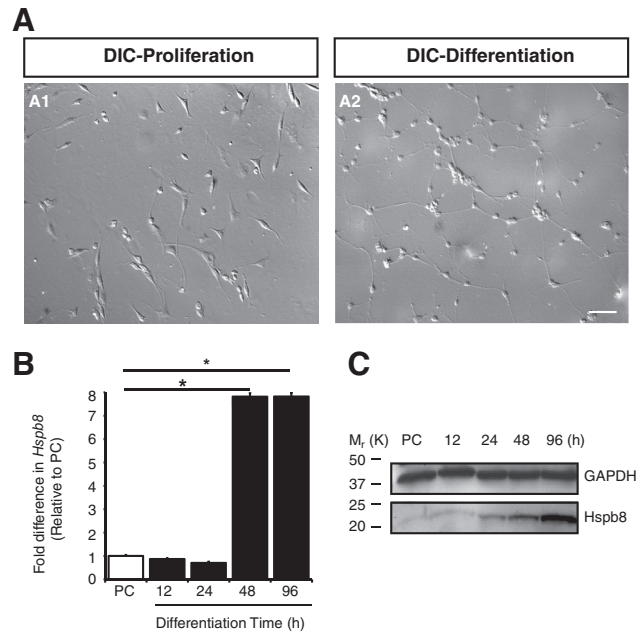


Figure 3. Expression changes of Hspb8 during precursor cell differentiation *in vitro*. **A**, Differential interference contrast (DIC) image of precursor cells cultured in the presence of growth factors is shown in **A1**, and precursor cells differentiated during 96 h are shown in **A2**. Images clearly show the morphological changes that occur before and after differentiation. Scale bar, 60 μ m. **B**, qRT-PCR of Hspb8 during the differentiation time course of precursor cells shows an increase in its expression level at 48 and 96 h after differentiation. Error bars indicate SEM. $*p < 0.0002$, PC versus 48 and 96 h, respectively. **C**, The expression pattern of Hspb8 protein was analyzed by Western blot before and after induction of differentiation, indicating an increase in the protein level after 24 h of differentiation. GAPDH was used as loading control.

We also analyzed the phenotype of surviving BrdU/GFP cells after Hspb8 overexpression (Fig. 7A) and found a significant 2.33-fold increase in the fraction of cells that coexpressed BrdU/GFP and the neuronal marker, NeuN (LVGFP, 1 ± 0.29 ; LV-Hspb8, 2.33 ± 0.60 ; $p = 0.026$, Fig. 7B). A similar but not statistically significant increase was seen in the number of BrdU/GFP/S100 β ⁺ astrocytes (LVGFP, 1 ± 0.58 ; LV-Hspb8, 1.5 ± 0.65 ; $p = 0.49$; Fig. 8).

The α crystallin domain is involved in the proneurogenic effects of Hspb8

Several small heat-shock proteins share a highly conserved functional domain, the α -crystallin domain. Both Hspb8 and Hspb5 contain this domain. To study the potential role of the domain in the observed effects on neurogenesis, we overexpressed a truncated form of Hspb8, which lacks 120–145 residues of the α -crystallin domain (Δ Hspb8) *in vitro* and *in vivo*.

In cultured precursor cells, overexpression of Δ Hspb8 prevented the effect of Hspb8 on neuronal differentiation ($\sim 34\%$, $p = 0.001$; Fig. 4C) and cell survival ($p = 0.007$; Fig. 4D).

Similarly, Δ Hspb8 did not show the effect on the survival of BrdU/GFP-labeled cells seen after transduction with Hspb8 *in vivo* (LV-Hspb8, 144 ± 17.66 ; LV- Δ Hspb8, 75 ± 5.74 ; $p = 0.006$; Fig. 6F).

Again, and consistent with the *in vitro* data (Fig. 4C), the effect of Hspb8 was specific to the survival of new neurons because Δ Hspb8 did not show this effect (LV-Hspb8, 2.33 ± 0.60 ; LV- Δ Hspb8, 1 ± 0.20 ; $p = 0.020$; Fig. 7B). In addition, the mean fraction of astrocytes appeared to be reduced by 50% after transduction with Δ Hspb8, but this change did not meet the criterion of conventional statistical significance (LVGFP, 1 ± 0.58 ; LV- Δ Hspb8, 0.5 ± 0.29 ; $p = 0.41$; Fig. 8B). These results further

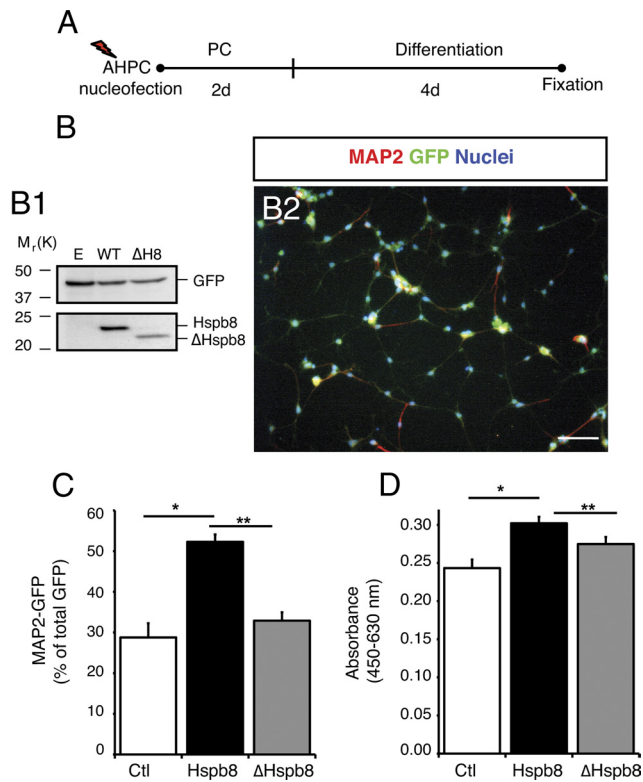


Figure 4. Overexpression of Hspb8 affects neuronal differentiation of precursor cells *in vitro*. **A**, Experimental time line for Hspb8 effect on precursor cell (AHPC) differentiation. **B**, Expression of Hspb8, Δ Hspb8, and GFP after nucleofection was verified by Western blot (**B1**). **B2**, Representative image of new neurons identified by the coexpression of GFP (green) and MAP2 (red). Nuclei were stained with DAPI (blue). Scale bar, 70 μ m. **C**, Precursor cells expressing Hspb8 or Δ Hspb8 were differentiated during 4 d. Quantification of MAP2/GFP neurons was done as described in Materials and Methods. There were significant differences in neuronal differentiation between groups. Hspb8 and Δ Hspb8 affect neuronal differentiation of precursor cells. Experiments were duplicated and repeated at least three times. Results represent the mean \pm SEM of total GFP. * p = 0.001, control versus Hspb8 (Tukey's *post hoc* test after one-way ANOVA). ** p = 0.001, Hspb8 versus Δ Hspb8 (Tukey's *post hoc* test after one-way ANOVA). **D**, Effect of Hspb8 overexpression on cell survival was done with Wst-1 assay, showing that Hspb8 significantly increased cell survival and that Δ Hspb8 affects this parameter. Wst-1 analysis from six wells per group was performed three times. Error bars indicate SEM. * p = 0.001, control versus Hspb8 (Tukey's *post hoc* test after one-way ANOVA). ** p = 0.007, Hspb8 versus Δ Hspb8 (Tukey's *post hoc* test after one-way ANOVA).

corroborate the involvement of the α -crystallin domain in the Hspb8-induced increase in survival of the newborn neurons.

Hspb8 regulates survival of differentiated precursor cells via Akt signaling

To get information about possible mechanisms by which Hspb8 exerts its effects on survival of precursor cells, we evaluated the activation of the key survival protein Akt, which is activated by Hspb8 in other cellular models (Sui et al., 2009). As previously reported, we also found that activated Akt (phospho-Akt) was downregulated in precursor cells that had been induced to differentiate (45%; p = 0.001; Fig. 9A,B) (Bracko et al., 2012). However, overexpression of Hspb8 caused a significant increase in the levels of phospho-Akt in differentiating precursor cells (91%; p < 0.001; Fig. 9C). To confirm the involvement of Akt signaling pathway in the survival of differentiating hippocampal precursor cells induced by Hspb8, we incubated precursor cells with a specific inhibitor (LY294002) for phosphatidylinositol-3-kinase (PI3K), a protein that is upstream of Akt in the survival pathway (Sui et al., 2009). We found a significant decrease in the levels of

phospho-Akt in precursor cells that were transfected with Hspb8 after the incubation with the PI3K inhibitor (164%; p < 0.001; Fig. 9C). Additionally, we found that silencing *Hspb8* significantly decreased the activation of Akt compared with controls (49%; p < 0.001; Fig. 9D). These data suggest that Hspb8 acts at least partly via the Akt-dependent signaling pathway to regulate survival and differentiation of precursor cells.

Hspb8 regulates survival of differentiating precursor cells in a cell-autonomous manner

We also sought to determine whether Hspb8 exerts a cell-autonomous or non-cell-autonomous effect on the survival of differentiating precursor cells. For these studies, we measured both proliferation and survival of wild-type precursor cells that were exposed to CM derived from precursor cells that overexpressed Hspb8 or had been transfected with siRNA-Hspb8 (Fig. 10A). The extent of BrdU incorporation was similar across precursor cells treated with the CM collected from all groups (p = 0.14; Fig. 10B). In a similar manner, the Wst-1 assay did not show differences in the viability of proliferative cells (p = 0.24; Fig. 10C). Wild-type precursor cells exposed to CM and induced to differentiation did not show significant changes in survival measured with the BrdU and Wst-1 assays (p = 0.15 and 0.22, respectively; Fig. 10D,E). These studies revealed that the effects of Hspb8 on survival of differentiating precursor cells *in vitro* cannot be attributed to factors secreted by transfected precursor cells or by differentiated cells derived from transfected precursor cells. Thus, our data rather suggest a possible cell-autonomous mechanism, by which Hspb8 promotes survival and possibly the differentiation of precursor cells. *In vivo* additional or alternative indirect mechanisms might be active.

Expression genetic analysis suggests an antiapoptotic role for Hspb8

We finally used our database of hippocampal gene expression in BXD mice to investigate the expression of Hspb8 and its variation in a genetic reference population (Overall et al., 2009). The BXD panel consists of mouse strains derived from inbreeding the progeny of an intercross between C57BL/6J and DBA/2J such that each resulting strain has a unique mix of both parental genotypes while being homozygous at every locus. The study described analyzed 69 of these strains, including the parental lines. The Affymetrix M430 version 2 microarray used in that study contains 3 probe sets targeting *Hspb8*, 1456434_x_at, 1417013_at, and 1417014_at. Because all three probe sets are of good quality (target-specific and expressed well in the hippocampus), we decided to use the first principal component (Hspb8-PC1, which explains >90% of the expression variance in these probe sets) as a meta-trait for further analysis. To address the genetic control of *Hspb8* in the hippocampus, we performed QTL mapping with Hspb8-PC1 to identify genomic loci influencing transcript expression. We found that expression of the *Hspb8* gene maps strongly to its own physical location in the genome (LOD score 17.5; Fig. 11A). This significant *cis*-QTL suggests that *Hspb8* is largely autoregulatory in the hippocampus and might exert a dominating effect in genetic networks in which it is involved.

We next searched for genes whose mRNA expression covaried with that of *Hspb8* in the hippocampus under the premise that genes regulated in a similar pattern are more likely to be under common genetic control or acting in the same biochemical pathways. To do this, we calculated the Pearson correlation coefficient for Hspb8-PC1 with every probe set in the expression dataset. The top 200 correlating probe sets were used for functional enrichment analysis

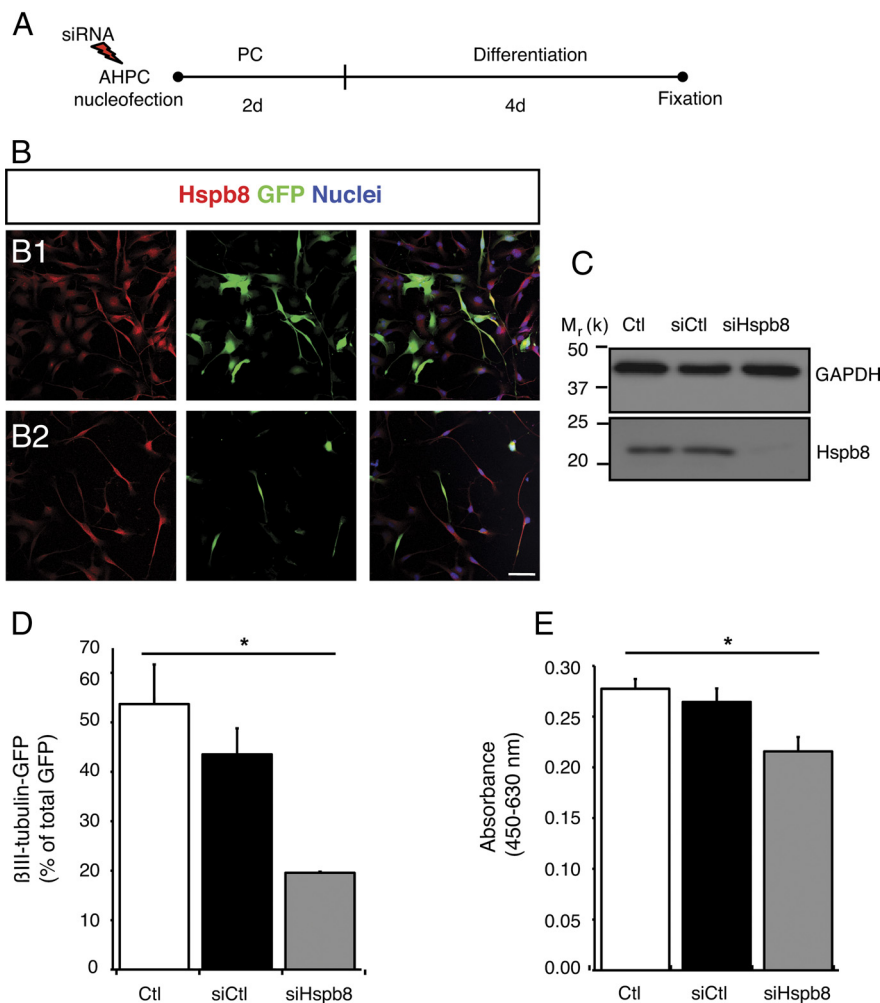


Figure 5. Knock-down of Hspb8 decreases neuronal differentiation and survival of precursor cells (AHPC) *in vitro*. **A**, Time line for the experiments in which Hspb8 was silenced to evaluate its effects on neuronal differentiation and survival. **B**, Immunofluorescence images of precursor cells nucleofected with control siRNA and Hspb8 siRNA sequences are shown in **B1** and **B2**, respectively. The decrease of Hspb8 was studied in cells that showed colocalization of the signals for Hspb8 (red) and GFP (green). **B2**, Merged images show cells with a decrease in Hspb8 expression without altered GFP expression after silencing. Scale bar, 30 μ m. **C**, The effect of Hspb8 knock-down was analyzed by immunoblotting. Silencing of Hspb8 (si-Hspb8) decreased the expression of Hspb8, whereas control sequences (si-Ctl) did not affect Hspb8 expression levels. GAPDH was used as loading control. **D**, **E**, Hspb8 knock-down significantly affected neuronal differentiation and survival of precursor cells. Control sequences did not significantly affect either parameter. Quantification of β III-tubulin/GFP neurons was performed as described in Materials and Methods. Experiments were as duplicates with three independent runs. Results represent the mean \pm SEM of total GFP. * $p = 0.011$, Ctl versus si-Hspb8 (Tukey's *post hoc* test after one-way ANOVA); $p = 0.44$, si-Ctl versus si-Hspb8 (Tukey's *post hoc* test after one-way ANOVA). **E**, Effect of Hspb8 overexpression on cell survival was also assessed with the Wst-1 assay. Wst-1 analysis from six wells per group was performed three times. * $p = 0.004$, Ctl versus si-Hspb8 (Tukey's *post hoc* test after one-way ANOVA); $p = 0.75$, Ctl versus si-Ctl (Tukey's *post hoc* test after one-way ANOVA).

(data not shown). Because there is strong linkage disequilibrium at this locus, which means that adjacent genes will be correlated because of genotype and not necessarily because of their functional expression pattern, we ignored probe sets from the Hspb8 linkage block (defined as the interval in which markers correlated with an $r^2 > 0.1$ with the markers adjacent to the Hspb8 gene). The remaining probe sets were split into genes positively or negatively correlating with Hspb8, and these two groups analyzed for enriched functional categories using the DAVID bioinformatics tool (Dennis et al., 2003; Huang da et al., 2009a, 2009b).

In the positively correlating group, we found enrichment for the categories “regulation of growth” (*Hopx*, *Ddr1*, *Fgfr1*, and *Ngf*) and “regulation of apoptosis” (*Bag3*, *Fgfr1*, *Ngf*, and *Ti-cam1*) (Fig. 11B). The negatively correlating group showed enrich-

ment for categories, including “intracellular signaling” (*Arhgef9*, *Rab14*, *Rap2a*, *Gnaq*, *Plcb1*, *Gm266*, *Spred2*, and *Usp8*), “apoptosis” (*Bcl2l11*, *Fem1b*, and *Peg3*) and “tissue morphogenesis” (*Acvr1*, *Fem1b*, and *Serpinb5*) (Fig. 11B).

We used the STRING online tool to identify a core network of interacting genes by submitting the gene names corresponding to the list of correlates described above and extending the network by adding the most-connected genes from the STRING database. The resulting core network (Fig. 11B) has been annotated to indicate the genes correlating with Hspb8-PC1. Positively correlating genes are highlighted green, negatively correlating genes in red. Two major clusters are apparent, one based around the nerve growth factor family of which several are positively correlated with Hspb8-PC1. Another group, positively interacting with the *Hspb8/Bag3* complex, is composed of members of the apoptotic machinery.

Discussion

In the present study, we show that small heat-shock protein Hspb8 is highly expressed in the course of adult hippocampal neurogenesis *in vivo* and during differentiation of adult hippocampal precursor cells *in vitro*. Overexpression of Hspb8 increased the survival of newborn neurons *in vitro* and *in vivo*. Our data indicate that the α -crystallin domain of Hspb8 is involved in the effects caused by Hspb8 and that Hspb8 promotes survival of new neurons *in vitro* through the activation of Akt suggesting, at least *in vitro*, a cell-autonomous mechanism.

Small heat shock proteins are differentially expressed in the brain of adult rodents (Quraishie et al., 2008; Kirbach and Golenhofen, 2011). Hspb8 is expressed in the hippocampus of adult mice and rats, but the expression of *Hspb8* mRNA is dependent on the developmental stage (Quraishie et al., 2008; Kirbach and Golenhofen, 2011). At embryonic and postnatal stages, *Hspb8* is

modestly or not at all expressed in the rat hippocampus, whereas in the adult, expression of Hspb8 increases considerably (Kirbach and Golenhofen, 2011). In nonpathological human brain, small heat shock proteins are also expressed. In such studies, Hspb8 was expressed in neurons and also in blood vessels (Wilhelmus et al., 2006). We confirmed that Hspb8 is widely expressed, including on blood vessels, and is detectable in the neurogenic subgranular zone (Figs. 1 and 2B,C). Hspb8 was increasingly expressed from type 2b cells to mature neurons (Fig. 1). Moreover, in cultured precursor cells, *Hspb8* mRNA and protein were expressed (Fig. 2B,C). These data first indicated that Hspb8 might be involved in survival and differentiation during adult neurogenesis *in vivo*, an idea supported by different lines of evidence suggesting an important role for Hspb8 in

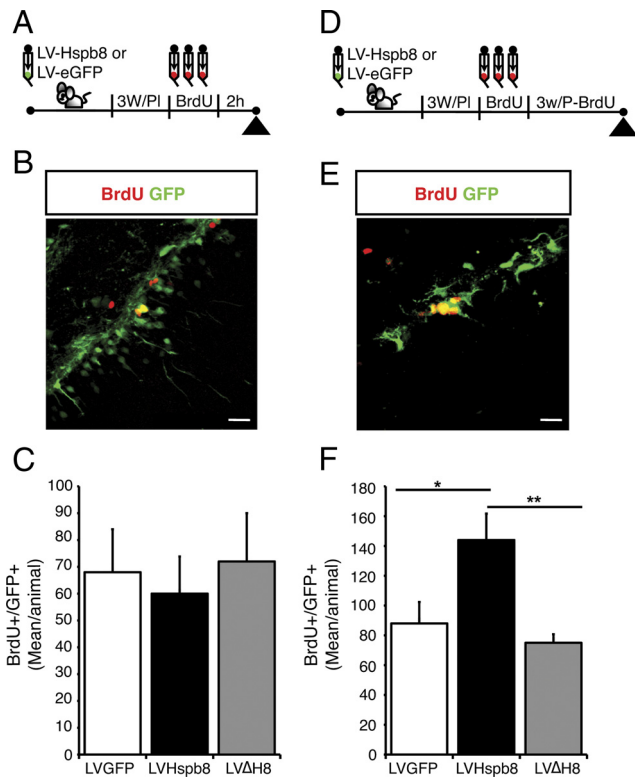


Figure 6. LV-mediated overexpression of LV-Hspb8 affects cell survival *in vivo*. **A**, Experimental design for the proliferation study in adult mice transduced with control viral vector (LVGFP), LV-Hspb8, or LV-ΔHspb8. After viral vector injection, proliferating cells were labeled by three sequential injections of BrdU (50 mg/kg). **B**, Cells in the proliferation phase were identified by the coexpression of BrdU/GFP in the transduced areas. Scale bar, 30 μm. **C**, Quantification of BrdU-labeled cells did not show significant changes in cell proliferation between LV-GFP, LV-Hspb8, and LV-ΔHspb8 groups. **D**, Three weeks after viral vector injection (LVGFP, LV-Hspb8, or LV-ΔHspb8), cells were labeled by three sequential injections of BrdU (30 mg/kg). Surviving cells were quantified 3 weeks after the last BrdU administration. **E**, Representative image of cells coexpressing BrdU/GFP in the transduced dentate gyrus. Scale bar, 30 μm. **F**, Quantification of BrdU-labeled cells indicated an increase in cell survival caused by LV-Hspb8 compared with LV-GFP. However, the truncated LV-ΔHspb8 did not affect cell survival caused by LV-Hspb8. *N* = 5 or 6 mice per group. Error bars indicate SEM. **p* = 0.022, LV-GFP versus LV-Hspb8 (Fisher's *post hoc* test after one-way ANOVA). ***p* = 0.006, LV-Hspb8 versus LV-ΔHspb8 (Fisher's *post hoc* test after one-way ANOVA).

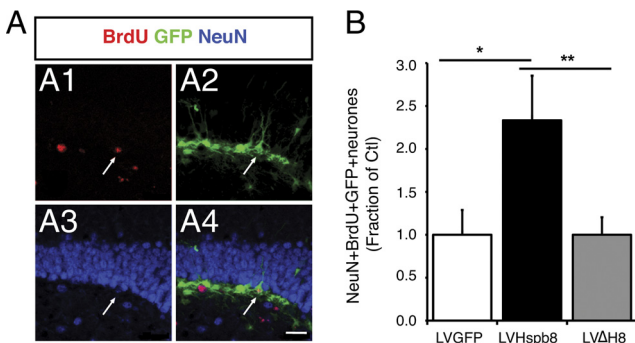


Figure 7. Hspb8 modulates neuronal differentiation *in vivo*. **A**, Neuronal differentiation was assessed by quantifying BrdU-labeled (**A1**, red) and NeuN-labeled (**A3**, blue) cells coexpressing GFP (**A2**, green) in the transduced areas (arrows). Scale bar, 40 μm. The merged image shows a granule cell triple labeled for BrdU/NeuN/GFP (**A4**). **B**, Significant increase in BrdU/NeuN/GFP-labeled cells in the dentate gyrus of mice injected with LV-Hspb8 related to LV-GFP control mice. The truncated LV-ΔHspb8 affects neuronal differentiation caused by LV-Hspb8. *N* = 5 or 6 mice per group. Error bars indicate SEM. **p* = 0.026, LV-GFP versus LV-Hspb8 (Fisher's *post hoc* test after one-way ANOVA). ***p* = 0.020, LV-Hspb8 versus LV-ΔHspb8 (Fisher's *post hoc* test after one-way ANOVA).

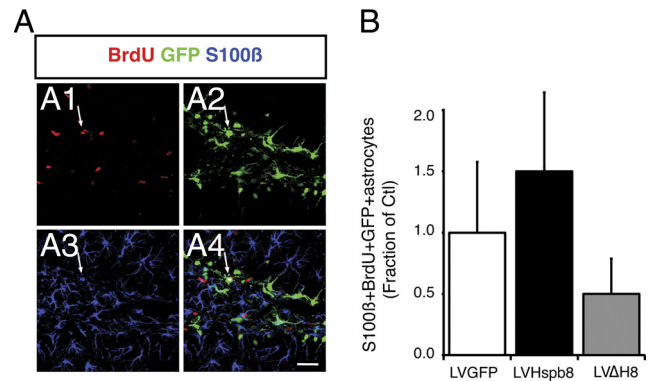


Figure 8. Hspb8 does not affect astrocytic differentiation *in vivo*. **A**, Astrocytic differentiation was assessed by counting BrdU-labeled cells (**A1**, red) and S100β-labeled cells (**A3**, blue) coexpressing GFP (**A2**, green) in the transduced areas (arrows). Scale bar, 40 μm. The merged image shows a triple-labeled cell for BrdU/GFP/S100β (**A4**). **B**, Slight increase in BrdU/GFP/S100β-labeled astrocytes in the dentate gyrus of mice injected with LV-Hspb8 in relation to LV-GFP control mice (not significant). In contrast, the truncated LV-ΔHspb8 decreased the fraction of astrocytes in relation to LV-GFP control mice. *N* = 5 or 6 mice per group. Error bars indicate SEM. *p* = 0.49, LV-GFP versus LV-Hspb8 or LV-Hspb8 versus LV-ΔHspb8 (Fisher's *post hoc* test after one-way ANOVA).

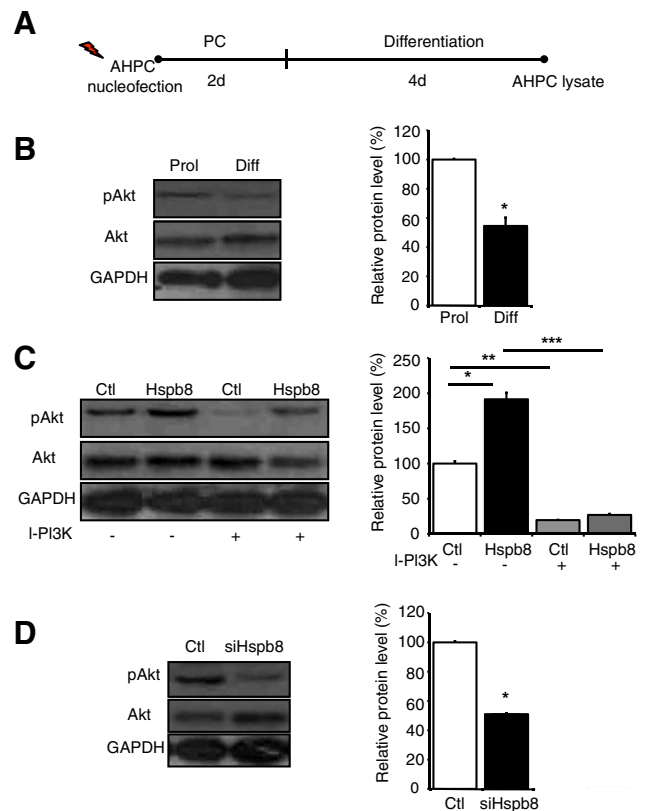


Figure 9. Overexpression of Hspb8 activates the Akt survival pathway during differentiation of precursor cells *in vitro*. **A**, Experimental time line for Hspb8 effects on precursor cell (AHPC) survival. **B**, Levels of phospho-Akt (p-Akt) decrease with differentiation of AHPC. **C**, Overexpression of Hspb8 increases levels of p-Akt, whereas cells that overexpressed Hspb8 and were incubated with PI3K inhibitor, LY294002 10 μM (I-P13K) showed a decrease in levels of p-Akt. **C**, Knock-down of Hspb8 also decreased Akt phosphorylation. **B–D**, Representative autoradiograms and densitometric analysis of p-Akt. **B**, Total Akt (unphosphorylated form). **D**, GAPDH autoradiograms. Data were normalized to total Akt and GAPDH levels. Densitometric analysis represents the mean ± SEM. **B**, *p* = 0.001. **C**, *p* < 0.001. *Control (Ctl) versus Hspb8. **Ctl versus Ctl + I-P13K. ***Hspb8 versus Hspb8 + I-P13K. **D**, *p* < 0.001. *Control (Ctl) versus siHspb8 (Tukey's *post hoc* test after one-way ANOVA).

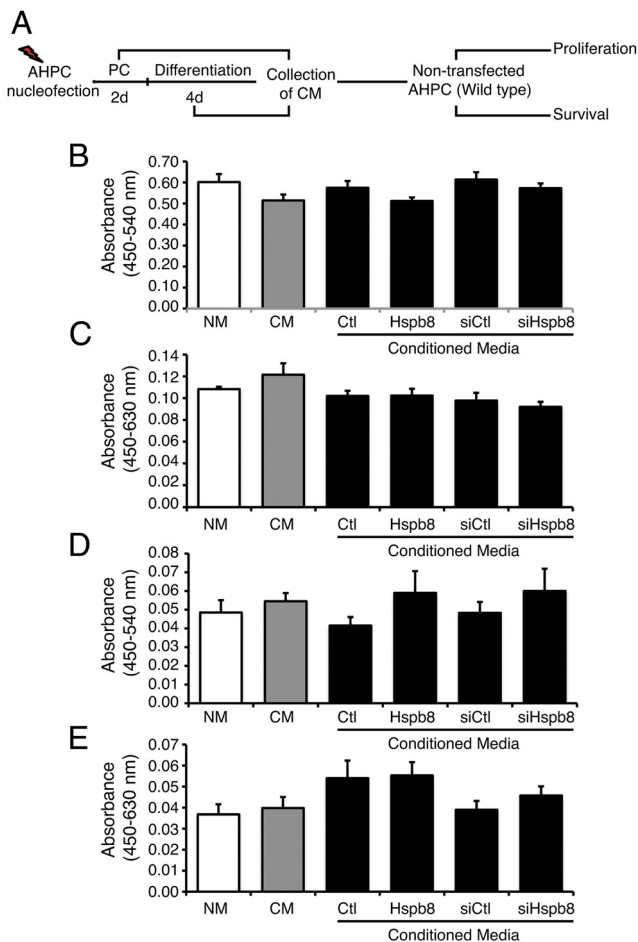


Figure 10. Soluble factors in CM from transfected precursor cells do not affect cell proliferation and survival of wild-type precursor cells *in vitro*. **A**, Time line for experiments, in which Hspb8 and siHspb8 were transfected to collect CM for subsequent treatment of nontransfected (wild-type) precursor cells (AHPC). **B, C**, CM from Hspb8- and siHspb8-expressing precursor cells did not affect cell proliferation (BrdU) and viability (Wst-1) of wild-type cells. Similar results were obtained with cells treated with normal proliferation media (NM) or with CM obtained from nontransfected cells. Error bars indicate SEM. **B, C**, $p = 0.14$ (BrdU) and $p = 0.24$ (Wst-1) (Tukey's *post hoc* test after one-way ANOVA). **D, E**, Wild-type precursor cells treated with CM from Hspb8- and siHspb8-expressing precursor cells did not lead to significant changes of survival. **D**, Wild-type precursor cells were prelabeled with BrdU before cells were switched to CM. Cells treated with NM or CM obtained from differentiated nontransfected cells did not show changes in cell survival. Error bars indicate SEM. **D, E**, $p = 0.15$ (BrdU) and $p = 0.22$ (Wst-1) (Tukey's *post hoc* test after one-way ANOVA). The BrdU and Wst-1 analysis from six wells per group was performed three times each.

survival of different cell types, including neurons (Wang et al., 2004; Yew et al., 2005; Depre et al., 2006; Quraishe et al., 2008; Gurusamy et al., 2009; Sui et al., 2009; Karch and Borchelt, 2010; Gonzalez-Malerva et al., 2011; Kirbach and Golenhofen, 2011). Accordingly, we saw an increase in Hspb8 mRNA and protein, when the differentiation of precursor cells was induced *in vitro* (Fig. 3 B, C).

Overexpression of Hspb8 in precursor cells favored survival and neuronal differentiation. This effect was lost when the fragment comprising amino acid residues 120–145 of the crystallin domain of Hspb8 was deleted.

To confirm these results *in vivo*, we overexpressed Hspb8 with a lentiviral vector. If injected into the hippocampus, these vectors predominantly (<85%) transduce precursor cells and immature neurons rather than mature granule cells (van Hooijdonk et al., 2009). However, this is not exclusive because, as in our experiment at 1 or 5 weeks after viral injection, transduced cells were

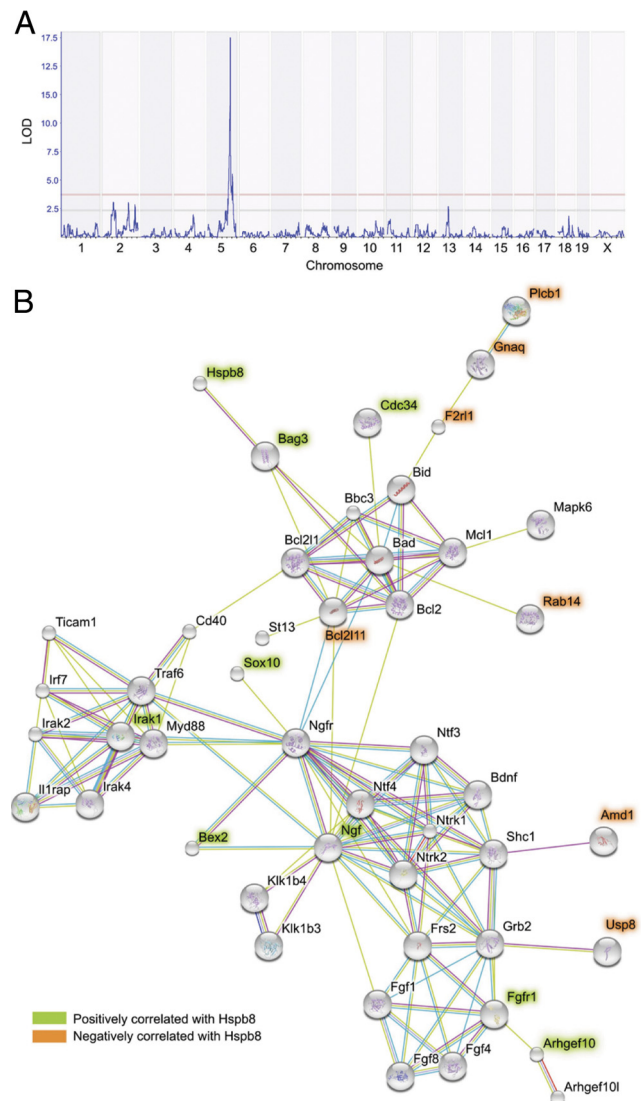


Figure 11. Genetic interactions of Hspb8. **A**, Genomic association mapping of Hspb8 mRNA expression. A composite trait comprised of the principal component of transcript expression measured by three microarray probes was mapped to the mouse genome. A very strong *cis*-QTL (quantitative trait locus at the same location as the Hspb8 gene, blue trace) was highly significant (LOD 17.5), indicating that a sequence variant at this locus strongly affects Hspb8 gene expression. Gray and red horizontal lines indicate genome-wide significance at $p = 0.63$ and $p = 0.05$, respectively. **B**, A subnetwork from the STRING database showing genes associated, directly or indirectly, with Hspb8. Colored connections indicate conceptual links between genes based on different sources of evidence, such as literature commentary, protein–protein binding, and microarray coexpression. Genes exhibiting expression correlation in a pertinent hippocampal microarray study are highlighted green for positive and red for inverse correlation.

found in the subgranular zone (van Hooijdonk et al., 2009; Fitzsimons et al., 2012). This might imply that the virus also infected the radial glia-like stem cells (type 1 cells).

The gain-of-function experiments confirmed that Hspb8 increased cell survival without affecting proliferation and promoted neuronal differentiation without significant changes in astrocytic differentiation. Hspb8-expression in astrocytes is consistent with a recent report (Seidel et al., 2012). Overexpressing Hspb8 with a truncated α -crystallin domain affected the effects of wild-type Hspb8. Surprisingly, the truncated form of the α -crystallin domain of Hspb8 that lacks the putative N-myristoylation site, the N-glycosylation, and protein kinase C motifs did not decrease baseline survival. However, in our con-

struct, other regions remained intact, such as hydrophobic sequences and phosphorylation motifs for Erk that are also important for the chaperon action of Hspb8 (Irobi et al., 2004; Shemetov et al., 2011). Thus, we cannot exclude that other regions of Hspb8 are modulating or cooperating with the deleted portion of the α -crystallin domain to promote survival. At the present time, it is not known which domain of Hspb8 is involved in protein interaction or whether Hspb8 is able to directly activate the kinases or promote the subcellular redistribution of proteins involved in survival. Further work is required to understand the contribution of each domain or regulatory site of Hspb8 to regulate adult hippocampal neurogenesis. However, silencing of Hspb8 confirmed that Hspb8 is relevant for cell survival and neuronal differentiation. This effect is also in line with reports that silencing Hspb8 caused cell death in MCF7 human breast adenocarcinoma cells (Gonzalez-Malerva et al., 2011).

Relatedly, missense mutations in residues of the crystallin domain of Hspb8 have caused neurodegeneration in cultured motor neurons without inducing cell death (Irobi et al., 2010). The α -crystallin is mutated in distal hereditary motor neuropathy and Charcot-Marie-Tooth neuropathy type 2. The effect of this mutation, however, is specific to motor neurons (Irobi et al., 2010). Our data indicate that the α -crystallin nevertheless has more general effects on neuronal survival and differentiation. These data are also in accordance with studies, in which overexpression of Hspb8 protected cardiomyocytes whereas missense mutations in Hspb8 reduced survival (Sanbe et al., 2009; Carra et al., 2010).

Of note, abnormal expression of α B-crystallin (HSPB5) in oligodendrocytes was suspected to underlie a familial syndrome characterized by atrophy of the dentate gyrus, dementia, and congenital cataract (Hudson and Munoz, 1997). A link to adult neurogenesis was proposed and has been discussed (Hudson and Munoz, 2003). However, no mutation has been found in the crystallin gene. In the heart, a mutation to Hspb8 causes desmin-related cardiomyopathy, in which desmin and Hspb8 form damaging amyloid. Intriguingly, this process is blocked by Hspb8 (Sanbe et al., 2009).

Consequently, the mechanisms underlying the survival-promoting effects of Hspb8 appear to be multifold and complex. In the heart, Hspb8 acts via the activation of key enzymes for cell survival, such as PI3K (Sui et al., 2009), accompanied by an increase in phosphorylated Akt. Akt is a serine/threonine protein kinase that acts downstream of PI3K and is involved in glucose metabolism, cell proliferation, apoptosis, transcription, cell migration, and dendritic growth through mTOR (Depre et al., 2006; Danan et al., 2007; Kim et al., 2009, 2012; Sui et al., 2009). Future studies should examine direct or indirect effects of Hspb8 on other aspects of adult neurogenesis, including neurite growth and maturation and the functional integration of the newborn cells.

Hspb8 apparently directly binds to Akt and 5AMP-activated protein kinase for promoting the nuclear translocation of both proteins to promote survival (Depre et al., 2006). Here, we show that Hspb8 acts via PI3K to activate Akt for promoting the survival of differentiating precursor cells (Fig. 9C), which is in line with the assumed role for Akt in modulating survival in the brain (Brazil et al., 2004). In addition, our *in vitro* data suggest that the effect of Hspb8 might be based on a cell-autonomous mechanism because soluble factors contained in CM from precursor cells or differentiated precursor cells transfected with Hspb8 or with siRNA oligonucleotides for Hspb8 did not affect cell proliferation and survival (Fig. 10). However, our study does not rule out changes mediated through Hspb8-dependent cell–cell interactions *in vivo*. Given that Hspb8 is also expressed in blood vessels

(Fig. 1), the *in vivo* experiments cannot fully exclude an extrinsic role for Hspb8. The vasculature presumably plays an important role for neurogenesis, given that the vascular niche presumably provides many regulators that could act as extrinsic cues for neurogenesis (Palmer et al., 2000). At least for the songbird system, such regulatory link is well established (Louissant et al., 2002).

Moreover, Hspb8 inhibits proapoptotic effectors, such as Bad, Foxo, and glycogen synthase kinase-3 β , and activates antiapoptotic effectors as endothelial NO synthase isoforms (Depre et al., 2006). In cardiomyocytes treated with HSP inducer geranylgeranylacetone, Hspb8 inhibited the activation of caspase-3 (Sanbe et al., 2009). Together, these data suggest multiple potential mechanisms and not a single mode of action.

Thus, our findings related to the prosurvival effect of Hspb8 are consistent with recent reports indicating that the promotion of survival is an important step in the stimulation of neurogenesis (Pieper et al., 2010; Sahay et al., 2011), which might consequently enhance the functionality of the dentate gyrus (Sahay et al., 2011).

In addition, we used our database of hippocampal gene expression in BXD mice to identify potential target genes in our system (Overall et al., 2009). This approach makes use of genetically determined variation in transcript expression to detect covariance as indicator of a shared direct or indirect causality.

Indeed, Hspb8 expression positively correlated with genes involved in cell growth (*Hopx*, *Ddr1*, *Fgfr1*, and *Ngf*). Some of these genes, such as *Ngf*, increase Bcl and decrease Bax to prevent neuronal apoptosis (Deckwerth et al., 1996; Putcha et al., 1999; Arthur et al., 2006). Consequently, *Hspb8* also positively correlated with other genes regulating apoptosis, such as *Ticam1* and *Bag3*. The correlation between *Hspb8* and *Bag3* is particularly interesting in the context of adult neurogenesis given that recent studies indicated that *Bag3* binds to Hspb8 to promote autophagy in different cell types (Carra et al., 2008a, 2008b; Carra, 2009; Fuchs et al., 2009; Gurusamy et al., 2009; McCollum et al., 2009; Arndt et al., 2010). Autophagy is a catabolic process involving lysosomal degradation and recycling of cytoplasmic constituents and occurs at low basal levels in all tissues. Autophagy contributes to tissue homeostasis (Levine and Kroemer, 2008) and is rapidly upregulated after damage. Nevertheless, in some circumstances, the upregulation of autophagy protects against the onset of apoptosis (Levine and Kroemer, 2008).

Similar to the anti-amyloidogenic effect of Hspb8 in cardiomyocytes, a prevention of β -sheet formation in Alzheimer disease and Huntingin aggregation in Huntington disease have also been suggested (Wilhelmus et al., 2006; Carra et al., 2009).

A related small heat-shock protein, Hspb4 (CryaA) is expressed in a highly region-specific manner and was discovered to be an essential survival factor for the dopaminergic interneurons in the olfactory bulb (Ninkovic et al., 2010). It thus seems that crystallins play important roles in neuronal survival, with different genes exerting more or less specific functions in different populations of cells. In conclusion, we here provide evidence that Hspb8 acts as an important factor in regulating survival in the adult hippocampal neurogenesis through the activation of a key survival pathway that involves PI3K and Akt.

References

- Arndt V, Dick N, Tawo R, Dreiseidler M, Wenzel D, Hesse M, Fürst DO, Saftig P, Saint R, Fleischmann BK, Hoch M, Höfeld J (2010) Chaperone-assisted selective autophagy is essential for muscle maintenance. *Curr Biol* 20:143–148. CrossRef Medline
- Arthur DB, Georgi S, Akassoglou K, Insel PA (2006) Inhibition of apoptosis by P2Y2 receptor activation: novel pathways for neuronal survival. *J Neurosci* 26:3798–3804. CrossRef Medline

- Babu H, Ramírez-Rodríguez G, Fabel K, Bischofberger J, Kempermann G (2009) Synaptic network activity induces neuronal differentiation of adult hippocampal precursor cells through BDNF signaling. *Front Neurosci* 3:49. [CrossRef Medline](#)
- Babu H, Claassen JH, Kannan S, Rünker AE, Palmer T, Kempermann G (2011) A protocol for isolation and enriched monolayer cultivation of neural precursor cells from mouse dentate gyrus. *Front Neurosci* 5:89. [CrossRef Medline](#)
- Bracko O, Singer T, Aigner S, Knobloch M, Winner B, Ray J, Clemenson GD Jr, Suh H, Couillard-Despres S, Aigner L, Gage FH, Jessberger S (2012) Gene expression profiling of neural stem cells and their neuronal progeny reveals IGF2 as a regulator of adult hippocampal neurogenesis. *J Neurosci* 10:3376–3387. [CrossRef Medline](#)
- Brazil DP, Yang ZZ, Hemmings BA (2004) Advances in protein kinase B signalling: AKTion on multiple fronts. *Trends Biochem Sci* 29:233–242. [CrossRef Medline](#)
- Carra S (2009) The stress-inducible HspB8-Bag3 complex induces the eIF2 α kinase pathway: implications for protein quality control and viral factory degradation? *Autophagy* 5:428–429. [CrossRef Medline](#)
- Carra S, Seguin SJ, Landry J (2008a) HspB8 and Bag3: a new chaperone complex targeting misfolded proteins to macroautophagy. *Autophagy* 4:237–239. [Medline](#)
- Carra S, Seguin SJ, Lambert H, Landry J (2008b) HspB8 chaperone activity toward poly(Q)-containing proteins depends on its association with Bag3, a stimulator of macroautophagy. *J Biol Chem* 283:1437–1444. [CrossRef Medline](#)
- Carra S, Brunsting JF, Lambert H, Landry J, Kampinga HH (2009) HspB8 participates in protein quality control by a non-chaperone-like mechanism that requires eIF2 α phosphorylation. *J Biol Chem* 284:5523–5532. [CrossRef Medline](#)
- Carra S, Boncoraglio A, Kanon B, Brunsting JF, Minoia M, Rana A, Vos MJ, Seidel K, Sibon OC, Kampinga HH (2010) Identification of the *Drosophila* ortholog of HSPB8: implication of HSPB8 loss of function in protein folding diseases. *J Biol Chem* 285:37811–37822. [CrossRef Medline](#)
- Cleveland DW, Fischer SG, Kirschner MW, Laemmli UK (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J Biol Chem* 252:1102–1106. [Medline](#)
- Crippa V, Sau D, Rusmini P, Boncoraglio A, Onesto E, Bolzoni E, Galbiati M, Fontana E, Marino M, Carra S, Bendotti C, De Biasi S, Poletti A (2010) The small heat shock protein B8 (HspB8) promotes autophagic removal of misfolded proteins involved in amyotrophic lateral sclerosis (ALS). *Hum Mol Genet* 19:3440–3456. [CrossRef Medline](#)
- Danan IJ, Rashed ER, Depré C (2007) Therapeutic potential of H11 kinase for the ischemic heart. *Cardiovasc Drug Rev* 25:14–29. [CrossRef Medline](#)
- David JC, Boelens WC, Grongnet JF (2006) Up-regulation of heat shock protein HSP 20 in the hippocampus as an early response to hypoxia of the newborn. *J Neurochem* 99:570–581. [CrossRef Medline](#)
- Deckwerth TL, Elliott JL, Knudson CM, Johnson EM Jr, Snider WD, Korsmeyer SJ (1996) BAX is required for neuronal death after trophic factor deprivation and during development. *Neuron* 17:401–411. [CrossRef Medline](#)
- Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA (2003) DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 4:P3. [CrossRef Medline](#)
- Depré C, Wang L, Sui X, Qiu H, Hong C, Hedhli N, Ginion A, Shah A, Pelat M, Bertrand L, Wagner T, Gaussin V, Vatner SF (2006) H11 kinase prevents myocardial infarction by preemptive preconditioning of the heart. *Circ Res* 98:280–288. [CrossRef Medline](#)
- Fitzsimons CP, van Hooijdonk LWA, Schouten M, Zalachoras I, Brinks V, Zheng T, Schouten TG, Saaltink DJ, Dijkmans T, Steindler DA, Verhaagen J, Verbeek FJ, Lucassen P, de Kloet ER, Meijer OC, Karst H, Joels M, Oitzl MS, Vreugdenhil E (2012) Knockdown of the glucocorticoid receptor alters functional integration of newborn neurons in the adult hippocampus and impairs fear-motivated behavior. *Mol Psychol* Advance online publication. Retrieved August 28, 2012. doi:10.1038/mp.2012.123. [CrossRef Medline](#)
- Fuchs M, Poirier DJ, Seguin SJ, Lambert H, Carra S, Charette SJ, Landry J (2009) Identification of the key structural motifs involved in HspB8/HspB6-Bag3 interaction. *Biochem J* 425:245–255. [CrossRef Medline](#)
- Galea LA (2008) Gonadal hormone modulation of neurogenesis in the dentate gyrus of adult male and female rodents. *Brain Res Rev* 57:332–341. [CrossRef Medline](#)
- Geraerts M, Eggermont K, Hernandez-Acosta P, Garcia-Verdugo JM, Baecklandt V, Debyser Z (2006) Lentiviral vectors mediate efficient and stable gene transfer in adult neural stem cells in vivo. *Hum Gene Ther* 17:635–650. [CrossRef Medline](#)
- Gonzalez-Malerva L, Park J, Zou L, Hu Y, Moradpour Z, Pearlberg J, Sawyer J, Stevens H, Harlow E, LaBaer J (2011) High-throughput ectopic expression screen for tamoxifen resistance identifies an atypical kinase that blocks autophagy. *Proc Natl Acad Sci U S A* 108:2058–2063. [CrossRef Medline](#)
- Gurusamy N, Lekli I, Gherghiceanu M, Popescu LM, Das DK (2009) BAG-1 induces autophagy for cardiac cell survival. *Autophagy* 5:120–121. [CrossRef Medline](#)
- Hagemann TL, Boelens WC, Wawrousek EF, Messing A (2009) Suppression of GFAP toxicity by α B-crystallin in mouse models of Alexander disease. *Human Mol Genet* 18:1190–1199. [CrossRef Medline](#)
- Huang da W, Sherman BT, Lempicki RA (2009a) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4:44–57. [CrossRef Medline](#)
- Huang da W, Sherman BT, Zheng X, Yang J, Imamichi T, Stephens R, Lempicki RA (2009b) Extracting biological meaning from large gene lists with DAVID. *Curr Protoc Bioinformatics* 13:11. [CrossRef Medline](#)
- Hudson AJ, Munoz DG (1997) A familial syndrome of congenital cataract, mental impairment, and dentate gyrus atrophy. *Ann Neurol* 41:512–520. [CrossRef Medline](#)
- Hudson AJ, Munoz DG (2003) Familial dementia with dentate atrophy and failure of neurogenesis. *Ann Neurol* 53:420–421; author reply 421. [CrossRef Medline](#)
- 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, Van Broeckhoven C, et al (2004) Hot-spot residue in small heat-shock protein 22 causes distal motor neuropathy. *Nat Genet* 36:597–601. [CrossRef Medline](#)
- Irobi J, Almeida-Souza L, Asselbergh B, De Winter V, Goethals S, Dierick I, Krishnan J, Timmermans JP, Robberecht W, De Jonghe P, Van Den Bosch L, Janssens S, Timmerman V (2010) Mutant HSPB8 causes motor neuron-specific neurite degeneration. *Hum Mol Genet* 19:3254–3265. [CrossRef Medline](#)
- Jessberger S, Aigner S, Clemenson GD Jr, Toni N, Lie DC, Karalay O, Overall R, Kempermann G, Gage FH (2008) Cdk5 regulates accurate maturation of newborn granule cells in the adult hippocampus. *PLoS Biol* 6:e272. [CrossRef Medline](#)
- Karch CM, Borchelt DR (2010) An examination of α B-crystallin as a modifier of SOD1 aggregate pathology and toxicity in models of familial amyotrophic lateral sclerosis. *J Neurochem* 113:1092–1100. [CrossRef Medline](#)
- Kempermann G, Jessberger S, Steiner B, Kronenberg G (2004) Milestones of neuronal development in the adult hippocampus. *Trends Neurosci* 27:447–452. [CrossRef Medline](#)
- Kempermann G, Chesler EJ, Lu L, Williams RW, Gage FH (2006) Natural variation and genetic covariance in adult hippocampal neurogenesis. *Proc Natl Acad Sci U S A* 103:780–785. [CrossRef Medline](#)
- Kim JY, Duan X, Liu CY, Jang MH, Guo JU, Pow-anpongkul N, Kang E, Song H, Ming GL (2009) DISC regulates new neuron development in the adult brain via modulation of AKT-mTOR signaling through kiaz1212. *Neuron* 63:761–773. [CrossRef Medline](#)
- Kim JY, Liu CY, Zhang F, Duan X, Wen Z, Song J, Feighery E, Lu B, Rujescu D, St Clair D, Christian K, Callicott JH, Weinberger DR, Song H, Ming GL (2012) Interplay between DISC1 and GABA signaling regulates neurogenesis in mice and risk for schizophrenia. *Cell* 148:1051–1064. [CrossRef Medline](#)
- Kirbach BB, Golenhofen N (2011) Differential expression and induction of small heat shock proteins in rat brain and cultured hippocampal neurons. *J Neurosci Res* 89:162–175. [CrossRef Medline](#)
- Koo JW, Russo SJ, Ferguson D, Nestler EJ, Duman RS (2010) Nuclear factor- κ B is a critical mediator of stress-impaired neurogenesis and depressive behavior. *Proc Natl Acad Sci U S A* 107:2669–2674. [CrossRef Medline](#)
- Leuner B, Caponiti JM, Gould E (2012) Oxytocin stimulates adult neuro-

- genesis even under conditions of stress and elevated glucocorticoids. *Hippocampus* 22:861–868. [CrossRef Medline](#)
- Levine B, Kroemer G (2008) Autophagy in the pathogenesis of disease. *Cell* 132:27–42. [CrossRef Medline](#)
- Lie DC, Colamarino SA, Song HJ, Désiré L, Mira H, Consiglio A, Lein ES, Jessberger S, Lansford H, Dearie AR, Gage FH (2005) Wnt signalling regulates adult hippocampal neurogenesis. *Nature* 437:1370–1375. [CrossRef Medline](#)
- Louissant A Jr, Rao S, Leventhal C, Goldman SA (2002) Coordinated interaction of angiogenesis and neurogenesis in the adult songbird brain. *Neuron* 34:945–960. [CrossRef Medline](#)
- Mastrangelo MA, Sudol KL, Narrow WC, Bowers WJ (2009) Interferon- γ differentially affects Alzheimer's disease pathologies and induces neurogenesis in triple transgenic-AD mice. *Am J Pathol* 175:2076–2088. [CrossRef Medline](#)
- McCollum AK, Casagrande G, Kohn EC (2009) Caught in the middle: the role of Bag3 in disease. *Biochem J* 425:e1–e3. [CrossRef Medline](#)
- Mueller AD, Pollock MS, Lieblich SE, Epp JR, Galea LA, Mistlberger RE (2008) Sleep deprivation can inhibit adult hippocampal neurogenesis independent of adrenal stress hormones. *Am J Physiol Regul Integr Comp Physiol* 294:R1693–R1703. [CrossRef Medline](#)
- Ninkovic J, Pinto L, Petricca S, Lepier A, Sun J, Rieger MA, Schroeder T, Cvekl A, Favor J, Götz M (2010) The transcription factor Pax6 regulates survival of dopaminergic olfactory bulb neurons via crystallin α A. *Neuron* 68:682–694. [CrossRef Medline](#)
- Overall RW, Kempermann G, Peirce J, Lu L, Goldowitz D, Gage FH, Goodwin S, Smit AB, Airey DC, Rosen GD, Schalkwyk LC, Sutter TR, Nowakowski RS, Whatley S, Williams RW (2009) Genetics of the hippocampal transcriptome in mouse: a systematic survey and online neurogenomics resource. *Front Neurosci* 3:55. [CrossRef Medline](#)
- Palmer TD, Willhoite AR, Gage FH (2000) Vascular niche for adult hippocampal neurogenesis. *J Comp Neurol* 425:479–494. [CrossRef Medline](#)
- Pawluski JL, Brummelte S, Barha CK, Crozier TM, Galea LA (2009) Effects of steroid hormones on neurogenesis in the hippocampus of the adult female rodent during the estrous cycle, pregnancy, lactation and aging. *Front Neuroendocrinol* 30:343–357. [CrossRef Medline](#)
- Peng Q, Masuda N, Jiang M, Li Q, Zhao M, Ross CA, Duan W (2008) The antidepressant sertraline improves the phenotype, promotes neurogenesis and increases BDNF levels in the R6/2 Huntington's disease mouse model. *Exp Neurol* 210:154–163. [CrossRef Medline](#)
- Perera TD, Coplan JD, Lisanby SH, Lipira CM, Arif M, Carpio C, Spitzer G, Santarelli L, Scharf B, Hen R, Rosoklija G, Sackeim HA, Dwork AJ (2007) Antidepressant-induced neurogenesis in the hippocampus of adult non-human primates. *J Neurosci* 27:4894–4901. [CrossRef Medline](#)
- Pieper AA, Xie S, Capota E, Estill SJ, Zhong J, Long JM, Becker GL, Huntington P, Goldman SE, Shen CH, Capota M, Britt JK, Kotti T, Ure K, Brat DJ, Williams NS, MacMillan KS, Naidoo J, Melito L, Hsieh J, De Brabander J, Ready JM, McKnight SL (2010) Discovery of a proneurogenic, neuroprotective chemical. *Cell* 142:39–51. [CrossRef Medline](#)
- Pinnock SB, Herbert J (2008) Brain-derived neurotrophic factor and neurogenesis in the adult rat dentate gyrus: interactions with corticosterone. *Eur J Neurosci* 27:2493–2500. [CrossRef Medline](#)
- Putcha GV, Deshmukh M, Johnson EM Jr (1999) BAX translocation is a critical event in neuronal apoptosis: regulation by neuroprotectants, BCL-2, and caspases. *J Neurosci* 19:7476–7485. [Medline](#)
- Quraishe S, Asuni A, Boelens WC, O'Connor V, Wytenbach A (2008) Expression of the small heat shock protein family in the mouse CNS: differential anatomical and biochemical compartmentalization. *Neuroscience* 153:483–491. [CrossRef Medline](#)
- Ramírez-Rodríguez G, Klempin F, Babu H, Benítez-King G, Kempermann G (2009) Melatonin modulates cell survival of new neurons in the hippocampus of adult mice. *Neuropsychopharmacology* 34:2180–2191. [CrossRef Medline](#)
- Ramírez-Rodríguez G, Ortiz-López L, Domínguez-Alonso A, Benítez-King GA, Kempermann G (2011) Chronic treatment with melatonin stimulates dendrite maturation and complexity in adult hippocampal neurogenesis of mice. *J Pineal Res* 50:29–37. [CrossRef Medline](#)
- Rossi C, Angelucci A, Costantin L, Braschi C, Mazzantini M, Babbini F, Fabbri ME, Tessarollo L, Maffei L, Berardi N, Caleo M (2006) Brain-derived neurotrophic factor (BDNF) is required for the enhancement of hippocampal neurogenesis following environmental enrichment. *Eur J Neurosci* 24:1850–1856. [CrossRef Medline](#)
- Sahay A, Scobie KN, Hill AS, O'Carroll CM, Kheirbek MA, Burghardt NS, Fenton AA, Dranovsky A, Hen R (2011) Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. *Nature* 472:466–470. [CrossRef Medline](#)
- Sanbe A, Daicho T, Mizutani R, Endo T, Miyauchi N, Yamauchi J, Tanonaka K, Glabe C, Tanoue A (2009) Protective effect of geranylgeranylacetone via enhancement of HSPB8 induction in desmin-related cardiomyopathy. *PLoS One* 4:e5351. [CrossRef Medline](#)
- Seidel K, Vinet J, Dunnen WF, Brunt ER, Meister M, Boncoraglio A, Zijlstra MP, Boddeke HW, Rub U, Kampinga HH, Carra S (2012) The HspB8-BAG3 chaperone complex is upregulated in astrocytes in the human brain affected by protein aggregation diseases. *Neuropathol Appl Neurobiol* 1:39–53. [CrossRef Medline](#)
- Shemetov AA, Seit-Nebi AS, Gusev NB (2011) Phosphorylation of human small heat shock protein HspB8 (Hsp22). *Mol Cell Biochem* 1:47–55. [CrossRef Medline](#)
- Snel B, Lehmann G, Bork P, Huynen MA (2000) STRING: a web-server to retrieve and display the repeatedly occurring neighbourhood of a gene. *Nucleic Acids Res* 28:3442–3444. [CrossRef Medline](#)
- Sui X, Li D, Qiu H, Gaussin V, Depre C (2009) Activation of the bone morphogenetic protein receptor by H11kinase/Hsp22 promotes cardiac cell growth and survival. *Circ Res* 104:887–895. [CrossRef Medline](#)
- Szklarczyk D, Franceschini A, Kuhn M, Simonovic M, Roth A, Minguez P, Doerks T, Stark M, Muller J, Bork P, Jensen LJ, von Mering C (2011) The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. *Nucleic Acids Res* 39:D561–D568. [CrossRef Medline](#)
- Tang D, Khaleque MA, Jones EL, Theriault JR, Li C, Wong WH, Stevenson MA, Calderwood SK (2005) Expression of heat shock proteins and heat shock protein messenger ribonucleic acid in human prostate carcinoma in vitro and in tumors in vivo. *Cell Stress Chaperones* 10:46–58. [CrossRef Medline](#)
- van Hooijdonk LW, Ichwan M, Dijkmans TF, Schouten TG, de Backer MW, Adan RA, Verbeek FJ, Vreugdenhil E, Fitzsimons CP (2009) Lentivirus-mediated transgene delivery to the hippocampus reveals sub-field specific differences in expression. *BMC Neurosci* 10:2. [CrossRef Medline](#)
- Wang L, Zajac A, Hedhli N, Depre C (2004) Increased expression of H11 kinase stimulates glycogen synthesis in the heart. *Mol Cell Biochem* 265:71–78. [CrossRef Medline](#)
- Wilhelmus MM, Boelens WC, Otte-Höller I, Kamps B, Kusters B, Maat-Schieman ML, de Waal RM, Verbeek MM (2006) Small heat shock protein HspB8: its distribution in Alzheimer's disease brains and its inhibition of amyloid- β protein aggregation and cerebrovascular amyloid- β toxicity. *Acta Neuropathol* 111:139–149. [CrossRef Medline](#)
- Yew EH, Cheung NS, Choy MS, Qi RZ, Lee AY, Peng ZF, Melendez AJ, Manikandan J, Koay ES, Chiu LL, Ng WL, Whiteman M, Kandiah J, Halliwell B (2005) Proteasome inhibition by lactacystin in primary neuronal cells induces both potentially neuroprotective and proapoptotic transcriptional responses: a microarray analysis. *J Neurochem* 94:943–956. [CrossRef Medline](#)
- Zhang L, Blomgren K, Kuhn HG, Cooper-Kuhn CM (2009) Effects of postnatal thyroid hormone deficiency on neurogenesis in the juvenile and adult rat. *Neurobiol Dis* 34:366–374. [CrossRef Medline](#)