Sirtuin 3 silencing improves oxaliplatin efficacy through acetylation of MnSOD in colon cancer

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Abstract

Sirtuin 3 (SIRT3) is the major mitochondria deacetylase and regulates ROS levels by targeting several key proteins, such as those involved in mitochondrial function and antioxidant defenses. This way, SIRT3 balances ROS production and scavenging and promotes cell survival. The aim of this study was to analyze the effect of SIRT3 silencing on the antioxidant response in SW620 colon cancer cell line, and whether this intervention could improve efficacy of oxaliplatin, a common drug used to treat colon cancer. For this purpose, we obtained stable clones of SW620 with SIRT3 knockdown and determined parameters such as ROS levels and ROS production, levels of several antioxidant enzymes, cell viability and apoptosis. Results showed that after SIRT3 silencing, both ROS levels and production were increased, and antioxidant enzymes gene expression was significantly reduced. Furthermore, manganese superoxide dismutase levels and enzymatic activity were reduced. Combination of SIRT3 knockdown with oxaliplatin treatment further increased ROS production and apoptosis, reducing cell viability. Finally, survival curves on colon cancer patients suggested that SIRT3 expression is related to a poorer prognosis. In conclusion, SIRT3 could be a target for colon cancer, since it regulates the antioxidant response and improves the efficacy of oxaliplatin treatment.

Introduction

Colorectal cancer is the third most common malignancy diagnosed worldwide and the fourth leading cause of death by cancer (Favoriti et al. 2016; Arnold et al. 2017). It has been reported that reactive oxygen species (ROS) and oxidative stress may play an important role during tumorigenesis and cancer progression, including colon cancer (Perše 2013; Galadari et al. 2017).

Cancer cells often show enhanced ROS production and accumulation compared to normal cells. These features confer cancer cells advantages in cell growth, survival and resistance to chemotherapy (Sullivan and Chandel 2014; Galadari et al. 2017). However, excessive levels of ROS lead to growth arrest and apoptosis (Hussain et al. 2003; Sainz et al. 2012). Indeed, some of the current anticancer therapies act increasing ROS levels, and therefore, it is important to consider the effective regulation of oxidative stress for tumor progression and response to therapy (Nogueira and Hay 2013; Lee et al. 2014).

Sirtuins are a family of seven proteins with NAD^+-dependent deacetylase activity and are involved in the regulation of several processes, such as metabolism, cellular proliferation and response to stress (Michan and Sinclair 2007; Torrens-Mas et al. 2017). Sirtuin 3 (SIRT3), the major mitochondrial deacetylase, plays a crucial role in modulating ROS production and scavenging (Finley and Haigis 2012; Alhazzazi et al. 2013; Torrens-Mas et al. 2017). One of the main targets of SIRT3 is the manganese superoxide dismutase or MnSOD, which detoxifies the radicals generated in the mitochondrial respiratory chain (Tao et al. 2010; Ozden et al. 2011; Bause and Haigis 2013).

Thus, SIRT3 could function as a protective mechanism in normal cells; however, its activity could also protect cancer cells from the deleterious effects of excessive oxidative stress. Different studies have found a significant correlation between SIRT3 levels and progression of several types of cancer, including colon cancer (Liu et al. 2014; Torrens-Mas et al. 2017). Furthermore, MnSOD has also been reported to enhance progression, invasion and metastasis in several types of cancer (Hempel et al. 2011).

The aim of this study was to analyze whether SIRT3 silencing in colon cancer cells could increase oxidative stress through regulation of MnSOD and other antioxidant enzymes, and thus increase cell death. For this purpose, a shRNA against SIRT3 was transfected into SW620 colon cancer cells to generate a stable cell line with SIRT3 knockdown. Parameters such as ROS levels, MnSOD levels, cell viability and apoptosis were analysed.

Materials and Methods

Reagents

Dulbecco's Modified Eagle's medium (DMEM) high glucose was purchased from GIBCO (Paisley, UK). Oxaliplatin ([SP-4-2-(1R-trans)]-(1,2-Cyclohexanediamine-N,N')[ethanedioata(2--)-O,O'] platinum or OXA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Routine chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA), Roche (Barcelona, Spain), Panreac (Barcelona, Spain) and Bio-Rad Laboratories (Hercules, CA, USA).

Cell culture and stable transfection

Human colon cancer cell line SW620 was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin and streptomycin at 37 °C with 5% CO_2.

Scrambled shRNA (TR20003) and shRNA targeting SIRT3 (TR309432) were purchased from Origene (Rockville, MD, USA). cDNA clones were amplified in *Escherichia coli* DH5αF' competent cells (Life Technologies, Paisley, UK) and isolated with MaxiPrep isolation kit (Life Technologies, Paisley, UK). SW620 were seeded in 6-well plates and the next day were transfected with either one of the plasmids and with Lipofectamine 2000 reagent (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Two days after transfection, cells were then selected with 4 µg/mL puromycin for 14 days. Multiple monoclones were isolated and checked for SIRT3 levels. The selected clones were maintained with a mild selective pressure of 2 µg/mL puromycin for all subsequent experiments.

Measurement of ROS levels by flux cytometry

Cells were seeded in 6-well plates at a density of 6×10^5 cells/well. The day after, cells were trypsinized, harvested into cytometer tubes and centrifuged at 16000 rpm for 5 min. Pellet was resuspended in 500 µL PBS with 10 µM 2',7'-dichlorofluorescein diacetate (DCFDA). After incubation for 15 min in the dark, cells were analyzed immediately using an Epics XL flow cytometer (Beckman–Coulter, Miami, FL, USA). The green fluorescence was measured using the FL-1 setting (log mode) after the cell debris was electronically gated out. Ten thousand events were acquired and analysis was performed with WinMidi software.

RT-PCR

Cells were seeded in 6-well plates at a density of 6×10^5 cells/well. Total RNA was isolated from cultured cells using TRI Reagent® (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's protocol and then quantified using a BioSpec-nano spectrophotometer (Shimadzu Biotech, Kyoto, Japan) set at 260 nm. For each sample, 1 µg of the total RNA was reverse transcribed to cDNA at 42°C for 60 min with 25 U MuLV reverse transcriptase in a 10 µL volume of retrotranscription reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 2.5 µM random hexamers, 10 U RNase inhibitor, and

500 µM each dNTP. Each cDNA was diluted 1/10 and aliquots were frozen (−20°C) until the PCR reactions were carried out.

PCR was performed using SYBR Green technology on a LightCycler 480 System II rapid thermal cycler (Roche Diagnostics, Basel, Switzerland). The genes, primers and temperatures for the annealing step are specified in Table 1. Total reaction volume was 10 µL, containing 7.5 µL Lightcycler® 480 SYBR Green I Master (containing 0.5 µM of the sense and antisense specific primers) and 2.5 µL of the cDNA template. The amplification program consisted of a preincubation step for denaturation of the template cDNA (5 min, 95°C), followed by 45 cycles consisting of a denaturation step (10 s, 95°C), an annealing step (10 s, temperature depending on primers; listed in Table 1), and an elongation step (12 s, 72°C min). A negative control lacking cDNA template was run in each assay.

The Ct values of the real-time PCR were analyzed, taking into account the efficiency of the reaction and referring these results to the total DNA amount, using the GenEx Standard Software (Multi-DAnalises, Sweden).

Enzymatic activities

Cells were seeded in 6-well plates at a density of 6×10^5 cells/well. Cells were harvested by scraping them into 200 µL of STE buffer (250 mM sucrose, 3.59 mM Trizma-Base, 16.4 Tris-HCl pH 7.4, 2mM EDTA, 40 mM KCl). Then, cells were disrupted by sonication at 40% amplitude for 10 seconds three times (VibraCell 75185) and centrifuged at 600x*g* for 10 min at 4°C to remove cell debris. Protein content (supernatant) was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Bonn, Germany) and the enzymatic assays were performed immediately after.

MnSOD (SOD, EC 1.15.1.1) activity was determined by following the reduction of cytochrome c by measuring the absorbance at 550 nm, as described by Quick et al (2000), and adding 1 mM KCN in the reaction mixture to inhibit the activity from cytoplasmatic CuZnSOD.

Western blot analysis

Cells were seeded in 6-well plates at a density of 6×10^5 cells/well. Cells were harvested by scraping them into 200 µL of RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 1 mM EDTA, 0.01 mM leupeptin, 0.01 M pepstatin, 2 mM PMSF, 1 mM NaF and 1 mM Na_3VO_4) and disrupted by sonication at 40% amplitude for 10

seconds three times. Samples were then centrifuged at 14000 ×*g* for 10 min at 4 °C and protein content (supernatant) was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Bonn, Germany).

Twenty µg of protein from cell lysate were separated on 12% SDS–PAGE gels and electrotransferred to 0.22 µm nitrocellulose membranes using the Trans-blot Turbo transfer system (Bio-Rad). Membranes were blocked with 5% non-fat powdered milk in Tris-buffered saline-Tween (TBS with 0.05% Tween-20) for 1h. Antisera against SIRT3 (Cell Signaling Technology Inc, Danvers, MA), acetylated MnSOD (acetyl K63; Abcam, Cambrigde, UK) GAPDH and MnSOD (Santa Cruz Biotechnology, CA, USA) were used as primary antibodies. Protein bands were visualized by Immun-Star® Western C® Kit reagent (Bio-Rad) Western blotting detection systems. The chemiluminiscence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and results were analyzed with Quantity One Software (Bio-Rad).

Cell viability assay

Cells were plated in 96-well plates at a density of 7×10^4 cells/well. Cell viability was determined by crystal violet assay. Briefly, cells were stained with 0.5% (p/v) crystal violet in 30% (v/v) acetic acid for 10 min. After washing in distilled water, 100 µL of methanol were added to solubilize the dye and absorbance *was measured at* 595 nm using a PowerWave XS Microplate Spectrophotometer (BioTek Instruments, Inc.).

Fluorometric measurement of ROS production

Cells were plated in 96-well plates at a density of 7×10^4 cells/well and treated with 5 µM OXA for 48 h. To test the effect of some ROS scavengers, cells were pre-treated for 3h with 250 µM ascorbic acid, and 400 µM N-acetyl-cysteine (NAC) was added at the same time of OXA. To measure ROS production, the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Eugene, Oregon, USA) was used. Briefly, 50 µM Amplex red reagent and 0.1 U/mL horseradish peroxidase were diluted in Krebs-Ringer phosphate buffer (145 mM NaCl, 4.86 mM KCl, 0.54 mM CaCl2, 1.22 mM $MgSO_4$, 5.5 mM glucose, 5.7 mM sodium phosphate, pH 7.4) and the reaction mixture was added to cells. Fluorescence measurement was recorded at times 0, 15, 30 and 60 minutes. An FLx800 microplate fluorescence reader (Bio-Tek Winooski, Vermont, USA) was used, set at excitation and emission wavelengths of 571 and 585, respectively. Values were normalized per number of viable cells determined by crystal violet assay.

Apoptosis fluorometric assay

Apoptosis was measured fluorometrically using Annexin V staining. Briefly, after cytotoxic treatment, cells were fixed with 2% paraformaldehyde (BD Biosciences, NJ, USA) in PBS for 30 minutes at room temperature. After washing cells twice with PBS, Annexin V/AlexaFluor350 (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) in annexin binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaOH and 2.5 mM $CaCl_2$) was added for 10 minutes in the dark at room temperature. Cells were rinsed twice with annexin binding buffer and fluorescence was measured. An FLx800 microplate fluorescence reader (Bio-Tek Winooski, Vermont, USA) was used and excitation and emission wavelengths were set at 346 and 442 nm. Values were normalized per number of viable cells determined by crystal violet assay.

Kaplan-Meier survival curves

Overall survival was assessed for 562 colon cancer patients from the data set GSE39582 (Marisa et al. 2013). Survival curves were generated using Kaplan-Meier analysis and assessed with Breslow test to check for statistical significance. Patients were split into high SIRT3 expression levels and low SIRT3 expression levels groups by selecting the 50% top and bottom expression patterns. The analysis was performed including all patients and evaluating overall survival and relapse-free survival, and the Breslow test was used to check for statistical significance.

Statistical analysis

The Statistical Program for the Social Sciences software for Windows (SPSS, version 21.0; SPSS Inc, Chicago, IL) was used for all statistical analyses. Results are presented as mean values ± standard error of the mean (SEM) from six independent experiments. The effects of SIRT3 silencing were assessed using the Student's t-test. The effects produced by cytotoxic agent oxaliplatin in combination with SIRT3 silencing were assessed by a two-way analysis of variance (ANOVA), and a post-hoc Student's t-test was performed when combinatory effects were found. Statistical significance was set at $P<0.05$.

Results

SIRT3 levels after stable knockdown

SIRT3 knockdown by shRNA transfection was determined by measuring both mRNA and protein levels in the selected clone as shown in Figure 1. Indeed, SW620 transfected cells showed a 69% decrease in SIRT3 mRNA levels (Figure 1A) and a 47% decrease in protein levels (Figure 1B). In Figure 1C are shown representative bands of the Western Blot.

To further check that SIRT3 knockdown was functional, ROS levels were measured by flow cytometry. As expected, ROS levels were increased by 78% after SIRT3 silencing, as shown in Figure 2.

Antioxidant gene expression was decreased in SIRT3-knockdown cells

Expression of several antioxidant genes was evaluated, including transcriptional factors such as NRF2 and Foxo3a, antioxidant enzymes such as superoxide dysmutases and catalase, and enzymes involved in the glutathione and peroxiredoxin/thyoredoxin systems. Figure 3 shows that all of the genes analyzed showed a significant decrease in their mRNA levels with SIRT3 knockdown.

SIRT3 knockdown compromises MnSOD expression and activity

The effects of SIRT3 knockdown on MnSOD, one of SIRT3 main targets, were further analyzed. First, both levels and acetylation of MnSOD were evaluated by Western Blot. As shown in Figure 4A, total levels of MnSOD were slightly decrease by SIRT3 knockdown. However, acetylated-MnSOD was significantly increased by 50% with SIRT3 knockdown, as shown in Figure 4B. Thus, as shown in Figure 4C, the ratio acetylated-MnSOD/total MnSOD was significantly higher in cells with SIRT3 silencing. Representatie bands of Western Blots are shown in Figure 4D. As a result, MnSOD activity dropped in these cells to 55% of control values, as seen in Figure 4E.

SIRT3 silencing affects response to oxaliplatin treatment

Effects of SIRT3 knockdown on cell viability, ROS production and apoptosis were evaluated alone and in combination with the cytotoxic treatment oxaliplatin. SIRT3 silencing reduced cell viability only by 17%, while oxaliplatin treatment decreased it by 45%, as shown in Figure 5A. The combination of SIRT3 knockdown and oxaliplatin treatment resulted in a 51% decrease in cell viability.

Figure 5B shows that ROS production is significantly higher after SIRT3 silencing and oxaliplatin treatment, 29% and 101% respectively. Combination of both resulted in a 147% increase in ROS production.

To check whether programmed cell death was also affected by SIRT3 silencing, an apoptosis assay was performed. As shown in Figure 5C, SIRT3 knockdown increased annexin V fluorescence by 112%, while oxaliplatin treatment significantly increased it by 342%. Both SIRT3 silencing and cytotoxic treatment resulted in a 657% increase in the apoptosis marker.

A treatment with different ROS scavengers was performed to prove a causal relationship between increased ROS production and lower cell viability. As seen in Figure 6A, cell viability in SIRT3-silenced cells was restored after OXA and NAC or ascorbic acid treatment, reaching comparable or even higher values to control cells after OXA treatment. Furthermore, ROS production was lowered by the addition of NAC or ascorbic acid in SIRT3-silenced cells after OXA treatment, as shown in Figure 6B.

SIRT3 expression is related to a poorer prognosis in colon cancer patients

SIRT3 expression was analyzed as a possible prognostic factor in colon cancer patients using the publicly available data set described under the Kaplan-Meier survival curves section of Materials and Methods. Figure 7A and 7B represent the overall survival (OS) and relapse free survival (RFS) in colon cancer patients expressing high or low levels of SIRT3, respectively. Although RFS did not reach a significant difference between both groups ($P<0.07$), OS was significantly lower in those patients with higher expression of SIRT3 ($P<0.009$).

Discussion

In this study, we have shown that SIRT3 silencing resulted in a downregulation of several antioxidant genes, as well as a reduction in MnSOD protein levels and activity. Furthermore, SIRT3 knockdown lead to higher ROS levels and ROS production, which

was accompanied by lower cell viability and an increase in apoptosis. Combination of SIRT3 silencing and cytotoxic treatment with oxaliplatin further increased ROS production and apoptosis. Finally, we show here, using bioinformatic tools, that high SIRT3 expression could be related to a poorer prognosis for colon cancer patients.

Growing evidence suggests that free radicals and oxidative stress play an important role in the development and progression of colon cancer (Perše 2013). In fact, it has been reported that human colorectal tumors present high levels of ROS and markers of oxidative stress such as catalase, glutathione peroxidase or MnSOD (Skrzycki et al. 2009; Perše 2013). However, the role of ROS remains controversial. A light increase in ROS production and mild oxidative stress may be beneficial for cell survival, while rising ROS levels above a certain threshold may trigger cell death (Trachootham et al. 2008; Panieri and Santoro 2016; Galadari et al. 2017).

SIRT3 is considered a crucial protein against oxidative stress, since it deacetylates and activates several proteins related to mitochondrial function and antioxidant enzymes (Alhazzazi et al. 2011a; Torrens-Mas et al. 2017). In this regard, we observed an increase in ROS levels and ROS production after SIRT3 knockdown, as has been widely described before (Kong et al. 2010; Finley et al. 2011; Finley and Haigis 2012; Kwon et al. 2015; Torrens-Mas et al. 2016). This increase has been attributed to the hyperacetylation, and thus inactivation, of SIRT3 targets, mainly MnSOD, which is one of the most important antioxidant enzymes of the cell (Ozden et al. 2011; Tao et al. 2014; Zou et al. 2016).

However, we have shown here that gene expression of several antioxidant enzymes was also affected by SIRT3 silencing. The downregulation of these antioxidant genes is presumably due to a reduction of transcriptional activity of FOXO3a, which is a target of SIRT3 and regulates the expression of crucial proteins involved in ROS scavenging and mitochondrial integrity, such as MnSOD and catalase (Sundaresan et al. 2009; Bause and Haigis 2013; Tseng et al. 2013; Rangarajan et al. 2015). Thus, SIRT3 silencing prevents FOXO3a deacetylation and leads to a reduction in the activity of this transcription factor.

To further study the effect of SIRT3 knockdown on antioxidant enzymes, we measured protein levels of MnSOD and acetylated MnSOD. As expected, the acetylation level of MnSOD was higher after SIRT3 silencing. Moreover, total protein levels of MnSOD were significantly reduced and the ratio acetylated MnSOD/total MnSOD almost doubled in cells with SIRT3 silencing. A previous study by our group also showed a reduction in MnSOD protein levels and other targets after treatment of MCF-7 breast cancer cell line with siRNA against SIRT3 (Torrens-Mas et al. 2016). The increase in acetylation in MnSOD resulted in a reduction of its enzymatic activity, as has also been described before (Tao et al. 2010; Ozden et al. 2011).

On the other hand, SIRT3 knockdown also reduced cell viability, which occurred with an increase in apoptosis. This may be due to the increase in ROS production that can lead to oxidative damage and cell death (Alhazzazi et al. 2011b; Papa and Germain 2014). These results are agreement with several studies that refer to SIRT3 as an oncogene, since SIRT3 limits ROS production and increases cellular resistance to oxidative stress, thus promoting cell proliferation and avoiding apoptosis (L. Zhang et al. 2013; Wang et al. 2015). However, some studies describe SIRT3 as a tumor suppressor in some types of cancer, such as breast cancer (Buler et al. 2012), ovarian cancer (Dong et al. 2016), hepatocellular carcinoma (B. Zhang et al. 2013; Liu et al. 2017) or B cell malignancies (Yu et al. 2016). In these reports, SIRT3 reduces cell proliferation of cancer cells and limits their metabolic reprogramming. Furthermore, patients with higher SIRT3 expression present a good outcome and an increased overall survival. These studies suggest a dual role for SIRT3 in cancer, which may depend on cellular type and cellular context (Torrens-Mas et al. 2017).

Finally, we observed that oxaliplatin increased apoptosis and ROS production, and when combined with SIRT3 knockdown, these parameters were further increased. As expected, the addition of ROS scavengers, NAC and ascorbic acid, reduced ROS production after OXA treatment. Accordingly, combination of OXA treatment and ROS scavengers produced a recovery in cell viability, reducing the cytotoxic effect of the increased ROS produced by OXA treatment.

Previous studies have shown that SIRT3 knockdown sensitizes cells to cytotoxic treatments and reduces cell proliferation (Alhazzazi et al. 2011b; George et al. 2016; Torrens-Mas et al. 2016). In this regard, our results show that SIRT3 silencing enhances the effectivity of oxaliplatin through increasing ROS production to excessive levels incompatible with cell viability. Moreover, when SIRT3 is silenced we observed an inability to induce the antioxidant defenses and counteract the harmful effects of ROS, which leads to an increase in the apoptosis process.

Taken together, these results support the role of SIRT3 as a crucial protein in limiting ROS production and promoting cell survival by preservation of proper mitochondrial function and integrity (Kim et al. 2010; Park et al. 2011). Furthermore, the survival analysis presented here suggests that when SIRT3 expression is high, tumor cells can balance ROS production activating the antioxidant response and could be more resistant to oxidative stress and even cytotoxic therapy (Papa and Germain 2014; Galadari et al. 2017). This way, patients with high expression of SIRT3 show a poorer survival.

In conclusion, SIRT3 silencing in colon cancer cells not only affects the activity of antioxidant enzymes, but also its expression at the mRNA level, which may reduce the antioxidant capacity of the cell and thus increasing cell death. In this regard, SIRT3 knockdown could be an adjuvant treatment for colon cancer, enhancing the effect of cytotoxic treatments such as oxaliplatin, which rely on increasing ROS production to induce cell death.

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References

362 Alhazzazi, T. Y., P. Kamarajan, E. Verdin, and Y. L. Kapila. 2011a. SIRT3 and cancer: tumor
363 promoter or suppressor? *Biochimica et biophysica acta* 1816: 80–8.
364 doi:10.1016/j.bbcan.2011.04.004.

365 Alhazzazi, T. Y., P. Kamarajan, N. Joo, J.-Y. Huang, E. Verdin, N. J. D'Silva, and Y. L. Kapila.
366 2011b. Sirtuin-3 (SIRT3), a novel potential therapeutic target for oral cancer. *Cancer* 117:
367 1670–8. doi:10.1002/cncr.25676.

368 Alhazzazi, T. Y., P. Kamarajan, E. Verdin, and Y. L. Kapila. 2013. Sirtuin-3 (SIRT3) and the
369 Hallmarks of Cancer. *Genes & Cancer* 4: 164–171. doi:10.1177/1947601913486351.

370 Arnold, M., M. S. Sierra, M. Laversanne, I. Soerjomataram, A. Jemal, and F. Bray. 2017. Global
371 patterns and trends in colorectal cancer incidence and mortality. *Gut* 66: 683–691.
372 doi:10.1136/gutjnl-2015-310912.

373 Bause, A. S., and M. C. Haigis. 2013. SIRT3 regulation of mitochondrial oxidative stress.
374 *Experimental Gerontology* 48: 634–639. doi:10.1016/j.exger.2012.08.007.

375 Buler, M., S.-M. Aatsinki, V. Izzi, and J. Hakkola. 2012. Metformin reduces hepatic expression of
376 SIRT3, the mitochondrial deacetylase controlling energy metabolism. Edited by Valdur
377 Saks. *PloS one* 7: e49863. doi:10.1371/journal.pone.0049863.

378 Dong, X. cai, L. min Jing, W. xiang Wang, and Y. xia Gao. 2016. Down-regulation of SIRT3
379 promotes ovarian carcinoma metastasis. *Biochemical and Biophysical Research*
380 *Communications* 475: 245–250. doi:10.1016/j.bbrc.2016.05.098.

381 Favoriti, P., G. Carbone, M. Greco, F. Pirozzi, R. E. M. Pirozzi, and F. Corcione. 2016. Worldwide
382 burden of colorectal cancer: a review. *Updates in Surgery* 68: 7–11. doi:10.1007/s13304-
383 016-0359-y.

384 Finley, L. W. S., and M. C. Haigis. 2012. Metabolic regulation by SIRT3: Implications for
385 tumorigenesis. *Trends in Molecular Medicine* 18: 516–523.
386 doi:10.1016/j.molmed.2012.05.004.

387 Finley, L. W. S., A. Carracedo, J. Lee, A. Souza, A. Egia, J. Zhang, J. Teruya-Feldstein, P. I.
388 Moreira, et al. 2011. SIRT3 Opposes Reprogramming of Cancer Cell Metabolism through
389 HIF1α Destabilization. *Cancer Cell* 19: 416–428. doi:10.1016/j.ccr.2011.02.014.

390 Galadari, S., A. Rahman, S. Pallichankandy, and F. Thayyullathil. 2017. Reactive oxygen species
391 and cancer paradox: To promote or to suppress? *Free Radical Biology and Medicine* 104:

144–164. doi:10.1016/j.freeradbiomed.2017.01.004.

George, J., M. Nihal, C. K. Singh, W. Zhong, X. Liu, and N. Ahmad. 2016. Pro-Proliferative Function of Mitochondrial Sirtuin Deacetylase SIRT3 in Human Melanoma. *Journal of Investigative Dermatology* 136: 809–18. doi:10.1016/j.jid.2015.12.026.

Hempel, N., P. M. Carrico, and J. A. Melendez. 2011. Manganese superoxide dismutase (Sod2) and redox-control of signaling events that drive metastasis. *Anticancer Agents Med Chem* 11: 191–201. doi:10.1007/s12020-009-9266-z.A.

Hussain, S. P., L. J. Hofseth, and C. C. Harris. 2003. Radical causes of cancer. *Nature Reviews Cancer* 3: 276–285. doi:10.1038/nrc1046.

Kim, H. S., K. Patel, K. Muldoon-Jacobs, K. S. Bisht, N. Aykin-Burns, J. D. Pennington, R. van der Meer, P. Nguyen, et al. 2010. SIRT3 Is a Mitochondria-Localized Tumor Suppressor Required for Maintenance of Mitochondrial Integrity and Metabolism during Stress. *Cancer Cell* 17: 41–52. doi:10.1016/j.ccr.2009.11.023.

Kong, X., R. Wang, Y. Xue, X. Liu, H. Zhang, Y. Chen, F. Fang, and Y. Chang. 2010. Sirtuin 3, a new target of PGC-1alpha, plays an important role in the suppression of ROS and mitochondrial biogenesis. *PLoS ONE* 5. doi:10.1371/journal.pone.0011707.

Kwon, D.-N., W.-J. Park, Y.-J. Choi, S. Gurunathan, and J.-H. Kim. 2015. Oxidative stress and ROS metabolism via down-regulation of sirtuin 3 expression in Cmah-null mice affect hearing loss. *Aging* 7: 579–594. doi:10.18632/aging.100800.

Lee, Y.-H., B. S. Kang, and Y.-S. Bae. 2014. Premature senescence in human breast cancer and colon cancer cells by tamoxifen-mediated reactive oxygen species generation. *Life Sciences* 97: 116–122. doi:10.1016/j.lfs.2013.12.009.

Liu, C., Z. Huang, H. Jiang, and F. Shi. 2014. The Sirtuin 3 Expression Profile Is Associated with Pathological and Clinical Outcomes in Colon Cancer Patients. *BioMed research international* 2014:87126.

Liu, Y., Y.-L. Liu, W. Cheng, X.-M. Yin, and B. Jiang. 2017. The expression of SIRT3 in primary hepatocellular carcinoma and the mechanism of its tumor suppressing effects. *European review for medical and pharmacological sciences* 21: 978–998.

Marisa, L., A. de Reyniès, A. Duval, J. Selves, M. P. Gaub, L. Vescovo, M.-C. Etienne-Grimaldi, R. Schiappa, et al. 2013. Gene expression classification of colon cancer into molecular

422 subtypes: characterization, validation, and prognostic value. *PLoS medicine* 10.
423 doi:10.1371/journal.pmed.1001453.

424 Michan, S., and D. Sinclair. 2007. Sirtuins in mammals: insights into their biological function.
425 *Biochemical Journal* 404: 1–13. doi:10.1042/BJ20070140.

426 Nogueira, V., and N. Hay. 2013. Molecular Pathways: Reactive Oxygen Species Homeostasis in
427 Cancer Cells and Implications for Cancer Therapy. *Clinical Cancer Research* 19.

428 Ozden, O., S.-H. Park, H.-S. Kim, H. Jiang, M. C. Coleman, D. R. Spitz, and D. Gius. 2011.
429 Acetylation of MnSOD directs enzymatic activity responding to cellular nutrient status or
430 oxidative stress. *Aging* 3: 102–107. doi:10.18632/aging.100291.

431 Panieri, E., and M. M. Santoro. 2016. ROS homeostasis and metabolism: a dangerous liason in
432 cancer cells. *Cell Death and Disease* 7: e2253. doi:10.1038/cddis.2016.105.

433 Papa, L., and D. Germain. 2014. SirT3 Regulates the Mitochondrial Unfolded Protein Response.
434 *Molecular and Cellular Biology* 34: 699–710. doi:10.1128/MCB.01337-13.

435 Park, S.-H., O. Ozden, H. Jiang, Y. I. Cha, J. D. Pennington, N. Aykin-Burns, D. R. Spitz, D. Gius, et
436 al. 2011. Sirt3, mitochondrial ROS, ageing, and carcinogenesis. *International journal of*
437 *molecular sciences* 12: 6226–39. doi:10.3390/ijms12096226.

438 Perše, M. 2013. Oxidative stress in the pathogenesis of colorectal cancer: Cause or
439 consequence? *BioMed Research International* 2013. doi:10.1155/2013/725710.

440 Quick, K. L., J. I. Hardt, and L. L. Dugan. 2000. Rapid microplate assay for superoxide scavenging
441 efficiency. *Journal of Neuroscience Methods* 97: 139–144. doi:10.1016/S0165-
442 0270(00)00179-5.

443 Rangarajan, P., A. Karthikeyan, J. Lu, E.-A. Ling, and S. T. Dheen. 2015. Sirtuin 3 regulates
444 Foxo3a-mediated antioxidant pathway in microglia. *Neuroscience* 311: 398–414.
445 doi:10.1016/j.neuroscience.2015.10.048.

446 Sainz, R. M., F. Lombo, and J. C. Mayo. 2012. Radical decisions in cancer: redox control of cell
447 growth and death. *Cancers* 4: 442–74. doi:10.3390/cancers4020442.

448 Skrzycki, M., M. Majewska, M. Podsiad, and H. Czeczot. 2009. Expression and activity of
449 superoxide dismutase isoenzymes in colorectal cancer. *Acta Biochimica Polonica* 56: 663–
450 670.

Sullivan, L. B., and N. S. Chandel. 2014. Mitochondrial reactive oxygen species and cancer. *Cancer & Metabolism* 2: 17. doi:10.1186/2049-3002-2-17.

Sundaresan, N. R., M. Gupta, G. Kim, S. B. Rajamohan, A. Isbatan, and M. P. Gupta. 2009. Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. *Journal of Clinical Investigation* 119: 2758–71. doi:10.1172/JCI39162.

Tao, R., M. C. Coleman, J. D. Pennington, O. Ozden, S.-H. Park, H. Jiang, H.-S. Kim, C. R. Flynn, et al. 2010. Sirt3-mediated deacetylation of evolutionarily conserved lysine 122 regulates MnSOD activity in response to stress. *Molecular cell* 40: 893–904. doi:10.1016/j.molcel.2010.12.013.

Tao, R., A. Vassilopoulos, L. Parisiadou, Y. Yan, and D. Gius. 2014. Regulation of MnSOD Enzymatic Activity by Sirt3 Connects the Mitochondrial Acetylome Signaling Networks to Aging and Carcinogenesis. *Antioxidants & Redox Signaling* 20: 1646–1654. doi:10.1089/ars.2013.5482.

Torrens-Mas, M., D. G. Pons, J. Sastre-Serra, J. Oliver, and P. Roca. 2016. SIRT3 Silencing Sensitizes Breast Cancer Cells to Cytotoxic Treatments through an Increment in ROS Production. *Journal of cellular biochemistry* 10: 1–10. doi:10.1002/jcb.25653.

Torrens-Mas, M., J. Oliver, P. Roca, and J. Sastre-Serra. 2017. SIRT3: Oncogene and tumor suppressor in cancer. *Cancers* 9. doi:10.3390/cancers9070090.

Trachootham, D., W. Lu, M. A. Ogasawara, R.-D. V. Nilsa, and P. Huang. 2008. Redox regulation of cell survival. *Antioxidants & redox signaling* 10: 1343–74. doi:10.1089/ars.2007.1957.

Tseng, A. H. H., S. S. Shieh, and D. L. Wang. 2013. SIRT3 deacetylates FOXO3 to protect mitochondria against oxidative damage. *Free Radical Biology and Medicine* 63: 222–234. doi:10.1016/j.freeradbiomed.2013.05.002.

Wang, Q., L. Li, C. Y. Li, Z. Pei, M. Zhou, and N. Li. 2015. SIRT3 protects cells from hypoxia via PGC-1α- and MnSOD-dependent pathways. *Neuroscience* 286: 109–121. doi:10.1016/j.neuroscience.2014.11.045.

Yu, W., R. A. Denu, K. A. Krautkramer, K. M. Grindle, D. T. Yang, F. Asimakopoulos, P. Hematti, and J. M. Denu. 2016. Loss of SIRT3 Provides Growth Advantage for B Cell Malignancies. *Journal of Biological Chemistry* 291: 3268–3279. doi:10.1074/jbc.M115.702076.

Zhang, B., L. Qin, C. J. Zhou, Y. L. Liu, H. X. Qian, and S. B. He. 2013. SIRT3 expression in hepatocellular carcinoma and its impact on proliferation and invasion of hepatoma cells. *Asian Pacific Journal of Tropical Medicine* 6: 649–652. doi:10.1016/S1995-7645(13)60112-1.

Zhang, L., X. Ren, Y. Cheng, K. Huber-Keener, X. Liu, Y. Zhang, Y. S. Yuan, J. W. Yang, et al. 2013. Identification of Sirtuin 3, a mitochondrial protein deacetylase, as a new contributor to tamoxifen resistance in breast cancer cells. *Biochemical Pharmacology* 86: 726–733. doi:10.1016/j.bcp.2013.06.032.

Zou, X., C. A. Santa-Maria, J. O'Brien, D. Gius, and Y. Zhu. 2016. Manganese Superoxide Dismutase Acetylation and Dysregulation, Due to Loss of SIRT3 Activity, Promote a Luminal B-Like Breast Carcinogenic-Permissive Phenotype. *Antioxidants & Redox Signaling* 25: 326–336. doi:10.1089/ars.2016.6641.

Figure legends

Figure 1. SIRT3 levels after shRNA SIRT3 transfection and clonal selection. (A) SIRT3 mRNA levels after SIRT3 silencing. (B) SIRT3 protein levels measured by Western Blot after SIRT3 silencing. (C) Representative bands of SIRT3 Western Blot and GAPDH as a loading control. Values are expressed as mean ± SEM (n=6) and normalized to control value. Cells transfected with control shRNA are shown in white, and cells with SIRT3 shRNA are shown in black. * indicates a significant difference between control and SIRT3-silecing cells ($P<0.05$).

Figure 2. SIRT3 silencing increased ROS levels measured by flow cytometry. Intracellular levels of ROS determined by DCF fluorescence. Values are expressed as mean ± SEM (n=6) and normalized as percentage of the control value. * indicates a significant difference between control and SIRT3-silecing cells ($P<0.05$).

Figure 3. SIRT3 silencing reduced antioxidant gene expression. NRF2: nuclear respiratory factor 2; Foxo3a: Forkhead box O3; MnSOD: manganese superoxide dismutase; CuZnSOD: copper-zinc superoxide dismutase; CAT: catalase; Gpx: glutathione peroxidase; GRd: glutathione reductase; Prx2-6: peroxiredoxine 2-6; Trx1-2: thioredoxin 1-2; TrxR2: thioredoxin reductase 2. Values are expressed as mean ± SEM (n=6) and values of control cells were set at 1. * indicates a significant difference between control and SIRT3-silecing cells ($P<0.05$).

Figure 4. SIRT3 silencing increased acetylated-MnSOD/total-MnSOD ratio and reduced MnSOD enzymatic activity. (A) Total protein levels of MnSOD as measured by Western Blot. (B) Acetylated-MnSOD levels measured by Western Blot. (C) Ratio of acetylated-MnSOD/total-MnSOD. (D) Representative bands of Western Blots against total MnSOD and acetylated MnSOD. (E) MnSOD enzymatic activity. Values are expressed as mean ± SEM (n=6) and normalized as percentage of the control value. * indicates a significant difference between control and SIRT3-silecing cells ($P<0.05$).

Figure 5. SIRT3 silencing increased oxaliplatin efficacy by increasing ROS production and apoptosis. (A) Cell viability after SIRT3 silencing and oxaliplatin treatment measured by crystal violet. (B) ROS production measured fluorometrically after SIRT3 silencing and oxaliplatin treatment. (C) Annexin V fluorescence, an apoptosis marker, measured after SIRT3 silencing and oxaliplatin treatment. Values are expressed as mean ± SEM (n=6) and normalized as percentage of the control value. S indicates a SIRT3-silencing effect, T indicates treatment effect, and SxT indicates a combinatory effect of both. A Student's t test was performed when combinatory effects were observed, where: * indicates a significant difference between control and SIRT3-silencing cells and # indicates statistical difference between vehicle- and oxaliplatin-treated cells ($P<0.05$).

Figure 6. ROS scavengers reduce ROS production and recover cell viability after SIRT3 silencing and OXA treatment. (A) Cell viability after SIRT3 silencing and oxaliplatin, NAC and ascorbic acid treatment measured by crystal violet assay. (B) ROS production was measured fluorometrically after SIRT3

530 silencing and oxaliplatin, NAC and ascorbic acid treatment. Values are expressed as mean ± SEM (n=6)
531 and normalized as percentage of the control value. * indicates a significant difference between control
532 and SIRT3-sileced cells (P<0.05).

533 **Figure 7.** Kaplan-Meier survival curves showed that high SIRT3 expression is correlated to a lower overall
534 survival for colon cancer patients. (A) Overall survival of colon cancer patients. (B) Relapse-free survival
535 of colon cancer patients.