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# The function of SIRT7 in Cellular Stress Response and Tissue Maintenance

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## Abstract

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Until the last few decades, aging was thought to be the stochastic accumulation of errors not subject to any active regulation. However, recent evidence suggests that aging is under regulatory control and is subject to modulation by classical signaling pathways. These pathways include the insulin/IGF-1 pathway, the mTOR pathway, and sirtuins, whose up- or downregulation can trigger diverse cell-protective mechanisms against environmental and physiological stress and lead to extension of lifespan.

Sirtuins have been shown to play a significant role in tissue maintenance by regulating cellular response to stresses such as genome instability, oxidative stress, and nutritional stress. However, since their role in maintaining proteostasis has not been studied yet, we set out to investigate if SIRT7, a nuclear member of the sirtuin family, functions in regulating protein homeostasis, particularly in the context of protein folding stress. Using a murine model, we focused on studying the function of SIRT7 in the liver and the hematopoietic system, two tissues that SIRT7 is most highly expressed in. We found that in the liver, SIRT7 relieves ER stress through suppressing translation by inhibiting the transcription of ribosomal proteins via Myc. In hematopoietic stem cells, SIRT7 regulates mitochondrial stress by repressing the activity of NRF-1 and inhibiting the transcription of mitochondrial ribosomal subunits. In a physiological context, loss of SIRT7 leads to a steatohepatitis phenotype in the liver and an aging phenotype in the hematopoietic stem cells. Thus, our study suggests the maintenance of stress by SIRT7 as an important mechanism for hepatic lipid metabolism and hematopoietic stem cell function.

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# Chapter 1. An Overview of Aging, Stress, and Sirtuins

Aging was once thought to be a passive, entropic process of deterioration that leads to a decline in tissue function. However, the field of aging research has progressed significantly over the past few decades. We know now that the aging process is under regulation by classical signaling pathways, including the insulin/IGF-1 pathway, the mTOR pathway, and sirtuins that are conserved over a broad evolutionary distance. In general, the identified conserved modifiers of longevity tend to mediate the response to environmental and physiological stress. In this chapter I review these conserved pathways and the link between stress response and aging.

## Molecular pathways that regulate aging

### Insulin/IGF-1 Signaling (IIS) Pathway

The first evidence suggesting the role of IIS pathway in aging was discovered in *C. elegans*, where loss-of-function mutations in *daf-2*, a homolog of mammalian insulin/IGF receptors, extended lifespan by more than 2-fold and cause the animal to remain active and youthful longer than normal (Kenyon et al., 1993). Further studies found that inhibiting IIS changes lifespan through changes in gene expression through DAF-16 (a FOXO transcription factor), HSF-1 (heat-shock transcription factor), SKN-1 (a Nrf-like xenobiotic-response factor). These transcription factor signaling is involved in the regulation of genes in stress-response such as antioxidant defense, mitochondrial function, proteostasis, and autophagy. Since IIS pathway senses nutrients and controls transcription of stress response genes, this pathway provides a molecular connection between dietary intake and cellular stress response pathways.

The IIS pathway's effect on longevity has been evolutionarily conserved and manipulations that reduce IIS extend the lifespan of a variety of model organisms such as flies, mice, and dogs (Kenyon, 2005) . In *Drosophila*, systemic inhibition of IIS or increasing the activity of FOXO specifically in adipose tissue increases lifespan (Yuan et al., 2009). In mice, mutations that inhibit the IIS signaling extends lifespan and inverse correlation between IGF-1 levels and lifespan was found (Bartke, 2008; Kappeler et al., 2008; Selman et al., 2008; Yuan et al., 2009). In humans, mutations known to impair IGF-1 receptor function have been identified in Ashkenazi Jewish centenarians (Suh et al., 2008). Variants of insulin signaling genes AKT1, FOXO1, and FOXO3a have also been linked to longevity in multiple centenarian cohorts (Flachsbart et al., 2009; Pawlikowska et al., 2009; Willcox et al., 2008).

Genetic analyses support the idea that IIS pathway mediates part of the beneficial effects of calorie restriction (CR) on longevity. In *C. elegans,* every-other-day feeding extends lifespan by inhibiting insulin/IGF-1 signalling (Honjoh et al., 2009). In flies, the life span extension by CR is partially mediated by FOXO activity (Giannakou et al., 2008). In addition, the already long lifespan of growth hormone receptor knockout mice cannot be further extended by CR, suggesting that CR may also extend mouse lifespan by reducing IIS (Bonkowski et al., 2009).

### mTOR Pathway

The mTOR kinase is a conserved amino acid and nutrient sensor that regulates growth, anabolic metabolism, and cell division (Cunningham et al., 2007; Wullschleger et al., 2006). mTOR kinase stimulates growth and blocks autophagy in nutrient-rich conditions and mTOR activity is suppressed under nutrient limitation. mTOR inhibition extends lifespan in many species, from yeast to mice and can extend life span when inhibited during a limited period of adult life in mice (Harrison, 2009; Kaeberlein, 2005; Kapahi, 2004; Vellai, 2003).

In mammals, mTOR kinase is part of two multiprotein complexes, mTORC1 and mTORC2. The mTORC1 complex consists of mTOR, RAPTOR, PRAS40, and mLST8 and regulates cell growth, protein synthesis, ribosome biogenesis, and autophagy through key downstream targets including ribosomal subunit S6 kinase (S6K) and 4E-BP (Guertin and Sabatini, 2007; Sengupta et al., 2010). The mTORC2 consists of mTOR, RICTOR, mSIN1, PROTOR, and mLST8. mTOR is thought to exert its effects on aging through modulation of protein synthesis and autophagy by regulating translation through 4E-BP, an inhibitor of translation, and ribosomal subunit S6 kinase (S6K) (Chen et al., 2007; Hansen, 2007; Kapahi, 2004; Medvedik et al., 2007; Steffen, 2008). Genetically modified mice with low levels of mTORC1 activity but normal levels of mTORC2 have increased lifespan (Lamming et al., 2012). When mTOR is inhibited, S6K activity decreases, and inhibition of S6K extends lifespan in extends lifespan in yeast, worms, flies, and mice (Fabrizio et al., 2001; Hansen, 2007; Kaeberlein, 2005; Kapahi, 2004; Selman, 2009). These studies suggest the downregulation of mTORC1/S6K1 as the critical mediator of mammalian lifespan. Inhibition of mTOR also stimulates autophagy, which is an important requirement for lifespan extension (Bjedov, 2010; Hansen, 2008; Toth, 2008). mTOR inhibition increases resistance to environmental stress, leading to a physiological shift towards tissue maintenance. mTOR signaling therefore stands at the crossroads of stress resistance and aging.

### AMP kinase Pathway

AMP kinase (AMPK) is a nutrient and energy sensor that is activated when the cell is in a low-energy state. Stressors that deplete cellular ATP such as glucose deprivation, hypoxia, and exercise lead to activation of AMPK (Kahn et al., 2005). AMPK activates transcriptional and posttranslational signaling responses that promotes catabolic pathways and represses anabolic pathways when the cell's AMP/ATP ratio rises. For example, AMPK phosphorylates and inhibits acetyl-CoA carboxylase, which is an important enzyme in fatty acid synthesis and in turn switches the cell's metabolism to fatty acid oxidation. There is evidence indicating that AMPK activation may mediate lifespan extension. Overexpression of AMPK extends lifespan in *C.elegans*, and treating mice with metformin extends lifespan in mice (Anisimov, 2008; Apfeld et al., 2004). In addition, the decline in AMPK activity in skeletal muscle with age and its association with insulin resistance suggest that a decrease in AMPK activity during aging may contribute to insulin resistance and metabolic syndrome.

### Sirtuin Pathway

Silent information regulator 2 (Sir2) proteins, or sirtuins, are highly conserved nicotinamide adenine dinucleotide dependent protein deacetylases found in organisms ranging from bacteria to humans (Matsubara et al.; North and Verdin, 2004). Sirtuins were initially described as histone deacetylases that functions in gene silencing in yeast, but are now known to have various cellular functions that regulates aging and age-related phenotypes in multiple organisms. Studies in model organisms suggest that sirtuin overexpression can extend life span (Kaeberlein et al., 1999; Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001). There are seven mammalian sirtuins, SIRT1-7, localized in various cellular compartments. Among them, only SIRT6 has provided evidence for sirtuin-mediated longevity. Mice deficient in SIRT6 exhibit accelerated aging phenotype (Mostoslavsky et al., 2006), and male transgenic mice overexpressing SIRT6 have extended lifespan (Kanfi et al., 2012). Nonetheless, many studies suggest that several of the seven mammalian sirtuins promote various aspects of healthy aging through regulating stress resistance.

SIRT1, the closest homolog to invertebrate Sir2 and the most-studied mammalian sirtuin, is best known for mediating the lifespan extension effects of calorie restriction (CR). SIRT1 activity is regulated by availability of nutrients and causes changes in cellular metabolic pattern and stress response pathways (Cohen et al., 2004). SIRT1 activity is induced during moderate undernutrition, such as fasting and calorie restriction. When activated, SIRT1 deacetylates many target proteins involved in metabolic regulation, stress response, and aging. For example, SIRT1 regulates lipid metabolism via PGC1a, acts as a tumor suppressor by maintaining genome integrity,

and regulates stress response through activating FOXO transcription factors (Brunet et al., 2004; Rodgers et al., 2005). These and many other studies support SIRT1's role in protecting against cellular stress and in delaying the onset of metabolic disease, neurodegeneration, and more thereby promoting health during aging (Haigis and Yankner, 2010).

In addition to SIRT1, studies of other sirtuins support the notion that this family of proteins regulates multiple aspects of stress resistance and metabolism. SIRT3, a mitochondrial sirtuin, functions in combating oxidative stress under CR by deacetylating and increasing the activity of superoxide dismutase 2 (SOD2), which scavenges reactive oxygen species (ROS) (Kim et al., 2010; Qiu et al., 2010; Someya et al., 2010). SIRT4, another mitochondrial member of the sirtuin family, is required for DNA damage-mediated blockage of glutamine metabolism (Jeong et al., 2013). SIRT6, a nuclear sirtuin, also plays a role in suppressing genome instability by upregulating DNA repair pathway (Kaidi et al., 2010; Mostoslavsky et al., 2006; Toiber et al., 2013).

## Stress response and aging

### Genotoxic Stress and Aging

DNA stability is continuously challenged by endogenous and exogenous chemical, physical, and biological agents (Hoeijmakers, 2009). Therefore, organisms have evolved a complex machinery of DNA repair, damage tolerance, and checkpoint pathways to counteract DNA damage (Lord and Ashworth, 2012). Accumulation of DNA damage has emerged as a major culprit in aging. Moreover, impaired sensing or repair of DNA damage underlying numerous premature aging diseases, such as Werner syndrome and Bloom syndrome, suggest an important role of genome maintenance in aging (Burtner and Kennedy, 2010; Ciccia and Elledge, 2010). Studies done in humans as well as other model organisms report accumulation of somatic mutations, copy number variations, and clonal mosaicism within aged cells (Forsberg et al., 2012; Moskalev et al., 2012). These alterations in DNA can lead to dysfunction in a cell by modulating essential genes and transcriptional pathways. If these alterations are not properly cleared by apoptosis or senescence, cell or tissue homeostasis will not be maintained and it can cause aging phenotypes.

### Loss of Proteostasis in Aging

Protein homeostasis, or proteostasis in a cell is maintained by a set of transcription factors and molecular chaperones that coordinates protein synthesis, folding, disaggregation, and degradation. In response to stress, this set of transcription factors

and chaperones sense and correct disorders in the proteome to restore homeostasis (Roth and Balch, 2011). Organelles such as endoplasmic reticulum (ER) and mitochondria have their own robust protein quality control systems due to the high volume of proteins and sensitivity to redox state, respectively (Buchberger et al., 2010). The unfolded protein response (UPR) in the ER is activated by loss of proteostasis due to accumulation of mis- or unfolded proteins. The ER-UPR is initiated by three ER transmembrane proteins, IRE1, PERK, and ATF6, which lead to the induction of chaperones and reduction of translation that can help restore the proteostatic balance (Walter and Ron, 2011). Mitochondrial UPR, a similar but less well-defined pathway, is induced by protein misfolding in mitochondria and functions to reestablish mitochondrial proteostasis (Heo et al., 2010).

Although the robustness and adaptability of the proteostasis network is remarkable, it is difficult to maintain proteostasis under chronic stress conditions, and proteotoxicity develops. With age, the ability of cells to maintain proteostasis under intrinsic and extrinsic stress declines (Powers et al., 2009). Many studies have demonstrated a tight relationship between proteostasis and aging (Koga et al., 2010). Interventions that modulate the activity of the proteostasis control in worms and mice extend their lifespans and healthspans. In addition, chronic expression of unfolded, misfolded, or aggregated proteins contributes to the development of several age-related diseases, such as Parkinson's disease and Alzheimer's disease (Powers et al., 2009).

### Oxidative Stress and Aging

The mitochondrial free radical theory of aging postulates that the increased production of ROS with age leads to the cumulative damage of mitochondria and mitochondrial DNA (Harman, 1956). In agreement with this theory, many studies have found that increased oxidative damage in aged tissues. Furthermore, in mice lacking SOD1, an antioxidant enzyme, show a 30% decrease in lifespan, while overexpression of SOD1/catalase extends lifespan in flies (Sun and Tower, 1999). Despite a large body of evidence supporting a correlation between oxidative damage and aging, there are also unexpected and contradicting data that challenges the mitochondrial free radical theory of aging (Hekimi et al., 2011). Decreasing SOD isoforms in worms increases ROS and lifespan (Van Raamsdonk and Hekimi, 2009). $SOD2^{+/-}$ mice show increase oxidative damage, but normal lifespan, and overexpression of antioxidant enzymes in mice, such as SOD1 or SOD2, does not extend lifespan (Jang et al., 2009). These contradicting data can be explained if ROS is regarded as stress-elicited survival signal. A little ROS might be good since it could give rise to the compensatory homeostatic response and promote survival. However, beyond a certain threshold, ROS could function to aggravate cellular damage (Lopez-Otin et al., 2013). Nevertheless, more studies are

needed to further clarify the connection between oxidative stress and mitochondrial dysfunction in aging.

# Chapter 2. SIRT7 Represses Myc to Suppress ER Stress and Prevent Fatty Liver Disease[1]

## Introduction

The endoplasmic reticulum (ER) responds to the accumulation of unfolded or misfolded proteins in ER lumen (ER stress) by activating a set of transcription factors and molecular chaperones – collectively called the unfolded protein response (UPR) that works to restore homeostasis. (Basseri and Austin, 2012; Cnop et al., 2012; Fu et al., 2012; Hotamisligil, 2010; Ozcan and Tabas, 2012). The UPR consists of three distinct pathways that are initiated by the stress sensing proteins : protein kinase RNA-like ER kinase (PERK), inositol-requiring protein 1α (IRE1α), and activating transcription factor 6 (ATF6) (Walter and Ron, 2011). The arms of the UPR are integrated to provide a response that remodels the secretory apparatus and aligns cellular physiology to the demands imposed by ER stress (Ron and Walter, 2007). The initial phase of the UPR is suppression of protein translation and increased production of molecular chaperones to promote protein folding, allowing the cells to cope with an increased protein-folding demand and restore protein homeostasis (Hetz, 2012; Walter and Ron, 2011). Prolonged ER stress has been implicated in the development of numerous diseases, including fatty liver disease (Hotamisligil, 2010; Ozcan and Tabas, 2012). Identification of crucial UPR regulators with precise ER-stress-relieving properties that are amenable for therapeutic targeting represents attractive opportunities for pharmacological intervention of fatty liver and a wide spectrum of human diseases.

Nonalcoholic fatty liver disease (NAFLD) affects one-third of adults and an increasing number of children in developed countries and is strongly associated with obesity and insulin resistance (Browning and Horton, 2004; Browning et al., 2004; Cohen et al., 2012). NAFLD begins with aberrant accumulation of triglyceride in the liver (steatosis). Hepatic steatosis can proceed to nonalcoholic steatohepatitis (NASH), a condition associated with hepatocyte injury, inflammation, and fibrosis. Steatohepatitis can further progress to cirrhosis and liver cancer (Argo and Caldwell, 2009; Starley et al.).

The sirtuin family of nicotinamide adenine dinucleotide ($NAD^+$)-dependent deacetylase is profoundly implicated in metabolic regulation (Bellet et al., 2012; Finkel et al., 2009; Gillum et al.; Hirschey et al., 2011; Houtkooper et al.; Imai and Guarente, 2010). Sirtuins are well sought-after drug targets for metabolic disorders, as their enzymatic activities

[1] Previously published material 'Shin, J., He, M., Liu, Y., Paredes, S., Villanova, L., Brown, K., Qiu, X., Nabavi, N., Mohrin, M., Wojnoonski, K., *et al.* (2013). SIRT7 represses Myc activity to suppress ER stress and prevent fatty liver disease. Cell Rep *5*, 654-665.' is included in Chapter 2.

are amenable for regulation (Baur et al., 2012). SIRT7, a histone H3 lysine 18 (H3K18) deacetylase that binds to the promoters of a specific set of gene targets for transcriptional repression (Barber et al., 2012), is the only mammalian sirtuin whose function in metabolic regulation remains unknown.

We set out to fill this gap of knowledge by asking whether SIRT7 governs metabolic homeostasis under physiological conditions. Here, we show that SIRT7 has a physiological function in metabolic regulation that occurs through a chromatin-dependent signaling pathway that maintains metabolic homeostasis. Finally, we show that SIRT7 can be targeted to restore metabolic homeostasis in animals with metabolic disorders.

## Results

### SIRT7 deficient mice develop steatosis resembling human fatty liver disease

SIRT7 is the most highly expressed in the liver, among the metabolic tissues (Ford et al., 2006). To gain insight into SIRT7 function, we generated SIRT7 knockout (KO) mice (Figure 1A). Livers of SIRT7 KO mice fed a chow diet appeared paler than the WT controls with 100% penetrance (Figure 1B). H&E staining showed that SIRT7 KO hepatocytes were markedly vacuolated, with the accumulated material staining positive for fat with the Oil Red O stain (Figure 1B). Quantification of the triglyceride extracted from the livers by a colorimetric assay showed that SIRT7 KO livers had a 2.5-fold increase in triglyceride content (Figure 1D). Compared to the WT controls, the livers of SIRT7 KO mice had increased expression of inflammatory markers (Figure 1D), and immunohistochemistry showed increased staining for F4/80 (Figure 1B), a marker for tissue macrophages, indicating the progression to steatohepatitis. Notably, although fatty liver disease is often associated with obesity(Cohen et al., 2012; Ozcan et al., 2006), SIRT7 KO mice were leaner than littermate controls (Figure 2A,B). Thus, SIRT7 deficiency results in fatty liver without obesity.

Next, we investigated the lipid metabolic pathways that are deregulated in SIRT7 KO livers. The expression of lipogenic genes was increased in SIRT7 KO livers compared to the WT controls, while the expression of genes in the fatty acid oxidation pathway was unchanged (Figure 1D), suggesting that increased lipogenesis may be a contributing factor for hepatosteatosis. Despite increased lipid content in the livers of SIRT7 KO mice (Figure 1C), the levels of plasma triglyceride were 4-fold lower in SIRT7 KO mice compared to WT controls (Figure 2C). Reduced plasma triglyceride in SIRT7 KO mice is not due to reduced food intake or malabsorption of lipid (Figure 2D,E), suggesting that SIRT7 KO mice may have reduced very-low-density lipoprotein (VLDL)

secretion, the lipoprotein responsible for hepatic lipid export. In a well-established VLDL-TG secretion assay, SIRT7 KO mice had a 50% reduction in VLDL-TG secretion (Figure 2F). Together, these data indicate that SIRT7 KO mice develop fatty liver due to increased lipogenesis and reduced VLDL secretion.

### Hepatic SIRT7 autonomously prevents the development of fatty liver

To investigate whether the fatty liver phenotype of SIRT7 KO mice is due to SIRT7 deficiency in the liver or systemic effects of SIRT7 deletion, we reintroduced SIRT7 specifically in the livers of SIRT7 KO mice via adeno-associated virus 8 (AAV8)-mediated gene transfer (Figure 3A). Strikingly, liver-specific reconstitution of SIRT7 in SIRT7 KO mice reversed the fatty liver phenotype (Figure 3B, C, D), suppressed hepatic inflammation and lipogenesis (Figure 3E), and rescued the VLDL-TG secretion defect (Fig. 3F). Importantly, this reversal of the hepatosteatosis was not due to SIRT7 overexpression, since the reconstituted expression of SIRT7 was comparable to endogenous levels (Figure 3A). Together, these data indicate that hepatic SIRT7 autonomously prevents the development of fatty liver.

### SIRT7 activation is a critical event of the UPR to alleviate ER stress

Activation of the UPR pathways induces inflammation (Hotamisligil, 2010), perturbs hepatic lipid metabolism by modulating lipogenesis and lipoprotein metabolism (Kammoun et al., 2009; Lee et al., 2008; Ota et al., 2008; Rutkowski et al., 2008; So et al., 2012; Wang et al., 2012; Zhang et al., 2011b), and results in the development of fatty liver, reminiscent of essential aspects of SIRT7 KO phenotype in the liver (Figure 1,2). We therefore hypothesized that SIRT7 might be an essential regulator of the UPR. ER stress triggers finely regulated signaling events and transcriptional activation of ER stress response target genes with well-defined cis-elements (Hetz, 2012; Walter and Ron, 2011). Analysis of the SIRT7 promoter using MATInspector identified potential binding elements for XBP1, a key regulator of the UPR, which preferentially binds to sequences containing an ACGT core (Figure 4A) (Acosta-Alvear et al., 2007; Sha et al., 2009). Thus, SIRT7 may be transcriptionally upregulated upon ER stress. Indeed, treatment with chemical inducers of ER stress, tunicamycin and thapsigargin, increased the expression of SIRT7 at both the mRNA and protein levels in various cell types (Figure 4B, C, D). However, ER stress did not induce SIRT7 expression in XBP1 KO mouse embryonic fibroblasts (MEFs) (Figure 4D). Furthermore, ectopic expression of the spliced XBP1 (XBP1s), the active form of XBP1, induced SIRT7 expression to the same degree as it induced Erdj4, a known XBP1s target (Figure 4F). Additionally, in a luciferase assay, XBP1s induced transcription driven by the SIRT7 promoter but not the SIRT3 promoter (Figure 4G). Thus, SIRT7 is induced transcriptionally by XBP1 upon ER stress.

Next, we tested the effects of altered SIRT7 levels on ER stress. Overexpression of SIRT7 substantially reduced ER stress in response to tunicamycin, as evidenced by the reduced eIF2$\alpha$ phosphorylation and expression of ER stress response genes (Figure 5A-C). A catalytically inactive SIRT7 mutant (H187Y) did not suppress ER stress (Figure 5A-C), linking the catalytic activity of SIRT7 to ER stress management. To determine whether endogenous SIRT7 prevents ER stress, we stably knocked down SIRT7 expression using two independent short hairpin RNAs that specifically target SIRT7. SIRT7 depletion led to constitutive induction of ER stress (Figure 5D). Similarly, ER stress response genes were also upregulated in SIRT7 KO MEFs (Figure 5E). ER stress induces a reduction in the polysome-to-monosome ratio, indicative of translational initiation blockade(Kawai et al., 2004). Ribosomal profiling via a sucrose gradient showed that in contrast to WT MEFs, which had a high polysome-to-monosome ratio, SIRT7 KO MEFs had a reduction in polysomes and an increase in low-molecular-weight monosomes, consistent with increased ER stress in SIRT7 KO MEFs (Figure 5F). SIRT7 also suppressed ER stress-induced cell death (Figure 5G,H). Together, these data suggest that SIRT7 activation is a critical event of the UPR to alleviate ER stress.

### Myc recruits SIRT7 to repress the expression of ribosomal proteins and to suppress ER stress

How does SIRT7 regulate the UPR? The initial phase of the UPR is suppression of protein translation and increased production of molecular chaperones to reestablish homeostasis(Hetz, 2012; Walter and Ron, 2011). SIRT7 deacetylates H3K18Ac at specific gene promoters to repress transcription, and a major class of SIRT7 target genes are involved in protein translation and ribosome biogenesis(Barber et al., 2012). Thus, SIRT7 may alleviate ER stress by suppressing ribosome biogenesis and translation. Although SIRT7 lacks known DNA binding motifs, previous work has shown that it is recruited to a subset of its target promoters via interaction with the transcription factor ELK4(Barber et al., 2012). However, ELK4 was dispensable for SIRT7 promoter occupancy at other target promoters, including the promoters of ribosomal protein genes, leaving open the question of how SIRT7 is recruited to such promoters.

Recently, Myc has been shown to coordinate the transcriptional control of ribosomal components and serve as a master regulator of ribosome biogenesis (Kim et al., 2000; van Riggelen et al., 2010). Chromatin remodeling is believed to be central to Myc function in modulating the expression of its target genes(Knoepfler et al., 2006; van Riggelen et al., 2010). Myc binding on target chromatin is associated with the H3K18Ac mark, a substrate of SIRT7(Martinato et al., 2008). We therefore hypothesized that Myc stabilizes SIRT7 at the promoters of ribosomal proteins to mediate chromatin remodeling and gene repression. To probe a potential connection between Myc and

SIRT7, we tested whether SIRT7 physically interacts with Myc in vivo. Western blot analysis of endogenous SIRT7 immunoprecipitates revealed a specific interaction with Myc (Figure 6A). Thus, SIRT7 physically interacts with Myc in cells.

To investigate whether SIRT7 is stabilized at the promoters of ribosomal proteins through its interaction with Myc, we assessed SIRT7 and Myc co-occupancy at these promoter regions. Chromatin immunoprecipitation (ChIP) was performed with anti-SIRT7 and anti-Myc antibodies to assess the genomic occupancy of endogenous SIRT7 and Myc, compared to IgG negative background control. SIRT7 bound to the core promoters of ribosomal proteins (Barber et al., 2012). Myc was also detected at the same regions as SIRT7 on the core promoters of ribosomal proteins (Figure 6B,C), consistent with the notion that Myc generally occupies the core promoter regions of actively transcribed genes(Lin et al., 2012). In contrast, Myc was not detected at the promoters of NME1, where SIRT7 binding is mediated through ELK4 (Figure 6D)(Barber et al., 2012). Myc depletion using siRNA led to a significant reduction in SIRT7 occupancy at the promoters of ribosomal proteins (Figure 6E,F). However, Myc depletion had no effect on SIRT7 occupancy at the promoters of NME1 (Figure 6G). These data suggest that Myc targets SIRT7 specifically to the promoters of ribosomal proteins.

The specific association of SIRT7 and Myc at the promoters of ribosomal proteins but not ELK4 target genes suggests that SIRT7 might specifically influence the expression of ribosomal proteins via Myc. Therefore, we examined the effects of Myc inhibition on SIRT7-mediated gene expression. As shown previously, SIRT7 KD led to increased expression of ribosomal proteins (Figure 6H)(Barber et al., 2012). Tunicamycin treatment induced SIRT7 expression and suppressed the expression of ribosomal proteins in control but not SIRT7 KD cells (Figure 6H), indicating that ER stress triggers the transcriptional silencing of ribosomal proteins and that this effect requires SIRT7. Strikingly, Myc inactivation via siRNA, abrogated the elevated expression of ribosomal proteins but not NME1 in SIRT7 KD cells (Figure 6H), demonstrating that the observations on ribosomal proteins are due to Myc-dependent effects of SIRT7 on gene expression. Together with the previous observation that SIRT7 deacetylates H3K18Ac at the promoters of ribosomal proteins for transcriptional repression(Barber et al., 2012), these data suggest that Myc targets SIRT7 specifically to the promoters of ribosomal proteins for transcriptional silencing.

The observation that ER stress represses ribosomal proteins in a SIRT7-dependent manner suggests that upon ER stress, the induction of SIRT7 might function to suppress ribosomal protein expression and protein translation, in order to relieve ER stress. To test this possibility, we assessed the effects of suppressing Myc-mediated

expression of ribosomal proteins on SIRT7-associated ER stress management. Myc inactivation by siRNA abrogated the increased ER stress in SIRT7 KD cells (Figure 6H). Myc inactivation also blunted the effects of SIRT7 on ER stress resistance (Figure 6I). Together, these results indicate that SIRT7 is targeted to the promoters of ribosomal proteins by interacting with Myc, and alleviates ER stress by countering Myc-dependent expression of these genes.

### ER stress underlies the development of fatty liver caused by SIRT7 deficiency

We next determined whether ER stress underlies the development of fatty liver in SIRT7 deficient mice. SIRT7 KO livers showed increased ER stress, as evidenced by the induction of e-IF2$\alpha$ phosphorylation and ER stress response genes, as well as a reduction in the polysome-to-monosome ratio (Figure 7A,B). Reintroduction of SIRT7 in the livers of SIRT7 KO mice via AAV8-mediated gene transfer reduced ER stress (Figure 7D) and reverted the fatty liver phenotype (Figure 7C). Moreover, treatment of SIRT7 KO mice with TUDCA, a small molecule chaperone that has been shown to alleviate ER stress in vivo (Ozcan et al., 2006), partially rescued the fatty liver phenotype (Figure 7C). Finally, liver-specific Myc knockdown in SIRT7 KO mice via AAV8-mediated gene transfer reduced ER stress (data not shown), suppressed hepatic inflammation and lipogenesis (Figure 7E), and reversed the fatty liver phenotype (Figure 7F). Thus, SIRT7 prevents the development of fatty liver by suppressing ER stress.

### SIRT7 reverts fatty liver associated with diet-induced-obesity

A high-fat high-calorie diet is associated with increased ER stress and the development of fatty liver disease(Oyadomari et al., 2008). We next asked whether SIRT7 could be targeted to alleviate high fat diet-induced ER stress and the development of fatty liver disease. We overexpressed SIRT7 specifically in the livers of mice fed a high fat diet via AAV8-mediated gene transfer. Consistent with previous reports(Oyadomari et al., 2008), high fat diet feeding led to an increase in ER stress markers (Figure 8A, B), accumulation of TG in the liver (Figure 7C-E) possibly due to decreased VLDL secretion and increased lipogenesis (Figure 8A, F), and increased inflammation (Figure 8A, D). Strikingly, SIRT7 overexpression in the livers of high fat diet fed mice suppressed ER stress (Figure 8A, B), VLDL secretion (Figure 8F), lipogenesis (Figure 8A), inflammation (Figure 8A, D), and rescued the fatty liver phenotype (Figure 8C-E). Thus, SIRT7 activation represents an attractive approach to revert ER stress-mediated fatty liver.

## Discussion

Our work uncovered a physiological role of SIRT7 in maintaining hepatic metabolic homeostasis, demonstrated the feasibility of targeting SIRT7 to restore metabolic homeostasis in animals with metabolic disorders, and revealed a regulatory branch of

the UPR that is amenable for therapeutic targeting. The canonical UPR leads to the phosphorylation of eIF2$\alpha$ and the suppression of translation to alleviate ER stress(Ozcan and Tabas, 2012; Walter and Ron, 2011). We show that SIRT7 is induced by XBP1 upon ER stress, and is recruited to the promoters of ribosomal proteins via Myc to repress gene expression and to alleviate ER stress (Figure 9A). In SIRT7 deficient cells, failure to engage SIRT7-mediated ER stress management results in constitutive ER stress, which induces the activation of the canonical UPR (phosphorylation of eIF2$\alpha$, blockade of polysome assembly and translational initiation, and induction of ER stress response genes), apoptosis, inflammation, and specifically in the liver, increased lipogenesis and reduced VLDL secretion (Figure 9B). Importantly, SIRT7 upregulation alleviates ER stress and restores hepatic metabolic homeostasis in diet-induced obese animals, providing an avenue to treat fatty liver disease and likely other ER stress-associated pathologies.

Our observation that SIRT7 opposes Myc-dependent gene regulation is intriguing. In addition to its role in fatty liver pathology, the dynamic balance between SIRT7 and Myc activities could play a pivotal role in the context of tumorigenesis. SIRT7 may keep the Myc oncogene in check to prevent tumorigenesis. However, under certain conditions, Myc can also trigger apoptosis as an evolved tumor defense mechanism(Evan et al., 1992; Soucek and Evan, 2010). Our study suggests that unopposed Myc activation in the absence of SIRT7 results in ER stress. This may be an underlying mechanism for Myc-induced apoptosis in cancer cells, which are particularly sensitive to ER stress(Ozcan and Tabas, 2012). Indeed, we find that SIRT7 prevents ER stress-induced cell death in a Myc-dependent manner (Figure 5G,H). These observations are consistent with previous findings that SIRT7 can promote cancer cell survival and maintain oncogenic transformation(Barber et al., 2012).

Our findings regarding the role of SIRT7 in ER stress management might also be important for aging biology. Indeed, SIRT7 has several potential links to aging. SIRT7 KO mice have shortened lifespans and exhibit phenotypes linked to aging(Vakhrusheva et al., 2008b). Furthermore, SIRT7 expression decreases in some aging tissues(Vakhrusheva et al., 2008b). Suppression of ribosomal proteins and ER stress leads to lifespan extension in yeast(Steffen et al., 2012). Thus, the interplay between SIRT7, ER stress, and protein translation may represent an evolutionarily conserved phenomenon in aging.

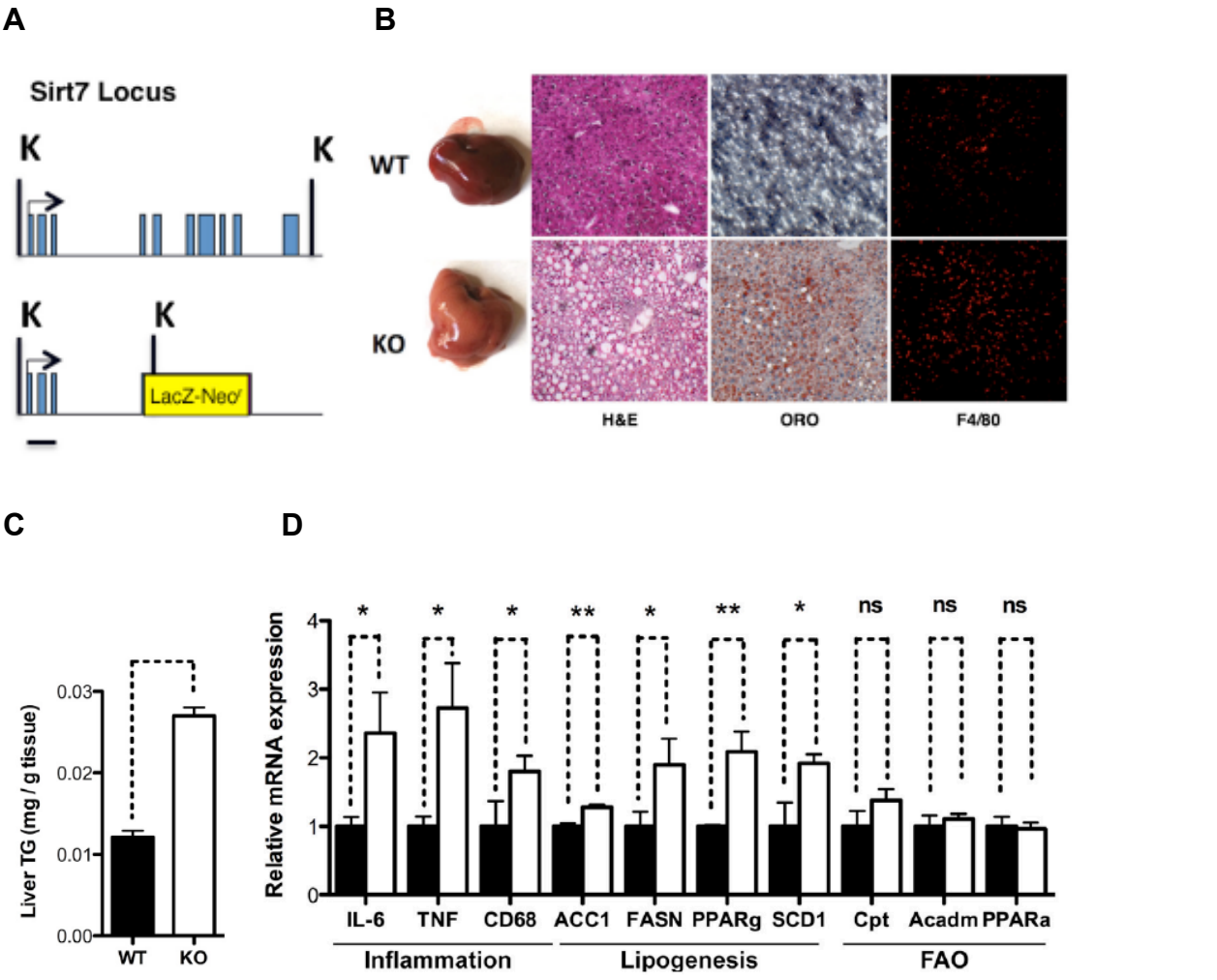


**Figure 1. SIRT7 prevents the development of fatty liver disease.**

**(A)** Schematic representation of the SIRT7 locus and the SIRT7 KO targeting vector.

**(B)** Morphology, H&E staining, Oil Red O staining, and F4/80 staining showing increased lipid accumulation and inflammation in the livers of SIRT7 KO mice compared to WT controls.

**(C)** Quantification of triglyceride extracted from livers in a colorimetric assay showing increased triglyceride content in SIRT7 KO livers. n=6.

**(D)** Gene expression analysis by quantitative PCR showing increased inflammation and lipogenesis but not fatty acid oxidation in SIRT7 KO livers. n=4.

Error bars represent S. E. M. *: $p<0.05$. **: $p<0.01$. ***:$p<0.001$. ns: $p>0.05$.

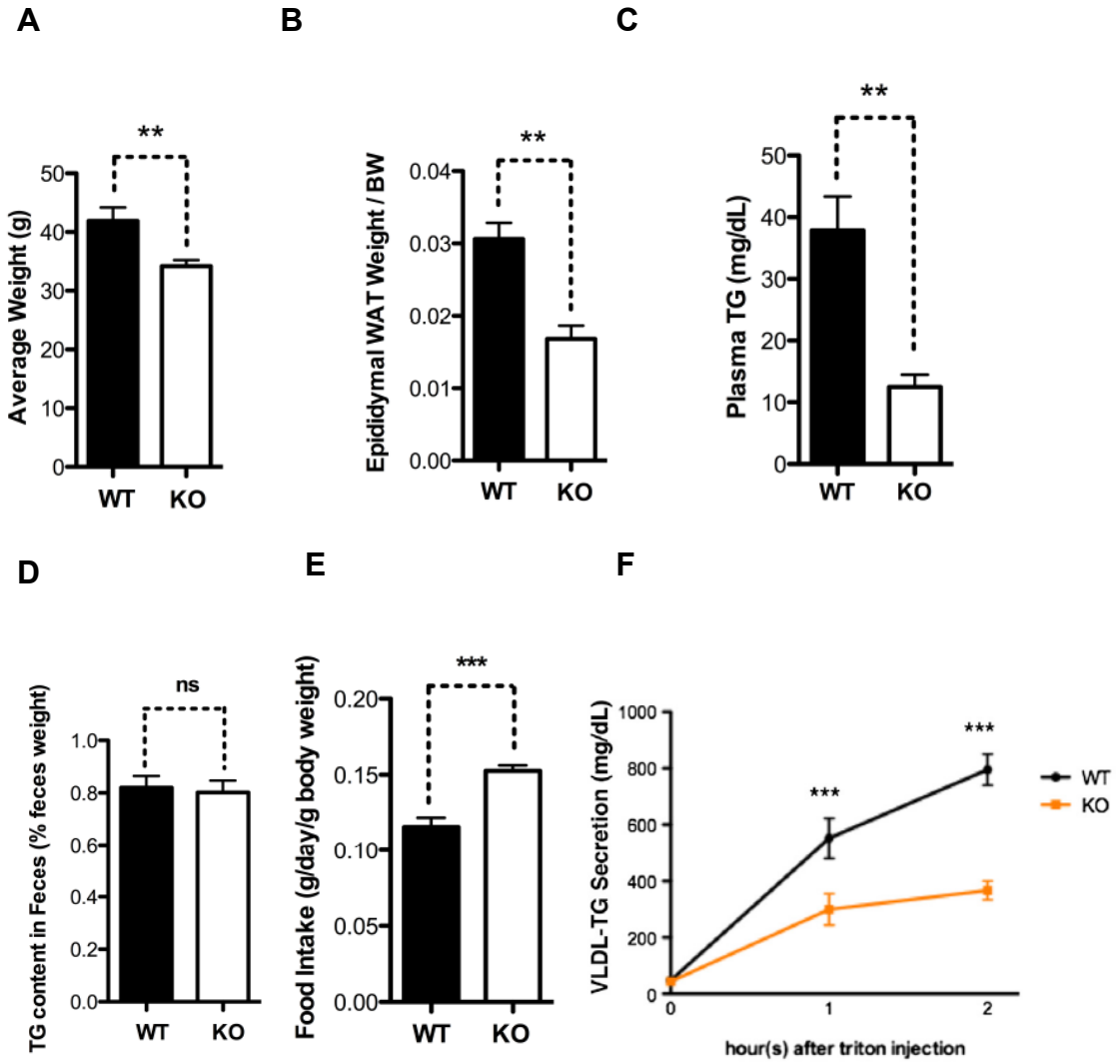


**Figure 2. Metabolic characterization of SIRT7 knockout mice.**

**(A)** Body weight.

**(B)** Epididymal white adipose tissue weight normalized against body weight.

**(C)** Blood triglyceride levels.

**(D)** Triglyceride content in feces.

**(E)** Food intake.

**(F)** A VLDL-TG secretion assay showing defective VLDL-TG secretion for SIRT7 KO mice

n=6. Error bars represent S. E. M. *: $p<0.05$. **: $p<0.01$. ***:$p<0.001$. ns: $p>0.05$.

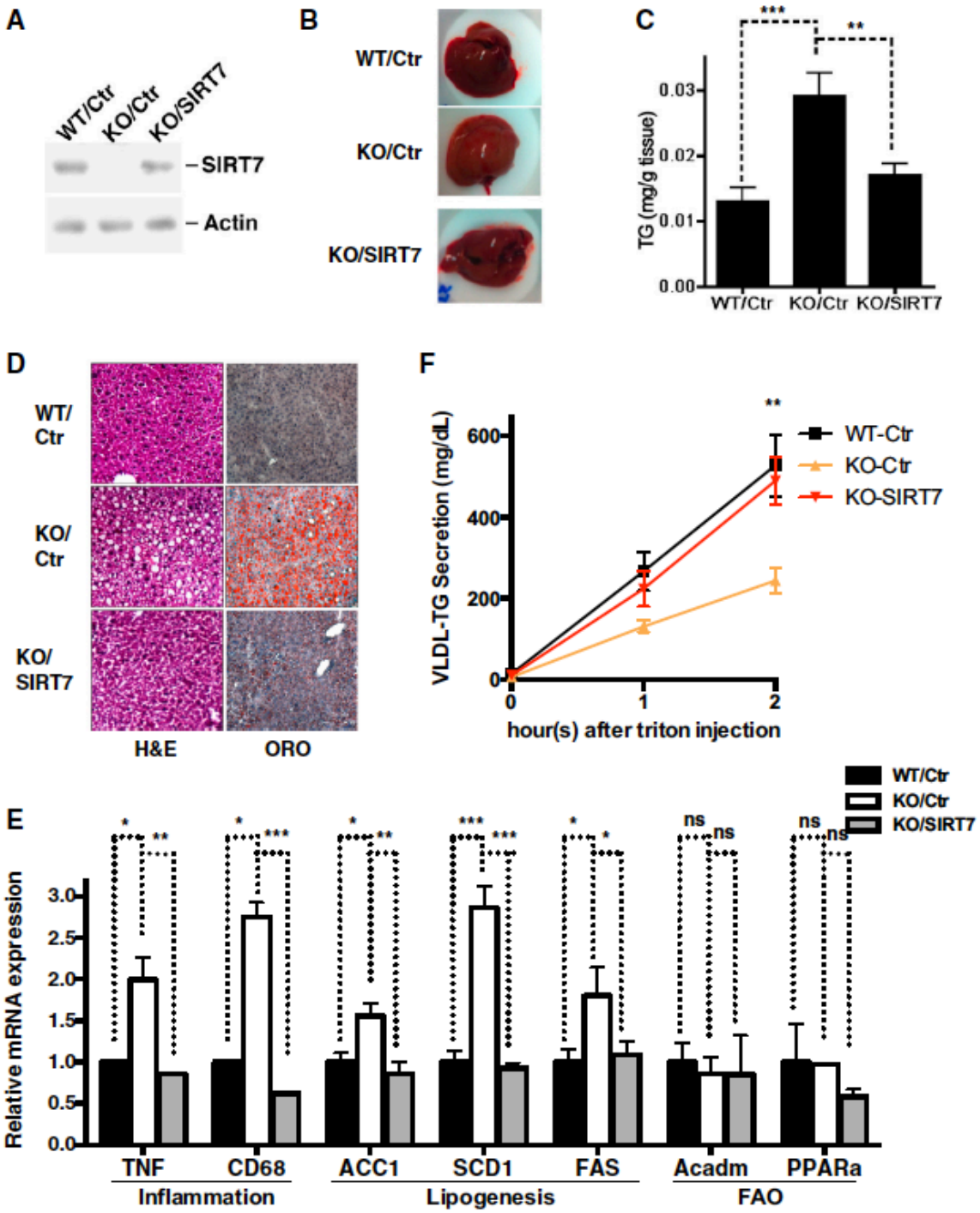


**Figure 3. Hepatic SIRT7 prevents the development of fatty liver autonomously.** Data shown are the comparison for the livers of WT, SIRT7 KO, and SIRT7 KO mice expressing SIRT7 in livers via AAV8-mediated gene transfer

**(A)** Western analysis for SIRT7 expression.

**(B)** Liver morphology.

**(C)** Liver triglyceride quantification.

**(D)** H&E and Oil Red O staining.

**(E)** Gene expression by quantitative PCR showing SIRT7 expression in liver suppresses hepatic inflammation and lipogenesis of SIRT7 KO mice.
**(F)** In a VLDL-TG secretion assay, SIRT7 expression in liver rescued VLDL-TG secretion defects of SIRT7 KO mice.
n=4. Error bars represent S. E. M. *: $p<0.05$. **: $p<0.01$. ***:$p<0.001$. ns: $p>0.05$.

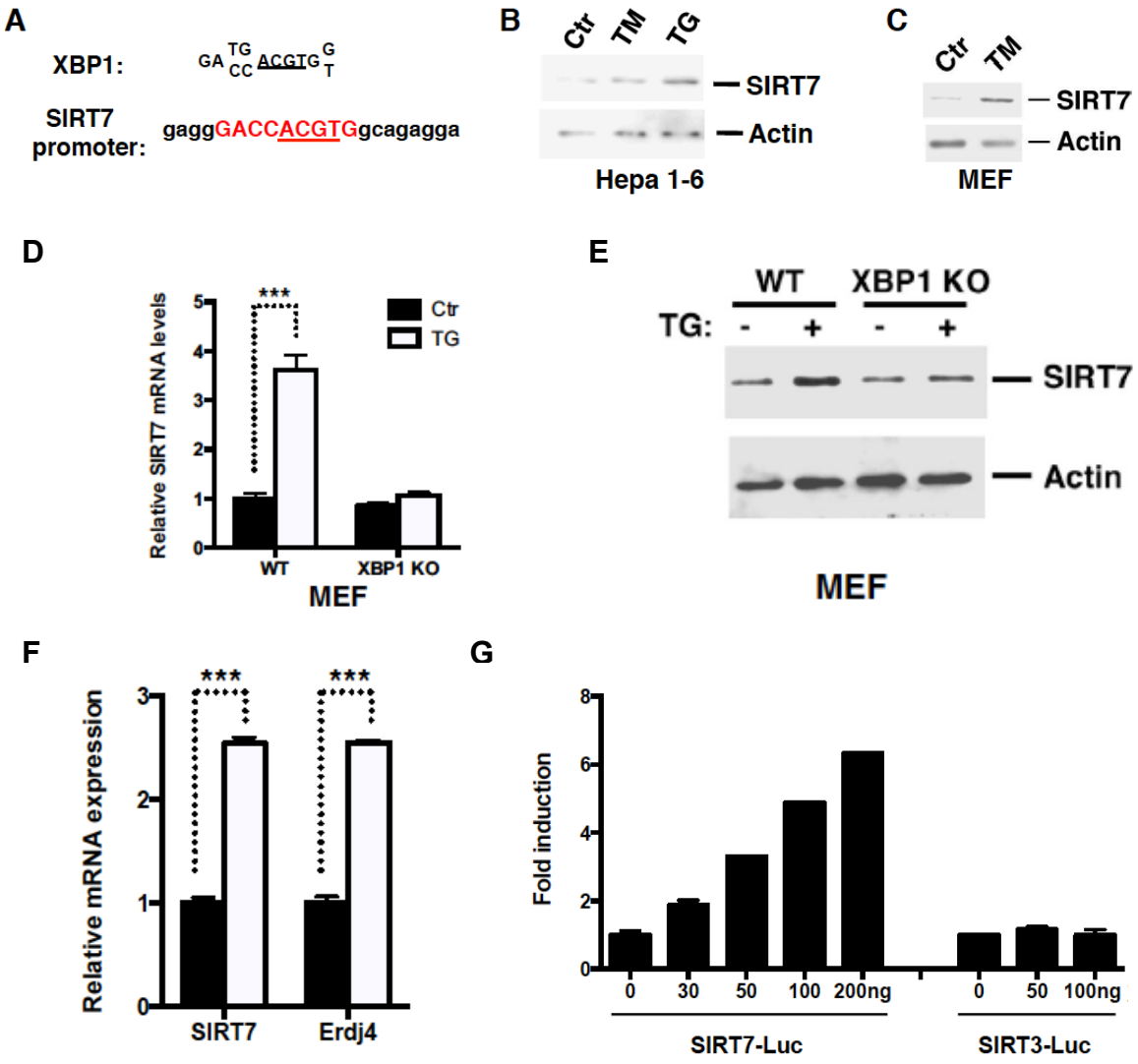


**Figure 4. SIRT7 is transcriptionally induced by ER stress**

**(A)** Comparison of the XBP1 consensus motif and the SIRT7 promoter sequenence.

**(B-C)** Gene expression analysis by western blotting showing increased SIRT7 expression upon treatment of ER stress inducers tunicamycin and thapsigargin.

**(D, E)** Gene expression analysis by quantitative PCR (D) and western blotting (E) showing increased SIRT7 expression upon treatment of ER stress inducer thapsigargin in WT but not XBP1 KO MEFs.

**(F)** Quantitative PCR showing spliced XBP1 (XBP1s), the active form of XBP1, induces transcription of SIRT7 to the same degree as Erdj4, a known XBP1s target.

**(G)** Luciferase assay showing XBP1 promotes the expression driven by SIRT7 promoter but not SIRT3 promoter.

Error bars represent S. E. M. *: $p<0.05$. **: $p<0.01$. ***:$p<0.001$.

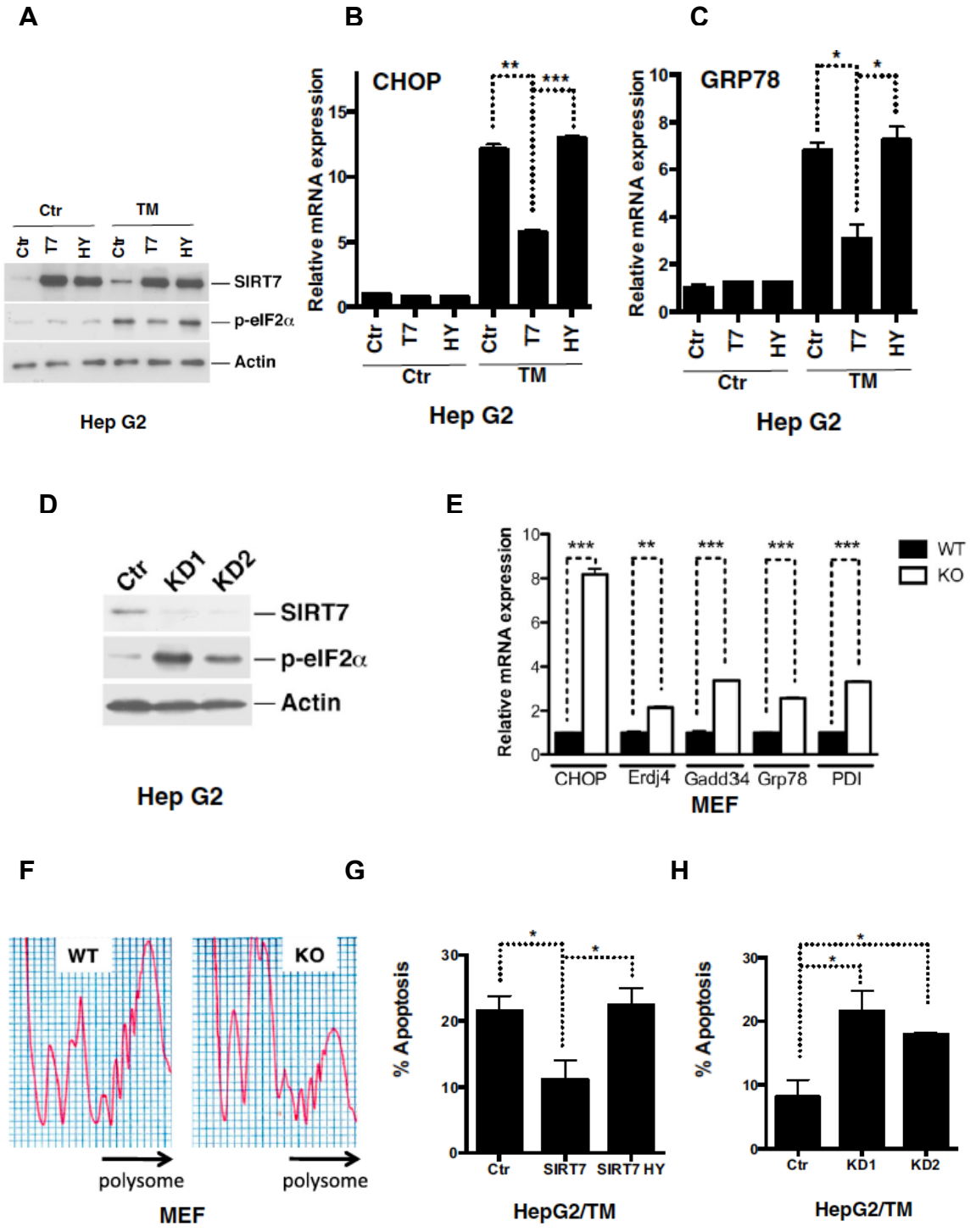


**Figure 5. SIRT7 suppresses ER stress.**

**(A-C)** Western blots (A) and quantitative PCR (B,C) showing reduced ER stress in tunicamycin-treated stable HepG2 cells overexpressing (OE) WT but not catalytically inactive (H187Y) SIRT7, as indicated by eIF2α phosphorylation levels and ER stress response gene expression.

**(D)** Western blots showing increased ER stress in SIRT7 knockdown (KD) stable HepG2 cells.
(**E,F)** Increased ER stress in SIRT7 KO MEFs. Quantitative PCR showing increased expression of ER stress-induced genes in SIRT7 KO MEFs (E). Polysome profiling showing reduced polysome-to-monosome ratio, an ER stress marker indicative of translational initiation blockade (F).
**(G,H)** SIRT7 prevents ER stress-induced cell death. Stable SIRT7 overexpression (OE) or knockdown (KD1 or KD2) cells used in Figure 3C, G were treated with tunicamycin (2ug/ml for J and 1ug/ml for K) for 24 hours. Apoptosis was scored with Annexin V staining.
Error bars represent S. E. M. *: $p<0.05$. **: $p<0.01$. ***:$p<0.001$.

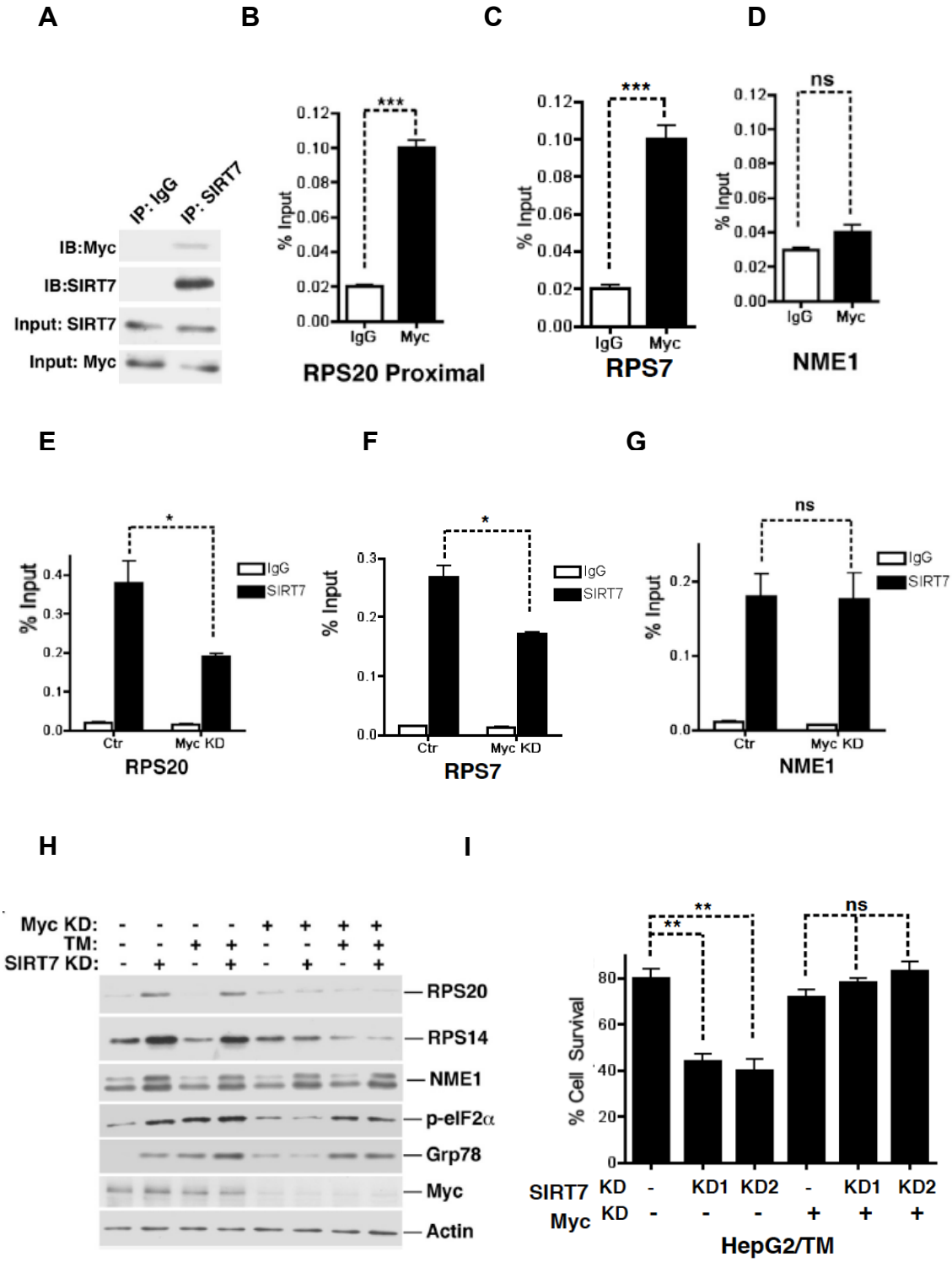


**Figure 6. Myc recruits SIRT7 to repress the expression of ribosomal proteins and to suppress ER stress.**

**(A)** Western analysis showing co-immunoprecipitation (co-IP) of endogenous SIRT7 and endogenous Myc in HepG2 cells.

**(B-D)** ChIP-qPCR (mean +/- S.E.M) showing Myc occupancy at the RPS20, RPS7 proximal promoter compared to IgG negative control samples. Myc occupancy at the NME1 promoters was used as negative controls. All samples were normalized to input DNA.
**(E-G)** Reduction of SIRT7 occupancy at the RPS20 and RPS7 but not the NME1 promoter in Myc knockdown cells determined by ChIP (mean +/- S.E.M).
**(H)** Western analysis showing Myc inactivation via siRNA abrogates ER stress and increased expression of ribosomal proteins but not NME1.
**(I)** Stable SIRT7 knockdown (KD1 or KD2) cells were treated with Myc siRNA, followed by tunicamycin treatment for 48 hours. Cell survival was scored with trypan blue staining.
Error bars represent S. E. M. *: $p<0.05$. ***:$p<0.001$. ns: $p>0.05$.

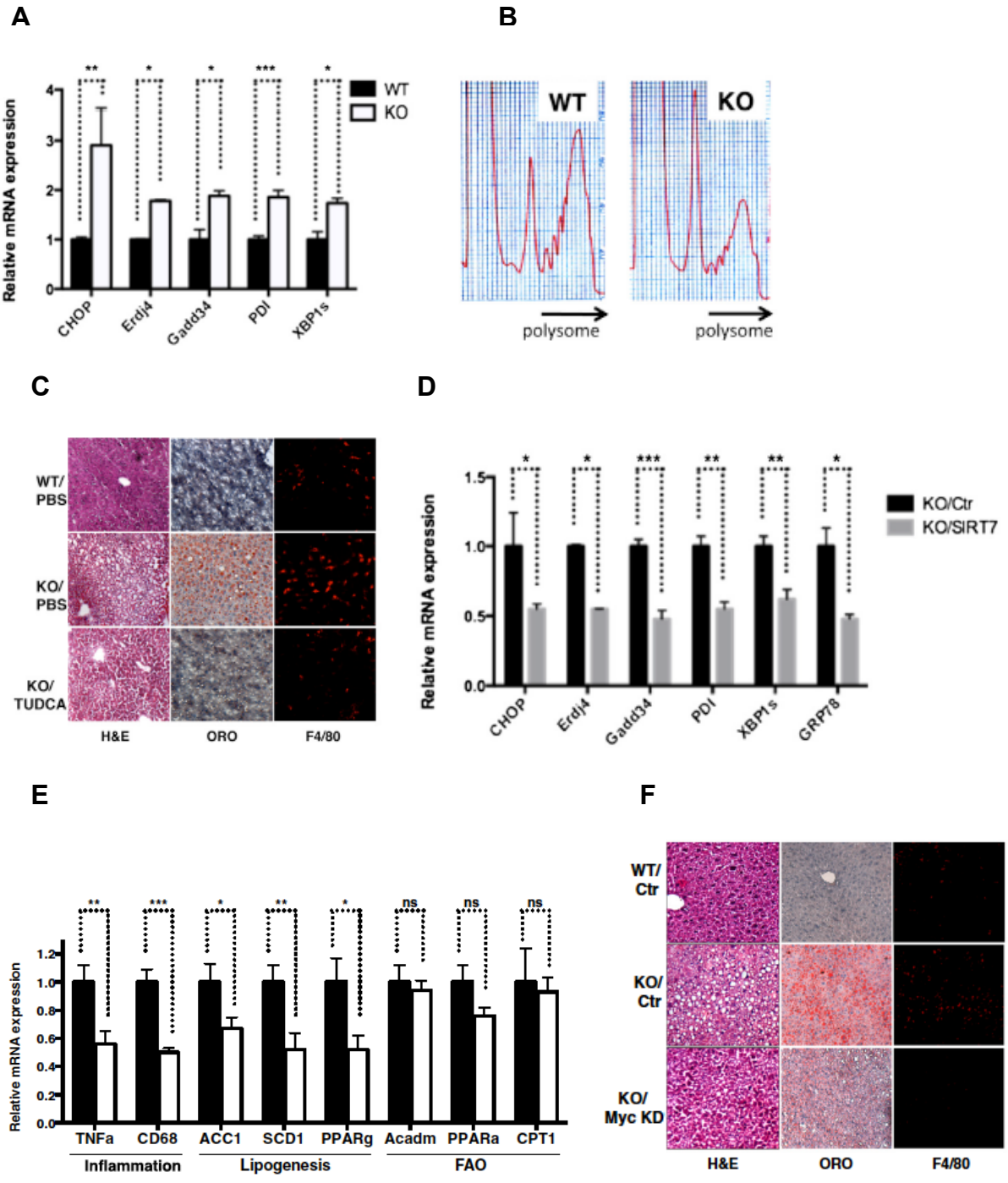


**Figure 7. SIRT7 prevents the development of fatty liver by suppressing ER stress and repressing Myc (A, B)** Quantitative PCR (A), and polysome profiling (B) showing increased ER stress in SIRT7 KO livers. n=6

**(C)** H&E, oil red O staining, and F4/80 staining of liver sections showing a small molecule chaperone TUDCA partially reverses fatty liver phenotype of SIRT7 KO mice. n=6

**(D)** Quantitative PCR analysis showing decreased expression of ER stress response genes in SIRT7 KO livers by reintroduction of SIRT7 via AAV8-mediated gene transfer.
**(E-F)** Data shown are the comparison of WT, SIRT7 KO, and SIRT7 KO mice with Myc knockdown in livers via AAV8-mediated gene transfer.
**(E)** Quantitative PCR analysis for the expression of inflammation, lipogenesis, and fatty acid oxidation genes. n=5
**(F)** H&E, oil red O staining, and F4/80 staining of liver sections. n=6
Error bars represent S. E. M. *: $p<0.05$. **:$p<0.01$. ***:$p<0.001$. ns: $p>0.05$.

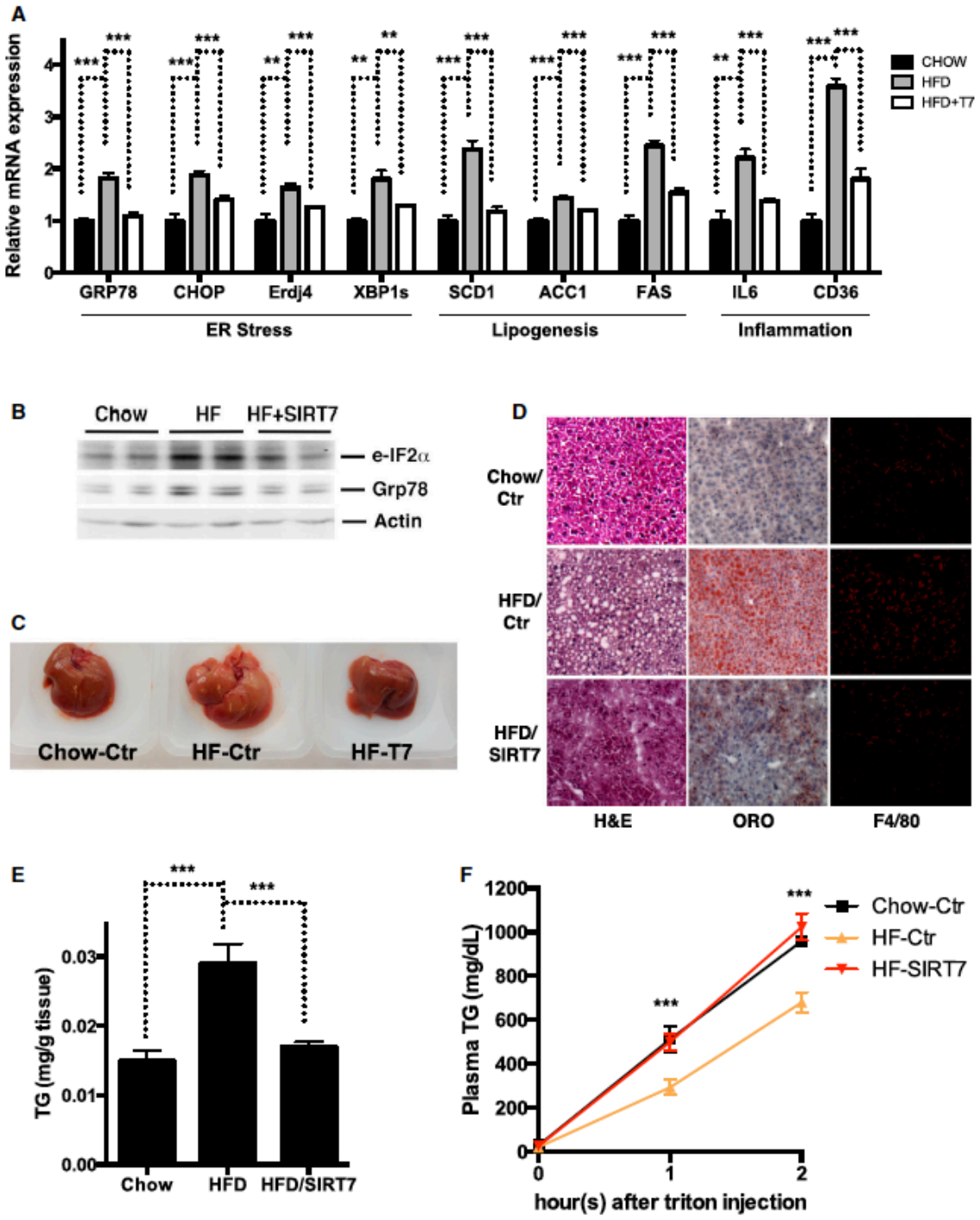


**Figure 8. SIRT7 rescues high fat diet-induced fatty liver.** Data shown are comparison of livers of mice fed a chow diet, a high fat diet, and a high fat diet with SIRT7 reintroduced specifically in the liver via AAV8-mediated gene transfer.

**(A)** Quantitative PCR analyses for gene expression of ER stress, inflammation, and lipogenesis.

**(B)** Western blotting for ER stress markers.

**(C)** Morphology.
**(D)** H&E staining, Oil Red O staining, and F4/80 staining.
(**E)** Quantification of liver triglyceride.
**(F)** A VLDL-TG secretion assay.
n=6. Error bars represent S. E. M. **:$p<0.01$. ***:$p<0.001$.

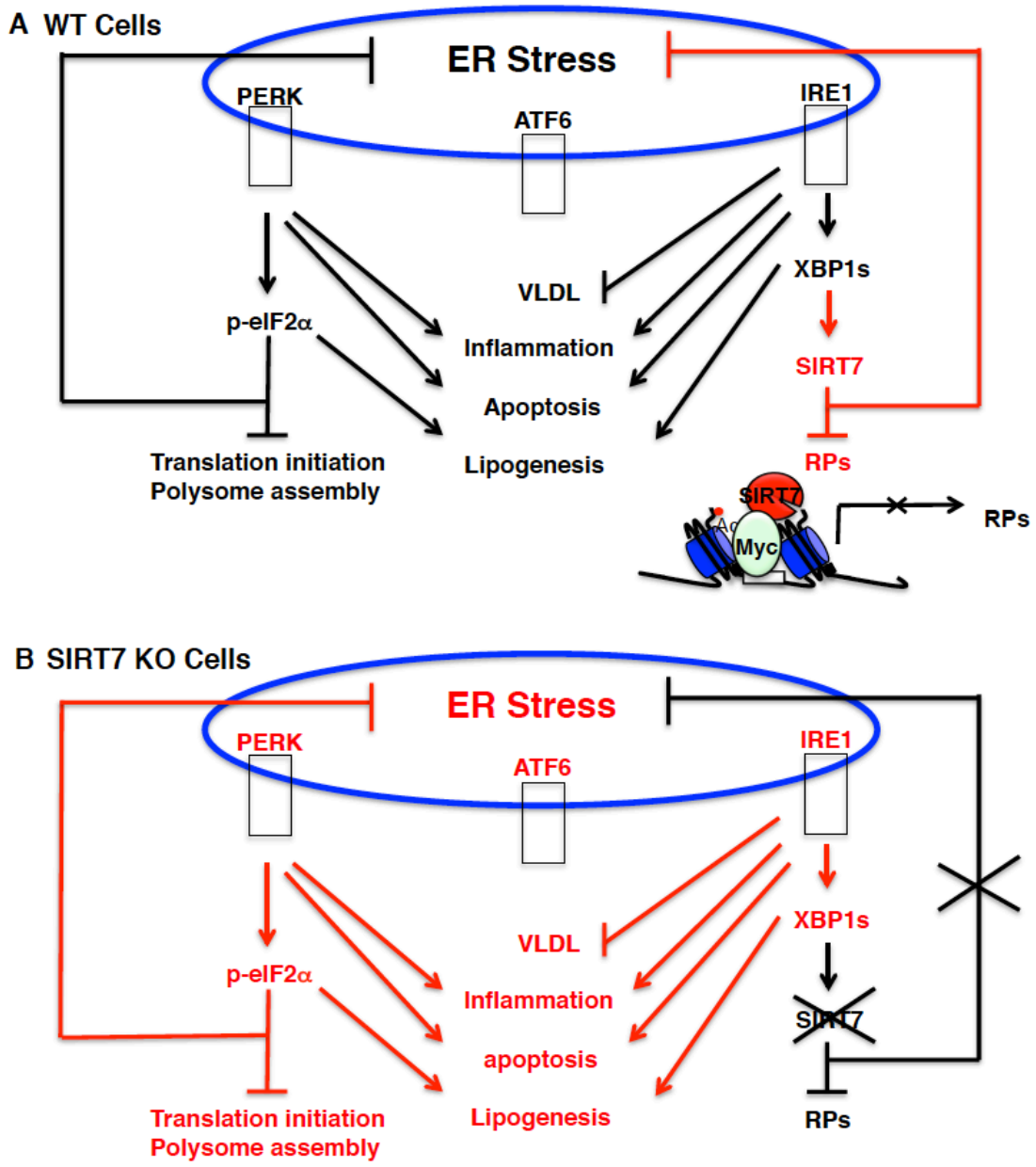


**Figure 9. A proposed model depicting SIRT7-mediated regulatory branch of ER stress response that prevents fatty liver disease.**

(A) In SIRT7 sufficient cells, SIRT7 is induced by XBP1 upon ER stress, and is stabilized at the promoters of ribosomal proteins through its interaction with Myc to silence gene expression and to relieve ER stress.

(B) In SIRT7 deficient cells, failure to engage SIRT7-mediated ER stress management results in constitutive ER stress, which in turn induces the activation of the canonical UPR, apoptosis, inflammation, and specifically in the liver, increased lipogenesis and reduced VLDL secretion.

# Chapter 3. Mitochondrial UPR Regulates a Metabolic Checkpoint in Hematopoietic Stem Cells[2]

## Introduction

Aging is a progressive degenerative process characterized by impaired physiological integrity and increased susceptibility to major human pathologies and mortality. Recent advancements in aging research are highlighted by the discovery that the rate of aging is controlled by evolutionarily conserved genetic pathways and biochemical processes (Fontana et al., 2010; Kenyon, 2010; Lopez-Otin et al., 2013). The general cause of aging is thought to be the chronic accumulation of cellular damage (Kirkwood, 2005; Lopez-Otin et al., 2013; Vijg and Campisi, 2008). This conceptual framework raises fundamental questions on aging: what are the origins of aging-causing damage? What is the cell- or tissue-specificity for sensing or responding to such damage? Are the effects of cellular damage on physiological aging reversible?

The stem cell theory of aging postulates that aging is the result of the failure of tissue specific stem cells to replenish tissues and to sustain tissue function (Rando, 2006). Stem cell function declines with age, consistent with the degeneration and dysfunction of aging regenerative tissues (Flach et al., 2014; Florian et al., 2013; Janzen et al., 2006; Oh et al., 2014; Rossi et al., 2008; Sahin and Depinho, 2010). Stem cell dysfunction is associated with premature aging (Scaffidi and Misteli, 2008; Zhang et al., 2011a) and evidence is emerging to suggest that stem cell manipulation can extend lifespan (Biteau et al., 2010; Lavasani et al., 2012; Rera et al., 2011). Longevity factors tend to be enriched or activated in stem cells compared to their differentiated progeny (Brown et al., 2013; Naka et al., 2010) and are essential for stem cell maintenance (Brown et al., 2013; Laplante and Sabatini, 2012; Miyamoto et al., 2007; Renault et al., 2009; Tothova et al., 2007; Webb et al., 2013), providing further support to the stem cell theory of aging.

Adult stem cells are maintained in a metabolically inactive quiescent state for prolonged periods of time (Folmes et al., 2012). Stem cell quiescence has long been viewed as a dormant state when there is little physiological demand for proliferation. However, recent advancements in stem cell biology favor the view that stem cell quiescence is an evolved adaptation to reduce metabolic byproducts, preserve genomic integrity, and

[2] Previously published material 'Mohrin, M., Shin, J., Liu, Y., Brown, K., Luo, H., Xi, Y., Haynes, C.M., and Chen, D. (2015). Stem cell aging. A mitochondrial UPR-mediated metabolic checkpoint regulates hematopoietic stem cell aging. Science *347*, 1374-1377.' is included in Chapter 3.

ensure stem cell maintenance (Bjornson et al., 2012; Cheung et al., 2012; Cheung and Rando, 2013; Liu et al., 2013; Rodgers et al., 2014). The signals that trigger stem cells to exit the cell cycle and enter quiescence, as well as the signal transduction leading to such a transition, remain elusive.

## Results

### SIRT7 Interacts with NRF1

SIRT7, a mammalian homolog of the longevity gene SIR2, is a chromatin binding protein that deacetylates H3K18 at specific gene promoters to repress transcription (Barber et al., 2012). SIRT7 lacks known DNA binding motifs and is recruited to its target promoters via its interaction with transcription factors. To gain insight into SIRT7 function, we took a proteomic approach to identify novel SIRT7-interacting transcription factors. We transfected 293T cells with Flag-tagged SIRT7, affinity-purified the Flag-tagged SIRT7 interactome, and identified SIRT7-interacting proteins by mass spectrometry. Among the potential SIRT7-interacting proteins was the transcription factor Nuclear Respiratory Factor 1 (NRF1), a master regulator of mitochondrial biogenesis, cellular respiration, and growth (Scarpulla, 2008). Western blot analysis of endogenous SIRT7 immunoprecipitates revealed a specific interaction with NRF1 (Figure 10A). Thus, SIRT7 and NRF1 interact in cells.

To determine whether NRF1 recruits SIRT7 to the promoters of its target genes for transcriptional repression, we performed chromatin immunoprecipitation (ChIP) with anti-SIRT7 and anti-NRF1 antibodies to assess the genomic occupancy of endogenous SIRT7 and NRF1 at the promoters of their target genes. SIRT7 bound to the proximal promoters of mitochondrial ribosomal proteins (mRPs) and mitochondrial translation factors (mTFs), but not other NRF1 targets (Figure 10B), consistent with a recent report (Barber et al., 2012). NRF1 was also detected at the same regions as SIRT7 at the proximal promoters of mRPs and mTFs (Figure 10C). Furthermore, NRF1 depletion using siRNA led to a robust reduction in SIRT7 occupancy at the promoters of mRPs and mTFs (Figure 10D). In contrast, NRF1 depletion had no effect on SIRT7 occupancy at the promoter of RPS20 (Figure 10D), where SIRT7 binding is mediated through Myc (Shin et al., 2013). Thus, NRF1 specifically recruits SIRT7 to the promoters of mRPs and mTFs.

### SIRT7 Limits Mitochondrial Activity and Cell Proliferation, and Promotes Nutritional Stress Resistance

Sirtuins are increasingly recognized as stress resistance genes, which are activated in response to various stresses and elicit diverse downstream events to alleviate stress

(Brown et al., 2013; Qiu et al., 2010). In response to the stress of nutrient deprivation, cells reduce mitochondrial activity, growth, and proliferation to prevent cell death (Inoki et al., 2003; Morita et al., 2013; Peng et al., 2002). SIRT7 expression was induced by nutrient deprivation (Figure 11A). The specific association of SIRT7 and NRF1 at the promoters of mRPs and mTFs suggests that SIRT7 might specifically suppress the expression of mRPs and mTFs, reduce mitochondrial activity and cell proliferation, and prevent cell death upon the stress of nutrient deprivation. We therefore examined the effect of SIRT7 inhibition on NRF1-mediated gene expression. We knocked down (KD) SIRT7 expression using two independent short hairpin RNAs. SIRT7 depletion led to increased expression of mRPs and mTFs (Figure 11B), consistent with the role of SIRT7 in transcriptional repression at these promoters. Notably, NRF1 inactivation via siRNA abrogated the elevated expression of mRPs and mTFs in SIRT7 KD cells (Figure 11B). In contrast, NRF1 inactivation did not affect the expression of RPS20 (Figure 11B). Together, these data suggest that NRF1 targets SIRT7 specifically to the promoters of mRPs and mTFs for transcriptional repression.

Transcriptional repression of mitochondrial and cytosolic (Barber et al., 2012; Shin et al., 2013) translation machinery components by SIRT7 suggests that SIRT7 might suppress mitochondrial activity and cellular proliferation. Mitotracker Green (MTG) staining revealed an increase in mitochondrial mass in SIRT7 KD cells (Figure 11C). Seahorse analysis showed that SIRT7 KD cells had increased oxygen consumption (Figure 11D). Thus, SIRT7 suppresses mitochondrial biogenesis and respiration.

SIRT7 KD resulted in a 40% increase in cellular proliferation rate (Figure 10E), consistent with previous reports (Lu et al., 2009; Vakhrusheva et al., 2008a). Furthermore, NRF1 inhibition via siRNA abrogated the increased mitochondrial activity and proliferation of SIRT7 KD cells (Figure 11F, G). Collectively, these data suggest that SIRT7 represses NRF1 activity to suppress mitochondrial activity and cellular proliferation.

We next asked whether SIRT7 plays a role in promoting nutritional stress resistance. Nutrient deprivation for 48 hours led to massive death of SIRT7 KD cells, while control cells remained viable (Figure 11H). Importantly, NRF1 inactivation by siRNA significantly improved the survival of SIRT7 KD cells upon nutrient deprivation (10I). Together, these data suggest that in response to the stress of nutrient deprivation, SIRT7 is induced to repress NRF-1 activity to suppress energy metabolism and cell proliferation. Failure to turn on this metabolic checkpoint results in cell death.

### SIRT7 Promotes Mitochondrial Protein Folding Stress Resistance

Perturbation of mitochondrial proteostasis, a form of mitochondrial stress, activates the mitochondrial unfolded protein response ($UPR^{mt}$), a retrograde signaling pathway leading to the transcriptional upregulation of mitochondrial chaperones, stress relief, and homeostasis reestablishment (Haynes and Ron, 2010; Yoneda et al., 2004; Zhao et al., 2002). Mitochondrial dysfunction also results in the attenuation of protein translation, which helps restore mitochondrial homeostasis by reducing the burden of incoming substrates on the mitochondrial protein folding environment (Haynes et al., 2013). SIRT7-mediated transcriptional repression of translation machinery suggests that SIRT7 may alleviate mitochondrial protein folding stress by suppressing translation.

We tested a role of SIRT7 in regulating mitochondrial protein folding stress. Treatment of cells with mitochondrial stress inducers led to the transcriptional upregulation of SIRT7 (Figure 12A), mirroring the increased transcription of canonical $UPR^{mt}$ genes (Zhao et al., 2002). Induction of mitochondrial protein folding stress via overexpression of an aggregation prone mutant mitochondrial matrix protein ornithine transcarbamylase (DOTC) results in upregulation of $UPR^{mt}$ genes and efficient clearance of misfolded DOTC (Zhao et al., 2002). However, in SIRT7 deficient cells, misfolded DOTC accumulated to a higher level than in SIRT7 proficient cells (Figure 12B). Furthermore, SIRT7 deficient cells displayed increased apoptosis upon mitochondrial stress (Figure 12C), but not general apoptosis induction (Shin et al., 2013). Thus, upon mitochondrial protein folding stress, SIRT7 is induced to alleviate mitochondrial stress and promote mitochondrial stress resistance.

Mitochondrial stress induced the expression of canonical $UPR^{mt}$ genes in SIRT7 deficient cells (Figure 12D, E), indicating that induction of SIRT7 and the canonical $UPR^{mt}$ genes are in separate branches of the $UPR^{mt}$. Furthermore, untreated SIRT7 KD cells also displayed an increase in the expression of canonical $UPR^{mt}$ genes (12D, 12E), but SIRT7 did not bind to their promoters (Figure 9B and (Barber et al., 2012)), indicating that SIRT7 deficiency results in constitutive mitochondrial unfolded protein stress and compensatory induction of canonical $UPR^{mt}$ genes.

To determine whether SIRT7 represses NRF1 to suppress mitochondrial translation and relieve mitochondrial unfolded protein stress, we assessed the effects of NRF1 inactivation on SIRT7-associated mitochondrial stress management. NRF1 inactivation via siRNA abrogated the increased mitochondrial protein folding stress in SIRT7 KD cells (Figure 12B, F, G). SIRT7 is also a regulator of the $UPR^{ER}$ that functions to suppress endoplasmic reticulum (ER) stress (Shin et al., 2013). However, NRF1 inactivation did not affect the increased ER stress in SIRT7 KD cells (Figure 12G). These data, together with the observations that SIRT7 represses NRF1 activity to reduce mitochondrial activity and cellular proliferation (Figure 11), suggest that the

interplay between SIRT7 and NRF1 constitutes a regulatory branch of the UPR$^{mt}$, functioning as the nexus of reduced mitochondrial translation for homeostasis reestablishment, suppressed energy metabolism, and repressed cell proliferation (Figure 12H).

### SIRT7 Limits Mitochondrial Protein Folding Stress, Mitochondrial Number, and Proliferation in HSCs

We next asked the physiological relevance of SIRT7 regulation of mitochondrial protein folding stress. In *C. elegans*, UPR$^{mt}$ is activated during the L3-L4 stage of larval development, when a burse of mitochondrial biogenesis takes place, and is attenuated when mitochondrial biogenesis subsides (Haynes and Ron, 2010; Yoneda et al., 2004). We hypothesize that SIRT7-mediated UPR$^{mt}$ may be particularly important for cells that experience bursts of mitochondrial biogenesis and convert between growth states with markedly different bioenergetic demands and proliferative potentials as a part of their normal physiology. Adult stem cells have very low mitochondrial content and primarily reside in a quiescent state, which are thought to be protective mechanisms that maintain their integrity (Folmes et al., 2012; Simsek et al., 2010). However, these cells exit quiescence upon physiological demands and increase mitochondrial biogenesis during proliferation and differentiation. This feature is conserved across species and is well preserved in humans (Piccoli et al., 2005; Romero-Moya et al., 2013).

We hypothesized that when stem cells convert from the quiescent state to the proliferative state, a burst of mitochondrial biogenesis is essential to meet the increased energy demand for proliferation, but may also result in increased mitochondrial protein folding stress. As a response, SIRT7 may repress NRF1 to reestablish mitochondrial protein homeostasis, suppress mitochondrial biogenesis, and exit the cell cycle. Thus, the UPR$^{mt}$-SIRT7-NRF1 axis may be an essential regulator of a metabolic checkpoint in stem cells. Since SIRT7 is highly expressed in the hematopoietic system (Figure 13A, B), we focused on HSCs (immunophenotypically defined as Lin$^-$c-Kit$^+$Sca1$^+$CD150$^+$CD48$^-$), which give rise to all mature blood cells through the process of hematopoiesis.

We asked whether SIRT7 limits mitochondrial protein folding stress, mitochondrial biogenesis, and proliferation in HSCs. We assessed the mitochondrial protein folding stress in HSCs isolated from SIRT7 knockout (KO) mice. SIRT7 deletion in HSCs led to an increase in mitochondrial stress (Figure 13C). We assessed how lack of SIRT7 affected mitochondrial mass and cell cycle status in various hematopoietic subpopulations. The levels of MTG staining in HSCs were 8-fold lower than myeloid progenitors (MP, Lin$^-$c-Kit$^+$Sca1$^-$), consistent with reduced mitochondrial mass in HSCs (Figure 13D). While there was no difference in the differentiated subpopulations

between the two genotypes, we observed a 60% increase in MTG levels in SIRT7 KO HSCs, indicating increased mitochondrial mass compared to WT controls (Figure 13D). Quantification of mitochondrial DNA confirmed increased mitochondrial numbers in SIRT7 KO HSCs (Figure 13E).

We next monitored HSC proliferation status in SIRT7 KO mice using *in vivo* BrdU incorporation. As expected, the frequency of BrdU positive HSCs was significantly lower than that of MPs, which are frequently in cycle (Figure 13F). The frequency of BrdU positive HSCs doubled in the absence of SIRT7 (Figure 13F). In contrast, no difference was detectable in differentiated subpopulations between WT and SIRT7 KO mice (Figure 13F).

In addition to the *in vivo* cell cycle analyses, we also monitored the propensity of HSCs and their differentiated progeny to enter the cell cycle upon *ex vivo* culture with cytokines using BrdU incorporation. As expected, we observed markedly lower levels of BrdU incorporation in HSCs than in MPs upon culture with cytokines (Figure 13G). While SIRT7 deficient HSCs had increased levels of BrdU staining compared to WT HSCs, differentiated progeny from both genotypes displayed similar staining (Figure 13G). Finally, animals with loss of HSC quiescence are particularly sensitive to the cell cycle specific myeloablative drug 5-Fluorouricil (5-FU) (Miyamoto et al., 2007). To exclude the possible effect of 5-FU on non-hematopoietic tissues, we repopulated the hematopoietic system of lethally irradiated recipients with either WT or SIRT7 KO bone marrow cells. Four months after transplantation, we challenged the reconstituted mice with weekly 5-FU treatments. Mice transplanted with SIRT7 KO bone marrow cells died sooner than those transplanted with WT controls (Figure 13H), consistent with the finding that SIRT7 deficient HSCs had reduced quiescence (Figure 13F,G). Collectively, these data suggest that SIRT7 is important for regulating mitochondrial mass and proliferation in the HSC compartment but not in differentiated progeny. SIRT7 is ubiquitously expressed in various subpopulations in the hematopoietic system (Figure 13B). However, HSCs have greatly reduced levels of NADH (Simsek et al., 2010), a competitive inhibitor of sirtuins (Lin et al., 2004). We speculate that this may lead to increased SIRT7 activity in the HSC compartment.

### SIRT7 Ensures HSC Maintenance

Reduced HSC quiescence causes compromised regenerative function (Baldridge et al., 2010; Liu et al., 2009; Tothova et al., 2007; Viatour et al., 2008; Zou et al., 2011). To determine whether loss of quiescence in SIRT7 deficient HSCs results in compromised regenerative function, we performed a competitive bone marrow transplantation assay. CD45.2 donor bone marrow cells were transplanted with an equal number of CD45.1 competitor bone marrow cells to reconstitute the hematopoietic compartment of lethally

irradiated recipient mice. HSCs derived from SIRT7 KO mice displayed a 40% reduction in reconstituting the hematopoietic system of recipient mice (Figure 14A).

HSCs can differentiate into lymphoid and myeloid lineages. To determine whether SIRT7 regulates lineage differentiation, we assayed donor-derived mature hematopoietic subpopulations in the peripheral blood of transplanted recipients. We observed an increase in the percentage of the myeloid lineage, such as granulocytes (Gr1+) and macrophages (Mac-1+), and a decrease in the percentage of the lymphoid lineage, such as B cells (B220+), in the transplant recipients of SIRT7 HSCs compared to the transplant recipients of WT HSCs (Figure 14B). Furthermore, SIRT7 KO mice also displayed myeloid biased differentiation in the peripheral blood under homeostatic conditions (data not shown). Thus, SIRT7 prevents the myeloid-biased differentiation profile.

To investigate whether the HSC defect of SIRT7 KO mice is due to SIRT7 deficiency in HSCs or the systemic effect of SIRT7 deletion, we reintroduced SIRT7 in SIRT7 KO HSCs via lentiviral transduction. Reintroduction of SIRT7 in SIRT7 KO HSCs improved the reconstitution capacity (Figure 14C) and rescued myeloid-biased differentiation (Figure 14D), indicating that SIRT7 regulates HSC maintenance cell-autonomously.

Although SIRT7 KO mice and WT controls had comparable HSC frequency in the bone marrow under steady-state conditions (Figure 14E), there was a 50% reduction in the frequency of HSCs derived from SIRT7 KO donors compared to WT donors in the bone marrow of transplant recipients (Figure 14F). In flow cytometry analyses of bone marrow cells derived from the transplant recipients using Annexin V staining, we observed increased percentage of apoptotic cells in SIRT7 KO HSCs compared to their WT counterparts (Figure 14G), which may account for compromised HSC engraftment. Collectively, these data suggest that upon mitochondrial protein folding stress, SIRT7 is required for HSCs to reduce mitochondrial biogenesis, exit cell cycle and return to quiescence. Failure to turn on this metabolic checkpoint results in cell death.

To determine whether mitochondrial protein folding stress underlies the functional defects of SIRT7 KO HSCs, we knocked down NRF1 in SIRT7 KO HSCs via lentiviral transduction. NRF1 inactivation in SIRT7 KO HSCs reduced mitochondrial protein folding stress (Figure 15A), engraftment (Figure 15B), and reconstitution (Figure 15C) and rescued myeloid-biased differentiation (Figure 15D). Together, these data suggest that SIRT7 represses NRF1 activity to alleviate mitochondrial protein folding stress and ensure HSC maintenance.

ROS production is intimately linked to mitochondrial activity and oxidative stress is known to drive HSCs out of quiescence (Ito et al., 2006; Miyamoto et al., 2007; Tothova et al., 2007). Suppression of mitochondrial protein folding stress by SIRT7 prompted us to test whether loss of quiescence in SIRT7 KO HSCs is due to increased oxidative stress. Quantification of cellular ROS levels using DCFDA staining showed comparable ROS levels in SIRT7 KO HSCs and WT controls under both homeostatic (data not shown) and transplantation conditions (data not shown). Thus, loss of quiescence and increased apoptosis in SIRT7 deficient HSCs do not correlate with increased ROS levels. Rather, these results are consistent with the notion that the $UPR^{mt}$ and ROS are uncoupled (Baker et al., 2012).

### HSC Aging is Regulated by SIRT7

The regenerative capacity of HSCs declines with age (Janzen et al., 2006; Rossi et al., 2008; Sahin and Depinho, 2010). Transcriptional profiling via microarray analyses showed that SIRT7 is one of the genes whose expression is reduced with age in HSCs (Chambers et al., 2007). We confirmed that SIRT7 expression in HSCs is reduced with age by qPCR (Figure 15E). Notably, SIRT7 deficiency resulted in decreased HSC reconstitution capacity (Figure 14A) and myeloid biased differentiation (Figure 14B), resembling essential aspects of HSCs from old animals (Janzen et al., 2006; Rossi et al., 2008; Sahin and Depinho, 2010). We next asked whether reduced SIRT7 expression in aged HSCs results in increased mitochondrial protein folding stress. HSCs isolated from old mice (2 years old) displayed increased mitochondrial protein folding stress compared to HSCs isolated from young mice (2-3 months old) (Figure 15F). This may contribute to increased apoptosis in aged HSCs under transplantation stress (Janzen et al., 2006). Importantly, reintroduction of SIRT7 in aged HSCs via lentiviral transduction reduced mitochondrial protein folding stress (Figure 15G), improved reconstitution capacity (Figure 15H), and rescued myeloid-biased differentiation (Figure 15I). NRF1 inactivation in aged HSCs also improved reconstitution capacity (data not shown) and rescued myeloid-biased differentiation (data not shown). Together, these observations suggest that SIRT7 downregulation results in increased mitochondrial protein folding stress in aged HSCs, contributing to their functional decline.

## Discussion

Collectively, our results establish mitochondrial protein folding stress as a trigger of a metabolic checkpoint that regulates HSC quiescence, establish the deregulation of the mitochondrial protein folding stress response as a contributing factor for HSC aging, and demonstrate the feasibility of alleviating mitochondrial protein folding stress to reverse HSC aging. The canonical $UPR^{mt}$ leads to the induction of mitochondrial

chaperones and proteases to alleviate mitochondrial protein folding stress. We show that in parallel to the induction of the canonical UPR$^{mt}$ genes, SIRT7 is also induced upon mitochondrial protein folding stress and is stabilized at the promoters of mitochondrial translation machinery components via NRF1 to repress gene expression and to alleviate mitochondrial protein folding stress. This regulatory branch of the UPR$^{mt}$ is coupled to reduced mitochondrial activity and cell proliferation. In SIRT7 deficient cells, failure to engage the mitochondrial protein folding stress management results in constitutive mitochondrial stress that induces activation of the canonical UPR$^{mt}$ genes, uncontrolled mitochondrial activity and cell proliferation, and apoptosis. Mitochondrial dysfunction is manifested in the metabolic tissues of SIRT7 deficient mice (Ryu et al., 2014). Specifically in HSCs, SIRT7 deficiency results in reduced regenerative capacity. Using a stress signal intrinsic to HSC proliferation as a messenger to return to quiescence may ensure the integrity of HSCs, which persist throughout the entire lifespan for tissue maintenance. The interplay between SIRT7, which is induced upon mitochondrial stress, and NRF1, a master regulator of mitochondria, is uniquely positioned to integrate mitochondrial stress to metabolic checkpoint regulation.

Mitochondrial protein folding stress emerges as an origin of cellular damage that causes HSC aging. The canonical UPR$^{mt}$ is upregulated and SIRT7 is repressed in aged HSCs. Therefore, repression of the SIRT7-mediated protective program contributes to the elevated mitochondrial protein folding stress in aged HSCs. As another possibility, ROS have been shown to induce robust mitochondrial protein unfolding stress (Baker et al., 2012; Runkel et al., 2013). ROS levels increase with age in HSCs and defective ROS management results in compromised HSC maintenance (Brown et al., 2013; Ito et al., 2006; Miyamoto et al., 2007; Paik et al., 2009; Renault et al., 2009; Tothova et al., 2007). It remains a possibility that high levels of ROS also contribute to elevated mitochondrial protein folding stress in aged HSCs and ROS-induced HSC defects are in part due to mitochondrial protein folding stress. It is interesting to note that SIRT3, a mitochondrial homolog of SIRT7, is highly enriched in HSCs where it regulates an oxidative stress response to reduce cellular ROS and is essential for HSC maintenance at an old age. Therefore, SIRT3 and SIRT7 converge at mitochondrial protection to ensure HSC maintenance.

One of the most interesting findings is that reintroduction of SIRT7 in aged HSCs can reduce mitochondrial protein folding stress and improve their regenerative capacity. Therefore, mitochondrial protein folding stress-induced HSC aging is reversible. It appears that HSC aging is not due to passive chronic accumulation of cellular damage over the lifetime, but rather regulated repression of cellular protective programs, giving hope for targeting the dysregulated cellular protective programs to reverse HSC aging and rejuvenate tissue homeostasis.

Our results indicate that SIRT7, a mammalian homolog of the longevity factor SIR2, may modulate the aging process by regulating stem cell quiescence and tissue maintenance. It will be of particular interest to establish whether other tissues use the same mechanism for maintaining stem cell quiescence. It will also be important to identify other genes that mediate mitochondrial protein folding stress to regulate stem cell quiescence. This knowledge will open new possibilities for regenerative medicine and treatment of diseases of aging.

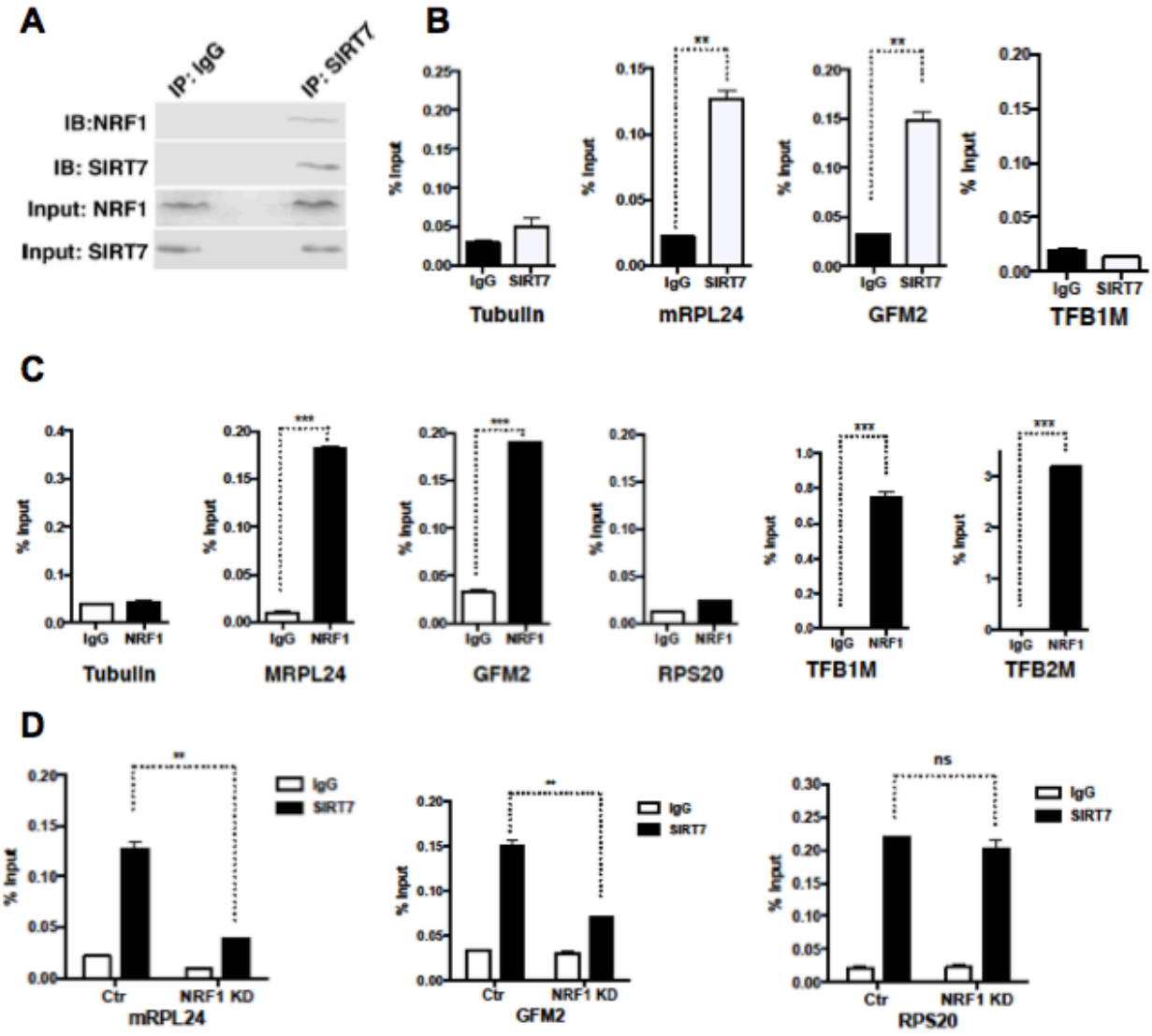


**Figure 10. NRF1 stabilizes SIRT7 at the promoters of mitochondrial translational machinery components.**

**(A)** Western blots showing co-IP of endogenous SIRT7 and NRF1 in 293T cells.

**(B)** ChIP-qPCR showing SIRT7 occupancy at the proximal promoters of mRPL24, GFM2, TFB1M compared to IgG control samples. Occupancy at the promoter of gamma tubulin was used as a negative control.

**(C)** ChIP-qPCR showing NRF1 occupancy at the proximal promoters of mRPL24 and GFM2, TFB1M, TFB2M but not RPS20, compared to IgG control samples. Occupancy at the promoter of gamma tubulin was used as a negative control.

**(D)** Reduction of SIRT7 occupancy at the promoters of mRPL24 and GFM2 but not the RPS20 promoter in NRF1 knockdown cells determined by ChIP, compared to IgG negative control samples. All samples were normalized to input DNA.

Error bars represent S. E. M. **: $p<0.01$. ***: $p<0.001$. ns: $p>0.05$.

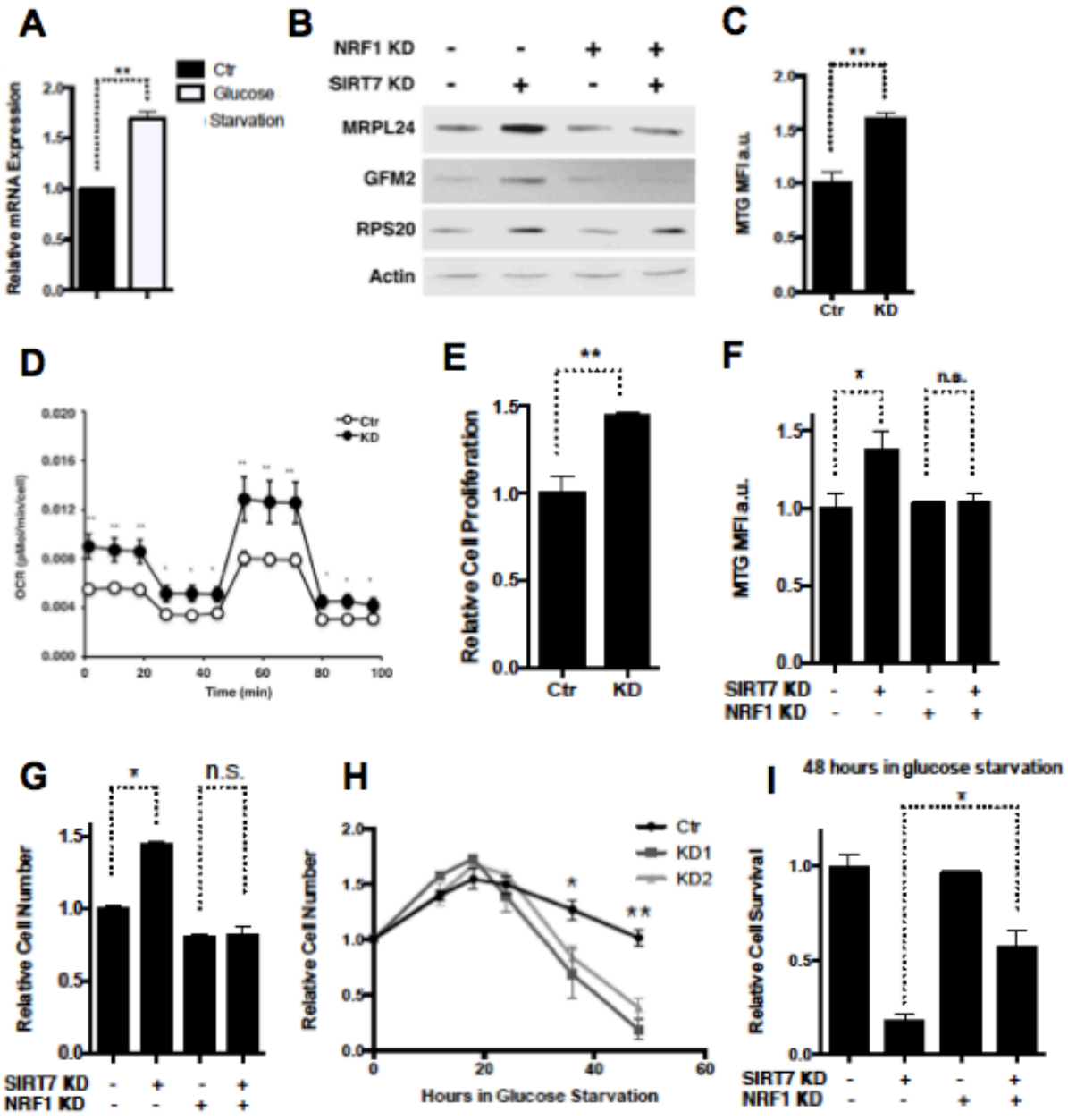


**Figure 11. SIRT7 limits mitochondrial activity and cell proliferation, and promotes nutritional stress resistance.**

**(A)** qPCR comparing SIRT7 expression in 293T cells growing in medium containing 25mM glucose and without glucose.

**(B)** Western analysis showing NRF1 inactivation via siRNA abrogates increased expression of GFM2 and MRPL24 but not RPS20 in SIRT7 knockdown 293T cells.

**(C)** MitoTracker Green staining showing increased mitochondrial mass in SIRT7 knockdown 293T cells.

**(D)** Seahorse analyses showing increased $O_2$ consumption in SIRT7 knockdown 293T cells. OCR, oxygen consumption rate.

**(E)** SIRT7 suppresses cell proliferation. Proliferation of SIRT7 knockdown 293T cells was determined using a Vi-Cell analyzer.
(**F)** Quantification of MTG staining showing NRF1 inactivation restores increased mitochondrial mass in SIRT7 knockdown 293T cells.
**(G)** NRF1 knockdown attenuates increased proliferation of SIRT7 knockdown 293T cells.
**(H)** SIRT7 promotes nutritional stress resistance. Reduced survival of SIRT7 knockdown 293T cells compared to control cells upon glucose starvation.
**(I)** SIRT7 increases nutrient starvation stress resistance. SIRT7 overexpressing 293T cells and control cells were deprived of glucose for 68 hours.
Error bars represent S. E. M. *: $p<0.05$. **: $p<0.01$. ***: $p<0.001$.

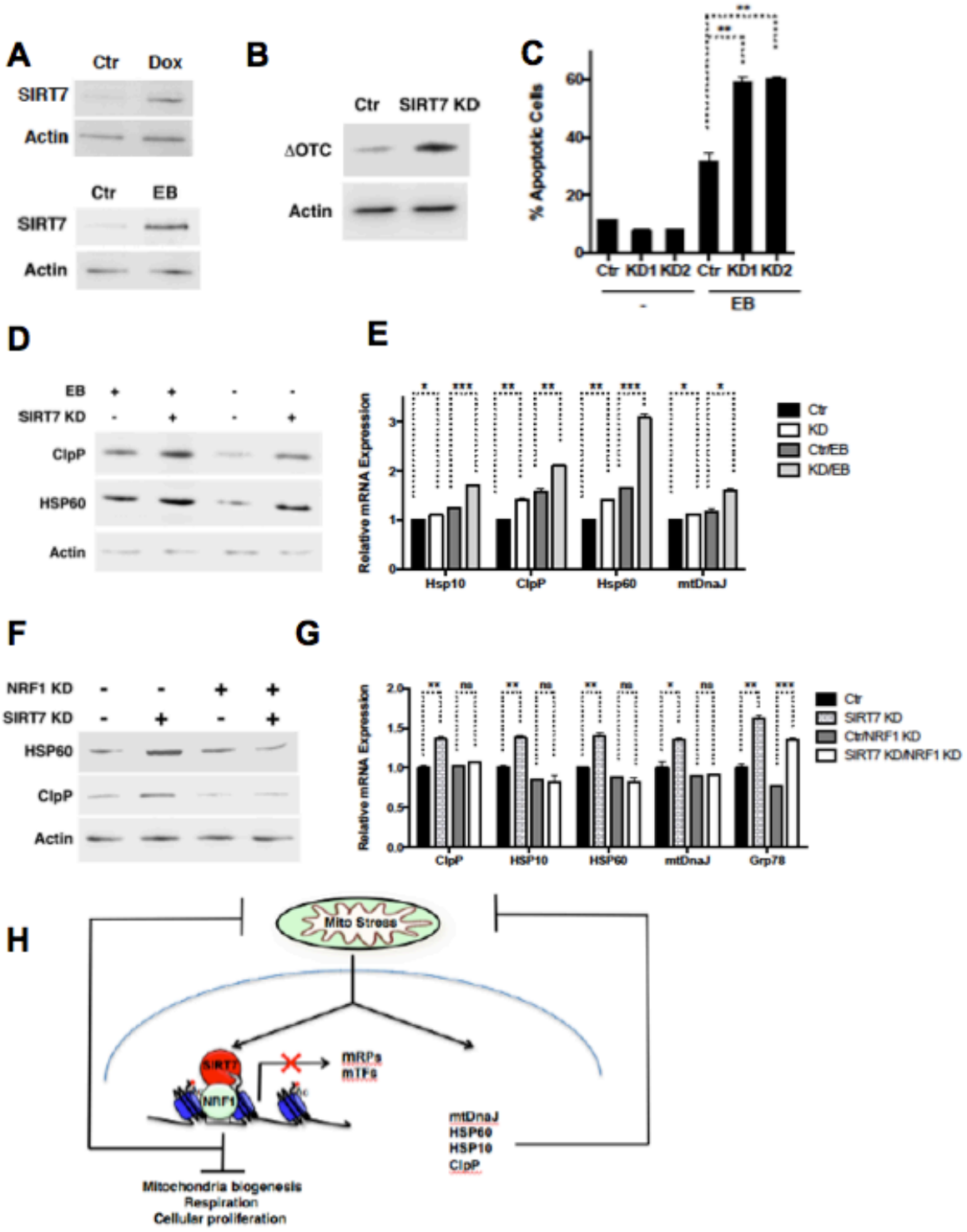


**Figure 12. SIRT7 represses NRF1 activity to suppress mitochondrial protein folding stress.**

**(A)** Western blots showing increased SIRT7 expression in 293T cells treated with mitochondrial stress inducers ethidium bromide (EB) and doxycycline (Dox).

**(B)** Western blots showing increased accumulation of misfolded DOTC in SIRT7 KD

cells compared to parental control 293T cells, which is rescued by NRF1 inactivation. Wild type OTC was used as a control.
**(C)** Annexin V staining showing increased apoptosis in SIRT7 knockdown 293T cells upon mitochondrial stress. Cells were treated with EtBr for 5 days.
**(D)** Western blots showing increased mitochondrial stress in EtBr-treated SIRT7 knockdown 293T cells.
**(E)** qPCR showing increased mitochondrial stress in SIRT7 knockdown 293T cells.
**(F)** Western blots showing NRF1 inactivation via siRNA abrogates increased mitochondrial stress in SIRT7 knockdown 293T cells.
**(G)** qPCR showing NRF1 inactivation via siRNA abrogates increased mitochondrial stress in SIRT7 knockdown 293T cells.
**(H)** A proposed model depicting a SIRT7-mediated regulatory branch of $UPR^{mt}$, which is coupled to repression of mitochondrial activity and cellular proliferation. Mitochondrial protein folding stress triggers a retrograde signaling pathway, leading to the induction of SIRT7. SIRT7 represses NRF1 activity and mitochondrial translation to reestablish mitochondrial homeostasis. Concomitantly, mitochondrial activity and cellular proliferation are suppressed.
Error bars represent S. E. M. *: $p<0.05$. **: $p<0.01$. ns: $p>0.05$.

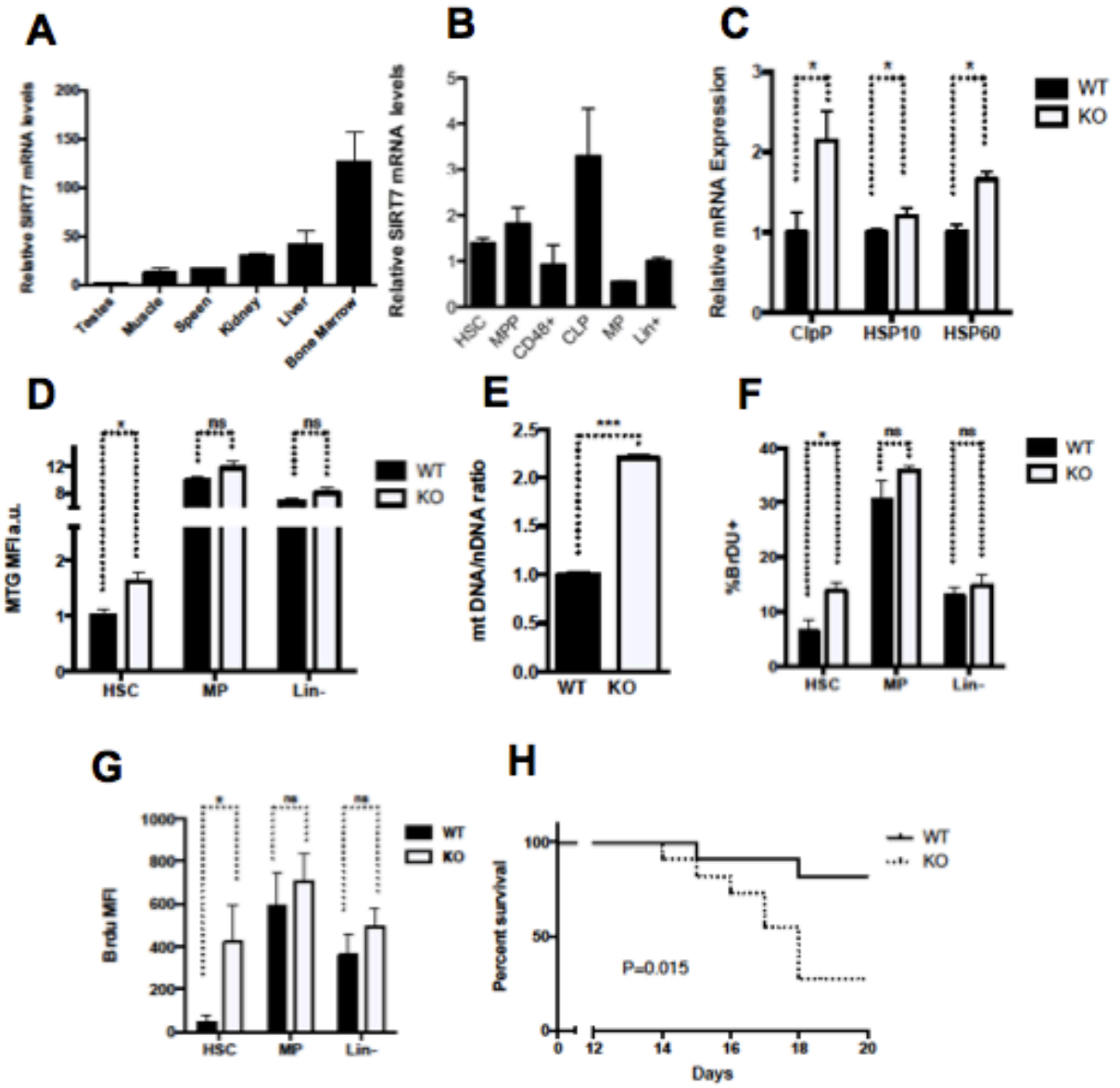


**Figure 13. SIRT7 suppresses mitochondrial protein folding stress, mitochondrial number, and proliferation of HSCs.**

**(A)** qPCR showing SIRT7 is highly expressed in the bone marrow.

**(B)** qPCR showing SIRT7 is ubiquitously expressed in various hematopoietic cellular compartments in the bone marrow. HSC, $Lin^{-}c\text{-}Kit^{+}Sca1^{+}CD150^{+}CD48^{-}$; multipotent progenitors (MPPs), $Lin^{-}c\text{-}Kit^{+}Sca1^{+}CD150^{-}CD48^{-}$; CD48+, $Lin^{-}c\text{-}Kit^{+}Sca1^{+}CD48^{+}$; myeloid progenitors (Thompson), $Lin^{-}c\text{-}Kit^{+}Sca1^{-}$; and differentiated blood cells, $Lin^{+}$

**(C)** qPCR showing increased mitochondrial stress in SIRT7 KO HSCs. n=4.

**(D, E)** MitoTracker Green staining (B) and quantification of the mitochondrial to nuclear DNA ratio (C) showing increased mitochondrial mass in SIRT7 KO HSCs but not MPs or $Lin^{-}$ cells. n=4.

**(F)** In vivo BrdU incorporation over a 16-h period showing increased proliferation of

SIRT7 knockout HSCs but not MPs or $Lin^-$ cells. n=4.

**(G)** Increased propensity of SIRT7 KO HSCs to enter cycle upon ex vivo culture with cytokines, as indicated by 1-hour BrdU pulse. n=4.

**(H)** SIRT7 deficiency results in increased sensitivity to myelotoxic stress. Lethally irradiated recipient mice transplanted with WT or SIRT7 KO bone marrow cells were subjected to weekly 5-FU injection 4 months posttransplantation. Survival rate was determined. n=12.

Error bars represent S. E. M. *: $p<0.05$. ***: $p<0.001$. ns: $p>0.05$.

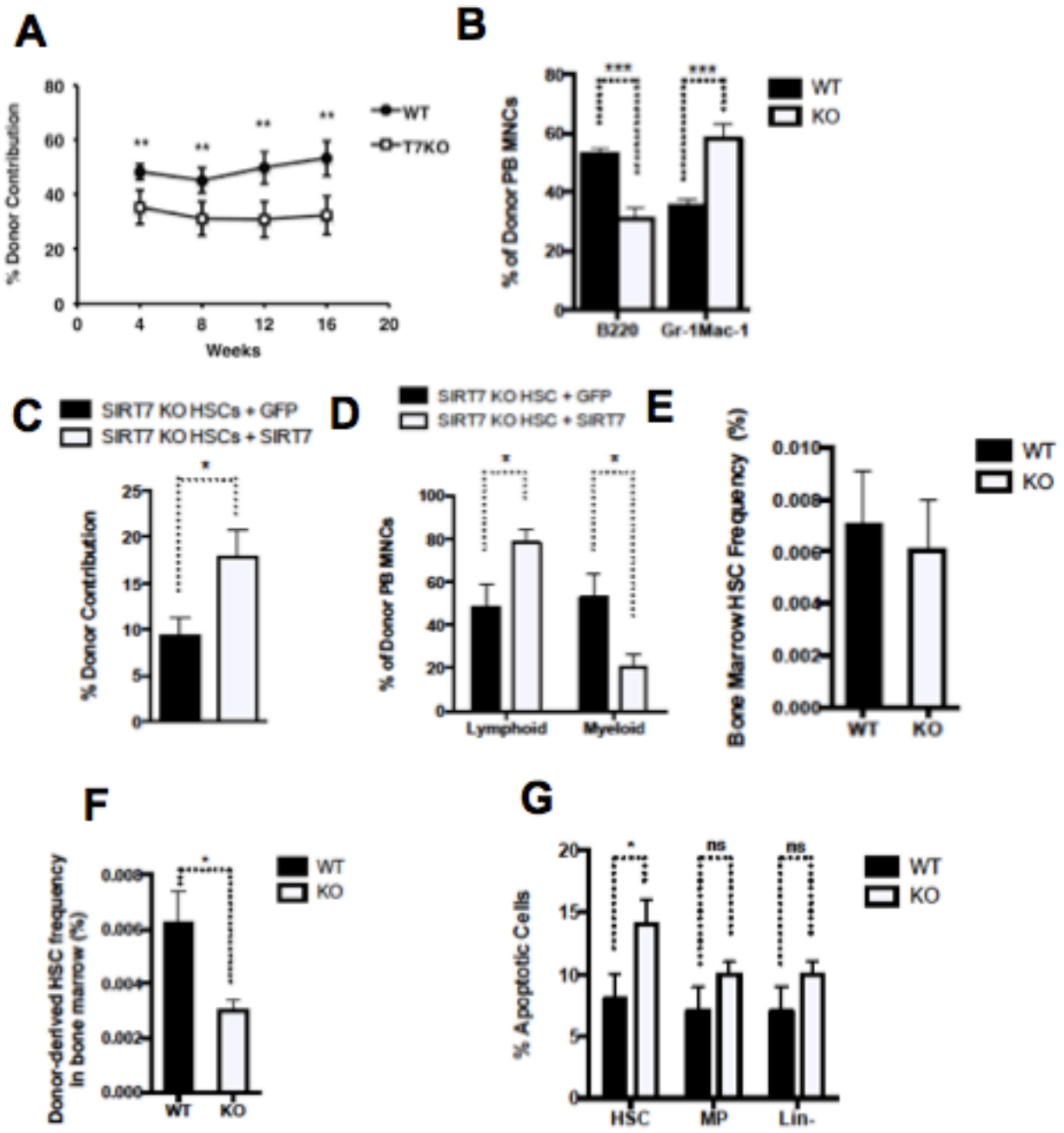


**Figure 14. SIRT7 ensures HSC maintenance.**

**(A**) Competitive transplantation using HSCs isolated from WT and SIRT7 KO mice as donors showing reduced reconstitution capacity of SIRT7 deficient HSCs. Data shown are the percentage of donor-derived cells in the peripheral blood of the recipients. Donors, n=3. Recipients, n=15. Data shown are representative of 5 independent transplants.

**(B)** Mice were transplanted with 250 FACS purified WT or SIRT7 KO HSCs (CD45.2) along with 500,000 WBM (CD45.1) cells. For blood analyses of every recipient at 4-

month posttransplantation, donor-derived cells were gated using the CD45.2 marker, and percentage of distribution for myeloid or lymphoid lineage within donor-derived cell population were determined by flow cytometry analyses. Data shown are the comparison of percentage of distribution for myeloid or lymphoid lineage derived from WT or SIRT7 KO donors. n=15.

**(C,D)** WT or SIRT7 KO HSCs transduced with SIRT7 lentivirus or control lentivirus were used as donors in a competitive transplantation assay. Data shown are the percentage of total donor-derived contribution (C) and donor-derived mature hematopoietic subpopulations (D) in the peripheral blood of recipients. n=7.

**(E)** The frequency of HSCs in the bone marrow of WT and SIRT7 KO mice was determined via flow cytometry. n = 4.

**(F)** The frequency of donor-derived HSCs in the bone marrow of WT and SIRT7 KO mice was determined via flow cytometry. n = 6.

**(G)** Annexin V staining showing increased apoptosis in SIRT7 KO HSCs under transplantation stress. n=7.

Error bars represent S. E. M. *: $p<0.05$. ***: $p<0.001$. ns: $p>0.05$.

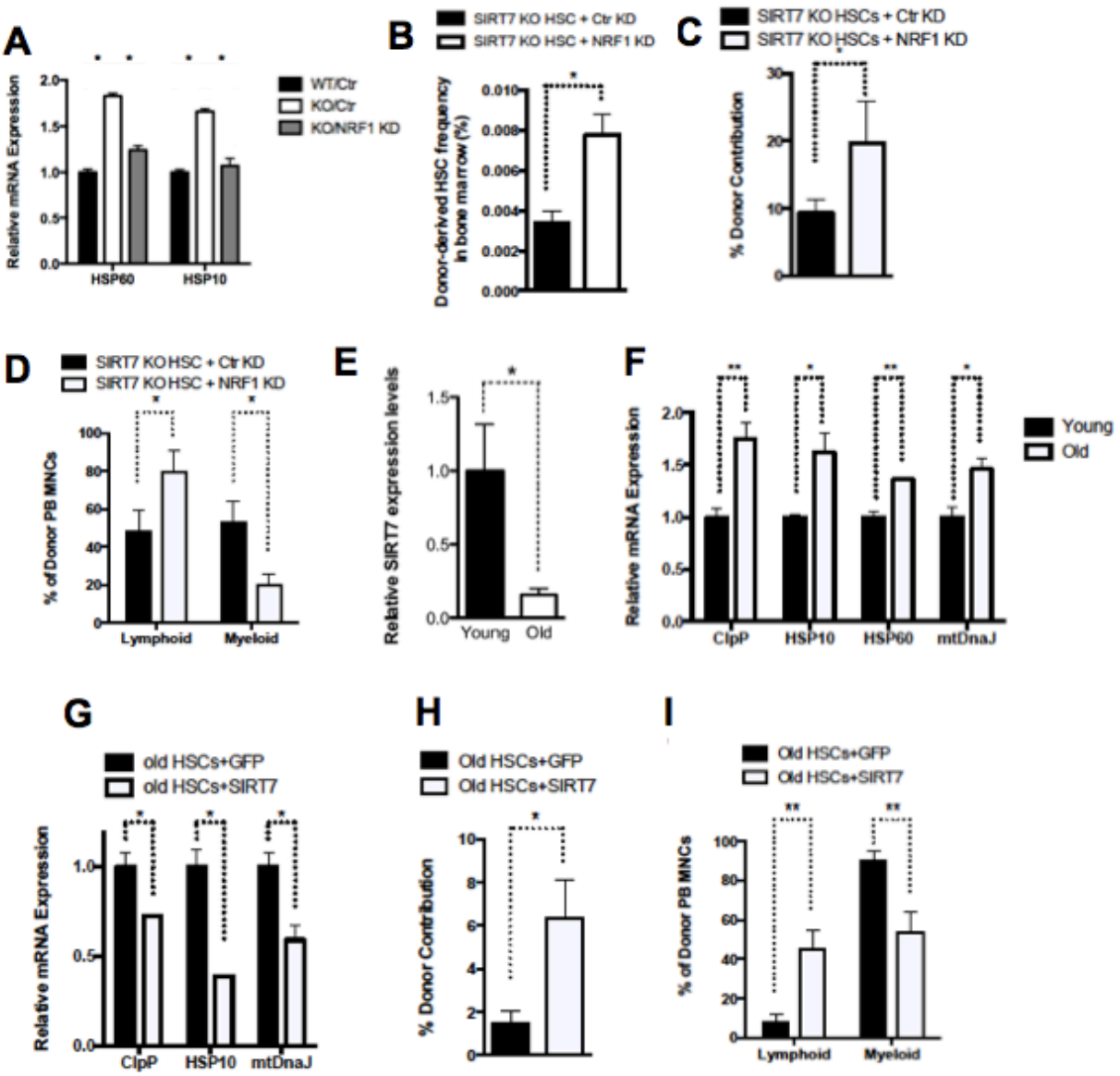


**Figure 15. HSC aging is regulated by SIRT7 via NRF1.**

**(A-D)** WT or SIRT7 KO HSCs transduced with NRF1 knockdown lentivirus or control lentivirus were used as donors in a competitive transplantation assay. Data shown are qPCR analyses of UPR$^{mt}$ gene expression (A), frequency of donor-derived HSCs in the bone marrow of recipient mice (B), the percentage of total donor-derived contribution (C) and donor-derived mature hematopoietic subpopulations (D) in the peripheral blood of recipients. n=7.

**(E)** qPCR showing reduced SIRT7 expression in aged HSCs. n=3.

**(F)** qPCR showing increased mitochondrial stress in aged HSCs. n=3.

**(G)** qPCR showing SIRT7 overexpression in old HSCs reduces mitochondrial stress.

**(H)** Competitive transplantation using aged HSCs transduced with SIRT7 virus or GFP

virus as donors showing SIRT7 overexpression increases reconstitution capacity and reverses myeloid-biased differentiation of aged HSCs. Data shown are the percentage of donor-derived cells in the peripheral blood of the recipients (D) and the percentage of lymphoid and myeloid cells in donor-derived cells in the peripheral blood of the recipients (E). n=7.
Error bars represent S. E. M. *: $p<0.05$. **: $p<0.01$.

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# Appendix: Materials and Methods

## Materials and Methods for Chapter 2

### Mice

Construction of the targeting vector and generation of SIRT7 deficient mice. Sirt7 -/- mice were produced by the VelociGene method (Valenzuela et al., 2003) (VelociGene Allele Identification Number: VG321). The SIRT7 KO targeting vector was constructed by replacing exons 4 to 11 with a LacZ gene inserted in frame after the first few bp of exon 4 (Figure 1A, B). Srf1 linearized targeting vector (30ug) was electroporated into CJ7,129SJ ES cells as described(Valenzuela et al., 2003). Positives clones were identified by Taqman analysis(Valenzuela et al., 2003) and confirmed by Southern blot analysis in which genomic DNA was digested with Kpn1 and the membranes were probed with a 500 bp fragment covering Exon 1-3. Chimeric mice were generated by injection of targeted ES clones into C57BL6/J Blastocysts. Male chimeras were mated with 129Sv females to generate F1 heterozygous mice, which were interbred to generate homozygous KO mice, which were screened by Southern blotting as described above (Figure S1A).

All mice were housed on a 12:12-hr light:dark cycle at 25°C. Experiments were performed using 4-6 month old male littermates. Mice were randomized into each of the groups. Samples were processed blindly during the experiments and the outcome assessment. All animal procedures were in accordance with the animal care committee at the University of California, Berkeley. The High-Fat diet was provided by OpenSource Diets (D12079B). For TUDCA treatment, mice received intraperitoneal injections of 500mg/kg/day TUDCA or PBS twice daily for 20 days as described(Cnop et al., 2012). VLDL-TG secretion assay was performed as described(Haeusler et al., 2010). Blood was collected from the mouse tail vein and kept on ice until centrifugation (1500*g*, 15 min at 4°C). VLDL concentration was quantified as described using a gas-phase differential electrical mobility analyzer(Caulfield et al., 2008). Plasma triglyceride and Free Fatty Acids were measured according to manufacturer's instruction (Wako Diagnostics). Triglycerides were extracted from liver tissues as described and were extracted from feces as described(Zadravec et al., 2010). Extracted triglyceride was quantified according to manufacturer's instruction (Wako Diagnostics). Liver tissues were processed for H&E staining, Oil Red O staining, and F4/80 staining as described(Sun et al., 2012).

For AAV-mediated gene transfer to the mouse liver, SIRT7 or Myc knockdown target sequence was cloned into dsAAV-RSVeGFP-U6 vector. dsAAV8 was produced by

triple-transfection and CsCl purification, and virus titer was determined as previously described(Gao et al., 2006). 3 x $10^{11}$ genome copies of virus were injected per mouse via tail vein. Mouse livers were harvested for analysis after 2 weeks.

**Cell culture, RNAi and viral transduction**

293T, Hepa 1-6, Hep G2 cells were acquired from the ATCC. WT and SIRT7 KO MEFs were generated according to standard procedures(Greber et al., 2007). XBP-1 KO MEFs were acquired from L. Glimcher (Lee et al., 2008). Cells were cultured in Advanced DMEM (Invitrogen) supplemented with 1% penicillin-streptomycin (Invitrogen) and 10% FBS (Invitrogen). For ER stress induction, cells were treated with tunicamycin (Sigma, 1ug/ml) or thapsigargin (Sigma, 0.1uM) for 24 hours for biochemical analysis or 48 hours for cell survival analysis. Cells were treated with 0.5 uM staurosporin for 48 hours for cell survival analysis. Cell survival was scored with trypan blue staining or Annexin V Staining (BD Biosciences). For Myc inactivation, cells were treated with 10058-F4 (Sigma, 2uM) for 24 hours before analysis. Cellular $NAD^+$ concentration was quantified using EnzyChrom$^{TM}$ NAD/NADH Assay Kit (Bioassay System).

SIRT7 knockdown target sequences are as follows, as previously described(Barber et al., 2012):
S7KD1, 5'-CACCTTTCTGTGAGAACGGAA-3';
S7KD2, 5'-TAGCCATTTGTCCTTGAGGAA-3',
Myc knockdown target sequences are as follows, as previously described(Caulfield et al., 2008):
Myc KD (human), 5'- GGACTATCCTGCTGCCAAG -3'
Myc KD (mouse), 5'- CCCAAGGTAGTGATCCTCAAA – 3'
Double-stranded siRNAs were purchased from Thermo Scientific and were transfected into cells via RNAiMax (Invitrogen) according to manufacture's instruction.

For lentiviral packaging, 293T cells were co-transfected with packaging vectors (pCMV-dR8.2 dvpr and pCMV-VSV-G) and the pSiCoR-SIRT7 knockdown or empty construct. For retroviral packaging, 293T cells were co-transfected with packaging vectors (pVPack-VSV-G, pVPack-GP) and pBABE-SIRT7, pBABE-SIRT7 H187Y, or empty construct. Viral supernatant was harvested after 48 hours. For transduction, cells were incubated with virus-containing supernatant in the presence of 8 μg/mL polybrene. After 48 hours, infected cells were selected with puromycin (1μg/mL).

**Co-immunoprecipitations**

Co-immunoprecipitations (Co-IPs) were performed as previously described(Qiu et al., 2010) with Flag-resin (Sigma) or Protein A/G beads (Santa Cruz) for SIRT7 IP. Elution

was performed with either Flag peptide (Sigma) or 100mM Glycine solution (pH3) for SIRT7 IP. Antibodies are provided in Supplementary Tables.

**ChIP and mRNA analysis**

Cells were prepared for ChIP as previously described(Dahl and Collas, 2007), with the exception that DNA was washed and eluted using a PCR purification kit (Qiagen) rather than by phenol-chloroform extraction. RNA was isolated from cells or tissue using the Trizol reagent (Invitrogen) and purified using the RNeasy Mini Kit (Qiagen). cDNA was generated using the qScript™ cDNA SuperMix (Quanta Biosciences). 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 ActB or GAPDH expression. Antibodies and PCR primer details are provided in Supplementary Tables.

**Polysomal profiling**

MEFs or liver tissues were collected for polysomal profiling as described(Zid et al., 2009). Briefly, 10 million MEF cells or 0.1g of liver were harvested and homogenized on ice in 400 µL of solublization buffer [300 mM NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 1 mM EGTA, 200 µg/mL heparin, 1 mM DTT, 400 U/mL RNAsin plus (Promega), 1X complete, Mini Protease Inhibitor Cocktail (Roche), 0.2 mg/mL cycloheximide, 1% Triton X-100, 0.1% Sodium deoxycholate]. 600 µL of additional solubilization buffer was added to a total of 1 mL. Cell lysate was placed back on ice for 10 min before centrifuging at 16, 000 g for 15 min at 4 °C. 1 mL of the supernatant was applied to the top of a 10-50% continuous sucrose gradient in high salt resolving buffer [140 mM NaCl, 25 mM Tris-HCl (pH 8.0), 10 mM MgCl2] and centrifuged in a Beckman SW41Ti rotor (Beckman Coulter, Fullerton, CA, USA) at 180,000 g for 90 min at 4 °C. Gradients were fractionated with continuous monitoring of absorbance at 254 nm.

**Statistical Analysis**

The number of mice chosen for each experiment is based on the principle that the minimal number of mice is used to have sufficient statistical power and is comparable to published literature for the same assays performed. No animals were excluded from the analyses. Student t test was used for data analysis. Error Bars represent standard errors. In all corresponding figures, * represents $p<0.05$. ** represents $p<0.01$. *** represents $p<0.001$. ns represents $p>0.05$.

## Materials and Methods for Chapter 3

### Cell culture and RNAi

293T cells were acquired from the ATCC. Cells were cultured in advanced DMEM (Invitrogen) supplemented with 1% penicillin-streptomycin (Invitrogen) and 10% FBS (Invitrogen). For mitochondrial stress induction, cells were treated with doxycycline (30 ug/mL) for 48 hrs, and ethidium bromide (50 ng/mL) for 7 days. Alternatively, cells were transfected with a construct expressing an aggregation prone mutant mitochondrial OTC protein from the Hoogenraad lab (Zhao et al., 2002). For nutrient deprivation, cells were cultured in glucose free medium (Invitrogen) or glutamine free medium (Invitrogen) for 48 hours. Cell proliferation and survival were scored using a Vi-Cell Analyzer (Beckman Coulter).

SIRT7 knockdown target sequences are as follows, as previously described (Shin et al., 2013):
S7KD1, 5'-CACCTTTCTGTGAGAACGGAA-3';
S7KD2, 5'-TAGCCATTTGTCCTTGAGGAA-3',
NRF1 knockdown target sequences are as follows:
NRF1 KD (mouse), 5'-GAAAGCTGCAAGCCTATCT-3'
NRF1 KD (human), 5'-CACCGTTGCCCAAGTGAATTA-3'
Double-stranded siRNAs were purchased from Thermo Scientific and were transfected into cells via RNAiMax (Invitrogen) according to manufacturer's instructions. Generation of SIRT7 knockdown and overexpressing cells was described previously (Shin et al., 2013). After puromycin selection, cells were recovered in puromycin free medium for a few passages before analyses.

### Measurements of mitochondrial mass, ATP, citrate synthase activity, and oxygen consumption

To measure mitochondrial mass, cells were stained with 100nM MitoTracker Green (Invitrogen) for 30 minutes in at 37°C, and analyzed with flow cytometry (BD Fortessa).

For oxygen consumption, $3X10^5$ cells were plated using CellTak (Kim et al.) and the oxygen consumption rate (OCR) was measured using a Seahorse XF24 instrument following the manufacturer's instructions (Seahorse Biosciences). OCR was measured under basal conditions, in the presence of the mitochondrial inhibitor oligomycin A (1 uM), mitochondrial uncoupler FCCP (1 uM) and respiratory chain inhibitor antimycin and rotenone (1 uM).

Citrate synthase activity was measured following the manufacturer's instruction (Biovision Citrate Synthase Activity Colorimetric Assay Kit #K318-100). To measure ATP, cells in suspension were mixed with an equal volume of CellTiterGlo in solid white luminescence plates (Grenier Bio-One) following the manufacturer's instructions (Promega). Luminescence was measured using a luminometer (LMAX II 384 microplate reader, Molecular Devices) to obtain relative luciferase units (RLU). Cell lines were plated at 30,000 cells per well of a 96-well dish in 100ul of media.

**Co-immunoprecipitations**

Co-immunoprecipitations (Co-IPs) were performed as previously described (Qiu et al., 2010) with Flag-resin (Sigma) or Protein A/G beads (Santa Cruz) for SIRT7 IP. Elution was performed with either Flag peptide (Sigma) or 100mM Glycine solution (pH 3) for SIRT7 IP. Antibodies are provided in Table S1.

**ChIP and mRNA analysis**

Cells were prepared for ChIP as previously described (Dahl and Collas, 2007), with the exception that DNA was washed and eluted using a PCR purification kit (Qiagen) rather than by phenol-chloroform extraction. RNA was isolated from cells or tissue using Trizol reagent (Invitrogen) and purified using the RNeasy Mini Kit (Qiagen). cDNA was generated using the qScript™ cDNA SuperMix (Quanta Biosciences). 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 ActB or GAPDH expression. Antibodies and PCR primer details are provided in Table S1-3.

**Mice**

SIRT7 knockout mice have been described previously (Shin et al., 2013). All mice were housed on a 12:12 hr light:dark cycle at 25°C. Oxygen consumption ($VO_2$) was measured using the Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus, OH). For 5-FU treatment study, $1x10^6$ bone marrow cells from WT or SIRT7 KO mice were transplanted into lethally irradiated recipient mice. 4 months posttransplantation, 5-FU was administrated to mice intraperitoneally at a dose of 150 mg/kg once per week, and the survival of the mice was monitored daily. All animal procedures were in accordance with the animal care committee at the University of California, Berkeley.

**Flow Cytometry and Cell Sorting**

Bone marrow cells were obtained by crushing the long bones with sterile PBS without calcium and magnesium supplemented with 2% FBS. 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, we used streptavidin conjugated to APC-Cy7, c-Kit-APC, Sca-1-Pacific blue, CD48-FITC, and CD150-PE (BioLegend). For congenic strain discrimination, anti-CD45.1 PerCP and anti-CD45.2 PE-Cy7 antibodies (BioLegend) were used. For assessment of apoptosis and cell cycle analysis, AnnexinV and Ki-67 (BioLegend) staining were performed respectively according to the manufacturer's recommendation after cell surface staining. For in vivo cell-cycle analysis, BrdU (Invitrogen) was incorporated over a 16-hour period. For ex vivo proliferation analysis, cultured bone marrow cells were pulsed with BrdU (Invitrogen) for one hour before flow cytometry analysis. For ROS levels and mitochondrial mass, bone marrow cells were incubated with 10 uM H2DCFDA (Invitrogen) or 100nM MitoTracker Green (Invitrogen) for 30 min at 37°C in the dark after cell surface staining (Miyamoto et al., 2007). All data were collected on a Fortessa (Becton Dickinson), and data analysis was performed with FlowJo (TreeStar). For cell sorting, lineage depletion or c-kit enrichment was performed according to the manufacturer's instructions (Miltenyi Biotec). Cells were sorted using a Cytopeia INFLUX Sorter (Becton Dickinson). Antibody details are provided in Table S1.

**Lentiviral Transduction of HSCs**

As previously described (Zhao et al., 2009), sorted HSCs were prestimulated for 24 hr in a 96 well U bottom dish in StemSpan SFEM (Stem Cell Technologies) supplemented with 10% FBS (Stem Cell Technologies), 1% Penicillin/Streptomycin (Invitrogen), IL3 (20ng/ml), IL6 (20ng/ml), TPO (50ng/ml), Flt3L (50ng/ml), and SCF (100ng/ml) (Peprotech).

SIRT7 was cloned into the pFUGw lentiviral construct. NRF1 shRNA was cloned into pFUGw-H1 lentiviral construct. Lentivirus was produced as described (Qiu et al., 2010), concentrated by centrifugation, and resuspended with supplemented StemSpan SFEM media. The lentiviral media were added to HSCs in a 24 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. After an additional 24 hr, HSCs were collected for gene expression analyses or transplantation.

**mtDNA/nDNA**

The mitochondrial DNA/nuclear DNA (mtDNA/nDNA) ratio was determined by isolating DNA from cells with Trizol (Invitrogen), as described previously (Lai et al., 2008). The ratio of mtDNA/nDNA was calculated as previously described (Brown et al., 2013; Venegas et al., 2011).

**Electron Microscopy**

40,000 HSCs were pelleted at 150g. Samples were fixed with 2% gluaraldehyde for 10minutes at room temperature while rocking. Samples were pelleted at 600g and

further fixed with 2% glutaraldehyde / 0.1M NaCacodylate at 4°C and were submitted to the UC Berkeley Electron Microscope Core Facility for standard transmission electron microscopy ultrastructure analyses.

**Transplantation Assays**

For bone marrow transplantations, $5x10^5$ bone marrow cells from WT or SIRT7 KO CD45.2 littermates was mixed with $5x10^5$ CD45.1 B6.SJL (Jackson Laboratory) competitor cells and injected into lethally irradiated (950 Gy) CD45.1 B6.SJL recipient mice. Alternatively, 250 sorted HSCs from WT or SIRT7 KO mice were mixed with $5x10^5$ CD45.1 B6.SJL competitor 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 by retroorbital bleeding. Red blood cells were lysed and the remaining blood cells were stained with CD45.2 FITC, CD45.1 PE, Mac1 PerCP, Gr1 Cy7PE, B220 APC, and CD3 PB (Biolegend). Antibody details are provided in Table S1.

**Statistical Analysis**

The number of mice chosen for each experiment is based on the principle that the minimal number of mice is used to have sufficient statistical power and is comparable to published literature for the same assays performed. Mice were randomized to groups and analysis of mice and tissue samples were performed by investigators blinded to the treatment of genetic background of the animals. Statistical analysis was performed with Excel (Microsoft) and Prism 5.0 Software (GraphPad Software). Means between two groups were compared with two-tailed, unpaired Student's t-test. Error Bars represent standard errors. In all corresponding figures, * represents $p<0.05$. ** represents $p<0.01$. *** represents $p<0.001$. ns represents $p>0.05$.