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Mitochondrial Stress-Initiated Aberrant Activation of the NLRP3 Inflammasome Regulates the Functional Deterioration of Hematopoietic Stem Cell Aging

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SUMMARY

Aging-associated defects in hematopoietic stem cells (HSCs) can manifest in their progeny, leading to aberrant activation of the NLRP3 inflammasome in macrophages and affecting distant tissues and organismal health span. Whether the NLRP3 inflammasome is aberrantly activated in HSCs during physiological aging is unknown. We show here that SIRT2, a cytosolic NAD^+-dependent deacetylase, is required for HSC maintenance and regenerative capacity at an old age by repressing the activation of the NLRP3 inflammasome in HSCs cell autonomously. With age, reduced SIRT2 expression and increased mitochondrial stress lead to aberrant activation of the

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AUTHOR CONTRIBUTIONS

D.C. conceived and supervised the project. H.L. performed the experiments and analyzed the data. W.-C.M. performed in vitro HSC response to treatments. R.K., T.-D.K., and W.-C.M. detected NLRP3 and caspase 1 expression in HSCs. H.-H.C. provided recipient mice. M.M. and J.J.S. assisted with transplants and data analyses. R.O. and J.J.S. provided SIRT7 KO mice. K.I. provided input on HSC biology. D.C. and H.L. wrote the manuscript with the assistance of all co-authors.

SUPPLEMENTAL INFORMATION

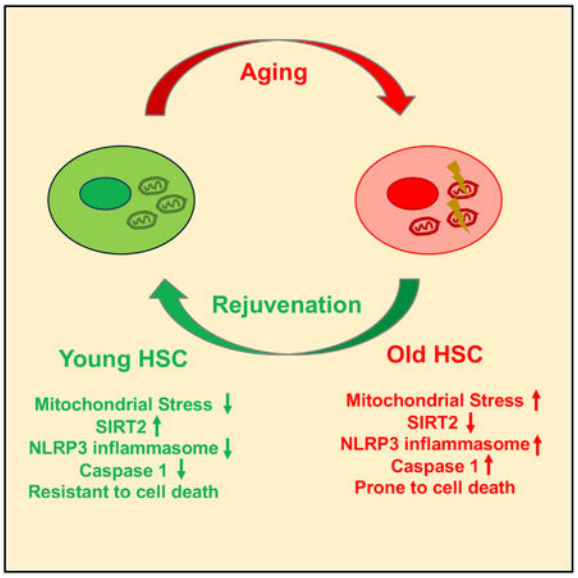
Supplemental Information includes six figures and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.12.101.

DECLARATION OF INTERESTS

D.C. and H.L. are inventors of a patent on inhibiting stem cell aging.

NLRP3 inflammasome in HSCs. SIRT2 overexpression, NLRP3 inactivation, or caspase 1 inactivation improves the maintenance and regenerative capacity of aged HSCs. These results suggest that mitochondrial stress-initiated aberrant activation of the NLRP3 inflammasome is a reversible driver of the functional decline of HSC aging and highlight the importance of inflammatory signaling in regulating HSC aging.

Graphical Abstract



In Brief

Luo et al. show that the NLRP3 inflammasome is activated in aged hematopoietic stem cells (HSCs) due to mitochondrial stress and SIRT2 inactivation, contributing to the functional decline of HSC aging. This study identifies methods for reversing HSC aging and highlights the importance of inflammatory signaling in regulating HSC aging.

INTRODUCTION

The degeneration and dysfunction of aging tissues are attributable to the deterioration of adult stem cells (López-Otín et al, 2013; Oh et al., 2014). Adult stem cells are maintained in a metabolically inactive quiescent state for prolonged periods of time as an evolved adaptation to ensure their survival (Cheung and Rando, 2013; Folmes et al., 2012). The transition from the quiescent state to proliferation is monitored by the restriction point that surveils mitochondrial health (Berger et al., 2016; Brown et al., 2013; Ito et al., 2016; Luchsinger et al., 2016; Mantel et al., 2015; Mohrin and Chen, 2016; Mohrin et al., 2015, 2018). The mitochondrial metabolic checkpoint is dysregulated in stem cells during physiological aging, contributing to their functional deterioration (Brown et al., 2013; Mohrin et al., 2015). How mitochondrial stress results in the loss of stem cell maintenance and regenerative potential is unknown.

Recent human studies have shown that aging is associated with the accumulation of somatic mutations in the hematopoietic system and expansion of the mutated blood cells, a phenomenon termed clonal hematopoiesis (Busque et al., 2012; Genovese et al., 2014; Jaiswal et al., 2014; McKerrell et al., 2015; Xie et al., 2014). Individuals with clonal hematopoiesis are at higher risk for not only blood diseases but also myocardial infarctions, strokes, vascular complications of type 2 diabetes, and earlier mortality (Bonnefond et al., 2013; Goodell and Rando, 2015; Jaiswal et al., 2014). Deficiency in the TET2 gene, which is frequently mutated in blood cells of the individuals with clonal hematopoiesis, results in clonal expansion and accelerates atherosclerosis development by inducing the inappropriate activation of the NLRP3 inflammasome in macrophages in mice (Fuster et al., 2017). In addition to atherosclerosis, aberrant activation of the NLRP3 inflammasome drives pathological inflammation in sterile inflammatory diseases associated with aging, such as Alzheimer's disease, Parkinson's disease, obesity, diabetes, multiple sclerosis, and cancer (Duewell et al., 2010; Guo et al., 2015; Heneka et al., 2013; Inoue et al., 2012; Jourdan et al., 2013; Yan et al., 2015). These observations support the notion that because the blood system supports all tissues, aging-associated defects in hematopoietic stem cells (HSCs) can be propagated in their progeny, including inappropriate activation of the NLRP3 inflammasome in macrophages, thereby having detrimental effects on distant tissues and organismal health span (Goodell and Rando, 2015). What remains unanswered is whether the NLRP3 inflammasome is aberrantly activated in HSCs during physiological aging and underlies aging-associated functional defects in HSCs.

Sirtuins are a family of protein deacylases that regulate diverse cellular pathways that control metabolism, stress resistance, and genome maintenance (Finkel et al., 2009; Giblin et al., 2014; Shin et al., 2013). SIRT2 is a mammalian sirtuin that resides in the cytosol and possesses deacetylase activity (North et al., 2003). We report that SIRT2 regulates the functional deterioration of HSCs at an old age by repressing the NLRP3 inflammasome activation. We show that the NLRP3 inflammasome is aberrantly activated in aged HSCs due to heightened mitochondrial stress and reduced SIRT2 activity. We demonstrate that functional deterioration of aged HSCs can be reversed by targeting the SIRT2-NLRP3-caspase 1 axis.

RESULTS

SIRT2 Is Required for HSC Maintenance in an Age-Dependent Manner

HSC aging is characterized by increased susceptibility to cell death upon stress, reduced per-cell repopulating capacity, and myeloid-biased differentiation (Janzen et al., 2006; Maryanovich et al., 2018; Rossi et al., 2008). At the molecular level, the epigenetic erosion with age leads to dysregulated control of gene expression, contributing to the decline of stem cell and tissue function (Goodell and Rando, 2015). Transcriptional profiling of young and old HSCs revealed that SIRT2 is among the most significantly repressed genes in old HSCs (Chambers et al., 2007). We validated this finding by assessing SIRT2 mRNA levels in highly enriched HSCs (immunophenotypically defined as $Lin^{-}c\text{-}Kit^{+}Sca1^{+}CD150^{+}CD48^{-}$) isolated from bone marrow of young (3 months old) and old (24 months old) wild-type (WT) mice by quantitative real-time PCR. SIRT2 mRNA levels were reduced by 3-fold in

HSCs isolated from old mice compared to those from young mice (Figures 1A and 1B). This observation prompted us to investigate the functional role of SIRT2 in HSCs.

We compared the quantity and quality of HSCs in WT and SIRT2 knockout (KO) mice. SIRT2 KO mice are born at the Mendelian ratio and are phenotypically normal (Bobrowska et al., 2012; Lo Sasso et al., 2014). Under homeostatic conditions, no difference in the number of immunophenotypically defined highly enriched HSCs was observed in the bone marrow of young WT and SIRT2 KO mice (Figure 1C). To determine whether SIRT2 deficiency affects HSC function, we performed a competitive bone marrow transplantation assay. CD45.2 HSCs isolated from donor mice were transplanted with CD45.1 competitor bone marrow cells to reconstitute the hematopoietic compartment of lethally irradiated recipient mice, and peripheral blood of the recipient mice was analyzed. HSCs isolated from young WT and SIRT2 KO mice were comparable in reconstituting the blood system of lethally irradiated recipient mice in competitive transplantation assays (Figures 1D and S1). To determine whether SIRT2 regulates lineage differentiation, we assayed mature hematopoietic subpopulations in the peripheral blood of WT and SIRT2 KO mice. No significant difference was observed in the percentage of lymphoid cells ($B220^+$ and $CD3^+$) and myeloid cells ($Mac\text{-}1^+Gr1^+$) in the peripheral blood of WT and SIRT2 KO mice (Figure 1E). Bone marrow cellularity was comparable between the two genotypes (Figure 1F).

However, at an old age, the number of HSCs in the bone marrow of SIRT2 KO mice was reduced compared to their WT littermates under homeostatic conditions (Figure 2A). The ability of HSCs isolated from aged SIRT2 KO mice to reconstitute the blood system of lethally irradiated recipient mice decreased by 2-fold compared to their WT counterparts (Figures 2B and S2A). In the peripheral blood of aged SIRT2 KO mice, the percentage of lymphoid cells was reduced and the percentage of myeloid cells was increased (Figure 2C). There was a mild reduction in the bone marrow cellularity in aged SIRT2 KO mice (Figure 2D). Together, these data suggest that SIRT2 has an age-dependent effect on HSC maintenance and hematopoiesis.

SIRT2 Prevents HSC Death upon Activation of the NLRP3 Inflammasome

Sirtuins are increasingly recognized as stress resistance genes (Brown et al., 2013; Mohrin et al., 2015; Qiu et al., 2010; Shin et al., 2013; Tao et al., 2010). We sought to determine whether the absence of SIRT2 affects cell survival to account for the age-associated changes in HSC number and repopulating capacity. In flow cytometry analyses of bone marrow cells derived from aged WT and SIRT2 KO mice using 7-aminoactino-mycin D (7AAD) to assess cell viability, we observed an increased percentage of cell death in the HSC population of SIRT2 KO mice compared to the WT controls (Figure 2E). However, WT and SIRT2 KO HSCs showed comparable staining for activated caspase 3 (Figure 2F), indicating that SIRT2 deficiency results in increased death of HSCs independent of caspase 3 activation.

In macrophages, pharmacological inhibition of SIRT2 enhances caspase 1 activation in response to the induction of the NLRP3 inflammasome by affecting the microtubule network (Misawa et al., 2013). In the innate immune system, inflammasomes are assembled upon cellular infection and stress that trigger the activation of the proteolytic enzyme caspase 1 to initiate innate immune responses (Lamkanfi and Dixit, 2014; Schroder and Tschopp, 2010).

The activation of caspase 1 induces pyroptosis, a form of programmed cell death that is caspase 1 dependent by definition and is independent of apoptotic caspases (Bergsbaken et al., 2009). We hypothesized that SIRT2 may prevent HSC death by repressing the NLRP3 inflammasome activation. NLRP3 has been extensively studied in macrophages. However, its expression in HSCs is readily detectable in a number of transcriptional profiling studies (Cabezas-Wallscheid et al., 2014; Månsson et al., 2007; Norddahl et al., 2011; Rossi et al., 2005; Sun et al., 2014). We further validated that NLRP3 expression in HSCs was about 15% of its expression in macrophages at the mRNA level and the protein level (Figures 3A and 3B). Consistently, based on BIOGPS, the expression level of NLRP3 in HSCs is about 20% of that in macrophages (http://biogps.org/#goto=genereport&id=216799).

We determined whether SIRT2 prevents HSC death upon the activation of the NLRP3 inflammasome. We isolated HSCs from WT and SIRT2 KO mice, which were primed with lipopolysaccharide (LPS) and were stimulated with ATP, a NLRP3 inflammasome inducer. Compared to WT HSCs, the treatment with LPS and ATP resulted in a reduction in the number of SIRT2 KO HSCs (Figure 3C) and an increase in the percentage of 7-AAD-positive SIRT2 KO HSCs (Figure 3D). The treatment with LPS and ATP did not have much effect on the frequency of differentiated hematopoietic cells (Figure 3E). Together, these data suggest that the activation of the NLRP3 inflammasome leads to cell death of HSCs in a cell-autonomous manner, which can be repressed by SIRT2. Consistent with a role of SIRT2 in repressing the NLRP3 inflammasome activation in HSCs *in vitro*, there was an increase in the activation of caspase 1 in HSCs derived from aged SIRT2 KO mice compared to those from WT control mice (Figures S2B and S2C), determined using a fluorescently labeled inhibitor of caspases (FLICA) probe that targets activated caspase 1 (Bruchard et al., 2015; Doitsh et al., 2014; Sheedy et al., 2013; Sokolovska et al., 2013), and increased caspase 1 activation in SIRT2 KO HSCs was blunted by NLRP3 inactivation (Figures S2D and S2E). The expression of NLRP3 and caspase 1 in WT and SIRT2 KO HSCs was comparable (Figures S2F and S2G), suggesting that SIRT2 likely regulates the NLRP3 activity at the post-transcriptional level.

The NLRP3 Inflammasome Regulates the Functional Decline of HSC Aging

Reduced SIRT2 expression in aged HSCs suggests that the NLRP3 inflammasome might be aberrantly regulated in HSCs during physiological aging. We assessed the activity of the NLRP3 inflammasome in HSCs of young and old WT mice. We isolated HSCs from young and old mice, which were primed with LPS and then stimulated with ATP. The activation of the NLRP3 inflammasome was assayed by western analyses of cleaved caspase 1. Treatment of old HSCs resulted in an increase in caspase 1 cleavage compared to young HSCs (Figure 4A). We further validated the activation of caspase 1 using FLICA probes. Under homeostatic conditions, no difference in the activation of caspase 3 was observed in young and old HSCs (Figure 4B), consistent with a previous report (Warr et al., 2013). However, aged HSCs exhibited increased activation of caspase 1 compared to young HSCs (Figure 4C). Thus, the NLRP3 inflammasome becomes aberrantly regulated in HSCs during aging.

To determine whether activation of the NLRP3 inflammasome is causal to the functional deterioration of aged HSCs, we knocked down NLRP3 or caspase 1 using short hairpin RNA

(shRNA) in old WT HSCs via lentiviral transduction. The functional capacity of the transduced HSCs was determined using a competitive transplantation assay. NLRP3 inactivation in aged HSCs increased the reconstitution capacity (Figures 4D, 4E, and S4A) and improved the differentiation into the lymphoid lineage (Figure 4F). Caspase 1 inactivation in aged HSCs also increased HSC engraftment and reconstitution capacity (Figures 5A–5C and S4B). Furthermore, HSCs from aged caspase 1 KO mice showed increased HSC engraftment, improved reconstitution capacity, and ameliorated myeloid-biased differentiation compared to WT controls (Figures 5D–5F). Together, these data suggest that activation of the NLRP3 inflammasome regulates the functional decline of aged HSCs.

SIRT2 Overexpression Reverses the Functional Decline of HSC Aging

Because SIRT2 expression reduces with age in HSCs (Figure 1B) (Chambers et al., 2007) and SIRT2 is required for the maintenance of aged but not young HSCs (Figures 1 and 2), we tested whether SIRT2 upregulation is sufficient to reverse the functional decline of aged HSCs. We overexpressed SIRT2 in young and aged WT HSCs via lentiviral transduction and examined their functional capacity using a competitive transplantation assay. Lentiviral transduction resulted in an 8-fold increase in SIRT2 expression in aged HSCs (Figure 6A) and a 2.7-fold increase in young HSCs (Figure S6A). Because SIRT2 expression is reduced by 3-fold in aged HSCs compared to young HSCs (Figure 1B), these data suggest that lentiviral transduction results in the comparable SIRT2 expression in aged and young HSCs. Compared to aged WT HSCs transduced with control lentivirus, reintroduction of SIRT2 in aged WT HSCs resulted in increased HSC engraftment and reconstitution capacity, and reversed myeloid-biased differentiation (Figures 6B–6E and S5). In contrast, SIRT2 overexpression did not significantly affect young HSCs (Figure S6).

Mitochondrial Stress Triggers Caspase 1 Activation in Aged HSCs

What are the stimuli of the NLRP3 inflammasome activation in aged HSCs? The NLRP3 inflammasome is unique among innate immune sensors, because it can be activated by endogenous damage signals in the absence of overt infection (Martinon et al., 2009; Strowig et al., 2012). Prominently, in macrophages, the mitochondria play an essential role in NLRP3 inflammasome activation by providing a platform for assembling the NLRP3 inflammasome complex and housing the effector molecules that directly activate the NLRP3 inflammasome (Wen et al., 2013). Because mitochondrial stress increases with age in HSCs and mitochondrial integrity is essential for HSC maintenance (Brown et al., 2013; Ito et al., 2016; Luchsinger et al., 2016; Mohrin et al., 2015, 2018), we assessed whether mitochondrial stress is a trigger of the NLRP3 inflammasome activation in HSCs during physiological aging. SIRT3, a mitochondrial deacetylase, promotes HSC maintenance by deacetylating critical lysine residues on the mitochondrial antioxidant superoxide dismutase 2 (SOD2), promoting the enzymatic activity of SOD2, and reducing mitochondrial oxidative stress (Brown et al., 2013; Qiu et al., 2010), while SIRT7, a histone deacetylase, enhances HSC maintenance by repressing the activity of the mitochondrial regulator nuclear respiratory factor 1 (NRF1) and suppressing mitochondrial protein folding stress (Mohrin et al., 2015). Therefore, SIRT3, SOD2 deacetylation, SIRT7, and NRF1 provide genetic tools to investigate the signaling events that mediate mitochondrial stress in HSCs. We examined

the effects of repressing mitochondrial stress on caspase 1 activation in aged HSCs *ex vivo*. Overexpression of SIRT3 or a constitutively active SOD2 mutant (SOD2 K53/89R) in aged HSCs via lentiviral transduction reduced the reactive oxygen species (ROS) level (Brown et al., 2013) and caspase 1 activation (Figure 7A). SIRT7 overexpression or NRF1 knockdown in aged HSCs reduced mitochondrial protein folding stress (Mohrin et al., 2015) and caspase 1 activation (Figures 7A and 7B).

In addition to the *ex vivo* examination, we further assessed the effects of mitochondrial stress on caspase activation in HSCs *in vivo*. SIRT7 KO mice show premature HSC aging phenotype (Mohrin et al., 2015). We therefore compared SIRT7 KO mice and their WT controls for caspase activation in HSCs. Caspase 1 activation was increased in HSCs of SIRT7 KO mice (Figure 7C), but the level of caspase 3 activation was unchanged (Figure 7D). Thus, mitochondrial stress initiates the aberrant activation of caspase 1 in aged HSCs. Notably, our findings that SIRT2 prevents the functional decline of aged HSCs and modulates the signaling of mitochondrial stress in HSCs are consistent with the recent observations that mitochondrial stress in HSCs leads to myeloid-biased differentiation and reduced repopulating capacity (Luchsinger et al., 2016; Mohrin et al., 2015).

DISCUSSION

Collectively, our results establish mitochondrial stress-initiated aberrant activation of the NLRP3 inflammasome as a trigger of the functional decline of HSC aging and highlight the importance of inflammatory signaling in regulating HSC aging (Figure 7E). In the absence of damage-associated molecular patterns, nuclear factor-κB (NF-κB) signaling primarily results in differentiation of HSCs (Figure S3) (Chen et al., 2018). However, in the presence of damage-associated molecular patterns, NF-κB activation leads to HSC death (Figure 3). These changes in HSC cell fate contribute to reduced HSC self-renewal potential of aged HSCs.

The HSC aging phenotype includes reduced repopulation capacity per cell, myeloid-biased differentiation, increased death upon stress, and paradoxically, increased HSC number based on cell surface markers (Janzen et al., 2006; Rossi et al., 2008), indicating that diverging mechanisms of HSC regulation shape the HSC aging phenotype. Several HSC aging mechanisms uncovered thus far, such as mitochondrial stress (Brown et al., 2013; Mohrin et al., 2015) and DNA damage (Rossi et al., 2007), result in cell death, reduced HSC number, and reduced HSC functionality. Increased HSC number with age is possibly attributable to TET2 mutation (Goodell and Rando, 2015). The SIRT2-NLRP3-caspase 1 axis transduces the signals from mitochondrial stress and contributes to the functional decline of aged HSCs (Figure 7E).

The NLRP3 inflammasome is activated in the periphery and the brain of aged animals (Youm et al., 2012, 2013). Inactivation of NLRP3 ameliorates systemic age-related inflammation and a wide array of aging-associated conditions (Youm et al., 2012, 2013). Our findings raise the possibility that, during physiological aging, aberrant activation of the NLRP3 inflammasome originated from HSCs may contribute to the NLRP3 inflammasome

activation in macrophages and influence systemic inflammation and the degeneration of distant tissues.

STAR★METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Danica Chen (danicac@berkeley.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice—SIRT2 knockout mice (C57BL/6) (Bobrowska et al., 2012), SIRT7 knockout mice (129) (Mohrin et al., 2015), and caspase 1 knockout mice (C57BL/6) (Kuida et al., 1995) have been described previously. All mice were housed on a 12:12 hr light:dark cycle at 25°C and received water and chow *ad libitum*. Young (3-5 month old) and aged (20-24 months old) littermates of the same sex were used. Both male and female mice were used. Animal procedures were performed in accordance with the University of California Berkeley animal care committee.

METHOD DETAILS

Flow Cytometry and Cell Sorting—Bone marrow cells were obtained by crushing the long bones with staining media (sterile PBS without calcium and magnesium supplemented with 2% FBS). Bone marrow cells were resuspended in staining media. The antibody mixture was added to bone marrow cells at 1:100 dilution. After staining for 20 minutes at 4°C, cells were washed with 3ml staining media. Lineage staining contained a cocktail of biotinylated anti-mouse antibodies to Mac-1 (CD11b), Gr-1 (Ly-6G/C), Ter119 (Ly-76), CD3, CD4, CD8a (Ly-2), and B220 (CD45R) (BioLegend). For detection or sorting of highly enriched HSCs, we used streptavidin, c-Kit, Sca-1, CD48, and CD150 antibodies (BioLegend). For congenic strain discrimination, anti-CD45.1 and anti-CD45.2 antibodies (BioLegend) were used. To determine cell death, 7AAD staining (Biolegend) was performed based on the manufacturer's instructions. To determine intracellular activation of specific caspases, fluorescent labeled inhibitors of caspases (FLICA) probe assays (ImmunoChemistry Technologies) and active caspase 3 detection kits (BD PharMingen) were used based on the manufacturer's instructions. For all the cell death and caspase activation analysis, bone marrow cells were stained for highly enriched HSC populations as described above followed by 7AAD or active caspase staining. For 7AAD staining, 5ul of 7AAD viability staining solution were added to 100ul of resuspended bone marrow cells and incubated for 15 minutes at 4°C. Samples were washed with staining media before flow cytometry analysis. For FLICA assays, 10ul of diluted FLICA reagents (1:5 dilution of stock in DMSO) were added to 290ul of bone marrow cells. After incubation for 1 hour at 37°C, samples were washed three times with 1ml 1X apoptosis buffer before flow cytometry analysis. For active caspase 3 staining, bone marrow cells were washed with ice cold PBS and then resuspended in BD cytofix/cytoperm solution. After incubation of 20 minutes at 4°C, cells were washed with BD 1X perm/wash buffer. 20ul of caspase 3 antibody were added to 100ul of resuspended bone marrow cells. Samples were then incubated for 30 minutes at room temperature. Cells were washed with 1X perm/wash buffer before flow

analysis. All data were collected on a Fortessa or LSRII (Becton Dickinson) and data analysis was performed with FlowJo (TreeStar). For cell sorting, c-kit enrichment was performed according to the manufacturer's instructions (Miltenyi Biotec). c-kit$^+$ cells were used for isolating enriched HSCs using markers (Lin$^-$Ckit$^+$Sca1$^+$) or highly enriched HSCs using markers (Lin$^-$Ckit$^+$Sca1$^+$CD150$^+$CD48$^-$). c-Kit$^-$ cells were used for Mac1(CD11b) antibody staining to isolate Mac1+ macrophage cells from mouse bone marrow. Cells were sorted using a Cytopeia INFLUX or Aria Fusion Sorters (Becton Dickinson). (Complete list of antibodies can be found in Key Resources Table)

Cell Culture—To prepare HSC media, StemSpan SFEM (StemCell Technologies) is supplemented with 10% ES-Cult FBS (StemCell Technologies), 1% Penicillin/Streptomycin (Invitrogen), IL3 (20ng/ml), IL6 (20ng/ml), TPO (50ng/ml), Flt3L (50ng/ml), and SCF (100ng/ml) (Peprotech). For *in vitro* inflammasome stimulation, freshly sorted HSCs were primed with 100ng/ml LPS for 20 hours and then stimulated with 3mM ATP for 30mins. For *in vitro* NF-kB signaling activation, freshly sorted HSCs were treated with or without 200ng/ml LPS and 1ug/ml Pam3CSK4 for 3 days. Cells were then stained for HSCs, differentiated blood cells, or 7AAD as described above. Proteins from culture media were trichloroacetic acid (TCA) precipitated for Western analyses of p20 caspase 1. Proteins from cell lysates were analyzed for pro-caspase 1. HEK293T cells were acquired from the ATCC. Cells were cultured in DMEM (Invitrogen) supplemented with 1% penicillin-streptomycin (Invitrogen) and 10% FBS (Invitrogen). All cells were maintained under standard growth conditions at 37°C and 5% CO_2 in a humidified atmosphere.

Immunoblotting—Proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked using 5% milk in PBST solution (PBS/0.1% Tween) for 30 minutes and incubated with primary antibodies for 1 hour at room temperature or 16 hours at 4°C. After washing with PBST three times (10 minutes each wash), membranes were incubated with secondary antibodies for 1 hour at room temperature. After washing with PBST three times (10 minutes each wash), membranes were developed using ECL (GE healthcare) following the manufacturer's instructions and imaged using ImageQuant™ LAS 4000 (GE healthcare). Antibody details are provided in Key Resources Table.

Lentiviral Transduction of HSCs—As previously described (Zhao et al., 2009), sorted HSCs were prestimulated for 5-10 hr in a 96 well U bottom dish in HSC media. Lentivirus was produced as described (Qiu et al., 2010). Briefly, 293T cells were transfected with lentiviral packaging plasmids together with pFUGw or pFUGw-H1 vectors using lipofectamine (Invitrogen) following the manufacturer's protocol. The oligonucleotide sequences used to clone Caspase1 or NLRP3 shRNA into pFUGw-H1 vectors were provided in Table S1. 24 hours after transfection, cells were changed to fresh media. 48 hours after transfection, virus was collected, filtered through a 45uM syringe filter. Lentivirus was then concentrated by centrifugation at 17,900 g for 90 min, and resuspended with 200ul HSC media. The lentiviral media were added to HSCs in a 96 well plate, spinoculated for 90 min at 270G in the presence of 8ug/ml polybrene. This process was repeated 24 hr later with a fresh batch of lentiviral media.

Transplantation Assays—250 freshly sorted HSCs from CD45.2 donor mice were mixed with 5×10^5 CD45.1 B6.SJL competitor bone marrow cells and injected into lethally irradiated B6.SJL recipient mice. To assess multilineage reconstitution of transplanted mice, peripheral blood was collected every month for 4 months. Red blood cells were lysed using 500ul 1X BD FACS lysing solution (BD Biosciences) for 5 minutes at room temperature, washed with 3ml PBS, and stained with antibody mixture consisting CD45.2, CD45.1, Mac1, Gr1, B220, and CD3 (Biolegend) for 20 minutes at 4°C. Cells were washed with 3ml PBS before flow analysis. Bone marrow cells were analyzed 4 months posttranplantation using CD45.2, CD45.1, Lineage cocktail, c-Kit, Sca-1, and CD150 antibodies. Antibody details are provided in Key Resources Table.

mRNA Analysis—RNA was isolated from cells using Trizol reagent (Invitrogen) following the manufacturer's instructions. cDNA was generated using the qScript cDNA SuperMix (Quanta Biosciences) following the manufacturer's instructions. Gene expression was determined by real time PCR using Eva qPCR SuperMix kit (BioChain Institute) on an ABI StepOnePlus system. All data were normalized to β-Actin expression. PCR primer details are provided in Table S1.

QUANTIFICATION AND STATISTICAL ANALYSIS

Mice were randomized to groups and analysis of mice and tissue samples was performed by investigators blinded to the treatment or the genetic background of the animals. Statistical analysis was performed with Student's t test (Excel). Data are presented as means and error bars represent standard errors. In all corresponding figures, * represents $p < 0.05$. ** represents $p < 0.01$. *** represents $p < 0.001$. ns represents $p > 0.05$. Replicate information is indicated in the figures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- SIRT2 is required for HSC maintenance at an old but not young age
- SIRT2 represses the activation of the NLRP3 inflammasome in HSCs
- NLRP3 inflammasome is activated in aged HSCs due to increased mitochondrial stress
- SIRT2 activation, NLRP3 inactivation, or caspase 1 inactivation reverses HSC aging

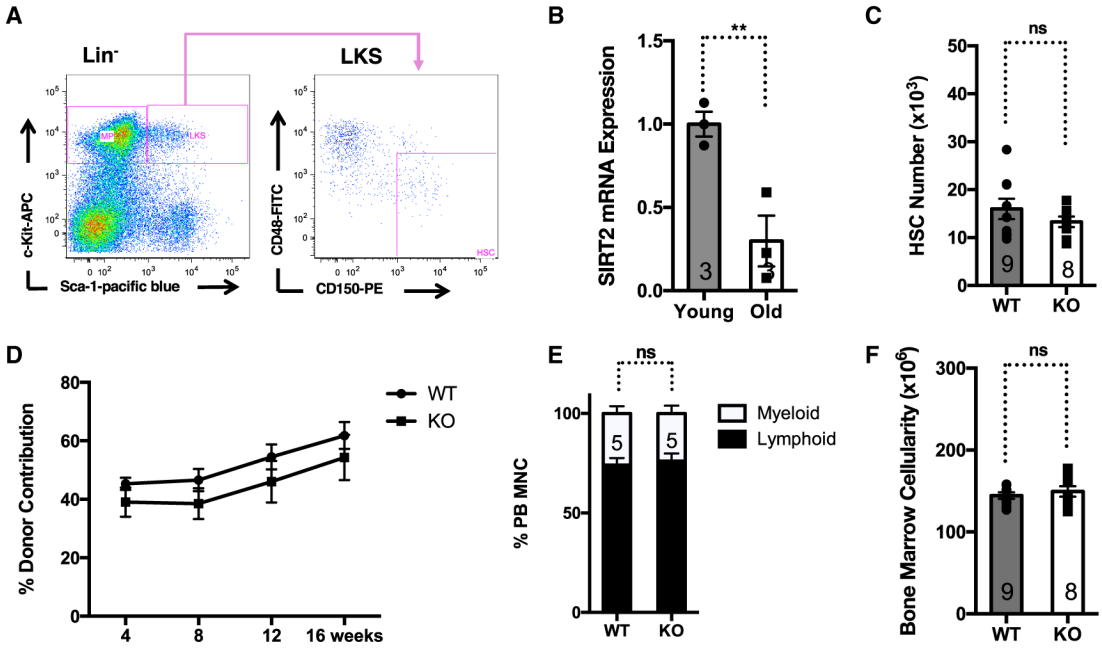

Figure 1.

SIRT2 Is Not Required for HSC Maintenance at a Young Age

(A) Gating strategy for HSCs of young mice. Enriched HSCs: Lin^{-}c-Kit^{+}Sca1^{+}. Highly enriched HSCs: Lin^{-}c-Kit^{+}Sca1^{+}CD150^{+}CD48^{-}.

(B) The expression of SIRT2 in HSCs isolated from young (3 months old) and old mice (2 years old) was quantified by qPCR. n = 3.

(C) HSC number in the bone marrow of 3-month-old WT and SIRT2 KO mice was determined via flow cytometry. n = 9 and 8.

(D) Competitive transplantation using HSCs isolated from 3-month-old WT and SIRT2 KO mice as donors. The percentage of total donor-derived cells in the peripheral blood (PB) of the recipients was determined via flow cytometry. n = 13.

(E) Lineage differentiation in the peripheral blood of 3-month-old WT and SIRT2 KO mice was determined via flow cytometry. MNCs, mononuclear cells. n = 5.

(F) Bone marrow cellularity in 3-month-old WT and SIRT2 KO mice. n = 9 and 8.

Error bars represent SE. **p < 0.01. ns, p > 0.05. Student's t test.

See also Figure S1.

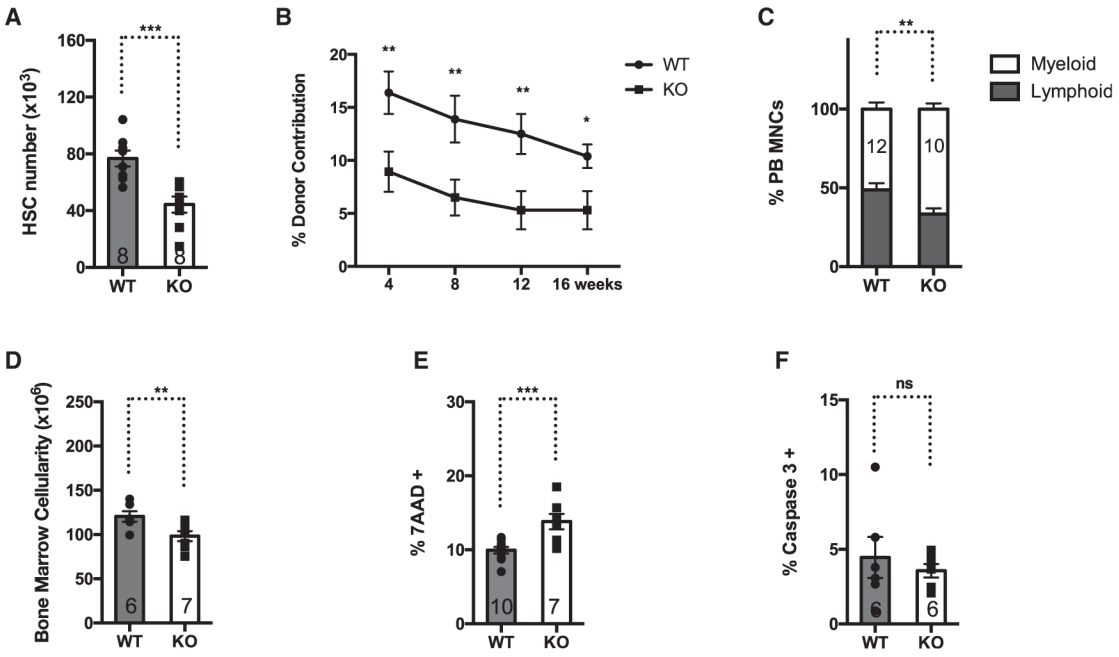

Figure 2.

SIRT2 Is Required for HSC Maintenance at an Old Age

(A) The number of HSCs in the bone marrow of 24-month-old WT and SIRT2 KO mice was determined via flow cytometry. n = 8.

(B) Competitive transplantation using HSCs isolated from 24-month-old WT and SIRT2 KO mice as donors. The percentage of total donor-derived cells in the peripheral blood (PB) of the recipients was quantified by flow cytometry. n = 12 and 11.

(C) The percentage of lymphoid and myeloid cells in the PB of 24-month-old WT and SIRT2 KO mice was determined by flow cytometry. MNCs, mononuclear cells. n = 12 and 10.

(D) Bone marrow cellularity of 2-year-old WT and SIRT2 KO mice. n = 6 and 7.

(E and F) Flow cytometry analyses of staining for 7-AAD and activated caspase 3 in HSCs of aged WT and SIRT2 KO mice. n = 10 and 7 (E). n = 6 (F).

Error bars represent SE. *p < 0.05. **p < 0.01. ***p < 0.001. ns, p > 0.05. Student's t test. See also Figure S2.

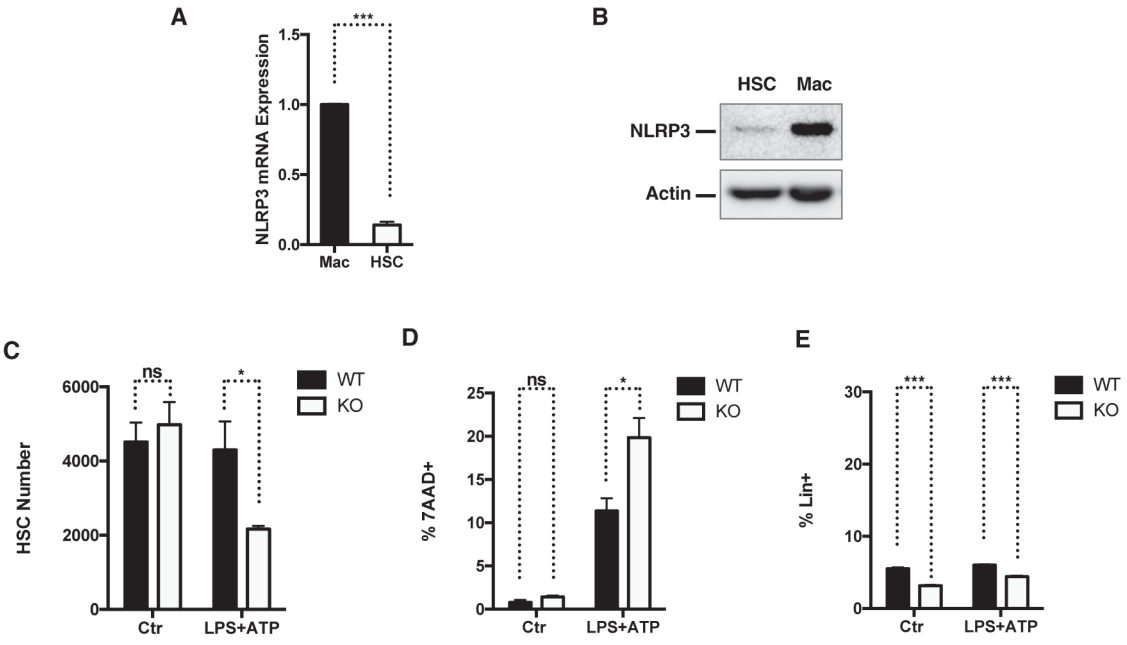

Figure 3.

SIRT2 Prevents HSC Death upon the NLRP3 Inflammasome Activation

(A and B) NLRP3 expression in macrophages and HSCs isolated from mouse bone marrow was determined by qPCR (A) and western analyses (B).

(C–E) HSCs isolated from young WT and SIRT2 KO mice were primed with LPS for 20 h, and stimulated with ATP, a NLRP3 inflammasome inducer, for 30 min. Cells were stained with 7AAD and analyzed with flow cytometry. Data shown are the number of HSCs (C), the percentage of 7AAD-positive cells in the gated HSC population (D), and the percentage of Lin^+ cells (E). n = 3.

Error bars represent SE. *p < 0.05. ***p < 0.001. ns, p > 0.05. Student's t test.

See also Figure S3.

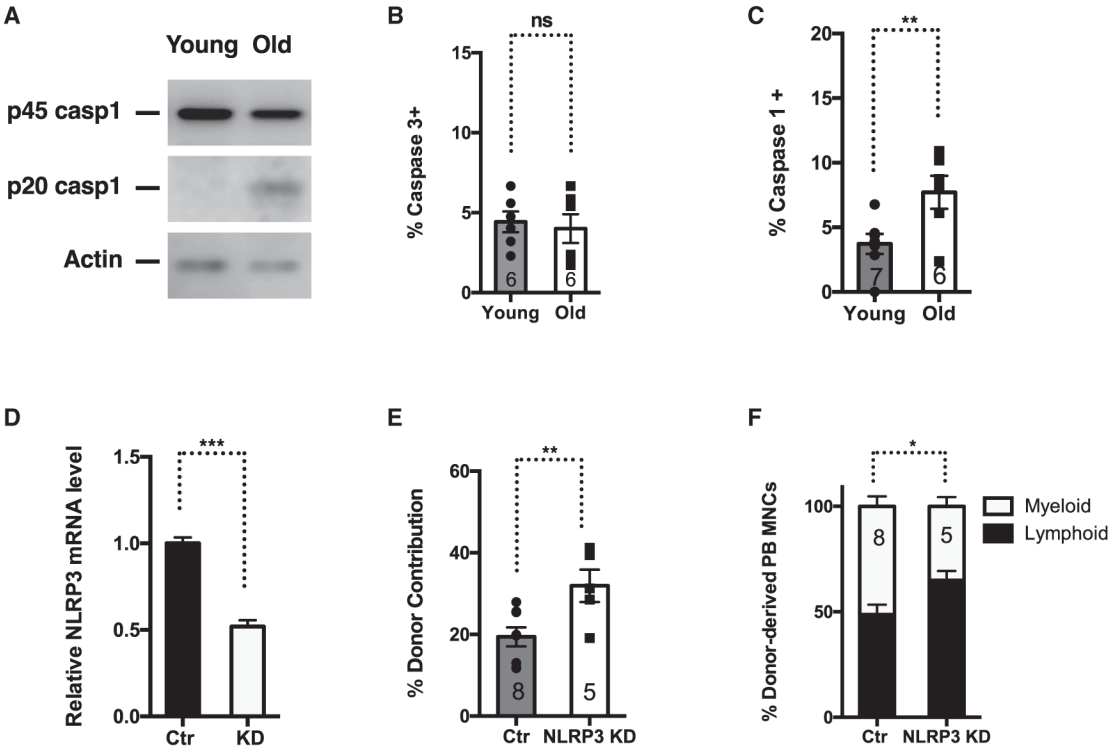

Figure 4.

The NLRP3 Inflammasome Regulates the Functional Decline of HSC Aging

(A) HSCs were isolated from young and old WT mice, primed with LPS, and then stimulated with ATP, a NLRP3 inflammasome inducer. Cell lysates were used for western analyses for pro caspase 1 and actin, and culture supernatants were used for cleaved p20 caspase 1 western analyses.

(B and C) Bone marrow cells of young and old WT mice were stained for activated caspases. Data shown are the percentage of caspase^{+} cells in HSCs. n = 6 (B). n = 7 and 6 (C).

(D) Aged HSCs were transduced with NLRP3 shRNA lentivirus or control virus. NLRP3 mRNA levels were quantified by qPCR. n = 3.

(E and F) Competitive transplantation using aged HSCs transduced with NLRP3 shRNA lentivirus or control virus as donors. The percentage of donor-derived cells in the peripheral blood of the recipients (E) and donor-derived lineage differentiation in the peripheral blood of the recipients (F) were determined by flow cytometry. n = 8 and 5.

Error bars represent SE. *$p < 0.05$. **$p < 0.01$. ns, $p > 0.05$. Student's t test.

See also Figure S4.

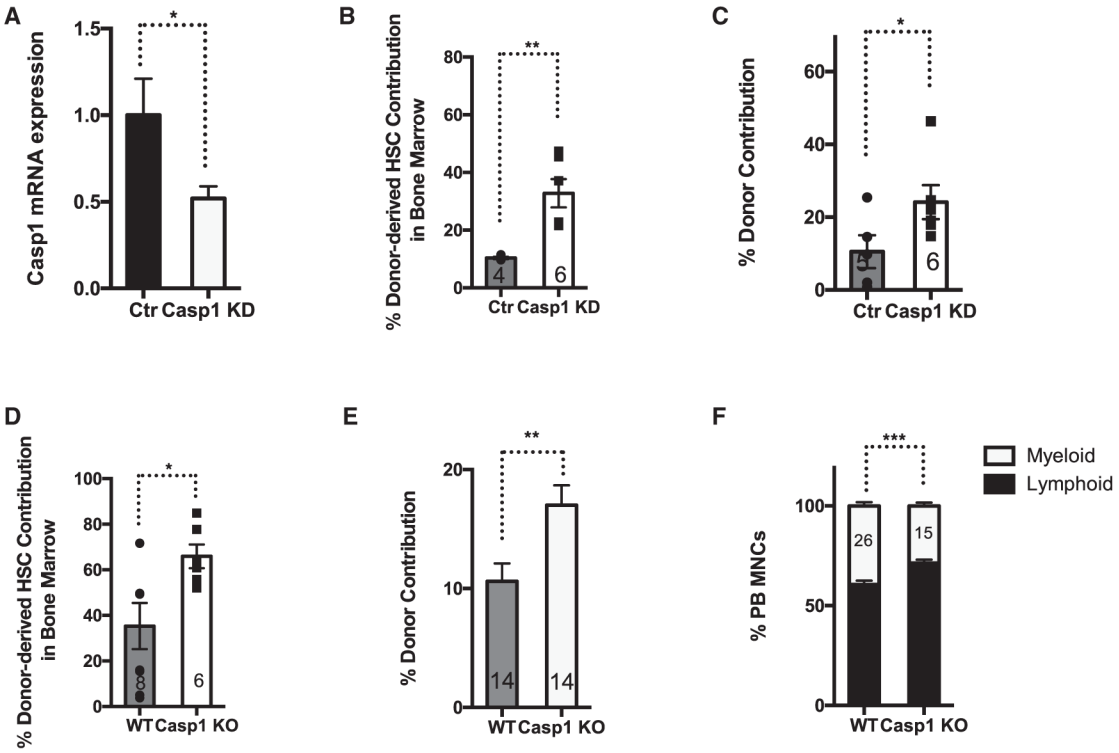

Figure 5.

HSC Aging Is Regulated by Caspase 1

(A–C) Competitive transplantation using aged HSCs transduced with caspase 1 shRNA lentivirus or control virus as donors. Gene expression of caspase 1 was determined by qPCR (A). The percentage of donor-derived HSCs in the bone marrow of the recipients (B) and the percentage of donor-derived cells in the peripheral blood of the recipients (C) were determined by flow cytometry. n = 3 (A). n = 4 and 6 (B). n = 5 and 6 (C).

(D and E) Competitive transplantation using HSCs from aged WT and caspase 1 KO mice as donors. Donor-derived HSC engraftment in the bone marrow (D) and the percentage of donor-derived cells in the peripheral blood of the recipients (E) were determined via flow cytometry. n = 8 and 6 (D). n = 14 (E).

(F) Lineage differentiation in the peripheral blood of aged WT and caspase 1 KO mice was determined by flow cytometry. n = 26 and 15.

Error bars represent SE. *p < 0.05. **p < 0.01. ***p < 0.001. Student's t test.

See also Figure S4.

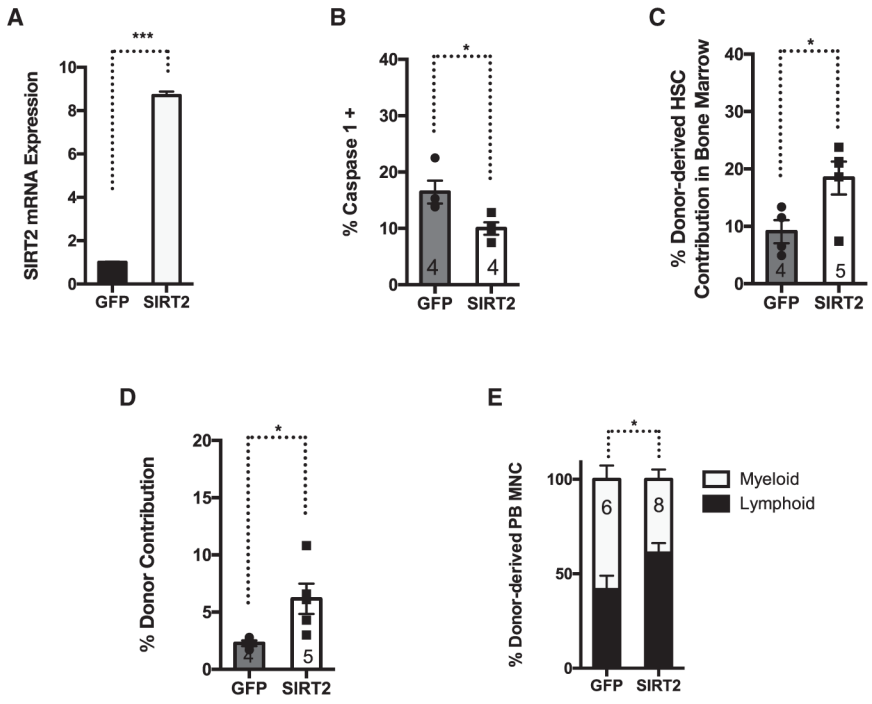

Figure 6.

SIRT2 Reverses the Functional Decline of HSC Aging

(A and B) Aged HSCs were transduced with SIRT2 or GFP lentivirus. SIRT2 mRNA levels were quantified by qPCR (A). Staining of activated caspase 1 was quantified by flow cytometry analyses (B). n = 3 (A). n = 4 (B).

(C–E) Competitive transplantation using aged HSCs transduced with SIRT2 or control lentivirus as donors. The donor-derived HSC engraftment in the bone marrow (C), the percentage of donor-derived cells in the peripheral blood (D), and donor-derived lineage differentiation in the peripheral blood (E) were determined by flow cytometry. n = 4 and 5 (C and D). n = 6 and 8 (E). Error bars represent SE. *$p < 0.05$. Student's t test. See also Figures S5 and S6.

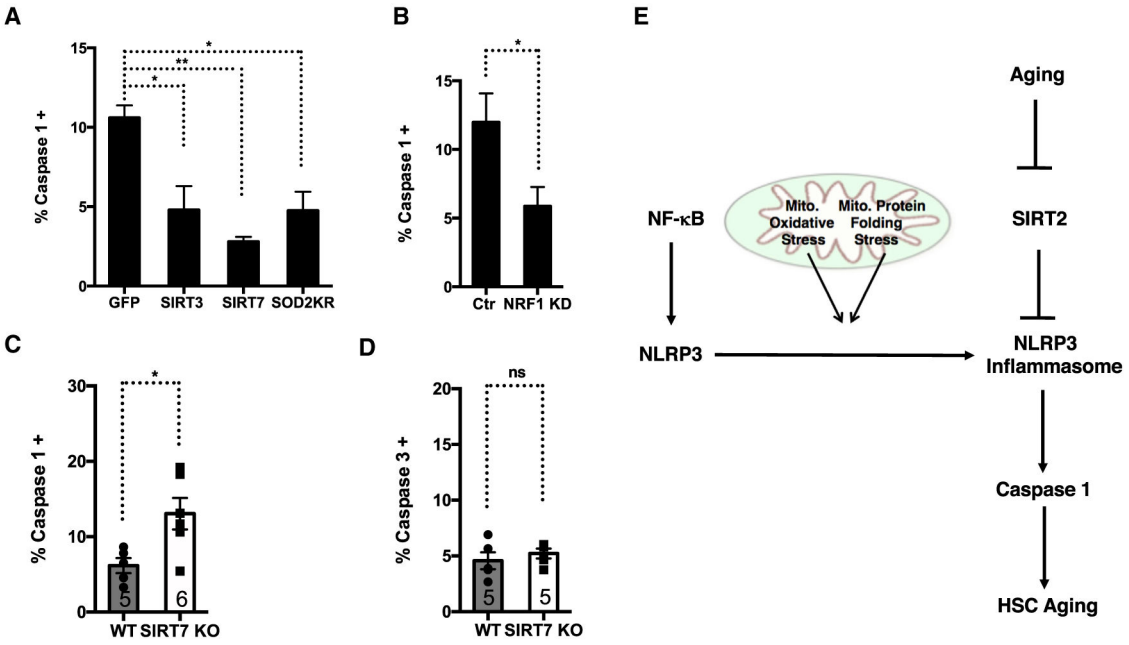

Figure 7.

Mitochondrial Stress Triggers Caspase 1 Activation in Aged HSCs

(A and B) Aged HSCs were transduced with indicated lentivirus: SIRT3, SIRT7, SOD2KR (A) and NRF1 shRNA(B). At 2 days post-transduction, cells were stained for activated caspase 1 and analyzed via flow cytometry. Data shown are activated caspase 1^+ cells in the gated HSC population. n = 3.

(C and D) Bone marrow cells of SIRT7 KO mice and their WT controls were stained for activated caspases. Data shown are the percentage of caspase$^+$ cells in HSCs. n = 5 and 6 (C). n = 5 (D).

(E) A proposed model. SIRT2 represses NLRP3 inflammasome activation in HSCs. In aged HSCs, reduced SIRT2 expression and increased mitochondrial stresses lead to activation of the NLRP3 inflammasome, reduced HSC survival, and reduced functionality.

Error bars represent SE. *p < 0.05. **p < 0.01. ns, p > 0.05. Student's t test.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Actin antibody produced in rabbit	Sigma-Aldrich	Cat# A2066; RRID: AB_476693
Caspase 1 Monoclonal Antibody (5B10), eBioscience(TM)	Thermo Fisher Scientific	Cat# 14–9832; RRID: AB_2016691
anti-NLRP3/NALP3 mAb (Cryo-2) antibody	AdipoGen	Cat# AG-20B-0014; RRID: AB_2490202
CD45.1 Pacific Blue	3iolegend	Cat# 110722; RRID: AB_492866
CD45.1 PerCP	3iolegend	Cat# 110726; RRID: AB_893345
Streptavidin PerCP	3iolegend	Cat# 405213
Mac1 PE	3iolegend	Cat# 101208; RRID: AB_312791
CD3 Pacific Blue	Biolegend	Cat# 100214; RRID: AB_493645
Sca1 Pacific Blue	Biolegend	Cat# 108120; RRID: AB_493273
Streptavidin APC-Cy7	Biolegend	Cat# 405208
c-Kit APC-Cy7	Biolegend	Cat# 105826; RRID: AB_1626278
CD3 APC-Cy7	Biolegend	Cat# 100222; RRID: AB_2242784
B220 APC-Cy7	Biolegend	Cat# 103224; RRID: AB_313007
Sca1 Cy7-PE	Biolegend	Cat# 122514; RRID: AB_756199
CD45.2 Cy7-PE	Biolegend	Cat# 109830; RRID: AB_1186098
CD150 Cy7-PE	Biolegend	Cat# 115914; RRID: AB_439797
Gr1 Cy7-PE	Biolegend	Cat# 108416; RRID: AB_313381
CD3 Biotin	Biolegend	Cat# 100304; RRID: AB_312669
CD48 Cy7-PE	Biolegend	Cat# 103424; RRID: AB_2075049
B220 Biotin	Biolegend	Cat# 103204; RRID: AB_312989
Gr1 Biotin	Biolegend	Cat# 108404; RRID: AB_313369
CD8a Biotin	Biolegend	Cat# 100704; RRID: AB_312743
Mac1 Biotin	Biolegend	Cat# 101204; RRID: AB_312787
Ter119 Biotin	Biolegend	Cat# 116204; RRID: AB_313705
CD4 Biotin	Biolegend	Cat# 100404; RRID: AB_312689
CD48 FITC	Biolegend	Cat# 103404; RRID: AB_313019
Gr1 FITC	Biolegend	Cat# 108406; RRID: AB_313371
CD150 PE	Biolegend	Cat# 115904; RRID: AB_313683
CD45.1 PE	Biolegend	Cat# 110708; RRID: AB_313497
c-Kit APC	Biolegend	Cat# 105812; RRID: AB_313221
B220 APC	Biolegend	Cat# 103212; RRID: AB_312997
CD45.2 APC	Biolegend	Cat# 109813; RRID: AB_389210
Gr1 PerCP-Cy5.5	Biolegend	Cat# 108428; RRID: AB_893558
Mac1 PerCP-Cy5.5	Biolegend	Cat# 101228; RRID: AB_893232
Gr1 APC-Cy7	Biolegend	Cat# 108424; RRID: AB_2137485
CD3 PerCP-Cy5.5	Biolegend	Cat# 100218; RRID: AB_1595492
Mac1 APC-Cy7	Biolegend	Cat# 101226; RRID: AB_830642
CD8a APC-Cy7	Biolegend	Cat# 100714; RRID: AB_312753

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ter119 APC-Cy7	Biolegend	Cat# 116223; RRID: AB_2137788
CD4 APC-Cy7	Biolegend	Cat# 100414; RRID: AB_312699
CD45.2 FITC	eBioscience	Cat#11–0454-85; RRID: AB_465062
Chemicals, Peptides, and Recombinant Proteins		
LPS	Invivogen	Cat# tlrl-eklps
ATP	Invivogen	Cat# tlrl-atpl
Pam3CSK4	Invivogen	Cat# tlrl-pms
Trichloroacetic acid (TCA)	Sigma	Cat# T0699
Dulbecco's Modification of Eagle's Medium	Invitrogen	Cat# 11965092
Fetal Bovine Serum	Invitrogen	Cat#10437–028
Penicillin Streptomycin Solution (100x)	Invitrogen	Cat# 15140122
0.25% Trypsin	Invitrogen	Cat# 25200056
TRIzol Reagent	Invitrogen	Cat#15596026
7AAD	Biolegend	Cat# 420404
Fixation buffer	Biolegend	Cat# 420801
Permeabilization wash buffer	Biolegend	Cat# 421002
CD117 (c-kit) MicroBeads, mouse	Miltenyi Biotec	Cat# 130–094-224
Stemspan SFEM	Stemcell technologies	Cat# 09600
ES-Cult FBS	Stemcell technologies	Cat# 06952
Murine IL3	Peprotech	Cat# 213–13
Murine IL6	Peprotech	Cat# 216–16
Murine Flt3 ligand	Peprotech	Cat# 250–31L
Murine TPO	Peprotech	Cat# 315–14
Murine SCF	Peprotech	Cat# 250–03
Critical Commercial Assays		
FAM-FLICA Caspase-1 Assay Kit	ImmunoChemistry Technologies	Cat# 98
FLICA 660 Caspase-1 Assay	ImmunoChemistry Technologies	Cat# 9122
PE Active Caspase-3 Apoptosis Kit	BD PharMingen	Cat# 550914
FITC Active Caspase-3 Apoptosis Kit	BD PharMingen	Cat# 550480
qScript cDNA SuperMix	Quanta Biosciences	Cat# 95048–100
Eva qPCR SuperMix kit	BioChain Institute	Cat# K5052200
Experimental Models: Cell Lines		
HEK293T	ATCC	CRL-3216
Experimental Models: Organisms/Strains		
Mouse: SIRT2 KO	Bobrowska et al., 2012	N/A
Mouse: Caspase1 KO	Kuida et al., 1995	N/A
Mouse: SIRT7 KO	Mohrin et al., 2015	N/A
Mouse: C57BL/6J	Charles Rivers Laboratories	C57BL/6NCrl

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse: B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1)	The Jackson Laboratories	JAX: 002014
Oligonucleotides		
Primers and shRNA cloning oligos	Table S1	N/A
Recombinant DNA		
pFUGw	Mohrin et al., 2015	Addgene #14883
pFUGw-H1	Mohrin et al., 2015	Addgene #25870