HHS Public Access

Author manuscript

Exp Hematol. Author manuscript; available in PMC 2016 April 01.

Published in final edited form as:

Exp Hematol. 2015 April ; 43(4): 319–330.e10. doi:10.1016/j.exphem.2014.12.005.

Reduced Levels of Hspa9 Attenuates Stat5 Activation in Mouse B-cells

Kilannin Krysiak[a,c], **Justin F. Tibbitts**[a,c], **Jin Shao**[a,c], **Tuoen Liu**[a,c], **Matthew Ndonwi**[a,c], and **Matthew J. Walter**[a,b,c,d,#]

[a]Department of Medicine, Washington University School of Medicine, St. Louis, MO, USA

[b]Department of Genetics, Washington University School of Medicine, St. Louis, MO, USA

[c]Division of Oncology, Washington University School of Medicine, St. Louis, MO, USA

[d]Siteman Cancer Center, Washington University School of Medicine, St. Louis, MO, USA

Abstract

HSPA9 is located on chromosome 5q31.2 in humans, a region that is commonly deleted in patients with myeloid malignancies [del(5q)], including myelodysplastic syndromes (MDS). *HSPA9* expression is reduced by 50% in patients with del(5q)-associated MDS, consistent with haploinsufficient levels. Zebrafish mutants and knockdown studies in human and mouse cells have implicated a role for *HSPA9* in hematopoiesis. To comprehensively evaluate the effects of Hspa9 haploinsufficiency on hematopoiesis, we generated an *Hspa9* knockout mouse model. While homozygous knockout of *Hspa9* is embryonic lethal, mice with heterozygous deletion of *Hspa9* (*Hspa9*$^{+/-}$) are viable and have a 50% reduction in *Hspa9* expression. *Hspa9*$^{+/-}$ mice have normal basal hematopoiesis and do not develop MDS. However, *Hspa9*$^{+/-}$ mice have a cell-intrinsic reduction in bone marrow CFU-PreB colony formation without alterations in the number of B-cell progenitors *in vivo*, consistent with a functional defect in *Hspa9*$^{+/-}$ B-cell progenitors. We further reduced *Hspa9* expression (<50%) using RNAi and observe reduced B-cell progenitors *in vivo*, indicating that appropriate levels (≥50%) of Hspa9 are required for normal B-lymphopoiesis *in vivo*. Knockdown of Hspa9 in an IL-7 dependent mouse B-cell line reduced Stat5 phosphorylation following IL-7 receptor stimulation, supporting a role for Hspa9 in Stat5 signaling in B-cells. Collectively, these data implicate a role for *Hspa9* in B-lymphopoiesis and Stat5 activation downstream of IL-7 signaling.

Keywords

Hspa9; hematopoiesis; knockout mouse; B-cell; Stat5

[#]Address correspondence to: Dr. Matthew J. Walter, M.D., Washington University School of Medicine, Campus Box 8007, 660 South Euclid Avenue, Saint Louis, MO 63110, (314) 362-9405, mjwalter@dom.wustl.edu.

INTRODUCTION

HSPA9 (GRP75/mortalin/mtHSP70) is a member of the highly conserved HSP70 family of proteins [1]. Across species, HSP70 family members play an essential role in protein homeostasis and cellular stress response [1,2]. Overexpression of HSP70 proteins results in increased cell survival and is associated with a multitude of cancers, while reduced expression increases apoptosis and susceptibility to cellular stresses [2,3]. Members of this family share a common protein binding function; however, unique expression patterns, subcellular localization and co-chaperone interactions have diversified the functions of the 8 well-described Hsp70 proteins expressed in mouse and human. To date, knockout mice have been described for 5 Hsp70 family members: *Hspa1a*, *Hspa1b*, *Hspa2*, *Hspa5* and *Hspa8* [1]. Homozygous knockout in mice is embryonic lethal for *Hspa5* and *Hspa8* [4,5].

Several HSP70 family proteins influence hematopoiesis through a variety of mechanisms, including their regulatory roles in cell signaling, cell cycle and glycolytic metabolism [6–11]. A role for *HSPA9*, the only HSP70 protein localized in the mitochondria, in hematopoiesis has been highlighted using multiple models. Zebrafish homozygous for a point mutation that results in a hypomorphic allele suffer from severe anemia and have defects in erythroid differentiation with increased apoptosis [12,13]. HSPA9 has also been implicated in erythropoiesis as a downstream mediator of erythropoietin signaling in a primary human CD34+ cell culture system [14]. RNAi-mediated knockdown of *HSPA9* in primary human CD34+ cells and *Hspa9* in a murine bone marrow transplant model disrupted erythroid differentiation, reduced mature B-cell numbers, reduced cellular proliferation, and increased apoptosis [15]. In a separate study, increased reactive oxygen species and reduced quiescence in hematopoietic stem cells was observed following RNAi-mediated knockdown of *Hspa9* in a mouse bone marrow transplant model [16]. Finally, *HSPA9* lies within a commonly deleted region affecting one allele of human chromosome 5 (del[5q]) that is lost in patients with myeloid malignancies [17–20]. Consistent with heterozygous loss, *HSPA9* mRNA expression is reduced by 50% in del(5q)-associated myelodysplastic syndrome (MDS) compared to non-del(5q) MDS and normal CD34+ cells, suggesting that haploinsufficiency may contribute to disease pathogenesis [21].

In order to precisely model *Hspa9* haploinsufficiency, we created a novel *Hspa9* knockout mouse model. Homozygous knockout of *Hspa9* is embryonic lethal, but heterozygous knockout mice ($Hspa9^{+/-}$) are viable and express 50% reduced levels of Hspa9 compared to wild-type mice. While $Hspa9^{+/-}$ mice do not develop MDS or leukemia and have normal erythropoiesis, we establish a role for *Hspa9* in B-lymphopoiesis through attenuation of Stat5 activation.

MATERIAL AND METHODS

Generation of $Hspa9^{+/-}$ mice

Normal karyotype C57Bl/6N embryonic stem (ES) cell clones containing a gene trap insertion in the third intron of *Hspa9* ($Hspa9^{+/Gt(IST14901H6)TIGM}$) were obtained from the Texas A&M Institute for Genomic Medicine [22]. Mice were maintained on a C57Bl/6N background, unless otherwise indicated. Southern blots were performed using standard

methods. PCR genotyping was performed as described previously and genotyping primer sequences are listed in Table S2 [22]. Mouse procedures were performed according to protocols approved by the Washington University Animal Studies Committee.

Western blot and RT-PCR analysis

Western blots were performed as previously described [15,23]. Antibodies used are described in Table S1. RNA was isolated using TRIzol LS reagent (Invitrogen), DNase treated (TURBO DNA-free kit, Ambion), converted to cDNA (SuperScript III First-Strand Synthesis System, Invitrogen), and analyzed using a StepOne Plus Real time PCR System (Applied Biosystems). Individual cDNA samples were normalized according to their levels of Gapdh or β-Actin. The relative standard curve method was used for analysis. Primers used are listed in Table S2.

Flow cytometry

Bone marrow, spleen or peripheral blood cells were isolated by standard methods and red cells were lysed with ACK lysis buffer. Antibodies used are listed in Table S1. For intracellular flow, cells were fixed in 2% paraformaldehyde and permeabilized in 100% methanol prior to intracellular staining. Fluorescent detection was performed by FACScan or Gallios cytometers (BD biosciences) and analyzed using FlowJo software. Cells were isolated using an iCyt Synergy flow sorter (Sony) or Beckman Coulter MoFlo.

Colony forming assays

Bone marrow or spleen cells were harvested, red cell lysed, and plated in methylcellulose media (Stem Cell Technologies) to detect CFU-C/CFU-E/BFU-E (M3434), mature BFU-E (M3234 supplemented with 3 or 6U/mL hEPO), and CFU-PreB (M3630) colonies per standard protocols, unless otherwise indicated. CFU-C and CFU-PreB colonies were scored on day 7–10. BFU-E/mature BFU-E colonies were scored on day 10–11. CFU-E colonies were scored on day 3 following benzidine staining.

Viral preparation, transduction, and bone marrow transplantation

HSPA9 cDNA was cloned from the previously described FLAG-Mortalin-WT vector into an MSCV-IRES-GFP vector [24]. For virus production, HEK293T cells were transfected with MSCV and EcoPack packaging plasmids by calcium phosphate transfection using CalPhos Mammalian Transfection Kit (Clontech) and viral supernatant was collected. Fcy-si control (shLUC) and Hspa9 knockdown (shHspa9 #3) shRNAs were generated as previously described [15]. For HSPA9 overexpression, an MOI of 1 was used. For Fcy-si experiments, lineage-depleted Ly5.2 bone marrow cells were transduced once with an MOI of 20 by spinoculation, as previously described [15]. On the following day, $0.75–1\times10^6$ cells were transplanted into lethally irradiated recipients (Ly5.1).

Microarray analysis

Cells were isolated from CFU-PreB media on day 7 and B220+ cells were sorted directly into TRIzol LS Reagent. Samples were hybridized to Mouse Gene 1.0 ST arrays and gene-level normalization and signal summarization was performed for all arrays using Expression

Console (Affymetrix). Differentially expressed individual genes were identified using Significance Analysis of Microarrays (SAM) [25]. Pathway analysis was performed on significantly altered genes using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) [26,27]. Microarray data will be deposited in GEO.

Cell culture

B7 (Ba/F3 cells stably transduced with MSCV-mIL7R-IRES-GFP) cells were maintained at a density <2 million cells/mL in media with 10ng/mL mIL-7 (Peprotech), as previously described [28]. B7 cells were electroporated with 1000nM *Hspa9*-targeting siRNAs (Thermo Scientific: D-057872-03 [siRNA 1] and D-057872-04 [siRNA 2]) or non-targeting control siRNA (Thermo Scientific: D-001206-14) using a Nucleofector Device (Lonza) program X-001 according to manufacturer's protocols.

Statistical analysis and visualization

Statistical analysis and graphing was performed using Prism (GraphPad).

RESULTS

Generation of *Hspa9*$^{+/-}$ mice

In order to evaluate the effect of *Hspa9* deletion on hematopoiesis *in vivo*, we created a mouse model with heterozygous inactivation of *Hspa9* (*Hspa9*$^{Gt(IST14901H6)TIGM}$, hereafter referred to as *Hspa9*$^{+/-}$) using ES cells containing a gene trap inserted in intron 3 of *Hspa9* (Fig. 1A). The gene trap insertion was confirmed by Southern blot and PCR (Fig. 1B and C). Heterozygous loss of *Hspa9* resulted in ~50% reduction in Hspa9 protein (Fig. 1D and Fig. S1) and mRNA (Fig. 1E) levels in *Hspa9*$^{+/-}$ mice compared to wild-type littermates.

Hspa9$^{+/-}$ mice are born at normal Mendelian ratios from *Hspa9*$^{+/-}$ x *Hspa9*$^{+/+}$ matings (N=261) (Table 1). However, intercrossing of *Hspa9*$^{+/-}$ mice did not produce homozygous mice (*Hspa9*$^{-/-}$), suggesting homozygous inactivation of *Hspa9* is embryonic lethal, independent of strain (N=73, C57Bl/6N background; N=139, outbred B6129F2 mice) (Table 1). Timed matings from *Hspa9*$^{+/-}$ x *Hspa9*$^{+/-}$ intercrosses (pure or mixed strain) failed to identify *Hspa9*$^{-/-}$ embryos after embryonic day 9.5, preventing analysis of hematopoietic cells from *Hspa9*$^{-/-}$ fetal livers (Table S3).

Basal and stress hematopoiesis is normal in *Hspa9*$^{+/-}$ mice up to 18 months of age

C57Bl/6N *Hspa9*$^{+/-}$ and *Hspa9*$^{+/+}$ littermate mice were evaluated at 2, 6, 9, 12 and 18 months of age. *Hspa9*$^{+/-}$ mice have normal peripheral blood and bone marrow morphology (data not shown), bone marrow and spleen cellularity (Fig. S2A), and complete blood counts (Fig. S2C). Immunophenotyping of mature and precursor hematopoietic cells in the peripheral blood, bone marrow and spleen of *Hspa9*$^{+/-}$ mice were normal (Fig. 2A–B and Fig. S3A–B) (see Table S4 for immunophenotypic markers). Immunophenotypic analysis of bone marrow progenitors (CMP, MEP, GMP) and stem cell-enriched populations (KLS, SLAM) between *Hspa9*$^{+/+}$ and *Hspa9*$^{+/-}$ mice at 2, 6 and 12 months of age revealed no difference (Fig. 2C and data not shown). Bone marrow and spleen myeloid (CFU-C) and erythroid progenitors (mature BFU-E/BFU-E/CFU-E), as evaluated by methylcellulose

colony-forming assays, were normal up to 18 months of age (Fig. 2D–E and Fig. S4). There was no difference in overall or leukemia-free survival between *Hspa9*$^{+/+}$ and *Hspa9*$^{+/-}$ mice up to 18 months of age (N=35 mice/genotype) (data not shown). Collectively, these results indicate that hematopoiesis in *Hspa9*$^{+/-}$ mice is largely normal.

Hspa9 is up-regulated in response to a number of cellular stresses to provide a cytoprotective effect [14,29–31]. Next, we tested recovery of *Hspa9*$^{+/-}$ and *Hspa9*$^{+/+}$ littermate mice following the induction of three different types of hematopoietic stress. First, we evaluated survival following weekly doses of 5-fluorouracil, which significantly reduces hematopoietic progenitors in normal mice (150mg/kg) (Fig. S5A) [32]. Second, recovery of red blood cells in the peripheral blood of older mice was evaluated for 15 days following phenylhydrazine-induced hemolytic anemia (2 doses of 30mg/kg) (Fig. S5B). Finally, radiation treatment was previously described to induce up-regulation of Hspa9 protein levels; therefore, we evaluated peripheral blood cell count recovery following a single dose of sublethal irradiation (500 Rads) (Fig. S5C) [33]. No differences in hematopoietic stress recovery were observed between *Hspa9*$^{+/-}$ and *Hspa9*$^{+/+}$ littermates for these assays.

CFU-PreB colonies are reduced in *Hspa9*$^{+/-}$ mice

As early as 2 months of age, the number of bone marrow CFU-preB methylcellulose colonies was significantly reduced in *Hspa9*$^{+/-}$ mice compared to *Hspa9*$^{+/+}$ littermates (Fig. 3A). The *Hspa9*$^{+/-}$ CFU-PreB colonies observed were also typically smaller in size (Fig. 3B). The same results were observed using CFU-PreB media containing recombinant mouse IL-7 (Fig. S6). To confirm the CFU-PreB phenotype was Hspa9-dependent, we expressed *HSPA9* in *Hspa9*$^{+/-}$ or *Hspa9*$^{+/+}$ bone marrow cells using a retrovirus. We observed an increase in the number of CFU-preB colonies only in *Hspa9*$^{+/-}$ bone marrow expressing HSPA9 (Fig. S7).

We next measured bone marrow B-cell progenitors and precursors using flow cytometry to determine whether the CFU-PreB colony reduction was due to a reduction in the total number of progenitors added to the media. There was no significant difference in the percent and absolute number of common lymphoid progenitors (CLP) or Hardy fractions A, B/C, D, E, or F in *Hspa9*$^{+/-}$ mice, indicating the reduction in colony number was not due to fewer numbers of *Hspa9*$^{+/-}$ progenitors (N=5–10/genotype) (Fig. 3C and D).

The reduction of CFU-PreB colonies is hematopoietic-cell intrinsic

We tested whether the reduction in CFU-PreB colonies was a hematopoietic cell-intrinsic phenotype or due to a defect in non-hematopoietic stromal cells by performing non-competitive bone marrow transplants of *Hspa9*$^{+/-}$ and *Hspa9*$^{+/+}$ cells into wild-type (*Hspa9*$^{+/+}$) or *Hspa9*$^{+/-}$ recipients. After long-term engraftment was established (>6 months), the number of CFU-PreB colonies from wild-type mice that received *Hspa9*$^{+/-}$ bone marrow was significantly reduced compared to mice that received *Hspa9*$^{+/+}$ bone marrow (N=7–9 mice/genotype, p=0.002) (Fig. 4A). There was no reduction in CFU-PreB colonies following transplantation of wild-type donor bone marrow cells into *Hspa9*$^{+/-}$ recipients (N=5) (Fig. 4A). These results indicate that the reduction in B-cell progenitors in *Hspa9*$^{+/-}$ mice is hematopoietic cell-intrinsic.

Next, we tested whether *Hspa9*$^{+/-}$ lymphoid progenitors are at a functional disadvantage compared to progenitors from control mice *in vivo* by performing competitive repopulation studies. Pools of donor bone marrow from C57Bl/6 (Ly5.2) *Hspa9*$^{+/-}$ or *Hspa9*$^{+/+}$ mice were mixed at a 1:1 ratio with a competitor bone marrow pool from wild-type C57Bl/6 (Ly5.1/Ly5.2) mice, and transplanted into wild-type C57Bl/6 recipient mice (Ly5.1). Analysis of donor-derived cells (Ly5.2) following competitive transplant revealed no significant difference in the number or percent of mature B-cells (Fig. 4B), CLPs, or Hardy fractions in primary or secondary recipients (data not shown). Collectively, the data indicate that *in vivo* homeostatic regulation may compensate for a functional defect in *Hspa9*$^{+/-}$ B-cell progenitors.

B-cell progenitors are reduced in *Hspa9* knockdown mice

In vivo B-cell differentiation and proliferation are regulated by a variety of extracellular signals. In contrast, cells in an *in vitro* CFU-PreB assay are exposed to few stimulatory signals, which may sensitize cells to the effects of *Hspa9* loss. In an attempt to overcome compensation and sensitize cells *in vivo*, we reduced *Hspa9* levels >50% acutely in adult bone marrow cells. Because homozygous knockout of *Hspa9* was lethal prior to fetal liver formation, preventing analysis of *Hspa9*$^{-/-}$ B-cell progenitors in the fetal liver, we knocked down *Hspa9* levels below the 50% level using RNAi. Bone marrow cells transduced with virus (YFP+) were sorted from recipient mice 8–14 weeks post transplant and the mRNA expression level of *Hspa9* in cells that received anti-*Hspa9* shRNA (shHspa9) was 37% of the level measured in cells that received the control knockdown construct (shLUC) (*i.e.*, 63% knockdown of *Hspa9*) (Fig. 5A). Consistent with *Hspa9*$^{+/-}$ mice, YFP+ *Hspa9* knockdown (shHspa9) bone marrow cells had significantly reduced CFU-PreB colony formation compared to YFP+ control (shLUC) cells ($p=0.017$, N=5–6 mice/genotype) (Fig. 5B).

The percent and absolute number of mature peripheral blood B-cells were significantly reduced, while T-cells and myeloid cells were not, as previously described [15]. Next, we interrogated B-cell progenitors and precursors not previously investigated. Acute knockdown of *Hspa9* >50% in adult bone marrow resulted in a significant reduction in the frequencies of common lymphoid progenitors (CLP) and all Hardy fractions versus the control knockdown, with the largest difference occurring in Hardy fraction D (5.75 fold-change, N=9 mice/group, $p<0.001$) (Fig. 5C–E), indicating *Hspa9* levels can affect B-cell progenitors *in vivo*. The percent of YFP+ Hardy fractions B–F in the spleen cells of mice that received the shHspa9 construct were also significantly reduced (1.7–3.5 fold, N=8 mice/group, $p<0.05$, Fig. S8B).

The mRNA expression of B-cell proliferation and activation genes is reduced in *Hspa9*$^{+/-}$ CFU-PreB colonies

Both cytokine availability and cytokine receptor expression can affect the ability of cells to respond to extracellular signals, and may contribute to *in vivo* compensation. Several cytokines, including IL-7 and Flt3-ligand, drive early B-cell proliferation and differentiation *in vivo*; however, IL-7 is the only cytokine supplement added to the methylcellulose media to promote CFU-PreB colony formation [34–36]. Levels of IL-7Rα expressed on the cell

surface of Hardy fractions A–D were not significantly different between *Hspa9*$^{+/-}$ and *Hspa9*$^{+/+}$ mice as measured by flow cytometry (N=5/genotype) (data not shown). To measure cytokine expression *in vivo*, we extracted RNA from femurs by flushing them with TRIzol. We observed no difference in mRNA expression levels of *IL-7* or *Flt3-ligand* between *Hspa9*$^{+/-}$ and wild-type littermates as measured by qRT-PCR (3 months old, N=3/genotype, data not shown).

To investigate whether downstream signaling is disrupted by heterozygous loss of *Hspa9*, we performed gene expression array profiling on colonies produced by *Hspa9*$^{+/-}$ bone marrow and compared them to wild-type colonies. Day 7 CFU-PreB colonies were collected and sorted for B220+ cells (N=5 mice/genotype) (Fig. S9A and B). Two hundred forty-two genes were significantly down-regulated and 169 were significantly up-regulated in *Hspa9*$^{+/-}$ colonies compared to controls in a supervised analysis (FDR<0.05). *Hspa9* expression was reduced in *Hspa9*$^{+/-}$ versus *Hspa9*$^{+/+}$ colonies, as expected (p=0.01) (Fig. S9C). Pathway analysis of down-regulated genes in *Hspa9*$^{+/-}$ colonies using DAVID identified high enrichment scores (>3 fold enrichment) in pathways that promote lymphocyte proliferation and activation. No pathway enrichment was observed in up-regulated genes in *Hspa9*$^{+/-}$ colonies (>1.5 fold enrichment) (Table 2). Microarray results suggest that the reduction in CFU-PreB colonies may be due to a blunted response to proliferation signals *in vitro.*

Knockdown of *Hspa9* reduces proliferation and Stat5 activation in an IL-7 dependent mouse B-cell line

Because expression levels of the IL-7 receptor and its activating ligand were not different, we determined whether knockdown of *Hspa9* altered the downstream cellular response to IL-7 stimulation. For this, we utilized the IL-7-dependent mouse B7 cell line. Knockdown of *Hspa9* using *Hspa9*-targeting siRNAs in B7 cells causes a reduction in cell numbers over time compared to control siRNA-treated B7 cells grown in 10 ng/mL of IL-7 (Fig. 6B). A similar reduction in cell numbers was observed when B7 cells are grown in low concentrations (≤1ng/mL) of IL-7 (Fig. S10).

We next investigated signaling downstream of the IL-7 receptor. Stat5 is indispensible for B-cell development and is rapidly activated by phosphorylation following IL-7 stimulation of the IL-7 receptor [37]. B7 cells were electroporated with *Hspa9*-targeting siRNAs, rested for two days, starved of IL-7 overnight, and stimulated with different concentrations of IL-7 (0.1, 1 or 10ng/mL). Cells were collected at 5, 10, 15 and 30 minutes following IL-7 stimulation and analyzed for Stat5 phosphorylation by flow cytometry. Following IL-7 stimulation, phosphorylation of Stat5 was reduced in B7 cells treated with *Hspa9* siRNA compared to control siRNA at all time-points and concentrations evaluated by flow cytometry (Fig. S11). These results were confirmed by Western blotting of B7 cells collected following 10 and 30 minutes of stimulation with 1ng/mL IL-7 (Fig. 6C).

DISCUSSION

The present study addresses the role of *Hspa9* in hematopoiesis and highlights the effects of *Hspa9* expression levels on B-lymphopoiesis. While basal and stress hematopoiesis was

normal in heterozygous knockout mice (*Hspa9*$^{+/-}$), we observed a significant reduction in CFU-PreB colony numbers in *Hspa9*$^{+/-}$ mice. Embryonic lethality prevented the study of *Hspa9* homozygous null mice; however, reduction of *Hspa9* expression >50% by RNAi-mediated knockdown led to a reduction in B-cell progenitors and precursors *in vivo.* Our results suggest that reduced levels of *Hspa9* impair Stat5 activation following IL-7 stimulation of B-cells. Collectively, these results indicate reduced *Hspa9* expression alters B-lymphopoiesis.

We report for the first time that homozygous knockout of *Hspa9* is embryonic lethal in mice, similar to knockout of *Hspa5* and *Hspa8*. This is consistent with other genetic models of *Hspa9* orthologs and indicates the importance of Hspa9 during embryogenesis [12,38–41]. Based on the altered hematopoiesis observed in Hspa9 mutant zebrafish, murine bone marrow shRNA transduction/transplantation models, and altered differentiation observed in human CD34+ cells, we predicted that *Hspa9* heterozygous knockout mice would have abnormal hematopoiesis, specifically affecting erythroid and B-cells[12,15,16]. However, constitutive heterozygous deletion of *Hspa9* did not alter erythroid or mature B-cell numbers, suggesting that >50% reduction of Hspa9 is necessary to perturb hematopoiesis in mice. It remains possible that the differences in phenotypes between these models are due to the level of Hspa9 expression and/or the constitutive deletion of *Hspa9* in the knockout model versus the acute reduction of Hspa9 that occurs using RNAi-mediated methods. Compensation for *Hspa9* deletion in the knockout model may occur during development. Similarly, acute RNAi-mediated knockdown of HSPA9 in primary human CD34+ cells may induce a phenotype not seen in the knockout mice, in addition to species-specific differences. Creation of an *Hspa9* conditional knockout mouse will be necessary to test this possibility.

We postulate that the reduction in CFU-PreB colony formation in *Hspa9*$^{+/-}$ mice is mediated through Stat5 activation. Our microarray analysis did not reveal dysregulation of the SOCS or phosphatase family of genes that could explain altered Stat5 activation. Alternatively, we propose that Hspa9 may regulate Stat5 activation through its co-chaperone Dnaja3, a negative regulator of Stat5b [42]. Hspa9 is an Hsp70 protein that relies on interactions with co-chaperones such as J-proteins for its normal functions. Dnaja3 is the J-protein associated with the non-mitochondrial import functions of Hspa9 and is responsible for recruiting substrates to Hspa9 [13]. Overexpression of Dnaja3 results in dose-dependent inhibition of Stat5 phosphorylation, cell growth, and expression of the Stat5 target Bcl-xl in two IL-7 responsive B-cell lines (human 697 pre-B and mouse mIL-7R expressing Ba/F3 cells) [42]. Hspa9 is expressed in excess of Dnaja3, a ratio that is important for normal chaperone and cellular functions [43,44]. Reducing the abundance of Hspa9 may increase the amount of unbound Dnaja3, increasing the Dnaja3 available to bind and inhibit the phosphorylation of Stat5b. Hspa9 and Dnaja3 have also been established as regulators of p53, through a similar mechanism [45–47].

Although CFU-PreB colonies were reduced *in vitro* from *Hspa9*$^{+/-}$ mice, there were no alterations in basal B-lymphopoiesis *in vivo*. This suggests that homeostatic regulation of B-cell numbers may account for *in vivo* compensation. IL-7 is the only supportive cytokine added to CFU-PreB media even though other cytokines such as Scf, Flt3-ligand and Cxcl12

regulate B-cell differentiation and proliferation *in vivo* [34–36]. Relying solely on the IL-7 signaling cascade in CFU-PreB culture conditions in the absence of these other cytokines may sensitize cells to subtle alterations in IL-7 signaling caused by heterozygous *Hspa9* loss. While we did not detect differences in bulk bone marrow *IL-7* expression, these results do not exclude the possibility that smaller, local bone marrow niche changes in IL-7 concentration exist. Additionally, increased expression of other B-cell supportive cytokines may exist. Genetic loss of *Hspa9* throughout the entire lifespan of the animal may allow compensation to occur and account for the difference between chronic heterozygous (knockout model) and acute (RNAi knockdown model) loss of *Hspa9* in mice. Indeed, further reduction of *Hspa9* (>50%) does disrupt B-lymphopoiesis *in vivo*; indicating a threshold level of *Hsap9* is required for normal B-cell development to proceed.

HSPA9 protects cells from a variety of stresses. Although $Hspa9^{+/-}$ mice respond similarly to $Hspa9^{+/+}$ littermates when challenged with hematopoietic stress inducers, it is possible that heterozygous loss of *Hspa9* impairs response to cellular stresses that we did not evaluate. Increased levels of reactive oxygen species (ROS) have been implicated in MDS pathogenesis [48,49]. HSPA9 haploinsufficiency in MDS patients could alter cellular function as increased levels of ROS occur in hematopoietic cells from Hspa9 mutant zebrafish and knockdown mice [12,16]. In other cellular systems, loss of Hspa9 has been associated with increased ROS generation while Hspa9 overexpression has been shown to protect from oxidative damage [29,31,50]. In addition, deletion of *RPS14*, a gene commonly deleted with *HSPA9* on del(5q), results in cellular stress due to ribosomal deficiency and p53 activation [51,52]. *HSPA9* haploinsufficiency may limit a cell's ability to appropriately regulate these stress states and may ultimately contribute to the ineffective hematopoiesis observed in del(5q) MDS. It is possible that an impaired ability of $Hspa9^{+/-}$ cells to respond to *ex vivo* cellular stresses may explain the observed reduction in CFU-PreB colony formation from $Hspa9^{+/-}$ bone marrow in the absence of *in vivo* reductions in B-cell progenitors.

HSPA9 is located in a commonly deleted region in myelodysplastic syndrome (MDS), del(5q), one of the most recurrent genetic abnormalities associated with the disease [17]. No single gene can explain the full spectrum of clinical phenotypes observed in MDS, suggesting that multiple del(5q) genes may contribute to pathogenesis [53]. Our studies suggest that *HSPA9* haploinsufficiency may contribute to B-cell progenitor alterations observed in patients with MDS. Studies of B-cells in MDS are limited, but alterations in B-cell progenitors have been described, including reductions in B-cell progenitors, increased levels of B-cell apoptosis, and reduced expression of B-cell signaling pathways [54–56]. Data we provide here indicate that loss of HSPA9 may contribute to these B-cell alterations in patients with del(5q). Additional studies are needed to better understand how loss of *Hspa9* attenuates Stat5 activation and B-cell development; however, this model provides evidence that *Hspa9* loss may alter B- lymphopoiesis. This model could be used for future studies investigating how simultaneous deletion of multiple genes contributes to myeloid disease pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Deepta Bhattacharya for kindly providing the IL-7Rα antibody as well as helpful scientific discussions, and Charles Mullighan for kindly providing the B7 cells. Research reported in this publication was supported by the National Cancer Institute and the National Heart Lung Blood Institute of the National Institutes of Health under Award Number F31CA165702 (K.K.) and Research Project Grant R01HL109336 (M.J.W.), respectively, as well as a Howard Hughes Medical Institute Physician-Scientist Early Career Award (M.J.W.) and a Siteman Cancer Biology Pathway Fellowship (K.K). Technical assistance was provided by the Alvin J. Siteman Cancer Center Flow Cytometry Core, which provided cell sorting; Tissue Procurement Core, which provided RNA processing for microarray analysis; Biostatistics Core, which provided informatics support; and GTAC for microarray hybridization, and are supported by an NCI Cancer Center Support Grant (P30CA91842). Additional technical assistance was provided by the Hope Center Viral Vectors Core, which is supported by a Neuroscience Blueprint Interdisciplinary Center Core Award (P30NS057105).

References

1. Daugaard M, Rohde M, Jäättelä M. The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. FEBS Lett. 2007 Jul 31; 581(19):3702–10. [PubMed: 17544402]
2. Murphy ME. The HSP70 family and cancer. Carcinogenesis. 2013 Jun; 34(6):1181–8. [PubMed: 23563090]
3. Hampton CR, Shimamoto A, Rothnie CL, Griscavage-Ennis J, Chong A, Dix DJ, et al. HSP70.1 and -70.3 are required for late-phase protection induced by ischemic preconditioning of mouse hearts. Am J Physiol Heart Circ Physiol. 2003 Aug; 285(2):H866–74. [PubMed: 12714332]
4. Luo S, Mao C, Lee B, Lee AS. GRP78/BiP is required for cell proliferation and protecting the inner cell mass from apoptosis during early mouse embryonic development. Mol Cell Biol. 2006 Aug; 26(15):5688–97. [PubMed: 16847323]
5. Florin L, Becker KA, Sapp C, Lambert C, Sirma H, Müller M, et al. Nuclear translocation of papillomavirus minor capsid protein L2 requires Hsc70. J Virol. 2004 Jun; 78(11):5546–53. [PubMed: 15140951]
6. Guo F, Sigua C, Bali P, George P, Fiskus W, Scuto A, et al. Mechanistic role of heat shock protein 70 in Bcr-Abl-mediated resistance to apoptosis in human acute leukemia cells. Blood. 2005 Feb 1; 105(3):1246–55. [PubMed: 15388581]
7. Ribeil J-A, Zermati Y, Vandekerckhove J, Cathelin S, Kersual J, Dussiot M, et al. Hsp70 regulates erythropoiesis by preventing caspase-3-mediated cleavage of GATA-1. Nature. 2007 Jan 4; 445(7123):102–5. [PubMed: 17167422]
8. Zou P, Yoshihara H, Hosokawa K, Tai I, Shinmyozu K, Tsukahara F, et al. p57(Kip2) and p27(Kip1) cooperate to maintain hematopoietic stem cell quiescence through interactions with Hsc70. Cell Stem Cell. 2011 Sep 2; 9(3):247–61. [PubMed: 21885020]
9. Miharada K, Karlsson G, Rehn M, Rörby E, Siva K, Cammenga J, et al. Cripto Regulates Hematopoietic Stem Cells as a Hypoxic-Niche-Related Factor through Cell Surface Receptor GRP78. Cell Stem Cell Elsevier. 2011 Oct; 9(4):330–44.
10. Mjahed H, Girodon F, Fontenay M, Garrido C. Heat shock proteins in hematopoietic malignancies. Exp Cell Res. 2012 Sep; 318(15):1946–58. [PubMed: 22652452]
11. Matsui H, Asou H, Inaba T. Cytokines direct the regulation of Bim mRNA stability by heat-shock cognate protein 70. Mol Cell. 2007 Jan 12; 25(1):99–112. [PubMed: 17218274]
12. Craven SE, French D, Ye W, de Sauvage F, Rosenthal A. Loss of Hspa9b in zebrafish recapitulates the ineffective hematopoiesis of the myelodysplastic syndrome. Blood. 2005 May 1; 105(9):3528–34. [PubMed: 15650063]

13. Goswami AV, Chittoor B, D'Silva P. Understanding the Functional Interplay between Mammalian Mitochondrial Hsp70 Chaperone Machine Components. J Biol Chem. 2010 Jun 11; 285(25): 19472–82. [PubMed: 20392697]

14. Ohtsuka R, Abe Y, Fujii T, Yamamoto M, Nishimura J, Takayanagi R, et al. Mortalin is a novel mediator of erythropoietin signaling. Eur J Haematol. 2007 Aug; 79(2):114–25. [PubMed: 17635236]

15. Chen TH-P, Kambal A, Krysiak K, Walshauser MA, Raju G, Tibbitts JF, et al. Knockdown of Hspa9, a del(5q31.2) gene, results in a decrease in hematopoietic progenitors in mice. Blood. 2011 Feb 3; 117(5):1530–9. [PubMed: 21123823]

16. Tai-Nagara I, Matsuoka S, Ariga H, Suda T. Mortalin and DJ-1 coordinately regulate hematopoietic stem cell function through the control of oxidative stress. Blood. 2014 Jan 2; 123(1):41–50. [PubMed: 24243970]

17. Haase D, Germing U, Schanz J, Pfeilstöcker M, Nösslinger T, Hildebrandt B, et al. New insights into the prognostic impact of the karyotype in MDS and correlation with subtypes: evidence from a core dataset of 2124 patients. Blood. 2007 Dec 15; 110(13):4385–95. [PubMed: 17726160]

18. Zhao N, Stoffel A, Wang PW, Eisenbart JD, Espinosa R, Larson RA, et al. Molecular delineation of the smallest commonly deleted region of chromosome 5 in malignant myeloid diseases to 1–1.5 Mb and preparation of a PAC-based physical map. Proc Natl Acad Sci USA. 1997 Jun 24; 94(13): 6948–53. [PubMed: 9192672]

19. Walter MJ, Shen D, Shao J, Ding L, White BS, Kandoth C, et al. Clonal diversity of recurrently mutated genes in myelodysplastic syndromes. Leukemia. 2013 Jun; 27(6):1275–82. [PubMed: 23443460]

20. Jerez A, Gondek LP, Jankowska AM, Makishima H, Przychodzen B, Tiu RV, et al. Topography, clinical, and genomic correlates of 5q myeloid malignancies revisited. J Clin Oncol. 2012 Apr 20; 30(12):1343–9. [PubMed: 22370328]

21. Graubert TA, Payton MA, Shao J, Walgren RA, Monahan RS, Frater JL, et al. Integrated genomic analysis implicates haploinsufficiency of multiple chromosome 5q31. 2 genes in de novo myelodysplastic syndromes pathogenesis. PLoS ONE Public Library of Science. 2009; 4(2):e4583.

22. Zambrowicz BP, Abuin A, Ramirez-Solis R, Richter LJ, Piggott J, BeltrandelRio H, et al. Wnk1 kinase deficiency lowers blood pressure in mice: a gene-trap screen to identify potential targets for therapeutic intervention. Proc Natl Acad Sci USA. 2003 Nov 25; 100(24):14109–14. [PubMed: 14610273]

23. Magee JA, Ikenoue T, Nakada D, Lee JY, Guan K-L, Morrison SJ. Temporal changes in PTEN and mTORC2 regulation of hematopoietic stem cell self-renewal and leukemia suppression. Cell Stem Cell. 2012 Sep 7; 11(3):415–28. [PubMed: 22958933]

24. Ma Z, Izumi H, Kanai M, Kabuyama Y, Ahn NG, Fukasawa K. Mortalin controls centrosome duplication via modulating centrosomal localization of p53. Oncogene. 2006 Apr 17; 25(39): 5377–90. [PubMed: 16619038]

25. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA. 2001 Apr 17; 98(9):5116–21. [PubMed: 11309499]

26. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009 Jan; 37(1):1–13. [PubMed: 19033363]

27. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009; 4(1):44–57. [PubMed: 19131956]

28. Mullighan CG, Collins-Underwood JR, Phillips LAA, Loudin MG, Liu W, Zhang J, et al. Rearrangement of CRLF2 in B-progenitor- and Down syndrome-associated acute lymphoblastic leukemia. Nat Genet. 2009 Nov; 41(11):1243–6. [PubMed: 19838194]

29. Liu, Y.; Liu, W.; Song, X-D.; Zuo, J. Molecular and cellular biochemistry. Vol. 268. Springer; 2005. Effect of GRP75/mthsp70/PBP74/mortalin overexpression on intracellular ATP level, mitochondrial membrane potential and ROS accumulation following glucose deprivation in PC12 cells; p. 45-51.

30. Williamson CL, Dabkowski ER, Dillmann WH, Hollander JM. Mitochondria protection from hypoxia/reoxygenation injury with mitochondria heat shock protein 70 overexpression. Am J Physiol Heart Circ Physiol. 2008 Jan; 294(1):H249–56. [PubMed: 17982016]

31. Orsini F, Migliaccio E, Moroni M, Contursi C, Raker VA, Piccini D, et al. The life span determinant p66Shc localizes to mitochondria where it associates with mitochondrial heat shock protein 70 and regulates trans-membrane potential. J Biol Chem. 2004 Jun 11; 279(24):25689–95. [PubMed: 15078873]

32. Zhang Y, Wong J, Klinger M, Tran MT, Shannon KM, Killeen N. MLL5 contributes to hematopoietic stem cell fitness and homeostasis. Blood. 2009 Feb 12; 113(7):1455–63. [PubMed: 18818388]

33. Sadekova S, Lehnert S, Chow TY. Induction of PBP74/mortalin/Grp75, a member of the hsp70 family, by low doses of ionizing radiation: a possible role in induced radioresistance. Int J Radiat Biol. 1997 Dec; 72(6):653–60. [PubMed: 9416787]

34. Dolence JJ, Gwin K, Frank E, Medina KL. Threshold levels of Flt3-ligand are required for the generation and survival of lymphoid progenitors and B-cell precursors. Eur J Immunol. 2011 Feb; 41(2):324–34. [PubMed: 21268003]

35. Funk PE, Varas A, Witte PL. Activity of stem cell factor and IL-7 in combination on normal bone marrow B lineage cells. J Immunol. 1993 Feb 1; 150(3):748–52. [PubMed: 7678620]

36. Egawa T, Kawabata K, Kawamoto H, Amada K, Okamoto R, Fujii N, et al. The earliest stages of B cell development require a chemokine stromal cell-derived factor/pre-B cell growth-stimulating factor. Immunity. 2001 Aug; 15(2):323–34. [PubMed: 11520466]

37. Goetz CA, Harmon IR, O'Neil JJ, Burchill MA, Farrar MA. STAT5 activation underlies IL7 receptor-dependent B cell development. J Immunol. 2004 Apr 15; 172(8):4770–8. [PubMed: 15067053]

38. Spradling AC, Stern D, Beaton A, Rhem EJ, Laverty T, Mozden N, et al. The Berkeley Drosophila Genome Project gene disruption project: Single P-element insertions mutating 25% of vital Drosophila genes. Genetics. 1999 Sep; 153(1):135–77. [PubMed: 10471706]

39. Craig EA, Kramer J, Kosic-Smithers J. SSC1, a member of the 70-kDa heat shock protein multigene family of Saccharomyces cerevisiae, is essential for growth. Proc Natl Acad Sci USA. 1987 Jun; 84(12):4156–60. [PubMed: 3035571]

40. Kimura K, Tanaka N, Nakamura N, Takano S, Ohkuma S. Knockdown of mitochondrial heat shock protein 70 promotes progeria-like phenotypes in caenorhabditis elegans. J Biol Chem. 2007 Feb 23; 282(8):5910–8. [PubMed: 17189267]

41. Amsterdam A, Nissen RM, Sun Z, Swindell EC, Farrington S, Hopkins N. Identification of 315 genes essential for early zebrafish development. Proc Natl Acad Sci USA. 2004 Aug 31; 101(35): 12792–7. [PubMed: 15256591]

42. Dhennin-Duthille I, Nyga R, Yahiaoui S, Gouilleux-Gruart V, Régnier A, Lassoued K, et al. The tumor suppressor hTid1 inhibits STAT5b activity via functional interaction. J Biol Chem. 2011 Feb 18; 286(7):5034–42. [PubMed: 21106534]

43. Finka A, Goloubinoff P. Proteomic data from human cell cultures refine mechanisms of chaperone-mediated protein homeostasis. Cell Stress Chaperones. 2013 Sep; 18(5):591–605. [PubMed: 23430704]

44. Laufen T, Mayer MP, Beisel C, Klostermeier D, Mogk A, Reinstein J, et al. Mechanism of regulation of hsp70 chaperones by DnaJ cochaperones. Proc Natl Acad Sci USA. 1999 May 11; 96(10):5452–7. [PubMed: 10318904]

45. Ahn BY, Trinh DLN, Zajchowski LD, Lee B, Elwi AN, Kim S-W. Tid1 is a new regulator of p53 mitochondrial translocation and apoptosis in cancer. Oncogene. 2010 Feb 25; 29(8):1155–66. [PubMed: 19935715]

46. Kaul S, Reddel RR, Mitsui Y, Wadhwa R. An N-terminal region of mot-2 binds to p53 in vitro. Neoplasia. 2001 Mar; 3(2):110–4. [PubMed: 11420746]

47. Kanai M, Ma Z, Izumi H, Kim S-H, Mattison CP, Winey M, et al. Physical and functional interaction between mortalin and Mps1 kinase. Genes Cells. 2007 Jun; 12(6):797–810. [PubMed: 17573779]

48. Rassool FV, Gaymes TJ, Omidvar N, Brady N, Beurlet S, Pla M, et al. Reactive oxygen species, DNA damage, and error-prone repair: a model for genomic instability with progression in myeloid leukemia? Cancer Res. 2007 Sep 15; 67(18):8762–71. [PubMed: 17875717]

49. Sallmyr A, Fan J, Rassool FV. Genomic instability in myeloid malignancies: increased reactive oxygen species (ROS), DNA double strand breaks (DSBs) and error-prone repair. Cancer Lett. 2008 Oct 18; 270(1):1–9. [PubMed: 18467025]

50. EQ, Liu X, Liu Y, Liu W, Zuo J. Over-expression of GRP75 inhibits liver injury induced by oxidative damage. Acta Biochim Biophys Sin (Shanghai). 2013 Feb; 45(2):129–34. [PubMed: 23287070]

51. Dutt S, Narla A, Lin K, Mullally A, Abayasekara N, Megerdichian C, et al. Haploinsufficiency for ribosomal protein genes causes selective activation of p53 in human erythroid progenitor cells. Blood. 2011 Mar 3; 117(9):2567–76. [PubMed: 21068437]

52. Barlow JL, Drynan LF, Hewett DR, Holmes LR, Lorenzo-Abalde S, Lane AL, et al. A p53-dependent mechanism underlies macrocytic anemia in a mouse model of human 5q– syndrome. Nat Med. 2009 Nov 22; 16(1):59–66. [PubMed: 19966810]

53. Ebert BL. Molecular dissection of the 5q deletion in myelodysplastic syndrome. Semin Oncol. 2011 Oct; 38(5):621–6. [PubMed: 21943668]

54. Sternberg A, Killick S, Littlewood T, Hatton C, Peniket A, Seidl T, et al. Evidence for reduced B-cell progenitors in early (low-risk) myelodysplastic syndrome. Blood. 2005 Nov 1; 106(9):2982–91. [PubMed: 16076868]

55. Amin HM, Jilani I, Estey EH, Keating MJ, Dey AL, Manshouri T, et al. Increased apoptosis in bone marrow B lymphocytes but not T lymphocytes in myelodysplastic syndrome. Blood. 2003 Sep 1; 102(5):1866–8. [PubMed: 12730116]

56. Pellagatti A, Cazzola M, Giagounidis A, Perry J, Malcovati L, Porta Della MG, et al. Deregulated gene expression pathways in myelodysplastic syndrome hematopoietic stem cells. Leukemia. 2010 Apr; 24(4):756–64. [PubMed: 20220779]

HIGHLIGHTS

- Homozygous knockout of *Hspa9* in mice is embryonic lethal
- Haploinsufficiency of *Hspa9* causes a significant reduction in CFU-PreB colony formation
- Knockdown of *Hspa9* >50% significantly reduces B-cell progenitor numbers
- Knockdown of *Hspa9* attenuates Stat5 activation following IL-7 stimulation of a mouse B-cell line

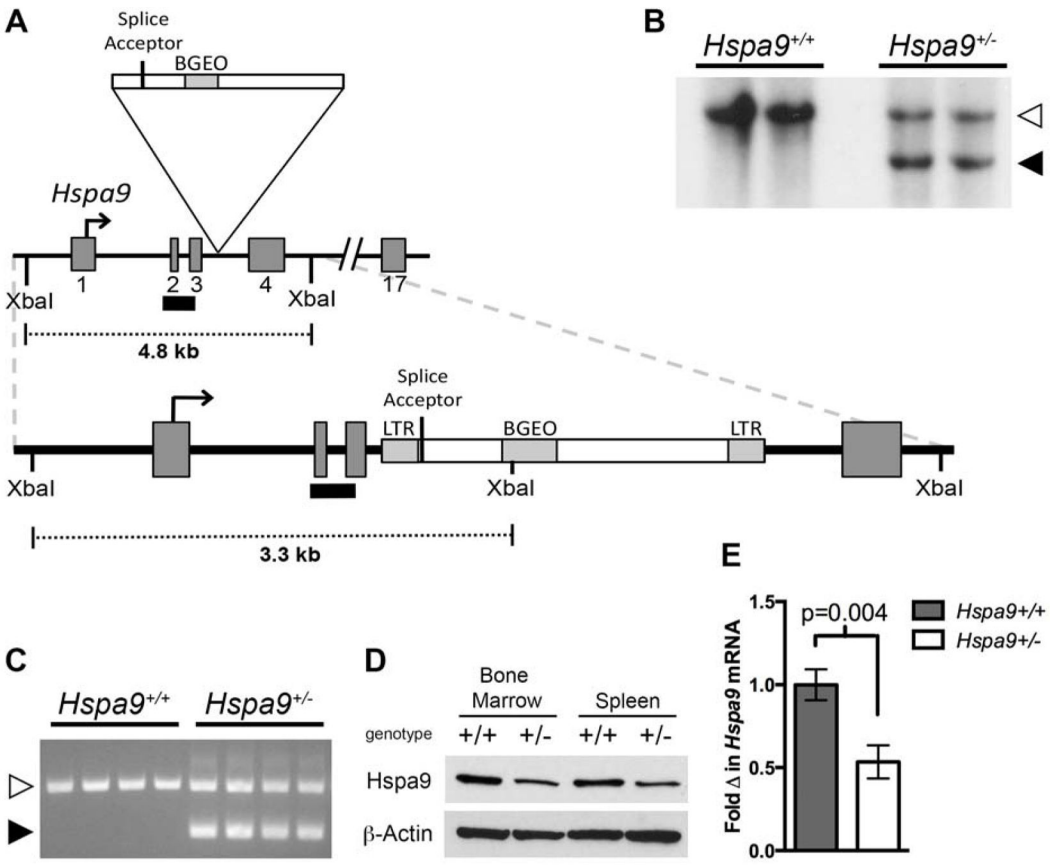

Figure 1. Generation of *Hspa9*$^{+/-}$ mice

A) Exons 1–4 of the *Hspa9* locus with gene trap insertion. A black bar indicates the Southern blot probe. **B)** Southern blot of tail DNA from *Hspa9*$^{+/+}$ and *Hspa9*$^{+/-}$ mice showing DNA fragments from a wild-type allele (*white arrow*, 4817 bp) and gene trap-disrupted allele (*black arrow*, 3303 bp). **C)** Results of 3 primer PCR amplification of tail DNA from *Hspa9*$^{+/+}$ and *Hspa9*$^{+/-}$ mice showing a band from the wild-type allele (*white arrow*, 453 bp) and gene trap-disrupted allele (*black arrow*, 307 bp). **D)** Expression of Hspa9 in bone marrow and spleen of littermates by C-terminal antibody with β-Actin loading control. **E)** RT-PCR expression of *Hspa9* mRNA in bone marrow of littermates, normalized to *Gapdh* (N=3/genotype). Statistical analysis by two tailed Student's t-test. Error bars represent mean ± SD.

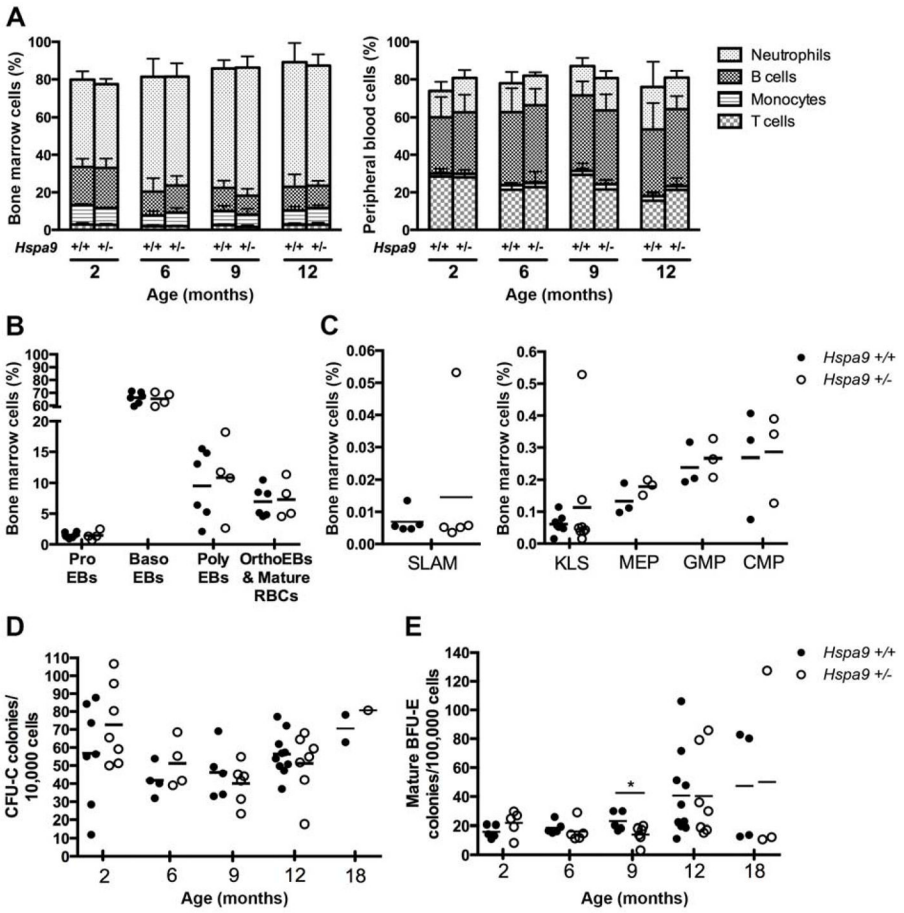

Figure 2. Immunophenotyping of bone marrow and peripheral blood cells in *Hspa9*$^{+/-}$ mice are normal compared to *Hspa9*$^{+/+}$ littermates

A) No difference was observed in bone marrow (*left panel*) or peripheral blood (*right panel*) of *Hspa9*$^{+/-}$ and *Hspa9*$^{+/+}$ littermates analyzed by flow cytometry for immunophenotypic markers for (*bars labeled top to bottom*) neutrophils (Gr1^{+}/CD115^{-}), B-cells (B220^{+}), monocytes (Gr1^{lo}/CD115^{+}) and T-cells (CD3e^{+}) (N=3–6/genotype at each time point). **B)** Red blood cell precursors evaluated by flow cytometry (CD71$^{+/-}$/Ter119^{+}) in bone marrow of *Hspa9*$^{+/-}$ and *Hspa9*$^{+/+}$ littermates at 12 months of age showing no difference between genotypes. **C)** Bone marrow cells from 12-month-old *Hspa9*$^{+/-}$ and wild-type littermates were stained with immunophenotypic markers for SLAM (Lin^{-}Sca^{+}cKit^{+}CD150^{+}CD48^{-}), KLS (cKit^{+}, Lin^{-}, Sca^{+}), megakaryocyte-erythrocyte progenitors (MEP, Lin^{-}Sca^{-}cKit^{+}FcγR^{lo}CD34^{-}), granulocyte-monocyte progenitors (GMP, Lin^{-}Sca^{-}cKit^{+}FcγR^{hi}CD34^{+}), and common myeloid progenitors (CMP, Lin^{-}Sca^{-}cKit^{+}FcγR^{lo}CD34^{+}). Bone marrow cells were isolated at indicated time points from *Hspa9*$^{+/+}$ and *Hspa9*$^{+/-}$ mice, **D)** plated in CFU-C media (10,000 cells/plate) and counted on day 7 or **E)** plated in mature BFU-E media containing only erythropoietin (100,000 cells/plate) and counted on day 10–11. (*Hspa9*$^{+/+}$, *filled circles; Hspa9*$^{+/-}$, *open circles*) ProEBs, proerythroblasts; BasoEBs, basophilic erythroblasts; PolyEBs, polychromatic erythroblasts; OrthoEBs, orthochromatic erythroblasts. Statistical analysis by two tailed Student's t-test. Error bars represent mean ± SD. *p<0.05

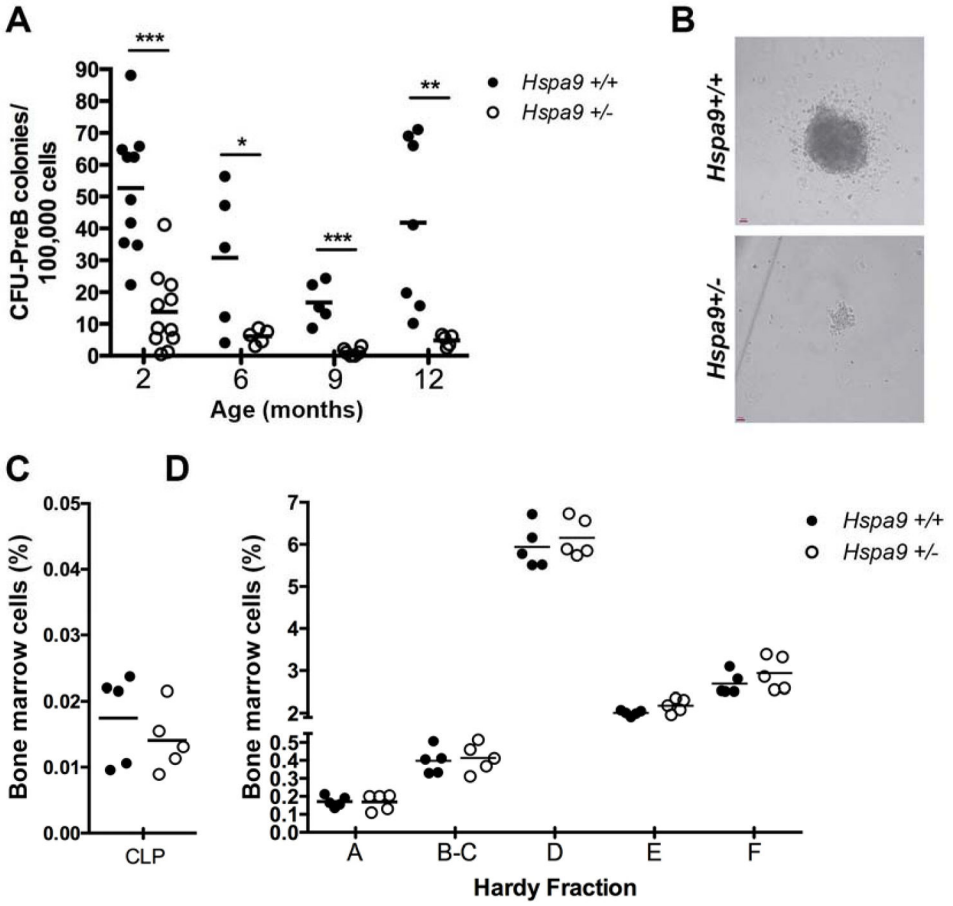

Figure 3. Colony forming ability of B-cell progenitors is significantly reduced in *Hspa9*$^{+/-}$ compared to *Hspa9*$^{+/+}$ mice

A) The number of *Hspa9*$^{+/-}$ CFU-PreB colonies counted on day 7 were significantly reduced compared to colonies from *Hspa9*$^{+/+}$ littermate bone marrow at all ages evaluated. **B)** Representative images of CFU-PreB colonies. Percentage of bone marrow cells that are common lymphoid progenitors **(C)** and Hardy fractions **(D)** were not significantly different between *Hspa9*$^{+/-}$ and *Hspa9*$^{+/+}$ littermate mice at 4–5 months of age. (*Hspa9*$^{+/+}$, *filled circles; Hspa9*$^{+/-}$, *open circles*) CLP, common lymphoid progenitor. Statistical analysis by two tailed Student's t-test. *p<0.05, **p<0.01, ***p<0.001

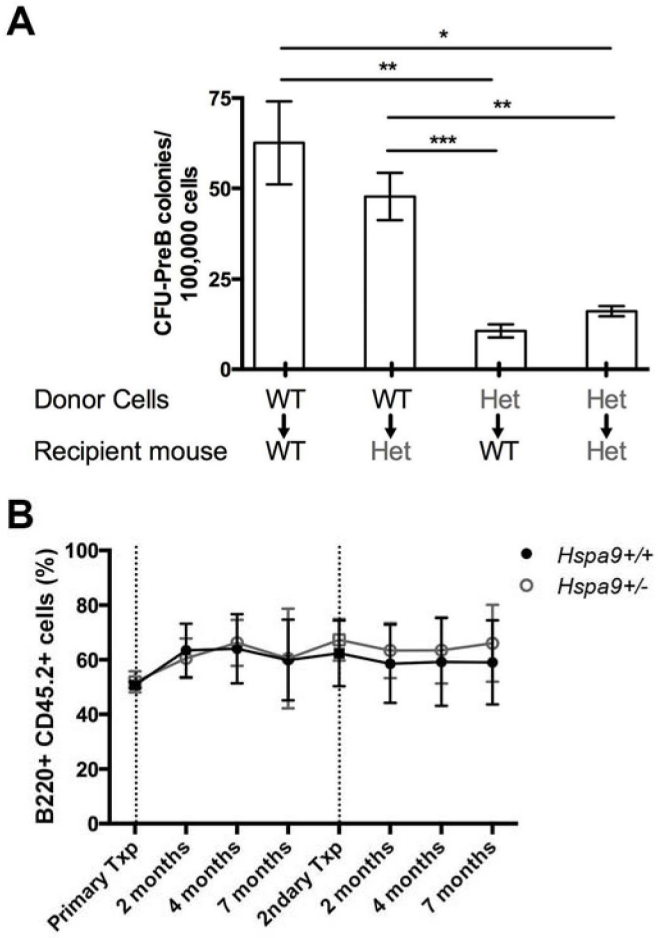

Figure 4. The reduction in *Hspa9*$^{+/-}$ CFU-PreB colony formation is hematopoietic cell-intrinsic

A) Donor bone marrow from *Hspa9*$^{+/+}$ (WT) or *Hspa9*$^{+/-}$ (HET) mice was transplanted into lethally irradiated *Hspa9*$^{+/+}$ (WT) or *Hspa9*$^{+/-}$ (HET) recipients. Bone marrow was harvested 6 months after transplant and plated in CFU-PreB promoting methylcellulose (N=4–9 mice/genotype). **B)** A ratio of 1:1 *Hspa9*$^{+/+}$ (*black lines*) or *Hspa9*$^{+/-}$ (*grey lines*) test cells (Ly5.2) and competitor bone marrow (Ly5.1/5.2) were transplanted into lethally irradiated recipients (Ly5.1). Mice were bled at intervals indicated after transplant and relative chimerism of B220+ peripheral blood cells were evaluated in recipients. Following long-term engraftment, bone marrow from recipients were pooled and transplanted into lethally irradiated secondary recipients. Data represents pooled results from two independently transplanted cohorts (N=10–15 mice/genotype). Txp, transplant. Statistical analysis by two tailed Student's t-test and an ANOVA. Error bars represent mean ± SD. *p<0.05, **p<0.01, ***p<0.001

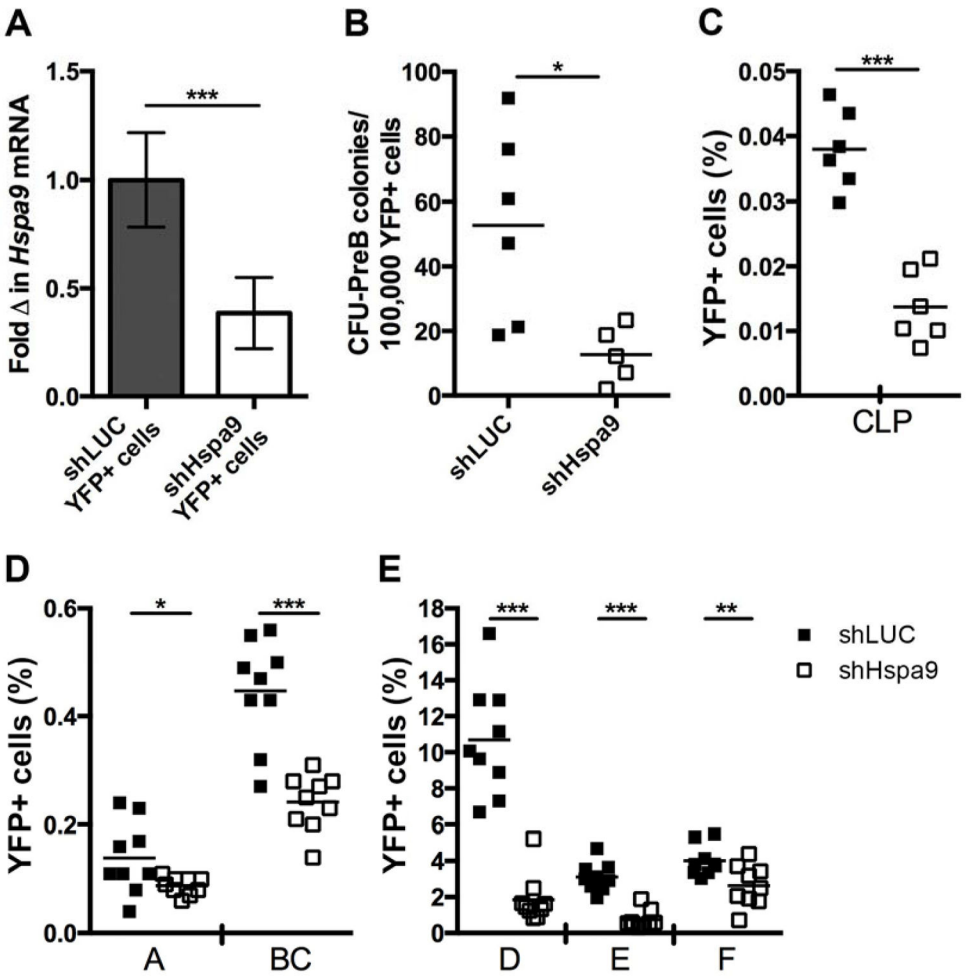

Figure 5. B-cell progenitors are significantly reduced in mice following >50% knockdown of *Hspa9*

A) Expression of *Hspa9* analyzed by RT-PCR in YFP+ bone marrow cells sorted from wild-type mice that received bone marrow transduced with shLUC or shHspa9 lentiviral constructs (N=7–8 mice/group, normalized to *Gapdh*). **B)** YFP+ bone marrow cells sorted from shLUC (*closed squares*) or shHspa9 (*open squares*) mice 10–12 weeks post-transplant were plated in CFU-PreB methylcellulose medium. CFU-PreB colonies were counted on day 7 and significantly reduced in shHspa9 bone marrow. The percent of CLPs **(C)** and Hardy fractions A–F **(D, E)** from the YFP+ bone marrow population of recipient mice is reduced. Statistical analysis by two tailed Student's t-test. Error bars represent mean ± SD. *p<0.05, **p<0.01, ***p<0.001

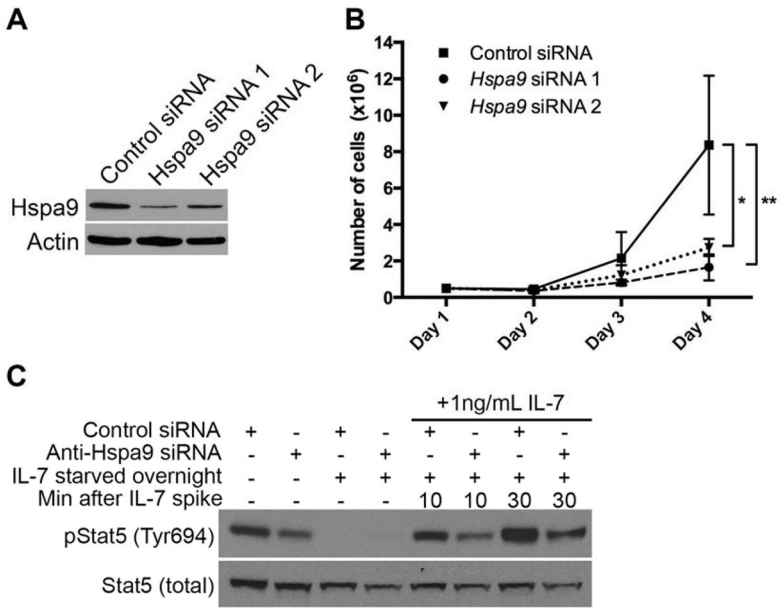

Figure 6. Knockdown of *Hspa9* inhibits growth and IL-7 receptor mediated Stat5 phosphorylation in B7 cells

B7 cells were electroporated with a non-targeting control siRNA or siRNA targeting *Hspa9*. The following day (day 1), cells were counted and plated at the same cell concentration. **A)** Hspa9 expression levels were evaluated by Western blot on day 2. **B)** Growth of B7 cells maintained in 10ng/mL IL-7 is significantly inhibited by knockdown of *Hspa9* by two independent *Hspa9*-targeting siRNAs (N=3/group; Control siRNA, *solid line*; siRNA 1, *dashed line*; siRNA 2, *dotted line*). **C)** Western blot of lysates from B7 cells treated with *Hspa9*-targeting siRNA 1 or non-targeting control were analyzed for total and phosphorylated (Tyr694) Stat5. Cells were grown in 10mg/mL IL-7 for 4 days (*Lane 1 and 2*). Cells were starved overnight starting on day 3 (*Lane 3 and 4*). Cells starved overnight were stimulated on day 4 with 1ng/mL IL-7 and lysates were collected after 10 minutes (*Lane 5 and 6*) and 30 minutes (*Lane 7 and 8*). Stat5 phosphorylation was reduced in cells treated with *Hspa9*-targeting siRNA. Representative data is shown from 4 biological replicates. Error bars represent mean ± SD. *p<0.05, **p<0.01, ***p<0.001

Table 1

Homozygous deletion of *Hspa9* is embryonic lethal

	***Hspa9*$^{+/+}$ x *Hspa9*$^{+/-}$**	***Hspa9*$^{+/-}$ x *Hspa9*$^{+/-}$ †**	
		Observed (Expected)	
Genotype	**Observed (Expected)**	**C57Bl6/N**	**B6129F2**
Hspa9$^{+/+}$	121 (130.5)	30 (18.25)	52 (34.75)
Hspa9$^{+/-}$	140 (130.5)	43 (36.5)	87 (69.5)
Hspa9$^{-/-}$	0 (0)	0 (18.25)*	0 (34.75)*
total	261	73	139

*p<0.0001

†C57Bl/6N *Hspa9*$^{+/-}$ mice were outcrossed to wild-type 129X1/SvJ mice (Jax: 000691) to generate *Hspa9*$^{+/-}$ B6129F1.

Table 2

Pathways significantly down-regulated in *Hspa9*$^{+/-}$ CFU-PreB colonies

Annotation Cluster 1: Enrichment Score 5.02				
Term		**# genes**	**P Value**	**Benjamini**
GO:0045321	leukocyte activation	13	5.56E-06	6.28E-03
GO:0046649	lymphocyte activation	12	8.65E-06	4.89E-03
GO:0001775	cell activation	13	1.80E-05	6.77E-03

Annotation Cluster 2: Enrichment Score 3.48				
Term		**# genes**	**P Value**	**Benjamini**
GO:0051249	regulation of lymphocyte activation	9	1.94E-04	5.36E-02
GO:0032944	regulation of mononuclear cell proliferation	7	2.51E-04	5.53E-02
GO:0050670	regulation of lymphocyte proliferation	7	2.51E-04	5.53E-02
GO:0070663	regulation of leukocyte proliferation	7	2.87E-04	5.28E-02
GO:0002694	regulation of leukocyte activation	9	3.07E-04	4.85E-02
GO:0050865	regulation of cell activation	9	3.35E-04	4.64E-02
GO:0050863	regulation of T cell activation	7	1.24E-03	1.31E-01