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SIRT3 regulates cellular iron metabolism and cancer growth by repressing iron regulatory protein 1

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Abstract

Iron metabolism is essential for many cellular processes including oxygen transport, respiration and DNA synthesis, and many cancer cells exhibit dysregulation in iron metabolism. Maintenance of cellular iron homeostasis is regulated by iron regulatory proteins (IRPs), which control the expression of iron-related genes by binding iron-responsive elements (IREs) of target mRNAs. Here, we report that mitochondrial SIRT3 regulates cellular iron metabolism by modulating IRP1 activity. SIRT3 loss increases reactive oxygen species production, leading to elevated IRP1 binding to IREs. As a consequence, IRP1 target genes, such as the transferrin receptor (TfR1), a membrane-associated glycoprotein critical for iron uptake and cell proliferation, are controlled by SIRT3. Importantly, SIRT3 deficiency results in a defect in cellular iron homeostasis. *SIRT3* null cells contain high levels of iron and lose iron-dependent TfR1 regulation. Moreover, *SIRT3* null mice exhibit higher levels of iron and TfR1 expression in the pancreas. We found that the regulation of iron uptake and TfR1 expression contribute to the tumor suppressive activity of SIRT3. Indeed, *SIRT3* expression is negatively correlated with *TfR1* expression in human pancreatic cancers. SIRT3 overexpression decreases TfR1 expression by inhibiting IRP1 and represses proliferation in pancreatic cancer cells. Our data uncover a novel role of SIRT3 in cellular iron metabolism through IRP1 regulation, and suggest that SIRT3 functions as a tumor suppressor, in part, by modulating cellular iron metabolism.

Keywords

SIRT3; Iron metabolism; TfR1; IRP1; pancreatic cancer

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INTRODUCTION

Cellular Iron homeostasis is critical for normal cell functions. Iron deficiency can cause growth arrest and cell death, whereas iron excess generates free radicals that damage DNA, lipid membranes and proteins.[1, 2] Thus, cellular iron content must be tightly controlled, and dysregulations of iron homeostasis are frequent causes of diseases, including neurodegenerative diseases, hereditary hemochromatosis and cancers.[1, 3]

Cellular iron homeostasis is achieved by the coordinated and balanced expression of proteins involved in iron uptake, storage and export. Maintenance of cellular iron homeostasis is regulated by iron regulatory proteins (IRPs: IRP1 and IRP2), which are activated under iron-deficient conditions.[2] IRPs control the expression of iron-related genes by binding to iron-responsive elements (IREs) of target mRNAs.[1] For example, transferrin receptor (TfR1), which has a key role in cellular iron uptake,[4] is regulated by IRPs binding to the IREs in the 3' untranslated region of its mRNA.[2] Although IRPs are regulated by cellular iron levels, recent studies demonstrate cellular reactive oxygen species (ROS) affect cellular iron metabolism by modulating IRP1 activity.[5, 6]

SIRT3 is a major mitochondrial deacetylase that targets many enzymes involved in cell metabolism. Beyond metabolic targets, SIRT3 has been shown to regulate the production of ROS from mitochondria by multiple mechanisms. For example, SIRT3 deacetylates and activates IDH2 and MnSOD,[7, 8] which maintain cellular ROS homeostasis. SIRT3 also deacetylates numerous components of the electron transport chain, suggesting that SIRT3 could directly suppress the production of ROS.[9] Indeed, SIRT3 loss increases cellular ROS levels and contributes to numerous age-related pathologies, including hearing loss and tumorigenesis.[10-12]

Due to its pivotal role as a regulator of ROS, we sought to probe the role of SIRT3 in modulating IRP1 activity and cellular iron metabolism. Here, we show that SIRT3 regulates cellular iron homeostasis by modulating IRP1 activity. *SIRT3* null cells display altered expression of iron-related genes and excess cellular iron content. The regulation of iron metabolism contributes to the tumor suppressive activity of SIRT3, suggesting the novel activity of SIRT3 in controlling cellular iron metabolism and tumor growth.

RESULTS

SIRT3 loss increases TfR1 expression and cellular iron uptake

Cellular ROS levels, in addition to changes in iron, have been shown to regulate cellular iron content and uptake by modulating IRP1 activity.[5, 6, 13] Because SIRT3 is a well-known inhibitor of ROS production and SIRT3 loss results in elevated cellular ROS levels,[9] we hypothesized that SIRT3 might regulate cellular iron metabolism. To test this hypothesis, we first assessed whether SIRT3 regulates the expression of TfR1 required for the uptake of transferrin (Tf)-bound iron. We found that TfR1 messenger RNA (mRNA) and protein levels were nearly doubled in SIRT3 knockout (KO) MEFs compared to wild-type (WT) MEFs (Figures 1a and b). Furthermore, SIRT3 KO cells expressed more TfR1 on their plasma membrane (Figure 1c). To test whether the increased TfR1 on SIRT3 KO cells was

functional in Tf uptake, cells were incubated with Alexa-conjugated transferrin for indicated times and the level of internalized fluorescence was measured. In SIRT3 KO cells, high levels of fluorescence were apparent compared to WT cells (Figure 1d). Consistent with elevation in transferrin uptake, nonheme iron content was also significantly increased in SIRT3 KO MEFs (Figure 1e), indicating that SIRT3 loss enhanced cellular iron content and uptake by increasing TfR1 expression.

Next, we observed that reconstitution with SIRT3 reversed the increased TfR1 mRNA and protein levels of SIRT3 KO cells (Figures 1f and g and Supplementary Figure 1a). The expression of TfR1 on membrane and the Tf uptake were also decreased in the KO cells reconstituted with SIRT3 (Figure 1h and Supplementary Figure 1b). Moreover, we found that reconstitution of KO cells with human SIRT3 can reverse the phenotype, whereas reconstitution with a catalytic mutant of SIRT3 cannot (Supplementary Figures 1c and d). Taken together, these data demonstrate that SIRT3 regulates cellular iron metabolism through TfR1.

SIRT3 regulates TfR1 through ROS

To examine the molecular mechanisms underlying the increased TfR1 expression in SIRT3 KO cells, we examined several pathways known to regulate TfR1 in SIRT3 WT and KO cells. It has been shown that TfR1 expression is transcriptionally regulated by hypoxia-inducible factor 1 (HIF1). The *TfR1* gene contains a hypoxia response element that binds HIF1, which regulates TfR1 expression under hypoxic conditions.[14, 15] As SIRT3 loss also promotes HIF1α stabilization,[12] we probed whether SIRT3 loss induced TfR1 through HIF1α. When SIRT3 WT and KO MEFs were cultured under 1% O_2 (hypoxia), we observed comparable TfR1 induction in both cell types (Supplementary Figure 2a), suggesting that SIRT3 KO cells have intact hypoxia-dependent TfR1 regulation. Next, to directly examine the requirement for HIF1α in the increased TfR1 in SIRT3 KO cells, we stably knocked down HIF1α in SIRT3 WT and KO MEFs by using lentiviral short hairpin RNAs (shRNAs) (Supplementary Figure 2b).[12] As reported,[12] SIRT3 KO MEFs exhibited an exaggerated response to hypoxia, measured as the change in a HIF1α target gene *Glut1* expression, compared to WT MEFs (Supplementary Figure 2c). However, SIRT3 WT and KO MEFs expressing shRNAs against HIF1α had comparable response to hypoxia. We found that knockdown of HIF1α did not reverse the increased TfR1 expression in SIRT3 KO cells (Figure 2a), indicating that SIRT3 regulates TfR1 in a HIF1α-independent manner.

Because SIRT3 has a pivotal role in regulating ROS production[9, 11] and SIRT3 KO MEFs exhibit increased cellular ROS levels (Figure 2b), we next considered the possibility that SIRT3 regulates TfR1 expression in a ROS-dependent manner. To do so, we treated SIRT3 WT and KO MEFs with the antioxidant N-acetylcysteine (NAC) in order to assess the model that suppressing ROS could block the effects of SIRT3 loss on TfR1 expression. We observed that NAC treatment completely abrogated the increased ROS production in SIRT3 KO cells (Figure 2b) and reduced TfR1 expression to comparable levels in SIRT3 WT and KO MEFs (Figures 2c and d and Supplementary Figures 2d). In addition, we observed similar results with a cell-permeable glutathione (GSH) analogue, an intracellular antioxidant that plays a critical role in cellular defense against oxidative stress[16]

(Supplementary Figure 2e). Finally, to probe further whether increased oxidative stress promotes TfR1 expression, we grew WT MEFs in media containing hydrogen peroxide (H_2O_2). We found that *TfR1* mRNA levels were significantly induced after H_2O_2 treatment and NAC rescued the increased TfR1, restoring its expression to untreated levels (Figure 2e). Thus, these data demonstrate that the regulation of ROS by SIRT3 contributes to TfR1 expression.

SIRT3 loss increases IRP1 activity and results in a defect in iron-dependent TfR1 regulation

Although TfR1 expression is regulated by multiple pathways, the control mediated by the IRE/IRP regulatory system has emerged to be central and essential.[17] Previous evidence demonstrated that IRP1 functions as a sensor of cellular ROS levels and its IRE binding activity is enhanced by ROS.[5, 6] Exposure to ROS, particularly nitric oxide (NO) and H2O2, leads to removal of the iron-sulfur cluster (ISC) and induction of the IRE-binding activity of IRP1.[5, 18] Moreover, ROS scavengers, such as catalase and NAC decrease IRP1 RNA binding activity.[13, 19] Given the essential role of ROS in the increased TfR1 phenotype in *SIRT3* null cells, we reasoned that the mechanism by which SIRT3 regulates TfR1 expression involves IRP1. To test this idea we first investigated the IRE-binding activity of IRPs in SIRT3 WT and KO MEFs. We observed that IRPs-binding activity of SIRT3 KO cells was much higher than that of SIRT3 WT cells (Figure 3a, left). Moveover, when we examined the IRE-binding activity in NAC treated SIRT3 WT and KO MEFs, we found that NAC treatment reduced the IRE-binding activity to comparable levels in both cells (Figure 3a, right). Next, to investigate directly the contribution of IRP1 to the increased TfR1 expression in KO cells, we stably knocked down IRP1 in SIRT3 WT and KO MEFs by using two shRNAs (Supplementary Figure 3a) and compared the expression of TfR1. Consistent with our hypothesis, knockdown of IRP1 almost completely abrogated the increase in TfR1 mRNA and protein levels in SIRT3 KO cells (Figures 3b and c).

In addition to IRP1, IRP2 is important for TfR1 expression and cellular iron metabolism.[5] Interestingly, recent studies showed that IRP2 is also regulated by cellular ROS levels and antioxidants inhibit IRP2 activity by promoting its turnover. [20] Indeed, SIRT3 KO cells have an increased IRP2 expression compared to WT cells (Supplementary Figure 3b). To determine whether the enhanced IRP2 expression contributes to the increased TfR1 expression and IRE-binding activity in SIRT3 KO cells, we down-regulated IRP2 expression in SIRT3 WT and KO cells using shRNAs for IRP2 (Supplementary Figure 3c). We found that SIRT3 KO cells still exhibited the increased TfR1 expression and IRE-binding activity after knockdown of IRP2 compared to WT cells (Supplementary Figures 3c and d), indicating that SIRT3 can regulate TfR1 expression in a IRP2-independent manner.

It is well known that TfR1 and ferritins are inversely regulated by IRPs.[2] Moreover, a transcriptome-wide analysis of the IRE/IRP regulatory network placed TfR1 and ferritins at the center of IRP-dependent regulation.[21] Thus, to further confirm that SIRT3 influences the cellular IRP1 activity, we examined the expression of ferritin in SIRT3 WT and KO cells. Consistent with our result demonstrating the increased IRP1 binding activity in SIRT3 KO cells, we observed that ferritin light chain (FTL) expression was significantly decreased in

KO cells compared to WT cells (Figure 3d and Supplementary Figure 3e). Like ferritins, ferroportin-1 (FPN), an iron exporter, is similarly regulated by IRPs. The binding of IRPs to FPN1 mRNA leads to inhibition of its protein expression.[1] We found that SIRT3 KO cells exhibited decreased FPN1 protein expression compared to WT cells (Figure 3e and Supplementary Figure 3f). Moreover, only the IRE-containing form of divalent metal transporter 1 (DMT1) (+ IRE) mRNA was up-regulated in SIRT3 KO cells compared to WT cells whereas we observed no increase in DMT1 mRNA without the IRE (-IRE) (Figure 3f). Furthermore, to probe the contribution of ROS, we cultured cells with NAC and measured the expression levels of FTL and DMT1+IRE. NAC treatment reversed the differential expression of these IRP1 target genes in SIRT3 KO cells (Supplementary Figures 3g and h). Taken together, these data illustrate that SIRT3 loss increases IRP1 activity through ROS, leading to enhanced TfR1 expression.

Cellular iron levels tightly regulate the expression of iron-related genes such as TfR1 and ferritins. Under conditions of high intracellular iron, IRPs become unavailable for IRE binding, resulting in decreased TfR1 and increased ferritins expression.[2, 5] However, we found that in SIRT3 KO cells the expression of TfR1 and FTL were inappropriately regulated despite the increased intracellular iron levels, suggesting that SIRT3 loss results in a defect in iron-dependent regulatory system. To test this idea we examined the influence of excess iron on TfR1 expression in SIRT3 WT and KO cells. We measured TfR1 expression in SIRT3 WT and KO cells in the presence of non-Tf-bound iron. It has been reported that this iron supplement could be taken up by cells through a TfR1-independent pathway.[22] As expected, treatment with iron decreased TfR1 expression in WT cells. Strikingly, we found that KO cells were unable to repress TfR1 expression in response to exogenous iron (Figure 3g). Taken together, these data illustrate that SIRT3 loss result in dysregulated activation of IRP1 as a cause of the defect in cellular iron homeostasis of *SIRT3* null cells.

SIRT3 inhibits tumor cell growth by regulating TfR1 expression

Cellular iron metabolism is crucial for cell proliferation partly due to the fact that iron serves as a cofactor for ribonucleotide reductase, the rate-limiting enzyme in DNA synthesis.[5] Furthermore, high TfR1 expression is observed in highly proliferative cells, including cancer cells.[3, 23] Previously, we reported that SIRT3 KO cells grow significantly faster than WT cells.[12] Thus, we hypothesized that the increased TfR1 expression also contributes to the proliferation phenotype of SIRT3 KO cells. First, to test whether cellular iron metabolism is important for the increased growth rate of SIRT3 KO cells, we grew SIRT3 WT and KO MEFs in media containing deferoxamine (DFO), an iron chelator, thereby diminishing intracellular iron content. Under these conditions, WT and KO cells grew at a similar rate (Figure 4a and b). Moreover, knockdown of TfR1 by using shRNAs abrogated the increased proliferation of SIRT3 KO cells (Supplementary Figures 4a and b), demonstrating that the enhanced iron metabolism and TfR1 expression are required for the increased proliferation of SIRT3 KO cells.

It has been shown that increased TfR1 expression enhances tumor cell proliferation and tumorigenesis.[24] To confirm the importance of TfR1 regulation by SIRT3 in tumorigenic properties of transformed cells, we suppressed TfR1 expression in KRAS-transformed

SIRT3 WT and KO MEFs (Supplementary Figure 4c) and assessed their clonogenic growth. As expected, KO transformed MEFs exhibited an increased cell proliferation compared to WT cells. Importantly, knockdown of TfR1 markedly diminished the clonogenic growth of both SIRT3 WT and KO cells to similar levels (Figure 4c and Supplementary Figure 4d). Next, to assess the anchorage independent growth of these cells, we grew them in soft agar. Consistent with our hypothesis, SIRT3 KO cells no longer exhibited the increased colony formation when TfR1 was reduced by shRNAs (Figure 4d).

We next asked whether increased expression of TfR1 supports cell proliferation. We overexpressed TfR1 in WT MEFs (Supplementary Figure 4e) and examined their growth. In line with our results, overexpression of TfR1 significantly increased cell proliferation (Figures 4e and f), highlighting the role of TfR1 in cell growth. Together, these data support the idea that increased TfR1 expression induced by SIRT3 loss contributes to increased proliferation and tumorigenic phenotypes of SIRT3 KO cells.

SIRT3 KO mice have enhanced in the pancreas

To determine whether SIRT3 regulates TfR1 expression *in vivo*, we examined TfR1 expression in tissues from SIRT3 WT and KO mice. Consistent with our *in vitro* results, we found that SIRT3 KO mice had higher levels of TfR1 mRNA and protein in the pancreas (Figures 5a and b) compared to WT mice, even though the mRNA levels of TfR1 were similar in other tissues (Supplementary Figure 5a). These results corresponded to an increase in pancreatic nonheme iron content (Figure 5c).

Previously, it has been reported that human pancreatic cancers exhibit high expression of TfR1 compared to normal pancreatic tissues, and TfR1 could be a marker of malignant transformation in the pancreas,[25] suggesting that SIRT3 may exert its tumor suppressive activity in part by regulating TfR1 expression in the pancreas. Consistent with previous findings, *TfR1* mRNA levels were increased in human pancreatic ductal adenocarcinomas (PDAC) compared to normal pancreas (Figure 5d, left), whereas *SIRT3* mRNA levels were significantly decreased in PDAC samples compared to normal pancreatic tissues (Figure 5d, right). Interestingly, paired t-test analysis of this dataset revealed that *SIRT3* mRNA levels were decreased in the PDAC samples compared to the normal pancreatic tissue from the same patient, whereas *TfR1* mRNA levels showed the opposite expression pattern (Supplementary Figure 5b). When we further analyzed the correlation between *SIRT3* and *TfR1* expression in PDAC samples, we found that *SIRT3* is inversely correlated with *TfR1* (Supplementary Figure 5c). Overall, the expression data provide a novel link between SIRT3 and TfR1 expression in human pancreatic cancers and are consistent with our model that SIRT3 may function as a tumor suppressor by regulating cellular iron metabolism.

SIRT3 regulates PDAC growth by inhibiting IRP1 activity

In order to examine whether SIRT3 can regulate TfR1 expression in PDAC, we stably overexpressed SIRT3 in two human PDAC cell lines; 8988T and Panc1. We found that SIRT3 overexpression repressed TfR1 expression in both PDAC cell lines (Figure 6a and Supplementary Figure 6a). We next examined whether SIRT3 represses TfR1 expression by inhibiting IRP1 binding activity in PDAC cells. In line with our results demonstrating

increased the IRP1 activity in SIRT3 KO cells, we observed that SIRT3 overexpression inhibited the IRP1 activity in PDAC cells (Figure 6b). Next, we tested the hypothesis that SIRT3-mediated control of TfR1 expression could influence cancer cell growth. We found that SIRT3 overexpression repressed the growth of PDAC cell lines (Figure 6c and Supplementary Figure 6b). Remarkably, control and SIRT3 overexpressing cells proliferated at similar rates when TfR1 was reduced by using shRNAs (Figure 6d and Supplementary Figure 6c). Finally, to examine whether SIRT3 overexpression inhibits TfR1 through ROS, we measured TfR1 expression in control and SIRT3 overexpressing PDAC cells, with or without NAC treatment. We found that SIRT3 overexpression no longer repressed TfR1 expression, when cells were treated with NAC, demonstrating that SIRT3 regulates TfR1 through the ROS regulation (Figure 6e). Taken together, these data illustrate that SIRT3 decreases TfR1 expression by inhibiting IRP1 activity, which represses proliferation of pancreatic cancer cells.

DISCUSSION

In this study we demonstrate that SIRT3 regulates cellular iron metabolism through IRP1 with important implications for tumor cell growth (Figure 6f). It has been shown that SIRT3 functions as a tumor suppressor that controls genomic stability and glycolytic metabolism.[11, 12] Our study reveals that SIRT3 exerts additional tumor suppressive function by regulating cellular iron metabolism. We find that the enhanced iron uptake and TfR1 expression are required for the increased proliferation of SIRT3 KO cells (Figure 4). This idea is further validated by the finding that SIRT3 overexpression inhibits IRP1 activity, leading to a decreased TfR1 expression, which suppresses proliferation of human pancreatic cancer cells. Previously, we reported that *SIRT3* is significantly deleted in about 20% of all human cancers, and up to 40% of breast and ovarian cancers.[12] Thus, our observations raise exciting possibilities regarding the targeting of TfR1 and/or iron metabolism as a potential therapeutic strategy against SIRT3-deleted human cancers.

Our study shows that SIRT3 regulates cellular iron content and uptake. We observed that the loss of SIRT3 results in iron accumulation in cells and mouse pancreas. Accumulation of excess iron is toxic, because it generates highly reactive radicals such as hydroxyl or lipid radicals that damage lipid membrane, proteins and DNA.[26] Iron also catalyzes the formation of ROS, and excess iron promotes ROS production and oxidative stress.[27] Given the function of SIRT3 in ROS production, we propose a detrimental feed-forward mechanism by which the loss of SIRT3 promotes the amplification of both ROS production and iron accumulation, leading to genomic instability.

Our studies reveal a novel connection between SIRT3 and IRP1. Interestingly, we observed that SIRT3 KO cells also have an increased IRP2 expression compared to WT cells (Supplementary Figure 3b). Although we found that the knockdown of IRP1 but not IRP2 diminished the increased TfR1 expression in SIRT3 KO cells (Figures 3b and c and Supplementary Figure 3c), it does not preclude the involvement of IRP2 in these phenotypes. At the low oxygen concentrations, IRP2 has high IRE-binding activity, whereas IRP1 assembled with the ISC mainly functions as a cytosolic aconitase and exhibits low IRE-binding activity.[18] Because the oxygen levels in mammalian tissues (3 – 6%) are lower

than those of cultured cells (20%), IRP2 may contribute to the increased TfR1 in the pancreas of SIRT3 KO mice (Figures 5a and b). In addition, the presence of IREs in a variety of mRNAs[21] indicates that the SIRT3-mediated IRPs regulation could extend to processes other than iron metabolism.

Our current work highlights the regulation of TfR1 expression by SIRT3. However, enhanced TfR1 expression has been observed mainly in highly proliferative cells, particularly cancer cells.[3, 23] Moreover, we found that SIRT3 KO mice had similar TfR1 expression in several tissues compared to WT mice, except the pancreas (Supplementary Figure 5a). Thus, it will be important for future work to examine how SIRT3 controls iron homeostasis in vivo, and also to determine whether SIRT3 is involved in iron metabolism in differentiated, non-proliferating cells.

In this study we demonstrate that SIRT3 is involved in iron metabolism by modulating IRP1 activity, suggesting that SIRT3 may influence cellular iron hemostasis. Indeed, we found that SIRT3 loss results in a defect in iron-responsive TfR1 regulation (Figure 3g) and SIRT3 KO mice exhibit pancreatic iron accumulation. Recently, it has been shown that IRP1 has a key role in erythropoiesis and dietary iron absorption.[28] Moreover, the activity of SIRT3 is inhibited in frataxin-deficient heart, which causes hyperacetylation of numerous cardiac mitochondrial proteins and may contribute the cardiomyopathy in Friedreich's ataxia (FRDA), an iron-related human disease.[29] Interestingly, FRDA cells exhibit an increased IRP1 activity and iron accumulation.[30] Thus, it will be interesting for future studies to examine the role of SIRT3 in systemic iron homeostasis and iron-related human diseases.

MATERIAL AND METHODS

Cell Culture

Immortalized WT and SIRT3 KO MEFs were maintained as previously described[12] and transformed with retrovirus expressing KRAS. 8988T and Panc1 human PDAC cell lines (kindly provided by Dr. Alec C. Kimmelman, Dana-Farber Cancer Institute (DFCI), Boston, MA) were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific) and penicillin/streptomycin (Invitrogen). Except for the hypoxic (1% O_2) experiments (Supplementary Figures 2a and c), all cells were cultured under normoxia.

Constructs and reagents

The following antibodies were used: TfR1 (Invitrogen), β-Actin (Sigma), FTL (abcam), Fpn1 (Novus Biologicals), IRP1 (abcam), IRP2 (Thermo Scientific), SIRT3 (Cell Signaling) and α-Tubulin (Santa Cruz). NAC, GSH, hydrogen peroxide, iron supplement, DFO were purchased from Sigma. Lentiviral shRNA against TfR1 and IRP1 were obtained from the RNAi facility of DFCI and IRP2 pLKO.1 shRNA vectors were purchased from Sigma. The mouse SIRT3, human SIRT3, human SIRT3 catalytic mutant (SIRT3-H248Y)[31] and mouse TfR1 were cloned into retroviral pBabe vector and used to generate stable cell lines.

Clonogenic Assay

Cells were plated in 6-well plates at 400 cells per well in 2 ml of media. After 7-10 days, colonies were fixed and stained with 0.2% crystal violet. Media was not changed throughout the course of the experiment.

Soft agar Assay

Anchorage-independent growth was assessed by plating cells in a two-layer agar system (Millipore), in which the final concentration of the bottom agarose layer was 0.8% and 0.4% for the top agarose layer that contained the cells.

Western Blotting

Cells were lysed with lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5 and 0.5% NP-40) supplemented with protease inhibitor cocktail (Roche). Cell lysates were separated by SDS-PAGE and immunoblotting.

Animal Studies

Animal studies were performed according to protocols approved by the institutional animal care and use committee, the standing committee on animals at Harvard. SIRT3 WT and KO mice[12] were used for all studies.

ROS determination

Cells were washed with phosphate-buffered saline (PBS) and labeled at 37 °C for 20 min in Hank's balanced salt solution (HBSS) (Gibco) containing 10 μM DCFDA (Invitrogen). Cells were trypsinized and resuspended in HBSS. Fluorescence was measured by flow cytometry using a FACSCalibur (BD Biosciences).

Flow Cytometry

Cells were incubated in serum-free media for 30 min to remove any residual transferrin and then were exposed to 5 μg/ml transferrin conjugated with Alexa Fluor 488 (Invitrogen) for the times indicated. Internalization was stopped by washing the cells with cold PBS. Bound transferrin was removed by washing with PBS at pH 5.0. Cells were harvested, pelleted by centrifugation and resuspended in PBS containing 3% FBS. The fluorescence intensity was measured by flow cytometry using a FACSCalibur (BD Biosciences).

Nonheme Iron Assay

Nonheme iron was measured as described.[32] Briefly, protein extracts were mixed with protein precipitation solution (1:1 1 N HCl and 10% trichloroacetic acid) and heated to 95°C for 1 hr. Iron was collected by centrifugation at 4°C at 13000 rpm for 10 min, the supernatant was mixed with the equal amount of chromogen solution (0.5 mM ferrozine, 1.5 M sodium acetate, 0.1% thioglycolic acid). The absorbance was measured with a microplate reader (Agilent Technologies).

RNA-EMSA

An RNA binding assay was performed with the LightShift Chemiluminescent RNA EMSA kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, cytosolic extracts were prepared by using NE-PER cytoplasmic extraction kit (Thermo Scientific) and used for measuring the IRE-binding activities. Biotinylated IRE RNAs (5'-UCCUGCUUCAACAGUGCUUGGACGGAAC-3'-Biotin) were used.

Quantitative RT-PCR

Total RNA was prepared with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. 1 μg of total RNA was reverse-transcribed using iScript cDNA synthesis kit (BioRad). Diluted cDNAs were analyzed by real-time PCR using Sybr Green I Mastermix on a Lightcycler 480 (Roche). The level of gene expression was normalized to *β-Actin*. Primers sequences were: ACTTGCCCAGATGTTCTCAG and GTATCCCTCTAGCCATTCAGTG for human *TfR1*; CTACGTCGCCCTGGACTTCGAGC and GATGGAGCCGCCGATCCACACGG for human *β-Actin*; AGTGTCAGAAAACCCAAGAGG and CGTTTCAGCCAGTTTCACAC for mouse *TfR1*; TGGCTCCCTATATCCCAATG and GGTCTGCTGGAACCCAGTAA for mouse *Hif1α*; TGCAGCCCAAGGATCTCTCT and CGGCTTGCCCGAGATCT for mouse *Glut1*; GGGTTGGCAGTGTTTGATTG and CTGGGCTGTTAGTCATCTGG for mouse *Dmt1-IRE*; GGGTTGGCAGTGTTTGATTG and TTCTGAGTTATCTTGGGCGTG for mouse *Dmt1+IRE*; AGCCATGTACGTAGCCATCC and CTCTCAGCTGTGGTGGTGAA for mouse *β-Actin*.

Statistical Analysis

Unpaired two-tailed Student's tests were performed unless otherwise noted. All experiments were performed at least two or three times.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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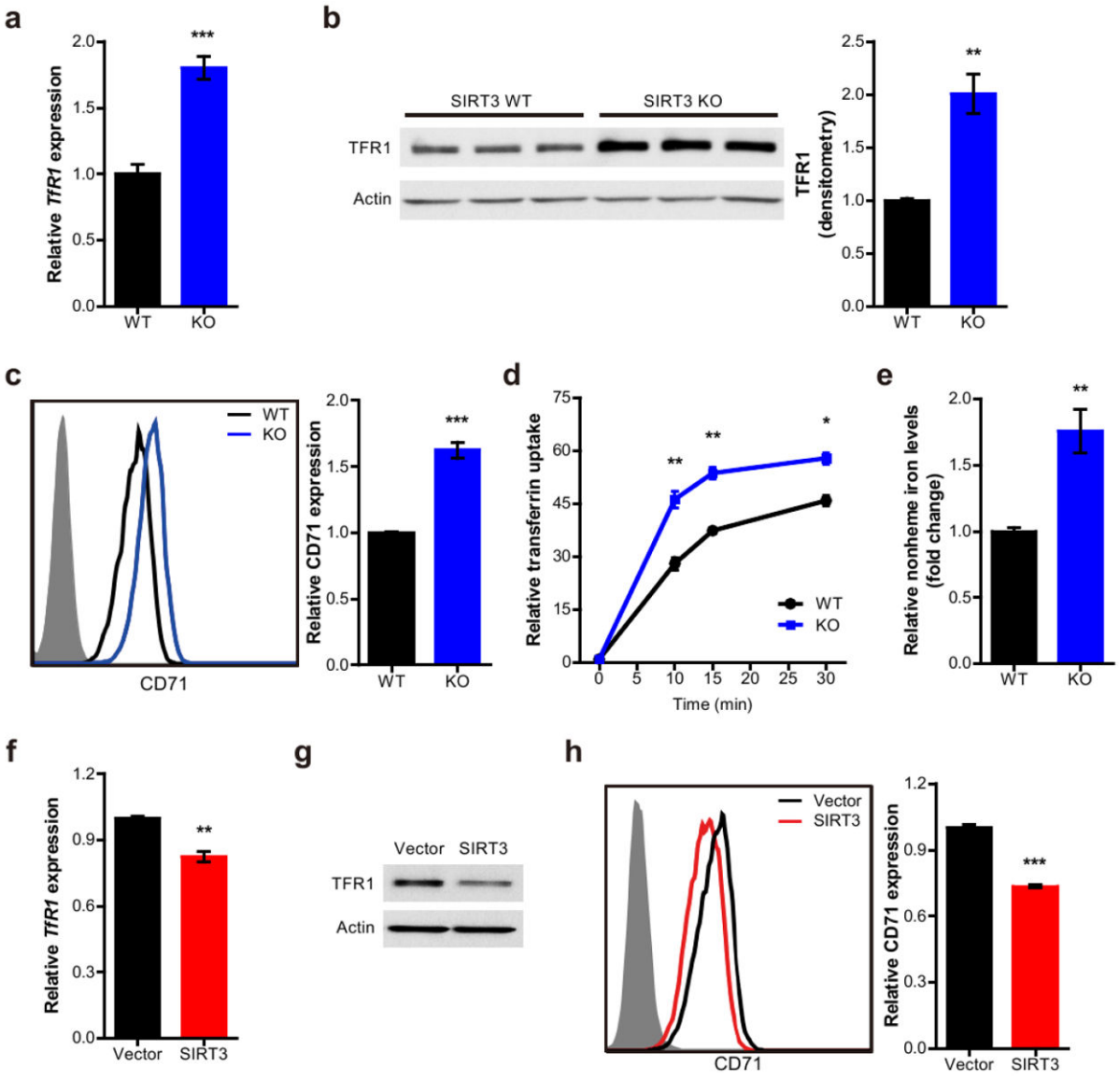

Figure 1.

Loss of SIRT3 increases TfR1 expression. (**a**) Relative TfR1 mRNA levels in SIRT3 WT and KO MEFs (n = 3). β-actin was used as an endogenous control for qRT-PCR. (**b**) TfR1 protein levels in whole cell lysates from SIRT3 WT and KO MEFs were detected by immunoblotting with anti-TfR1 antibody. β-actin serves as a loading control. (**c**) Expression levels of TfR1 (CD71) on the surfaces of SIRT3 WT and KO MEFs. Cells were either left unstained (gray) or stained with the anti-CD71 antibody (n = 3). (**d**) Relative transferrin uptake was measured in SIRT3 WT and KO MEFs. Cells were incubated with Alexa488-conjugated transferrin for indicated times and the intensities of fluorescence were measured by using flow cytometry (n = 3) (Data represent mean ± SD). (**e**) Nonheme iron content in SIRT3 WT and KO MEFs (n = 4). (**f**) Relative TfR1 mRNA levels in SIRT3 KO MEFs reconstituted with SIRT3 (n = 3). (**g**) TfR1 protein levels in whole cell lysates from SIRT3 KO MEFs reconstituted with SIRT3. β-actin serves as a loading control. (**h**) Expression

levels of TfR1 on the surfaces of SIRT3 KO MEFs reconstituted with SIRT3 (n = 3). Data represent mean ± SEM. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

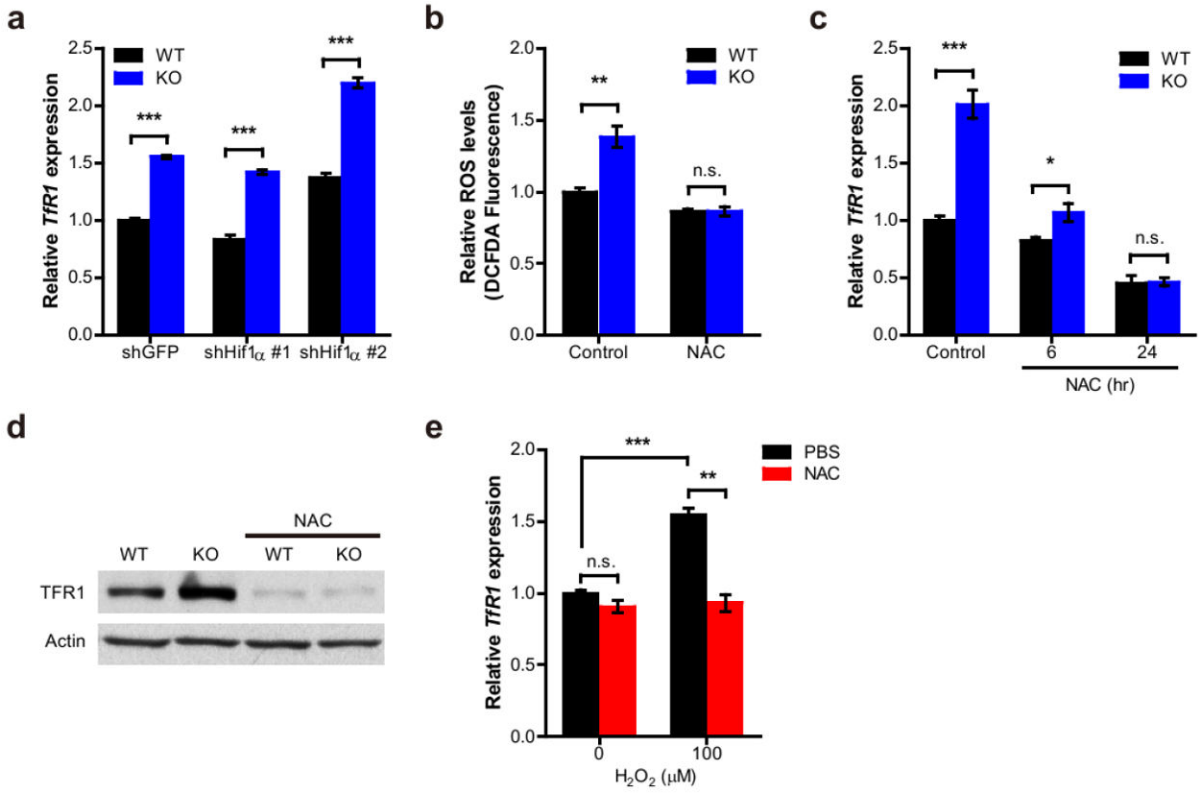

Figure 2.

SIRT3 regulates TfR1 through ROS. (**a**) Relative *TfR1* mRNA levels in SIRT3 WT and KO MEFs expressing control shRNA (shGFP) or shRNA targeted HIF1α (shHif1α #1 and shHif1α #2) (n = 3). (**b**) Relative ROS production in SIRT3 WT and KO MEFs incubated with or without 10 mM NAC for 24 hr (n = 3). (**c**) Relative *TfR1* mRNA levels in SIRT3 WT and KO MEFs incubated with or without 10 mM NAC for 6 or 24 hr (n = 3). (**d**) TfR1 protein levels in whole cell lysates from SIRT3 WT and KO MEFs incubated with or without 10 mM NAC for 24 hr. β-actin serves as a loading control. (**e**) Relative *TfR1* mRNA levels in H_2O_2 (100 μM) treated WT MEFs incubated with or without NAC (5 mM) for 4 hr (n = 3). Data represent mean ± SEM. n.s., not significant. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

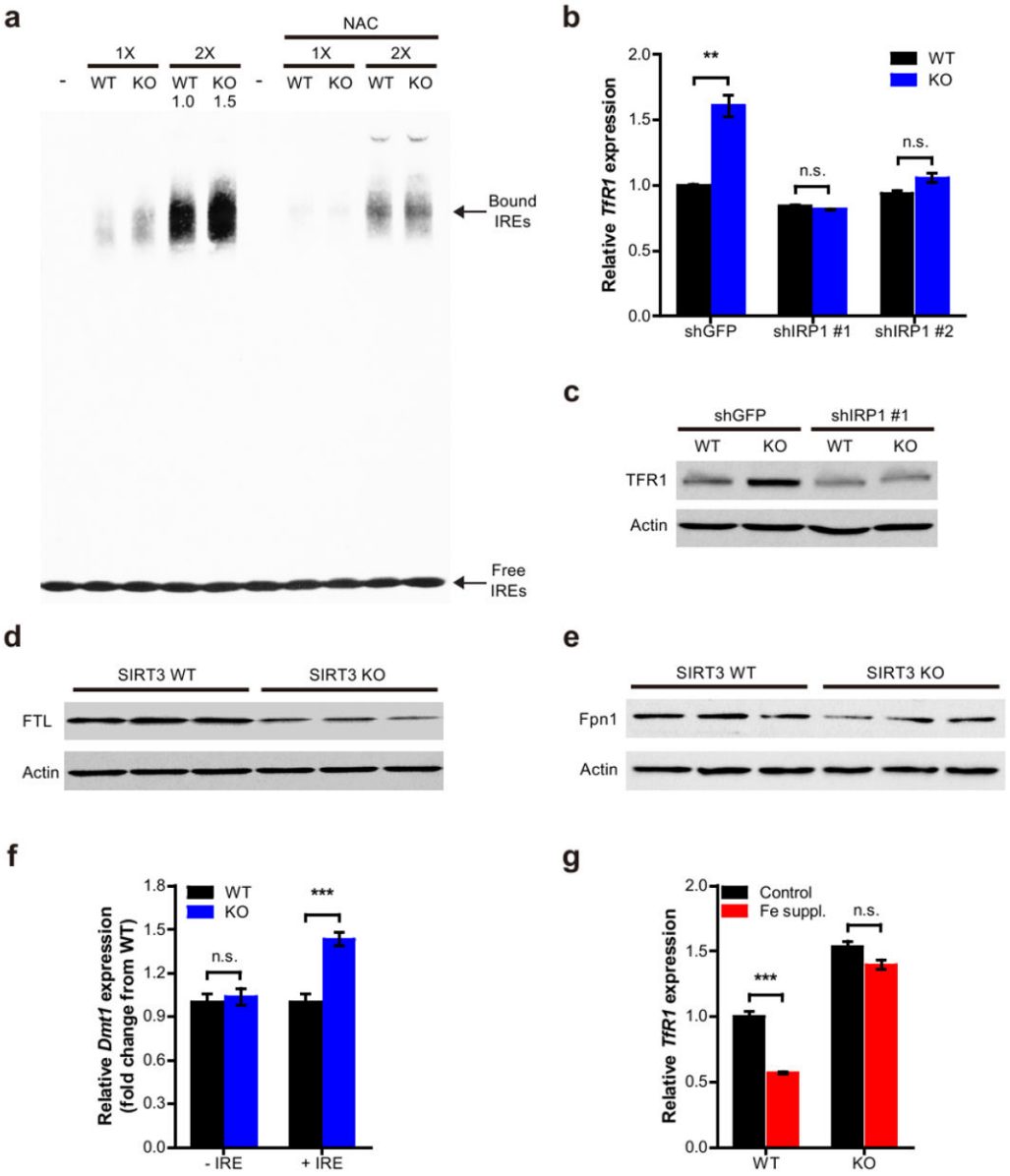

Figure 3.

Loss of SIRT3 increases the IRP1 activity (**a**) Electrophoretic mobility shift assays with cytosolic lysates from SIRT3 WT and KO MEFs with or without 10 mM NAC for 24 hr. Result is a representative of n = 2 experiments. (**b**) Relative *TfR1* mRNA levels in SIRT3 WT and KO MEFs expressing control shRNA (shGFP) or shRNA targeted IRP1 (shIRP1 #1 and shIRP1 #2) (n = 3). (**c**) TfR1 protein levels in whole cell lysates from SIRT3 WT and KO MEFs in SIRT3 WT and KO MEFs expressing control shRNA (shGFP) or shRNA targeted IRP1 (shIRP1 #1). (**d**) L-ferritin protein levels in whole cell lysates from SIRT3 WT and KO MEFs. β-actin serves as a loading control. (**e**) Ferroportin-1 protein levels in whole cell lysates from SIRT3 WT and KO MEFs. β-actin serves as a loading control. (**f**) Relative *DMT1-IRE* and *DMT1+IRE* mRNA levels in SIRT3 WT and KO MEFs (n = 3). (**g**) Relative *TfR1* mRNA levels in SIRT3 WT and KO MEFs incubated with or without iron

supplement (1:1000) for 30 hr (n = 3). Data represent mean ± SEM. n.s., not significant. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

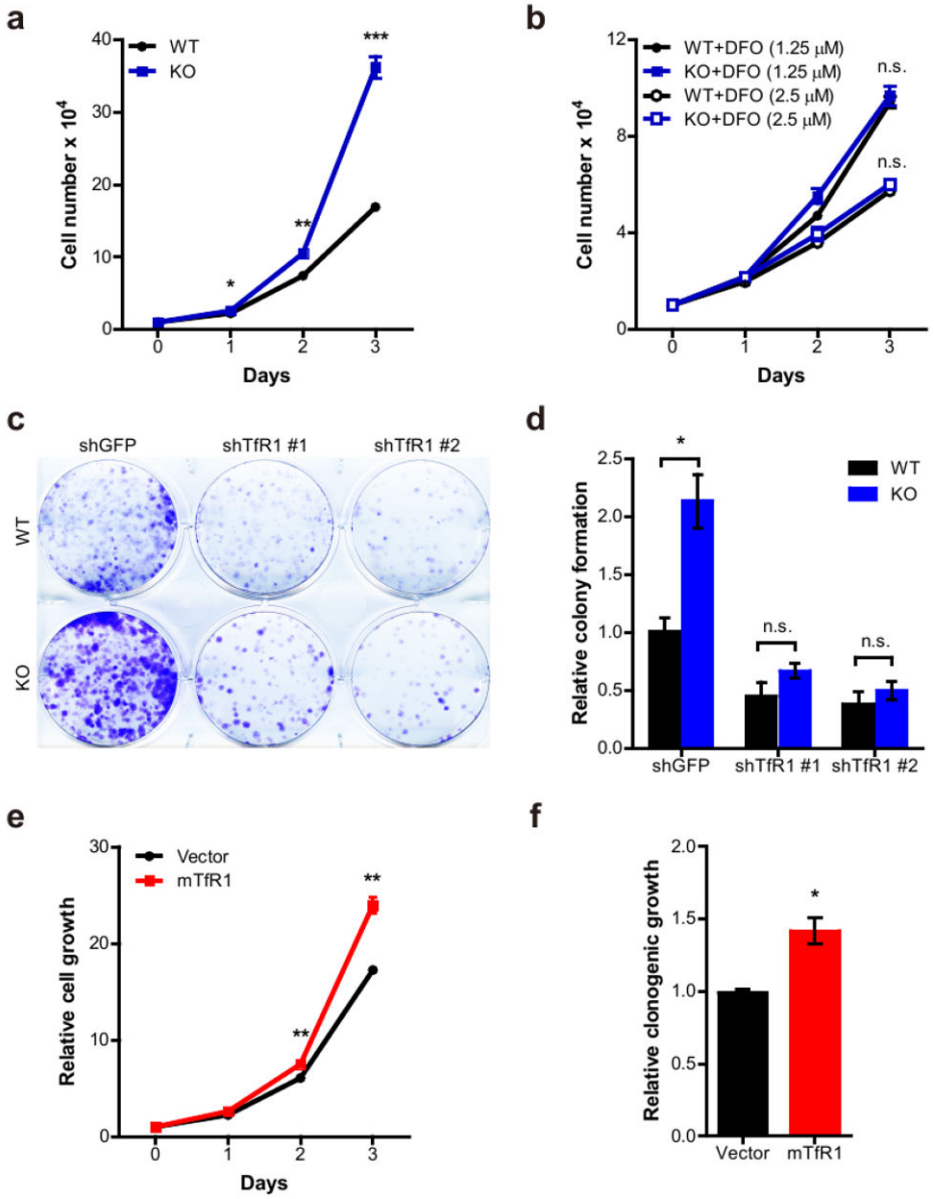

Figure 4.

SIRT3 regulates tumor cell growth through TfR1. (**a, b**) Growth curves of SIRT3 WT and KO MEFs cultured in standard media (a) or media supplemented with 1.25 or 2.5 μM DFO (b) for the indicated number of days and counted (n = 3). (**c**) Clonogenic assays with transformed SIRT3 WT and KO MEFs expressing control shRNA (shGFP) or shRNA targeted TfR1 (shTfR1 #1 and shTfR1 #2). Representative wells of the clonogenic growth experiment. (**d**) Soft agar assays using transformed SIRT3 WT and KO MEFs expressing control shRNA (shGFP) or shRNA targeted TfR1 (shTfR1 #1 and shTfR1 #2) (n = 4). (**e**) Growth curves of WT MEFs expressing vector or mTfR1 for the indicated number of days and counted (n = 3) (Data represent mean ± SD). (**f**) Relative clonogenic growth of WT MEFs expressing vector or mTfR1 (n = 3). Data represent mean ± SEM. n.s., not significant. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

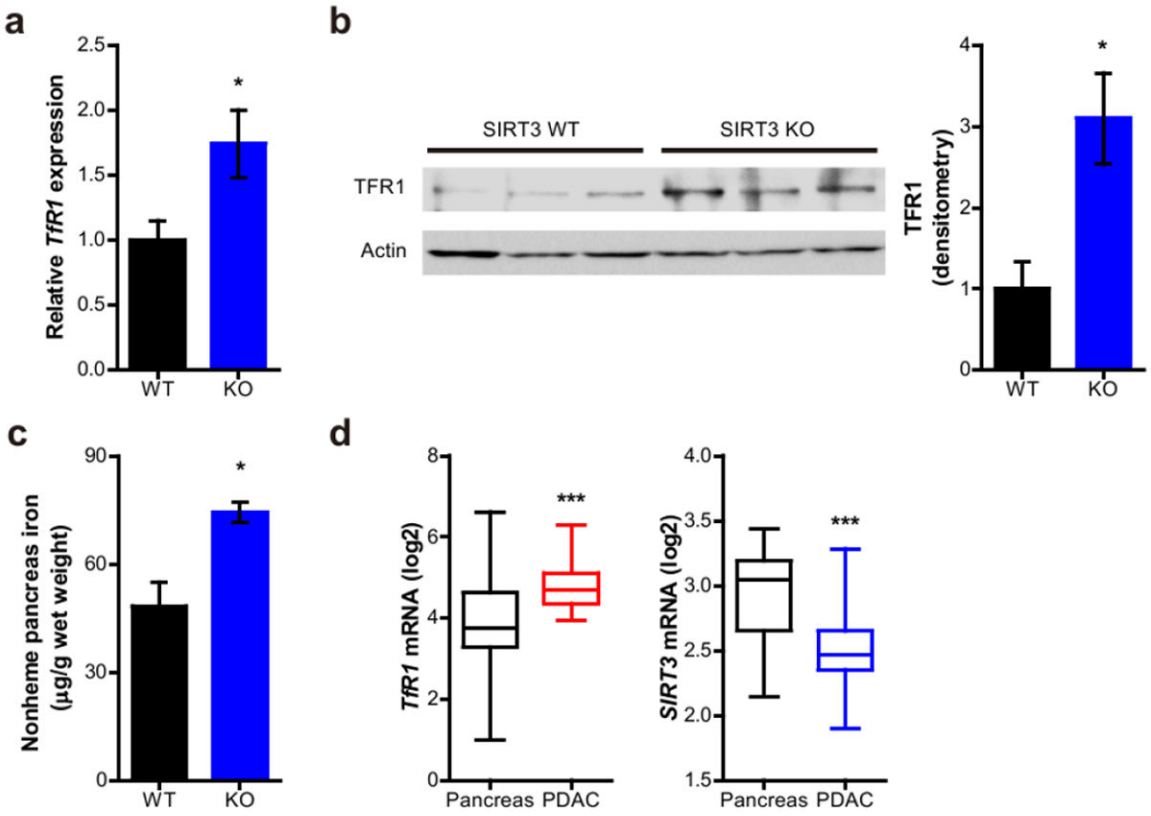

Figure 5.

SIRT3 loss increases TfR1 expression in pancreas. (**a**) Relative *TfR1* mRNA levels in SIRT3 WT and KO pancreas (n = 5). β-actin was used as an endogenous control for qRT-PCR. (**b**) TfR1 protein levels in SIRT3 WT and KO pancreas were detected by immunoblotting with anti-TfR1 antibody. β-actin serves as a loading control. (**c**) Nonheme iron content in the pancreas of SIRT3 WT and KO mice (n = 3). (**d**) *SIRT3* (left) and *TfR1* (right) mRNA levels were determined using the Oncomine microarray database (http://www.oncomine.org) in normal versus pancreatic ductal adenocarcinoma (PDAC). The boxes represent the interquartile range; whiskers represent the 10^{th}-90^{th} percentile range; bar represent the median. Data represent mean ± SEM. n.s., not significant. **P* < 0.05 and ****P* < 0.001.

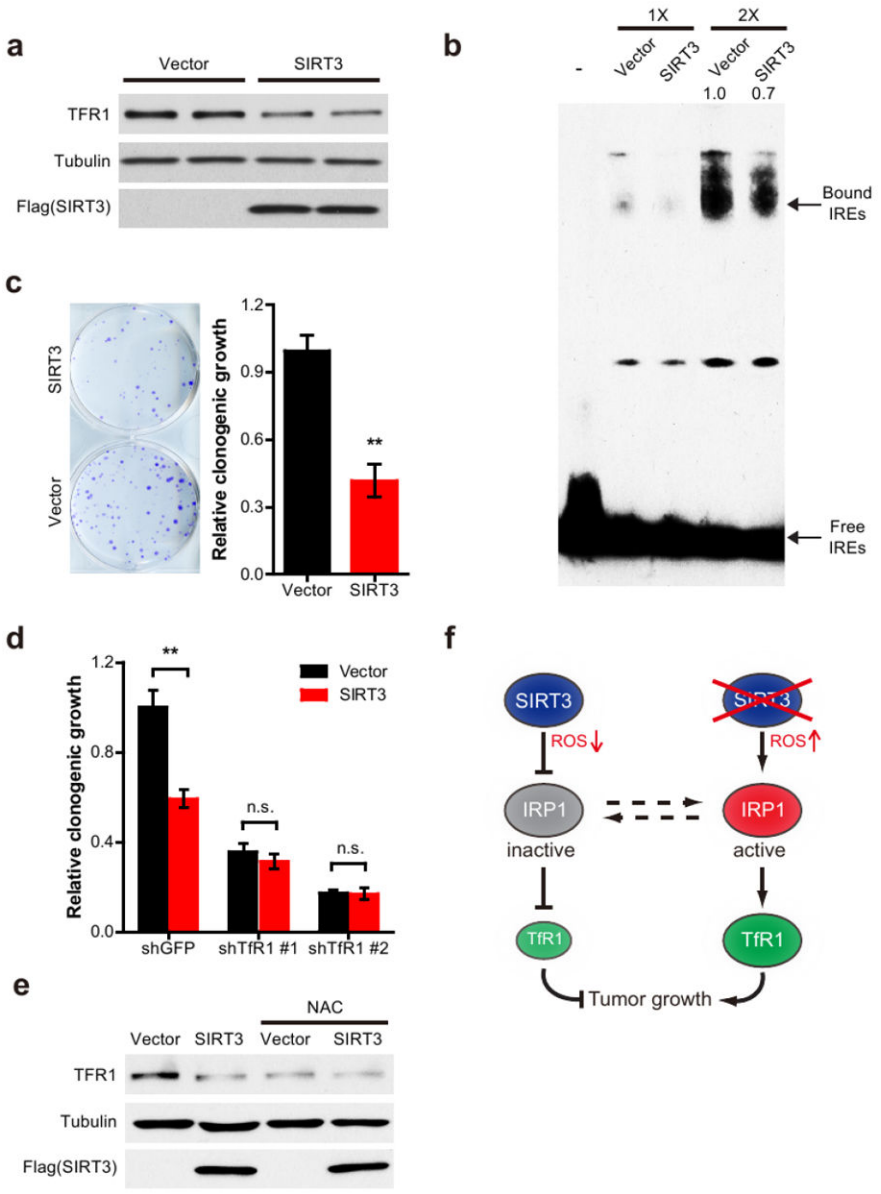

Figure 6.

SIRT3 regulates PDAC growth through TfR1. (**a**) TfR1 protein levels in 8988T cells stably expressing empty vector (Vector) or SIRT3 (SIRT3). α-Tubulin serves as a loading control. (**b**) Electrophoretic mobility shift assays with cytosolic lysates from 8988T cells expressing empty vector or SIRT3. Result is a representative of n = 2 experiments. (**c**) Relative clonogenic growth of 8988T cells expressing empty vector or SIRT3. Representative wells of the clonogenic growth experiment (left). The number of colonies was counted (right) (n = 3). (**d**) Relative clonogenic growth of Vector and SIRT3 8988T cells expressing control shRNA (shGFP) or shRNA targeted TfR1 (shTfR1 #1 and shTfR1 #2) (n = 3). (**e**) TfR1 protein levels in Vector and SIRT3 8988T cells incubated with or without 10 mM NAC for

24 hr. α-Tubulin serves as a loading control. (**f**) Schematic of the regulation of TfR1 and tumor growth by SIRT3. Data represent mean ± SEM. n.s., not significant. ***P* < 0.01.