TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2015) 39: 380-387
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doi:10.3906/biy-1408-40

Changes in gene expression in *SIRT3* knockout liver cells

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Received: 19.08.2014 • **Accepted/Published Online:** 07.11.2014 • **Printed:** 15.06.2015

Abstract: The sirtuin *(SIRT)* gene family is reported to regulate critical intracellular processes from aging to cellular metabolism and repair. *SIRT3* knockout (*SIRT3*$^{-/-}$) mice develop receptor positive mammary tumors starting at 13 months and *SIRT3* expression is decreased in human breast cancer samples as well as several other diseases. It is established that carcinogenesis results from the accumulation of multiple aberrant genetic events including the activation of oncogenes and inactivation of tumor-suppressor genes. To determine the potential early genomic events that may play a role in the tumor-permissive phenotype observed in *SIRT3*$^{-/-}$ cells, we compared gene expression profile in *SIRT3*$^{-/-}$ and wild-type (*SIRT3*$^{+/+}$) mouse livers. Differences between the expression profiles of genes important in the p53 and apoptosis pathway and signal transduction pathways, as well as genes involved with insulin and cholesterol metabolism, were determined. These results demonstrate that the expression of several oncogenes including *Cdkn1*, *Myc*, and *Nos2* are increased. In contrast, several genes shown to be downregulated in human breast cancer including *Btg2*, *Egr-1*, *Fos*, *Jun*, *Gadd4*, and *Wnt1* had decreased expression. The current work demonstrates that the loss of function of SIRT3 results in a cellular environment permissive for carcinogenesis and is characterized by altered metabolism.

Key words: *SIRT3*, mitochondria, gene expression, cancer, metabolism, liver

1. Introduction

The sirtuins (SIRTs) are a family of evolutionarily conserved protein deacetylases that regulate critical intracellular processes from bacteria to mammals (Brachmann et al., 1995; Schwer and Verdin, 2008; Park et al., 2011). Silent information regulator 2 (Sir2), which was first identified in *Saccharomyces cerevisiae* (yeast), is the founding member of the SIRTs (Guarente, 1999; Houtkooper et al., 2014). Sir2 has been shown to increase the life span of yeast (Kaeberlein et al., 2002), *Caenorhabditis elegans* (Tissenbaum and Guarente, 2001), and *Drosophila melanogaster* (Rogina and Helfand, 2004). There are seven SIRTs (SIRT1 to SIRT7) in mammals that function as NAD^+-dependent deacetylases or ADP-ribosyltransferases and are found in different cellular compartments such as the nucleus (SIRT1, -6, and -7), the cytoplasm (SIRT2), and the mitochondria (SIRT3, -4, and -5) (Michishita et al., 2005; Longo and Kennedy, 2006; Ozden et al., 2011). SIRTs are also involved in processes important to cellular defense against oxidative stress, suggesting that cellular repair and organelle renewal may be the mechanism by which these genes regulate aging and life span (Okawara et al., 2007; Tao et al., 2010; Ozden et al., 2011; Houtkooper, et al., 2012).

In humans and mice, SIRT3 is a mitochondrially localized protein deacetylase that is thought to regulate proteins important in maintaining the integrity of the organelle (Jacobs et al., 2008). SIRT3 is particularly interesting to study because of its localization to the mitochondria, where the majority of the reactive oxygen species (ROS) are produced in the cell as a consequence of aerobic respiration. SIRT3 is also the main protein deacetylase in the mitochondria (Lombard et al., 2007) and acetylation of lysine residues has emerged as an important means by which mitochondrial proteins undergo posttranslational regulation (Ahn et al., 2008; Choudhary et al., 2009; Lu et al., 2009). Hence, studying this particular SIRT protein may provide important mechanistic information behind the relationships among oxidative stress, mitochondrial renewal, and aging (Someya et al., 2010).

SIRT3$^{-/-}$ mice do not develop liver tumors under normal conditions. However, SIRT3 is a stress-responsive protein, and in response to a stress condition, such as radiation

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exposure or change in diet, SIRT3$^{-/-}$ mice have been reported to develop severe hepatocellular damage, which might make these animals more susceptible to liver tumor development (Tao et al., 2010; Green and Hirschey, 2013). Moreover, SIRT3 has been found to be downregulated in hepatocellular carcinoma (HCC) cell lines and human cancer hepatocytes (Zhang et al., 2013; Wang et al., 2014). Decrease in SIRT3 expression significantly correlates with poor outcome of HCC patients (Zhang et al., 2012). These results further suggest that deletion of SIRT3 leads to predisposition to liver cancer. It has been reported that one of the functions of SIRT3 is to indirectly maintain the stability of p53 through Mdm2; consequently, it inhibits hepatocellular carcinoma cell growth (Zhang and Zhou, 2012). Since SIRT3 has numerous substrates in different pathways, the combination of different complex cellular activities of SIRT3 has a protective impact against HCC tumor formation.

Beyond recognizing that SIRT3 functions as a deacetylase enzyme, the specific mechanism by which SIRT3 regulates mitochondrial integrity and metabolism is not well understood. There is evidence that SIRTs regulate a number of different transcription factors such as FOXO (Jacobs et al., 2008), p53 (Peck et al., 2010), and NF-kB (Yu et al., 2013), though it is not clear whether SIRT3 alters the gene expression profile of the cell. Thus, we are further interested in how SIRT3 may regulate the gene expression of different transcription factors important in cancer pathways in addition to genes important in cellular metabolism. To study whether SIRT3 regulates genes important in metabolic and signal transduction pathways, we compare gene expression in *SIRT3*$^{-/-}$ and *SIRT3*$^{+/+}$ mouse livers. We examine whether there are differences between the expression profiles of genes important in the p53 and apoptosis pathway and signal transduction pathways, as well as genes involved with insulin and cholesterol metabolism.

2. Materials and methods

2.1. Mice and tissue samples

The *SIRT3*$^{-/-}$ mice (Sirt3GT218Lex strain) were obtained from the Mutant Mouse Regional Resource Center at the University of North Carolina, Chapel Hill, USA. Age-matched *SIRT3*$^{-/-}$ and *SIRT3*$^{+/+}$ mice were sacrificed and their livers harvested. The mice were euthanized by CO_2 and cervical dislocation. The livers were excised and rinsed in PBS while the gallbladder was removed. The tissue was flash-frozen for storage.

2.2. RNA isolation

RNA was extracted from the harvested liver tissue using the TRIzol protocol (Invitrogen, Carlsbad, CA, USA). Briefly, tissue samples were homogenized with the TRIzol reagent. The homogenate was centrifuged at 12,000 × *g* for 10 min and the cell debris was removed. Chloroform was added to the homogenate to perform phase separation. RNA was precipitated from the phase separation solution and washed with 75% ethanol. The purity of the RNA was verified by ultraviolet spectrophotometry and agarose gel electrophoresis.

2.3. Real-time reverse transcription-polymerase chain reaction

First-strand cDNA synthesis from total RNA was performed according to the manufacturer's instructions (RT^2 First Strand Kit; SABiosciences, Frederick, MD, USA). A genomic DNA elimination mixture was added to each RNA sample. The cDNA synthesis reactions were incubated at 42 °C for 15 min for reverse transcription (RT) and then heated at 95 °C for 5 min to inactivate the reverse transcriptase. Real-time RT-PCR was performed using a commercially available kit that profiles gene expression pathways (RT^2 Profiler PCR Array System SYBR Green; SABiosciences). Commercial primers of p53, signal transduction, insulin, and lipoprotein and cholesterol pathway genes were used (SABiosciences) to compare the relative expression of pathway genes between *SIRT3*$^{-/-}$ and *SIRT3*$^{+/+}$ liver samples. The samples were run in a thermocycler (7500 Real-Time PCR System; Applied Biosystems, Foster City, CA, USA) for 40 cycles of 15 s at 95 °C and the fluorescence signal was measured. The expression of the pathway genes was normalized to β-actin and the results were analyzed by the comparative Ct method. Real-time RT-PCR was performed three times and fold differences in *SIRT3*$^{-/-}$ compared to *SIRT3*$^{+/+}$ were calculated. Differences were compared using the unpaired t-test and results were considered significant at $P < 0.05$.

2.4. Western blotting

Western blotting on PVDF membrane was performed using the Trans-Blot Transfer System (Bio-Rad, Hercules, CA, USA). PVDF membranes were incubated with anti-c-Jun, anti-SIRT3, and anti-c-fos (Cell Signaling) and anti-actin (Abcam) primary antibodies for 16 h at 4 °C.

3. Results

3.1. Changes in p53 gene expression pathway in *SIRT3*$^{-/-}$ mouse livers

In the p53 gene expression pathway, 7 genes were found to have a greater than 2-fold change in liver tissue of the *SIRT3*$^{-/-}$ mice compared to the *SIRT3*$^{+/+}$ mice (Figure 1).

The genes with a greater than 2-fold increase included *Cyclin-dependent kinase inhibitor 1A P21* (*Cdkn1a*), *Myelocytomatosis oncogene* (*Myc*), and *Transformation related protein 73* (*Trp73*). The gene with the highest increase in the *SIRT3*$^{-/-}$ livers was *Myc* with a 3.73-fold change, though the results were not quite statistically significant ($P = 0.10$). *Cdkn1a* had a 2-fold increased expression and has been recently implicated in mechanisms of carcinogenesis,

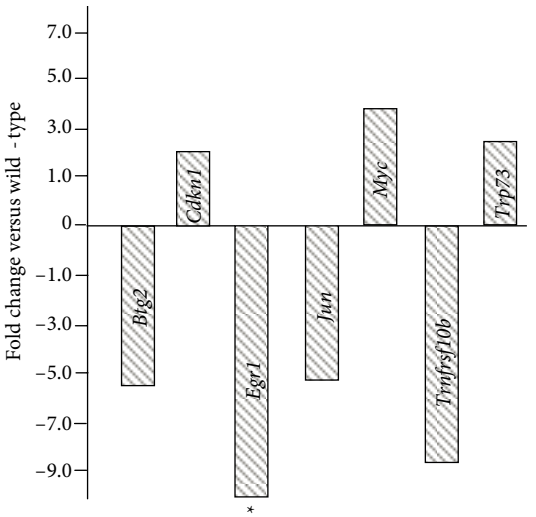

Figure 1. Changes in p53 gene expression pathways in *SIRT3*$^{-/-}$ livers compared to *SIRT3*$^{+/+}$ mouse livers. Livers from *SIRT3*$^{+/+}$ and *SIRT3*$^{-/-}$ mice were harvested as previously described. These samples were used with the Mouse p53 Signaling Pathway RT^2 Profiler PCR Array that quantifies the expression levels of 84 genes related to p53 signaling (SABiosciences). Asterisk indicates $P < 0.05$ by t-test.

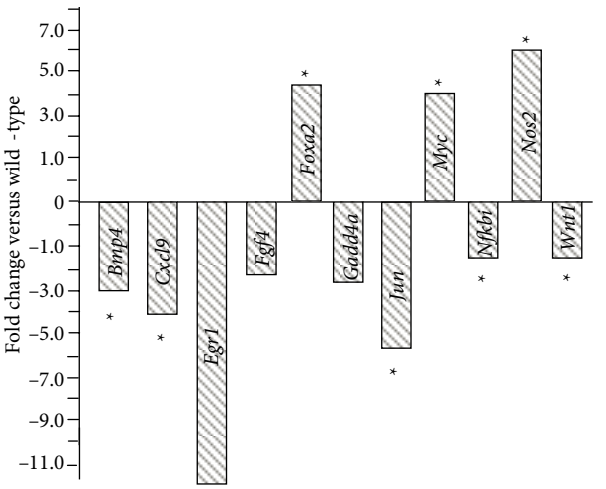

Figure 2. Changes in the expression of signal transduction pathway genes in *SIRT3*$^{-/-}$ livers compared to *SIRT3*$^{+/+}$ mouse livers. The samples from the *SIRT3*$^{-/-}$ and *SIRT3*$^{+/+}$ mouse livers were also analyzed using the Mouse Signal Transduction Pathway Finder RT^2 Profiler PCR Array that quantifies the expression levels of 84 genes that represent 18 different signal transduction pathways (SABiosciences). Data are presented as fold change in RNA levels between the knockout livers as compared to the wild-type liver data. Results from these experiments were done in triplicate. For all the experiments in this figure, error bars around data points represent one standard deviation about the arithmetic mean. Asterisk indicates $P < 0.05$ by t-test.

although it has been characterized as a tumor suppressor (Roninson et al., 2005). There is emerging evidence that *Cdkn1a* may not be a classic tumor suppressor as very few human cancers display loss-of-function mutations of this gene (Roninson et al., 2005) and it is overexpressed in human cancers including breast (Winters et al., 2001), liver (Wagayama et al., 2002), and squamous cell carcinomas (Sarbia et al., 1998).

B-cell translocation gene 2, anti-proliferative (*Btg2*); *Early growth response 1* (*Egr1*); *Jun oncogene* (*Jun*); and *Trnfrsf10b* all had decreased expression in the *SIRT3*$^{-/-}$ liver tissue. The most significant change was seen in *Egr1*, which had a 9.87-fold lower level of expression in the *SIRT3*$^{-/-}$ livers ($P = 0.04$). *Egr1* encodes a zinc-finger transcription factor that regulates diverse cellular processes such as growth and differentiation (Pagel and Deindl, 2011).

3.2. Changes in signal transduction gene expression pathway in *SIRT3*$^{-/-}$ mouse livers

The pathways represented by the commercial Mouse Signal Transduction Pathway Finder RT2 ProfilerTM PCR Array were TGFβ, WNT, JAK/STAT, Notch, Hedgehog, PPAR, oxidative stress, and hypoxia. There were a number of genes with greater than 2-fold change in the signal transduction pathway: *Bmp4*, *Cxcl9*, *Egr1*, *Fgf4*, *Gadd4a*, *Jun*, *Nfkbi*, and *Wnt1* all exhibited decreased expression and *Foxa2*, *Myc*, and *Nos2* exhibited increased expression (Figure 2). Notably, *Egr1* again had the greatest change with a 11.38-fold level of decreased expression in the *SIRT3*$^{-/-}$ livers ($P = 0.05$). *Myc* expression in the *SIRT3*$^{-/-}$ livers increased by 3.96-fold and here the difference was statistically significant ($P = 0.0075$). Another gene with a notable increase was *Nos2*, which had a 5.91-fold increase in *SIRT3*$^{-/-}$ livers ($P = 0.02$).

3.3. Changes in insulin and lipoprotein-cholesterol gene expression pathways in SIRT3$^{-/-}$ mouse livers

The insulin pathway genes with greater than 2-fold change all had lower expression in the *SIRT3*$^{-/-}$ livers, including *Fos*, *IGFBP-1*, *Jun*, and *Serpine1* (Figure 3a). *IGFBP-1* had the greatest change with a 17.06-fold decrease in expression ($P = 1.0 \times 10^{-7}$). Insulin-like growth factor (IGF)-binding protein-1 (IGFBP-1) is best characterized as the main protein regulating the bioavailability of IGF-1 (Lee et al., 1997).

Like in the p53 and signal transduction pathways, *Jun* exhibited a lower level of expression in the *SIRT3*$^{-/-}$ livers (–8.87-fold change, $P = 0.0063$). In addition, *Fos* had a 15.2-fold decrease in expression. The Jun and Fos family of proteins make up the heterodimeric activating protein 1 (AP-1), a transcription factor known to regulate many genes and biological processes involving cell proliferation, differentiation, and transformation (Shaulian and Karin, 2001). Since Jun and Fos proteins have important roles in tumor formation as SIRT3, the protein levels of Fos and

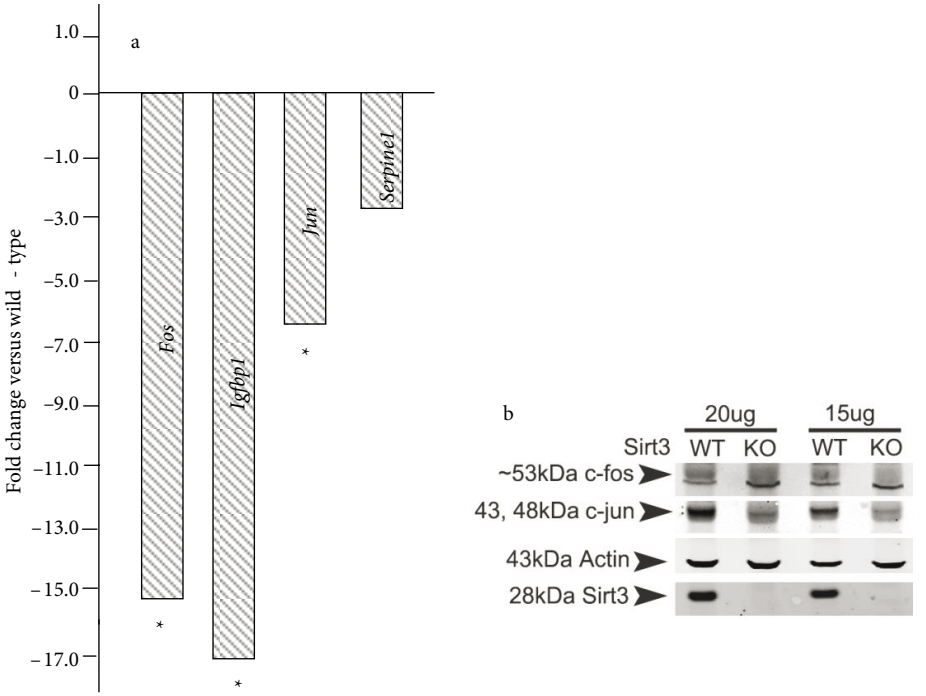

Figure 3. Changes in insulin signaling pathway in *SIRT3*$^{-/-}$ livers compared to *SIRT3*$^{+/+}$ mouse livers. **A-** Livers from *SIRT3*$^{+/+}$ and *SIRT3*$^{-/-}$ mice were harvested (as above) and these samples were used with the Mouse Insulin Signaling Pathway PCR array RT^2 Profiler PCR Array that quantifies the expression levels of 84 genes related to insulin signaling (SABiosciences). **B-** Western blot analysis. Livers from *SIRT3*$^{+/+}$ and *SIRT3*$^{-/-}$ mice were harvested (as above) and cells were lysed using lysis buffer including 1% NP-40, protease, and phosphatase inhibitors. Proteins were resolved using 10% SDS PAGE.

Jun were also investigated using western blot analysis to examine if their gene expression levels corresponded with their protein expression. Consistent to their gene expression, Jun and Fos protein expression levels were also decreased in *SIRT3*$^{-/-}$ livers (Figure 3b).

The fourth gene that exhibited decreased expression in the *SIRT3*$^{-/-}$ livers was *Serpine1*. This gene encodes plasminogen activator inhibitor 1 (PAI-1), a regulatory protein in the plasminogen activator (fibrinolytic) system that is part of the serine protease inhibitor (SERPIN) family (Dellas and Loskutoff, 2005).

Six genes were found to have greater than 2-fold change in the lipoprotein and cholesterol pathway: *Mvd*, *Nr0b2*, *Olr1*, *Osbpl5*, *Scarf1*, and *Vldlr* (Figure 4). These genes were not observed to have a significant difference between the *SIRT3*$^{-/-}$ and *SIRT3*$^{+/+}$ livers in the previous pathways. Except for Mvd, the expression of the genes significantly decreased in *SIRT3*$^{-/-}$ relative to *SIRT3*$^{+/+}$ livers.

4. Discussion

The SIRT3 protein is a major mitochondrial deacetylase that regulates various key metabolic pathways, decreases ROS production, and controls stress response. SIRT3 has numerous substrates that are involved in the regulation of metabolism such as acetyl-CoA synthetase, long-chain acyl-CoA dehydrogenase, and 3-hydroxy-3-methylglutaryl-CoA synthase 2. Such metabolic regulation increases oxidative metabolism, fatty acid oxidation, amino acid oxidation, ketone body synthesis, and ATP generation (Finley et al., 2012; Green and Hirschey, 2013). These metabolic directive roles of SIRT3 can contribute to metabolic homeostasis of hepatocytes, especially in various stress conditions, and prevent tumor growth by preventing the switch of metabolism in favor of cancer cells (Zhu et. al., 2014). Additionally, SIRT3 has been reported to activate the manganese superoxide dismutase enzyme, which might decrease mitochondrial superoxide levels, and protect cells against DNA damaging effects of ROS (Tao et al., 2010). In this manuscript, in addition to posttranslational roles of SIRT3, we investigated the changes in gene expression in SIRT3$^{-/-}$ mouse liver.

4.1. p53 gene expression pathway

Decreased *Egr1* mRNA has been shown in human, mouse, and rat breast carcinoma cell lines and overexpression of the transcription factor has been shown to suppress growth of tumors in mice (Huang et al., 1994; Calogero et al., 2004). There is also evidence that *Egr1* is deleted in estrogen receptor-negative human breast cancer but

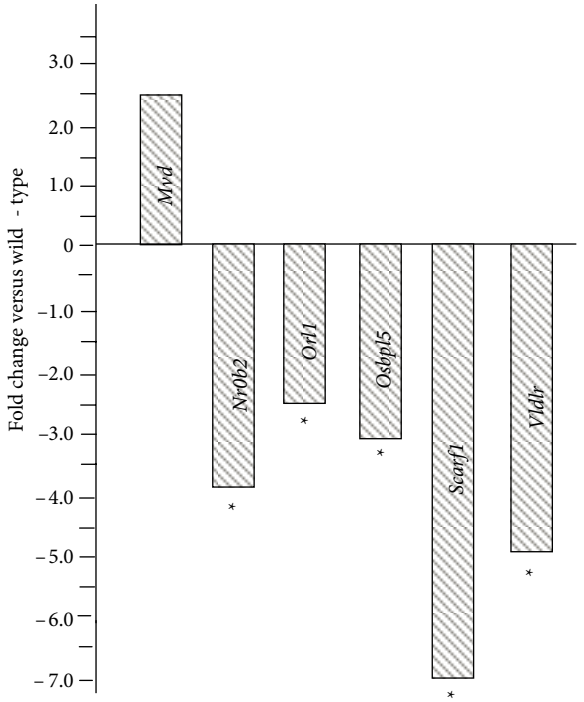

Figure 4. Changes in lipoprotein and cholesterol pathway genes in SIRT3$^{-/-}$ livers compared to SIRT3$^{+/+}$ mouse livers. The samples from the *SIRT3*$^{-/-}$ and *SIRT3*$^{+/+}$ mouse livers were also analyzed using the Mouse Lipoprotein and Cholesterol Metabolism Pathway Finder RT^2 Profiler PCR Array that quantifies the expression levels of 84 genes involved in lipoprotein transport and cholesterol metabolism (SABiosciences). Data are presented as fold change in RNA levels between *SIRT3*$^{-/-}$ livers as compared to the *SIRT3*$^{+/+}$ liver data. Results from these experiments were done in triplicate. For all the experiments in this figure, error bars around data points represent one standard deviation about the arithmetic mean. Asterisk indicates $P < 0.05$ by t-test.

not estrogen receptor-positive breast cancer (Ronski et al., 2005), whereas *SIRT3*$^{-/-}$ mice develop estrogen and progesterone receptor-positive mammary tumors (Kim et al., 2010). The decreased expression of *Egr1* in *SIRT3*$^{-/-}$ livers in this study suggests that these genes may share a common mechanism in the development of breast cancer, though the pathways differ in the pathogenesis specific to receptor positive versus negative breast cancer.

The overexpression of *Myc* is consistent with our previous results showing that lentiviral infection of *Myc* complements *SIRT3* and results in the immortalization and transformation of *SIRT3*$^{-/-}$ mouse embryonic fibroblasts (Kim et al., 2010).

4.2. Signal transduction gene expression pathway

Nos2 codes for one of the three isoforms of nitric oxide synthase (NOS2, also known as iNOS) and is rapidly inducible by inflammatory cytokines and microbial endotoxins (Kanwar et al., 2009). It has been well demonstrated that tumor cells express high levels of Nos2 and that the nitric oxide produced from NOS2 can promote tumorigenesis in a variety of cancers including gastric carcinomas (Nam et al., 2004), lung cancer (Kisley et al., 2002), and breast cancer (Ellies et al., 2003). NOS2 in breast cancer has been shown to enhance cellular migration and invasion (Orucevic et al., 1999) and its overexpression in human breast cancer is correlated with worse histologic grade and clinical stage of the tumor (Reveneau et al., 1999). Consistent to previous findings, NOS2 expression was significantly unregulated in *SIRT3* deficient mouse livers in our study.

The forkhead box transcription factor A2 (Foxa2) has roles in glucose and fat metabolic homeostasis and in differentiation of the pancreas and liver. Recent evidence suggests that deacetylation of FOXA2 by SIRT1 decreases its protein stability by promoting its proteasomal degradation in a nutrient-dependent manner (van Gent et al., 2014). To date, there has been no report on FOXA2 and SIRT3 association, but increased expression of FOXA2 in *SIRT3*$^{-/-}$ mice suggests that the SIRT3 protein may have a function similar to that of SIRT1 on FOXA2 stability.

4.3. Insulin and lipoprotein-cholesterol gene expression pathways

The regulation of *IGFBP-1* transcription is mainly controlled by insulin under basal conditions (Heemskerk et al., 1999) and its expression is upregulated in response to a number of inflammatory conditions (Lee et al., 1997; Scharf et al., 2004). It has been demonstrated that *SIRT1*$^{-/-}$ mouse livers express high levels of *IGFBP-1* (Lemieux, 2005) and *IGFBP-1* transgenic mice share a similar phenotype with mice lacking *SIRT1* (Gay et al., 1997; Ben Lagha, 2006). Interestingly, the upregulation of *IGFBP-1* is also mediated by age-related oxidative stress and depletion of glutathione, one of the main antioxidants in the liver that precipitously declines with age (Vericel et al., 1994). In humans, decreased expression of *IGFBP-1* has been shown in nonalcoholic steatohepatitis, a disease marked by increased hepatocyte apoptosis and mitochondrial dysfunction (Younossi et al., 2005). These studies demonstrate that IGFBP-1 is important in the stress response of the liver and protects hepatocytes from metabolic and oxidative damage. Here, we show that *SIRT3*$^{-/-}$ livers have significantly decreased expression of *IGFBP-1*, suggesting that SIRT3's protective effect as a mitochondrial fidelity protein is connected to the actions of IGFBP-1, as both proteins have emerged as important regulators of mitochondrial function.

The *Fos* and *Jun* gene families have characteristics of both protooncogenes and tumor suppressors depending on the cellular microenvironment, cell lineage, and differentiation stage (Eferl and Wagner, 2003). In

hepatocytes, AP-1 is upregulated during liver regeneration in response to injury and mice lacking liver-specific *Jun* fail to regenerate the liver after partial hepatectomy (Behrens et al., 2002). *Jun* has also been implicated in the early development of liver tumor formation by antagonizing the actions of p53 to prevent apoptosis (Eferl, 2003). *Fos* has been characterized as a protooncogene that activates neoplastic transformation in chondroblasts and osteoblasts (Jochum et al., 2001; Shaulain and Karin, 2001). However, it acts as a tumor suppressor in hepatocytes by inhibiting cell-cycle progression, repressing in vitro Ras-driven anchorage-independent cell growth, and suppressing Ras-driven tumor formation in vivo (Mikula et al., 2003). The downregulation of *Fos* in *SIRT3*$^{-/-}$ livers demonstrated in this study may help explain the mechanism by which *SIRT3*$^{-/-}$ cells are transformed and immortalized by *Ras*.

Small heterodimer partner (SHP, NR0B2) has been reported to have some tumor suppressor activities in the development of liver cancer and to play roles in cholesterol and lipid metabolism and glucose and energy homeostasis (Zhang and Wang, 2011). Oxidized low-density lipoprotein receptor 1 (Olr1) protein may be involved in the regulation of Fas-induced apoptosis and has been associated with cirrhosis, cardiovascular disease, and diabetes (Musso et al., 2011). Oxysterol binding protein-like 5 (Osbpl5) plays an important role in the maintenance of cholesterol balance in the cells (Du et al., 2011). Scavenger receptor (SCARF1) mediates the clearance of apoptotic cells from the tissues. In addition, it can bind and mediate the uptake of acetylated low-density lipoprotein into endothelial cells (Armengol et al., 2013). Very low density lipoprotein receptor (VLDLR) has been reported to play roles in lowering the triglyceride levels in the liver and to have various protective roles against obesity, insulin resistance, premature heart disease, tumor growth, inflammation, and angiogenesis (Gao et al., 2014).

No histological difference has been observed between the livers of *SIRT3*$^{+/+}$ and *SIRT3*$^{-/-}$ mice in normal conditions (Tao et al., 2010; Zhang et al., 2012). However, in response to a stress stimulus, such as ionizing radiation or drug exposure, the livers of *SIRT3*$^{-/-}$ mice display higher sensitivity to these agents and show damage in their hepatocytes (Tao et al., 2010; Lu et al., 2011; Gholami et al., 2014).

To conclude, *SIRT3*$^{-/-}$ mice develop mammary tumors at 13 months and human breast cancer tissue samples display decreased *SIRT3* expression, providing evidence that *SIRT3* is the first characterized mitochondrial tumor suppressor gene (Kim et al., 2010). Additional studies have shown that *SIRT3* also plays an important role in metabolism, as *SIRT3*$^{-/-}$ mice livers have decreased levels of ATP and respiration (Ahn et al., 2008) and exhibit aberrant fatty-acid oxidation (Hirschey et al., 2010). In this study, we also showed that loss of *SIRT3* changes the expression of many genes involved in metabolic and signal transduction pathway.

Acknowledgment

We thank Nancy Krett, PhD, for scientific editorial assistance.

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