SIRT3 Is a Mitochondria-Localized Tumor Suppressor Required for Maintenance of Mitochondrial Integrity and Metabolism during Stress

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SUMMARY

The sirtuin gene family (SIRT) is hypothesized to regulate the aging process and play a role in cellular repair. This work demonstrates that SIRT3−/− mouse embryonic fibroblasts (MEFs) exhibit abnormal mitochondrial physiology as well as increases in stress-induced superoxide levels and genomic instability. Expression of a single oncogene (Myc or Ras) in SIRT3−/− MEFs results in in vitro transformation and altered intracellular metabolism. Superoxide dismutase prevents transformation by a single oncogene in SIRT3−/− MEFs and reverses the tumor-permissive phenotype as well as stress-induced genomic instability. In addition, SIRT3−/− mice develop ER/PR-positive mammary tumors. Finally, human breast and other human cancer specimens exhibit reduced SIRT3 levels. These results identify SIRT3 as a genomically expressed, mitochondria-localized tumor suppressor.

INTRODUCTION

An emerging theme in aging research is that sirtuin genes appear to regulate longevity in a wide variety of living systems from yeast to mammals (Sinclair, 2005; Tissenbaum and Guarente, 2001). Sirtuin genes are the human and murine homologs of the Saccharomyces cerevisiae Sir2 gene, which has been shown to regulate both replicative and overall life span (Guarente and Kenyon, 2000). The sirtuin genes are also central to the regulation of longevity in C. elegans and D. melanogaster (Rogina and Helfand, 2004). The mammalian sirtuin family consists of seven NAD+-dependent protein deacetylases that are localized to the nucleus (SIRT1, SIRT6, and SIRT7), mitochondria (SIRT3, SIRT4, and SIRT5), and cytoplasm (SIRT2), respectively, and that regulate a wide range of intracellular process (Haigis and Guarente, 2006).

The incidence of human malignancies increases exponentially as a function of aging, suggesting a mechanistic connection between aging (longevity) and carcinogenesis (Finkel et al., 2009). Mammalian cells contain fidelity proteins or tumor

SIGNIFICANCE

The incidence of human malignancies increases significantly with age, suggesting a mechanistic connection between aging (longevity) and carcinogenesis. One aspect of that connection is impaired mitochondrial function, which is observed in both aging cells and cancer cells as aberrant oxidative metabolism. Sirtuin family genes regulate longevity in yeast, C. elegans, and D. melanogaster, and in mammals, three of the seven sirtuin genes are localized to the mitochondria, including SIRT3. These observations led us to hypothesize that SIRT3 might be a tumor suppressor that protects against carcinogenesis by maintaining mitochondrial integrity and efficient oxidative metabolism. The current work demonstrates that the loss of function of SIRT3 results in a cellular environment permissive for carcinogenesis and characterized by aberrant oxidative metabolism.
suppressor (TS) genes, such as p53, and loss of function of these proteins results in a damage-permissive cell phenotype (Sherr, 2004). As such, the loss of function of these fidelity proteins is considered an early event in carcinogenesis. Because cancer is a disease of aging, and sirtuin genes appear to play a role in repair of cellular damage during aging, it is reasonable to propose that sirtuin genes may also have an anticarcinogenic role and function as TSs (Saunders and Verdin, 2007; Wang et al., 2002; Schwer et al., 2002) and is the primary mitochondrial protein deacetylase (Lombard et al., 2007). In this regard, it is proposed that SIRT3 is ideally situated to function as a mitochondrial fidelity protein, and by extension, loss of function could result in a damage-permissive and tumorigenic cellular environment.

**RESULTS**

**SIRT3 Knockout MEFs Exhibit Increased Superoxide Levels and Chromosomal Instability in Response to Exogenous Stress**

We have previously shown that HCT116 cells genetically altered to express a deacetylation-null mutant SIRT3 gene (SIRT3dn) have difficulty responding to increased reactive oxygen species (Jacobs et al., 2008). In addition, it has previously been shown that SIRT3−/− livers and MEFs have decreased total ATP levels and mitochondrial respiration (Ahn et al., 2008). As such, steady-state levels of superoxide were determined in SIRT3+/+ and SIRT3−/− MEFs at passage 2, 6, and 10, and mtDNA primers that amplify either the 10 kb amplicon used for PCR. All the results in this figure are from at least three separate experiments. Data are presented as the average ± SD; *p < 0.05 by t test. See also Figure S1.

Figure 1. SIRT3 Knockout MEFs Exhibit Increased Superoxide Levels, Aneuploidy in Response to Exogenous Stress, and Decreased Mitochondrial Integrity with Increasing Age

(A) Superoxide levels were elevated in SIRT3 knockout cells exposed to agents that induce mitochondrial damage. SIRT3+/+ and SIRT3−/− MEFs were cultured in 6% oxygen and exposed to either 5 Gy of IR or 5 μM antimycin A for 3 hr, and superoxide levels were monitored by DHE oxidation as compared to control, untreated cells (Cont). For all DHE oxidation experiments, the results were the normalized mean fluorescence intensity (MFI) for three independent replicates.

(B) SIRT3−/− superoxide levels were elevated when cultured in 21% oxygen. SIRT3+/+ and SIRT3−/− MEF cells were cultured at 21% O2 for 6 hr, and superoxide levels were monitored by DHE oxidation, as compared with control cells grown at 6% O2.

(C) Mitochondrial superoxide levels were elevated in SIRT3 knockout MEFs and increase following exogenous stress. Mitochondrial superoxide levels were determined by the addition of Mitosox (3 μM) to the culture medium and cells were incubated for an additional 10 min before trypsinization and resuspension. Fluorescence was measured via flow cytometry, and 20,000 and 40,000 cells were counted for each sample.

(D) SIRT3 knockout MEFs exhibited aneuploidy following exposure to IR. SIRT3+/+ and SIRT3−/− MEFs were exposed to either 2 or 5 Gy. Whole-mount chromosomes were counted in a blinded fashion. Bars show the mean chromosome number per cell from 100 separate counts.
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in MEFs from a transgenic mouse expressing SIRT3\textsuperscript{dn}, a roughly 2-fold increase in superoxide levels was observed (see Figure S1A available online).

In contrast, a difference was observed in cells treated with two stress-inducing exogenous agents. Ionizing radiation (IR) and antimycin A, a mitochondrial electron transport chain (Complex III) inhibitor, represent genotoxic and metabolic stresses, respectively, that have been hypothesized to cause an increase in mitochondrial superoxide levels (Aykin-Burns et al., 2009). Exposure of SIRT3\textsuperscript{−/−} MEFs to either IR or antimycin A significantly increased intracellular superoxide levels, while only a comparatively modest increase was observed in SIRT3\textsuperscript{+/+} cells (Figure 1A). In addition, SIRT3\textsuperscript{−/−} MEFs exhibited significantly higher intracellular superoxide levels when cultured at 21% O\textsubscript{2} for 6 hr (Figure 1B), compared with either SIRT3\textsuperscript{+/+} or SIRT3\textsuperscript{−/−} MEFs that are routinely grown at 6% O\textsubscript{2} or SIRT3\textsuperscript{+/+} MEFs cultured at 21% O\textsubscript{2}.

Mitochondrial superoxide levels (measured using Mito-SOX oxidation) were elevated in the SIRT3\textsuperscript{−/−} MEFs and significantly increased following exposure to either IR or antimycin A (Figure 1C). In contrast, a much smaller increase in mitochondrial superoxide levels was observed in irradiated or antimycin-A-treated SIRT3\textsuperscript{+/+} MEFs. An increase in mitochondrial superoxide level was also observed in the SIRT3\textsuperscript{−/−} MEFs at 21% O\textsubscript{2} (Figure S1B). These results suggest that loss of SIRT3 may allow exogenous stressing agents to more readily disrupt oxidative metabolism, leading to increased steady-state levels of superoxide.

Cellular exposure to exogenous genotoxic stress, such as IR, has previously been shown to induce chromosomal aberrations, and one mechanism accounting for this observation has been hypothesized to involve increased intracellular superoxide levels (Spitz et al., 2004). Chromosome analysis of at least 100 metaphases from SIRT3\textsuperscript{−/−} or SIRT3\textsuperscript{+/+} MEFs showed a chromosome complement of 40 ± 2 in both knockout and wild-type cells. In contrast, a relatively modest dose of radiation (2 and 5 Gy) caused a significant increase in chromosome number in the SIRT3\textsuperscript{−/−} MEFs after 72 hr (Figure 1D), suggesting that loss of SIRT3 results in chromosomal instability induced by genotoxic stress.

SIRT3 Knockout Livers and MEFs Have Decreased Mitochondrial Integrity with Age

It has previously been suggested that nuclear sirtuins may function as fidelity proteins that play a role in the maintenance of genomic integrity (Wang et al., 2008). Because SIRT3 is localized to the mitochondria (Oyano et al., 2002; Schwer et al., 2002), it seemed logical to investigate if it might also play a role in the maintenance of mitochondrial DNA (mtDNA) integrity. Livers from SIRT3 knockout mice at 20, 36, and 58 weeks showed a gradual decrease in mtDNA integrity, as measured by the amplification efficiency for a large (10,095 bp) fragment of mtDNA, compared with isogenic wild-type mice (Figure 1E). SIRT3 knockout MEFs also showed a decrease in mtDNA integrity that was first observed at passage number 6 (Figure 1F) and was further decreased at passage number 10, as compared with SIRT3\textsuperscript{+/+} MEFs. The amplification efficiency of a small control 117 bp fragment was unchanged in both the livers (Figure S1C) and MEFs (Figure S1D).

SIRT3 Knockout MEFs Do Not Spontaneously Immortalize

It is well established that mitochondrial abnormalities, including those associated with altered mitochondrial metabolism, are observed in tumor cells in vitro and in human malignancies (Singh, 2006; Warburg, 1956). We therefore determined if SIRT3 knockout MEFs would exhibit altered growth characteristics as compared with SIRT3\textsuperscript{+/+} MEFs. SIRT3\textsuperscript{−/−} and SIRT3\textsuperscript{+/+} MEFs at passage 3 were cultured identically and at passage 8 exhibited increased doubling times, and neither was able to divide beyond cell passage 15 (data not shown). These primary cells have identical doubling times (data not shown). Thus, SIRT3\textsuperscript{+/+} and SIRT3\textsuperscript{−/−} MEFs do not have the ability to divide beyond passage 15, and as such cannot spontaneously immortalize (see Experimental Procedures for further description).

These MEFs at passage three were also measured for loss of contact inhibited cell growth via colony-formation assays. This assay measures the ability of tissue culture cells plated to clonally to spontaneously form colonies, which are defined as concentrated nests of cells that pile up and grow on top of each other. MEFs were plated at 10\textsuperscript{4} in a 100 mm dish and the medium was changed every 2 days until 28 days, when cells were stained with crystal violet. SIRT3\textsuperscript{−/−} MEFs stained after 28 days formed a greater number of colonies (Figures 2A and 2B, bars 1 and 2), relative to SIRT3\textsuperscript{+/+} MEFs. In addition, SIRT3\textsuperscript{−/−} MEFs exhibited decreased stress-induced apoptosis in response to either IR (2 and 5 Gy) or camptothecin (Figure S2A) and these results are in agreement with previously published results (Allison and Milner, 2007) suggesting SIRT3 is a general proapoptotic factor. These results suggest that SIRT3\textsuperscript{−/−} MEFs have decreased stress-induced apoptosis as well as relatively low frequency of contact inhibition; however, these cells cannot grow beyond passage 15, and therefore did not spontaneously immortalize.

SIRT3\textsuperscript{−/−} MEFs Expressing a Single Oncogene Display a Transformation-Permissive Phenotype

It was shown over 20 years ago that primary immortalized cells can be transformed in vitro by the cooperation of at least two oncogenes, such as Ras and Myc (Land et al., 1986; Parada et al., 1984), validating the Knudson two-hit model for carcinogenesis (Knudson, 1971). This idea has been extended to show that inactivation or deletion of a TS gene can complement the activation of a single oncogene, resulting in cellular transformation (Sherr, 2004).

To determine if loss of SIRT3 results in an in vitro transformation-permissive phenotype, SIRT3\textsuperscript{+/+} and SIRT3\textsuperscript{−/−} MEFs were infected with lentivirus expressing either Myc or Ras. It has been previously shown that overexpression of Myc in primary cells results in massive programmed cell death, whereas overexpression of Ras induces premature senescence (Sebastian et al., 2005; Serrano et al., 1997). Consistent with previous findings, SIRT3\textsuperscript{+/+} MEFs exhibited in vivo immortalization (see Experimental Procedures for description) after infection with both Myc and Ras, but not with either Myc or Ras alone (Table 1). In contrast, SIRT3\textsuperscript{−/−} MEFs infected with either Myc or Ras alone became immortalized, as well as cells infected with both genes (Table 1). In addition, MEFs from a transgenic mouse expressing SIRT3\textsuperscript{dn} (amino acid 248 changed from histidine to tyrosine) and...
lacking SIRT3 (SIRT3\textsuperscript{+/−}−SIRT3\textsuperscript{−/−}) were also immortalized by a single oncogene. In contrast, SIRT3\textsuperscript{+/−}−SIRT3\textsuperscript{−/−} MEFs required both Myc and Ras (Table 1). Polymerase chain reaction (PCR) and western analysis confirmed viral integration and expression of Myc and Ras (data not shown). These results suggest that SIRT3 may act as a TS by substituting for one of the two oncogenes required for in vitro immortalization (see Experimental Procedures for description).

SIRT3\textsuperscript{+/−}, SIRT3\textsuperscript{+/−} Myc/Ras, SIRT3\textsuperscript{−/−}, SIRT3\textsuperscript{−/−} Myc, SIRT3\textsuperscript{−/−} Ras, and SIRT3\textsuperscript{−/−} Myc/Ras (referred to hereafter as “the panel”) MEFs were plated at 1 × 10^5/100 mm dish for a total of 28 days and contact inhibited cell growth was determined. SIRT3\textsuperscript{−/−} Ras and SIRT3\textsuperscript{−/−} Myc/Ras cells displayed a sizeable increase in focal colony formation (Figure 2B, bars 4 and 6), while SIRT3\textsuperscript{−/−} Myc and SIRT3\textsuperscript{+/+} Myc/Ras (bars 3 and 5) showed a slight increase, as compared with SIRT3\textsuperscript{+/−} and SIRT3\textsuperscript{−/−} MEFs (bars 1 and 2). These results reveal that cells lacking SIRT3 display a significant loss of contact inhibition in response to oncogene expression.

Another cell biological criterion of in vitro transformation is the ability of cells to form colonies when plated at very low cell densities, which is a measure of increased mitotic activity or reproductive integrity. As such, the panel of MEFs was plated at either 100 or 250 cells per well in 60 mm six-well culture plates and stained with crystal violet after 10 days (Figure 2C). The results of these experiments show that cells lacking SIRT3 and expressing Myc, Ras, or Myc/Ras form more colonies than SIRT3 wild-type cells expressing both Myc and Ras. The transformed SIRT3\textsuperscript{−/−} Myc, SIRT3\textsuperscript{−/−} Ras, and SIRT3\textsuperscript{−/−} Myc/Ras cells also exhibit less basal apoptosis than the SIRT3\textsuperscript{+/−} Myc/Ras cells (Figure S2B). Finally, SIRT3\textsuperscript{−/−} Myc/Ras, SIRT3\textsuperscript{−/−} Ras, and SIRT3\textsuperscript{−/−} Myc cells exhibited a more transformed morphology as shown by random cell orientation, changes in cellular architecture, and nuclear to cytoplasmic ratios (data not shown).

### Loss of SIRT3 Results in an Invasive and Tumorigenic Phenotype

The frequency of aneuploidy and/or polyploidy has been suggested as one of many biomarkers that may be proportional to the degree of malignancy of a tumor (Deng, 2006). Fluorescence-activated cell sorting (FACS) analysis demonstrated that SIRT3\textsuperscript{−/−} Myc, SIRT3\textsuperscript{−/−} Ras, and SIRT3\textsuperscript{−/−} Myc/Ras cells have significantly more polyploid cells (Figure 3A) and chromosomal analysis showed more aneuploidy (Figure 3B) than measured in the SIRT3\textsuperscript{+/−}, SIRT3\textsuperscript{+/+} Myc/Ras, or SIRT3\textsuperscript{−/−} cell lines. The results of these cell biological experiments suggest that cells lacking SIRT3 and overexpressing at least one exogenous oncogene exhibit a more in vitro transformed phenotype than wild-type SIRT3 cells expressing two oncogenes (Myc and Ras).

### Table 1. Immortalization of SIRT3\textsuperscript{−/−} MEFs Only Requires a Single Oncogene

<table>
<thead>
<tr>
<th>MEFs</th>
<th>Control</th>
<th>Myc</th>
<th>Ras</th>
<th>Myc/Ras</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIRT3\textsuperscript{+/−}</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>immort</td>
</tr>
<tr>
<td>SIRT3\textsuperscript{−/−}</td>
<td>none</td>
<td>immort</td>
<td>immort</td>
<td></td>
</tr>
<tr>
<td>SIRT3\textsuperscript{−/−} + Lenti-MnSOD</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>immort</td>
</tr>
<tr>
<td>SIRT3\textsuperscript{−/−},SIRT3\textsuperscript{+/−}</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>immort</td>
</tr>
<tr>
<td>SIRT3\textsuperscript{−/−},SIRT3\textsuperscript{−/−}</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>immort</td>
</tr>
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</table>

None, no MEF immortalization; immort, immortalization. Lenti-MnSOD, lentiviral-MnSOD 10 MOI. Immortalization experiments were done in triplicate.
Cell growth in soft agar and athymic nude mice are well-established systems in which to assess anchorage independent growth and tumorigenesis, respectively. It has been demonstrated that MEFs immortalized with two oncogenes, such as Myc and Ras, have altered growth properties but do not grow in soft agar or form allograft tumors in nude mice unless an additional genetic event occurs (Land et al., 1986). Anchorage-independent growth was determined by examining cell growth in soft agar, and these experiments showed that SIRT3 \(^{-/-}\) Ras, SIRT3 \(^{-/-}\) Myc/Ras, and, to a smaller degree, SIRT3 \(^{-/-}\) Myc cells have an anchorage-independent phenotype (Figure 3C). To determine in vivo tumorigenesis, 10\(^6\) cells were implanted into the hind limbs of nude mice. After 3 weeks, the SIRT3 \(^{-/-}\) Myc/Ras cell lines were able to grow (in 6/6 mice) allograft tumors (Figure 3D) consistent with a poorly differentiated sarcoma (Figure 3E), while no tumors were observed for either the SIRT3 \(^{+/+}\) Myc/Ras or the other cell lines (0/6 mice). These results suggest that loss of SIRT3, when combined with Myc and Ras, provides a necessary genetic event resulting in tumorigenesis.

**SIRT3 Knockout Transformed MEFs Display Altered Intracellular Metabolism**

We have shown that SIRT3 knockout MEFs demonstrate normal steady-state levels of superoxide under unstressed conditions, but exhibit a stress-induced increase in superoxide levels. As such, superoxide levels were measured in transformed SIRT3 knockout cells to determine whether the genetic loss of SIRT3 combined with oncogene transformation created an increased pro-oxidant intracellular environment. SIRT3 \(^{-/-}\) Ras, SIRT3 \(^{-/-}\) Myc/Ras, and, to a lesser extent, SIRT3 \(^{-/-}\) Myc cells exhibited higher steady-state levels of total cellular superoxide (Figure 4A) as well as mitochondrial superoxide levels (Figure S3A). The SIRT3 \(^{-/-}\) Ras and SIRT3 \(^{-/-}\) Myc/Ras cells exhibit increased total cellular ATP levels (Figure 4B) when compared with the SIRT3 \(^{+/+}\) Myc/Ras or untransformed SIRT3 \(^{+/+}\) MEFs (data not shown). Surprisingly, the SIRT3 \(^{-/-}\) Ras and SIRT3 \(^{-/-}\) Myc/Ras cells had significantly decreased mitochondrial ATP levels (Figure 4C, Figure S3B). These results suggest that the transformed SIRT3 knockout cells are more metabolically active but are generating this energy from sources other than mitochondrial oxidative phosphorylation, and are either producing more reactive oxygen species or have a decreased ability to scavenge superoxide.

**SIRT3 Knockout Transformed MEFs Display Increased Glycolysis and Decreased Oxidative Phosphorylation**

It is well established that tumor cells consume glucose at a much greater rate than nontransformed cells, and this is referred to as the Warburg effect (Warburg, 1956). As such, glucose metabolism was monitored in the panel of MEF cell lines. Figure 4D shows that SIRT3 knockout cells infected with Myc, Ras, or Myc and Ras consume increased amounts of glucose.

One potential mechanism accounting for the observed increases in intracellular superoxide and glycolysis in the SIRT3 \(^{-/-}\) Ras and SIRT3 \(^{-/-}\) Myc/Ras cells might involve changes in the level of oxidative phosphorylation. As such, the activities of electron transport complexes I, II, III, and IV were determined in the SIRT3 wild-type and knockout transformed cell lines. These experiments showed a significant decrease in complex I and complex III activity in the SIRT3 \(^{-/-}\) Ras and...
SIRT3−/− Myc/Ras cells, as compared with the SIRT3+/− Myc/Ras cells (Figure 4E). These results confirm that loss of SIRT3 alters complex I activity (Ahn et al., 2008) (Figure S3C) and suggest a role for SIRT3 in the regulation of complex III. This also suggests that the SIRT3−/− Myc/Ras and SIRT3−/− cells have a decreased ability to generate sufficient ATP by oxidative phosphorylation to keep up with the increased demands of proliferation. This is consistent with the decrease in mtATP levels observed in the SIRT3−/− Ras and SIRT3−/− Myc/Ras cells (Figure 4C). Therefore, these cells may enhance metabolism of glucose in glycolysis to increase the production of ATP. Alternatively, glucose metabolism could also be increased to generate reducing equivalents to detoxify hydroperoxides formed from higher steady-state levels of superoxide and hydrogen peroxide (Aykin-Burns et al., 2009). In addition, the decreased activities of complexes I and III could increase the residence times of electrons at sites where univalent reduction of O2 to form superoxide could occur (Spitz et al., 2004).

SOD Decreases SIRT3−/− Ras- and Myc/Ras-Induced Growth Properties and Prevents Transformation of SIRT3−/− MEFs by a Single Oncogene

SIRT3−/− Ras and SIRT3−/− Myc/Ras cells formed larger colonies when plated at low densities (Figure 2C), suggesting that these cells have increased growth rates. These results were confirmed by cell growth assays demonstrating that SIRT3 knockout cells expressing Myc and/or Ras proliferate faster than wild-type cells. These cells fell into two distinct groups: SIRT3−/− Ras and SIRT3−/− Myc/Ras cells had shorter doubling times than SIRT3−/− Myc and SIRT3+/− Myc/Ras cells (Figure 5A). To investigate the idea that increased reactive oxygen species, and specifically superoxide, may be proproliferative in SIRT3 knockout transformed cells, an adenoviral vector that causes enforced overexpression of manganese superoxide dismutase was utilized (AdMnSOD) (Aykin-Burns et al., 2009). AdMnSOD, which decreases steady-state levels of superoxide (Figure S4A), was used to overexpress MnSOD in transformed SIRT3 knockout cells. AdMnSOD increased the cell doubling time of the SIRT3−/− Ras and SIRT3−/− Myc/Ras but not the SIRT3+/− Myc/Ras cells (Figure 5B), suggesting that excess superoxide favors increased cell proliferation.

To determine if increased mitochondrial superoxide was required for the immortalization of SIRT3−/− MEFs, Myc and Ras infections were repeated in the presence a lentivirus expressing MnSOD (lenti-MnSOD, a kind gift from Dr. Rezvani, Columbia University). Infection of lenti-MnSOD 24 hr prior to exposure to IR prevented the increase in IR-induced mitochondrial superoxide levels (Figure S4B) that was observed in the SIRT3−/− MEFs (Figure 1C). Although immortalization of SIRT3−/− cells infected with both Myc and Ras was not affected, coinfection with lenti-MnSOD prevented immortalization of SIRT3−/− cells infected with only one oncogene (Table 1). Expression of the lentiviral exogenous MnSOD was confirmed (data not shown). The results of these experiments suggest that elevated superoxide levels in SIRT3 knockout cells play a central role in immortalization.

We have shown earlier that SIRT3−/− cells have increased stress-induced superoxide levels (Figures 1A and 1B), including when these MEFs routinely cultured at 6% O2 were subsequently grown at 21% O2 (Figure 1B), suggesting that superoxide may be at least one mechanism promoting cellular transformation (Table 1). Thus, it seemed reasonable to determine whether long-term growth at 21% O2 would increase the loss of contact inhibition phenotype as determined by colony-formation assays.
protein levels in SIRT3−/− Myc/Ras-transformed MEFs. SIRT3−/− Myc/Ras cells were infected with virus, and 48 hr later cells were harvested and extracts were isolated and 20 μg protein were separated by SDS-PAGE, transferred onto nitrocellulose, and immunoblotted using an anti-MnSOD antibody (Cell Signaling Technology, Inc.).

(F) Infection of lent-SIRT3-wt but not lent-SIRT3-dn in SIRT3−/− Myc/Ras transformed MEFs reverses the increase in mitochondrial superoxide levels. Mitochondrial superoxide levels were determined by the addition of Mito-SOX (3 μM) to the culture medium and cells were incubated for an additional 10 min before being trypsinized and resuspended. Fluorescence was measured via flow cytometry, and 20,000 and 40,000 cells were counted for each sample. Results are from at least three separate experiments. Data are presented as the average ± SD; *p < 0.05 and **p < 0.01 by t test. See also Figure S4.

(Figures 2A and 2B). As such, SIRT3+/+ and SIRT3−/− MEFs were cultured at 21% O2 for 28 days to determine if these conditions would increase cell contact inhibition. A roughly 5-fold increase in loss of contact inhibition, as determined by colony formation, was observed in the SIRT3−/− MEFs grown at 21% O2 (Figure 5C, bar 2 versus 4), whereas no difference was observed in the SIRT3+/+ MEFs (bar 1 versus 3). Similar to the results observed in Table 1, the addition of lent-MnSOD reversed the increase in colonies formed when SIRT3−/− cells were grown at 21% O2 for 28 days (Figure 5D, bar 2 versus 4) as well as the overall density of the colonies (data not shown). Finally, we previously showed that SIRT3−/− MEFs exposed to IR displayed an increase in total cellular superoxide levels (Figure 1A) and genomic instability (Figure 1D). When these experiments were repeated in the presence of MnSOD (Figure 5E), the IR-induced increase in aneuploidy was prevented.

These results suggest that the increase in superoxide observed in the SIRT3−/− MEFs plays a role, at least in part, in the tumor-permissive phenotype. To address this idea, MnSOD expression was determined in wild-type and SIRT3 knockout
mouse livers at 5, 9, and 13 months. A slight decrease in MnSOD expression was observed at 9 months that became statistically significant at 13 months (Figure 5F, Figure S4C). In contrast, no significant change in MnSOD expression was observed in the wild-type mice. A decrease in MnSOD expression was also observed in the transformed SIRT3+/−/Myc/Ras cells (Figure S4D), which have previously been shown to have increased superoxide levels compared to the SIRT3+/−/Myc/Ras cells (Figure 4A). Finally, similar to the SIRT3+/−/MEFs, MnSOD+/−/MEFs (a kind gift from Prabhat Goswami, University of Iowa) are also immortalized by a single oncogene (Table S1), suggesting that loss of MnSOD may also result in an immortalization-permissive phenotype.

Chromatin immunoprecipitation analysis of SIRT3+/+ and SIRT3−/− mice showed decreased binding of two primary transcription factors that regulate MnSOD, FOXO3a and NF-κB (Figure S4E), to the MnSOD promoter at 13 months but not 5 months (data not shown). No change in total (Figure S4F) or mitochondrial (data not shown) FOXO3a or NF-κB was observed in SIRT3+/+ or SIRT3−/− age-matched mice. However, SIRT3 deacetylates FOXO3a (Figure S4G) and there is a significant increase in phospho-FOXO3a levels in cells expressing SIRT3+/+, as compared with cells expressing the wild-type SIRT3 (Figure S4H, lower panel). These results are consistent with recently published data (Sundaresan et al., 2009). Finally, cells expressing SIRT3+/+ contain decreased nuclear FOXO3a protein levels (Figure S4I), as shown by others (Sundaresan et al., 2009). These results suggest that loss of SIRT3 deacetylation activity decreases FOXO3a nuclear localization.

Finally, transfection with a vector expressing a constitutively active FOXO3a dominant positive gene (pCMV-N-FOXO3a), which increases nuclear FOXO3a protein levels (Jacobs et al., 2008), prevented immortalization of SIRT3−/− MEFs by a single (Myc or Ras) oncogene (Table S1). These results suggest that nuclear import of FOXO3a may play a role, at least in part, in an immortalization-permissive phenotype.

**SIRT3 Wild-Type, But Not a Deacetylation-Null Mutant Gene, Induces MnSOD Gene Expression and Decreases Mitochondrial Superoxide Levels**

MnSOD protein levels are decreased in the SIRT3−/− Myc/Ras (Figure 5G, bar 1), as compared with the SIRT3+/+ Myc/Ras (bar 2) cells. As such, SIRT3−/− Myc/Ras cells were infected with lentivirus expressing either a wild-type SIRT3 (lenti-SIRT3-wt) or a deacetylation null mutant SIRT3 (Ahn et al., 2008) gene (lenti-SIRT3-dn). These experiments showed that the wild-type (Figure 5G, bar 1 versus 3), but not the deacetylation null mutant SIRT3 (bar 4) gene, increased MnSOD protein levels to those observed in the wild-type SIRT3+/+ Myc/Ras MEFs (bar 2). Exogenous Myc tagged SIRT3 expression was confirmed by western blotting (data not shown). In addition, lenti-SIRT3-wt, but not lenti-SIRT3-dn, reversed the increase in mitochondrial superoxide levels observed in the SIRT3−/− Myc/Ras cells (Figure 5H). Finally, SIRT3+/+ Myc/Ras MEFs cells infected with retroviruses expressing two different SIRT3 shRNAs also decreased MnSOD expression (Figure S4J). These results suggest a more direct link between SIRT3 deacetylation and MnSOD expression as well as altered mitochondrial metabolism.

**SIRT3 Is a Mitochondria-Localized Murine Tumor Suppressor**

SIRT3 knockout MEFs are immortalized and transformed in vitro by the expression of a single oncogene, suggesting that loss of SIRT3 results in a tumor-permissive phenotype. Thus, we investigated whether SIRT3 knockout mice developed tumors. SIRT3+/− mice were healthy, and no outwardly observed phenotype was noted (Lombard et al., 2007); however, 7 of 20 female mice developed mammary gland tumors (Figure 6A) over 24 months (Figure 6B), while 0 SIRT3+/− mice developed mammary tumors during the same period. Histological H&E examination of these mammary tumors showed a characteristic invasive ductal carcinoma (Figure 6C). In addition, single, but not double, positive immunohistochemistry (IHC) staining, was observed for cytokeratin 14 (CK14), a basal epithelial cell marker, or CK18, a luminal epithelial cell marker (Figure 6D), suggesting a well differentiated histological pathology. IHC identified these tumors as estrogen receptor and progesterone receptor (ER/PR) positive (Figure 6E). These results parallel a well-differentiated, receptor-positive histological characteristic that is commonly observed in breast malignancies in older women.

SIRT3−/− mouse livers exhibit increased mtDNA damage and decreased MnSOD expression with age and develop mammary tumors after 12 months, suggesting that cellular reactive oxygen species might increase with age in the SIRT3−/− mice. Mammary tissue isolated from SIRT3+/+ and SIRT3−/− mice were stained with an anti-nitrotyrosine antibody as a marker for increased protein damage caused by intracellular reactive oxygen/nitrogen species, because increased nitrotyrosine formation on proteins is believed to reflect increased formation of ONOO−, which is the reaction product of nitric oxide and superoxide. SIRT3 knockout mouse mammary ductal cells exhibited increased anti-nitrotyrosine staining (StressMarq Biosciences Inc.) at 12 months (Figure 6F, Figure S5), while no differences were detected at 5 months (data not shown). This suggests that increased oxidative/nitrosative damage to proteins is occurring in the mammary tissues of SIRT3−/− animals as a function of age.

**SIRT3 Is a Potential Human Tumor Suppressor**

The hypothesis that SIRT3 serves as a tumor suppressor in vivo was further supported by the observation that SIRT3 expression is decreased in breast cancer specimens from a commercially available tissue array (US Biomax, Inc.), as compared with normal breast tissue samples (Figure 7A). In addition, zero of nine metastatic lymph nodes positively stained for SIRT3 (data not shown). IHC staining also confirmed that SIRT3 localizes to normal mammary ductal cells (Figure 7B). SIRT3 RNA expression is also decreased in stage IIA, IIB, and III malignancy breast samples (TissueScan Breast Cancer Panel 1, Origene) (Figure 7C).

The Oncomine cancer microarray database (Rhodes et al., 2007) was subsequently used to determine if SIRT3 expression is decreased in human malignancies. SIRT3 was decreased in breast tumors as compared with normal breast (Figure 7D) and as a function of both Elston Grade (G-1, G-2, or G-3) (Figure 7E, Figure S6A) and pathological differentiation (well, moderately, or poorly differentiated) (Figure 7F, Figure S6B). Finally, SIRT3 expression was also decreased in several other human
malignancies including testicular (Figure S6C), glioblastoma multiforme (Figure S6D), prostate (Figure S6E), head and neck squamous cell (Figure S6F), clear cell renal (Figure S6G), and hepatocellular (Figure S6H) cancers.

**DISCUSSION**

Although there is no universal definition of a TS gene, for the purposes of this work, we defined a TS gene as one that: (1) protects a tissue culture cell from one step on the path to in vitro transformation; (2) results in tumorigenesis in murine models lacking expression; (3) is decreased in human malignancies; and (4) results in the loss of functional organelle integrity or the accumulation of cellular damage to critical biomolecules either spontaneously or following exposure to stress. Based on these criteria, we propose that SIRT3 is a mitochondrial TS.

In this regard, we demonstrated that SIRT3−/− MEFs infected with a single oncogene become immortalized as well as in vitro transformed, and that they exhibit anchorage-independent growth. Furthermore, SIRT3−/− MEFs expressing Myc and Ras grew in nude mice, suggesting that loss of SIRT3 also results in an in vivo tumorigenic phenotype. In addition, SIRT3 knockout mice spontaneously form mammary tumors later in life. Finally, SIRT3 protein levels are decreased in human breast cancers as well as several other human malignancies. In our murine model, SIRT3 appears to be limited to the mitochondria. Thus, we propose that SIRT3 is a genomically expressed, mitochondrially localized TS.

Interestingly, the murine mammary tumors we assayed were well-differentiated, ER/PR positive. In humans, these markers are most often seen in tumors from women who develop breast cancer later in life. Because sirtuins are the human homologs of longevity genes in *C. elegans* and yeast (Tissenbaum and Guarente, 2001), this result suggests that the SIRT3 knockout mouse may be useful as a model for an ER/PR-positive subtype of breast cancer that is more commonly observed in women over 65 years of age.

In this work we have also shown that the transformed SIRT3−/− Myc/Ras cells, but not the wild-type Myc/Ras cells, have significant increases in glucose metabolism, superoxide levels, and total cellular ATP levels, but decreased ATP from oxidative phosphorylation. However, it is unclear if this was due to loss of SIRT3 or the natural process of transformation. In addition, the activity of complexes I and III of the electron transport chain was significantly decreased in the SIRT3−/− Myc/Ras and SIRT3−/− Ras cells, as compared with SIRT3+/+ Myc/Ras cells. This could explain why the addition of lentivirus MnSOD

![Figure 6. SIRT3 Is a Mitochondria-Localized Murine Tumor Suppressor](image-url)
prevented the immortalization of SIRT3−/− cells by Myc or Ras alone; however, expression of both Myc and Ras provided an additional proliferative event that pushed the cells over the edge toward immortalization, transformation, and tumorigenesis. Finally, the in vivo situation might be more complex than in cultured cell model, because that data showed that the decreased MnSOD expression was only observed in older SIRT3 mutant mice. This issue is potentially interesting and will be investigated in future studies.

Eukaryotic cells contain fidelity proteins that function to monitor the integrity of critical intracellular processes, and deletion or mutation of the corresponding genes results in a cellular environment permissive for the accumulation of DNA damage (Hunter, 1997). Thus, it seems like a logical extension that the environment permissive for the accumulation of DNA damage or mutation of the corresponding genes results in a cellular monitor the integrity of critical intracellular processes, and dele-

ROS (Figure 7G). This may create a cellular environment per-

in an increase in mitochondrial superoxide and perhaps other

results in a decrease in MnSOD as a function of age resulting

the integrity of the mitochondria. In this regard, loss of SIRT3

mitochondria would also contain fidelity proteins to maintain

be investigated in future studies.

Experimental Procedures

Cell Lines

MEFs were isolated from E14.5 isogenic SIRT3+/+ and SIRT3−/− mice and maintained in a 37°C incubator with 5% CO2 and 6% oxygen except when otherwise noted. SIRT3+/+ or SIRT3−/− MEFs were infected at passage 3 with lentivirus expressing either Myc, Ras, or MnSOD were made by Applied Biological Materials, Inc. (Richmond, British Columbia), and pooled selected cells were used for all experiments. For this study, our definition of spontaneous immortalization of primary MEFs is the ability to continue dividing past passage 15 and subsequently divide indefinitely. For in vitro immortalization experiments via enforced genetic expression of one or two oncogenes, MEFs at passage 3 were infected with a lentivirus containing Myc, Ras, or both. Cells were cultured and split every 2 days to prevent confluency and plated into a new 100 mm dish at 3.0 × 10^5 cells. After 17 additional passages (20 total), cells were considered immortalized if they continued to divide. MEFs were infected with 5 MOI virus per 10 cm/plate. Levels of Myc and Ras were confirmed by western blot analysis, and PCR analysis (data not shown). Soft agar and colony formation assays were done as previously described (Supplemental Experimental Procedures).

Statistical Analysis

Data were analyzed by Student’s t test, and results were considered significant at p < 0.05. Results are presented as mean and standard deviation (±SD).

Measurement of Intracellular Superoxide Levels

Superoxide production was determined as described elsewhere (Slane et al., 2006) using the fluorescent dye dihydroethidium (DHE), obtained from Molecular Probes (Eugene, OR). Mitochondrial superoxide levels were determined...
by the addition of Mito-SOX (3 μM) to the cells, cultured as described above and incubated for an additional 10 min before being trypsinized and resuspended and measured by fluorescence (Molecular Probes). See Supplemental Experimental Procedures for more detail.

Measurement of Glucose Consumption, ATP Levels, and Oxygen Consumption
Glucose consumption per cell was measured by plating 300,000 cells on a 60 mm plate. Cells were given fresh medium at time zero. Cells were counted and medium samples were obtained at 48 hr and analyzed using a YSI glucose meter. Glucose consumption was determined by subtracting glucose content at 48 hr from the time zero samples and dividing by the number of cells.

Total ATP levels were monitored using a CellTiter-Glo Luminescent Cell Viability Assay as per the manufacturer's instructions (Promega). CellTiter-Glo was added to 10^6 cells and placed on an orbital shaker to induce cell lysis, and samples were read on a chemiluminescence plate reader (Tecan Safire; integration time of 1 s). Mitochondrial ATP was determined by incubating MEFs in 20 mM 2DG and 5 mM pyruvate for 4 and 24 hr prior to using an adenosine 5'-triphosphate bioluminescent somatic cell assay kit (Supplemental Experimental Procedures).

Oxygen consumption was measured using the YSI oxygen monitor containing a Clark-type electrode as per the manufacturer's instructions. The cell pellet was resuspended at a density of 2.5 × 10^6 cells/ml in phosphate-buff ered saline (PBS) containing 5 mM glucose. For each sample, a 3 ml sample was placed in the electrode chamber and allowed to equilibrate with air for 3 min. Oxygen consumption was then recorded for 15 min. The data were normalized to cell number.

Chromosome Aberrations
MEFs were exposed at passage four to irradiation and harvested after 72 hr. Whole-mount chromosomes were counted in a blinded fashion. Individual spreads were deemed countable if all chromosomes were clearly defined and clearly visible within the ghost of a single cytoplasm.

Real-Time Long PCR Assay for mtDNA Damage
mtDNA damage was measured as amplification efficiency for a large (10,095 bp) fragment relative to a short (117 bp) Amplicon of mtDNA in wild-type and knockout mice (see Supplemental Experimental Procedures).

Allograft and Tissue Specimen
Cells (2 × 10^6 or 1 × 10^6) in PBS for a total volume of 100 μl/ injection site were injected subcutaneously in right and left flanks of 8-week-old male athymic mice (Jackson Laboratories). Tumor sizes were measured twice weekly in two dimensions (width, W, and length, L) with calipers. Average tumor volume (V) was calculated as V = 0.5 × W × L^2. At the termination of the experiment, mice were sacrificed; tumors were excised and weighed. All animal care followed approved institutional guidelines of the National Institutes of Health. All experiments were approved by the animal care and use committee of the National Institute Diabetes and Digestive and Kidney Diseases (K069-GDDB-08) or the National Cancer Institute (ROB-118).

Normal and breast cancer tissue slides were purchased from (US Biomax, Inc.) and analyzed by IHC. RNA samples from normal breast (NB) and malignant breast were purchased (TissueScan Breast Cancer Panel 1 Origene) and analyzed by the supplier's instructions. As these samples were obtained commercially without accompanying patient identifying data, they are considered “exempt” according to HHS guidelines.

Oxidative Phosphorylation Enzyme Activities
Oxidative phosphorylation enzyme activities were measured on total cellular protein including complex I (Supplemental Experimental Procedures). Complex II, III, and IV methods are presented in the Supplemental Experimental Procedures section.

SUPPLEMENTAL INFORMATION
Supplemental Data include Supplemental Experimental Procedures, six figures, and one table, and can be found with this article online at doi:10.1016/j.ccr.2009.11.023.

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